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β-adrenoceptor subtypes involved in myocardial preconditioning and postconditioning

A thesis submitted to Cardiff University in partial fulfilment of the requirements for the degree of *Philosophiae Doctor*

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Summary

Ischaemia and reperfusion in the heart leads to necrotic cell death (infarction) and contractile dysfunction (stunning). Ischaemic preconditioning (non-lethal periods of ischaemia prior to a longer ischaemic episode) and postconditioning (modified reperfusion) both reduce infarct size and are thought to act via activation of reperfusion injury salvage kinase (RISK) pathways to prevent the opening of the mitochondrial permeability transition pore (MPTP) at reperfusion. Catecholamines are released centrally and locally during myocardial ischaemia and activate adrenoceptors. This investigation was concerned with the roles of β-adrenoceptor activation in preconditioning and postconditioning.

Studies utilising isolated paced atrial and ventricular tissues demonstrated that preconditioning against stunning could be achieved by ischaemic preconditioning, or pre-treatment of tissues with isoprenaline, a β-adrenoceptor agonist. Both forms of protection were blocked by propranolol, a β-adrenoceptor antagonist. Postconditioning was not protective in this model.

A Langendorff model of regional ischaemia was used to determine effects of β-adrenoceptor agonists and antagonists on infarct size. In this model, ischaemic postconditioning was blocked by timolol, a non-selective β-adrenoceptor antagonist. Formoterol, a β_2-adrenoceptor agonist, given at reperfusion, mimicked postconditioning. The non-selective adrenoceptor agonist, adrenaline, when applied at reperfusion, worsened reperfusion contracture but had no effect on infarct size. The application of a selective β_1-adrenoceptor antagonist (CGP-20712A) at reperfusion led to a reduction of infarct size whereas β_2 (ICI-118,551) and β_3 (SR-59230A) antagonists had the opposite effect.

If the results are replicable in man *in vivo* they would be of clinical relevance because a commonly used class of drugs (β-adrenoceptor antagonists) have the potential to abrogate the protection of postconditioning. Another widely available class of drugs (β-adrenoceptor agonists) can have cardioprotective effects at reperfusion.

Declaration

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

Signed ..... (candidate) Date 16/5/2014

This thesis is being submitted in partial fulfilment of the requirements for the degree of Ph.D.

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This thesis is the result of my own independent work/investigation, except where otherwise stated.

Other sources are acknowledged by explicit references.

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I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

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Statement regarding my personal involvement in experiments

The experiments described in Chapter 4 –Part 1 were carried out under my supervision by Mr Jason R. Bell M.Pharm., a final year project student in my laboratory in 2008. I designed the experiments and set up the preparations, he carried out the protocols described.

Western Blotting was carried out by me under the helpful guidance of Dr Dwaine Burley and Mr David Elsey. The buffers I used had already been prepared in their laboratory.

Dr Emma Kidd supervised me whilst I performed the lactate dehydrogenase assay and gave guidance regarding the HPLC and Western blotting experiments.

All other experiments were entirely my own work.

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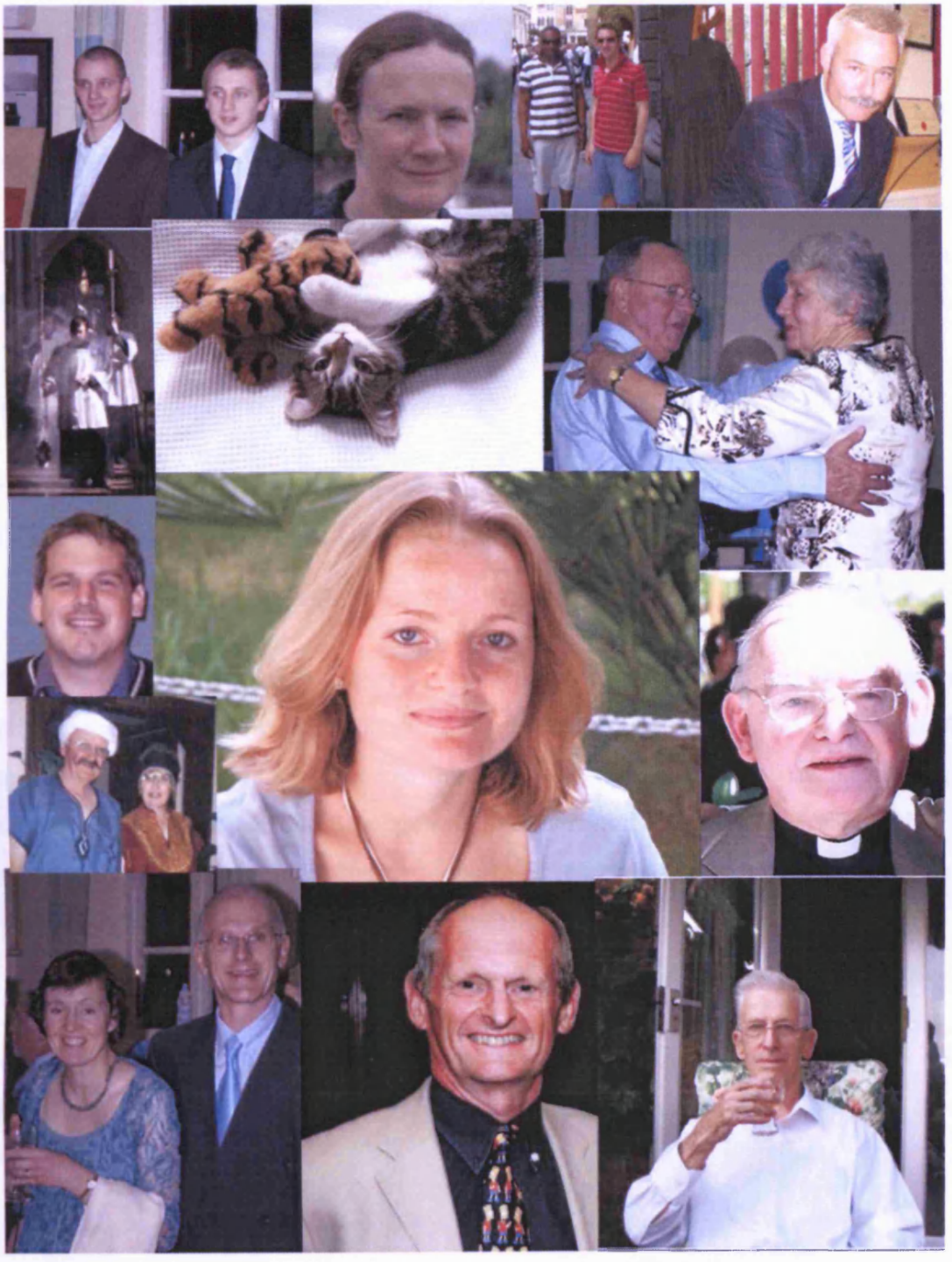
Gurnemanz: "Woher brachtest du dies?"

Kundry: "Von weiter her als du denken kannst."

Richard Wagner, Parsifal (First performance: Bayreuth, 26th July 1882)

Dedication

"To my friends pictured within"



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Contents

A list of figures and tables in this thesis.

Publications arising from this work

Research papers

PENSON, P.E., FORD, W.R., KIDD, E.J. & BROADLEY, K.J. (2008). Activation of β-adrenoceptors mimics preconditioning of rat-isolated atria and ventricles against ischaemic contractile dysfunction. *Naunyn-Schmiedeberg's Archives of Pharmacology*, **378,** 589-97.

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Abstracts

PENSON, P.E., FORD, W.R., KIDD, E.J. & BROADLEY, K.J. (2008). Protective role of β_2- and β_3-adrenoceptors at reperfusion in isolated rat heart. *Journal of Molecular and Cellular Cardiology*, **44,** 719.

PENSON, P.E., FORD, W.R., KIDD, E.J. & BROADLEY, K.J. (2007). Infarct-size reduction induced by ischaemic postconditioning is dependent on β2-adrenoceptor activation in the Langendorff perfused rat heart. *Proceedings of the British Pharmacological Society (PA2 Online)*.

PENSON, P.E., FORD, W.R., KIDD, E.J. & BROADLEY, K.J. (2006). Preconditioning by the β-adrenoceptor agonist, isoprenaline, in the rat isolated atrium but not ventricle. *Proceedings of the British Pharmacological Society (PA2 Online)*.

Abbreviations

AAR	Area at risk
ADP	Adenosine diphosphate
Akt	A Serine-threonine protein kinase also known as protein kinase B (PKB)
ATP	Adenosine triphosphate
CF	Coronary flow
CPP	Coronary perfusion pressure
DNA	Deoxyribose nucleic acid
DT	Developed tension
Erk 1/2	Extracellular signal regulated kinase 1/2
GDP	Guanosine diphosphate
GISSI	Gruppo Italiano per lo Studia Della Streptochinasi nell'Infarcto Miocardio
GTP	Guanosine triphosphate
INT	2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride
K_{ATP}	ATP-sensitive potassium channel
LVDP	Left ventricular developed pressure
MAP Kinase	Mitogen activated protein kinase

mK_{ATP}	Mitochondrial ATP-sensitive potassium channel
mPTP	Mitochondrial permeability transition pore
n	The number of replicate observations in an experimental group
PKA	Protein Kinase A
PKB	Protein Kinase B (also known as Akt, see above)
PVDF	Polyvinylidine difluoride
RISK	Reperfusion injury salvage kinase.
RPP	Rate Pressure Product
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis

Chapter 1 -General introduction

1.1 Historical background

"Adrenalin is liberated normally in fear, rage, asphyxia and pain"

Walter B. Cannon (1914)

The late nineteenth and early twentieth century was a time of enormous discovery in the fields of physiology and pharmacology. Much of what was discovered at that time is directly relevant to the work presented in this thesis. Thirty years prior to Cannon's classic description of the physiological effects of adrenaline (Cannon, 1914), Cohnheim described the development of infarction in the heart following artery occlusion (Fye, 2002). At the turn of the last century, crude extracts of adrenaline were achieved independently by Abel and (the appropriately named) Takamine (Sears & Lotvall, 2005). Later, in what was arguably the first, albeit theoretical, description of a drug receptor, Dale discussed the possibility of a 'receptive substance' for adrenaline (Dale, 1906).

This work was continued throughout the twentieth century. Cannon went on to develop his own theory of sympathetic neurotransmission involving two sympathetic neurotransmitters, Sympathin E (mediating excitatory effects) and Sympathin I (mediating inhibitory effects). However, Von Euler showed that there was one sympathetic neurotransmitter, noradrenaline (Von Euler, 1951). Ahlquist invented the concept of α- and β-adrenoceptors to account for the

various effects of catecholamines in different tissues (Ahlquist, 1948). This neat explanation turned out to be a physiological reality.

Adrenaline has been thought of a 'stress hormone' ever since Cannon's famous description of its actions preparing the body for 'fight or flight' (Cannon, 1929; Cannon, 1914). This thesis examines the effects of adrenoceptors in one particular situation of extreme physiological stress – myocardial ischaemia and reperfusion.

Ischaemia, from the Greek *ischo* meaning 'to hold back' and *haima* meaning 'blood' (Opie, 1997) refers to a condition whereby a tissue is inadequately perfused with blood. Ischaemia ultimately leads to cell death and infarct formation. The process of infarct formation was extensively studied by Reimer and Jennings in the 1970s (Reimer & Jennings, 1979; Reimer *et al.*, 1977). Rapid restoration of blood flow (reperfusion) is necessary to salvage ischaemic myocardium. However, reperfusion is associated with damage over and above that sustained during ischaemia leading to the classic description of reperfusion as a 'double edged sword' (Braunwald & Kloner, 1985).

The first major advances in protecting the myocardium against the damage sustained during ischaemia and reperfusion came with the discovery that short periods of non-lethal ischaemia primed the myocardium such that, a later more severe ischaemic insult led to the development of a much smaller infarct than in untreated controls (Murry *et al.*, 1986). This phenomenon, known as 'ischaemic preconditioning', has been widely studied ever since.

More recently, it has been discovered that alternating short periods of ischaemia and reperfusion at the end of a period of ischaemia reduced infarct size to a similar extent to preconditioning. This phenomenon is known as postconditioning (Na *et al.*, 1996; Zhao *et al.*, 2003). Early clinical data have demonstrated that postconditioning occurs in man and that it holds promise as a therapeutic strategy (Darling *et al.*, 2007; Sivaraman *et al.*, 2007; Staat *et al.*, 2005; Thibault *et al.*, 2008)

Adrenoceptors and their ligands have important roles during ischaemia and reperfusion. Catecholamines are released from the adrenal medulla in response to stress (Cannon, 1929; Cannon, 1914) and are released locally in the myocardium in response to ischaemia (Kuroko *et al.*, 2007; Lameris *et al.*, 2000). Furthermore, since 1896, adrenaline has been administered to patients and experimental animals undergoing resuscitation from cardiac arrest in order to cause peripheral vasoconstriction, and to direct the blood-flow generated by cardiopulmonary resuscitation to the heart and brain (Penson *et al.*, 2007).

Activation of adrenoceptors at reperfusion can worsen ischaemic damage, as demonstrated by the fact that β-adrenoceptor antagonists can be protective when administered at reperfusion (Feuerstein *et al.*, 1998). Conversely, treatment of tissues with β-adrenoceptor agonists prior to ischaemia, can mimic the protection of ischaemic preconditioning (Frances *et al.*, 2003). Nothing is yet known about the roles of β-adrenoceptors during postconditioning. These facts and questions have prompted this re-evaluation of the roles of β-adrenoceptor activation before and during ischaemia and at reperfusion.

This introduction will begin with a discussion of ischaemia and reperfusion, followed by a section regarding adrenoceptors in the heart. The final section will discuss the roles of β-adrenoceptors in cardioprotection, the rationale, aims and hypotheses of the study.

1.2 Ischaemia and reperfusion

1.2.1 Definitions of ischemia and reperfusion

Ischaemia has been defined as *'a reduction in local blood flow such that the oxygen supply is less than the oxygen demand required for function'* (Parums, 1999). Whilst correct, this definition is over-simplistic and overlooks some important points. Ischaemia occurs either as a result of occlusion of a blood vessel – termed 'supply ischaemia' or by the increased metabolic demands of an organ or tissue above a level at which oxygen and nutrients can be supplied. This is called 'demand ischaemia' (Zucchi *et al.*, 2007). Thus ischaemia can be relative; 'low-flow ischaemia' or absolute; 'no-flow ischaemia' (Pantos *et al.*, 2006). Ischaemia should be differentiated from hypoxia. The latter refers only to a deficit in oxygen, whilst the former involves a deficit of blood-flow and therefore results in the accumulation of waste products of metabolism with consequent pathological effects (Allen & Orchard, 1987).

Prolonged no-flow ischaemia leads to well characterised consequences in the heart. Necrotic cell death occurs in the region distal to the occlusion forming an infarct. The infarct begins in the subendocardium and progresses in a 'wave-front' phenomenon towards the subepicardium until the entire area supplied by the blocked vessel is infarcted (Reimer *et al.*, 1979; Reimer *et al.*, 1977). Reperfusion, the restoration of blood flow to an ischemic region, is absolutely

essential to salvage myocardium. The Gruppo Italiano per lo Studia Della Streptochinasi nell'Infarcto Miocardio (GISSI) clinical trial in the 1980s demonstrated the survival benefit of revascularisation, now a mainstay of clinical therapy of myocardial ischaemia (GISSI Investigators, 1986). However, the rapid reintroduction of blood into ischemic tissue results in further damage known as reperfusion injury (Braunwald *et al.*, 1985; Heyndrickx *et al.*, 1975; Jennings *et al.*, 1960). Reperfusion injury is an important consideration from a clinical perspective because whilst it is almost impossible to predict when myocardial infarction or cardiac arrest will occur, reperfusion usually occurs as a result of a medical intervention (e.g. defibrillation and thrombolysis) and is therefore more amenable to therapeutic interventions than the damage which occurs during ischaemia (Piper *et al.*, 2004).

1.2.2 Pathophysiology of ischaemic damage and reperfusion injury

All the studies in this thesis involve experimentally-induced acute ischaemia and reperfusion in tissues taken from healthy animals. However, in man, myocardial ischaemia occurs as the result of pathological processes which occur over long periods of time. Therefore, a discussion of these processes is relevant prior to a discussion of the cellular mechanisms of ischaemic damage and reperfusion injury.

1.2.2.1 Coronary heart disease and ischaemia

Coronary heart disease kills 101,000 people in the United Kingdom every year, making it the most common cause of death. One in five men and one in six women die from the disease. It is also the biggest cause of premature mortality (death in people less than 75 years old) (Allender

et al., 2007). Coronary heart disease is a chronic disorder, resulting from the deposition of atherosclerotic plaques in the coronary arteries. 'Athera' is the Greek for porridge (Aaronson *et al.*, 2004) and in this context describes the consistency of the plaques. Early manifestations of atherosclerosis can be seen in children and adolescents, with lipoprotein accumulation in the arterial intima, forming fatty streaks. These develop to form atherosclerotic lesions by middle age. The lesions reduce the diameter of the artery lumen. The development of the plaque involves inflammation, increased endothelial permeability, the invasion of leucocytes and monocytes (which release cytokines and recruit macrophages) and smooth muscle proliferation. The smooth muscle cells produce collagen which contributes to the formation of the fibro-lipid plaque. This process has been extensively reviewed elsewhere (Camm, 2002; Hansson, 2005; Libby, 2001). Stable plaques reduce the luminal diameter of arteries and cause the symptoms of stable angina. Plaques are prone to rupture leading to acute myocardial infarction (see Section 1.2.2). Results from the Framingham Heart Study demonstrated that increasing age, hypertension, diabetes, smoking and plasma total and LDL cholesterol are important risk factors for the development of coronary artery disease (Wilson *et al.*, 1998).

1.2.2.2 Pathophysiology of acute myocardial infarction

Acute myocardial infarction occurs as a result of coronary thrombosis (DeWood *et al.*, 1980). Atherosclerotic plaque rupture and damage to the vascular endothelium are important triggers for thrombus formation as both pathologies expose collagen in the arterial wall to which platelets can bind and become activated. Fibrin is formed at the site of the rupture and can occlude the vessel. The region of myocardium distal to the blockage is no longer adequately perfused with blood, thus does not receive oxygen or nutrients. Additionally, waste products of

metabolism cannot be removed and accumulate locally. If unresolved, this leads to cell death in the ischaemic region; the area of dead tissue is called the infarct. As described above, the infarct begins in the subendocardium and progresses in a 'wave-front phenomenon' towards the subepicardium until the entire area supplied by the blocked vessel is infarcted (Reimer *et al.*, 1979; Reimer *et al.*, 1977). Coagulation necrosis occurs in the infarcted myocardium, and neutrophils enter the region after 18 hours. It was at one time argued that neutrophil entry was pathological; however, it is now understood to be a response to the damage. The most convincing evidence of this, being that all forms of ischaemic damage and reperfusion injury can be demonstrated in neutrophil-free systems such as the isolated perfused heart (Baxter, 2002).

Over a period of several days, dead tissue is replaced by granulation tissue and scar formation takes place. The infarcted myocardium is liable to develop further pathologies. Mitral regurgitation can occur if the papillary muscles have been damaged. Injury to node or conducting tissue leads to arrhythmias and free wall and rupture of the inter-ventricular septum can also occur.

These pathologies have been reviewed elsewhere (Yang *et al.*, 2006). Ventricular remodelling and progression to heart failure are also common complications of myocardial infarction (Hellermann *et al.*, 2002; Sampson & Hutchinson, 1967). As a result of cell-death, the pump efficiency of the heart is reduced and ventricular emptying is impaired. Initially Starling's law provides a compensatory increase in cardiac contractility; however over a long period of time,

pathological ventricular dilation occurs (Pfeffer & Braunwald, 1990; Sweetman, 2002). The failing heart is unable to pump sufficient blood to meet the metabolic demands of the tissues. The fall in cardiac output and reduced blood-flow to tissues leads to reflex sympathetic activation which increases β-adrenoceptor mediated tachycardia and increased force of contraction. β-adrenoceptors have been implicated in the pathophysiology of heart failure and studies indicate that chronic release of endogenous catecholamines stimulates β1-adrenoceptors to induce myocyte hypertrophy and apoptosis, whereas β_2-adrenoceptors have an opposing protective action (Xiao et al., 2004).

Another compensatory mechanism which is activated in response to the fall in cardiac output after myocardial infarction is the renin-angiotensin-aldosterone system. Angiotensin II causes vasoconstriction, increased cardiac output and promotes sodium and water retention by activation of AT1 receptors in vascular smooth muscle, the heart and kidneys and by causing the release of aldosterone from the kidneys. In the short term this restores blood pressure and organ perfusion. Long-term activation of the renin-angiotensin system is however detrimental and is an important element in the pathophysiology of the disease. Angiotensin II stimulates myocardial hypertrophy and interstitial fibrosis, and promotes noradrenaline release from nerve endings. Thus the renin-angiotensin-aldosterone cascade has important roles in pathological ventricular remodelling and angiotensin converting enzyme inhibitors, which prevent the production of angiotensin II, reduce mortality in heart failure (SOLVD Investigators, 1992). The interested reader is directed to review articles on this topic (Ford, 2009; Opie & Pfeffer, 2009).

1.2.3 Cellular mechanisms of ischaemic damage

Blood-flow through the capillary beds in tissues serves to deliver oxygen and metabolic substrates and to remove the waste products of metabolism. The metabolic and functional changes which occur during ischaemia are a result of the cessation or impairment of these functions. Consequent biochemical and metabolic changes lead either to ischaemic cell death, or if reperfusion occurs, provide the milieu in which reperfusion pathologies occur.

Under normal physiological conditions the metabolic energy demands of the heart are met mainly by fatty acid catabolism. However glucose is also a substrate and plays an important role in supplying additional energy when myocardial workload is increased above basal levels (Chaitman *et al.*, 2004; Depre *et al.*, 1999; Kantor *et al.*, 2000; Stanley *et al.*, 1997). Both fatty acids and glucose feed into the Krebs' (citric acid) cycle and oxidative phosphorylation. Metabolism of glucose begins in the cytosol with the process of glycolysis which for each molecule of glucose, produces two molecules of pyruvate and converts two molecules of ADP to ATP. Oxygen is required for the next stage in metabolism, the production of acetyl coenzyme A from pyruvate which feeds into the Krebs' cycle and thereafter into oxidative phosphorylation, producing approximately 30 molecules of ATP from ADP, for each molecule of glucose metabolised. The oxygen-dependent reactions of the Krebs' cycle and oxidative phosphorylation cease soon after the onset of ischaemia. However, in the absence of oxygen, energy can be produced by the conversion of pyruvate to lactic acid. This is an inefficient process, producing a net of only 2 molecules of ATP from each molecule of glucose. For reviews see Stryer (1995) or Ingwall (2002). Upon the onset of ischaemia, glycolysis is initially stimulated to provide sufficient energy to sustain the metabolic processes of the cell. Under

anoxia alone, the cell could continue to produce ATP in this manner, however during ischaemia there is an absence of blood-flow and anaerobic metabolites remain within the cytosol. The accumulating lactic acid decreases intracellular pH and suppresses glycolysis. Some ATP can be synthesised from creatine phosphate. This compound has a high energy phosphate bond which can be hydrolysed; the free energy liberated being used to produce ATP from ADP and Pi. The cellular content of creatine phosphate declines during ischaemia as a result of ATP production, eventually ATP levels fall Creatine kinase catalyses the transfer of a phosphoryl group from creatine phosphate to ADP (Ingwall, 2002).

ATP is required to sustain all metabolic processes within the cell. A high concentration of ATP in relation to its breakdown products (ADP and Pi) drives ATPase reactions. The reduction in the ATP/ADP ratio during ischaemia leads to the failing of the contractile machinery of the myocyte and disruption of active transport of ions and solutes across the plasma membrane (Bers, 2001; Kammermeier, 1987; Kammermeier *et al.*, 1990).

1.2.3.1 Effects of ischaemia on membrane transport function

Changes in ion-channel function as a result of ischaemia are shown below (Figure 1-1). Under normal physiological conditions, calcium is removed from the cell via a transporter protein in the plasma membrane which is dependent upon ATP for its activity (Figure 1-1 A), thus under ischaemic (low ATP) conditions, the transport is disrupted and the cytosolic calcium concentration increases. The smooth endoplasmic reticulum calcium ATPase is also inactivated. Additionally the sodium-potassium ATPase is inhibited leading to an increase in cellular sodium

levels and a decrease in cytosolic potassium. The anaerobic metabolism of pyruvate into lactate causes a decrease in cytosolic pH (an increase in the concentration of hydrogen ions). These changes in cytoplasmic ion concentrations lead to a reversal of the sodium-hydrogen exchanger, which under ischaemic conditions removes hydrogen ions from the cytoplasm and allows sodium ions to enter the cells from the extracellular milieu. The increased osmotic load as a result of raised intracellular sodium draws water into the cell. (Figure 1-1 B) The calcium increase in the cytosol is also aided by entry via L-type channels (Smart *et al.*, 1997). The intracellular ionic alterations during ischaemia have been reviewed extensively elsewhere (Ingwall, 2002; Moens *et al.*, 2005; Murphy & Steenbergen, 2008a; Murphy & Steenbergen, 2008b; Pantos *et al.*, 2006; Solaini & Harris, 2005; Suleiman *et al.*, 2001). Calcium is an important regulator in many cellular processes; therefore derangement of cellular calcium leads to many abnormalities. During ischaemia, calcium activates proteases and phospholipid-dependent protein kinases which can damage a wide variety of cellular structures including mitochondrial and sarcolemmal membranes. Damage to the sarcolemmal membrane causes more calcium to enter the cell. High cytosolic calcium concentrations favour the opening of the mitochondrial permeability pore at reperfusion (Crompton & Andreeva, 1994; Crompton *et al.*, 1988) (Figure 1-1 C) resulting in cell death (See section 1.2.4.1 for further details).

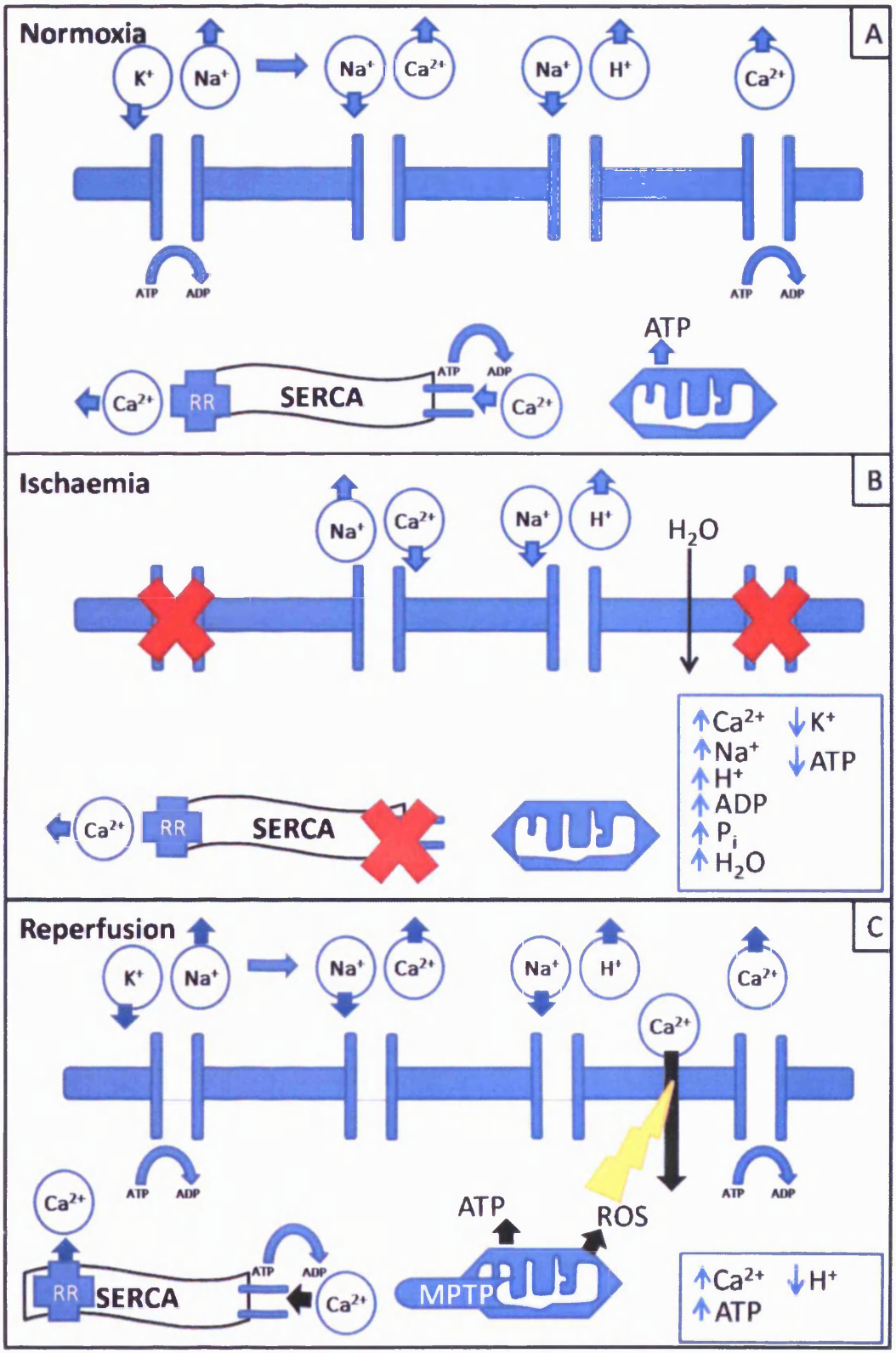

Figure 1-1: Disruption of ion channels during ischaemia and reperfusion. Modified from diagrams in numerous sources (Ingwall, 2002; Murphy *et al.*, 2008b; Pantos *et al.*, 2006)

Metabolic processes in the myocyte during A, normoxia; B, ischaemia and C, reperfusion. (A) Under normal physiological conditions, calcium is removed from the cell via a transporter protein which is dependent upon adenosine triphosphate (ATP) for its activity, thus under ischaemic (low ATP) conditions (B), the transport is disrupted and the cytosolic calcium concentration increases. The smooth endoplasmic reticulum calcium ATPase is also inactivated. Additionally the sodium-potassium ATPase is inhibited leading to an increase in cellular sodium levels and a decrease in cytosolic potassium. The anaerobic metabolism of pyruvate into lactate causes a decrease in cytosolic pH (an increase in $[H^+]$). These changes in cytoplasmic ion concentrations lead to a reversal of the sodium-hydrogen exchanger, which under ischaemic conditions removes hydrogen ions from the cytoplasm and allows sodium ions to enter the cells from the extracellular milieu. The increased osmotic load as a result of raised intracellular sodium draws water into the cell. The calcium increase in the cytosol is also aided by entry via L-type channels. The intracellular ionic alterations during ischaemia have been reviewed extensively elsewhere. Calcium is an important regulator in many cellular processes; therefore derangement of cellular calcium leads to many abnormalities. During ischaemia, calcium activates proteases which damage a wide variety of cellular structures including mitochondrial and plasma membranes causing more calcium to enter the cell. At reperfusion (C) High cytosolic calcium concentrations favour the opening of the mitochondrial permeability pore (MPTP) resulting in cell death. RR=ryanodine receptor, SERCA = Smooth endoplasmic reticulum calcium ATPase.

1.2.3.2 Effects of ischaemia on contractile function

Contractile dysfunction occurs rapidly after the onset of ischaemia, manifest by a reduction of contractility as a result of a decreased ratio of ATP/ADP and decreased intracellular pH. Contractile function is compromised when cellular ATP content is still relatively high (Stapleton & Allshire, 1998).

1.2.3.3 Ischaemic contracture

Ischaemic contracture refers to the phenomenon whereby myocyte shortening occurs during ischaemia. In experimental models of myocardial ischaemia, this is seen as an increase in diastolic tension. Ischaemic contracture causes only a small amount of damage to the myocardial cytoskeleton. This is in contrast to hypercontracture which develops upon reperfusion (See Section 1.2.4.1). It has been suggested that the onset of contracture in the ischaemic period is concurrent with depletion of glycogen. This has been discussed by Pantos (2006) in an excellent review.

1.2.3.4 Fate of ischaemic cells

The extent of the biochemical disturbance within the ischaemic cells will determine the fate of the cells upon reperfusion. However, if reperfusion does not occur, ischaemic cell death ensues by necrotic mechanisms. Necrosis under these conditions is also known as oncosis (Majno & Joris, 1995). The mechanism of cell death is probably sarcolemmal membrane rupture (Reimer & Ideker, 1987) as a result of osmotic increases of water in the cytosol and damage to the sarcolemmal membrane by calcium-activated enzymes (Buja, 2005; Buja *et al.*, 1993; Buja & Entman, 1998). There is debate as to the extent of apoptotic cell death during ischaemia. Initially it was thought that the contribution of apoptosis to ischaemic cell death was minimal (Gottlieb *et al.*, 1994), perhaps because of the ATP dependence of apoptosis. However a later study estimated to apoptosis to account for 86 % of ischaemic cell death after 2 hours ischaemia in the rat heart (Anversa *et al.*, 1998). This question is likely to remain controversial. Since the first description of apoptosis (Kerr *et al.*, 1972) evidence has emerged to suggest that different forms of cell death are not as distinct as was thought (Eefting *et al.*, 2004; Leist & Jaattela, 2001). Furthermore, different methods of quantifying apoptosis give markedly varying results (Eefting *et al.*, 2004). Of particular concern is the technique whereby broken strands of deoxyribose nucleic acid (DNA) are quantified as a measure of apoptosis. This technique is known as terminal deoxy-nucleotidyl transferase [TdT]-mediated deoxyuridine triphosphate [dUTP]-biotin nick end-labeling (TUNEL). However TUNEL has been shown to give a positive results in necrotic cells when minor damage to DNA has occurred (Jugdutt & Idikio, 2005).

1.2.4 Reperfusion injury

Reperfusion injury is best described by a series of seemingly paradoxical observations. The paradox that reperfusion is both necessary for survival and damaging has led to it being described as a 'double-edged sword' (Braunwald *et al.*, 1985). Reperfusion injury is manifest in four distinct pathologies: i) Infarct development, known as lethal reperfusion injury. ii) Reversible contractile dysfunction known as stunning (Heyndrickx *et al.*, 1975; Jennings *et al.*, 1960). iii) Reperfusion arrhythmias, such as ventricular tachycardia, premature ventricular beats and ventricular fibrillation (Manning & Hearse, 1984b) iv) The 'no-reflow phenomenon', whereby regions of ischaemic myocardium are not adequately reperfused when the blood supply is returned, due to endothelial dysfunction and capillary plugging with neutrophils (Kloner *et al.*, 1974).

The concept of the 'oxygen paradox' was by first described by Latham (1951) and later developed by Hearse, who realised that whilst hypoxia was an important pathophysiological feature of ischaemia, the rapid reintroduction of oxygen to an ischaemic tissue resulted in further cell death (Hearse *et al.*, 1973). It is thought that the combination of the partially damaged respiratory chain (Lesnefsky *et al.*, 2004; Sack, 2006) and xanthine oxidase (Hearse *et al.*, 1986; Manning *et al.*, 1984a; Yellon *et al.*, 1985) lead to pathologically high levels of reactive oxygen species within the cell. These cause DNA damage, lipid peroxidation of the sarcolemmal membrane and widespread damage to proteins and enzymes (Hausenloy & Yellon, 2008)

The calcium paradox is another important concept involved in the pathology of ischaemic damage and reperfusion injury. It was first noted in the 1960s that if calcium was removed from the solution perfusing a heart, cell death accompanied by morphological changes appeared when calcium was returned (Zimmerman & Hulsmann, 1966; Zimmerman *et al.*, 1967). An analogous situation occurs at reperfusion after ischaemia. Cytosolic concentrations of calcium are greatly increased during early reperfusion due to damage to the sarcoplasmic reticulum and sarcolemmal membrane (Figure 1-1 C).

Thus, the increase in intracellular calcium and reactive oxygen species are largely responsible for the reperfusion pathologies described below.

1.2.4.1 Lethal reperfusion injury

"Lethal injury is defined as injury caused by restoration of blood flow after an ischemic episode leading to death of cells that were only reversibly injured during that preceding ischemic episode" (Piper et al., 1998).

Perhaps the most controversial aspect of the study of reperfusion injury has surrounded the existence of lethal reperfusion injury (Ambrosio & Tritto, 1997; Bauer & Deeg, 1997; Bolli, 1997; Cohen *et al.*, 1997; Flaherty & Zweier, 1997; Reimer, 1997; Venturini & Schaer, 1997). Reperfusion was initially thought to simply hasten the death of cells which had been irreversibly injured during ischaemia (Jennings *et al.*, 1960). However, because interventions at reperfusion

can reduce necrosis, lethal reperfusion injury is likely to be an important pathophysiological entity (Piper *et al.*, 1998).

Cell death at reperfusion occurs as a result of the opening of large non specific channels in the inner mitochondrial membrane. This 'mitochondrial permeability transition' was first described in the 1970s (Haworth & Hunter, 1979; Hunter & Haworth, 1979a; Hunter & Haworth, 1979b; Hunter *et al.*, 1976) and it has long been known that the transition could be prevented by cyclosporine A (Crompton *et al.*, 1988). However, the phenomenon has only recently been attributed to a specific channel, the mitochondrial permeability transition pore (MPTP). The molecular identity of the pore is uncertain (Halestrap *et al.*, 2007) but may be formed of an aggregation of cyclophilin D (Lim *et al.*, 2007b), the adenine nucleotide transporter and the voltage-dependent anion channel (Halestrap, 2006). It is thought that cyclosporine acts by preventing cyclophilin D interacting with other pore components (Javadov & Karmazyn, 2007; Woodfield *et al.*, 1998).

When the MPTP is opened the mitochondrial potential gradient is lost, and thus ATP generation is halted. Pro-apoptotic mediators such as cytochrome C may also be released from the mitochondrial matrix into the cytoplasm (Halestrap, 2006), however, because the cell loses its capacity to generate ATP upon MPTP opening, it has been suggested that cell death following MPTP opening occurs predominantly due to necrosis rather than apoptosis, which is an energy-dependent process (Murphy *et al.*, 2008b). Other studies however have demonstrated an

important role for apoptosis in reperfusion injury (Gottlieb *et al.*, 1994). For the reasons outlined above, this controversy is difficult to resolve.

During early reperfusion, intracellular pH returns to normal levels due to the removal of cytoplasmic lactic acid. This is paradoxically damaging because low pH prevents the formation of the MPTP (Halestrap, 2006). This phenomenon is known as the 'pH paradox' (Bond *et al.*, 1994; Lemasters *et al.*, 1996). The high intracellular calcium concentrations which occur during early reperfusion also promote MPTP opening (Halestrap, 2006). The MPTP does not open during ischaemia (Levraut *et al.*, 2003).

Another contributor to cell death at reperfusion is hypercontracture. Contracture can be enhanced during reperfusion to such an extent that mechanical damage to the myocyte occurs. This is called hypercontracture and is caused by calcium overload as a result of cytosolic calcium oscillations (Pantos *et al.*, 2006). Hypercontracture can cause cell membrane rupture and lead to cell death. This form of myocardial damage is associated with specific morphological features referred to as contraction band necrosis (Duflou *et al.*, 2006; Hutchins & Silverman, 1979; Rodriguez-Sinovas *et al.*, 2007; Virmani *et al.*, 1996).

1.2.4.2 Stunning

"Postischemic dysfunction, or myocardial stunning, is the mechanical dysfunction that persists after reperfusion despite the absence of irreversible damage" (Bolli, 1990).

"(1) stunning is a transient, fully reversible abnormality, provided that sufficient time is allowed for recovery; (2) stunning is a mild, sublethal injury that must be kept apart from the irreversible damage occurring in myocardial infarction; and (3) stunned myocardium has a normal or near normal coronary flow" (Ferrari et al., 1999).

The term stunning was first used in 1982, to describe post ischaemic contractile dysfunction (Braunwald & Kloner, 1982). However, the phenomenon of post-ischaemic dysfunction had been recorded earlier (Heyndrickx *et al.*, 1975) although not afforded much interest, because it was believed that, outside of the laboratory setting, reperfusion was a rare event (Bolli & Marban, 1999).

It has been demonstrated in a canine model that fifteen minutes of ischaemia can lead to subnormal myocardial function for twenty-four hours (Moens *et al.*, 2005), This has potential clinical implications, although because stunning is reversible, it is probably more important as a cause of morbidity than mortality. However, in certain patients with low-cardiac output following ischaemia, stunning may be life threatening (Ferrari *et al.*, 1999). Stunning may also occur repeatedly after episodes of angina and lead to ventricular failure (Bolli, 1990). Thus, the

prevention of stunning is an important target in the treatment of ischemia and reperfusion and stunning is worthy of experimental and clinical study.

The mechanism of stunning has been attributed to two mechanisms which are not mutually exclusive. The oxyradical hypothesis and the calcium overload hypothesis. It is thought that high cytosolic levels of these two injurious mediators causes sarcoplasmic reticulum dysfunction and inhibits the contractile machinery of the cell, leading to excitation-contraction uncoupling (Bolli, 1990; Bolli *et al.*, 1999).

1.2.4.3 No-reflow phenomenon

"...under some circumstances, restoration of arterial flow into the previously ischemic tissue either does not occur or is greatly impeded. This so-called "no-reflow" phenomenon..." (Kloner et al., 1974)"

Reperfusion injury also occurs to the endothelium of the coronary blood vessels, resulting in increased cellular permeability and reduced ability of the endothelium to mediate vasodilator responses (Dauber *et al.*, 1990). Damage to the microvasculature and neutrophil infiltration also occur causing plugging and preventing reperfusion of some areas of tissue on the restoration of blood-flow. This has been described as the 'no-reflow' phenomenon (Kloner *et al.*, 1974). These factors have been extensively reviewed elsewhere (Carden & Granger, 2000; Honda *et al.*, 2005; Jassem *et al.*, 2002; Moens *et al.*, 2005; Piper *et al.*, 2003; Suleiman *et al.*, 2001).

1.2.4.4 Reperfusion arrhythmias

"Dysrhythmia associated with reperfusion may ... play a critical role in sudden cardiac death" (Corr & Witkowski, 1983)

Arrhythmias such as ventricular tachycardia, premature ventricular beats and ventricular fibrillation often occur following reperfusion (Manning *et al.*, 1984b) and are associated with sudden cardiac death (Corr *et al.*, 1983). The arrhythmias are caused by ionic disturbances and the presence of reactive oxygen species at reperfusion (Pantos *et al.*, 2006).

1.2.5 Amelioration of reperfusion injury

Various pharmacological agents including adenosine and opioids have been demonstrated to attenuate reperfusion injury in animal models. This has been extensively reviewed by Gross and Gross (2006). Ischaemic postconditioning (Staat *et al.*, 2005; Thibault *et al.*, 2007a; Thibault *et al.*, 2008) and its pharmacological mimic, cyclosporine (Piot *et al.*, 2008) have additionally been demonstrated to be of benefit in man. Such strategies have the potential to be applied at the time of medical interventions which lead to reperfusion in order to minimise reperfusion injury. Possible uses would include thrombolysis and angioplasty after acute coronary thrombosis/ myocardial infarction and at defibrillation after cardiac arrest.

1.3 Cardioprotection by pre-and post-conditioning

In Reimer's first paper describing 'ischaemic preconditioning', it was demonstrated that short periods of non-lethal ischaemia primed the myocardium such that, a later more severe ischaemic insult (Figure 1-2) led to the development of a much smaller infarct than in untreated controls (Murry *et al.*, 1986). This phenomenon has been widely studied ever since. In addition to reduction of infarct size, other benefits of preconditioning are seen, including improved post-ischaemic cardiac function (Urabe *et al.*, 1993) and reduction in the frequency of reperfusion arrhythmias (Li *et al.*, 1992). Reimers' study provided the first demonstration that the heart could be protected against the damage sustained during ischaemia-reperfusion and generated great excitement. However, the clinical utility of ischaemic preconditioning is limited by a number of factors: i) The onset of ischaemia in coronary heart disease cannot be reliably predicted. ii) The protective intervention must be administered prior to the ischaemic insult. iii) The protection of preconditioning is short-lived (although there is a later phase of protection, see 1.3.2.1). iv) The application of ischaemic preconditioning is invasive and potentially dangerous. However, preconditioning has been shown to be of benefit when administered prior to cardiac surgery (Yellon *et al.*, 1993).

Recently, it has been discovered that alternating short periods of ischaemia and reperfusion at the end of a period of ischaemia (postconditioning) reduced the frequency of arrhythmias (Na *et al.*, 1996) and reduced infarct size to a similar extent as preconditioning (Zhao *et al.*, 2003) (Figure 1-2). This phenomenon is known as postconditioning. Early clinical data have demonstrated that postconditioning occurs in man and that it holds promise as a therapeutic strategy (Darling *et al.*, 2007; Sivaraman *et al.*, 2007; Staat *et al.*, 2005; Thibault *et al.*, 2008).

It can be helpful to call the protective cycles of ischaemia 'conditioning ischaemia' and the long insult, against which protection is sought, the 'index ischaemia'. The optimal length of the 'conditioning' cycles of ischaemia and reperfusion appear to be shorter for postconditioning than preconditioning. Postconditioning must be administered in the early minutes of reperfusion to be protective, whereas protection can be preserved even when the delay between preconditioning and the index ischaemia is up to two hours.

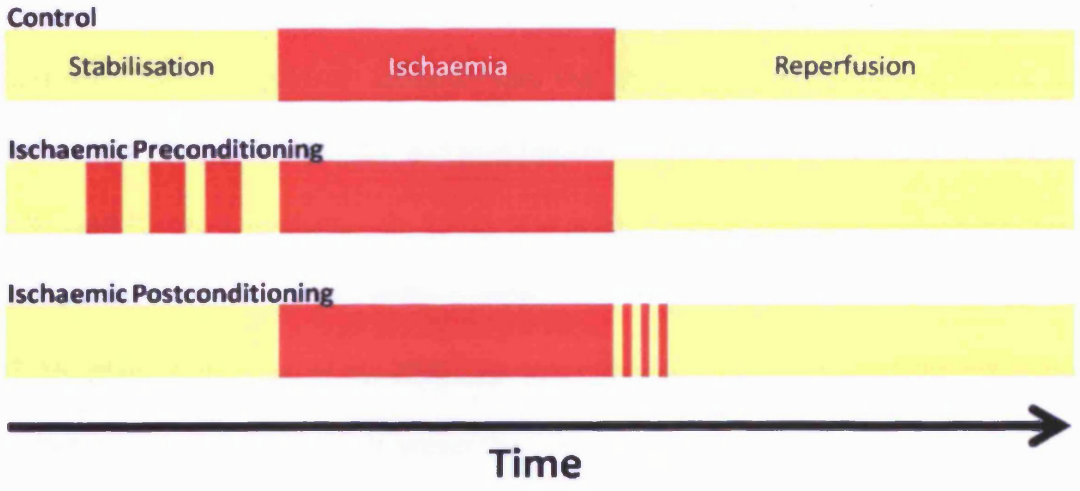

Figure 1-2. Typical experimental protocols used in the study of ischaemic pre- and post-conditioning. Time is shown along the abscissa. Red areas represent ischaemia, yellow areas represent normal perfusion. It should be noted that the cycles of ischaemia for postconditioning are generally shorter than those employed in preconditioning protocols. Postconditioning also requires to be employed in the first minutes of reperfusion whereas a longer delay is tolerated between the end of preconditioning and the beginning of the index ischaemia.

1.3.1 Mechanisms of protection by preconditioning and postconditioning

Since the discovery of pre- and post-conditioning, a great deal of effort has been invested in elucidating the mechanism by which these phenomena exert their protective effects and in attempts to replicate them pharmacologically. Although the mechanisms involved have not been fully elucidated, it is believed that both strategies confer protection by acting at reperfusion to prevent MPTP opening and consequent cell death (Garcia-Dorado *et al.*, 2006; Hausenloy & Yellon, 2003; Hausenloy & Yellon, 2007b; Lim *et al.*, 2007a).

Studies from Gross' laboratory demonstrated that the protection of preconditioning in dogs could be blocked by glibenclamide, and thus the importance of K_{ATP} channels in preconditioning was realised (Gross & Auchampach, 1992). It was later realised that the channel involved was the mitochondrial K_{ATP} channel (mK_{ATP}) rather than its sarcolemmal equivalent (Garlid *et al.*, 1997; Liu *et al.*, 1998; Sato *et al.*, 2000). For some time it was thought that the mK_{ATP} was the end-effector of preconditioning, however this was later questioned (Oldenburg *et al.*, 2002) and prevention of MPTP opening is now believed to hold that role (Garcia-Dorado *et al.*, 2006; Hausenloy *et al.*, 2003; Hausenloy *et al.*, 2007b; Lim *et al.*, 2007a). It is believed that mK_{ATP} opening causes the release of reactive oxygen species from the mitochondria into the cytoplasm (Andrukhiv *et al.*, 2006) which activate Reperfusion Injury Salvage Kinase signalling pathways (see below) which exert their protective effects at reperfusion, although they also play a role in the conditioning (trigger) phase of preconditioning.

Pre- and post-conditioning both prevent MPTP opening by the activation of RISK pathways at the time of reperfusion (Hausenloy & Yellon, 2004). These pathways consist of the Akt pathway and the Erk 1/2 pathways (Hausenloy *et al.*, 2003; Lecour *et al.*, 2005; Solenkova *et al.*, 2006). RISK pathways are the mediators between the stimuli for preconditioning and postconditioning, and the end effector (currently thought to be prevention of MPTP opening). It would appear that evolution has furnished the cell with manifold ways of achieving protection such that there are a number of different mechanisms capable of activating the pathways and that redundancies exist such that cardioprotection may be achieved by numerous means.

Early studies demonstrated that autacoids such as adenosine (Liu *et al.*, 1991), catecholamines (Banerjee *et al.*, 1993) and bradykinin (Goto *et al.*, 1995) are released from the cell during the preconditioning ischaemia and that these trigger protection. These activate signalling pathways which converge on the mK_{ATP} channel. Numerous G-protein coupled receptors can activate protective signalling pathways, however the important steps appear to be the activation of PI3-K/Akt which results in the production of nitric oxide from nitric oxide synthase which in turn activates protein kinase G (PKG) via guanylyl cyclase. PKG activates an isoforms of protein kinase C (PKCε) which is thought to activate the mK_{ATP} channel (Jaburek *et al.*, 2006), and thus lead to cardioprotection. The mechanism by which activation of PKC before the index ischemia leads to protection at reperfusion is a matter of intense research. One well supported hypothesis is that upon reperfusion PKC interacts with the adenosine A_{2B} signalling cascade, making it more sensitive to endogenous adenosine and leading to RISK pathway activation (Cohen & Downey, 2008) (Figure 1-3).

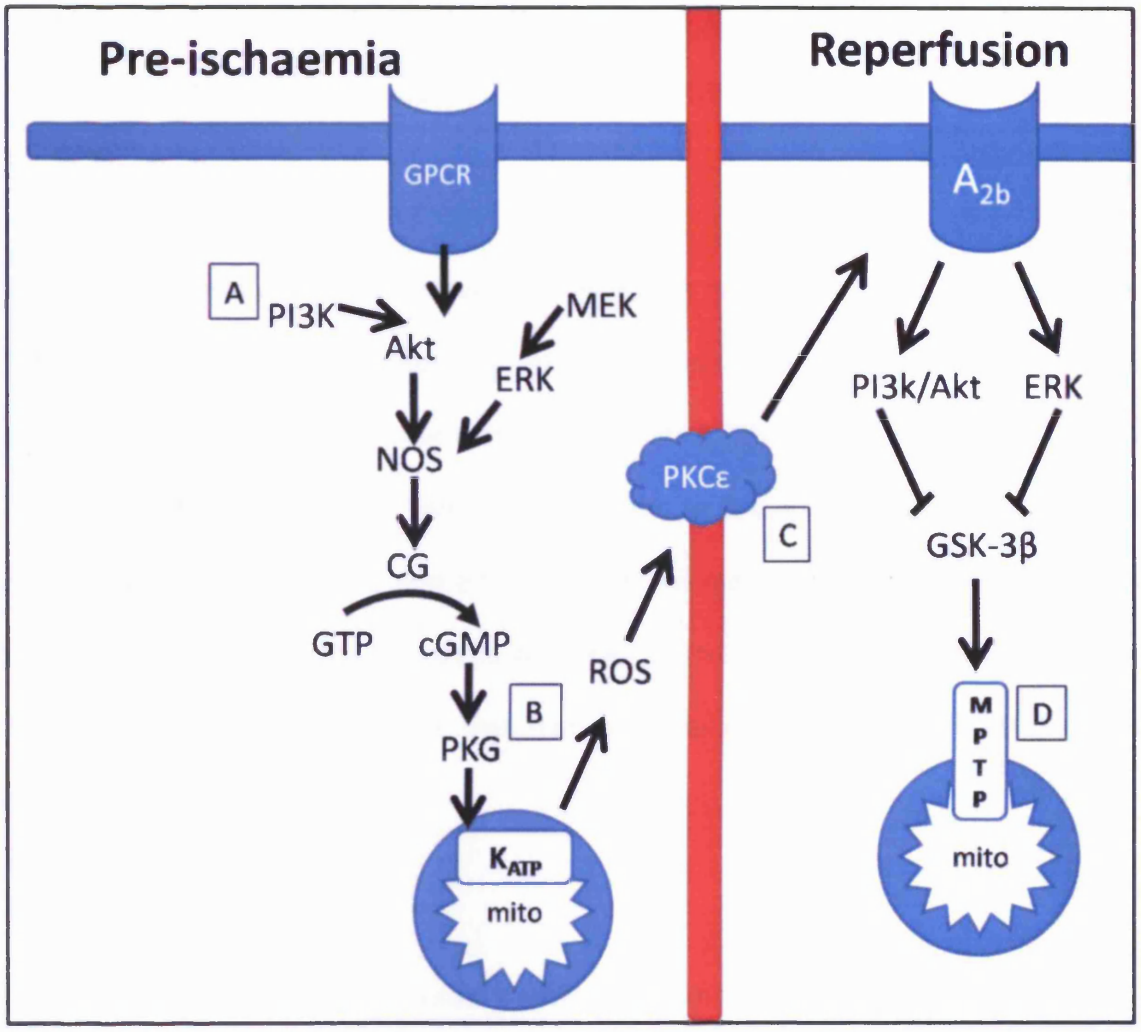

Figure 1-3 Mechanisms of preconditioning. A) Autacoids released during ischaemia activate G-protein coupled receptors which activate PI3K-Akt and Mek-Erk signalling and eventual activation of protein kinase G (PKG). B) This results in the opening of the mitochondrial K_{ATP} channel and the release of reactive oxygen species (ROS). ROS activate protein kinase C (PKC). C) PKC activates Reperfusion Injury Salvage Kinase (RISK) pathways at reperfusion via the adenosine A_{2b} receptor leading to the prevention of mitochondrial permeability transition pore (MPTP) opening (D). This diagram draws from ideas presented in numerous sources (see text) and in particular from Cohen and Downey (2008). NOS, nitric oxide synthase; GSK-3β glycogen synthase kinase 3β.

Elucidating the mechanism by which postconditioning exerts protection is in some ways less problematic that preconditioning, because postconditioning does not require a 'memory'. In order to achieve protection, RISK pathways must be activated at reperfusion. It appears that

adenosine may be responsible for this effect and that postconditioning delays washout of adenosine from the extracellular space (Kin *et al.*, 2005; Philipp *et al.*, 2006).

Adenosine is a purine nucleoside with widespread actions throughout the body (Drury & Szent-Gyorgyi, 1929). Perhaps more than any other endogenous mediator, adenosine has been implicated in the mechanism of preconditioning (Liu *et al.*, 1991) and postconditioning (Kin *et al.*, 2005). Adenosine is a vasodilator and is involved in the normal regulation of coronary vascular tone. It is released in response to increased myocardial oxygen requirements (Berne *et al.*, 1983; Foley *et al.*, 1978). Adenosine is formed in the heart from ATP during ischaemia (Meghji *et al.*, 1988). Thus it can be seen that there are some parallels with adenosine and catecholamines during ischaemia.

1.3.2 Phenomena related to preconditioning and postconditioning

1.3.2.1 Second window of preconditioning

The protective effects of preconditioning are transient and are lost in most studies within two hours after the preconditioning ischaemia. However protection against is seen once again at around 24 hours after reperfusion. This phenomenon is called the second window of preconditioning (Kuzuya *et al.*, 1993; Marber *et al.*, 1993) and involves de novo synthesis of cardioprotective proteins within the cardiomyocyte (Das & Maulik, 2006).

1.3.2.2 Graded reperfusion

For some time it has been known that the consequences of reperfusion can be minimised by restoring blood-flow to ischaemic tissues in a graded manner (Okamoto *et al.*, 1986). This phenomenon is known as graded reperfusion or stepped reperfusion and has been demonstrated to reduce infarct size in dogs (Sato *et al.*, 1997). Whether the protective mechanisms involved in graded reperfusion are the same as those of postconditioning is not yet known.

1.3.2.3 Remote preconditioning and postconditioning

Remote preconditioning is the phenomenon whereby preconditioning of one organ or region of tissue can afford protection in another. The phenomenon was first described after it was discovered that preconditioning occlusions of dog circumflex artery protected the heart against ischaemia induced by occlusion of the left anterior descending artery (Przyklenk *et al.*, 1993). Later it was discovered that ischaemic myocardial energy metabolism could be improved by prior renal ischaemia in rabbits (Takaoka *et al.*, 1999) and that a similar technique in rats could reduce infarct size (Weinbrenner *et al.*, 2002). Remote ischemic postconditioning has been achieved in pigs by inflation of a blood-pressure cuff over a limb at reperfusion. This led to a reduction in infarct size, assessed by creatinine kinase release (Andreka *et al.*, 2007). This technique offers exciting possibilities for eliciting cardioprotection in a non-invasive manner. Remote ischaemic preconditioning has been demonstrated to be effective in humans, blood pressure cuff inflation over a limb, prior to coronary artery bypass graft, led to reduced troponin-T release in treated patients compared to controls (Hausenloy *et al.*, 2007a). Recently,

remote postconditioning against endothelial dysfunction in the forearm has also been demonstrated (Loukogeorgakis *et al.*, 2007).

1.3.2.4 Effects of disease states on preconditioning and postconditioning

Most laboratory experiments are carried out in healthy animals, whereas preconditioning and postconditioning are likely to be used clinically in a population with pre-existing pathologies. Emerging evidence suggests that the protection afforded by preconditioning may be altered under these conditions. For example, three cycles of ischaemia and reperfusion are required to elicit protection in perfused hearts from diabetic hearts whereas two cycles are protective in hearts from healthy animals (Tsang *et al.*, 2005). Other studies have demonstrated that the protective effects of preconditioning are lost in hearts taken from aged rats (18-20 months old) (Schulman *et al.*, 2001) and hypertensive rats (Ebrahim *et al.*, 2007b). However, in hearts taken from rats with DOCA-salt hypertensive left ventricular hypertrophy, infarct reduction by bradykinin was attenuated, however the protective effects of ischaemic preconditioning were unaffected (Ebrahim *et al.*, 2007a). There have been few studies to date investigating the effects of disease states upon postconditioning, however the available evidence has been collated in a comprehensive review dealing with the interaction of cardiovascular risk factors with ischaemia-reperfusion injury, preconditioning and postconditioning (Ferdinandy *et al.*, 2007). An understanding of how disease processes affect pre-and postconditioning is essential to the successful translation of these strategies to the clinic.

1.3.2.5 Pharmacological preconditioning and postconditioning

Cardioprotection by preconditioning and postconditioning are both limited by the fact that induction of ischaemia is invasive and potentially dangerous. Thus many research groups have spent time searching for pharmacological mimetics of these phenomena. A number of pharmacological mimetics of preconditioning have been found, including adenosine (Downey *et al.*, 1993), nitric oxide (Cohen *et al.*, 2006) and anaesthetics (Cason *et al.*, 1997). Clinically a pharmacological mimetic of postconditioning would be extremely useful and cyclosporine (Piot *et al.*, 2008), adenosine A_3 receptor agonists (Gardner *et al.*, 2004) and recently B-type natriuretic peptide (Burley & Baxter, 2007) have shown promise in this regard.

Current understanding of the mechanisms of cardioprotection would predict a number of ways in which drugs could mimic pre- and post-conditioning. This could be achieved by activation of RISK pathways, opening of the mK_{ATP} channel, or direct inhibition of the MPTP

This thesis will investigate the possibility that agonists of β-adrenoceptors may be able to mimic myocardial pre- and post-conditioning. Current evidence for pharmacological preconditioning by β-adrenoceptors is described in Section 1.5 below.

1.3.2.6 Late pharmacological postconditioning

On the basis of studies investigating protocols and mechanisms of postconditioning, it has been suggested that protective strategies must be employed very early in reperfusion to be protective (Piper *et al.*, 2004). However, there is also evidence that reperfusion injury develops

in a wave-front phenomenon over a period of several hours, similar to ischaemic damage (Zhao *et al.*, 2000). This progressive damage presents a therapeutic target. Administration of TG100-115, an inhibitor of pro-inflammatory PI3K isoforms (PI3K γ and PI3K δ), reduced infarct size, even when administered 3 hours after reperfusion (Doukas *et al.*, 2006).

1.4 Cardiac β-adrenoceptors

Ahlquist first proposed the existence of α- and β-adrenoceptors in the 1940s to account for the various effects of adrenaline-related compounds in different tissues (Ahlquist, 1948). Lands and colleagues went on to suggest subpopulations of β-adrenoceptors. They described β_1-adrenoceptors as being responsible for lipolysis and cardiostimulation and β_2-adrenoceptors as mediating bronchodilatation and vasodilatation (Lands *et al.*, 1967). Today, the structures of nine adrenoceptors are known, the chromosomal locations of their genes have been mapped, and a great deal is known about their function. The predominant adrenoceptor found in the human heart is β_1, although β_2- adrenoceptors are present to a lesser extent and β_3-adrenoceptors are found in very small numbers. Whilst the total density of β-adrenoceptors is homogenous throughout the organ, there exist regional differences in the ratio of β_1- and β_2-subtypes. In human atria, the β_1/β_2 ratio is 7:3 whereas in the ventricles it is 4:1 (Brodde *et al.*, 2006; Brodde & Michel, 1999).

Based on empirical observations which did not fit the accepted pattern of $\beta_1/\beta_2/\beta_3$ pharmacology, the existence of a β_4 adrenoceptor was proposed for some time. It was shown that CGP 12177, a β_1/β_2 non-selective antagonist, at high concentrations, caused positive

inotropic effects in isolated left atria. This effect was not susceptible to propranolol and was not abolished in β_3 knockout mice hence a new receptor subtype was proposed (Kaumann & Molenaar, 1997; Kaumann *et al.*, 1998). However, this idea has lost favour since it was demonstrated that putative β_4 responses were abolished in β_1 knockout mice, suggesting that responses ascribed to β_4-adrenoceptors occurred by ligands interacting with an alternative binding site on the β_1-adrenoceptor (Kaumann *et al.*, 2001; Konkar *et al.*, 2000).

Because rats will be used for all the experiments in this study, it is important to understand the similarities and difference between β-adrenoceptors in the model species, and in man. There is 89% amino-acid sequence homology between the rat β_1-adrenoceptor and the human β_1-adrenoceptor. The sequence identity is most similar for β_2 at 93%. The β_3 receptor is the most different between the species with only 88% homology (Hieble, 2000).

1.4.1 β-adrenoceptor signalling

Adrenoceptors belong to the family of rhodopsin-like (Family A) seven transmembrane membrane domain receptors (Bylund *et al.*, 1994). The β_2-adrenoceptor was the first such receptor to be cloned after bacteriorhodopsin (Dixon *et al.*, 1986). β-adrenoceptors have between 408 and 477 amino acids with an extracellular N-terminus and an intracellular C-terminus (Table 1-1) (Bylund *et al.*, 1994; International Union of Basic and Clinical Pharmacology, 2008).

Table 1-1 Characteristics of human β-adrenoceptors (International Union of Basic and Clinical Pharmacology, 2008)

	Number of amino acids	Chromosome location	Gene name
β_1-adrenoceptor	477	10q24-q26	ADR B1
β_2-adrenoceptor	413	5q31-q32	ADR B2
β_3-adrenoceptor	408	8p12-p11.2	ADR B4

The transmembrane alpha helices group together and form a pocket into which ligands bind (Tota & Strader, 1990). The 7-transmembrane coupled receptors couple to heterotrimeric guanine nucleotides binding proteins (referred to as 'G-proteins) through which they activate intracellular signalling. Gilman and Rodbell were awarded the 1989 Nobel Prize in Physiology of Medicine "*for their discovery of G-proteins and the role of these proteins in signal transduction in cells*". When the receptor is inactive, the G-protein binds guanosine diphosphate (GDP). Upon agonist binding GDP is exchanged for GTP and the G-protein dissociates into α and βγ subunits which mediate cellular effects, particularly via modulation of adenylyl cyclase. Signalling is terminated by the intrinsic GTPase activity of the G-protein which converts GTP back to GDP. (Gilman, 1994; Rodbell, 1994).

Numerous varieties of G-protein exist and they are classified by their α subunits (Table 1-2).

Table 1-2 G protein families and their cellular effects (Goldsmith & Dhanasekaran, 2007; Rodbell, 1994).

G protein family	Cellular effects
G_s	Increased activity of adenylyl cyclase
G_i	Decreased activity of adenylyl cyclase
G_q	Increased activity of phospholipase C
G_{12}	Increased activity of mitogen activated protein (MAP) kinases

Classic β-adrenoceptor signalling is via G_s. This was the first G-protein to be discovered and was designated 's' because the α-subunit stimulates adenylyl cyclase as opposed to G_i which inhibits adenylyl cyclase (Rodbell, 1994). Adenylyl cyclase functions to increase the production of cAMP which in turn activates the cAMP-dependent protein kinase, protein kinase A (PKA). This effect can be mimicked pharmacologically by forskolin, a direct activator of adenylyl cyclase (Figure 1-4).

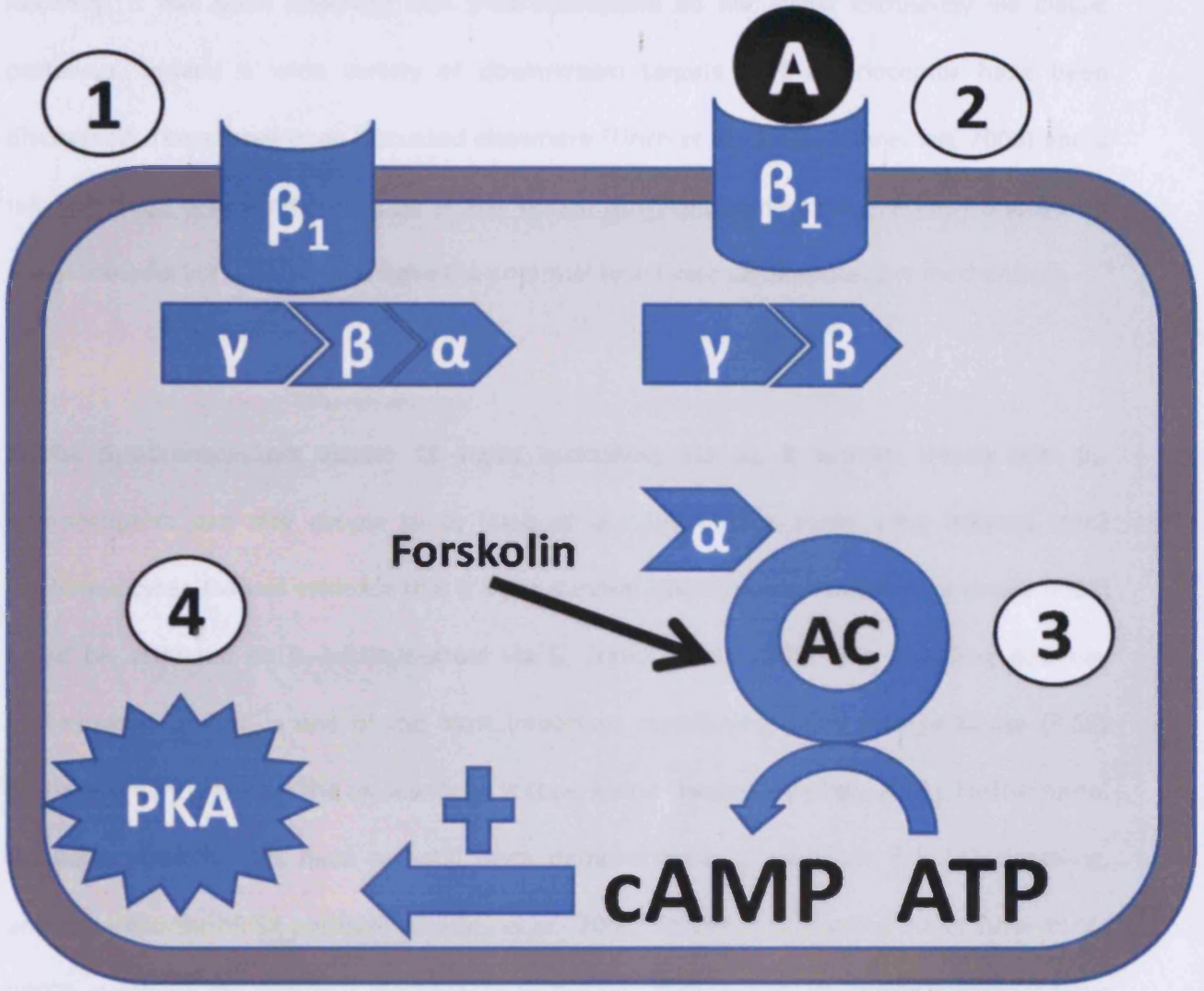

Figure 1-4 β-adrenoceptor signalling via adenylyl cyclase and protein kinase A. 1) In the absence of agonist, the Agonist binding to the β_1-adrenoceptor-G-protein complex causes a conformational change leading to the dissociation of the α and the βγ subunits. The α-subunit activates the enzyme adenylyl cyclase (AC) which catalyses the production of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). Protein Kinase A is activated by cAMP and has numerous downstream cellular effects. Forskolin is an experimental compound which activates AC directly.

Activation of protein kinase A has widespread actions throughout the heart including positive inotropic (increased force of contraction), lusitropic (increased rate of relaxation), dromotropic (increased excitability) and bathmotropic (increased conductivity) actions (Brodde *et al.*, 1999; Michel & Insel, 2006).

Recently, it has been observed that β-adrenoceptors do not signal exclusively via classic pathways. Indeed a wide variety of downstream targets of β-adrenoceptor have been discovered. These have been discussed elsewhere (Finch *et al.*, 2006; Minneman, 2006) and a full discussion is beyond the scope of this thesis. However, some of the recently discovered signal transduction mechanisms have the potential to activate cardioprotective mechanisms.

Whilst β_1-adrenoceptors appear to signal exclusively via G_s, it is now known that β_2-adrenoceptors can also couple to G_i (Xiao *et al.*, 1995). One study using isolated H9c2 cardiomyocytes showed evidence that the pro-survival kinase, phosphoinositide 3-kinase (PI3K) could be activated by β_2-adrenoceptors via G_i (Yano *et al.*, 2007). The signalling pathway downstream of PI3K is one of the most important reperfusion injury salvage kinase (RISK) pathways, which protect the myocardium at reperfusion (Hausenloy *et al.*, 2005). Furthermore, β-adrenoceptor ligands have recently been demonstrated to modulate Erk 1/2 signalling, another important RISK pathway (Chesley *et al.*, 2000; Galandrin & Bouvier, 2006; Tutor *et al.*, 2007). However, the picture is complicated by the fact that agonists for classic β-adrenoceptor signalling do not necessarily act as activators of Erk 1/2 signalling, in fact they may possess properties of a neutral antagonist or an inverse agonist (Ciccarelli *et al.*, 2007; Galandrin *et al.*, 2006). To date, only a very small number of β-adrenoceptor agonists have been investigated for their effects on Erk 1/2 signalling so this represents an emerging field.

1.4.2 β-adrenoceptor polymorphisms

A number of genetic polymorphisms occur for each subtype of adrenoceptor, some have been suggested to be of potential functional importance. Much of this work in this field was carried out in the laboratory of the late Otto Brodde. Mutations with likely functional consequences are summarised below (Table 1-3).

Table 1-3 Phenotypic effects of β-adrenoceptor polymorphisms. Data is a summary of that presented by Brodde (Leineweber *et al.*, 2004)

Receptor	Mutation	Comments
β_1	Ser49Gly	Gly49 leads to greater binding affinity of agonists, larger maximal increase in adenylyl cyclase by isoprenaline and greater agonist-induced receptor downregulation in HEK293 cells (Levin *et al.*, 2002)
β_1	Gly389Arg	Arg389 results in greater hypotensive effect of β-adrenoceptor antagonists *in* vivo (Liu *et al.*, 2003; Sofowora *et al.*, 2003)
β_2	Arg16Gly	Gly16 results in lower responsiveness of cardiac muscle and vascular smooth muscle to salbutamol *in vivo* (Gratze *et al.*, 1999)
β_2	Gln27Glu	Glu27 causes raised peripheral vasodilator responses to isoprenaline *in vivo* (Cockcroft *et al.*, 2000; Dishy *et al.*, 2001).
β_2	Thr164Ile	Ile164 leads to a blunted inotropic and chronotropic responses to terbutaline *in vivo* (Brodde *et al.*, 2001).
β_3	Trp16Arg	Arg16 results in lower basal autonomic nervous system and increased autonomic response upon standing *in vivo* (Shihara *et al.*, 1999; Shihara *et al.*, 2001).

Table 1-4 : Binding affinities and adenylyl cyclase responses (% isoprenaline response) for a range of ligands at human β-adrenoceptors expressed in Chinese hamster ovary cells. All data are taken from Hoffmann (2004) except that marked with * which was taken from (Baker, 2005).

Compound	β_1-adrenoceptor		β_2-adrenoceptor		β_3-adrenoceptor	
	Ki (nM)	Efficacy	Ki (nM)	Efficacy	Ki (nM)	Efficacy
Noradrenaline	3,570.0	123.0	26,400.0	103.0	4,300.0	122
Adrenaline	3,970.0	133.0	735.0	110.0	126,000.0	106
Isoprenaline	224.0	100.0	458.0	100.0	1,570.0	100
Fenoterol	13,600.0	66.0	719.0	76.0	55,700.0	110
Salbutamol	2,440.0	2.0	2,170.0	33.0	53,700.0	87
Salmeterol	1,600.0	-33.0	24.6	44.0	7,180.0	-13
Formoterol	1,710.0	58.0	2,570.0	?	8,090.0	139
Terbutaline	31,300.0	6.0	15,400.0	41.0	79,800.0	45
BRL-37344	37,900.0	-5.0	9,170.0	-7.0	430.0	28
Alprenolol	5.8	-24.0	1.2	?	35.0	11
Pindolol	2.6	-25.0	4.8	-34.0	44.1	13
Carvedilol	1.7	-28.0	1.1	-30.0	247.0	2
Atenolol	388.0	-23.0	8,140.0	-26.0	65,100.0	7
Bisoprolol	22.4	-33.0	1,150.0	-30.0	9,070.0	-1
Metoprolol	47.0	-24.0	2,960.0	-34.0	10,100.0	2
S-Propranolol	1.8	-35.0	0.8	-35.0	186.0	-4
CGP-20712A	4.7	-25.0	4,040.0	-30.0	2,360.0	-20
SR-59230A	16.4	-19.0	61.9	?	122.0	5
CGP-12177	4.5	-19.0	4.3	-32.0	77.1	36
ICI-118,551	49.5	-22.0	0.7	-32.0	611.0	-30
Broxaterol	1,310.0	-11.0	1,290.0	20.0	3,990.0	42
Timolol*	5.4	?	0.2	?	158.4	?

1.4.3 Effects of ischaemia on cardiac β-adrenoceptors

The effects of ischaemia on adrenoceptor density are complex. Widely varying results have been reported by different laboratories (albeit in different experimental systems). It is difficult to draw conclusions from such a wide variety of models, however there appears to be a trend towards reduction of β_1 adrenoceptor density during ischaemia in cell culture, and the opposite effect in isolated organs and in vivo, although this is not true for all studies.

Table 1-5 A summary of published studies investigating the effects of ischaemia on cardiac β-adrenoceptors.

Species	Model	Ischaemia/ hypoxia length (min)	Effects of Ischaemia on β-adrenoceptors	Investigators
HEK293T	Cell culture /simulated ischaemia	30	Reduction of surface β_1 assessed by radioligand binding	(Iwatsubo *et al.*, 2003)
Dog	Coronary ligation *in vivo*	60	Increased levels of β-adrenoceptors assessed by radioligand binding	(Mukherjee *et al.*, 1982)

Species	Model	Ischaemia/ hypoxia length (min)	Effects of Ischaemia on β-adrenoceptors	Investigators
Rat	Langendorff Global ischaemia	15	Increased levels of β-adrenoceptor in cell membrane (radioligand binding). Increased sensitivity of adenylyl cyclase system to forskolin	(Strasser *et al.*, 1990)
		50	Further increase in β-adrenoceptor levels but decreased adenylyl cyclase activity	
Dog	Coronary artery ligation *in vivo*	60	Increased in β-adrenoceptor density(radioligand binding) decreased adenylyl cyclase activity	(Vatner *et al.*, 1988)
Dog	Coronary artery ligation *in vivo*	60 120	No change in β-adrenoceptor density at either time-point. Both led to decline in adenylyl cyclase activity	(Karliner *et al.*, 1989)

Species	Model	Ischaemia/ hypoxia length (min)	Effects of Ischaemia on β-adrenoceptors	Investigators
Chick	Embryonic ventricular cells exposed to hypoxia	120	Decreased surface β-adrenoceptor density	(Marsh & Sweeney, 1989)
Rat	Neonatal ventricular myocytes exposed to hypoxia	120	Decreased cytosolic β-adrenoceptor levels. Increased cytosolic β-adrenoceptor levels	(Rocha-Singh *et al.*, 1991)
Rabbit	Coronary ligation in vivo	10,20,60	Reduction in surface β-adrenoceptor levels at all time-points.	(Iwase *et al.*, 1993)

1.4.4 Cardiovascular clinical uses of drugs acting upon β-adrenoceptors

Adrenoceptors are present throughout the body and respond to circulating adrenaline and sympathetic nerve stimulation by noradrenaline. Because of their many functions in different organs, adrenoceptors have become a target for drug development in several conditions affecting diverse organs. These have been extensively reviewed elsewhere (Michel *et al.*, 2006).

James Black (now Sir James) working at I.C.I. in the 1960s developed the first selective β-adrenoceptor antagonists with the rationale that by reducing the workload of the heart, the severity of ischaemic attacks could be reduced in patients with angina pectoris (Black, 1988; Black *et al.*, 1964). The antihypertensive effects of these agents were observed later (Black, 1988; Black & Stephenson, 1962; Prichard, 1964; Prichard *et al.*, 1963; Prichard & Gillam, 1969). Initially β-adrenoceptor antagonists were thought to be contraindicated in heart failure because of their negative inotropic effects, and β-adrenoceptor agonists were used acutely in the treatment of heart failure. However, it is now realised that heart failure is associated with adrenergic overload and β-adrenoceptor desensitisation and is susceptible to treatment with β-adrenoceptor antagonists (Bond, 2001; Bond *et al.*, 2007; Waagstein *et al.*, 1975).

Adrenaline and adrenoceptor agonists are used clinically to mimic the 'fight or flight' (Cannon, 1929; Cannon, 1914) actions of this hormone. Thus adrenaline is given by the intramuscular route to patients suffering acute anaphylactic shock. The rationale is to reverse the bronchospasm and hypotension associated with this condition (Mehta, 2006). In an example of adrenoceptor ligand use from outside the cardiovascular system, selective β_2-adrenoceptor agonists are used for their bronchodilator effects in asthmatics (Mehta, 2006). Similarly, during cardiac arrest, intravenous adrenaline is administered in order to cause peripheral vasoconstriction (an α-adrenoceptor mediated effect) and direct any cardiac output generated by chest cardiopulmonary resuscitation to the heart and brain. There is very little evidence for the effectiveness of this approach, indeed no clinical trial has shown adrenaline to be more effective than placebo leading to the suggestion that a non-selective agent such as adrenaline exerts detrimental effects upon the heart at reperfusion which outweigh the benefits achieved by vasoconstriction (Penson *et al.*, 2007). This issue is considered in greater detail in Chapter 4.

1.5 Adrenoceptors and cardioprotection

Preconditioning has been demonstrated by short periods of ischaemia (Murry *et al.*, 1986) alterations in temperature (Khaliulin *et al.*, 2007), rapid pacing of the heart (Vegh *et al.*, 1991) and stretching of myocardial fibres (Gysembergh *et al.*, 1998). These stimuli have in common that they are all stressful to cardiac cells but do not result in irreversible damage when applied for short periods of time. It is likely that these stimuli result in the release of an endogenous mediator which activates a protective signalling cascade. Catecholamines are released from the adrenal medulla in response to stress (Cannon, 1929; Cannon, 1914) and are released locally in the myocardium in response to ischaemia (Kuroko *et al.*, 2007; Lameris *et al.*, 2000) and are therefore potential mediators of preconditioning (Broadley & Penson, 2004).

1.5.1 β-adrenoceptor mediated preconditioning

Several groups have demonstrated that a preconditioning-like adapted state can be induced by transient treatment with an agonist of the β-adrenoceptor (Lange *et al.*, 2006; Lochner *et al.*, 1999; Marais *et al.*, 2001; Moolman *et al.*, 2006b; Moolman *et al.*, 2006c; Nasa *et al.*, 1997; Robinet *et al.*, 2005; Tong *et al.*, 2005; Yabe *et al.*, 1998; Yates *et al.*, 2003) (Table 1-6).

Table 1-6 Receptors and signalling pathways implicated in mediating preconditioning by β-adrenoceptor agonists.

Species	Model	Endpoint	Receptor	Mediators	Investigators
Mouse	Langendorff	LVDP ↑ Infarct Size ↓	β_2	Switch from G_s to G_i signalling	(Tong *et al.*, 2005)
Rat	Langendorff	MCF LVEDP ↑ RPP ↑ CK release ↓	β_1	PI3-K, PKC, PKA,	(Robinet *et al.*, 2005)
Rat	Langendorff	LVDP ↑ CK release ↓	?	?	(Nasa *et al.*, 1997)
Rat	Langendorff	RPP ↑ CK release ↓	$\beta_1>\beta_2$	?	(Frances *et al.*, 2003)
Rat	Langendorff	Apoptosis ↓ Infarct Size ↓	?	P38 MAPK	(Moolman *et al.*, 2006a)
Rat	Langendorff	Infarct Size ↓	?	Adenosine A_3 receptor PI 3-K	(Moolman *et al.*, 2006c)

Species	Model	Endpoint	Receptor	Mediators	Investigators
Rat	Working Heart	Aortic output ↑	?	PKA	(Lochner *et al.*, 1999)
Rat	Working Heart	Cardiac Output ↑	?	P38 MAPK	(Marais *et al.*, 2001)
Guinea-pig	Isolated atria.	↓Stunning	?	?	(Yates *et al.*, 2003)

Table 1-7 Evidence for β-adrenoceptor involvement in ischaemic preconditioning

Species	Model	Endpoint	Conclusion	Investigators
Rabbit	*In vivo*	↓Infarct Size	No β-adrenoceptor involvement	(Iliodromitis *et al.*, 2004)
Rabbit	Langendorff	↓Infarct Size	β_1-adrenoceptor required for preconditioning	(Spear *et al.*, 2007)
Rat	Langendorff	RPP ↑	Endogenous catecholamines not necessary for preconditioning	(Frances *et al.*, 2003)
Mouse	Langendorff	↓Infarct Size RPP ↑	Preconditioning abolished in β_2 knockouts.	(Tong *et al.*, 2005)

Catecholamine release and subsequent activation of β-adrenoceptor has also been implicated in the mechanism of cardioprotection achieved by administration of opioids (β_2) (Huang *et al.*, 2007) and anaesthetics (β_1) (Lange *et al.*, 2006).

Controversy remains as to whether activation of β-adrenoceptors is an essential part of ischaemic preconditioning, or whether there are redundancies in the pathway which leads to protection – that is to say that preconditioning could be activated by β-adrenoceptors, but equally well by unrelated receptors (Table 1-7). This is an important question in the clinic, where β-adrenoceptor antagonists are administered for the treatment of angina, hypertension

and heart failure (Mehta, 2006). It is possible that minor 'silent' periods of ischaemia could induce preconditioning, and blockade of this protection could result in more serious consequences during longer ischaemic episodes such as occur during myocardial infarction.

In rabbits in vivo it has been demonstrated that blockade of β_1-adrenoceptors by atenolol or esmolol had no effect on the infarct-size limitation afforded by ischaemic preconditioning (Iliodromitis *et al.*, 2004). However, a study in Langendorff perfused rabbit hearts showed protection of ischaemic preconditioning against necrosis was blocked by the β_1-adrenoceptor antagonist CGP-20712A (Spear *et al.*, 2007). In a rat Langendorff model of ischaemic preconditioning, depletion of endogenous catecholamines by reserpine did not affect the improved functional recovery in hearts that had undergone ischaemic preconditioning (Frances *et al.*, 2003). In a mouse Langendorff heart model, preconditioning protection (improved functional recovery and reduced infarct size) were abolished in knockout animals lacking the β_2-adrenoceptor (Tong *et al.*, 2005). It would seem likely that the reliance of ischaemic preconditioning on β-adrenoceptor activation varies between species and indeed different models, and the varying conditions and endpoints used in studies account for the apparently contradictory results observed.

1.5.2 Mechanism of protection by β-adrenoceptors

1.5.2.1 Induction of demand ischaemia

It is possible that positive inotropes such as isoprenaline could result in preconditioning by causing the energy demands of the tissue to exceed the capacity of diffusion to supply the substrates, and remove the waste products of metabolism. Indeed it has been noted that rapid pacing of the heart can mimic preconditioning by inducing ischaemia (Vegh *et al.*, 1991).

1.5.2.2 Activation of RISK pathways

There is a large body of evidence, from in-vivo and Langendorff isolated heart experiments, that activation of protein kinases cascades as a result of β-adrenoceptor activation results in the heart undergoing adaptive changes and becoming resistant to later ischaemic damage. Protein Kinase A (PKA) has been often implicated in the preconditioning achieved by β-adrenoceptor agonists (Table 1-6), this is to be expected, as PKA is also an essential mediator in classic β-adrenoceptor signalling leading to positive inotropic and chronotropic effects of β-adrenoceptor agonists. It has been suggested that β-adrenoceptor activation could lead to PKCε activation via the βγ subunit of the G-protein directly activating PI3-K signalling. (Robinet *et al.*, 2005) thus activating RISK pathways. Other mechanisms by which β-adrenoceptors could lead to RISK activation have been discussed previously (Section 1.4.1)

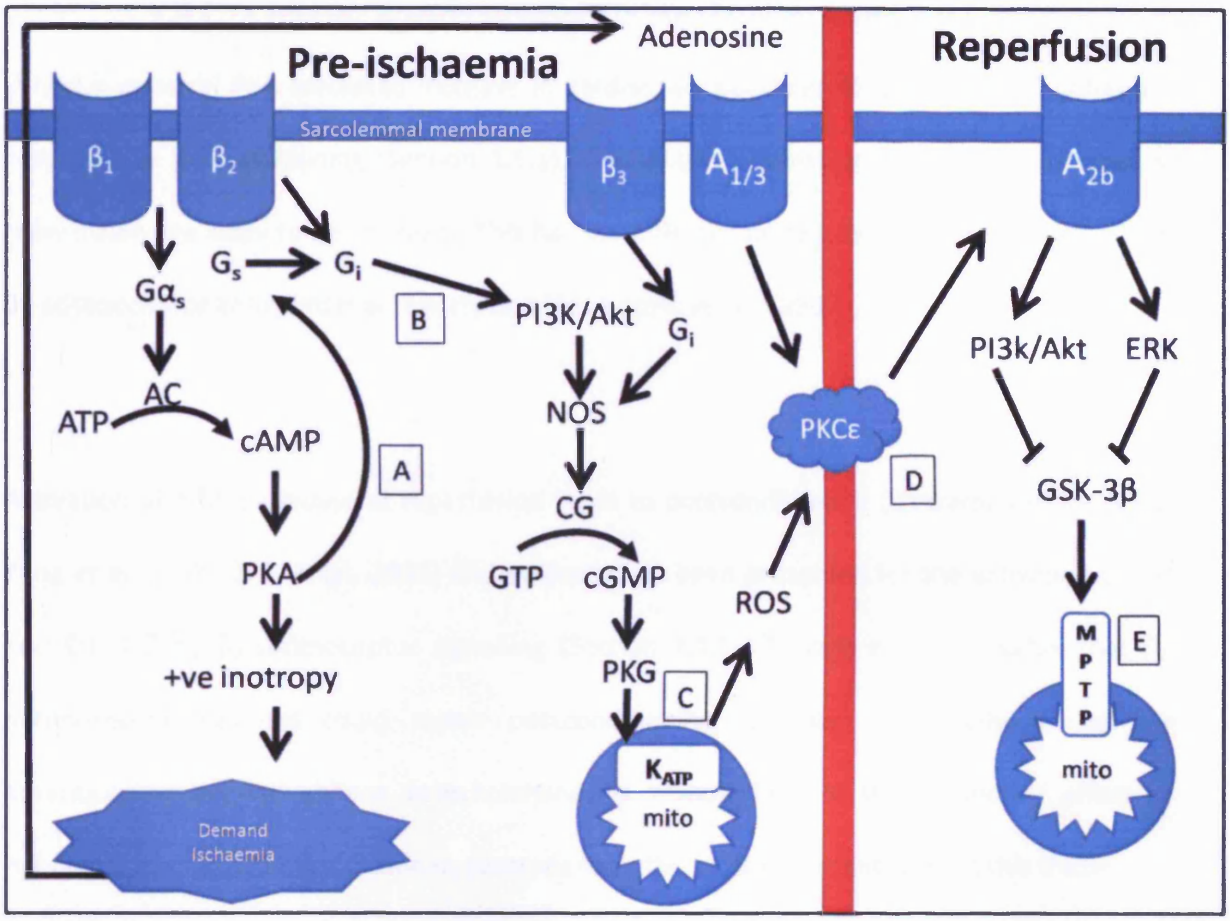

Figure 1-5 Possible mechanisms of preconditioning by β-adrenoceptor agonists. A) β_2-adrenoceptor-mediated Protein Kinase A (PKA) production causes a switch from signalling via G_s to G_i. B) G_i activates PI3K and subsequent downstream targets. C) K_{ATP} is opened causing the release of Reactive oxygen species (ROS). D) ROS activate protein kinase C (PKC). E) PKC activates Reperfusion Injury Salvage Kinase (RISK) pathways at reperfusion via the adenosine A_{2b} receptor leading to the prevention of mitochondrial permeability transition pore (MPTP) opening. This diagram draws from ideas presented in numerous sources (see text) and in particular from Cohen and Downey (2008)

It is well established that activation of β_1 adrenoceptors (and therefore activation of adenylyl cyclase) at reperfusion is detrimental because selective β_1-adrenoceptor antagonists reduce infarct size (Feuerstein *et al.*, 1998; Spear *et al.*, 2007). Thus, β-adrenoceptor mediated cardioprotection is likely to be mediated by a non G_s pathway, and possibly via G_i. These links between β-adrenoceptors and RISK pathways may predict protective effects of β_2-adrenoceptor stimulation at reperfusion.

1.5.3 A role for β-adrenoceptors in postconditioning?

Whilst a classical PKA mediated increase in cardiac workload resulting in demand ischaemia may lead to preconditioning (Section 1.5.1), mechanisms increasing myocardial workload at reperfusion are likely to be injurious. This has been demonstrated by the protective effects of β_1-adrenoceptor antagonists at reperfusion (Feuerstein *et al.*, 1998).

Activation of RISK pathways at reperfusion leads to postconditioning (Sivaraman *et al.*, 2007; Yang *et al.*, 2005; Zhu *et al.*, 2006) and evidence has been presented for the activation of Akt and Erk 1/2 by β_2-adrenoceptor signalling (Section 1.4.1). Therefore, it is possible that β_2-adrenoceptor agonists could mimic postconditioning, or even that activation of β_2-adrenoceptor by endogenous catecholamines is responsible for the protective effect of ischaemic postconditioning. These hypotheses form the most important work in this thesis.

1.6 Aims of this thesis

The overall aim of this thesis is to investigate the effects of β-adrenoceptor activation, by endogenous and exogenous agonists, before myocardial ischaemia and at reperfusion. Administration of drugs at reperfusion (i.e. postconditioning) will be the main focus because of the clinical relevance and scientific novelty.

1.7 General Hypothesis

Postconditioning, and to a lesser extent, preconditioning have remarkable potential to reduce the burden of mortality and morbidity which result from ischaemic heart disease. These phenomena exert their protective effects in the first moments of reperfusion when high concentrations of catecholamines are present in the ischaemic myocardium.

Catecholamines are able to mimic preconditioning. Nothing is known about their roles in postconditioning. By their actions on adrenoceptors, these compounds have the potential to alter the extent of reperfusion injury. Knowledge of signalling pathways downstream of β-adrenoceptors allows predictions to be made about how different subtypes may act at reperfusion. It is expected that activation of adrenoceptor subtypes which increase myocardial workload at reperfusion (β_1) will have detrimental effects on infarct size when applied at reperfusion, whereas activation of β_2 and β_3 which have the potential to activate kinases involved in RISK pathways, may have protective effects.

Chapter 2 General methods

In order to facilitate ease of reading the text, the suppliers of all drugs and chemicals are given at the end of the chapter (Table 2-1). However, the suppliers of equipment are mentioned in the text.

2.1 Choice of species and strain

Rats were chosen for these experiments for a number of reasons. The rat has been extensively used as a model of ischaemic damage and reperfusion injury. Stunning, infarction, preconditioning and postconditioning can all be demonstrated in this species. There are commercially available antibodies to the mediators of cardioprotective transduction pathways. The rat is a small species which makes handling easy and keeps costs to a minimum. Importantly, the rat is deficient in coronary collateral blood vessels (Maxwell *et al.*, 1987), making the reproducible induction of regional ischaemia possible. Furthermore, the Sprague-Dawley strain was readily available and has been extensively used by a number of research groups studying cardioprotection.

2.2 Choice of experimental techniques

The data described in this thesis have been obtained from two complementary experimental models: isolated paced cardiac tissues and isolated bicarbonate-buffer-perfused hearts prepared according to a modification of Langendorff's method (the 'Langendorff heart'). The Langendorff heart is an excellent model for studying ischaemic damage and reperfusion injury, as both functional data and infarct-size data can be obtained. However, the study of the effects

of β-adrenoceptor activation during ischaemia and reperfusion is likely to be complicated by the well documented positive inotropic and chronotropic effects of these agents and their vasodilator actions in the coronary circulation (Ahlquist, 1948). Therefore, electrically paced isolated cardiac tissues were additionally employed. This allowed the study of the direct effects of ischaemia, preconditioning, postconditioning and drug treatment on contractile function in different regions of the heart without interference from coronary vascular effects.

2.3 Animals and husbandry

These studies complied with the guidelines for the care and use of laboratory animals according to the Animals (Scientific Procedures) Act 1986. Male Sprague-Dawley rats were purchased from either Harlan (Bicester, UK) or B & K Universal (Bristol, U.K). See individual chapters for details of supplier and the weight ranges used. Rats were stored in cages containing between two and six animals. After arriving in the departmental animal facility, animals were allowed to acclimatise for at least one week before being used in experiments. Rats had *ad libitum* access to water and to food which was supplied in the form of Teklad Global 14% Protein Rodent Maintenance Diet supplied by Harlan. Temperature was maintained at 21 ± 2 °C and humidity at 55% ± 10%. Rats were exposed to 12 hour cycles of light and darkness.

2.4 Isolated atria and ventricular strips

2.4.1 Overview of technique

Isolated paced atria and ventricular tissues have been used for many years in the study of cardiac physiology and the pharmacological actions of drugs upon the heart (Blinks & Koch-

Weser, 1961; Koch-Weser & Blinks, 1962; Koch-Weser & Blinks, 1963; Perry, 1970). Isolated right atria beat spontaneously providing the sino-atrial node has not been damaged during dissection. However, non-automatic tissue preparations such as the left atria and right ventricular strips used in this study require electrical pacing. Using low voltages and punctuate stimulation (as opposed to electrical stimulation field stimulation) contraction can be achieved without significant release of noradrenaline. This was an important consideration in this study in which the effects of adrenoceptor activation were studied. The tissues are tied at one end to a bipolar platinum electrode through which stimulation is achieved. The other end of the tissue is tied to an isometric force transducer. These preparations were chosen for this investigation because they allow the study of β-adrenoceptor activation on the myocardium without the vascular effects of β-adrenoceptor ligands.

2.4.2 Animal preparation

Rats were killed by a blow to the head followed by cervical dislocation. The abdominal cavity was opened using curved scissors and lateral incisions were made on each side of the rib cage to expose the heart. The pericardium was removed using a small pair of scissors and the heart was clamped at the apex using a pair of Spencer-Wells forceps.

2.4.3 Atrial and ventricular preparations

The left atrial appendage was lifted using a pair of curved forceps and a 5-0 suture was inserted through the tip and tied off for attachment to an isometric transducer (Pioden dynamometer UF1 range ± 55g). Another suture was inserted at the atrio-ventricular junction for securing the

tissue to a bipolar platinum electrode. The atrial appendage was then cut free from the ventricle and immediately placed in ice-cold modified Krebs-Henseleit buffer (KHB) (composition (in mM): NaCl 118.4; $MgSO_4$ 1.2; KCl 4.7; $CaCl_2.6H_2O$ 2.5; KH_2PO_4 1.2; $NaHCO_3$ 25.0 and glucose 11.7) gassed with 95% O_2 in CO_2. A suture was then inserted into the apical end of the right ventricular muscle and tied off for attachment to an isometric transducer. A strip was then cut by dissecting up to the top of the ventricle where another suture was put through the tissue for attaching the tissue to an electrode. Both tissues were submerged in ice-cold KHB whilst they were being tied on to the electrodes before being transferred to a 20 ml organ bath containing KHB at 37°C gassed with 5% CO_2 in O_2. Both tissues received punctuate electrical stimulation via a bipolar platinum electrode at 2Hz at 150% of the threshold voltage required to cause contraction. Square-wave pulses lasting 50 ms were given. Stimulation by this method does not cause significant release of neurotransmitters (Koch-Weser *et al.*, 1963). The apparatus was adjusted so that there was an initial resting tension of 1g (±0.1g) on atrial preparations and 1.5g (±0.1g) on ventricles. All preparations were left for one hour to stabilise, during which time they were frequently washed.

2.4.4 Induction of simulated ischaemia

Ischaemia was simulated by replacing the 95% O_2 / 5% CO_2 with 95% N_2/CO_2 and by bathing the tissues in a modified KHB without glucose, but containing 7.0 mM choline chloride in order to keep a constant osmolarity in the absence of glucose (Carr *et al.*, 1997; Walker *et al.*, 1994; Walker *et al.*, 1995)

2.4.5 Recording and calculation of data

Absolute tension was measured via transducers as described above, the signal was amplified using a Grass model 79D EEG polygraph data recording system (Grass instrument Co. Quincy, Mass., U.S.A.), converted from analogue to digital data using a Powerlab 200 (ADInstruments, and passed to a computer (Hardware: iMac, Apple Macintosh; . Software: AD instruments Chart v5.1 sampling frequency 200 Hz).

From the recorded tension values, the following parameters were calculated throughout the experiment using the Chart software 'cyclic variables' calculation facility which allows analysis of periodic waveforms (ADInstruments, 2008). *Diastolic tension* was defined as the minimum tension during each contraction cycle. *Developed tension* was calculated by subtracting the diastolic tension from the maximum contraction in each cycle.

2.5 Langendorff heart preparations

2.5.1 Overview of technique

The isolated perfused mammalian heart was first described by Langendorff following on from the work of Cyon, Ludwig and Martin (Zimmer, 1998). The Langendorff heart can be perfused with blood or with a crystalloid buffer (as is the case in this study). The aortic arch is perfused in a retrograde fashion, such that the aorta is cannulated and perfusate flows down the aorta until it reaches the aortic valve which remains closed under the pressure of the fluid which flows through the coronary ostia and into the coronary circulation in an anterograde fashion. A fluid-filled balloon can be inserted into the left ventricle to measure ventricular pressure and give an

indication of cardiac contractile function. With careful technique, the heart can be kept viable for several hours after excision and commencement of perfusion. Drugs can be added to the perfusate to investigate their effects on the heart. Ischaemia can be induced globally, by stopping the flow of perfusate, or regionally, by occluding a coronary artery. Histological techniques can be applied to the heart after ischaemia to investigate the magnitude of infarct (Doring & Dehnert, 1987 ; Skrzypiec-Spring *et al.*, 2007; Sutherland & Hearse, 2000).

2.5.2 Set-up

Male Sprague-Dawley rats 300-450g were anesthetised with an overdose of pentobarbitone sodium (54mg per rat) given via the intraperitoneal route. At the same time heparin (10 IU per rat) was given by the same route. Lack of consciousness and pain sensitivity was confirmed by the absence of pedal withdrawal reflex. Rats were laid in a supine position. Using a large pair of scissors and a large pair of rat-tooth forceps, a central lateral incision was made from below the rib-cage to the neck, removing skin and fur. The abdominal cavity was opened just below the diaphragm using scissors, and the diaphragm was carefully cut away. The ribcage was loosened by bilateral incisions and then folded back to reveal the thoracic cavity. The heart was gently lifted, and connective tissue and blood vessels gently cut away exposing the thoracic aorta which was cut approximately 1/3 of the way down the cavity, and then gently dissected away from the cavity wall. The heart was then removed with lungs attached and immediately placed in ice-cold KHB prior to mounting of the aorta on a cannula through which pre-warmed (37°C) KHB (containing NaCl 118mM, KCl 4.7mM, KH_2PO4 1.2mM, $MgSO_4$ 1.2mM, $CaCl_2$ 2.5mM, glucose 11mM and insulin 100mU.l^{-1} and continuously bubbled with 95% O_2/CO_2) was initially pumped at 10 ml.min^{-1}. The heart was held in place temporarily by a small artery clip. The lungs

and connective tissue were carefully dissected away. A small slit was made in the pulmonary artery to allow egress and collection of perfusate. The heart was then tied firmly on to the cannula with suture and the artery clip removed. The left atrial appendage was removed and a fluid-filled latex balloon inserted into the left ventricle for the measurement of intra-ventricular pressure. A 5-0 suture was placed around the left main coronary artery, the ends of which were threaded through a small piece of tubing which could be pulled tight against the heart and clamped to occlude the vessel in a reversible manner.

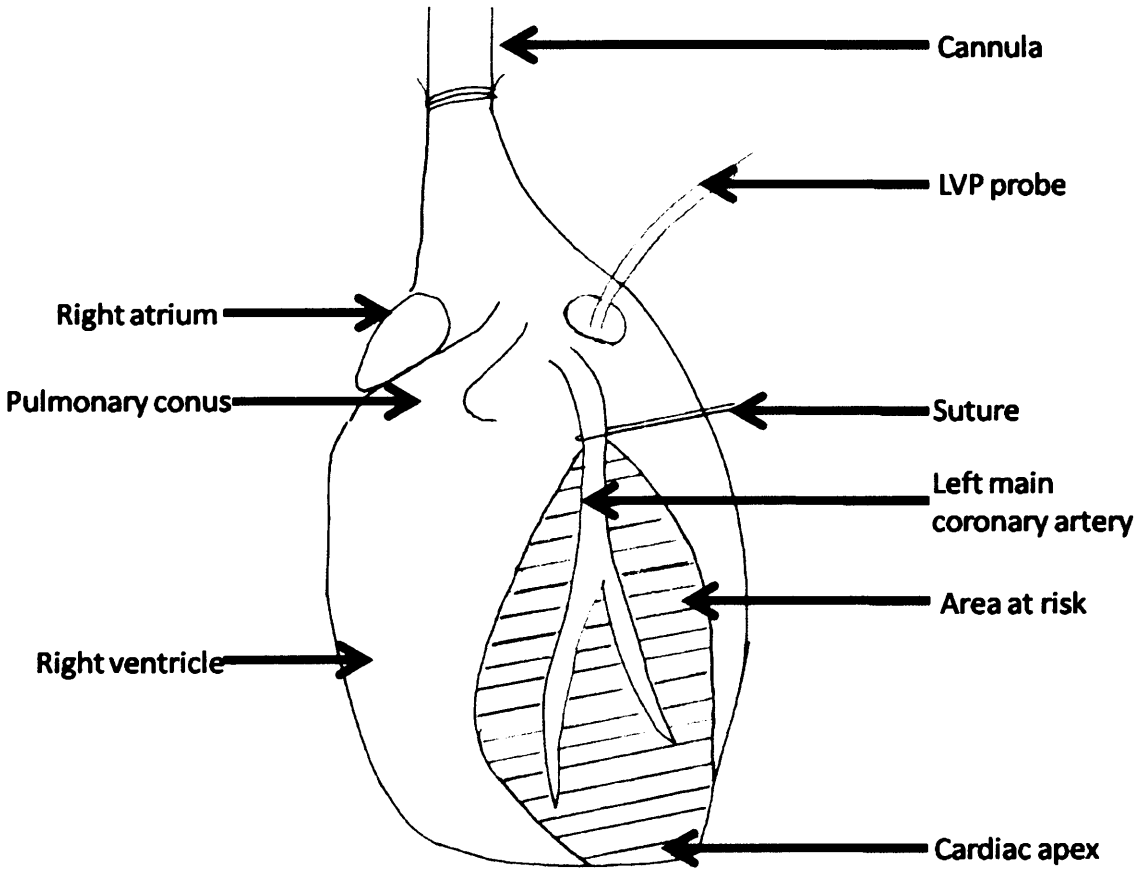

Figure 2-1 A drawing of the ventral view of the heart during regional ischaemia. A 5-0 suture was passed around the left main coronary artery. The ends of the suture were passed through a short piece of tubing and were held in place by a clamp made from two p200 pipette tips placed one inside the other with the sharp end cut away (not shown). The area at risk is shaded.

The heart was then jacketed in a small water bath containing KHB to maintain normothermic conditions throughout the experiment. Coronary perfusion pressure was measured by means of a pressure transducer attached to a side arm located directly above the cannula. The flow-rate was then adjusted to give a constant coronary perfusion pressure of 60 (±2) mmHg which was maintained throughout the experiment. This was achieved by means of the peristaltic pump (Gilson Minipuls 3)) which was driving the KHB through the system. The pump was regulated by a pump controller (ADI STH Pump controller) which received the pressure signal from the CPP transducer and relayed a negative feedback signal to the pump in response to changes in pressure (Figure 2-2) Functional data were converted to digital signal using Powerlab/8SP (ADI instruments) and displayed and recorded using a personal computer (iMac, Apple Macintosh, software Chart 5, ADI instruments). Left ventricular pressure, coronary perfusion pressure and coronary flow were monitored continuously. The latter measurement was achieved by recording the voltage signal from the pump controller which determined the speed of the pump rotation. This was calibrated daily to give a reading in $ml.minute^{-1}$. Left ventricular developed pressure, diastolic tension and rate were monitored from the left ventricular pressure signal. Rate pressure product (RPP) was obtained by multiplying the rate at any given point by the developed tension.

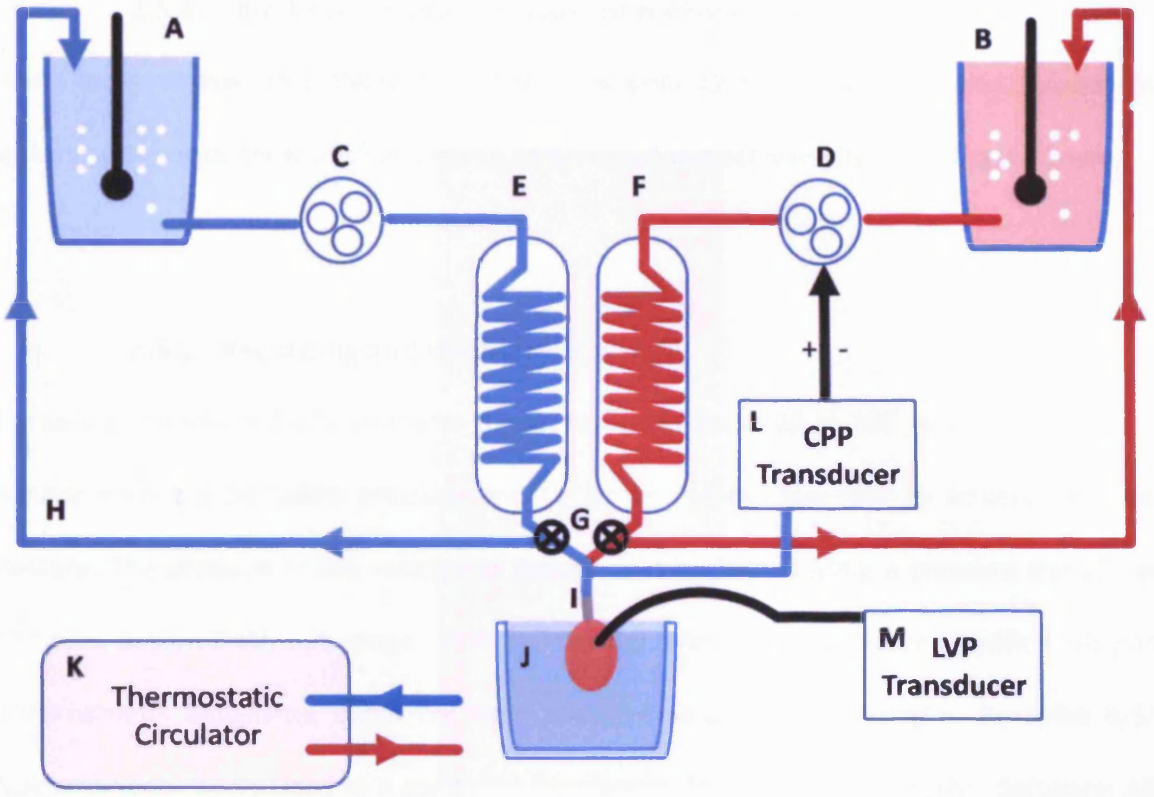

Figure 2-2: A schematic diagram of the Langendorff apparatus used in these experiments. The system is designed to enable rapid switching between standard Krebs-Henseleit buffer (KHB) (A) and KHB containing the experimental drug which is drawn from a separate reservoir (B). KHB in both reservoirs is bubbled with 95% O2 / 5% CO2 through a sintered glass gassing rod. . A peristaltic pump (C,D) is used to pump KHB from each reservoir to a separate warming coil (E,F). The warming coils also serve as bubble traps. Three-way taps (G) allow warmed KHB to be recirculated to the reservoir (H) or directed to the heart via a stainless-steel cannula (I). The heart is immersed in KHB in a heart chamber (J), which in common with the warming coils, is maintained at 37°C by a thermostatic circulator (K). Coronary perfusion pressure (CPP) is continually monitored by a pressure transducer attached by a fluid-filled tube attached to a sidearm directly above the cannula (L). The voltage signal is used to control the peristaltic pump and keep average pressure constant. A fluid-filled balloon is inserted into the left ventricle and is attached to a pressure transducer used to monitor left ventricular pressure (LVP).

2.5.3 Inclusion criteria

Hearts which had a left ventricular developed pressure of >60 mmHg and a rate of >200 beats.min^{-1} at the end of the stabilisation period were included in the study and were randomised to a treatment group. Hearts which underwent prolonged ventricular fibrillation during reperfusion were excluded from analysis. Induction of ischaemia was confirmed by at least a 30% drop in coronary flow after coronary ligation. Hearts which did not achieve this reduction in flow were excluded.

2.5.4 Stabilisation and induction of regional ischaemia

Hearts were allowed to stabilise for 20 minutes prior to ischaemia which was induced by tightening the coronary snare (see above). Reperfusion was achieved by loosening the snare.

2.5.5 Recording and calculation of data

A pressure transducer (ADInstruments model MLT844, range -20 to 300 mmHg) was used to monitor coronary perfusion pressure and to control pump flow-rate to achieve constant pressure. The pressure in the ventricular balloon was measured using a pressure transducer (DTX Plus, Becton Dickinson, range -30 to 300 mmHg). Pressure signals were amplified using an ADInstruments Bridgeamp, converted from analogue to digital data using a Powerlab 8/SP (ADInstruments, and passed to a computer (Hardware: iMac, Apple Macintosh; . Software: AD instruments Chart v.5.1 sampling frequency 1 KHz).

From the recorded pressure values, the following parameters were calculated throughout the experiment using the Chart software 'cyclic variables' calculation facility. *Diastolic pressure* was defined as the minimum pressure during each contraction cycle. *Developed pressure* was calculated by subtracting the diastolic pressure from the maximum pressure in each cycle. *Rate* was calculated as the number of contractions per minute.

2.5.6 Double staining to determine infarct size

At the end of the reperfusion period, the left main coronary artery was re-occluded and the heart was perfused with a 1% solution of Evans blue dye, to enable differentiation of the ischaemic area at risk from the normally perfused myocardium. Thus the area at risk is the non-blue region (Figure 2-3). Hearts were then frozen overnight before being sliced into six sections and placed into a 1% solution of 2,3,5-triphenyltetrazolium chloride at 37˚C for 30 minutes and then placed in 10% formalin (phosphate buffered formaldehyde solution, pH 7.3) at 4°C overnight. This procedure resulted in infarcted areas of tissue appearing white, whilst non-damaged tissue was seen as brick-red (Figure 2-3). This is because dehydrogenase enzymes in viable tissues react with tetrazolium to form a bright red formazan pigment (Nachlas & Shnitka, 1963).The sections were placed between two glass plates, separated by a 2.5mm gap.

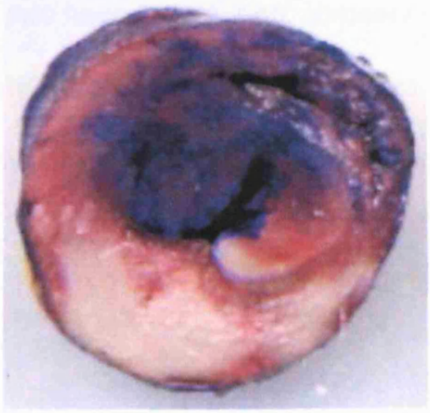

Figure 2-3: A photograph of heart sections after dual staining with Evans' blue dye and 2,3,5-triphenyltetrazolium chloride. Blue areas are regions which were normally perfused throughout the experiment (Evans Blue Positive). Red (Tetrazolium Positive)areas show viable tissue within the area which was made ischaemic. White areas (Tetrazolium negative) are regions of infarct.

The area of the sections and the infarcted and non-damaged regions was traced onto a clear piece of film and the areas were measured using computer aided planimetry (ImageJ 1.38x , NIH) allowing the volume at risk and the infarct volume to be calculated from the known area and depth of the sections. Infarct size was measured blinded to the treatment group.

2.5.7 Stability of Langendorff preparations

In order to test the viability of Langendorff preparations over the time periods used in these experiments, time controls were carried out whereby preparations were left for the duration of ischaemia. After stabilisation, time control hearts were left for two hours and 35 minutes, equivalent to the length of time spent in ischaemia and reperfusion in experimental hearts. At a time point equivalent to 35 minutes ischaemia and one hour reperfusion, there had been a modest decrease in coronary flow to 74±12 % of baseline and a similar fall in LVDP to 77±8 % (n=4). After the equivalent of two hours reperfusion, coronary flow had fallen to 66±9 % and LVDP to 61± 10% of baseline (Figure 2-4). These hearts were therefore considered suitably robust and stable for studies involving ischaemia and reperfusion.

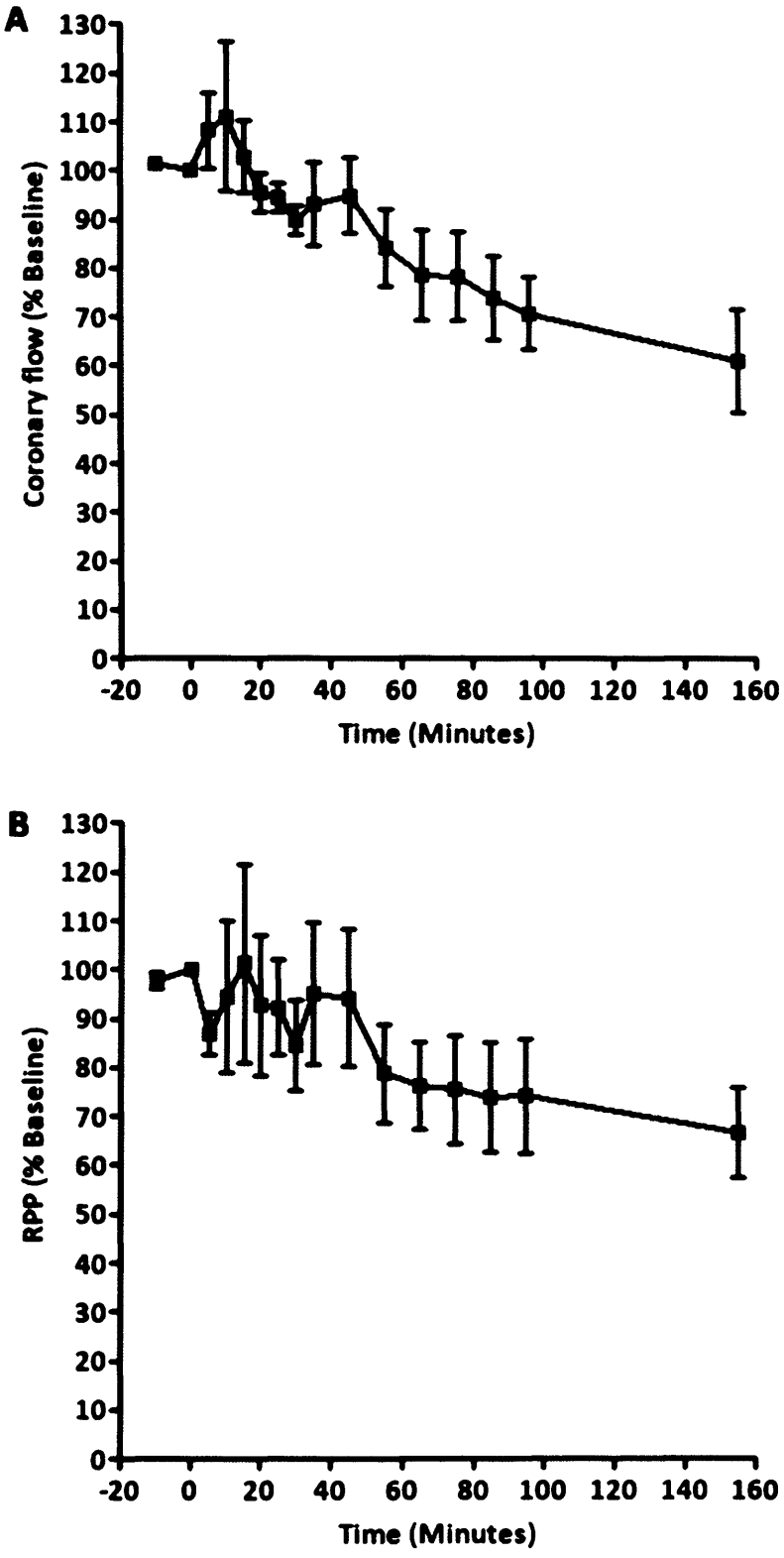

Figure 2-4. The deterioration in A) coronary flow and B) rate pressure product over time in Langendorff heart preparations not exposed to ischaemia and reperfusion. n=4, Initial RPP = 18730 ± 3476 mmHg.min^{-1}, Initial flow = 16.59±2.02 ml.min^{-1} 0 is the time at which ischaemia was induced in experimental groups.

2.6 Colourimetric assay for lactate dehydrogenase

2.6.1 Overview of technique

The detection and quantification of LDH in solution can be achieved by a spectrophotometric method which relies on the catalytic conversion of lactate to pyruvate, by LDH and the consequent reduction of NAD+ to NADH/H+. Diaphorase then catalyses the transfer of H/H+ from NADH/H+ to 2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride (INT), a pale yellow tetrazolium salt, causing its reduction to formazan, which is red.

The degree of colour change can be measured by a spectrophotometer which shines light through the solution and measures light absorbance. Using samples of LDH of known concentration, a standard curve can be drawn. Interpolation of the absorbance values of unknown samples onto standard curve allows the quantification of LDH concentration.

The extent of cell death in isolated paced ventricles was estimated by taking samples of KHB from the organ bath (see Chapter 3 for details of sampling) and measuring the concentration of lactate dehydrogenase. A pipette was used to remove 0.5 ml of KHB which was sealed in a microcentrifuge tube and frozen at -20°C until analysis. Enzymes such as lactate dehydrogenase cannot cross the sarcolemma and their detection implies that membrane breakdown and cell death have occurred. A commercially available assay kit was used (Cytotoxicity detection kit^{Plus} (LDH)®, Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions (Roche Applied Science, 2006). Reaction mixture was made up immediately prior to the experiment. Samples of Krebs (100 µL), and standard solutions were added to a 96-well

plate after which reaction mixture (100 μL) was added to each well. The plate was covered in aluminium-foil to exclude light and was gently rotated on a plate shaker for 30 minutes prior to spectrophotometric analysis. Absorbance was read at λ_{490} nm by a MRX TC Spectrophotometer using Revelation V 4.22 software (Dynex Technologies Limited, Worthing, UK). Samples were compared to a standard curve made up of concentration of l-lactate dehydrogenase from rabbit muscle lactate dehydrogenase (0.5, 1, 5, 25, 50, and 100 units.ml-1) in KHB.

2.7 High performance liquid chromatography with electrochemical detection for measurement of catecholamines in coronary perfusate

2.7.1 Overview of technique

Reversed-phase high performance liquid chromatography (HPLC) with electrochemical detection is a method which enables the separation and quantification of the components of a mixture in solution. Separation occurs prior to detection and is achieved by adding samples to a polar solvent, the 'mobile phase' which is pumped through a non polar, solid, porous 'stationary phase' (the chromatographic column). The physiochemical interactions between the compounds of interest, the stationary phase and the mobile phase determine how long they are retained on the column. The retention time for a particular molecule is a constant (assuming constant experimental conditions). Thus unknown compounds can be identified by comparing their retention times with standard samples of known identity.

Detection is achieved by an electrode at the end of the chromatographic column which is charged and (in the experiments described in this study) leads to the oxidation of the catechol ring of catecholamines. The resultant electrical signal is used to detect the presence of catecholamines. As larger concentrations of catecholamines give a larger electrical signal, a standard curve can be constructed using standard samples. Interpolation onto this curve can be used to estimate the concentration of unknown samples.

2.7.2 Sample collection and storage

Samples of coronary perfusate were collected using a 200 µl pipette at various time points from the pulmonary artery of hearts undergoing ischaemia and reperfusion (See Chapter 6 for details). The samples were immediately decanted into micro-centrifuge tubes and snap-frozen by immersion in liquid nitrogen. The samples were then stored at -80°C until analysis.

2.7.3 Analysis

The catecholamines, noradrenaline and adrenaline, were separated by their differential rates of elution through a reversed-phase chromatographic column (Genesis C_{18}; 4µm particle diameter; 120 Å pore size) which was held at 20°C . The mobile phase used was a modification of that described by Wester (1987a; 1987b) and consisted of an aqueous solution of 100mM sodium citrate, 0.3 mM disodium ethylenediaminetetraacetic acid (EDTA), 0.43 mM octane sulphonic acid and 5.5% v/v acetonitrile, adjusted to pH 2.45 using o-phosphoric acid. The mobile phase was filtered via a Whatman 5 µm filter and was sonicated for 30 minutes prior to use in order to degas the solution. The mobile phase was pumped through the column at a rate

of 1.2 $ml.min^{-1}$, using a SpectraSYSTEM P1000 HPLC pump. Samples of noradrenaline and adrenaline (0.01, 0.1, 1, 10, 100, 1000 nM) were prepared In KHB and a standard curve was plotted in order to calculate the concentration of samples. Standards and samples were injected in 20µl boluses. A signal/noise ratio of 3 gave a detection limit of 0.1 nM for adrenaline and noradrenaline.

2.8 Western blotting for components of cellular signalling pathways

2.8.1 Overview of technique

Western blotting (immuno-blotting) involves the separation of proteins in a biological sample by gel electrophoresis. Proteins are applied to a gel across which a potential difference is applied. They move at a rate determined by their mass and charge and thus separation of different proteins is achieved. Proteins are transferred onto a nitrocellulose membrane and identified by interaction of proteins of interest and specific antibodies. The strength of the antibody signal can be used as a semi-quantitative measure of the amount of protein present. Where proteins are modified by phosphorylation and specific antibodies have been raised against the phosphorylated and dephosphorylated forms of the protein then the proportion of the total protein which is in the phosphorylated state can be estimated.

2.8.2 Sample collection

Hearts were prepared according to Langendorff as described above and exposed to various treatments (See Chapter 6 for details) They were then removed from the Langendorff apparatus, arrested in cold KHB and sliced into sections using a scalpel. The apex was removed first and divided into left and right sections. Two samples each of left and right ventricular tissue were also taken. Samples were placed into micro-centrifuge tubes (Eppendorf Ltd, Cambridge, UK) and were snap-frozen in liquid nitrogen and stored at -80°C until analysis. It was not possible to perform infarct-size measurements in the same hearts which were used for Western blotting experiments, thus experiments were repeated. Functional data were collected for all hearts used for Western blotting to ensure that these hearts conformed to the inclusion criteria.

2.8.3 Analysis

Tissue samples (from the left side of the apex) were pulverised in liquid nitrogen using a pestle and mortar and homogenised using lysis buffer, designed to encourage the solubilisation of the proteins. The buffer contained a commercially available protease inhibitor cocktail in order to prevent protein digestion by proteases present in the sample. Homogenates underwent sonication for 3 seconds and centrifugation at 13,000 g for 15 minutes after which the supernatant was removed.

A commercial assay kit, Bicinchonic acid (BCA)^{TM} Protein Assay Kit (Pierce, UK) was used to determine the total protein concentration in each sample. Absorbance was read at λ_{540} nm by

MRX TC Spectrophotometer using Revelation V 4.22 software (Dynex Technologies Limited, Worthing, UK). Samples were compared to a standard curve made up of concentrations (0, 25, 250, 500, 1000, 2000, 4000, 10000 $\mu g.ml^{-1}$) of bovine serum albumin in lysis buffer.

The supernatant was divided into 20µl aliquots. To each aliquot, 20µl of Laemmli sample buffer was added and the samples were stored at -20°C until further analysis. The composition of Laemmli sample buffer was (4% sodium dodecyl sulphate, 20% glycerol, 10% 2-mercaptoethanol, 0.0004% bromophenol blue, 0.215 M Tris HCl).

Protein separation was achieved by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) using a mini-PROTEAN 3® cell and tank system (Bio-Rad Laboratories Inc., Hemel Hempstead, UK). 10 µg of each protein sample was loaded. Separated proteins were then transferred to a polyvinylidine difluoride (PVDF) membrane (Amersham Biosciences Ltd.). The membrane was agitated in a 5% solution of milk powder in Tris buffered saline-tween (TBS-Tween) for 3 hours to prevent non-specific binding of proteins. The PVDF membranes were initially probed with a rabbit anti-phospho-(ser473)-Akt primary antibody (1:500 dilution) at 4°C overnight. They were then washed in TBS-Tween (three 15 minute washes) after which the membranes were probed with a goat anti-rabbit-horseradish peroxidase conjugate secondary antibody (1:10000 dilution), followed by a further three 15 minute washes. The membrane was then coated with Western Blotting Luminol Reagent (Santa Cruz Biotechnology Inc., USA), and then exposed to photographic film (Hyperfilm™, Amersham Biosciences Ltd., Amersham, UK) for 15 minutes. The films were developed, fixed and dried and

then converted to digital images using an Epson perfection 2480 photo scanner (Epson Ltd. Hemel Hempstead, UK).

Membranes were then stripped of antibody using Restore™ Western blotting stripping reagent and then re-probed using a total Akt antibody (1:500 dilution). The procedure was exactly as described in the previous paragraph, except that the time for which the membrane was exposed to the photographic film was ten seconds.

Images were analysed using the software ImageJ v1.38x (National Institute of Health, USA). Mean grey values were obtained for bands. These were converted into optical density measurements using a calibrated step tablet (Kodak). Protein loading was controlled for by comparing total and phosphorylated Akt on the same blot (See chapter 6 for details)

2.9 Solutions and chemicals

2.9.1 Water

Water used throughout this experiment was double distilled (analytical grade) obtained from a Millipore Elix 10 still (Millipore Billerica, MA)

2.9.2 Krebs Henseleit buffer (KHB)

A buffer based on that first described by Krebs (Krebs & Henseleit, 1932), was used in Langendorff heart preparations and in experiments utilising isolated atria and ventricles. A

tenfold stock solution was made weekly containing NaCl (1.184 μM); $MgSO_4$ (12 mM); KCl (47 mM) and KH_2PO_4 (12 mM) in water. This was stored in a refrigerator at 4°C.

KHB was made up freshly before each experiment. To an appropriate volume of stock solution was added a freshly made solution of $NaHCO_3$ and glucose. The volume was then adjusted to approximately 90% of the final required volume using distilled water. $CaCl_2$ was dissolved separately and added last to the mixture, before a final adjustment of the volume achieved by the addition of water. Final concentrations of reagents in the KHB were as follows: NaCl 118mM, KCl 4.7mM, KH_2PO4 1.2mM, $MgSO_4$ 1.2mM, $CaCl_2$ 2.5mM, glucose 11mM.

To the KHB used in Langendorff perfused heart experiments only was added insulin $100mUl^{-1}$ because of the problems of delivering sufficient energy substrate to the heart by glucose alone (Sutherland *et al.*, 2000).

KHB was filtered through a Whatman 5 μm filter to remove particulate impurities prior to use in Langendorff preparations (Sutherland *et al.*, 2000).

KHB was gassed with 95% O_2 / 5% CO_2 for approximately 20 minutes prior to the commencement of the experiment. In bicarbonate buffers, CO_2 is required to maintain the pH of the buffer at 7.4 (Sutherland *et al.*, 2000).

2.9.3 Pentobarbitone solution and preparation of injections

Pentobarbitone solution was made up weekly to a final concentration of 60 $mg.ml^{-1}$. This was achieved by first dissolving 600 mg in 2ml of pure ethanol. This was made up to 10 ml with 0.9% sodium chloride solution which had been warmed to approximately 30°C. This solution was stored in a refrigerator at 4°C.

Injections were prepared freshly, shortly before use. Into a 1ml syringe with needle attached 0.1 ml of a $100U.ml^{-1}$ solution of heparin was drawn up followed by 0.9 ml of the pentobarbitone solution. Injections were given via the intraperitoneal route close to the right hind limb.

2.9.4 2,3,5-triphenyltetrazolium chloride

A 1% solution of 2,3,5-triphenyltetrazolium chloride in KHB was prepared immediately prior to use. The solution was warmed to 37°C prior to addition of the heart slices.

2.9.5 Evans blue

Evans blue dye was prepared as a 10 % solution in water and was stored in the refrigerator (2-8 °C) until immediately before use when an appropriate volume was diluted with KHB to form a 1% solution.

2.9.6 Insulin

Insulin was supplied as a 100Uml^{-1} solution and was diluted to 1U.ml^{-1} with water. This stock solution was kept in a refrigerator at 4°C and 0.1 ml was added to every litre of KHB to give a final concentration of 100 mU.l^{-1}.

2.9.7 Lysis buffer

Lysis buffer contained Tris-HCl (pH 7.6),20 mM; EDTA (pH 8.0), 10 mM; Triton X-100, 0.25 %, NaCl, 100 mM; KCl, 10mM and $MgCl_2.6H_2O$, 3mM.

2.9.8 Running buffer (x10 concentrated)

Running buffer consisted of an aqueous solution of trizma base, 10.25M; glycine, 1.92 M and SDS, 1% (w/v).

2.9.9 Transfer buffer

Transfer buffer consisted of an aqueous solution of tris(hydroxymethyl)aminomethane (TRIS), 25mM; glycine, 192mM and methanol 20% (v/v). pH was adjusted to 8.3.

2.9.10 Tris buffered saline (TBS) (concentrated) 1 L

TBS (concentrated) consisted of an aqueous solution of TRIS, 0.2 M and NaCl, 1.37 M. pH was adjusted to 7.6.

2.9.11 TBS-tween 1 L

TBS-tween consisted of an aqueous solution of TBS (concentrated), 10% and Tween-20, 0.1%.

2.9.12 Running gel

Running gel was freshly prepared with the following components: water (46.0%); 30% conjugated acrylamide, (26.7%); 15M Tris (pH 8.8), (25.3%); 10% SDS, (1.0%); 10% ammonium persulphate (0.1g/ml), (1%) and Tetramethylethylenediamine (TEMED), (0.06%).

2.9.13 Stacking gel

Each stacking gel was freshly prepared with the following components: water, (68.3%); 30% conjugated acrylamide, (17.9 %); 15M Tris (pH 8.8), (12.7 %); 10% SDS, (1%); 10% ammonium persulphate, (1.0%) and TEMED, (0.1%).

2.10 Cleaning of glassware and equipment

At the end of each experiment (Langendorff or isolated tissue), glassware was washed firstly with very hot tap water. This was followed by soaking in a dilute solution of hydrochloric acid followed by thorough rinsing with distilled water. The organ baths which had contained the isolated atria and ventricles were washed in a similar manner. The Langendorff apparatus was cleaned by running very hot water through the tubing, followed by distilled water and lastly hydrochloric acid (approx 1 M) which was left in the apparatus until shortly before the next use, when it was thoroughly washed out with distilled water.

2.11 Sources of drugs and chemicals

Table 2-1 A list of drugs and chemicals used in this thesis and the suppliers

Drug/Chemical	Manufacturer
95% N_2 / 5% CO_2	British Oxygen Company, Guildford, Surrey, GU2 7XY
95% O_2 / 5% CO_2	British Oxygen Company, Guildford, Surrey, GU2 7XY
Acetonitrile	Fisher Scientific, Loughborough, LE11 5RG
Acrylamide (30% conjugated)	Fisher Scientific, Loughborough, LE11 5RG
(-)-Adrenaline (+)- bitartrate	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH
Akt antibody (Phospho-Ser473)	Cell Signalling Technology Inc, 3 Trask Lane, Danvers, MA 01923
Akt antibody (Total)	Cell Signalling Technology Inc, 3 Trask Lane, Danvers, MA 01923, USA
Ammonium persulphate	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH
Bovine Serum Albumin	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH
BRL-37344 sodium	Tocris Bioscience, Avonmouth, Bristol, BS11 0QL
Calcium chloride dihydrate	Fisher Scientific, Loughborough, LE11 5RG
CGP-20712A dihydrochloride	Tocris Bioscience, Avonmouth, Bristol, BS11 0QL
Choline chloride	Acros Organics, Geel, Belgium.

Drug/Chemical	Manufacturer
CL-316243	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH
R(-)-Denopamine	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH
EDTA (disodium salt)	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH
Ethanol	Fisher Scientific, Loughborough, LE11 5RG
Evans Blue	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH
Forskolin	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH
Formoterol	Tocris Bioscience, Avonmouth, Bristol, BS11 0QL
Glucose	Fisher Scientific, Loughborough, LE11 5RG
Glycine	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH
Goat-anti Rabbit antibody HRP conjugate	Upstate (Millipore) 290 Concord Road, Billerica, MA 01821, USA
Heparin	CP Pharmaceuticals, Wrexham, Clwyd, U.K.
Hydrochloric acid	Fisher Scientific, Loughborough, LE11 5RG
Hypurin porcine insulin	AAH hospital supplies, Coventry, UK.
ICI-118551 hydrochloride	Tocris Bioscience, Avonmouth, Bristol, BS11 0QL

Drug/Chemical	Manufacturer
(-)-isoprenaline (+)-bitartrate	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH
Laemmli buffer	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH
l-lactate dehydrogenase from rabbit muscle	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH
Magnesium sulphate (analytical grade)	Fisher Scientific, Loughborough, LE11 5RG
Marvel™(original) Dried Skimmed Milk	Premier International Foods, Spalding, UK
Methanol	Fisher Scientific, Loughborough, LE11 5RG
N_2 (liquid)	British Oxygen Company, Guildford, Surrey, GU2 7XY
L-(−)-Noradrenaline (+)-bitartrate salt monohydrate	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH
Octane sulphonic acid sodium	Fisher Scientific, Loughborough, LE11 5RG
Pentobarbitone sodium	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH
Phentolamine mesylate	Alliance Pharmaceuticals, Chippenham, Wiltsire.
Potassium chloride (analytical grade)	Fisher Scientific, Loughborough, LE11 5RG
Potassium dihydrogen orthophosphate (analytical grade)	Fisher Scientific, Loughborough, LE11 5RG
Procaterol hydrochloride	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH

Drug/Chemical	Manufacturer
(±)-propranolol	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH
Protease inhibitor cocktail	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH
Restore™ – Western blotting stripping reagent	Pierce (Thermo-Fisher) PO Box 117, Rockford, IL 61105, USA)
Salbutamol hemisulphate	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH
Sodium chloride	Pierce (Thermo-Fisher) PO Box 117, Rockford, IL 61105, USA)
Sodium chloride solution 0.9%	
Sodium citrate	Fisher Scientific, Loughborough, LE11 5RG
Sodium dodecyl sulphate	Baxter Healthcare, Newbury, Berkshire, RG20 7QW
Sodium hydrogen carbonate	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH
SR-59230A hydrochloride	Tocris Bioscience, Avonmouth, Bristol, BS11 0QL
TEMED	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH
Timolol maleate	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH

Drug/Chemical	Manufacturer
2,3,5-triphenyltetrazolium chloride	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH
TRIS	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH
Triton X-100	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH
Trizma base	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH
Tween-20	Sigma-Aldrich Company Ltd, Poole, Dorset, BH12 4QH

2.12 Statistics and treatment of data

When two groups of data were compared a t-test was used (paired or unpaired as appropriate, see individual chapters for details. Three or more groups were compared by 1-Way Analysis of Variance (ANOVA) followed by the Student Newman-Keuls post-hoc test. This test assumes equal variance between experimental groups. This was confirmed using Bartlett's Test of Equal Variance. ANOVA also assumes a near-Gaussian distribution of data. Each data set was thus subjected to D'Agostino & Pearson's Omnibus Normality Test. Coronary flow, LVDP and RPP were compared using repeated-measures ANOVA. All analyses were performed using GraphPad Prism® Version 4.02, GraphPad software, Inc.

Chapter 3 - Can ischaemic preconditioning, postconditioning and β-adrenoceptor-mediated preconditioning protect against stunning in rat isolated atria and ventricles?

3.1 Introduction

Ischaemic preconditioning is an extremely powerful cardioprotective phenomenon whereby transient periods of ischaemia protect the heart against later more severe ischaemic insult. The phenomenon, first discovered by Reimer's group (Murry *et al.*, 1986) protects the heart against arrhythmias (Ravingerová *et al.*, 2002) and importantly prevents lethal cell injury and reduces infarct size (Murry *et al.*, 1986). However, controversy exists as to whether preconditioning reduces stunning (a component of reperfusion injury consisting of reversible contractile dysfunction). Whilst protection against stunning has been demonstrated *in vitro* by classic ischaemic preconditioning (Fralix *et al.*, 1993) and pharmacological preconditioning (Gardner *et al.*, 2004; Yates *et al.*, 2003), this has been difficult to replicate in vivo (Jennings, 1996; Kloner & Jennings, 2001a; Kloner & Jennings, 2001b; Ovize *et al.*, 1992). Conversely, the protection of late preconditioning (second window of preconditioning) against stunning is much more robust (Przyklenk & Heusch, 2003; Sun *et al.*, 1995; Tang *et al.*, 1996). Using *in vivo* experimental models with recovery of animals, stunning can easily be distinguished from lethal reperfusion injury, because of its reversal over time. However, using *in vitro* preparations which are stable only for several hours, it can be difficult to distinguish whether post-ischaemic contractile dysfunction is caused by stunning or cell death. Furthermore, any intervention which improves post-ischemic contractile function may do so by ameliorating either stunning or cell death (or

both). For the same reasons it has been difficult to determine whether postconditioning improves contractile function after reperfusion as a result of reducing cell death or whether it truly improves stunning (Penna *et al.*, 2008). Although a reduction of infarct size is the most important outcome in improving life expectancy after ischaemia in the clinic, reduction of stunning may also be important in reducing morbidity (See Section 1.2.4.2).

Several groups of investigators have demonstrated that a preconditioning-like adapted state can be induced by transient pre-treatment of hearts or other tissues with a β-adrenoceptor agonist (Frances *et al.*, 2003; Iliodromitis *et al.*, 2004; Spear *et al.*, 2007; Tong *et al.*, 2005). However, it is unclear whether or not ischaemic preconditioning is dependent on β-adrenoceptor activation (Tune *et al.*, 2004). Studies investigating β-adrenoceptor-mediated preconditioning have demonstrated functional improvements (improved left ventricular developed pressure) in β-adrenoceptor-preconditioned hearts over controls, possibly representing a reduction in stunning (Frances *et al.*, 2003; Iliodromitis *et al.*, 2004; Spear *et al.*, 2007; Tong *et al.*, 2005). These studies have been carried out in Langendorff or working hearts which are excellent models for studying ischaemic damage and reperfusion injury, as both functional data and infarct-size data can be obtained. However, in these experiments, improved functional recovery was associated with reduced cell death as assessed by visualisation of infarct size or measurement of cardiac enzyme release which may also be responsible for the improved contractile function.

A further limitation of experiments investigating β-adrenoceptor preconditioning in isolated hearts and indeed *in vivo* procedures is that these experiments may be complicated by the fact that activation of β_2-adrenoceptors in the coronary vasculature, leads to coronary vasodilatation potentially increasing contractile force via the Gregg effect (Gregg, 1963). Furthermore, activation of β_1-adrenoceptor in the heart causes a positive chronotropic effect (Ahlquist, 1948).

One study, has demonstrated protection against post ischaemic dysfunction in isolated paced guinea-pig atria exposed to 30 minutes simulated ischaemia (Yates *et al.*, 2003). This provides evidence that β-adrenoceptor-mediated preconditioning can protect against stunning. Electrically paced isolated cardiac tissues allow the study of the direct effects of ischaemic preconditioning and drug treatment on contractile function in different regions of the heart. As the tissues are not supplied by coronary flow but by diffusion, true ischaemia cannot be induced. Ischaemia is simulated by using a hypoxic buffer containing no glucose. Reperfusion is simulated by replacing normal buffer and reoxygenating the tissues (Carr *et al.*, 1997; Gardner *et al.*, 2004; Sivaraman *et al.*, 2007; Walker *et al.*, 1994; Walker *et al.*, 1995). It has been suggested that when tissues are exposed to short periods of simulated ischaemia (such as 30 minutes used in this study) contractile dysfunction is predominantly due to stunning, whereas with longer periods, cell death becomes more important (Walker *et al.*, 1994).

Thus by using a short (30 minute) period of ischaemia in isolated paced tissues, it is anticipated that the effects interventions on stunning can be investigated without the confounding factors

associated with cell death and without complications caused by vascular properties of β-adrenoceptor ligands.

The aim of this study was to use this model to test the hypothesis that ischaemic preconditioning and β-adrenoceptor-mediated preconditioning could be demonstrated in isolated paced cardiac tissues which provide a coronary vascular-independent model of myocardial ischaemia. One would expect β-adrenoceptor-mediated preconditioning to be susceptible to a β-adrenoceptor antagonist, a hypothesis which was tested. However, it was also an aim of this study to test the hypothesis that ischaemic preconditioning is dependent on β-adrenoceptor activation in this model. Furthermore, investigations were carried out to determine whether ischaemic postconditioning could protect against stunning.

3.2 Hypotheses

- Ischaemic preconditioning will lead to improved post-ischaemic contractile function in isolated cardiac tissues

- Activation by β-adrenoceptor agonists prior to ischaemia will mimic this protection

- The protection elicited by ischaemic preconditioning will be blocked by a non-selective β-adrenoceptor antagonist.
- Ischaemic postconditioning will improve post-ischaemic contractile function in isolated cardiac tissues

3.3 Methods

3.3.1 Atrial and ventricular preparations

Male Sprague-Dawley rats (Harlan, Bicester, U.K.) were used throughout and weighed 250-350g at the time of killing. Atrial and ventricular preparations were set up as described in section 2.4. All preparations were left for one hour to stabilise, during which time they were frequently washed. Initial diastolic tension was 1g for atrial tissues and 1.5g for ventricular tissues.

3.3.2 Experimental protocols

After the stabilisation period, the tissues were randomised to one of the following treatment groups (Figure 3-1). **Control**; tissues underwent a further thirty minutes normoxia, followed by 30 minutes simulated ischaemia (the index ischaemia), achieved as described in Section 2.5.4. Normoxic glucose-containing KHB was then restored and the contractile function of the tissues was monitored for one hour, during which time the KHB was replaced every fifteen minutes. **Ischaemic preconditioning (IPC)**; tissues underwent ten minutes simulated ischaemia and ten minutes normal oxygenation prior to the index ischaemia. **Ischaemic preconditioning in the presence of propranolol (IPC + Pro)**. Ischaemic preconditioning was performed as described as above, in the presence of 10^{-6} M propranolol which was added twenty-five minutes prior to the preconditioning ischaemia and maintained throughout simulated ischaemia and throughout the experiment after reoxygenation and glucose replacement. **β-adrenoceptor mediated preconditioning (βP)**. Tissues were exposed to isoprenaline (10^{-6} M) under normoxic conditions for 10 minutes, followed by washout. Simulated ischaemia begun twenty minutes later. **Propranolol (Pro)** Tissues were treated as controls, apart from the presence of 10-6 M propranolol which was present in the bath throughout the experiment. **β-adrenoceptor**

mediated preconditioning in the presence of propranolol (βP + Pro). Tissues were exposed to propranolol (10-6 M) for fifteen minutes prior to isoprenaline treatment as described above. Propranolol was maintained throughout the experiment. **Ischaemic postconditioning (Postcon)** – Tissues were treated as for controls, except at reoxygenation, tissues were exposed to 3 cycles of 1 minute normoxia followed by 1 minute simulated ischaemia. The protocols for ischaemic and pharmacological preconditioning were chosen based on preliminary data to find the optimum protocols for protection in this model (Table 3-1).

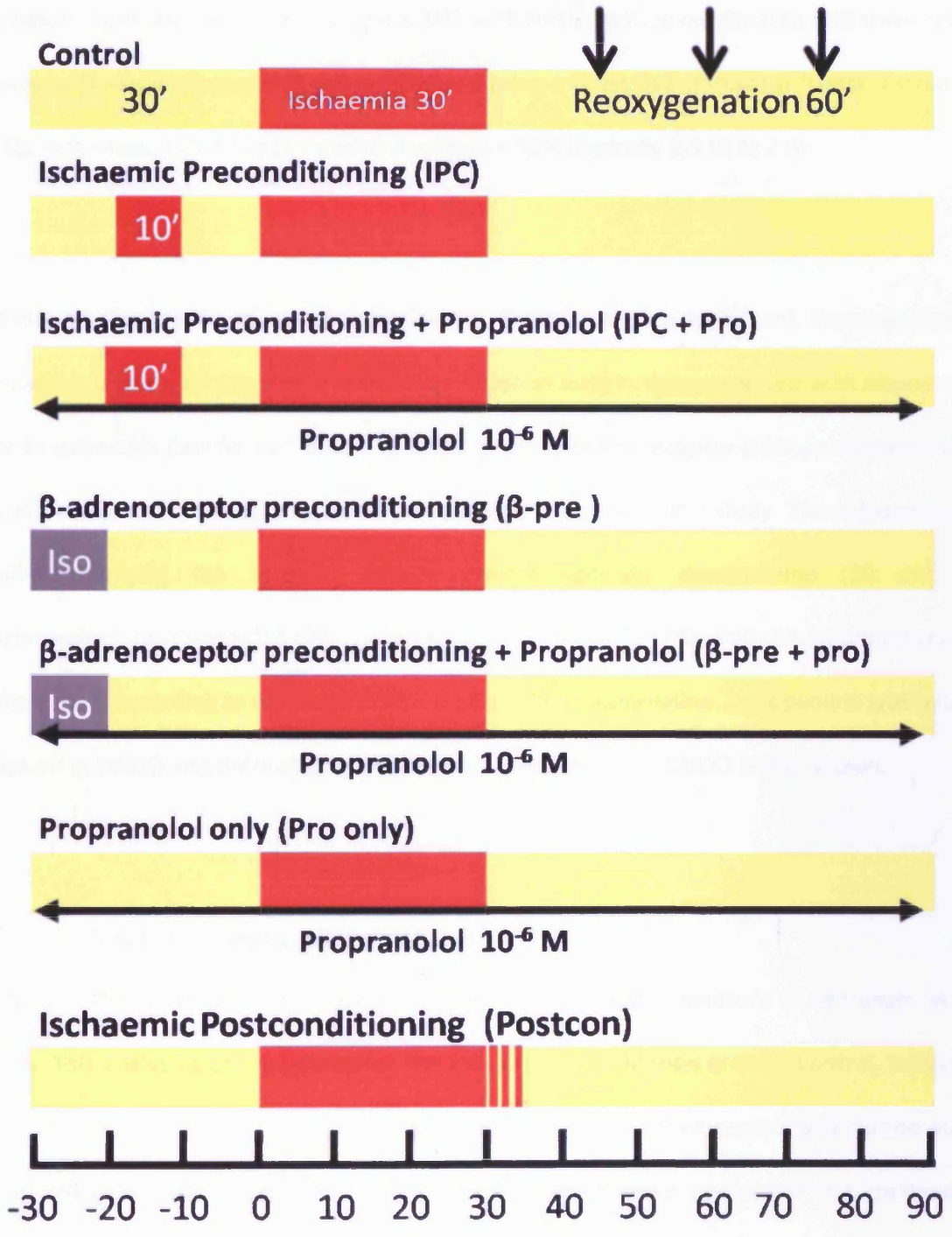

Figure 3-1 Experimental Protocols. Time (minutes) is shown along the abscissa with the onset of simulated ischaemia designated 0. Yellow regions correspond to normoxic conditions, red: simulated ischaemia and grey, drug treatment. Vertical arrows indicate washes during reperfusion.

The tissues were washed by replacing the KHB with fresh warmed oxygenated KHB three times. Tissues were paced throughout the experiment using a Grass S48 stimulator (Grass instrument Co. Quincy, Mass., U.S.A.) with threshold voltage + 50% (typically 1-5 V) at 2 Hz.

Analysis of the results of these experiments demonstrated a significant improvement of contractile function 60 minutes after reoxygenation in atria in tissues treated with isoprenaline prior to ischaemia (See Section 3.4.6). In order to discover the receptor subtype responsible for this effect, three further experimental groups were included in the study. These experiments involved applying the selective β-adrenoceptor agonists, **denopamine** (10 μM, β_1-adrenoceptor), **procaterol** (10 μM, β_2-adrenoceptor) and **CL-316243** (100 nM, β_3-adrenoceptor) to the tissues according to the same protocol as used for isoprenaline. Denopamine was initially dissolved in DMSO and thereafter in KHB. The vehicle control for DMSO is also shown.

3.3.3 Cell death/LDH assay

Analysis of the concentration of LDH in the KHB was used as a measure of cell death in the tissues. This analysis was performed for the following experimental groups: Control, Ischaemic preconditioning, β-adrenoceptor preconditioning. In addition a time control was carried out in which cell death was monitored in naïve tissues which were paced but not exposed to ischaemia. Samples of Krebs (0.5 ml) were removed from the organ bath immediately prior to simulated ischaemia, 15 minutes into ischaemia, at the end of ischaemia and every 15 minutes during recovery. Samples were stored and analysed as described in Section 2.6. Data are

presented as units of LDH per ml of KHB. Statistical analysis was carried out by ANOVA followed by Student-Newman-Keuls post-hoc test

3.3.4 Data analysis

Prism version 4.02 for windows (GraphPad Software, Inc) was used for production of graphs and statistical analyses. Tension was monitored continuously, enabling calculation of diastolic (baseline) and developed tension. The degree of ischaemic contracture was quantified by measuring the increase in diastolic tension during simulated ischaemia at its maximum point. The time taken to reach maximal contracture was also recorded. Cardiac function was assessed at fifteen and sixty minutes post reoxygenation. Developed tension at these time points was calculated as a percentage of developed tension at the end of the stabilization period for each preparation. Groups of data were compared by one-way ANOVA followed by Student-Newman-Keuls *post hoc* test.

3.4 Results

3.4.1 Preliminary data

Table 3-1 A summary of preconditioning protocols used in preliminary studies to obtain optimal conditions for ischaemic and pharmacological preconditioning. Time proceeds along the abscissa. White regions correspond to normoxic conditions; black indicates simulated ischaemia and grey indicates exposure to isoprenaline (10-6 M). The magnitude of the developed tension of the tissues (± SEM) is given for 15 and 60 minutes after reoxygenation and is expressed as a % of the baseline (prior to preconditioning). The length of time in minutes of each stage of the protocol is shown. * Indicates significant ($p<0.05$) difference from respective controls, as determined by one-way ANOVA with Student-Newman-Keuls post hoc test.

Preconditioning Protocol	Atria		Ventricles	
	15 min.	60 min.	15 min.	60 min.
30′ Ischaemia	29.3±1.7% n=11	47.9± 2.8 n=11	22.4 ± 2.8 n=11	44.4 ± 3.8 n=11
15′ 5′ 10′ 30′ Ischaemia	42.9±5.0 n=5	49.2±0.9 n=5	35.3+4.0 n=5	53.4±10.5 n=5
10′ 20′ 30′ Ischaemia	27.2 ± 4.1 n=6	75.8±16.6* n=6	19.4±2.8 n=6	41.8±4.1 n=6
10′ 10′ 10′ 30′ Ischaemia	47.0 ± 4.0* n=6	59.3±3.9 n=6	39.0±5.2* n=6	50.12±8.4 n=6
15′ 5′ 10′ 30′ Ischaemia	41.79±3.0 n=6	63.4±5.0 n=6	21.98±2.7 n=6	43.8±5.9 n=6
5′ 10′ 5′ 10′ 30′ Ischaemia	39.6±5.8 n=6	54.6±6.8 n=6	16.2±2.7 n=6	28.5±3.4 n=6

3.4.2 Baseline cardiodynamics

Table 3-2 Mean baseline values of developed tension for experimental groups in this study.

	Atria		Ventricles	
	n	Baseline (g)	n	Baseline (g)
Control	11	0.32 ± 0.05	12	1.27 ± 0.15
Ischaemic preconditioning (IPC)	6	0.34 ± 0.04	6	1.55 ± 0.13
IPC + Propranolol (IPC+ Pro)	6	0.34 ± 0.11	6	1.45 ± 0.08
Propranolol only (Pro)	6	0.40 ± 0.04	5	1.18 ± 0.25
β-adrenoceptor preconditioning (βP)	6	0.43 ± 0.07	7	1.41 ± 0.18
βP + propranolol (βP + Pro)	6	0.42 ± 0.13	5	1.27 ± 0.35
Postconditioning (Postcon)	5	0.50 ± 0.06	5	1.54 ± 0.99
Denopamine	3	0.51 ± 0.12	N/A	N/A
Procaterol	4	0.47 ± 0.07	N/A	N/A
CL-316243	5	0.47 ± 0.08	N/A	N/A
DMSO	4	0.39 ± 0.03	N/A	N/A

3.4.3 General observations

In control preparations in both atria and ventricles, simulated ischaemia led to a rapid reduction in developed tension, which fell to near-zero levels after five minutes. Diastolic tension rose during simulated ischaemia (indicative of contracture) reaching a maximum between 20 and 25 minutes and fell thereafter. Peak contracture was 0.65 ± 0.1g in atria and 1.95 ± 0.18 g in ventricles. Upon reoxygenation, recovery of developed tension occurred rapidly during the first 15 minutes, and more slowly thereafter (Figure 3-2). Therefore contractile function was compared at 15 and 60 minutes post-reoxygenation. Preconditioning protocols were optimised by carrying out preliminary studies (Table 3-1).

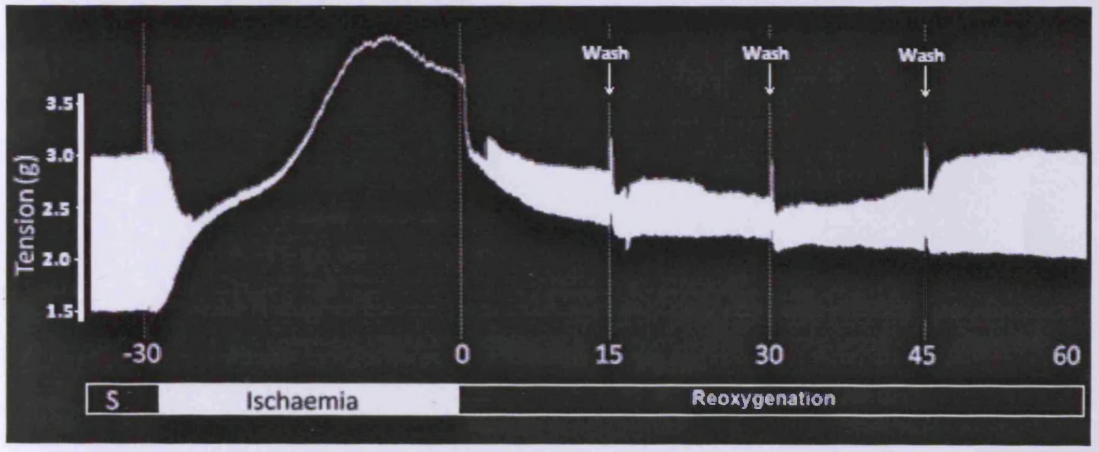

Figure 3-2 A representative trace from a ventricular preparation exposed to simulated ischaemia and reoxygenation. S represents the final minutes of the stabilisation period. Time (minutes) from reoxygenation are shown along the abscissa. Note the rapid fall in developed tension during simulated ischaemia and the increased diastolic tension indicating ischaemic contracture. Diastolic and developed tension both recover rapidly over the first 15 minutes of reoxygenation and more slowly thereafter.

3.4.4 Effects of ischaemic and β-adrenoceptor-mediated preconditioning on ischaemic contracture

Ischaemic preconditioning in ventricular tissues led to a significant ($p<0.05$) reduction in maximal ischaemic contracture from 1.95 ± 0.18g in controls to 0.92 ± 0.28g. A similar trend was evident in atrial tissues although this did not reach significance. β-adrenoceptor-mediated preconditioning did not alter the maximal contracture compared to controls (Figure 3-3).

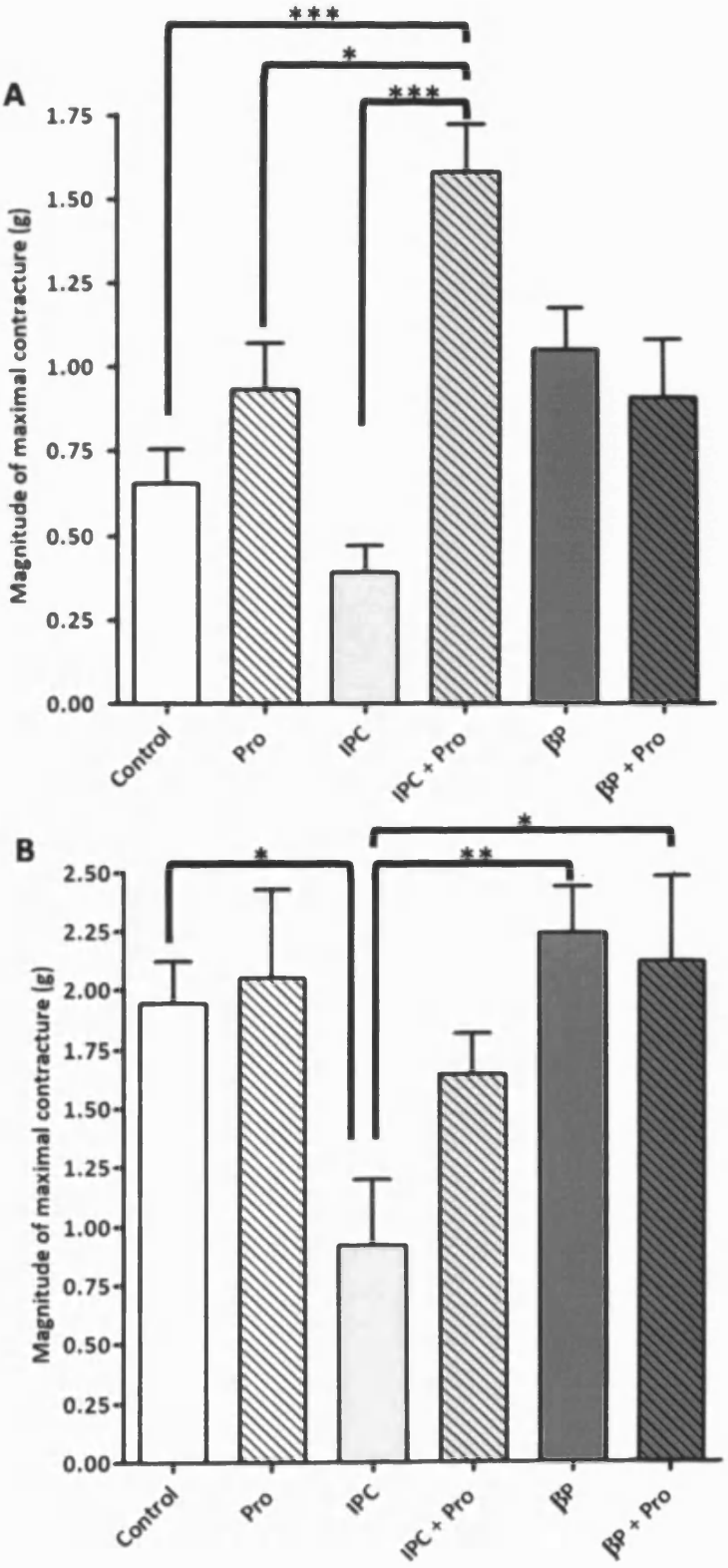

Figure 3-3 Magnitude of ischaemic contracture (maximum increase in baseline/diastolic tension (g) during simulated ischaemia) in a) atria and b) ventricular strips. * indicates $p<0.05$, **indicates $p<0.01$; *** indicates $p<0.001$.

Ischaemic preconditioning led to a significant ($p<0.01$) reduction in the time taken to achieve maximal contracture in ventricular tissue from 22.45 ± 3.5 minutes in controls to 16.33 ± 1.5 minutes. β-adrenoceptor-mediated preconditioning did not alter the time taken to reach maximal contracture (Figure 3-4).

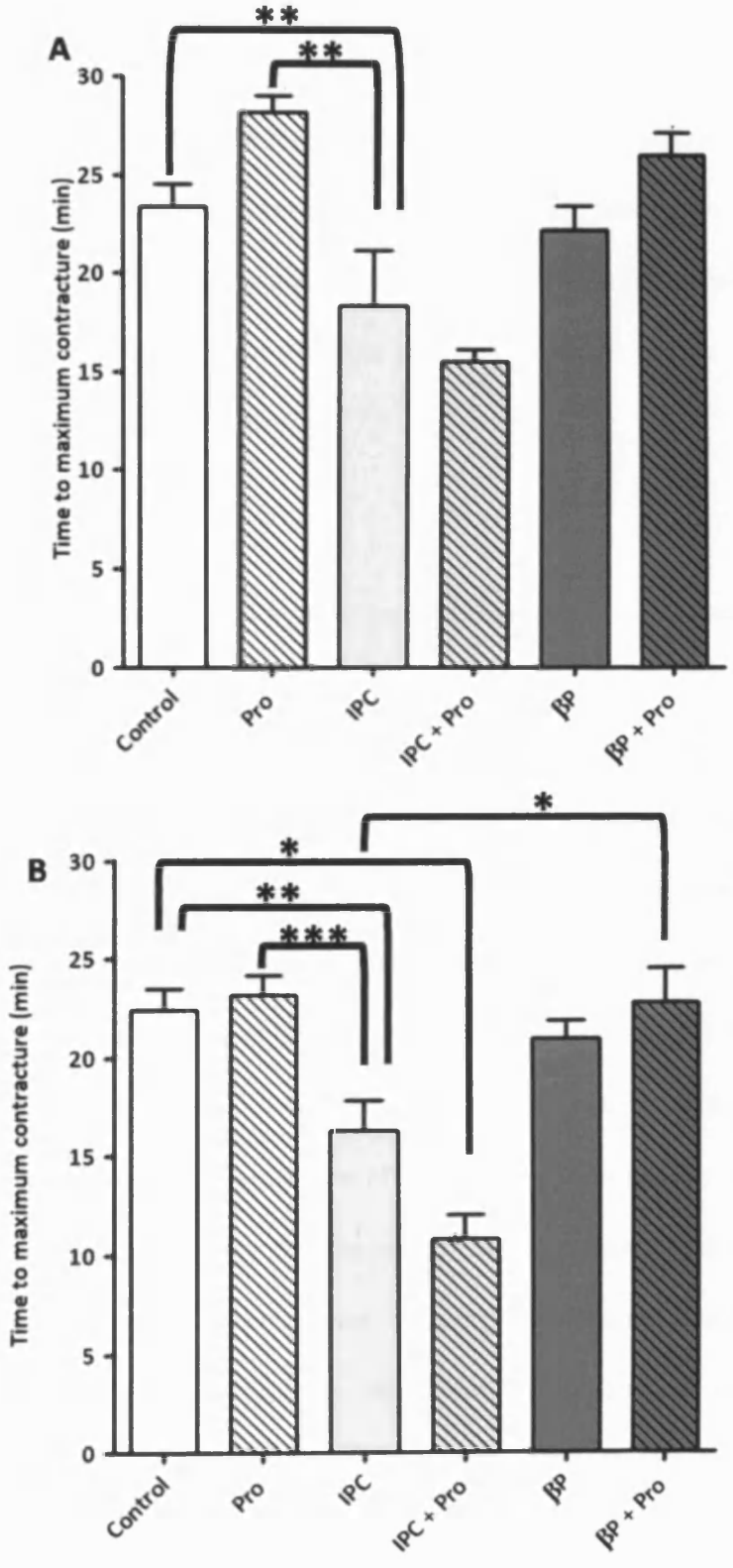

Figure 3-4 Time (minutes) into simulated ischaemia at which maximum contracture was reached in a) atria and b) ventricular strips. * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001.

Propranolol alone had no significant effect on either the magnitude of the maximal contracture, or the time taken to achieve this contracture. When ischaemic preconditioning was carried out in the presence of propranolol, the magnitude of ischaemic contracture was not significantly different from either control or preconditioned ventricular tissues. In atria however, the combination of ischaemic preconditioning and propranolol led to an ischaemic contracture of 1.58 ± 0.36g which was significantly ($p<0.001$) greater than either control atria or those which had undergone ischaemic preconditioning.

3.4.5 Effects of ischaemic preconditioning and postconditioning on contractile recovery

Ischaemic preconditioning led to an improved recovery of developed tension in both atrial and ventricular preparations compared to controls when compared fifteen minutes after reoxygenation. By this time point, developed tension in control (untreated) atrial tissues had recovered to 29.3 ± 1.7 % of the developed tension measured at the end of the stabilization period. Recovery of function was significantly ($p<0.05$) improved to 47 ± 4.0 % in tissues that had undergone ischaemic preconditioning. When ischaemic preconditioning was carried out in the presence of propranolol, the protective effect was lost. Tissues thus treated recovered to 26.4 ± 5.1 % which was not significantly different to control, however was significantly ($p<0.01$) smaller than recovery of atria which had undergone ischaemic preconditioning without propranolol. Propranolol alone had no significant effect on the recovery of tissues. Postconditioning did not significantly affect the recovery of atria (Figure 3-5 A).

A similar picture was observed in ventricular tissues. Untreated tissues recovered to 22.4 ± 2.8 % of pre-ischaemic values in the first fifteen minutes of reoxygenation. Ischaemic preconditioning significantly ($p<0.05$) increased this value to 39.0 ± 5.2 %. Propranolol had no significant effect on recovery when given alone, however when combined with ischaemic preconditioning, recovery at this time point was significantly ($p<0.01$) less than ischaemic preconditioning alone, and not significantly different to control. Postconditioning did not affect the recovery of ventricular tissues (Figure 3-5 B).

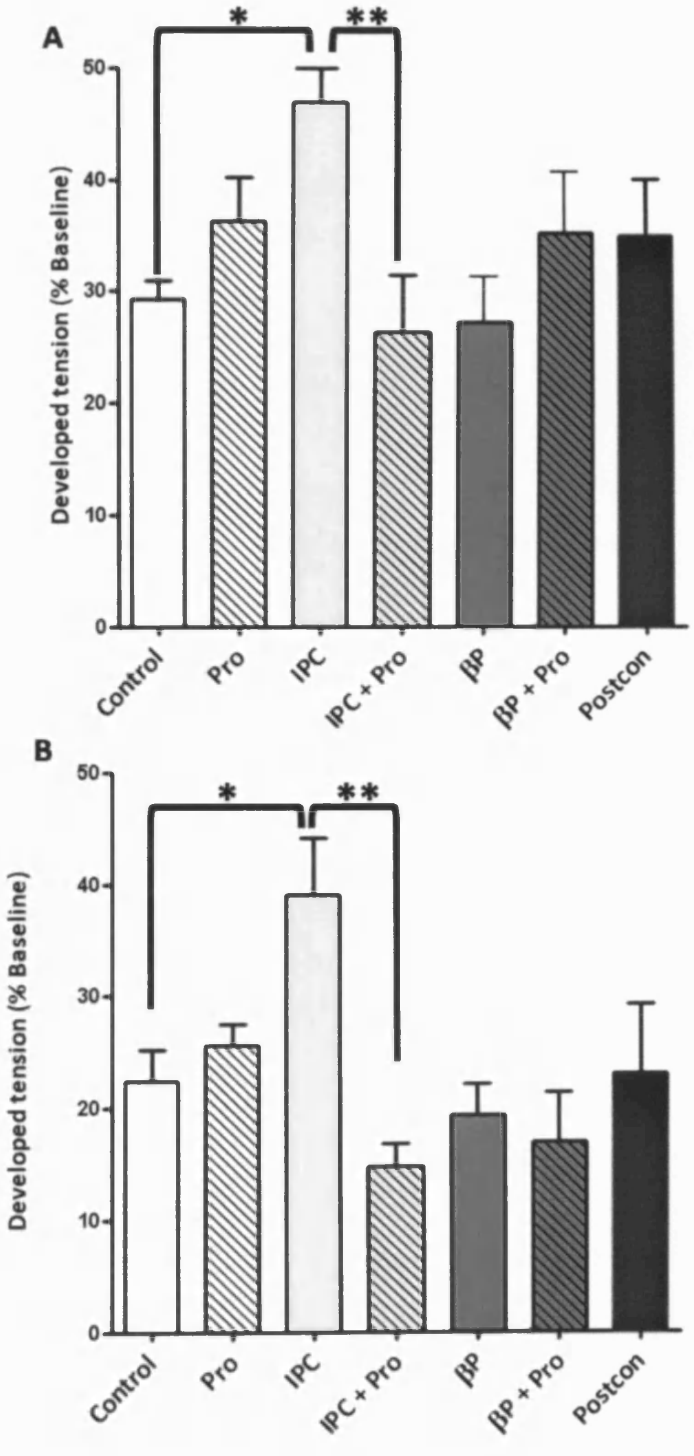

Figure 3-5: Developed tension after 15 minutes reoxygenation in a) atria and b) ventricular strips. Developed tension is expressed as a percentage of the developed tension achieved at the end of the stabilisation period. * indicates $p<0.05$ ** indicates $p<0.01$.

No significant effect of ischaemic preconditioning or postconditioning upon recovery was seen in either tissues 60 minutes after reoxygenation (Figure 3-6).

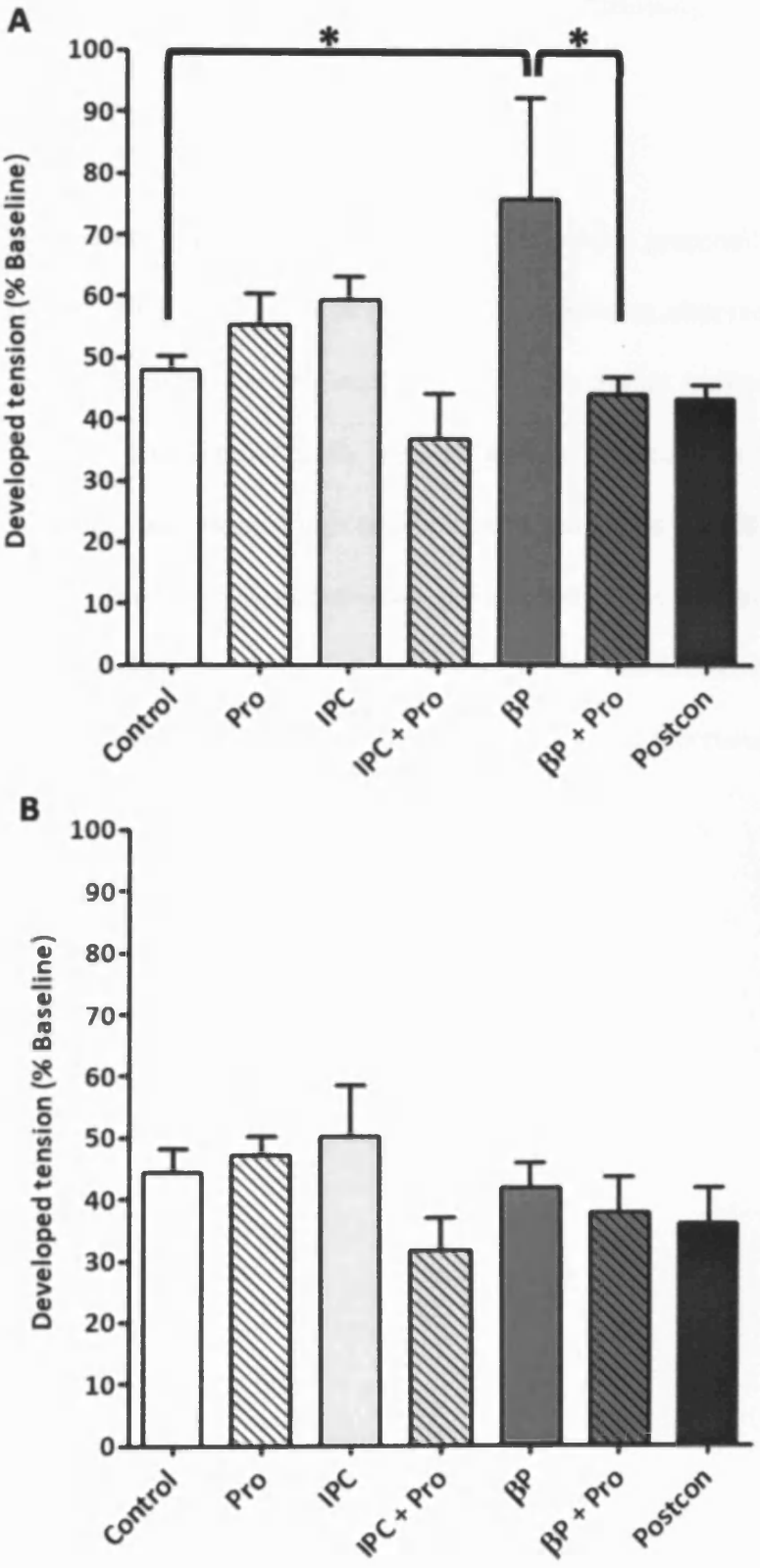

Figure 3-6: Developed tension after 60 minutes reoxygenation in a) atria and b) ventricular strips. Developed tension is expressed as a percentage of the developed tension achieved at the end of the stabilisation period. * indicates $p<0.05$.

3.4.6 β-adrenoceptor-mediated preconditioning: effects after reoxygenation

Recovery of developed tension in tissues which had received a preconditioning exposure of isoprenaline was not different to control in either tissue 15 minutes after reoxygenation (Figure 3-5). However, after 60 minutes reoxygenation in atria, the tissues treated with isoprenaline had recovered to 75.8 ± 16.6, significantly ($p<0.05$) greater than controls which recovered to 47.9 ± 2.3. This increase was blocked in propranolol treated tissues to 44.0 ± 3.0 % which was significantly ($p<0.05$) smaller than isoprenaline preconditioned tissues, and not significantly different to controls. Propranolol alone had no significant effect on recovery at this time period (Figure 3-6 A). Isoprenaline treatment did not improve recovery of function in ventricular tissue (Figure 3-6 B).

3.4.7 β-adrenoceptor subtype

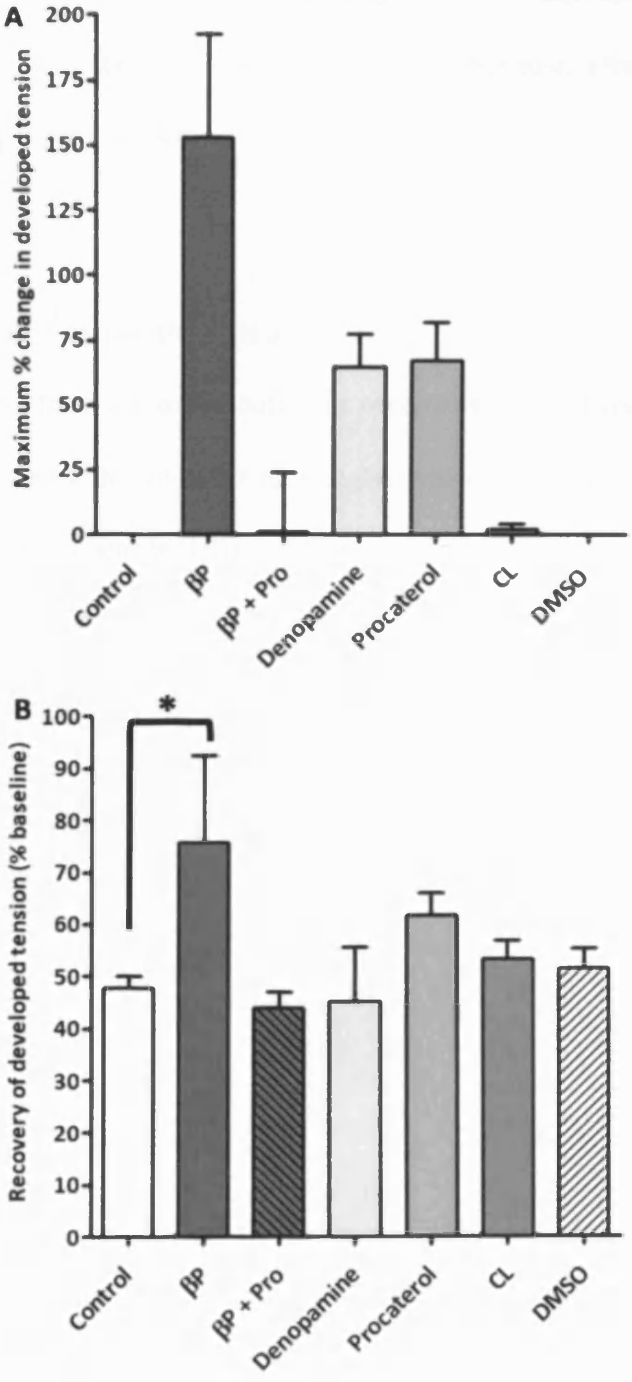

Figure 3-7 A) Maximal change in developed tension (as a % of baseline) caused by β-adrenoceptor agonist preconditioning in isolated atria and B) Recovery of developed tension at 60 minutes after reoxygenation in atria exposed to preconditioning by β-adrenoceptor agonists. * indicates a significant difference $p<0.05$.

None of the selective β-adrenoceptor agonists led to improved post-ischaemic contractile recovery in the same way as isoprenaline (Figure 3-7 B). Successful application of denopamine and procaterol was confirmed by the positive inotropic effects of these agents during preconditioning (Figure 3-7 A).

3.4.8 Cell death/LDH assay

LDH in KHB taken from the organ bath was measured during ischaemia and during reperfusion for control, ischaemic preconditioning and β-adrenoceptor preconditioning groups. There was no significant difference in LDH release between groups either during ischaemia or at reperfusion. There was a trend in all experimental groups towards larger LDH release during reperfusion than during ischaemia (Figure 3-8).

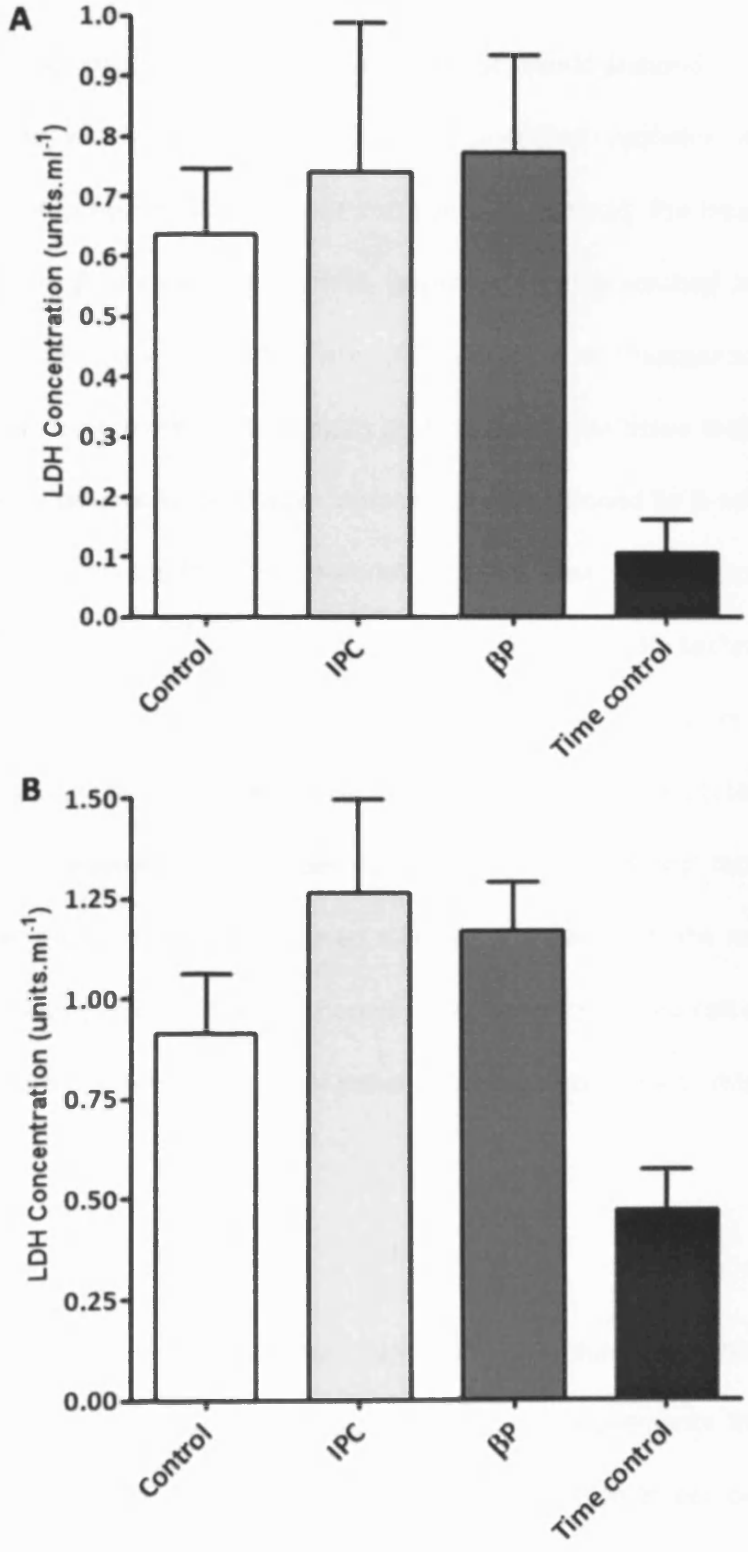

Figure 3-8 LDH release from ventricles during A) ischaemia and B) reperfusion

3.5 Discussion

This study has demonstrated cardioprotection by ischaemic preconditioning in both atria and ventricles. This was manifest by improved functional recovery at 15 minutes after reoxygenation in preconditioned tissues compared to controls. Pre-treatment of tissues with the non-selective β-adrenoceptor agonist, isoprenaline, also resulted in improved functional recovery, although this was manifest later (60 minutes after reoxygenation). The failure of β-adrenoceptor-mediated preconditioning to protect ventricular tissue in this model is unlikely to reflect the fact that ventricular tissue cannot be preconditioned by β-adrenoceptor activation, because isolated heart models have demonstrated that post ischaemic cardiac function can be improved by β-adrenoceptor preconditioning (Frances *et al.*, 2003; Lochner *et al.*, 1999; Marais *et al.*, 2001; Moolman *et al.*, 2006b; Moolman *et al.*, 2006c; Nasa *et al.*, 1997; Robinet *et al.*, 2005; Tong *et al.*, 2005; Yabe *et al.*, 1998), a fact which would necessitate a protective effect on the left ventricle. However, as discussed above, improved functional recovery may be a result of reduced cell death rather than reduced stunning. Furthermore, the mechanisms of reduced stunning by preconditioning may be different to those which reduce cell death. Thus the failure to demonstrate protection in this study either reveals a peculiarity of this model of stunning or of the right ventricle.

In this series of experiments, LDH data support the hypothesis that this model is a model of stunning rather than a cell death model because improvements in cardiac function in preconditioned groups were not accompanied by a reduction in cell death assessed by LDH measurements. Despite an improvement in post-ischaemic function in ischaemic preconditioned and β-adrenoceptor preconditioned tissues, there was no difference in LDH

release between groups, either during ischaemia or at reperfusion. As LDH is only released from cells when the sarcolemma is damaged, LDH release is a good surrogate marker for cell death. The similar amount of cell death in all groups supports the hypothesis that any improvement in function was due to an anti-stunning effect.

However, because LDH could be measured in the KHB, cell death was evidently occurring to some extent in all experimental groups, indeed cell death could be observed in time controls which were not made ischaemic. The cell death may well have been most prevalent in the core of the tissue which is likely to be hypoxic because it relies on diffusion for the delivery of oxygen. Continuous cell death is a limitation of the model used. The cell death data were collected for the ventricular preparations only, because the mass of atrial preparations was extremely small in comparison to the size of the organ bath and therefore any LDH release was diluted to very low concentrations which could not be reliably measured using the assay employed in this study.

It has been suggested that ischaemia-induced release of catecholamines in the heart is a mechanism by which ischaemic preconditioning induces a protective state (Huang *et al.*, 2007). Catecholamine release and subsequent activation of β-adrenoceptor has also been implicated in the mechanism of cardioprotection achieved by administration of opioids (β_2) (Lange *et al.*, 2006) and anaesthetics (β_1) (Carr *et al.*, 1997). However, controversy remains as to whether activation of β-adrenoceptors is an essential part of ischaemic preconditioning, or whether there are redundancies in the pathway which lead to protection by alternative pathways (see

Section 1.5.1). This is an important question in the clinic, where β-adrenoceptor antagonists are administered for the treatment of angina, hypertension and heart failure (Kloner & Rezkalla, 2006). Ischaemic preconditioning can be used as a protective strategy prior to cardiac surgery. It is thought that angina attacks may also induce preconditioning that can be protective against the damage sustained during a later myocardial infarction (Iliodromitis *et al.*, 2004). Therefore, it is important to understand how these protective phenomena may be modified by commonly used β-blocking medication.

In this study, the beneficial effects of ischaemic preconditioning on post-ischaemic recovery were blocked by the non-selective β-adrenoceptor antagonist, propranolol, suggesting an important role for β-adrenoceptors in ischaemic preconditioning in this model. However, the timecourse of β-adrenoceptor mediated and ischaemic preconditioning was different, ischaemic preconditioning led to improved contractile function early after reoxygenation (15 minutes) but not after 60 minutes. At this later time point, however β-adrenoceptor –agonist pre-treatment led to improved function. Furthermore, tissues preconditioned by ischaemia and by β-adrenoceptor stimulation behaved differently from one another during ischaemia. Ischaemic preconditioning led to a reduction in the magnitude of ischaemic contracture which is consistent with a protective effect. Ischaemic contracture is of the rigor type, and develops under conditions where ATP concentrations are insufficiently high to break actin-myosin cross-links (Kolocassides *et al.*, 1995). Rigor contracture can lead to damage to the cytoskeleton and thus any reduction in the magnitude of contracture is likely to be protective. In contrast, β-adrenoceptor stimulation by isoprenaline did not reduce the magnitude of contracture in either atria or ventricles even in tissues where there was improved recovery at 60 minutes. Thus,

ischaemic preconditioning may involve wider ranging beneficial effects than those seen with β-adrenoceptor-mediated preconditioning.

In agreement with studies in whole hearts (Kolocassides *et al.*, 1995; Spear *et al.*, 2007; Tong *et al.*, 2005), ischaemic preconditioning in ventricular preparations reduced the time taken to achieve maximum contracture. It is difficult to predict how ischaemic tissues will recover based on the profile of ischaemic contracture (Pantos *et al.*, 2006). However, the difference seen in these experiments between ischaemic and β-adrenoceptor-mediated preconditioning suggests that different mechanisms may be involved in each.

It is possible that positive inotropes such as isoprenaline could result in preconditioning by causing the energy demands of the tissue to exceed the capacity of diffusion to supply the substrates, and remove the waste products of metabolism. Indeed it has been noted that rapid pacing of the heart can mimic preconditioning by inducing ischaemia (Lochner *et al.*, 1999; Robinet *et al.*, 2005). Such an effect might be exaggerated in an a model such as that used in this study, in which there is no flow of buffer through blood vessels in the tissue and the core of the preparation is entirely dependent on diffusion to receive oxygen and nutrients. In this study none of the subtype-selective β-adrenoceptor agonists mimicked preconditioning in the same way as isoprenaline, but these agents also failed to induce positive inotropy to the same extent as isoprenaline. However, there is a large body of evidence, from in vivo and Langendorff experiments, in which the myocardium is inevitably better supplied with perfusate via the coronary vasculature and therefore do not depend on the diffusion of metabolic substrates

over large distances, that activation of protein kinases cascades as a result of β-adrenoceptor activation results in the heart undergoing adaptive changes and becoming resistant to later ischaemic damage.

No beneficial effects of postconditioning against stunning were seen in either tissue. The protocol (3 x 1 minute normoxia followed by 1 minute simulated ischaemia) was chosen largely due to methodological constraints, because the process of inducing simulated ischaemia was complicated using the available apparatus and 1 minute was the shortest period for which this could be feasibly achieved. However other groups have demonstrated improved functional recovery by postconditioning with a similar protocol (4 x 1 minute normoxia followed by 1 minute simulated ischaemia) in paced human atrial trabeculae (Sivaraman *et al.*, 2007). However that study utilised a longer period of ischaemia (90 minutes) and therefore improved recovery was probably due to reduced cell death whereas this study was designed as a stunning model (Walker *et al.*, 1994). Thus the failure to see postconditioning protection in this study may have been a result of: i) The inability of postconditioning to protect against stunning in the rat (postconditioning has already been shown to be ineffective against stunning in the rabbit and dog) (Couvreur *et al.*, 2006). ii) The use of insufficient cycles of postconditioning (3 v. 4). iii) Species differences in the algorithm required to elicit postconditioning.

The present study is limited by the fact that the model uses atrial appendage tissue and right ventricular strips rather than left ventricle which is of more functional importance. However

these tissues were chosen, because of their thin walls, in order to minimise the limitations to the tissue caused by reliance on diffusion for the delivery of oxygen and nutrients.

In terms of physiological relevance, this model falls between isolated whole hearts and cell culture, but is not subject to the problems of cell differentiation seen in culture and is not complicated by the effects upon coronary flow which would be expected when β-adrenoceptor agonists are administered in a Langendorff preparation. Indeed, the effects seen in this model must occur at the level of the myocardium. The most clinically relevant finding of this study was that the functional improvements of ischaemic preconditioning were blocked by propranolol. This finding mirrors the result of other studies (Laskey, 2005) in which β-adrenoceptor activation has been shown necessary for the protection of ischaemic preconditioning. This study has proved inconclusive in determining the receptor subtype involved in these effects, and this issue requires further investigation.

In conclusion, it has been demonstrated that isolated left atria and right ventricles can be used as a convenient model of ischaemic and pharmacological preconditioning against stunning. Ischaemic- and β-adrenoceptor-mediated preconditioning both led to an improvement in post ischaemic contractile function. The protective effects in both cases were abrogated by propranolol. However, the timecourse of protection during ischaemia and reoxygenation was different in each case suggesting that ischaemic preconditioning involved more complex mechanisms than the simple release of catecholamines and subsequent signalling activation of β-adrenoceptors.

3.5.1 Clinical relevance

Because many patients with cardiovascular diseases take β-adrenoceptor antagonists, these drugs may have the potential to abrogate the effects of ischaemic preconditioning induced prior to cardiac surgery (Kloner *et al.*, 2006) or as a result of angina attacks (Hieble, 2000). This may therefore be an undesirable consequence of β-adrenoceptor antagonist therapy

3.6 Conclusions

- Ischaemic preconditioning can protect against stunning in isolated rat atria and ventricles. This protection is dependent on β-adrenoceptor activation.

- β-adrenoceptor preconditioning can protect against stunning in isolated rat atria.

- The protocol of ischaemic postconditioning employed in this study does not protect against stunning.

Chapter 4 What effects do endogenous and exogenous adrenaline have upon the heart at reperfusion?

4.1 Introduction

The activation of adrenoceptors by catecholamines has important physiological and therapeutic roles in myocardial ischaemia and at reperfusion. The onset of myocardial ischaemia triggers the release of adrenaline from the adrenal medulla, as a result of fear and anxiety. An increase in sympathetic discharge also results in the release of noradrenaline from sympathetic neurones within the heart (Foley *et al.*, 1987; Kern *et al.*, 1989; Lindner *et al.*, 1996a). In ischaemic regions, metabolic alterations during prolonged ischaemia lead to the emptying of neuronal noradrenaline vesicles into the sarcoplasm. A reversal of the noradrenaline uptake$_1$ transporter releases the neurotransmitter into the synaptic cleft where it is accumulates. This noradrenaline release is independent of neuronal activity. Some synaptic noradrenaline spills over into the circulation and can be measured (Schoemig & Richard, 1991).

One consequence of severe myocardial ischaemia is cardiac arrest. This occurs when myocardial damage is so severe that the rhythmic control of the heart is lost and the heart enters a non-pumping state, either ventricular fibrillation, pulseless electrical activity, asystole or pulseless ventricular tachycardia (International Liaison Committee on Resuscitation, 2005). The massive fall in blood pressure accompanying cardiac arrest leads to further catecholamine release (Schoemig *et al.*, 1991). Thus, cardiac arrest results in a raised plasma catecholamine

state. Raised catecholamine concentrations are maintained long after cardiopulmonary resuscitation (Niemann & Garner, 2005). The treatment of cardiac arrest includes intravenous injections of adrenaline, designed to cause peripheral vasoconstriction. The administration of adrenaline during cardiac arrest is therefore a mimic of the natural response and both endogenous and exogenous adrenaline can be present in the heart during ischaemia and reperfusion and thus have the potential to modify the process of ischaemic damage and reperfusion injury. High endogenous levels of catecholamines during cardiopulmonary resuscitation are correlated with poor survival (Lindner *et al.*, 1996a). This raises the possibility that increasing catecholamine levels further through exogenous administration is detrimental.

In Britain, it is recommended that 1 mg of adrenaline should be given intravenously every three minutes during cardiac arrest (Mehta, 2006) although international guidelines suggest dosing every three to five minutes (International Liaison Committee on Resuscitation, 2005). This disparity probably reflects the fact that the guidelines are based on best practice and collective wisdom rather than evidence-based medicine.

4.1.1 Rationale for the use of adrenaline in cardiopulmonary resuscitation

During a cardiac arrest, patients are treated using cardiopulmonary resuscitation. This includes chest compressions and artificial ventilation. Adrenaline is administered intravenously (International Liaison Committee on Resuscitation, 2005). The rationale for the use of this agent is that it causes an increase in blood pressure by increasing peripheral resistance. Crile and

Dolley (1906) stated *"The value of adrenalin in raising the blood pressure, by its action upon the vascular walls in the state of suspended animation".*

Vasopressors also prevent arterial collapse under the increased intrathoracic pressure caused by chest compressions (Michael *et al.*, 1984). Aortic diastolic pressure, the main determinant of coronary blood-flow is increased (Brown & Werman, 1990; Michael *et al.*, 1984). Maintenance of coronary blood-flow is the most important factor in enabling return of spontaneous circulation (ROSC) or successful defibrillation (Babbs *et al.*, 2001; Kern *et al.*, 1988; Paradis *et al.*, 1990; Paradis *et al.*, 1991; Zhong & Dorian, 2005)

The peripheral vasoconstrictor effects of adrenaline are as a result of the activation of α-adrenoceptors, particularly in arterioles in the kidney, mucosa and skin and also in many veins (Hoffman, 2001; Hoffman & Taylor, 2001). Catecholamines such as adrenaline do not penetrate the blood-brain-barrier (Dahlgren *et al.*, 1980) and cannot act on central adrenoceptors when given systemically. Changes in systemic blood pressure, therefore, largely dictate alterations in cerebral blood flow (Dahlgren *et al.*, 1980; Hoffman, 2001). Thus, the increased blood pressure achieved by peripheral vasoconstriction improves blood-flow in the coronary and cerebral circulation, protecting the most vital organs from ischaemic damage and improving the chances of achieving ROSC.

However, in addition to its vascular effects, adrenaline also acts upon adrenoceptors in the heart where it has the potential to cause harmful effects which may account for the poor

results with this agent in the clinic. In particular adrenaline increases the energy demand of the fibrillating heart (Ditchey & Lindenfeld, 1988; Ditchey *et al.*, 1994a; Ditchey & Slinker, 1994b; Monroe & French, 1960; Monroe & French, 1961) thus worsening ischaemia and its positive inotropic and chronotropic actions (Ahlquist, 1948) will increase the energy demands of the heart if normal cardiac rhythm is restored.

4.1.2 Clinical evidence for the (in)effectiveness of adrenaline

Despite its widespread use, a large body of experimental evidence from animal studies suggests that adrenaline may have no effect, or even a detrimental effect on various measures of well-being and survival during resuscitation (Brown *et al.*, 1988; Klouche *et al.*, 2003; Niemann *et al.*, 2005; Schwartz & Lagranha, 2006; Wenzel *et al.*, 1999). High doses do not improve survival and may increase adverse effects (Ditchey *et al.*, 1988; Hilwig *et al.*, 2000; Hornchen *et al.*, 1993; Lindner *et al.*, 1991a; Voelckel *et al.*, 2000). Poor survival after resuscitation with adrenaline has also been seen in several clinical trials (Barton & Callaham, 1991; Behringer *et al.*, 1998; Brown *et al.*, 1992; Callaham *et al.*, 1991; Callaham *et al.*, 1992; Gonzalez *et al.*, 1989; Gueugniaud *et al.*, 1998; Herlitz *et al.*, 1995; Holmberg *et al.*, 2002; Lindner *et al.*, 1991b; Lipman *et al.*, 1993; Marwick *et al.*, 1988; Michael *et al.*, 1984; Olson *et al.*, 1989; Paradis *et al.*, 1990; Paradis *et al.*, 1991; Sherman *et al.*, 1997; Woodhouse *et al.*, 1995). Trials have been designed to answer one or more of the following questions:

- Is adrenaline more effective than placebo control in cardiopulmonary resuscitation?

- Does high dose adrenaline produce better results than the standard dose?

- Are subtype-specific adrenoceptor agonists superior to adrenaline?

- Is the response to adrenaline improved when specific antagonists are given concurrently?

- Do non-adrenergic vasopressors produce better results than adrenaline?

Although a large number of studies have been carried out, interpretation of the results is difficult because of the variety of endpoints used in studies. These have ranged from the most basic studies which used the restoration of spontaneous circulation as the endpoint (taking no account of the fate of the patient thereafter) to complex studies which have monitored survival over a number of months and have included measures of patients neurological function. Most studies have been conducted on a small scale, presumably due to limited funding and facilities. These studies therefore have had limited power to detect differences between treatment groups. Many studies have included inherent scientific weaknesses, such as not being blinded to the physician or not including a placebo control. Several studies that were blinded included a direction that adrenaline be administered to the patient if the trial drug failed. These directions have been included on the basis of sound ethical reasoning; however, they make it difficult to draw definitive conclusions.

Numerous studies have examined the effects of high and standard doses of adrenaline. The current guidelines for the administration of adrenaline allow for the repeated administration of the drug, and this too was the case for experimental agents. Where doses of drug are cited in the text below, they refer to the dose given in each individual bolus rather than the total dose administered except where stated otherwise.

4.1.2.1 Comparison of standard-dose adrenaline and placebo

For ethical reasons, no blinded randomised trials of adrenaline in the resuscitation setting have included a placebo control group (American Heart Association, 2005). Several investigators have compared the outcome of groups of patients treated with adrenaline with those not treated with adrenaline. This comparison is inherently biased, however, because patients treated with adrenaline are more likely to be complicated cases that do not respond to initial defibrillation. This makes interpretation of the data very difficult.

A retrospective study was carried out in Sweden, examining the role of adrenaline in treating out-of-hospital cardiac arrest over a 12-yr period. The records of 1360 patients were examined. Adrenaline was given to 35% of patients and did not result in any change in the survival of patients to hospital discharge compared to untreated patients. However, as the authors conceded the trial was not randomised, and a major confounding factor was that the patients treated with adrenaline were generally those who presented with more complicated clinical conditions (Herlitz *et al.*, 1995) Later, a larger (10,966 patients) prospective evaluation of adrenaline in cardiopulmonary resuscitation was carried out by the same group of researchers.

A more clinically useful endpoint, survival to one month after cardiac arrest, was employed. Using multivariate analysis, the use of adrenaline was found to be independently correlated with poor survival, especially in patients presenting with non-shockable cardiac rhythms (Holmberg *et al.*, 2002). This study was also limited by the lack of randomisation and differences in baseline variables between patient groups. An Australian group compared both high and standard-dose adrenaline with placebo in cardiac arrest. They found no difference in survival to hospital discharge between the groups, and the overall hospital discharge rate was 0.9%. This trial in 339 patients probably represents the best attempt at conducting a placebo-controlled clinical trial of adrenaline in cardiopulmonary resuscitation to date. However, the results must be taken with caution, as the trial was not well randomised and nearly half the patients were treated with un-blinded standard-dose adrenaline (Woodhouse *et al.*, 1995).

One study recruited 199 patients in ventricular fibrillation who had failed to respond to 1 defibrillation attempt. The patients were randomised to receive endotracheal adrenaline or lidocaine to compare the effectiveness of these drugs in resuscitation. These patients were compared to a group of 630 historical controls, treated with only sodium bicarbonate. The results of this study are hard to determine, as the number of patients available for analysis was reduced by poor compliance with study protocol. Survival to hospital discharge was significantly smaller (16%) in patients treated with adrenaline than in patients treated with sodium bicarbonate or no drug (38%) (Olson *et al.*, 1989).

In conclusion, no trial has identified a benefit of adrenaline over placebo in cardiopulmonary resuscitation, although it should be borne in mind that the quality of the investigations carried out to date is rather poor.

4.1.2.1 High-dose adrenaline

The standard dose of 1 mg adrenaline was taken directly from early studies in dogs conducted by Redding and Pearson (Pearson & Redding, 1964a; Pearson & Redding, 1963; Pearson & Redding, 1964b; Ralston & Babbs, 1985). More recently, concern was raised that due to the difference in mass between the dogs used in the study and an adult human, the dose of adrenaline used in the study was too small. Additionally, it was shown that at doses above 1 mg, a dose-dependent vasopressor relationship existed for adrenaline in human beings during cardiopulmonary resuscitation (Gonzalez *et al.*, 1989). In other words, 1 mg is a sub-maximal vasopressor dose. In dogs, it was shown that the peripheral vasoconstrictor effects of adrenaline were responsible for the beneficial effects (Michael *et al.*, 1984). Thus, numerous trials have been carried out to compare the effects of high-dose adrenaline (up to 10 mg) with standard protocol. Some of these trials are of a high standard, being both randomised and blinded.

In the 1990s Barton and Callaham published a series of papers investigating treatment of cardiac arrest with standard-dose and high-dose adrenaline. They initially carried out a small trial on 49 patients. Apart from its small size, the trial suffered considerable weaknesses, in that it was not blinded and not randomised and the data were gathered retrospectively. However, a

trend towards a higher rate of restoration of spontaneous circulation was seen in high-dose-treated patients (Barton *et al.*, 1991).

These investigators set out to determine whether high-dose adrenaline was associated with a higher incidence of complications than normal dose. Sixty-eight patients with non-traumatic cardiac arrest were recruited into the study. The study was not randomised, and physicians treated as they saw fit. Patients were retrospectively allocated to the high-dose or low-dose group, depending on whether they had received more or less than 2.8 $\mu g\ kg_{-1}\ min^{-1}$. Additionally those patients who had received a bolus of 50 $\mu g\ kg_{-1}$ were included in the high-dose group (Callaham *et al.*, 1991). The study was therefore somewhat flawed, as it was not comparing 2 distinct doses rather it was looking at a continuous scale with an arbitrary dividing line. The only significant difference between the 2 trials in terms of complications was a greater depression of serum calcium in the high-dose adrenaline group. No significant difference was seen in survival to hospital discharge, although 30% of patients in the standard-dose group survived compared to 18% of the high-dose treated patients. Although no conclusions can be drawn in the absence of statistical significance, the trial was probably underpowered to detect a survival difference and the results suggest better survival in the low-dose-treated group.

The same group of investigators went on to carry out a randomised clinical trial of high-dose adrenaline and standard dose adrenaline in pre-hospital cardiac arrest. The study enrolled 816 patients. Noradrenaline was also examined as a vasopressor in this trial, and the results of this part of the study are discussed elsewhere. In addition to restoration of spontaneous circulation,

additional outcome measures were included. These were survival to hospital admission, survival to hospital discharge, and their Cerebral Performance Category score (a measure of mental function and capacity) measured whilst the patients were in hospital. High-dose adrenaline increased the probability of achieving restoration of spontaneous circulation. In the high-dose group, circulation was restored in 13% of patients compared to 8% for standard-dose adrenaline. However, no statistically significant increase to hospital discharge was noted, although this trial was probably underpowered to detect any such change (Callaham *et al.*, 1992).

At around the same time, another group carried out a randomised trial of standard- and high-dose adrenaline, specifically during asystole and electromechanical dissociation. The trial included 68 adult patients and was blinded. Survival to hospital discharge was the primary endpoint. There was a trend towards a higher rate of discharge in the high-dose adrenaline group although this did not reach statistical significance. Blood pressure was higher at one and five minutes post resuscitation and the rate of initial restoration of circulation was better in high-dose-treated groups (57% v. 15%) and there was no difference in the incidence of adverse effects between groups (Lindner *et al.*, 1991a).

Paradis' group have long been interested in resuscitation. In one early study they examined the effects of coronary perfusion pressure on the restoration of spontaneous circulation. The trial was relatively small, recruiting 100 patients. In addition to the main focus of the study, the investigators also analysed some data relating to patients in the study who had been treated

with high-dose rather than standard-dose adrenaline. No difference was seen in terms of achievement of restoration of spontaneous circulation and no patients in either group survived to hospital discharge. Results from the trial gave some indication that high coronary perfusion pressures were correlated with good rates of restoration of spontaneous circulation, confirming the data seen elsewhere in animal models. It was tentatively suggested that a coronary perfusion pressure of 15mmHg might be a cut-off point, below which restoration of spontaneous circulation was unlikely or impossible (Paradis *et al.*, 1990). The group therefore went on to carry out a small clinical trial (32 patients) designed to address specifically the question of how high- and low-dose adrenaline affected coronary perfusion pressure during cardiopulmonary resuscitation. They discovered that high-dose adrenaline was more effective than standard dose at raising the coronary perfusion pressure above their previously defined critical level; and hence, they suggested that high-dose adrenaline may be more effective at achieving restoration of spontaneous circulation than the standard dose (Paradis *et al.*, 1991).

The Multicenter High-Dose Epinephrine Study Group performed a large trial comparing standard- and high-dose adrenaline in cardiac arrest outside the hospital. 1280 patients were randomised to receive either 0.02 $mg.kg^{-1}$ or 0.2 $mg.kg^{-1}$ given intravenously. The study included patients with any presenting rhythm and examined multiple endpoints including restoration of spontaneous circulation, survival to hospital admission, survival to hospital discharge, and neurological outcome. No difference could be found between the two groups with respect to any of these outcomes. The authors attributed this failure to the delay in reaching the patient and the extensive ischaemic damage that had already occurred (Brown *et al.*, 1992).

At one hospital, the radical alteration of resuscitation protocols allowed a comparison to be made. Initially no adrenaline was used in resuscitation; however, the revised guidelines directed physicians to use high dose adrenaline (10mg). A retrospective analysis was then carried out, investigating the success of resuscitation before and after the protocol was changed. Immediate survival was 43% in patients who did not receive adrenaline but only 22 % in high-dose-treated patients. This difference was statistically significant (Marwick *et al.*, 1988). The weakness of the study was that, as a retrospective analysis, it could not be randomised; however, the study focused on patients presenting with ventricular fibrillation after failure to respond to initial defibrillation, and was thus more focussed than other studies which recruited patients with numerous presenting rhythms and causes of cardiac arrest. Of 210 patients, 77 received high-dose adrenaline and the rest received no drug. Multiple logistic regression analysis showed adrenaline to be an independent predictor of poor outcome but no significant difference was seen in survival to hospital discharge. The authors suggested that this might be due to the very small number of patients who survived to be discharged. One study attempted to determine whether high-dose adrenaline could exert any beneficial effects after the failure of standard therapy (Sherman *et al.*, 1997). Although small (140 patients), this study had the advantage of being blinded and randomised. No benefit of high-dose adrenaline was seen in terms of any of the outcome measures. It should be remembered that the ischaemic damage to the heart and other organs are likely to be very high after initial treatments have been tried and have failed. It would be surprising if any intervention could be of benefit at this stage.

The European Epinephrine Study Group conducted a large multicentre randomised trial (3327 patients), comparing the use of high-dose (5 mg) and standard-dose (1 mg) adrenaline in

cardiac arrest outside the hospital (Gueugniaud *et al.*, 1998). The investigators found a small increase in the likelihood of restoring circulation when high-dose adrenaline was used (40.4% vs. 36.4 %). Analysis of subgroups revealed that high dose adrenaline improved the chances of restoring circulation in patients with asystole but not when ventricular fibrillation was the presenting rhythm. No difference was seen between the groups in terms of survival to hospital discharge or in terms of neurological outcome. One group carried out a randomised, blinded trial, comparing standard (1 mg) and high (10 mg) doses of adrenaline in asystole. The investigators did not find any difference in survival between the groups in terms of survival although the study was extremely small, recruiting only 40 patients and was thus vastly underpowered to detect any such changes (Lipman *et al.*, 1993).

A retrospective study of 178 patients in ventricular fibrillation showed that high cumulative doses of adrenaline given during resuscitation were correlated with poor neurological outcome as assessed by the cerebral performance score (Behringer *et al.*, 1998).

In conclusion, it seems that high-dose adrenaline is more efficient at achieving a restoration of spontaneous circulation than the standard dose, but no trial has shown an increase in survival to hospital discharge or any improvement in long-term neurological function in survivors. On this basis, the use of high-dose adrenaline may be unwarranted; the benefit to the patient of achieving restoration of spontaneous circulation but dying soon after certainly seems negligible. One interpretation of this data is that the increased benefit from the additional

vasopressor effect of high-dose adrenaline is counterbalanced by increased cardiac adverse effects.

4.1.2.2 Other adrenergic agents

Noradrenaline has been compared to adrenaline in one randomised clinical trial of out-of-hospital resuscitation. No significant difference was seen between standard-dose adrenaline and noradrenaline (11 mg). However, there was a trend towards a better initial achievement of restoration of spontaneous circulation but worse neurological recovery in patients treated with noradrenaline (Callaham *et al.*, 1992).

One randomised study including 102 patients set out to compare adrenaline with the α_1-adrenoceptor selective agonist, methoxamine, in resuscitation from ventricular fibrillation (Olson *et al.*, 1989). The study was carefully designed and equipressor doses of each drug were given. Methoxamine was less effective at achieving restoration of spontaneous circulation than was adrenaline. However this study was very interesting, since the dose of adrenaline given was 0.5 mg. This is half the clinically recommended dose. However, 19.6% of patients in the adrenaline group were discharged alive from hospital, a reasonably high figure by the standards of resuscitation trials. This result should be followed up with a larger comparison of standard- and low-dose adrenaline and ideally a placebo group to discover, firstly, whether or not adrenaline is beneficial in resuscitation, and, if this is the case, to enable the optimal dose of adrenaline to be found.

4.1.2.3 Non-adrenergic vasopressors

After initial successes in animal models and the observation that vasopressin can increase coronary perfusion pressure during human cardiopulmonary resuscitation (Morris *et al.*, 1997), several studies of resuscitation using vasopressin in humans have been carried out despite the fact that this drug is known to be a coronary vasoconstrictor (Martinez *et al.*, 2003). A small, early study (8 patients), suggested that vasopressin may be more effective than adrenaline at achieving restoration of spontaneous circulation from cardiac arrest (Lindner *et al.*, 1996b). These researchers went on to echo their findings in a larger study (40 patients; (Lindner *et al.*, 1997)) which recruited patients in ventricular fibrillation and showed a benefit of vasopressin over adrenaline in initially restoring circulation. Stiell carried out a randomised, triple blind, controlled trial, comparing adrenaline (1 mg), and vasopressin (40 U) in cardiac arrests which occurred in the hospital. Two hundred patients were recruited into the study, which found no difference between the patient groups in terms of 1-hr survival or survival to hospital discharge. The authors concluded that vasopressin should not be recommended for cardiopulmonary resuscitation (Stiell *et al.*, 2001). In the biggest comparison of vasopressin and adrenaline carried out so far, 1219 patients who had suffered cardiac arrest outside the hospital were randomised to receive either adrenaline (1 mg) or vasopressin (40 U). One of the endpoints used was survival to hospital admission. Although no overall difference was seen between the groups, the large number of patients recruited into the study enabled analysis of subgroups to be carried out. It was found that when the presenting rhythm was asystole, patients treated with vasopressin were more likely to survive to hospital discharge than adrenaline-treated patients (Wenzel *et al.*, 2004).

4.1.2.4 Vasopressor combinations

One study including 298 patients found that administering adrenaline together with vasopressin resulted in a greater rate of success than when adrenaline was used alone. The authors suggested that the synergistic vasopressor effects of the 2 drugs allowed smaller than usual doses of each to be given, thus reducing adverse effects. In fact, in this trial the same total dose of adrenaline (3.8 mg) was given to both groups of patients (Guyette *et al.*, 2004).

4.1.2.5 Conclusions from clinical trials

There are no studies that show adrenaline to be better than placebo in terms of survival. Higher than standard adrenaline doses do not lead to better results and may in fact lead to worse outcomes.

4.1.3 Possible reasons for the ineffectiveness of adrenaline

Adrenaline is a non-selective α and β adrenoceptor agonist, released by the adrenal medulla in response to stress. The cardiovascular effects of adrenaline are complex, due to the fact that several subtypes of adrenoceptors are present in the heart and in the vasculature and physiological responses are usually the result of a combination of effects.

In ventricular myocardium and the sino-atrial node, adrenaline acts predominantly on β_1-adrenoceptors to mediate positive chronotropic and inotropic effects. This leads to an increase in oxygen demand in the myocardium. In the ischaemic heart, activation of β-adrenoceptors

can therefore lead to a worsening of ischaemia. During ventricular fibrillation, adrenaline also increases the energy demands of the myocardium (Monroe *et al.*, 1960; Monroe *et al.*, 1961).

The stimulation of α_1-adrenoceptors may also be undesirable in resuscitation because they are also found in the heart, where they mediate a positive inotropic effect, leading to an increased oxygen demand in the myocardium. This has been demonstrated both in the rat (Broadley *et al.*, 1999; Williamson & Broadley, 1989) and in human subjects (Landzberg *et al.*, 1991). Although it should be noted, that when a non-selective adrenoceptor agonist such as adrenaline is given, the predominant effect on inotropy is likely to be through β-adrenoceptors. Despite the apparent contraindications to α_1 stimulation, some investigators have studied the possibility of using methoxamine, an α_1-adrenoceptor agonist in the resuscitation setting. Two studies compared the use of this agent with adrenaline. In a canine model of resuscitation, methoxamine was found to be superior to adrenaline (Roberts *et al.*, 1990), however in a clinical study in man adrenaline was superior (Olson *et al.*, 1989).

The cardiodynamics of adrenaline are complicated because it can cause vasoconstriction (α-adrenoceptors) and vasodilatation (β-adrenoceptors). However high-doses of adrenaline can result in coronary vasoconstriction (Karch, 1989). This presents a major limitation to the use of adrenaline in resuscitation.

Adrenaline can be oxidised *in vivo* by ceruloplasmin, a plasma α-globulin, by polymorphonucleocytes and by cytochrome oxidase (Behonick *et al.*, 2001). Oxidation products of adrenaline, of which adrenochrome is the main example (Green & Richter, 1937) have been

demonstrated to inhibit catechol-o-methyl transferase, an enzyme responsible for the inactivation of catecholamines (Abbs *et al.*, 1967). This has the potential to further increase the concentrations of active catecholamines.

The oxidation products of catecholamines have been known for some time to cause damage to myocardial tissue (Dhalla *et al.*, 1978; Yates & Dhalla, 1975) although it is not known if they are more or less harmful than adrenaline itself (Behonick *et al.*, 2001; Bindoli *et al.*, 1992). It has been demonstrated that oxidation of catecholamines is partially responsible for the increased levels of reactive oxygen species observed during ischaemia. High levels of oxygen radicals are partly responsible for post-ischaemic contractile dysfunction because they cause peroxidation of membrane phospholipids, disrupting the structure of the membrane and increasing its permeability. This leads to cell death by necrotic mechanisms (Lazzarino *et al.*, 1994; Moens *et al.*, 2005).

The aim of this study was therefore to investigate the effects of exogenous adrenaline on stunning (Chapter 4, Part 1) and infarct size (Chapter 4, Part 2) in order to explore the possibility that the poor outcome of cardiopulmonary resuscitation when adrenaline is used is due to cardiac actions of adrenaline. Additionally the effects of endogenous catecholamines were examined by the use of selective and non-selective antagonists in the stunning model (Chapter 4 Part 1). Experiments investigating the effects of adrenoceptor antagonists on infarct size are described later in the thesis (Chapter 5).

Chapter 4 Part 1: How does exposure to adrenaline and adrenoceptor antagonists at reperfusion affect stunning in isolated rat atria and ventricles ?

4.2 Hypotheses

- Exogenously applied adrenaline will have a detrimental effect upon contractile recovery when applied at reperfusion.

- β-adrenoceptor antagonists applied at reperfusion will improve contractile recovery after ischaemia by preventing the pharmacological actions of endogenous catecholamines.

4.3 Methods

Male Sprague Dawley rats were purchased from B and K universal, (Grimston UK) and weighed 300-450g at the time of killing. Isolated left atrial and right ventricular preparations were prepared as described above (Section 2.4). Initial diastolic tension was 1g for atrial tissues and 1.5g for ventricular tissues. Tissues were allowed to stabilise for 20 minutes before induction of simulated ischaemia. Reoxygenation and return to standard KHB occurred after 30 minutes. Preparations were left to recover for one hour during which time the KHB was replaced every fifteen minutes. Preparations were exposed to a range of β-adrenoceptor ligands with different actions and selectivities (Table 4-1). The drugs were administered 10 minutes prior to

reperfusion. At reperfusion, the drugs were replaced in the organ bath and remained there for fifteen minutes.

Table 4-1 A summary of the pharmacological actions of the agents used in this study and the concentrations at which they were used

Drug	**Concentration**	**Pharmacological action**
Adrenaline	10 μM	Non-selective adrenoceptor agonist
Timolol	1μM	Non-selective β-adrenoceptor antagonist
CGP-20712A	10nM	Selective β_1-adrenoceptor antagonist
ICI-118,551	10nM	Selective β_2-adrenoceptor antagonist

Timolol was used as a non-selective adrenoceptor antagonist in these experiments and hereafter in place of propranolol. This was because of the suggestion by a colleague that the 'membrane stabilising' effects of propranolol might confound the results. The study of a list of the pharmacological properties of numerous β-adrenoceptor antagonists (Broadley, 1996) revealed that timolol i) is a non-selective β-adrenoceptor antagonist ii) does not display membrane stabilising effects iii) does not display partial agonist effects (intrinsic sympathomimetic activity). This agent was thus chosen as the non-selective agent to be used in this study and in future experiments.

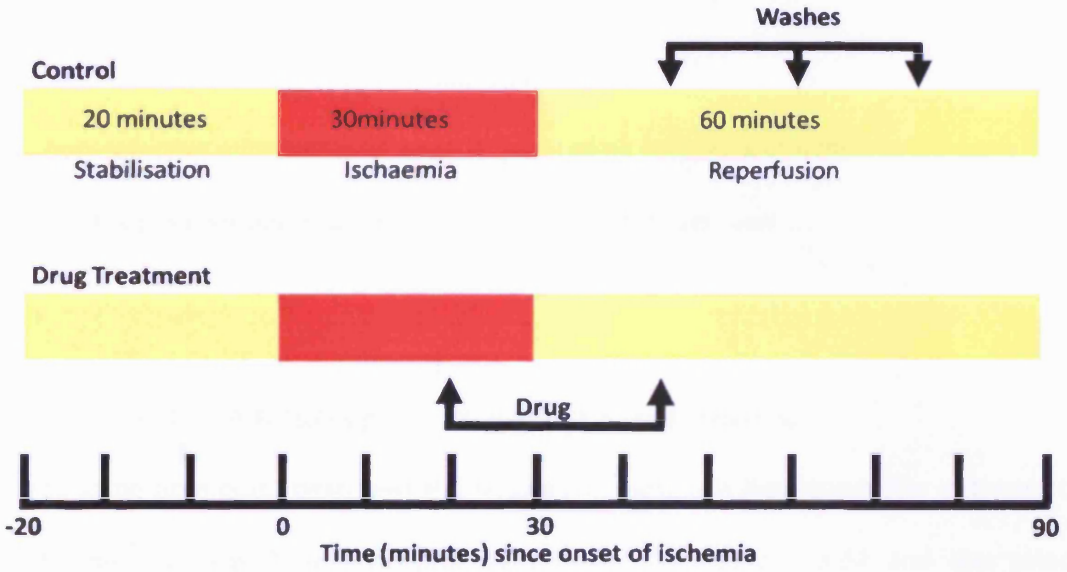

Figure 4-1 Experimental protocols used in this series of experiments

4.3.1 Experimental protocols

Controls received no treatment other than the ischaemic insult. The effects of administering each of the adrenoceptor ligands at reperfusion were investigated (Table 4-1). Adrenaline was administered to mimic the treatment of cardiac arrest, and selective and non-selective adrenoceptor antagonists were used to investigate the effects of endogenous catecholamines. After 20 minutes of simulated ischaemia, a dose of drug was added. The same dose was replaced at the time of reperfusion. The drug was removed from the organ bath by the washing of the tissue at the standard 15 minute intervals according to the protocol described above.

4.3.2 Adrenaline at reperfusion

The non-selective adrenoceptor agonist, adrenaline (10μM) was added to the bath for the final 10 minutes of ischaemia and the first 15 minutes of reperfusion

4.3.3 Adrenoceptor antagonists at reperfusion

At the same time point described above, the non-selective β-adrenoceptor antagonist timolol, 1μM; the selective β_2-adrenoceptor antagonist ICI-118551, 10nM and the selective β_1-adrenoceptor antagonist CGP-20712A, 10nM were added to the bath

4.3.4 Adrenaline at reperfusion in the presence of timolol

The effects of administering adrenaline in the presence of the non-selective adrenoceptor antagonist timolol were examined. Timolol was administered to the tissues at the same time as the adrenaline (10 minutes prior to reperfusion) and was washed out at the same time.

4.3.5 Analysis of data

Prism version 4.02 for windows (GraphPad Software, Inc) was used for production of graphs and statistical analyses. Tension was monitored continuously, enabling calculation of diastolic (baseline) and developed tension. Cardiac function was assessed at fifteen and sixty minutes post reoxygenation. Developed tension at these time points was calculated as a percentage of developed tension at the end of the stabilization period for each preparation. Groups of data were compared by one-way ANOVA followed by Student-Newman-Keuls *post hoc* test.

4.4 Results

4.4.1 Baseline functional data

The baseline developed tension for each experimental group in this study is shown (Table 4-2). There were no statistically significant differences between groups.

Table 4-2.Baseline developed tension for the experimental groups used in this study.

	Atria		Ventricle	
	n	developed tension	n	developed tension
Control	5	0.43±0.07	6	0.84±0.17
Adrenaline	5	0.34 ±0.08	4	0.62±0.13
Timolol	6	0.42±0.04	6	0.76±0.14
Adrenaline + timolol	6	0.46±0.08	5	0.78±0.13
ICI-118,551	3	0.44±0.08	4	0.91±0.11
CGP	5	0.55±0.04	4	1.05±0.06

4.4.2 Contractile profile of control tissues

The contractile profile of a control atrial and ventricular preparations were consistent with data seen previously (Chapter 3 in this thesis).

Measurements of contracture are not reported here, as in the previous chapter, as this study involved only interventions at reperfusion which therefore cannot alter ischaemic contracture. Developed tension increased throughout the recovery period although did not reach pre-ischaemic levels. In ventricular control studies, after 30 minutes of ischaemia, the mean developed tension was 5.7 ± 1.3 % (n=6). Reperfusion caused mean developed tension to rise to a plateau with a maximum of 86.2 ± 6.9 % after 60 minutes (Figure 4-3 B). In atrial control studies, after 30 minutes of ischaemia, the mean developed tension was 9.9 ± 1.11 % (n=5). Reperfusion caused mean developed tension to rise constantly with a maximum of 47.8 ± 4.5 %after 60 minutes of reperfusion (Figure 4-3 A).

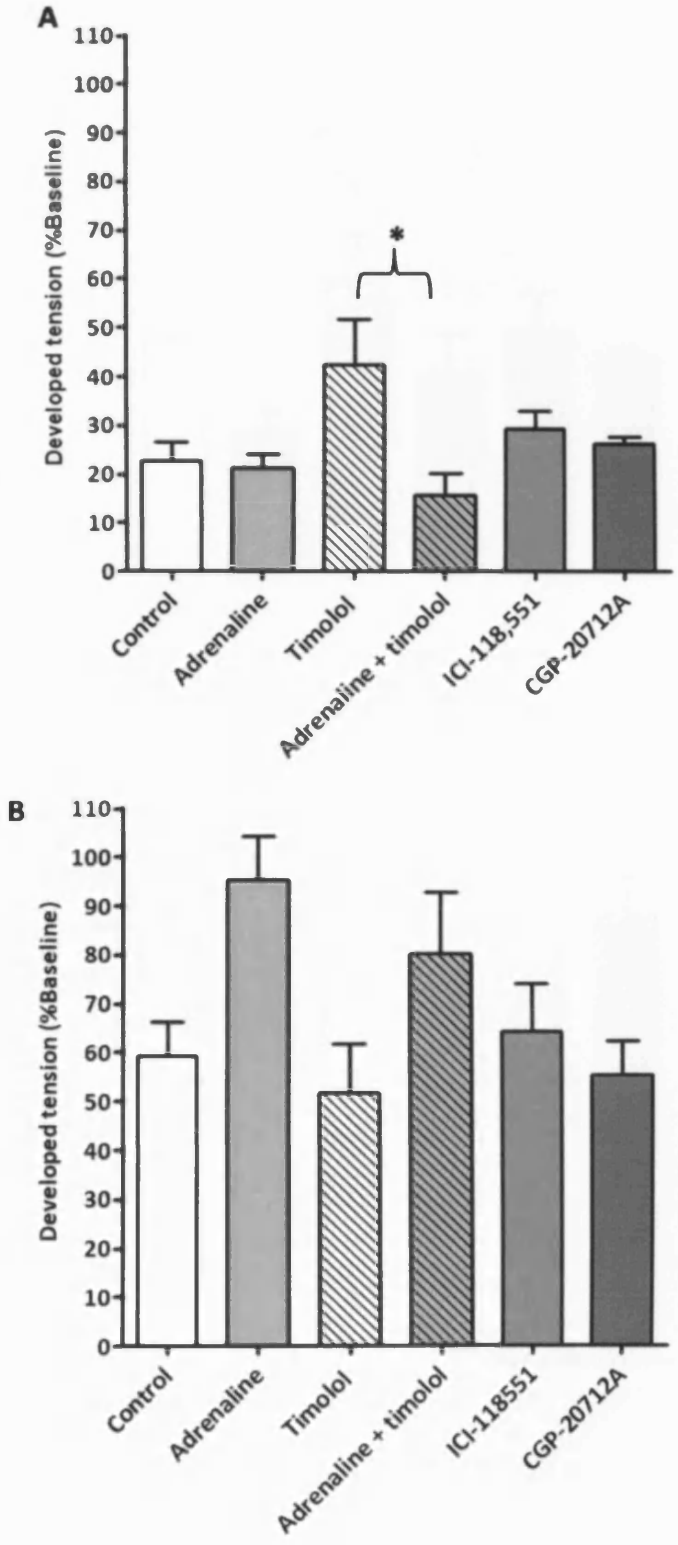

Figure 4-2 Developed tension after 15 minutes reoxygenation in A) atria and B) ventricular strips. Developed tension is expressed as a percentage of the developed tension achieved at the end of the stabilisation period. * indicates $p<0.05$.

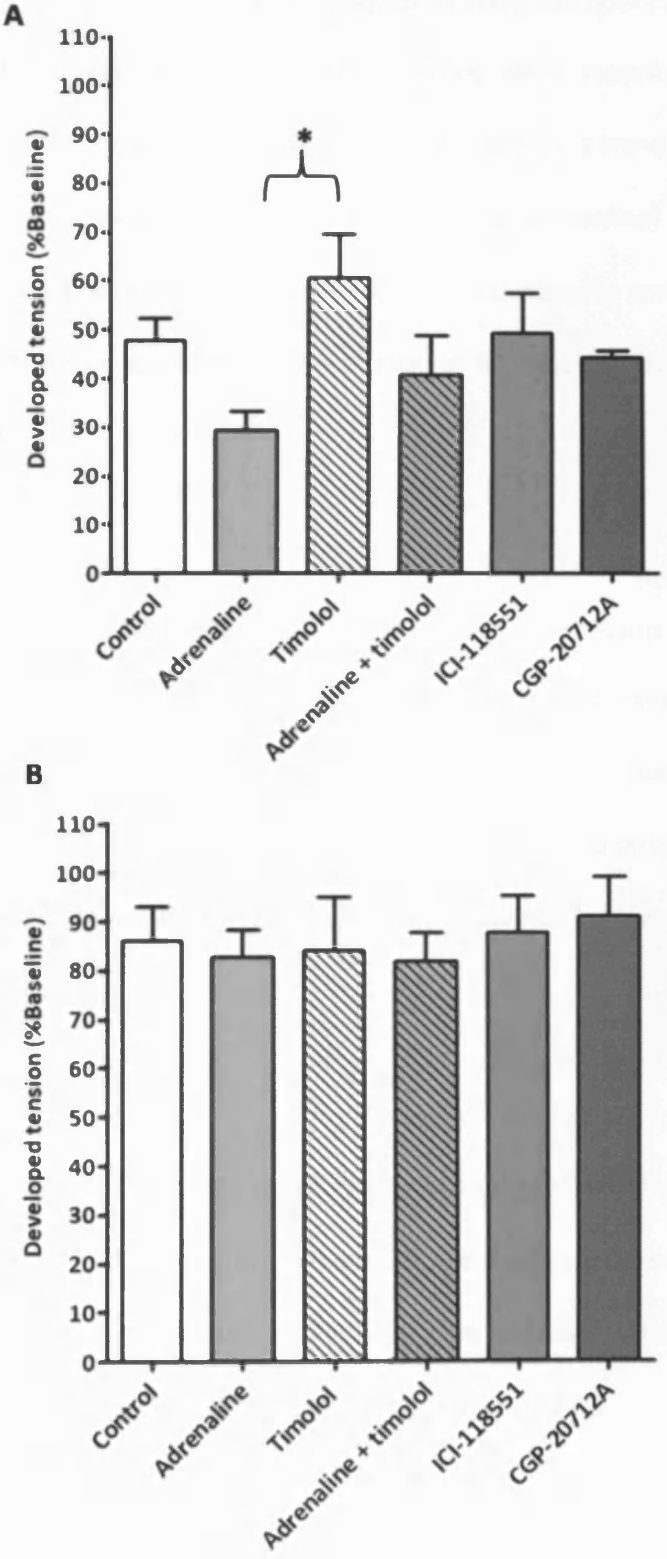

Figure 4-3 Developed tension after 60 minutes reoxygenation in A) atria and B) ventricular strips. Developed tension is expressed as a percentage of the developed tension achieved at the end of the stabilisation period. * indicates $p<0.05$.

4.4.3 Effects of adrenaline administration at reperfusion

Adrenaline caused a positive inotropic effect during early reperfusion in both atria and ventricles. This was still apparent as a non-statistically-significant trend in ventricles, 15 minutes after reoxygenation (developed tension was 95.3 ± 9.0 % of baseline) but not atria (developed tension was 21.2 ± 2.9 % of baseline) (Figure 4-2 B). Compared to controls, adrenaline did not significantly alter the recovery of stunning achieved at 60 minutes after reoxygenation in either tissue (Figure 4-3).

4.4.4 Effects of timolol administered at reperfusion

Timolol did not significantly affect the recovery of stunning at either time-point in atria or ventricles. However, there was a trend towards better recovery than controls in both time-points in atria. At 15 minutes after reoxygenation, timolol treated atria had recovered to 42.5 ± 9.1 % of baseline (Figure 4-2 A), and by 60 minutes, developed tension had reached 60.5 ± 9.0 % of baseline (Figure 4-3 A).

ANOVA did reveal a significant difference between timolol treated atria and tissues treated with both adrenaline and timolol at 15 minutes after reoxygenation. The latter group had recovered significantly ($p<0.05$) less well. Developed tension in tissues treated with both adrenaline and timolol was 15.7 ± 4.5 % of baseline (Figure 4-2 A).

At 60 minutes after reperfusion, a significant ($p<0.05$) difference was observed between the developed tension in atria treated with timolol (60.5 ± 9.0 % of baseline) and tissues treated with adrenaline (29.3 ± 3.9% of baseline) (Figure 4-3 A).

4.4.5 Effects of adrenaline administered at reperfusion in the presence of timolol

Administration of adrenaline in the presence of timolol at reoxygenation had no effect upon stunning in either tissue at either 15 or 60 minutes after reoxygenation when compared to controls (Figure 4-2, Figure 4-3).

4.4.6 Effects of β_1-adrenoceptor antagonist administered at reperfusion

Administration of the β_1-adrenoceptor antagonist, CGP-20712A, at reoxygenation had no effect upon stunning in either tissue at either 15 or 60 minutes after reoxygenation when compared to controls. Additionally, the CGP-20712A treated tissues were not significantly different from any other experimental group (Figure 4-2, Figure 4-3).

4.4.7 Effects of β_2-adrenoceptor antagonist administered at reperfusion

Administration of the β_2-adrenoceptor antagonist, ICI-118,551, at reoxygenation had no effect upon stunning in either tissue at either 15 or 60 minutes after reoxygenation when compared to controls. Additionally, the ICI-118,551 treated tissues were not significantly different from any other experimental group (Figure 4-2, Figure 4-3).

4.5 Discussion

Whilst none of the experimental groups in this study demonstrated a statistically significant change in recovery of contractile recovery when compared to control groups, there were some interesting differences between groups which may give some indication as to the effect of β-adrenoceptor activation on stunning.

The most important finding from these experiments was that endogenous and exogenous adrenaline may mediate a detrimental effect at reperfusion as demonstrated by the fact that atria treated with timolol recovered to a greater extent than those treated with adrenaline at 60 minutes after reoxygenation. However, this improvement was not seen with either the selective β_1 antagonist, CGP-20712A or the selective β_2-antagonist ICI-118,551. It is thus not possible to determine which β-adrenoceptor subtype is responsible for this effect. It is possible that activation of both subtypes is necessary for the effect, or that the concentrations of these agents used were insufficient to overcome the effects mediated by endogenous catecholamines. Higher concentrations could not be used, however or selectivity of the drugs would have been lost.

Any effect of antagonist given at this time-point is quite remarkable because there was probably insufficient time for the antagonist to reach equilibrium at its receptor. However this timing was chosen to ensure that we were studying effects of β-adrenoceptors at reperfusion and not during ischaemia. Additionally, in some preliminary experiments whereby timolol was present in the KHB throughout the entire experiment, the baseline developed tensions were

unsurprisingly significantly lower than in controls. It was not known how this effect may affect the recovery of tissues after ischaemia and so this approach was avoided.

These results also demonstrated a detrimental effect of adrenaline administration upon atrial stunning at 15 minutes after reoxygenation. Tissues treated with adrenaline alone had recovered less well than those treated with adrenaline and timolol.

Because the myocardium in this model was subjected neither to circulating catecholamines nor sympathetic nerve stimulation, the effects of endogenous catecholamines are likely to have been underestimated. This is a very important limitation in this model. In atria at 60 minutes after reperfusion, there was a trend towards improved recovery of timolol treated tissues when compared to controls. It is interesting to speculate whether this trend would have reached statistical significance, had the endogenous catecholamine concentrations been larger.

Although high, the concentration of adrenaline used in these experiments is clinically relevant. Clinical studies in which plasma concentrations of catecholamines were measured after successful resuscitation in patients with out-of-hospital cardiac arrest found concentrations as high as 2.17μM (Prengel *et al.*, 1992) . In the experiments reported here, a slightly higher concentration (10μM) was used in order to account for the fact that the adrenaline had to diffuse from the KHB into the tissue.

The strength and weaknesses of this model have been previously discussed (Chapter 3). However it should be noted that as β-adrenoceptor ligands were administered at reperfusion in this study, the fact that this model excludes any coronary or peripheral vascular effects of drugs applied to the preparation is even more relevant.

No intervention had any effect on stunning in ventricles at 60 minutes after reoxygenation. However recovery was very good in controls and all experimental groups had a recovery averaging above 80 %. It is probably very difficult to show any improvement above this level. The level of recovery seen in these experiments was higher than was observed in similar experiments carried out in the previous chapter (18 months previously). Cardiff University had changed its supplier of rats during this time period, although this is unlikely to account for the change. The difference may however be due to the equilibration time prior to experimentation. In the early experiments described in the previous chapter, preparations were allowed to equilibrate for 60 minutes before use, however it was noted that in most cases, a stable level of contraction had been achieved after 20 minutes. Conscious of the fact that such preparations deteriorate with time and that cell death occurs continuously as demonstrated in Chapter 3, the decision was made to reduce the equilibration time for these later experiments .

Because no intervention significantly altered post-ischemic recovery when compared to controls, the possibility remains that this model does not allow for protective interventions at reperfusion. In a similar model to that used in this study, it was demonstrated that the administration at reperfusion of IB-MECA, a selective agonist of the adenosine A_3 receptor,

improved functional recovery in guinea-pig paced papillary muscles and left atria (Gardner *et al.*, 2004). However, this study was carried out in a different species (guinea-pig) and the tissues were challenged only with hypoxia in normal Krebs, whereas in this study an attempt was made to simulate ischaemia by additionally using glucose-free KHB.

It is also possible that a higher number of experimental replicates would be required to turn trends into statistically significant differences. However the number of replicates used in this study was comparable to that used in Chapter 3 where differences were seen. Thus it was decided to devote further time and resources to the studies described in later chapters.

It is important to understand the effects of adrenoceptor activation upon myocardial stunning because of the high concentrations of catecholamines which the heart is exposed to during ischaemia and reperfusion. During myocardial ischaemia the non ischaemic region of myocardium will be exposed to high levels of catecholamines released from the adrenal medulla in response to pain and stress. Although the ischaemic region is not exposed to circulating catecholamines until reperfusion, the local concentrations are nonetheless high due to local release of catecholamines from sympathetic nerve endings. Noradrenaline is released during ischaemia as a result of the reverse function of the uptake$_1$ transporter (Schoemig *et al.*, 1991). Circulating adrenaline is taken up into sympathetic nerve endings and can be released along with noradrenaline in response to nerve stimulation (Esler *et al.*, 1991a; Esler *et al.*, 1991b; Esler *et al.*, 1995; Johansson *et al.*, 1997; Lameris *et al.*, 2000; Lameris *et al.*, 2002; Majewski *et al.*, 1981; Peronnet *et al.*, 1993). Additionally, adrenaline can be synthesised and

stored in the heart and released from intrinsic cardiac adrenergic cells in response to ischaemia (Kuroko *et al.*, 2007). Indeed, increased myocardial interstitial concentrations of adrenaline, noradrenaline and dopamine are all seen after ligation of the left anterior descending coronary artery in pigs. Adrenaline concentrations do not rise in non-ischaemic regions suggesting a myocardial rather than a circulating adrenaline source (Lameris *et al.*, 2000).

4.5.1 Clinical relevance

These results do not demonstrate any significant detrimental effect of adrenaline upon post-ischaemic stunning. However there is some limited evidence that activation of β-adrenoceptor at reperfusion might worsen stunning. However based on these results, the poor clinical outcome of patients who have been treated with adrenaline during cardiac arrest cannot be attributed to an effect of adrenaline on stunning.

4.6 Conclusion

- Stunning was not significantly affected by application of exogenous adrenaline at reoxygenation.

- Endogenous catecholamines do not play an important role in causing stunning because stunning was neither affected by selective nor non-selective adrenoceptor antagonists.

Chapter 4 Part 2: What effect does exogenous adrenaline have upon infarct size after regional ischaemia in the rat Langendorff heart?

4.7 Hypotheses

- Adrenaline, administered at reperfusion, will lead to an increased infarct size compared to control hearts.

- Adrenaline, administered at reperfusion, will lead to worsened post-ischaemic contractile recovery.

4.8 Methods

Rats were obtained from B&K international and weighed 250-350g at the time of killing. Langendorff heart preparations were set up as previously described (Chapter 2). After 20 minutes stabilisation, hearts were exposed to 35 minutes regional ischaemia achieved by ligation of the left main coronary artery after which the hearts were reperfused for two hours before undergoing staining to determine infarct size. Control hearts were otherwise untreated. Treatment groups were exposed to a range of concentrations of adrenaline (1nM, 100nM, 10μM) for the final 10 minutes of ischaemia and the first ten minutes of reperfusion (Figure 4-4).

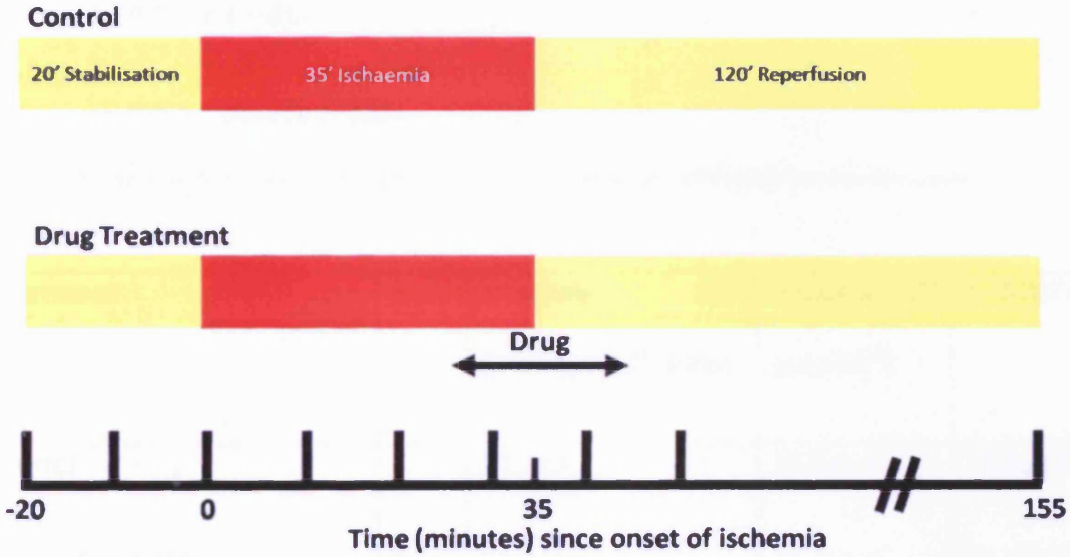

Figure 4-4 Experimental Protocols used in this study. Time is shown along the abscissa. Yellow areas denote normal perfusion and red areas indicate ischaemia.

4.9 Results

4.9.1 Baseline data

Table 4-3 Baseline cardiodynamics. There were no statistically significant differences between the groups.

Treatment	n	Baseline RPP ($mmHg.min^{-1}/1000$)	Baseline CF ($ml.min^{-1}$)	AAR(%Total)
Control	6	32.7±5	13.6±1.6	24.2±3.5
Adrenaline 1 nM	6	23.2±2	14.4±1.3	31.4±3.4
Adrenaline 100 nM	4	27.0±2	12.8±4.3	31.9±3.6
Adrenaline 10 μm	5	23.8+4	11.4±0.9	27.4±4.7

There were no statistically significant differences between experimental groups in terms of baseline coronary flow or rate-pressure product. Evans blue staining revealed no significant difference in the percentage of the heart made ischaemic by the coronary ligation (Table 4-3).

4.9.2 Infarct size

None of the concentrations of adrenaline had any statistically significant effect on infarct size when added to the tissues at reperfusion (Figure 4-5).

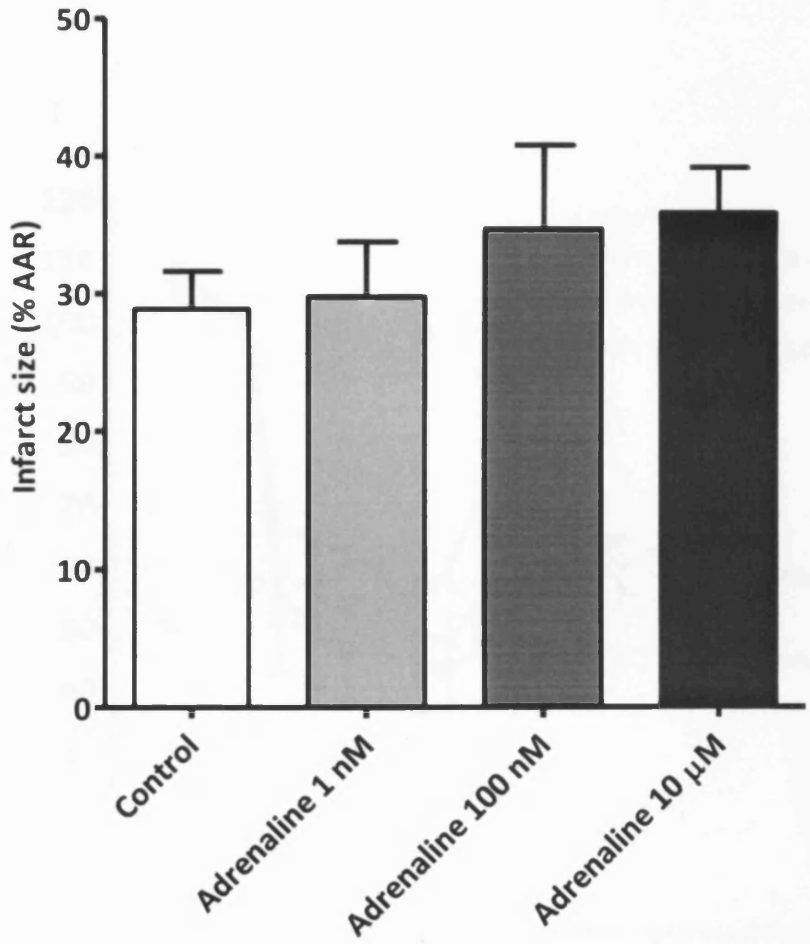

Figure 4-5: Infarct sizes in control hearts and hearts exposed to adrenaline at reperfusion.

4.9.3 Functional data

None of the treatment groups had any significant effect on the timecourse of coronary flow throughout the experiment (Figure 4-6).

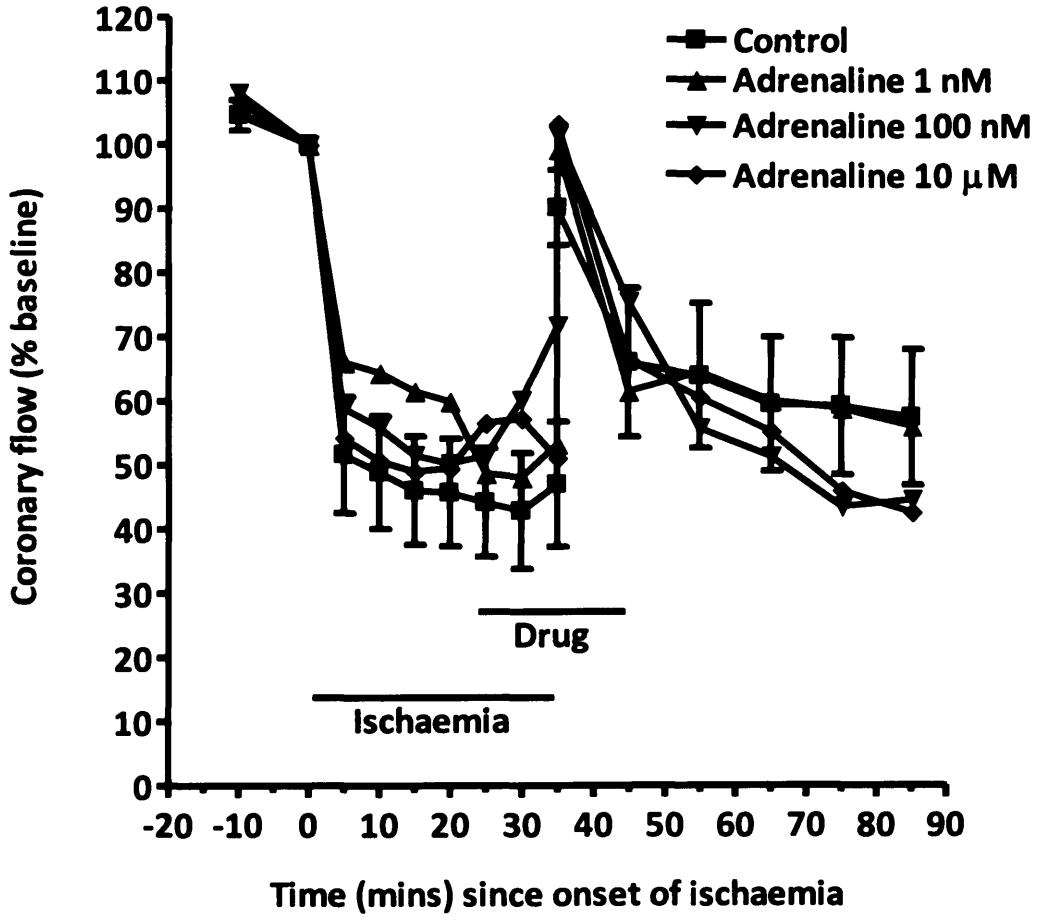

Figure 4-6 The timecourse of coronary flow (expressed as a percentage of the baseline value) throughout the experiment in each of the experimental groups. Error bars have been removed from all groups except for controls to improve clarity.

None of the treatment groups had any significant effect on the timecourse of rate pressure product (Figure 4-7) or left ventricular developed pressure (Figure 4-8) throughout the experiment.

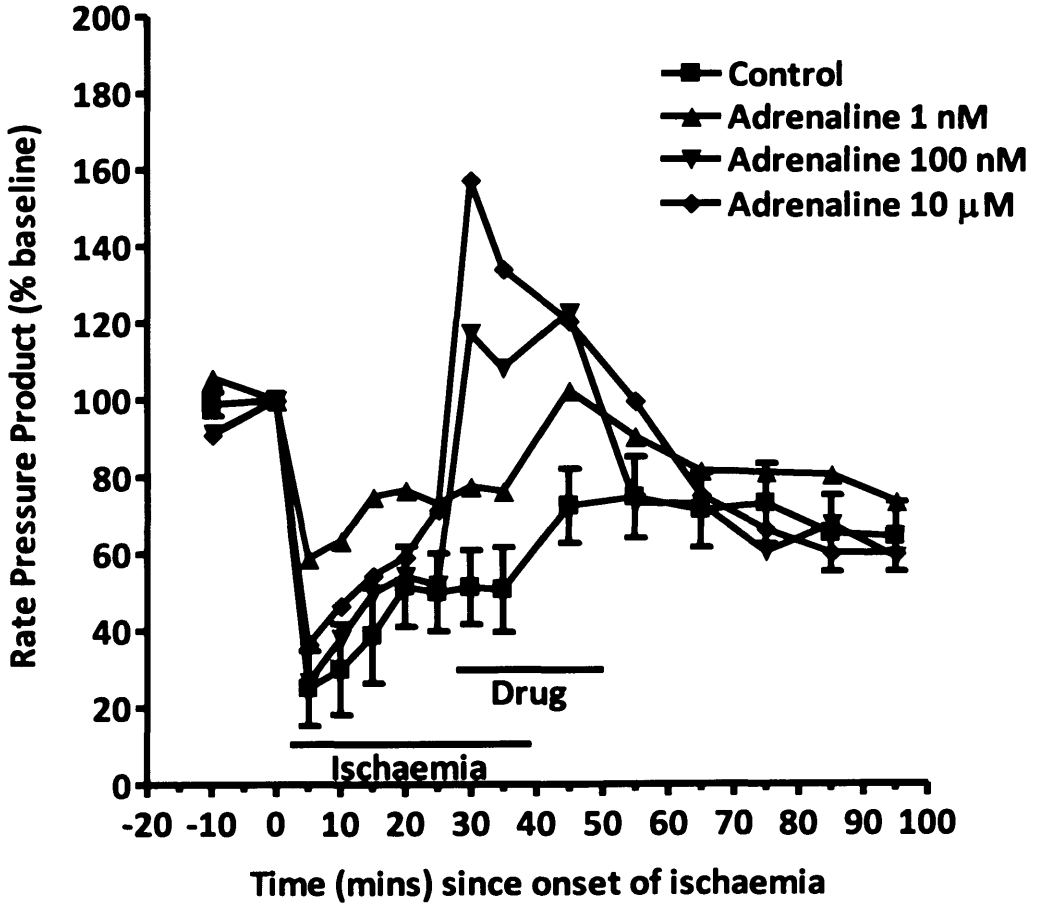

Figure 4-7 The timecourse of rate pressure product (expressed as a percentage of the baseline value) throughout the experiment. Error bars have been removed from all groups except for controls to improve clarity.

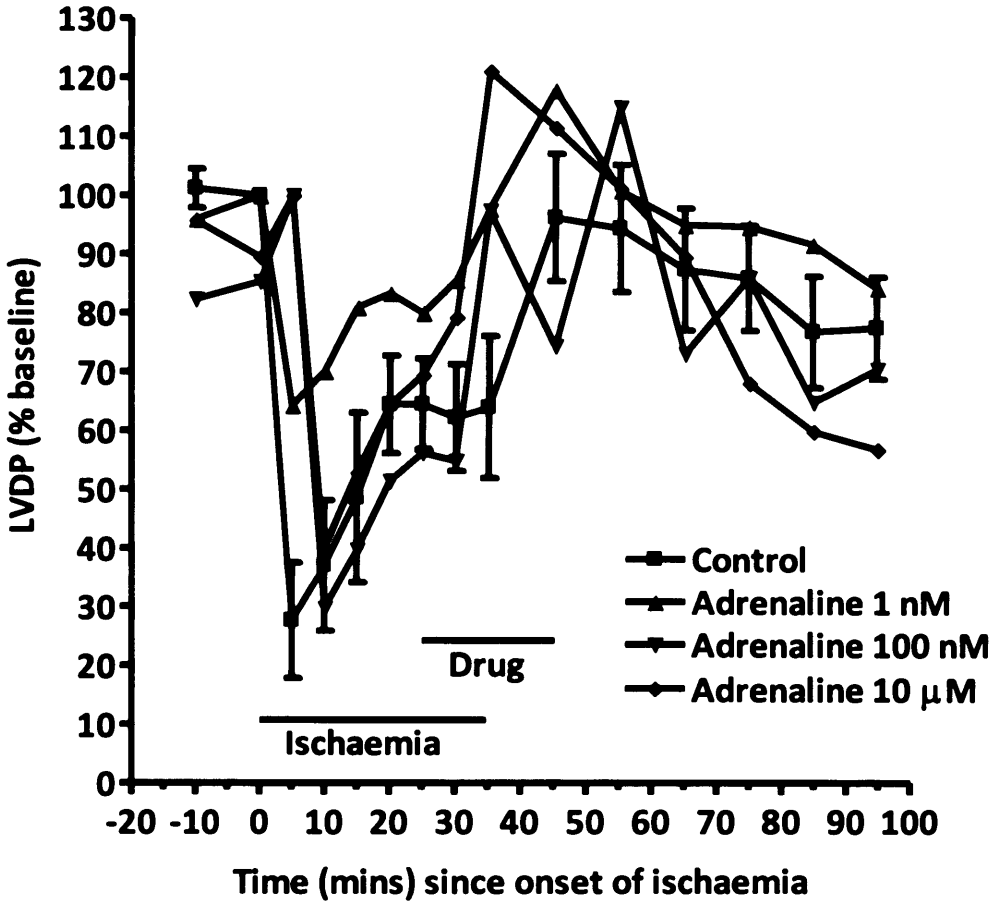

Figure 4-8 The timecourse of left ventricular developed pressure (expressed as a percentage of the baseline value) throughout the experiment. Error bars have been removed from all groups except for controls to improve clarity.

The administration of adrenaline at reperfusion caused a concentration-dependent increase in diastolic tension immediately after reperfusion (Figure 4-9). In all tissues, the highest diastolic tension occurred 10 minutes after reperfusion (45 minutes after the onset of ischaemia). This difference reached statistical significance at the highest concentration (10 μm) of adrenaline (Figure 4-10). At this concentration of adrenaline, the diastolic tension increased to 28.9±7.8 mmHg above baseline, compared to 3.0±4.1 in controls.

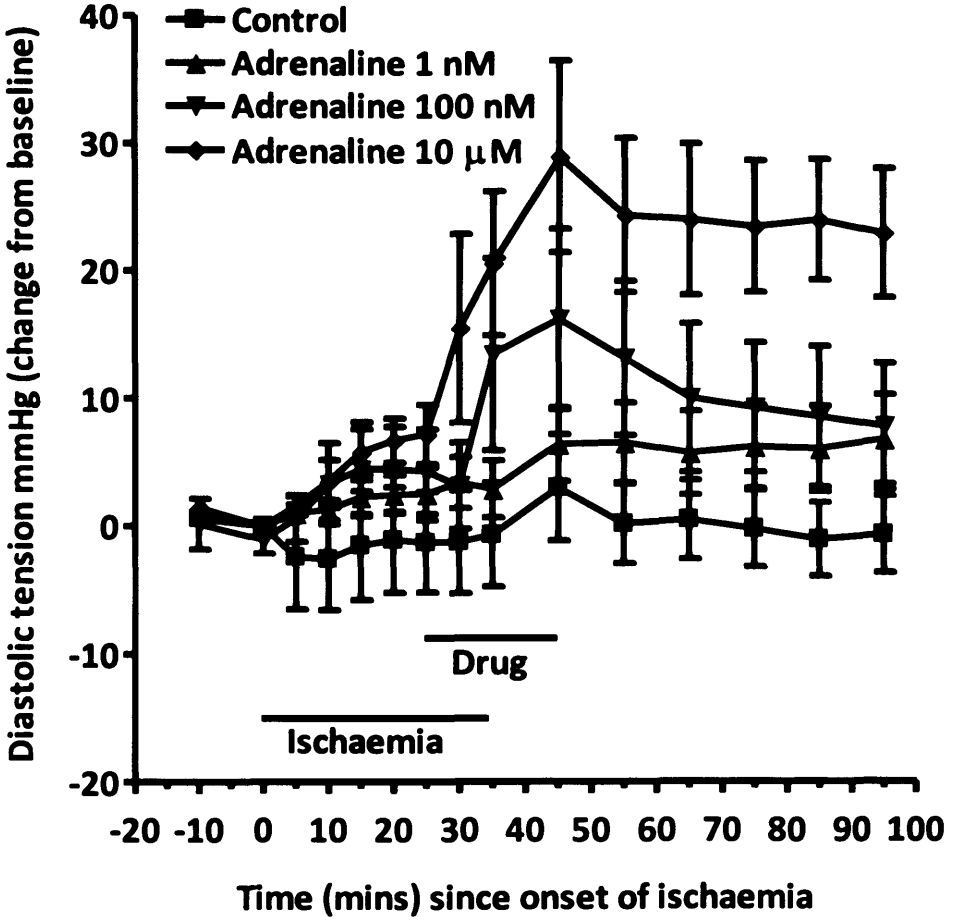

Figure 4-9 The change in diastolic tension throughout the experiment from baseline values.

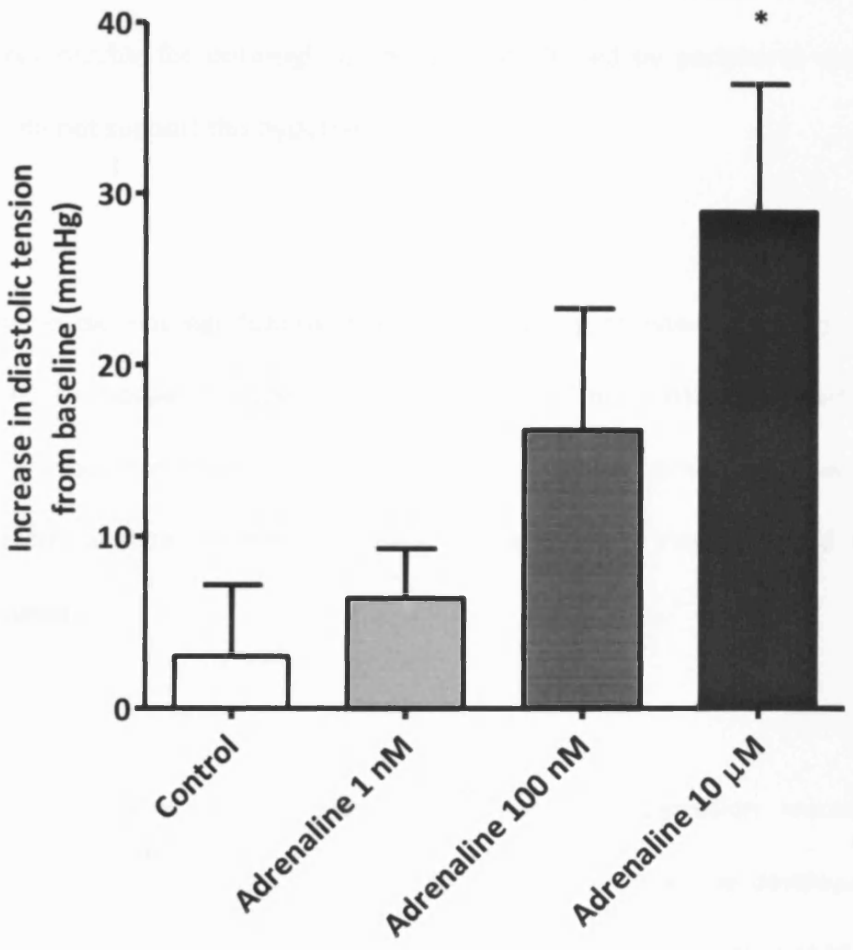

Figure 4-10 The maximum diastolic tension after reperfusion in each of the experimental groups. In each group, the largest diastolic tension occurred at 10 minutes post reperfusion. Units are mmHg change from baseline values.

4.10 Discussion

It has been demonstrated that, at the concentrations used in this study, adrenaline does not affect infarct size. Furthermore, recovery of rate pressure product and coronary flow were unaffected by adrenaline. This was contrary to expectations. The very poor survival rate after cardiac arrest with adrenaline treatment led to the hypothesis that cardiac effects of adrenaline were responsible for outweighing the benefit elicited by peripheral vasoconstriction. These results do not support this hypothesis.

Adrenaline did not significantly affect the recovery of rate-pressure-product. There was an expected dose-dependent trend towards increasing rate pressure product in early reperfusion, due to the positive chronotropic and inotropic effects of adrenaline. However, due to the high degree of variability inherent in these measurements, this trend did not reach statistical significance.

It is interesting that adrenaline administered at reperfusion worsened post-ischaemic contracture (hypercontracture). It has been suggested that the development of reperfusion contracture and post-ischaemic necrosis are causally related (Pantos *et al.*, 2006). Reperfusion contracture is responsible for contraction band necrosis (Fujiwara *et al.*, 1989) and in severe cases the stone heart' phenomenon (Pantos *et al.*, 2006). However, the increased contracture in adrenaline-treated hearts did not result in increased cell death in this model over two hours of reperfusion, neither was it associated with a worsening of contractile recovery.

This study used a regional no-flow model of ischaemia to replicate the cardiac conditions during cardiac arrest. However, when cardiac arrest is accompanied by cardiopulmonary resuscitation, it could be argued that a global low-flow model of ischaemia would better replicate the pathophysiological conditions. The regional ischaemia model was however chosen for this study for a number of reasons: i) Cardiac arrest most commonly occurs secondary to myocardial ischaemia (Engdahl *et al.*, 2003; Fischer *et al.*, 1997; Grubb *et al.*, 1995; Kuisma & Alaspaa, 1997; Weston *et al.*, 1997) and thus there are probably regions of low-flow and regions of zero-flow in the arrested heart undergoing cardiopulmonary resuscitation. ii) When myocardial ischaemia is the cause of cardiac arrest, resuscitation guidelines allow thrombolysis as part of the resuscitation therapy (International Liaison Committee on Resuscitation, 2005), thus reperfusion after regional no-flow ischaemia occurs. iii) Infarct size (the most important factor in determining survival) cannot be reliably visualised after global ischaemia. Other investigators have however used low-flow models of ischaemia to monitor myocardial function and at the same time have collected coronary perfusate and measured cardiac enzyme release as a surrogate marker of cell death. It would be interesting to test the effects of adrenaline at reperfusion in that model and compare the results with those presented here. iv) Later chapters investigate the effects of endogenous adrenaline upon infarct size in regional ischaemia and reperfusion, by the use of β-adrenoceptor antagonists. Use of the same model in each set of experiments aids comparisons.

The failure to demonstrate a change in infarct size by any of the treatment groups is not a feature of the model used. The same experimental techniques were utilised in Chapters 5 and 6 where both beneficial and detrimental effects on infarct size are provoked by treatments.

One possibility which has not been investigated in this study is that adrenaline mediates detrimental cardiac effects in the resuscitation setting by increasing the frequency of lethal reperfusion arrhythmias. High concentrations of adrenaline are known to be highly arrhythmogenic (Tisdale *et al.*, 1995). However, perhaps the most likely explanation for the ineffectiveness of adrenaline is that resuscitation after several minutes cardiac arrest in patients who are likely to have underlying pathologies and are in most cases elderly is very difficult. Thus it is almost impossible to show improved recovery with any treatment. However, if the rationale for the use of adrenaline in cardiac arrest is that it causes peripheral vasoconstriction, it should be compared in well-controlled clinical trials with more selective vasopressor agents.

4.10.1 Clinical implications

Adrenaline did not have any effect on the development of infarct size at any of the concentrations used. The poor clinical outcome of patients in cardiac arrest treated with adrenaline is probably not due to an effect of adrenaline on infarct size. This field needs a great deal more experimental and clinical studies to determine why adrenaline is ineffective at resuscitation, and whether any alternative compound is more effective.

4.11 Conclusions

- Adrenaline, applied at reperfusion, increases the magnitude of reperfusion contracture in a dose-dependent manner.

- Adrenaline, at the concentrations used in this study, has no effect on infarct size when applied at reperfusion.

- The effects of adrenaline when administered in the clinic during resuscitation are probably a result of the overall haemodynamic actions of this drug

Chapter 5 Is the activation of β-adrenoceptors necessary for postconditioning after regional ischaemia in the rat Langendorff heart?

5.1 Introduction

Ischaemic preconditioning and postconditioning act through similar mechanisms to activate RISK pathways and to prevent MPTP opening at reperfusion (Hausenloy *et al.*, 2007b). See Section 1.3 for further details. Ischaemia can cause the release of the catecholamines noradrenaline (Schoemig *et al.*, 1991) and adrenaline (Kuroko *et al.*, 2007) in the myocardium from sympathetic nerve endings and intrinsic cardiac adrenergic cells . These can activate β-adrenoceptors on the cardiomyocytes and therefore ischaemic catecholamine release has been proposed as a mechanism by which ischaemic preconditioning exerts protection. The data regarding this hypothesis are conflicting (See Section 1.5) however in Chapter 3, it was demonstrated that activation of β-adrenoceptors is required for the reduction in stunning caused by ischaemic preconditioning in isolated paced atria and ventricles. Others have shown that preconditioning-induced reduction of infarct size can be mimicked by application of β-adrenoceptor agonists, (Lange *et al.*, 2006; Lochner *et al.*, 1999; Marais *et al.*, 2001; Moolman *et al.*, 2006b; Moolman *et al.*, 2006c; Nasa *et al.*, 1997; Robinet *et al.*, 2005; Tong *et al.*, 2005; Yabe *et al.*, 1998; Yates *et al.*, 2003) suggesting that β-adrenoceptor agonists can activate RISK pathways. Recently, some direct evidence that β-adrenoceptor can activate PI3K (Yano *et al.*, 2007). and Erk 1/2 (Ciccarelli *et al.*, 2007; Galandrin *et al.*, 2006; Tutor *et al.*, 2007) has been presented (See Section 1.5).

Because β-adrenoceptor activation can initiate signalling via kinases involved in RISK pathways, agonists of these receptors have the potential to mimic postconditioning when applied in the early minutes of reperfusion. It has been proposed that postconditioning acts by mechanically delaying the washout of adenosine from the ischaemic region, allowing it to activate protective signalling pathways. It is possible that the same could be true of catecholamines which also accumulate in ischaemic regions and are presumably washed out soon after reperfusion.

To date, there have been no reported investigations into the possibility that ischaemic postconditioning exerts its protective effects by this mechanism. If this is the case, it would be expected that the protection of postconditioning could be attenuated by a β-adrenoceptor antagonist. It may also be possible to measure released catecholamines in coronary perfusate collected from isolated hearts during the postconditioning phase.

Studies have demonstrated that β_1-adrenoceptor antagonists applied at reperfusion lead to infarct size reduction (Feuerstein *et al.*, 1998; Gao *et al.*, 2000), suggesting a detrimental role for activation of these receptors by endogenous catecholamines at reperfusion. It is therefore unlikely that β_1-adrenoceptors are involved in the protection of postconditioning. However, signalling downstream of β_2-adrenoceptors and β_3-adrenoceptors has the potential to activate RISK pathways (See Section 1.5.3) and therefore to mimic postconditioning

5.2 Hypotheses

- Protection afforded by postconditioning will be abrogated by a non-selective β-adrenoceptor antagonist.

- Postconditioning is mediated by the endogenously released catecholamines noradrenaline and adrenaline.

- Protection afforded by postconditioning will be blocked by β_2 or β_3 adrenoceptor antagonists

- Antagonists of β_1 adrenoceptors at reperfusion will confer cardioprotection.

5.3 Methods

5.3.1 Langendorff protocols

Male Sprague-Dawley Rats 280-360g were obtained from B&K international (Grimston, UK). Langendorff perfused hearts were prepared as described previously (Chapter 2). Hearts which fulfilled inclusion criteria were randomised to one of ten experimental groups. Control hearts were exposed to 35 minutes ischaemia and 120 minutes reperfusion. At the end of the 35 minute ischaemic episode, postconditioned tissues were reperfused for 30 seconds, after which the coronary artery was re-occluded for 30 seconds. This cycle of reperfusion / re-occlusion was repeated twice more (Figure 5-1). Hearts were also exposed to postconditioning in the

presence of selective and non-selective adrenoceptor antagonists (Table 5-1) and to each of these antagonists alone (without postconditioning). All drugs were applied for the final ten minutes of ischaemia and the first ten minutes of reperfusion. Studies of cardioprotection have consistently cited early reperfusion as the crucial target of protection (Piper *et al.*, 2004). Thus, it was decided to apply the drug for the first 10 minutes of reperfusion. Although the lag volume in this system was kept to a minimum (≈5 ml) it was decided to switch to drug perfusion ten minutes before reperfusion to ensure that the first KHB to reach the reperfused tissue contained the required concentration of drug. Had the tissue been exposed to the tissue for longer than 10 minutes during ischaemia, problems such as receptor desensitisation may have been more likely. Additionally it would have been more difficult to determine whether protective drugs exerted their actions during ischaemia or at reperfusion.

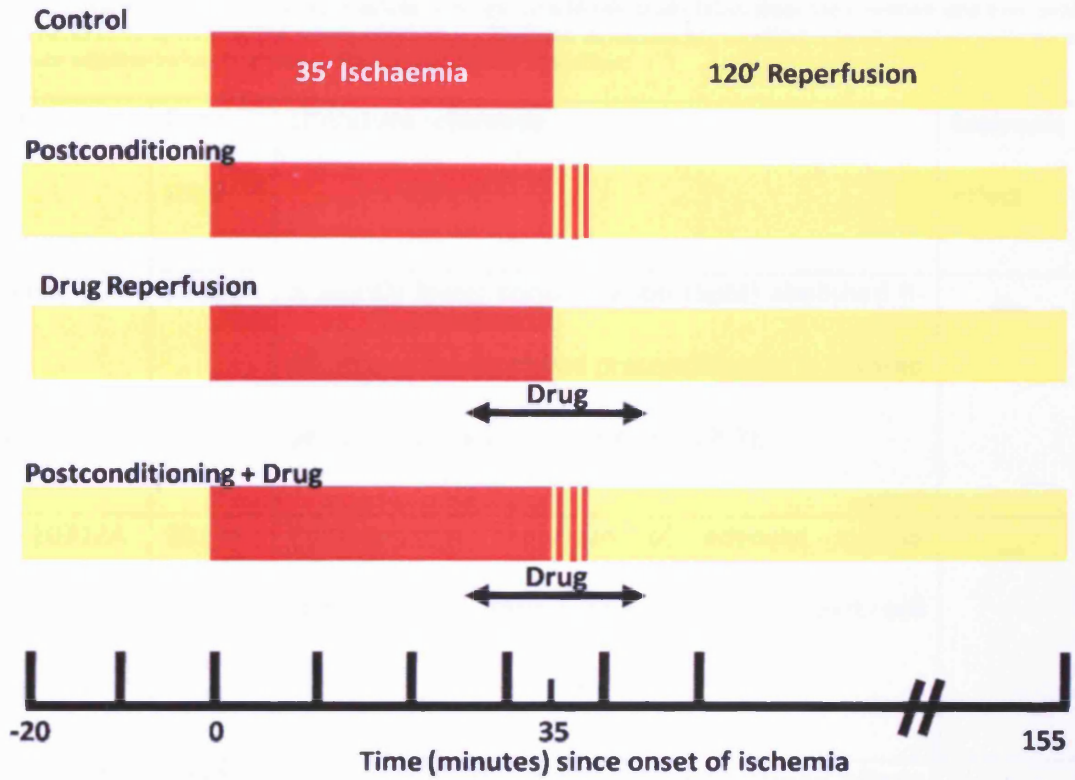

Figure 5-1 Experimental Protocols used in this study. Dark regions indicate ischaemia and light regions, normal perfusion.

Table 5-1 Pharmacological agents and their concentrations used in this study.

Drug	Concentration	Pharmacological action
Timolol	10μM	Non-selective β-adrenoceptor antagonist
CGP-20712A	10nM	Selective β_1 antagonist
ICI-118,551	10nM	Selective β_2 antagonist
SR-59230A	100nM	Selective β_3 antagonist

Table 5-2 Justifications for the concentrations of drugs used in this study taken from the literature and from preliminary experiments in naïve tissues, the results of which are reported as follows NE, no effect; - small negative inotropic effect; -- moderate negative inotropic effect; --- large negative inotropic effect.

Drug	**Conc. used**	**Literature reference**	**Inotropic effect**
Timolol	10 μM	A slightly lower concentration (5μM) abolished β-adrenoceptor-mediated preconditioning in isolated perfused rat hearts (Nasa *et al.*, 1997).	--
CGP-20712A	10 nM	Near-maximal reduction of adenylyl cyclase activation by isoprenaline in a rat- heart cell membrane model (Arnold *et al.*, 1993).	--
ICI-118,551	10 nM	This concentration blocks residual inotropic effects of isoprenaline in sheep ventricular muscle after β_1-adrenoceptor blockade (Borea *et al.*, 1992).	-
SR-59230A	100 nM	This dose causes a rightward shift of the chronotropic effects of the selective β_3-adrenoceptor agonist ZD 7114 in rat atria (Sterin-Borda *et al.*, 2006)	NE

5.3.2 Separation and detection of catecholamines in coronary perfusate

Coronary perfusate was collected at the end of the stabilisation period, at the end of ischaemia and at 3 minutes after reperfusion and was stored and analysed according to the method described in Section 2.7.

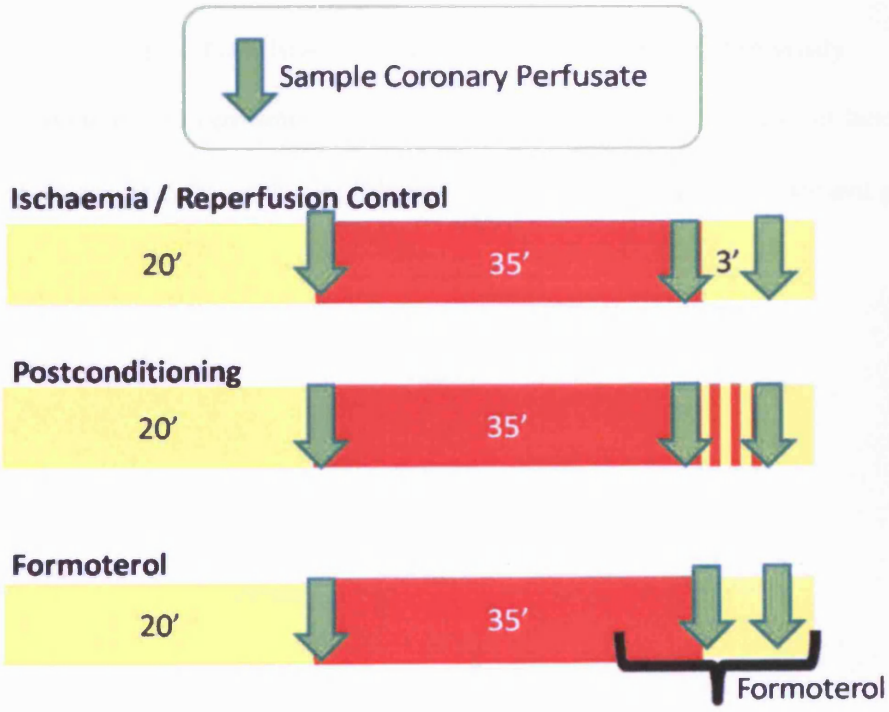

Figure 5-2 Protocol for sampling of coronary perfusate

5.3.3 Presentation of data and statistics

This chapter describes a single study in which the order of the experiments was randomised. Statistical analysis was carried out by comparing all ten groups using a single one-way ANOVA, however in order to improve the clarity of the results section, the results are presented as a series of graphs comparing each intervention to the same control and postconditioning group.

5.4 Results

5.4.1 Baseline cardiodynamics for infarct size study

The baseline cardiodynamics for hearts used in this study are shown below (Table 5-3). There were no statistically significant differences between any of the treatment groups.

Table 5-3 Baseline cardiodynamics and Area at risk as a percentage of total area for hearts used in these studies. RPP, Rate pressure product; CF, Coronary flow; AAR, Area at risk.

Treatment	n	Baseline RPP ($mmHg.min^{-1}/1000$)	Baseline CF ($ml.min^{-1}$)	AAR (%Total area)
Control	11	21.6 ± 2	14.0 ± 0.7	35.0 ± 3.3
Postconditioning	6	21.6 ± 3	19.8 ± 1.7	49.8 ± 3.3
Timolol	6	18.8 ± 4	16.9 ± 1.7	42.0 ± 10.1
Timolol + Postconditioning	5	20.2 ± 3	18.1 ± 2	47.1 ± 6.2
CGP-20712A	5	24.6 ± 4	16.0 ± 0.9	36.6 ± 8.8
CGP20712A+Postconditioning	3	26.4 ± 3	15.5 ± 3.2	26.3 ± 0.7
ICI-118,551	5	20.5 ± 3	11.4 ± 6.3	48.9 ± 5.3
ICI-118,551+Postconditioning	7	26.4 ± 3	12.0 ± 1.6	46.6 ± 2.1
SR-59230A	5	25.8 ± 4	12.7 ± 1.3	31.8 ± 5.8
SR-59230A+Postconditioning	4	22.5 ± 5	10.2 ± 0.5	34.6 ± 8.0

5.4.2 Infarct size reduction by postconditioning and functional parameters – Effects of timolol.

Infarct size, expressed as a percentage of the area at risk, in postconditioned hearts (13.07 ± 3.0 %) was significantly ($p<0.01$) smaller than in control hearts (41.1 ± 5.1), representing a 68 % relative reduction (Figure 5-3). In tissues treated with the non-selective β-adrenoceptor antagonist, timolol alone, infarct size was not different to controls (45.1 ± 3.9 %). When timolol was combined with postconditioning, the infarct size (73.7 ± 7.2) was significantly larger than postconditioned ($p<0.001$) and control ($p<0.01$) hearts. These effects occurred without significantly affecting the timecourse of coronary flow (Figure 5-4) throughout the experiment, or the recovery of contractile function as assessed by rate pressure product (Figure 5-5) or left ventricular developed pressure (Figure 5-6).

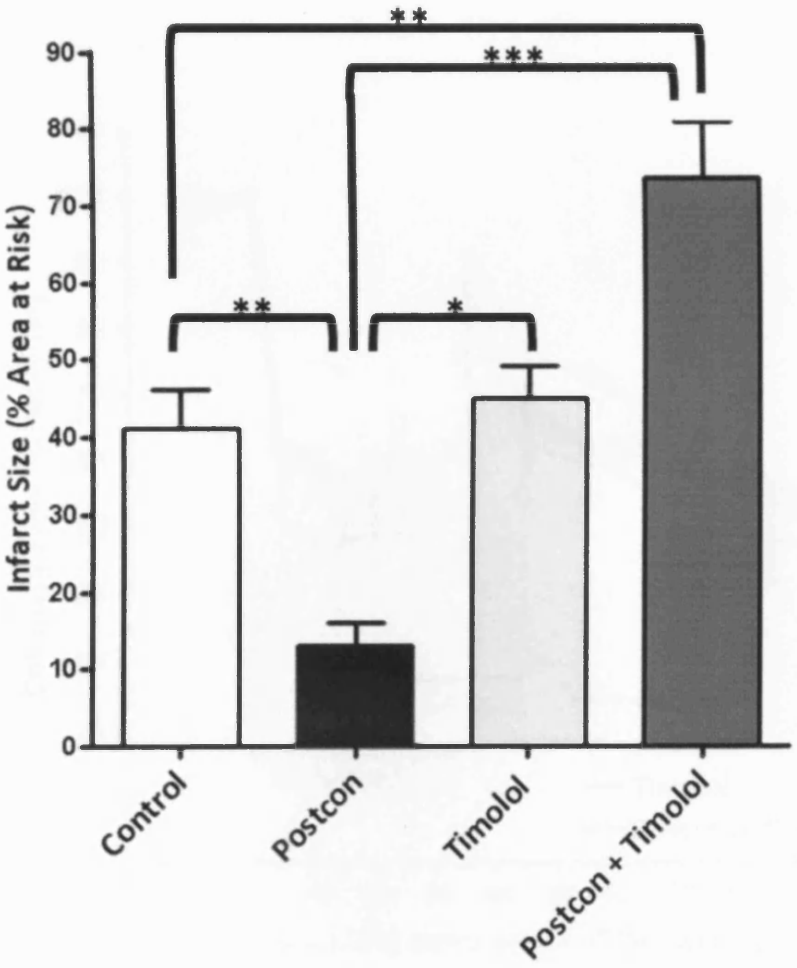

Figure 5-3 The effect of the non-selective β-adrenoceptor antagonist, timolol on infarct size reduction by postconditioning. *p<0.05 ** p<0.01 *** p<0.001.

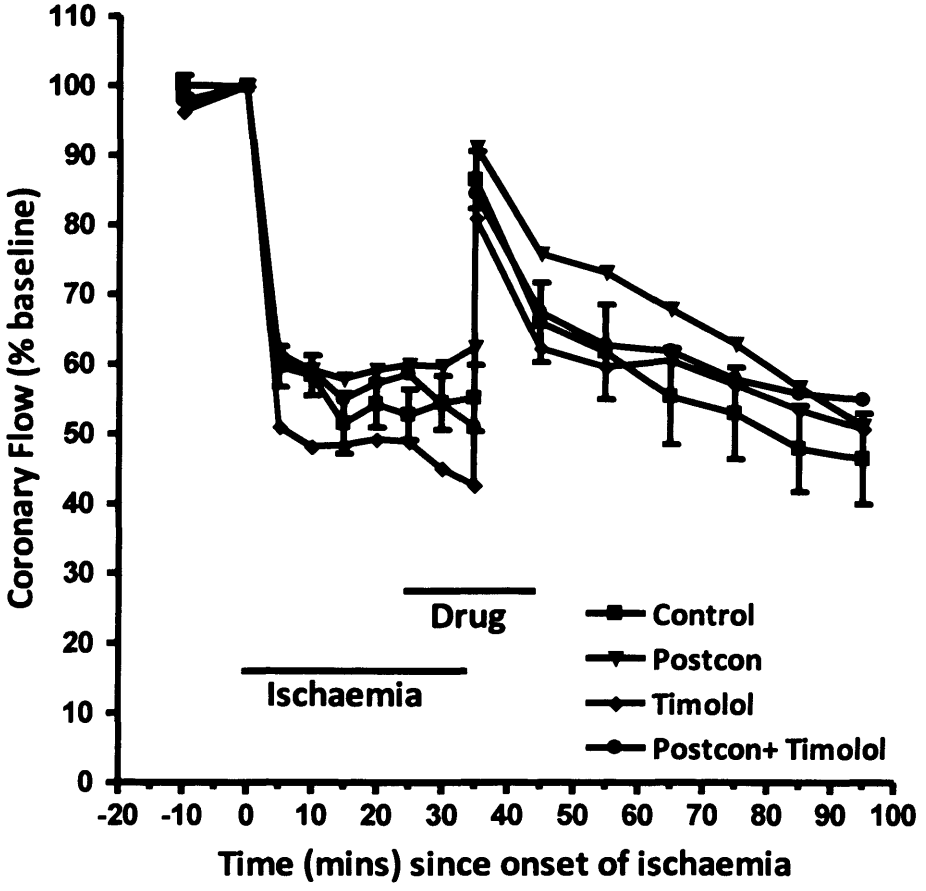

Figure 5-4 The timecourse of coronary flow for each experimental group in experiments to determine the effect of the non-selective β-adrenoceptor antagonist, timolol on infarct size reduction by postconditioning. There were no significant differences between groups. Error bars have been removed from all groups except for controls in order to improve clarity.

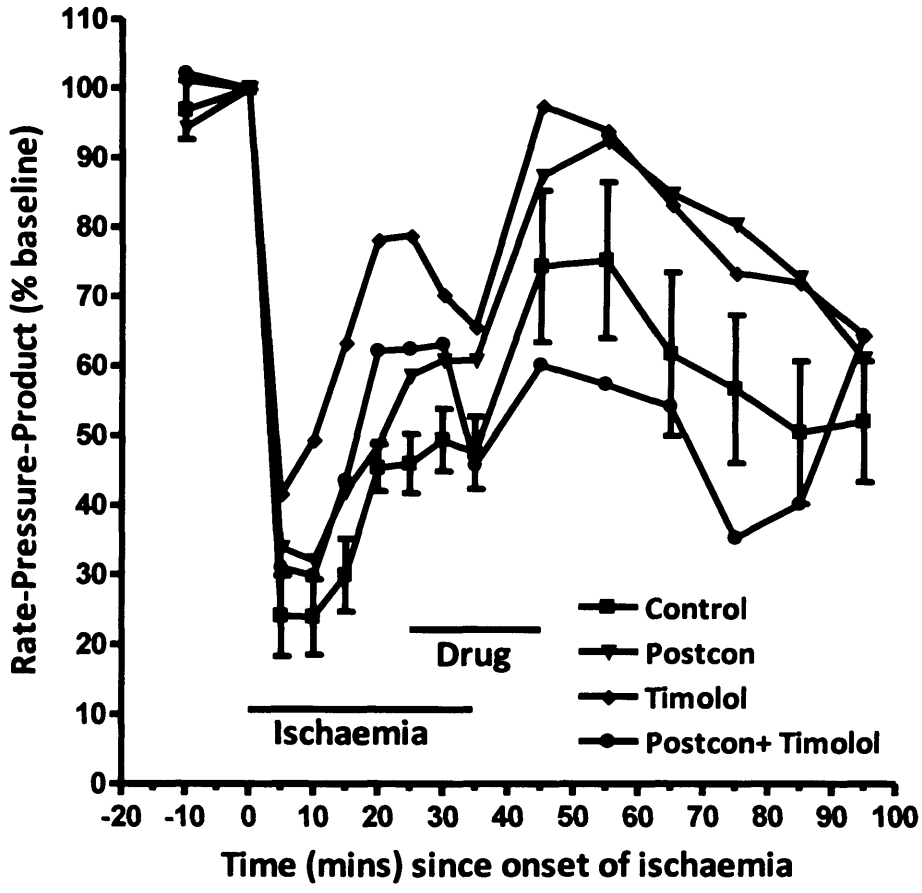

Figure 5-5 The timecourse of rate-pressure product for each experimental group in experiments to determine the effect of the non-selective β-adrenoceptor antagonist, timolol on infarct size reduction by postconditioning. There were no significant differences between groups. Error bars have been removed from all groups except for controls in order to improve clarity.

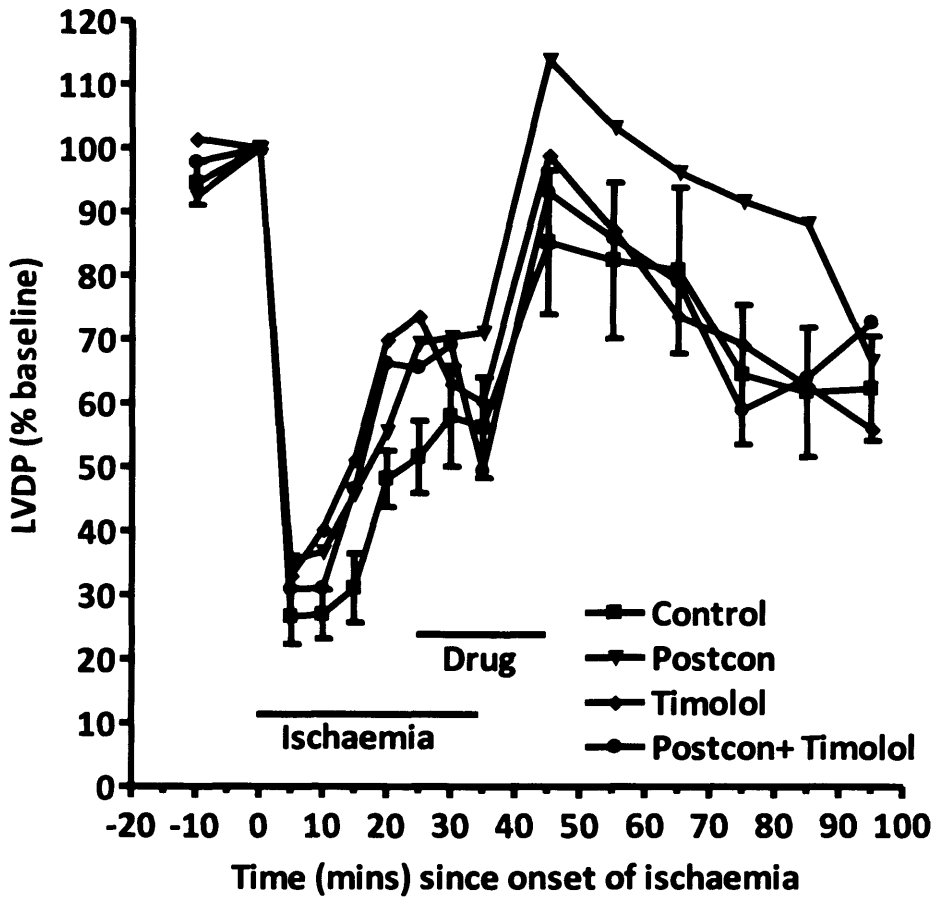

Figure 5-6 The timecourse of left ventricular developed pressure for each experimental group in experiments to determine the effect of the non-selective β-adrenoceptor antagonist, timolol on infarct size reduction by postconditioning. There were no significant differences between groups. Error bars have been removed from all groups except for controls in order to improve clarity.

5.4.3 Infarct size and functional parameters – CGP-20712A

When hearts were treated with the selective β_1-adrenoceptor antagonist CGP-20712A at reperfusion, the infarct size (12.6 ± 3.3) was significantly ($P<0.05$) smaller than control hearts and a 69 % relative reduction in infarct size (Figure 5-7). When postconditioning and CGP-20712A were combined, there was a trend towards a smaller infarct size than control hearts, although this did not reach statistical significance. These effects occurred without significantly affecting the timecourse of coronary flow (Figure 5-8) throughout the experiment, or the recovery of contractile function as assessed by rate pressure product (Figure 5-9) or left ventricular developed pressure (Figure 5-10). However there did appear to be a trend towards improved function in CGP-20712A treated hearts and indeed ANOVA of the final time-point of the LVDP graph (60 minutes after reperfusion) demonstrated a significant difference between controls and CGP-20712A treated hearts.

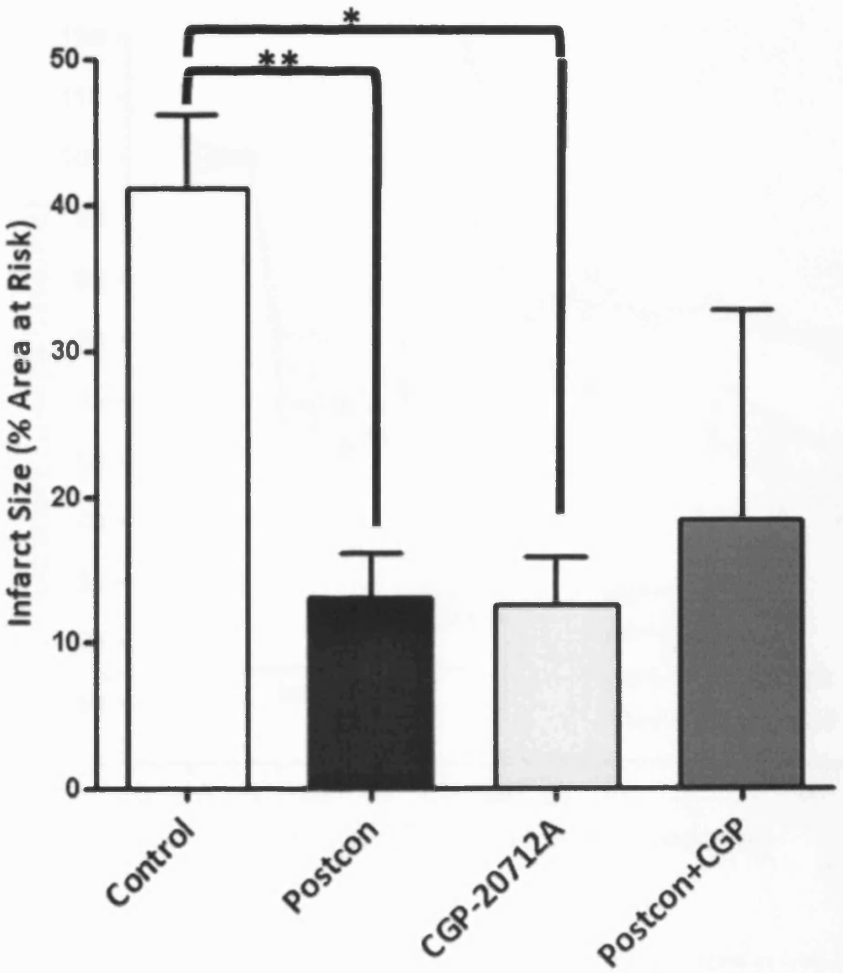

Figure 5-7 The effect of the selective β_1-adrenoceptor antagonist, CGP-20712A on infarct size reduction by postconditioning. * $p<0.05$ ** $p<0.01$.

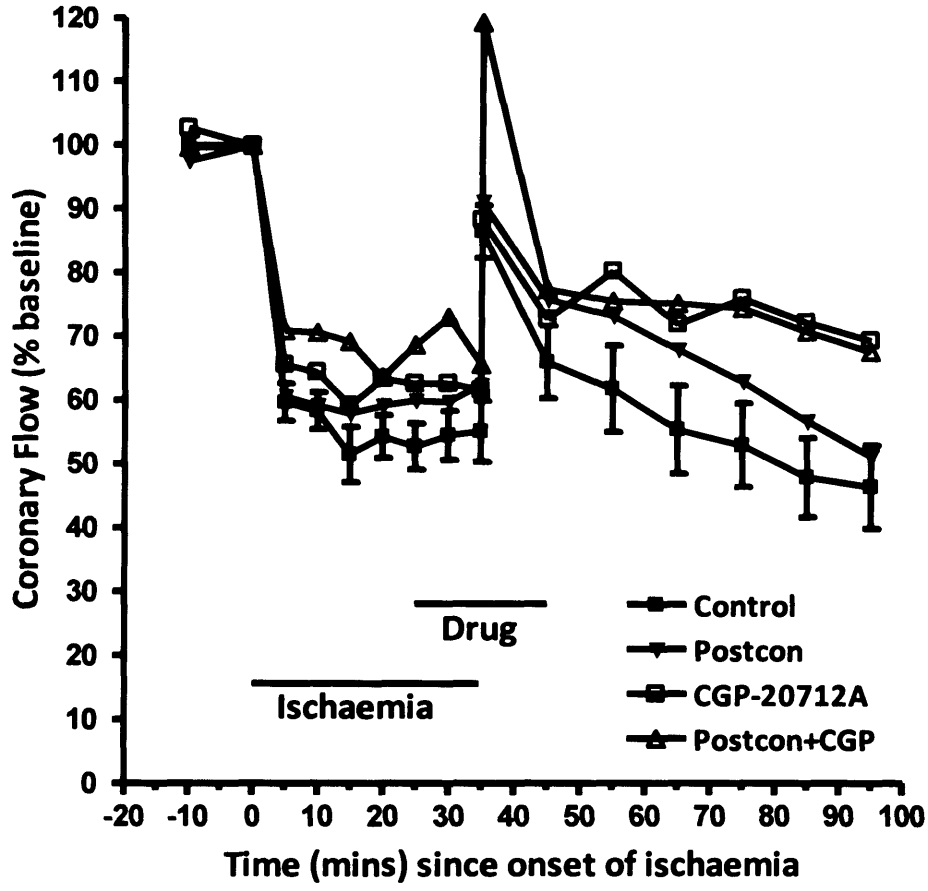

Figure 5-8 The timecourse of coronary flow for each experimental group in experiments to determine the effect of the selective β_1-adrenoceptor antagonist, CGP-20712A on infarct size reduction by postconditioning. There were no significant differences between groups. Error bars have been removed from all groups except for controls to improve clarity.

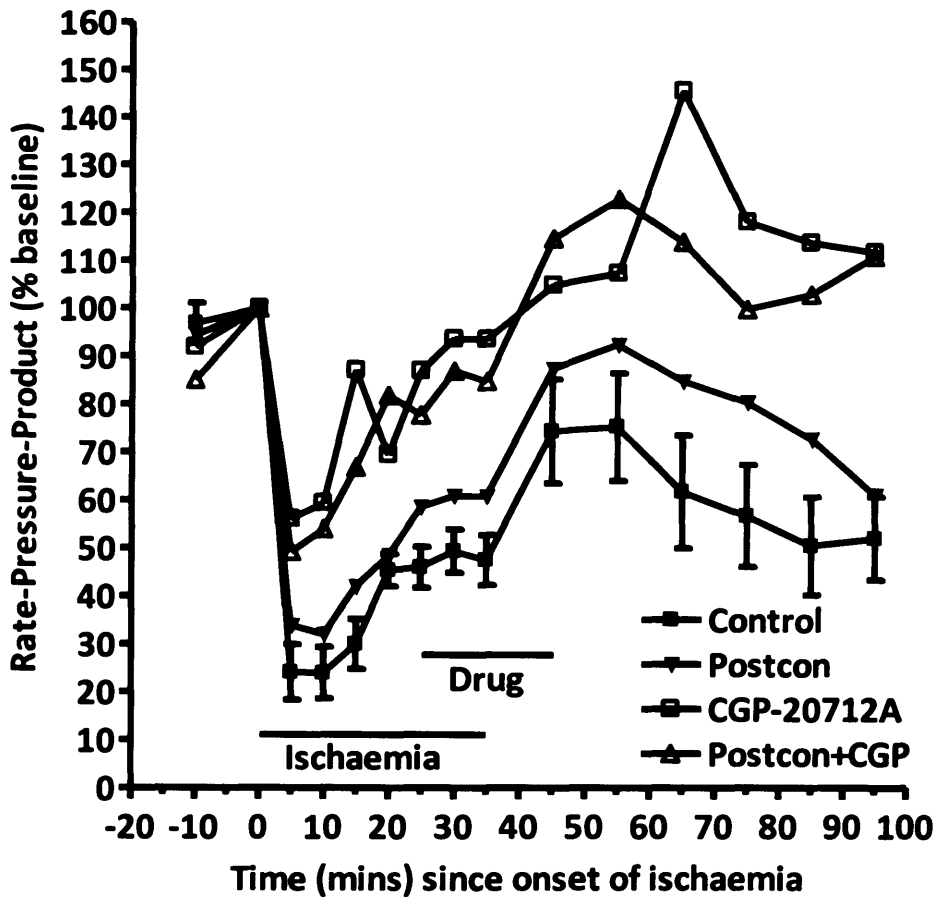

Figure 5-9 The timecourse of rate-pressure-product for each experimental group in experiments to determine the effect of the selective β_1-adrenoceptor antagonist, CGP-20712A on infarct size reduction by postconditioning. There were no significant differences between groups. Error bars have been removed from all groups except for controls to improve clarity.

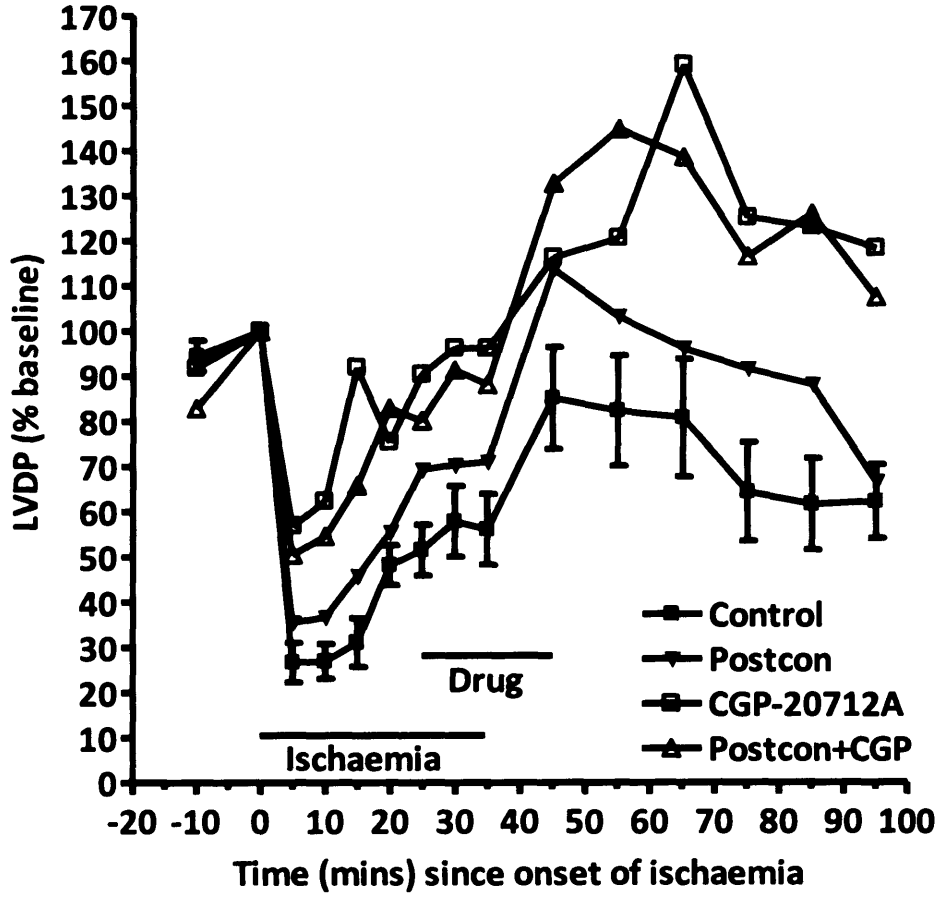

Figure 5-10 The timecourse of left ventricular developed pressure for each experimental group in experiments to determine the effect of the selective β_1-adrenoceptor antagonist, CGP-20712A on infarct size reduction by postconditioning. There were no significant differences between groups. Error bars have been removed from all groups except for controls to improve clarity.

5.4.4 Infarct size and functional parameters – ICI-118,551

When hearts were treated with the selective β_2-adrenoceptor antagonist ICI-118,551 at reperfusion, the infarct size (79.7 ± 5.3) was significantly ($P<0.01$) larger than control hearts, a 94 % relative increase in infarct size (Figure 5-11). When postconditioning and ICI-118,551 were combined, the infarct size was (76.8 ± 6.7), significantly larger than both control ($p<0.01$) and postconditioned ($p<0.01$) hearts. These effects occurred without significantly affecting the timecourse of coronary flow (Figure 5-12) throughout the experiment, or the recovery of contractile function as assessed by rate pressure product (Figure 5-13) or left ventricular developed pressure (Figure 5-14).

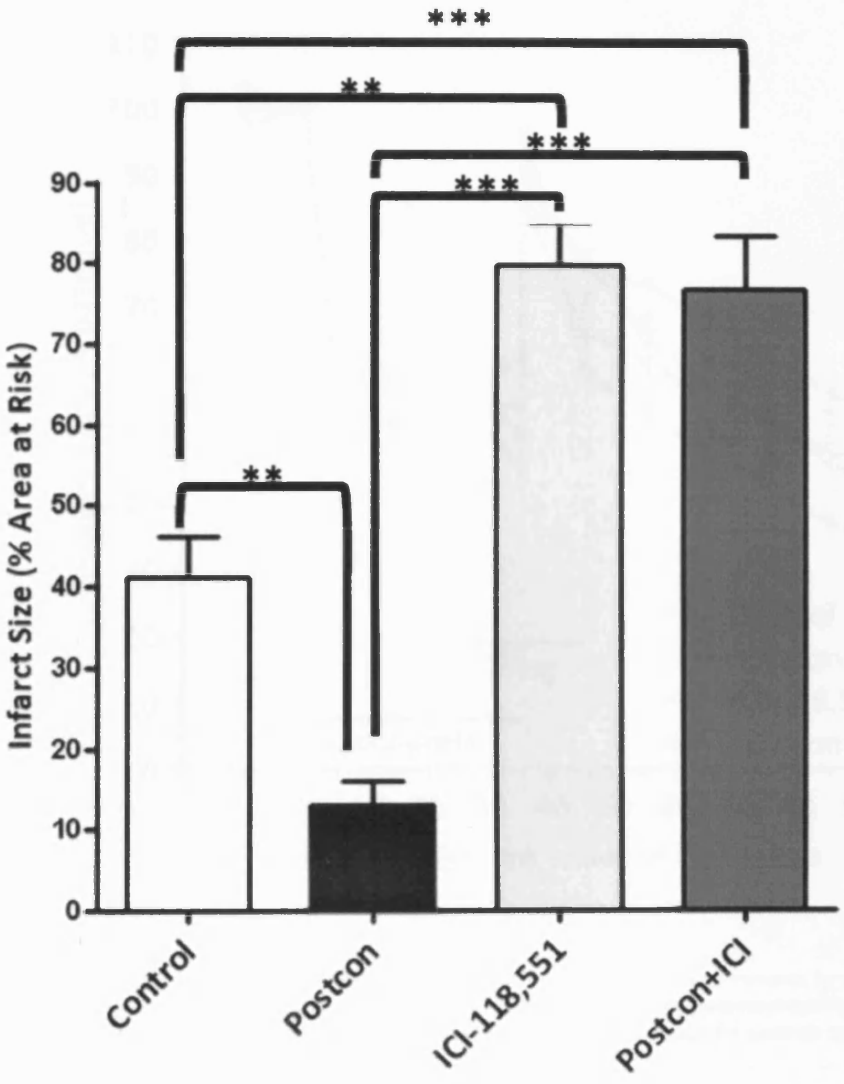

Figure 5-11 The effect of the selective β_2-adrenoceptor antagonist, ICI-118,551 on infarct size reduction by postconditioning. ** p<0.01 , *** p<0.001.

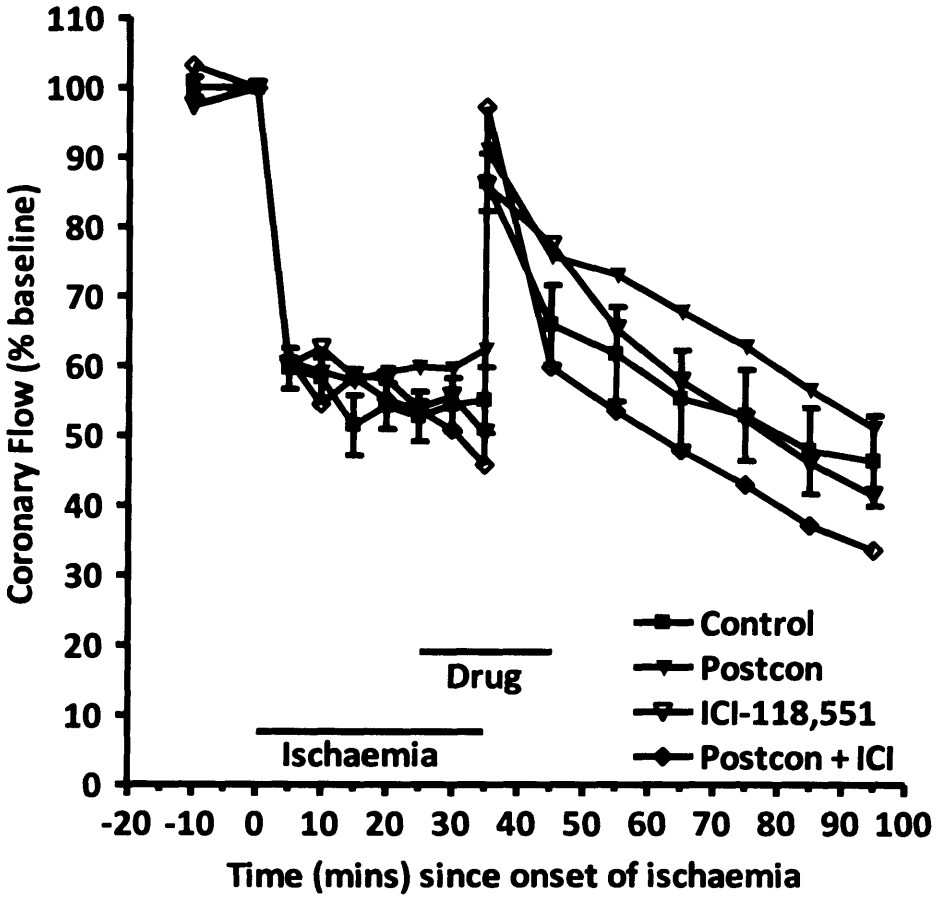

Figure 5-12 The timecourse of coronary flow for each experimental group in experiments to determine the effect of the selective β_2-adrenoceptor antagonist, ICI-118,551 on infarct size reduction by postconditioning. There were no significant differences between groups. Error bars have been removed from all groups except for controls to improve clarity.

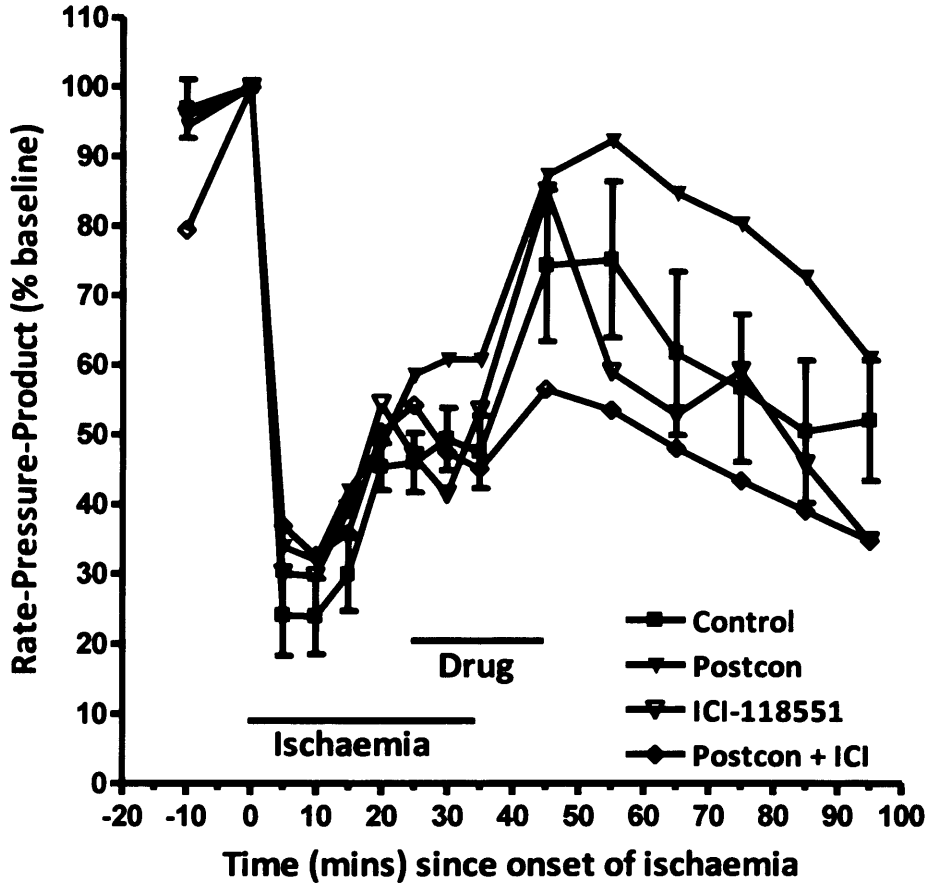

Figure 5-13 The timecourse of rate-pressure product for each experimental group in experiments to determine the effect of the selective β_2-adrenoceptor antagonist, ICI-118,551 on infarct size reduction by postconditioning. There were no significant differences between groups. Error bars have been removed from all groups except for controls to improve clarity.

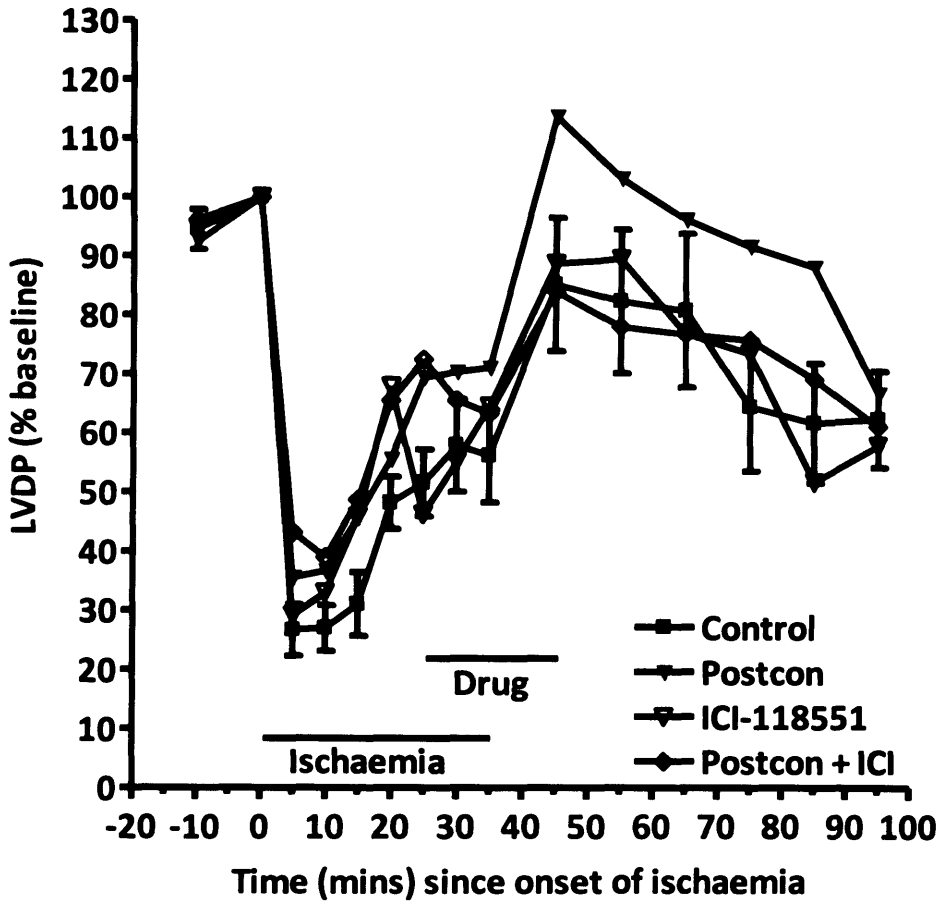

Figure 5-14 The timecourse of rate-pressure product for each experimental group in experiments to determine the effect of the selective β_2-adrenoceptor antagonist, ICI-118,551 on infarct size reduction by postconditioning. There were no significant differences between groups. Error bars have been removed from all groups except for controls to improve clarity.

5.4.5 Infarct size and functional parameters – SR-59230A

When hearts were treated with the selective β_3-adrenoceptor antagonist SR-59230A at reperfusion, the infarct size (66.3 ± 12.9) was significantly ($P<0.05$) larger than control hearts, a 61 % relative increase in infarct size (Figure 5-15). When postconditioning and SR-59230A were combined, the infarct size was (61.6 ± 12.9), significantly larger than postconditioned ($p<0.001$) hearts, but not significantly different from controls. These effects occurred without significantly affecting the timecourse of coronary flow (Figure 5-16) throughout the experiment, or the recovery of contractile function as assessed by rate pressure product (Figure 5-17) or left ventricular developed pressure (Figure 5-18).

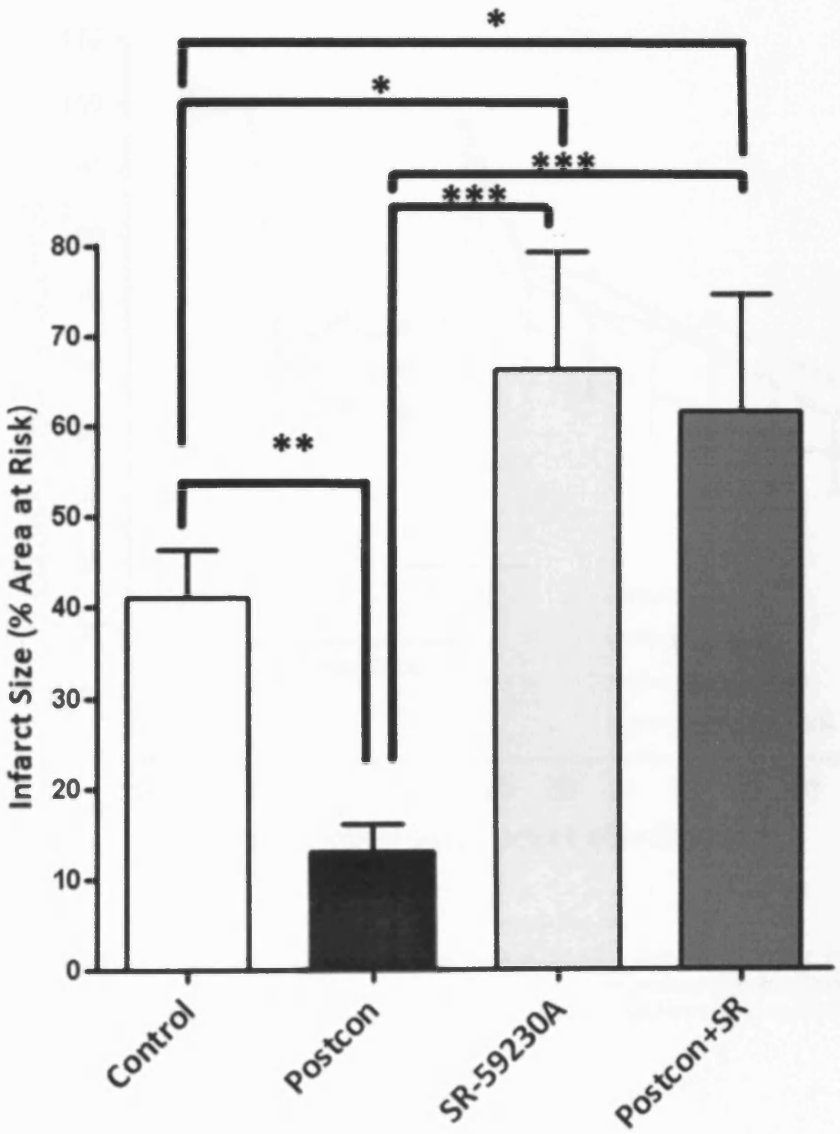

Figure 5-15 The effect of the selective β_3-adrenoceptor antagonist SR-59230A 223 on infarct size reduction by postconditioning. * p<0.05,** p<0.01, *** p<0.001.

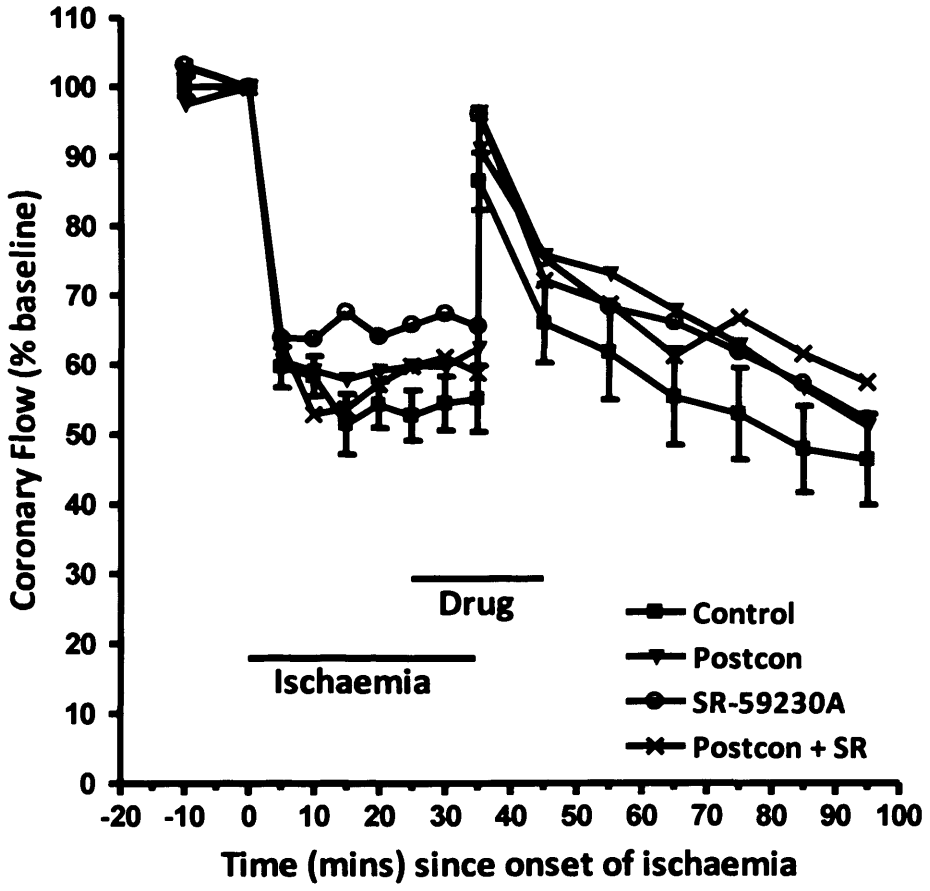

Figure 5-16 The timecourse of coronary flow for each experimental group in experiments to determine the effect of the selective β_3-adrenoceptor antagonist, SR-59230A on infarct size reduction by postconditioning. There were no significant differences between groups. Error bars have been removed from all groups except for controls to improve clarity.

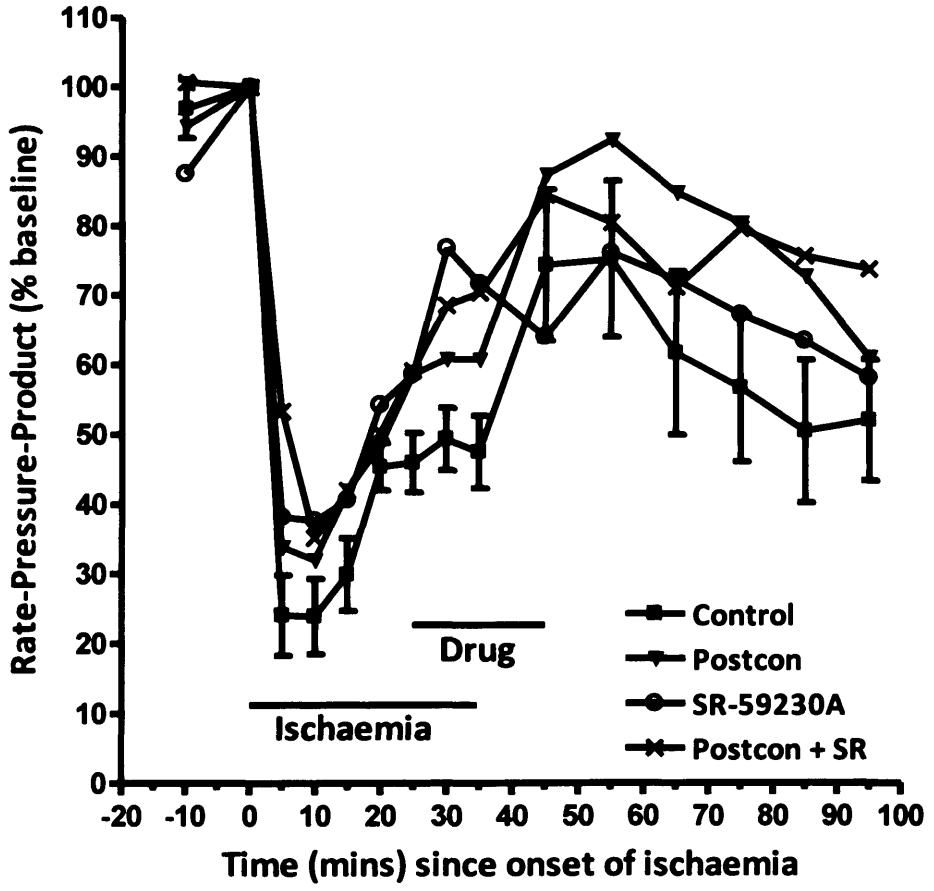

Figure 5-17 The timecourse of rate-pressure-product for each experimental group in experiments to determine the effect of the selective β_3-adrenoceptor antagonist, SR-59230A on infarct size reduction by postconditioning. There were no significant differences between groups. Error bars have been removed from all groups except for controls to improve clarity.

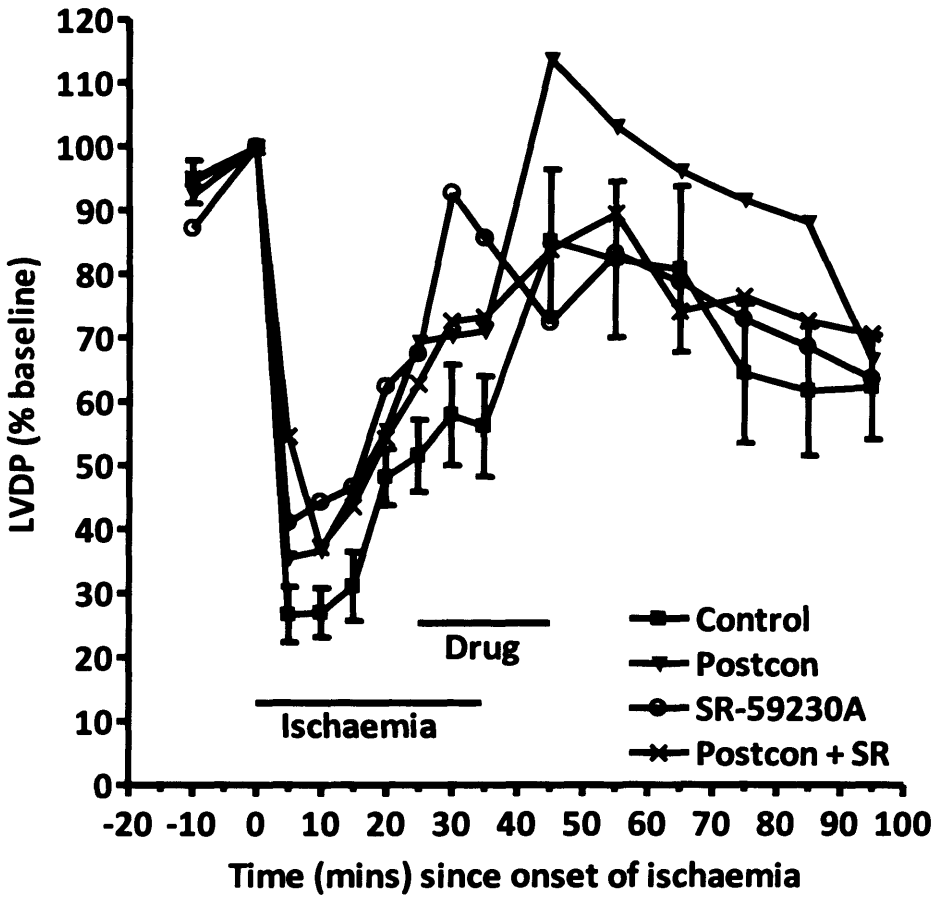

Figure 5-18 The timecourse of left-ventricular developed pressure for each experimental group in experiments to determine the effect of the selective β_3-adrenoceptor antagonist, SR-59230A on infarct size reduction by postconditioning. There were no significant differences between groups. Error bars have been removed from all groups except for controls to improve clarity.

5.4.6 Baseline cardiodynamics for catecholamine release study

Baseline cardiodynamics for both treatment groups are shown below (Table 5-4). There were no significant differences between the groups. Parameters were compared using an unpaired t-test.

Table 5-4 Baseline cardiodynamics for catecholamine release study

Treatment	n	Baseline RPP ($mmHg.min^{-1}/1000$)	Baseline CF ($ml.min^{-1}$)
Control	4	22.9 ± 3	12.3 ± 1.3
Postconditioning	4	19.0 ± 3	11.3 ± 0.8

5.4.7 Catecholamine release at reperfusion

Adrenaline and noradrenaline were successfully separated using HPLC and detected using electrochemical detection. A sample trace is shown below (Figure 5-19) Retention times were 154 seconds for noradrenaline and 190 seconds for adrenaline. End stabilisation values of both catecholamines were approximately 1nM. Using a signal/noise ratio of 3, 0.1 nM of adrenaline or noradrenaline could be detected using this method.

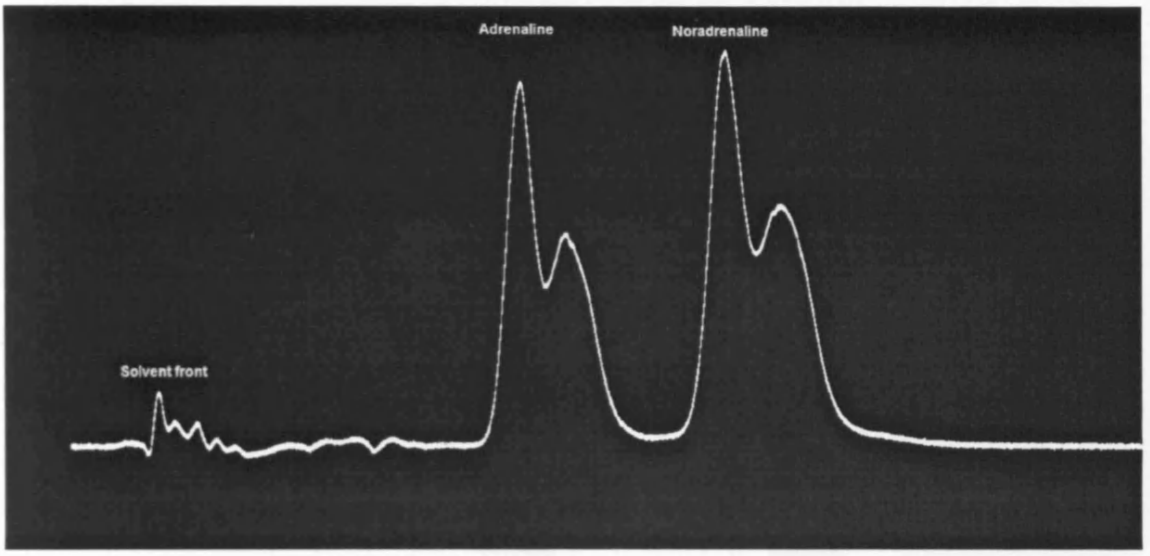

Figure 5-19. A trace from a HPLC experiment demonstrating peak resolution between adrenaline and noradrenaline. The shoulders probably represent a breakdown product of catecholamines. Further work would be required for formal identification. Equal concentrations (10 nM) of adrenaline and noradrenaline standards were injected into the column simultaneously.

Neither noradrenaline nor adrenaline concentrations were significantly altered by ischaemia or reperfusion (Figure 5-20).

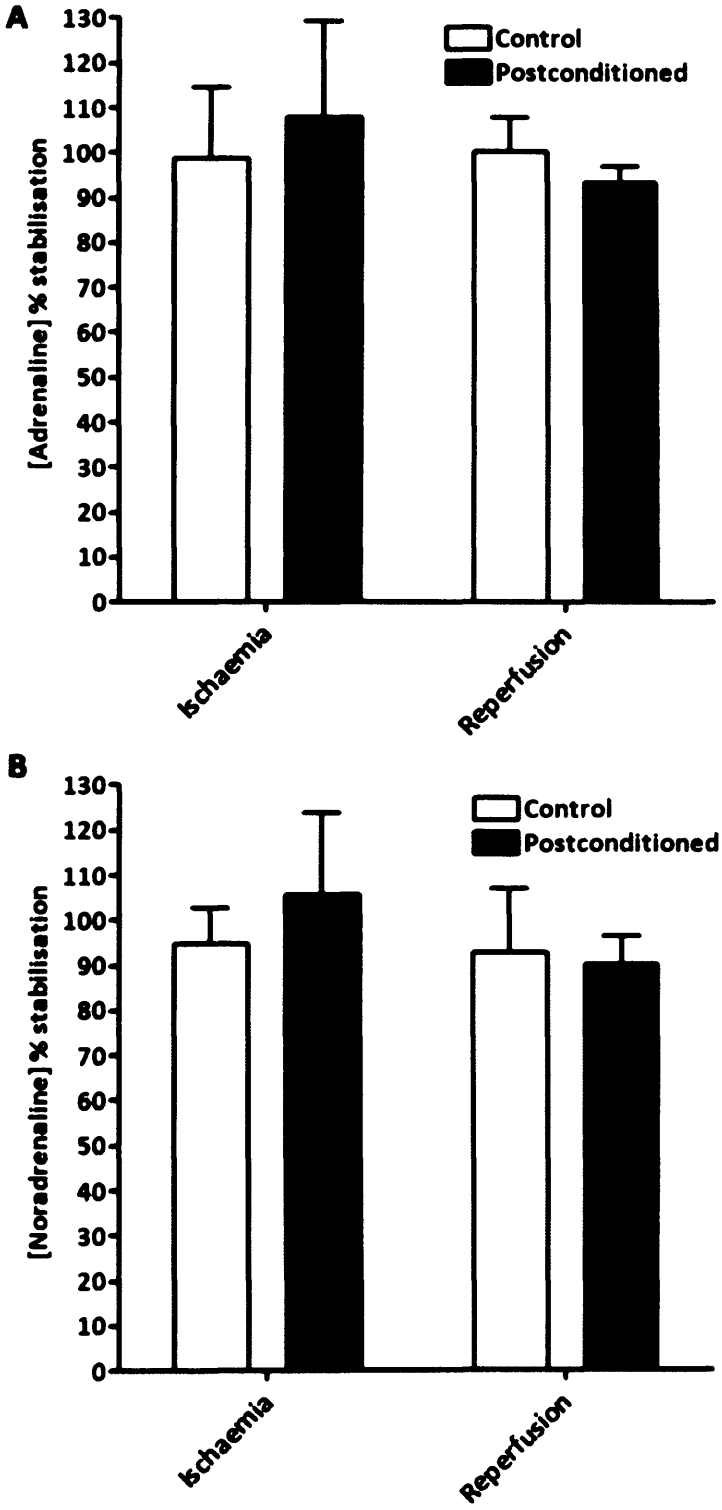

Figure 5-20 Concentration of adrenaline (A) and noradrenaline (B) in coronary perfusate at the end of ischaemia and after 3 minutes reperfusion in control and postconditioned tissues expressed as a percentage of end-stabilisation values which were approximately 1nM for both catecholamines.

5.5 Discussion

This study presents the first evidence that β-adrenoceptor activation is required for postconditioning. The non-selective β-adrenoceptor antagonist, timolol prevented infarct size reduction by postconditioning. When timolol was administered to tissues which endured ischaemia and reperfusion without postconditioning, infarct size was comparable with untreated controls. This suggests that timolol had no effect on reperfusion injury when applied alone at reperfusion, however studies designed to identify the receptor subtype responsible for the protective effect of postconditioning, suggest that when a non-selective antagonists such as timolol is applied, the outcome is a combination of its effects on different β-adrenoceptor subtypes. These multi-factorial effects may go some way towards explaining the surprising result whereby timolol and postconditioning combined gave a larger infarct size than seen in controls which would otherwise be difficult to explain.

In common with other investigators (Feuerstein *et al.*, 1998; Gao *et al.*, 2000) it was found that administration of a selective β_1-adrenoceptor antagonist (in this case, CGP-20712A) at reperfusion led to a significant reduction in infarct size. The infarct size reduction was of a similar magnitude to that achieved by postconditioning. When postconditioning and CGP-20712A were combined, the result was neither significantly different from control nor from postconditioned tissues. This is probably due to the fact that a number of experiments in this group were excluded for technical reasons, leaving a small number of replicates. However, these results suggest that activation of β_1-adrenoceptors by endogenous catecholamines at reperfusion has a detrimental effect on survival which can be abrogated by use of a selective antagonist. This presents a therapeutic strategy in the reduction of reperfusion injury. It was

interesting to note the trend towards improved recovery of coronary flow and rate-pressure-product in tissues treated with CGP-20712A. Although no significant difference was seen when the recovery curves were compared using repeated-measures ANOVA, a t-test comparing the LVDP at 60 minutes after reperfusion showed a significantly improved function at this time-point, in addition to the reduction of infarct size. Although the validity of performing such a statistical test may be questioned, it is nonetheless interesting because the idea of administering a negatively inotropic agent to a post-ischemic heart may be questioned by conventional wisdom, even if it results in a reduction of infarct size, because of its potential to depress cardiac function at a critical time when cardiac output may be compromised. However, these results demonstrate that cardiac function is not compromised by this agent, and suggest that it may even be improved.

Conversely, an increase in infarct size above control was observed in both postconditioned and non-postconditioned tissues treated with the selective β_2-adrenoceptor antagonist, ICI-118,551. This is interesting in light of a study which demonstrated that ICI-118,551 caused cell death by apoptosis in cultured myocytes (Communal *et al.*, 1999). The detrimental effects of a β_2-adrenoceptor antagonist at reperfusion suggest that endogenous catecholamines are acting upon these receptors and conferring innate protection. The identity of the catecholamine is not immediately clear however. Noradrenaline is released from sympathetic nerve endings during ischaemia as a result of the reverse function of the uptake$_1$ transporter (Schoemig & Richard, 1991). However noradrenaline preferentially activates β_1-adrenoceptors, whereas adrenaline preferentially activates β_2-adrenoceptor (Lands et al., 1967). Thus adrenaline is more likely to

mediate this effect. Indeed, adrenaline was detected in the coronary effluent from hearts in this study indicating its release within the heart.

The concentrations of adrenaline and noradrenaline at the end of stabilisation were similar, and were not altered by ischaemia. This may be interpreted as a failure to demonstrate ischaemia-induced catecholamine release. However, given that these experiments were carried out in isolated tissues in which the reserves of adrenaline and noradrenaline are limited and constantly depleting, the fact that the concentration at the end of ischaemia is the same as at the beginning despite this depletion is remarkable and might be indicative of the release of catecholamines. This question could be answered with the use of non-ischaemic time control tissues which were unfortunately not employed in this study. Catecholamine levels at reperfusion were not different in control and postconditioned hearts. Therefore these results do not support the hypothesis. However, it remains possible that the difference in catecholamine levels is too small to be detected by the assay employed, or that the washout of the catecholamines had already occurred by the perfusate samples were taken at the end of postconditioning.

The hypothesis that postconditioning prevents the immediate washout of endogenous mediators at reperfusion allowing activation of RISK pathways, is an attractive one. The hypothesis was first proposed for adenosine (Kin *et al.*, 2005; Philipp *et al.*, 2006). However any autacoid which can activate RISK pathways at reperfusion should be able to mimic postconditioning. The importance of different mediators may vary between experimental

models. In this study, an important role for β-adrenoceptors in postconditioning has been demonstrated.

ICI-118,551 is the only available selective β_2-adrenoceptor antagonist, thus it could be argued that an off-target toxic effect is responsible for the infarct-potentiating effect. This cannot be ruled out, however emerging evidence in other models of cellular injury has demonstrated potentiation of damage in β_2-adrenoceptor knockout strains. Patterson (2004) conducted studies in which isoprenaline was infused into β_1-adrenoceptor and β_2-adrenoceptor knockout mice; there was greater mortality and increased myocyte apoptosis in the β_2-adrenoceptor knockout mice. Thus it had been suggested that β_1-adrenoceptor signalling is pro-apoptotic and β_2-adrenoceptor signalling is anti-apoptotic (Xiao *et al.*, 2004). How this relates to cell death at reperfusion which is probably predominantly necrotic, is not yet known.

Postconditioning was unable to reverse the increase in infarct size caused by ICI-118,551. Indeed, there was not even a trend towards smaller infarct sizes in hearts exposed to postconditioning and ICI-118,551. This suggests that either β_2-adrenoceptor activation is necessary for postconditioning to be protective, or that antagonism of β_2-adrenoceptors, in the absence of concomitant β_1-adrenoceptor antagonism at reperfusion is so devastating to the cell that cell death is inevitable and postconditioning cannot confer any protection.

Thus, the absence of an effect of timolol alone on reperfusion may in fact reflect functional antagonism between a protective β_1-adrenoceptor antagonist and a detrimental β_2-adrenoceptor antagonist at reperfusion.

It was also interesting that the experiments utilizing the β_3-adrenoceptor antagonist, SR-59230A, gave very similar results to the β_2-adrenoceptor antagonist. It caused an increase in infarct size above that seen in controls and this damage could not be reversed by postconditioning. It is known that β_3-adrenoceptors couple to G_i and are in that respect similar to β_2-adrenoceptor. Little is known about the function of β_3-adrenoceptors in the heart, however, they are present in far smaller density than β_2-adrenoceptors. In light of this, it would seem surprising that a β_3-adrenoceptor antagonist could have such a marked effect on infarct size. This result could be due to non-selective interactions of SR-59230A which was the first commercially available β_3-adrenoceptor antagonist (Nisoli *et al.*, 1996), however some studies have suggested that SR-59230A is not selective for β_3-adrenoceptors, causing antagonism of other β-adrenoceptor subtypes (Candelore *et al.*, 1999) and α-adrenoceptors (Leblais *et al.*, 2004). It also appears that there are considerable differences between human and rodent β_3-adrenoceptors (Alexander *et al.*, 2008). Thus, it is difficult to determine from these results alone whether this represents a true β_3-adrenoceptor-mediated effect or whether the effect was mediated via β_2-adrenoceptor antagonism. The use of β_3-adrenoceptor knockout strains could help to clarify this result in the future.

If these results were indicative of the situation in humans *in vivo* they would be of extreme relevance in the clinic, where β-adrenoceptor antagonists are administered for the treatment of angina, hypertension and heart failure (Mehta, 2006). It would appear that antagonists which act upon the β_2-adrenoceptor or β_3-adrenoceptor are detrimental at reperfusion and consequently β_1-adrenoceptor antagonists are likely to be more protective than non-selective antagonists, at least acutely at reperfusion. Mechanical postconditioning induced by repeated inflations and deflations of an angioplasty balloon have been shown to reduce creatine kinase release (a marker of infarct size) from human hearts undergoing revascularisation therapy after myocardial infarction (Staat *et al.*, 2005). Recently, long term clinical benefits have also been reported (Thibault *et al.*, 2008). Postconditioning is therefore moving from an experimental observation to a clinical therapy. Thus it is important to investigate whether commonly used drugs have the potential to prevent protection induced by postconditioning (or worse, to cause increased infarct size in non-postconditioned tissues).

Pharmacological mimetics of postconditioning are being actively sought (Vinten-Johansen *et al.*, 2005a; Vinten-Johansen *et al.*, 2005b). This study demonstrated that β_2-adrenoceptor antagonists had detrimental effects when administered at reperfusion. This leaves open the possibility that agonists at these receptors may have the opposite effect and could be used to induce postconditioning. Alternatively, β_2-adrenoceptors may be maximally stimulated by endogenous agonists in which case no effect of additional exogenous agonists would be expected. Even if this is the case, it would appear that administration of a β_1-adrenoceptor antagonist reduces infarct size

The relevance of these results to the clinical situation is limited by the *ex vivo* nature of the preparation. This model lacks sympathetic stimulation and circulating catecholamines, so the concentrations of the catecholamines in the myocardium may have been far removed from physiological normal levels.

Because of the well documented vascular effects of β-adrenoceptor ligands, α-adrenoceptors mediating vasoconstriction and β-adrenoceptors mediating vasodilatation (Ahlquist, 1948), it is important to exclude the possibility that adrenoceptor-mediated variations in blood flow are responsible for any protective or detrimental effects seen at reperfusion. In this study, there was no significant difference between experimental groups in the timecourse of coronary flow throughout ischaemia and reperfusion. However, this method was unable to demonstrate any alterations in regional blood-flow. This is worthy of further investigation. It is not clear whether activation of β-adrenoceptors at reperfusion would cause a homogenous vasodilatation throughout the heart, or whether vasodilatation would occur preferentially in tissue which had not previously been ischaemic causing coronary 'steal'(Reivich *et al.*, 1961) and directing blood-flow away from damaged regions. A final possibility is that blood would be diverted towards the area at risk. Thus, an important topic for future investigation would be the measurements of regional coronary flow during ischaemia and reperfusion. This could be achieved using fluorescently labelled microspheres (Chien *et al.*, 1995).

5.5.1 Clinical implications

This study has confirmed the results seen elsewhere that β_1-adrenoceptor antagonists at reperfusion lead to a reduction in infarct size and thus clinical benefit may be gained by the use of antagonists selective against β_1-adrenoceptors at reperfusion. Many patients with cardiovascular disease are prescribed β-adrenoceptor antagonists, including a number of non-selective agents such as carvedilol and propranolol. The results from this study suggest that these agents have the potential to abrogate the protective effects of postconditioning. Because postconditioning has been demonstrated in man *in vivo* (Staat *et al.*, 2005) and has been proposed as a therapeutic strategy, a thorough understanding of any factors likely to blunt its protection is essential. Antagonists of β_2-adrenoceptors and β_3-adrenoceptors may also blunt protective effects mediated by endogenous catecholamines in the absence of postconditioning. This has important implications for the use of non-selective antagonists.

5.6 Conclusions

- The postconditioning protocol used in this series of experiments significantly reduces infarct size.

- The protection afforded by postconditioning is abrogated by a non-selective β-adrenoceptor antagonist.

- The protection afforded by ischaemic postconditioning is abrogated by antagonism of β_2 adrenoceptors.

- No detectable differences in the concentrations of adrenaline and noradrenaline in the coronary perfusate of control and postconditioned tissues were observed.

Chapter 6 – Can β-adrenoceptor agonists mimic postconditioning in the rat Langendorff heart ?

6.1 Introduction

In Chapter 5, it was demonstrated that the protection afforded by ischaemic postconditioning was lost in the presence of a non-selective β-adrenoceptor antagonist (timolol), suggesting that β-adrenoceptor activation is necessary for postconditioning to exert its effect. Furthermore it was demonstrated that the protection is probably mediated via the β_2-adrenoceptor or the β_3-adrenoceptor, because selective antagonists for these receptors (ICI-118,551 and SR-59230A respectively), when applied at reperfusion, led to larger infarct sizes than seen in controls, implying a protective effect of endogenous catecholamines on these receptors.

Further evidence of the infarct-sparing effects of β_2-adrenoceptor and β_3-adrenoceptor activation could be provided by applying selective agonists of these receptors at reperfusion and examining whether the protective effects of postconditioning are mimicked. This chapter will thus examine the effects of the administration of selective and non-selective β-adrenoceptor agonists at reperfusion.

It was also demonstrated in Chapter 5 that the selective β_1-adrenoceptor antagonist, CGP-20712A was protective when applied at reperfusion, suggesting that activation of β_1-adrenoceptors and subsequent activation of the pathway involving adenylyl cyclase, cAMP, and

protein kinase A at reperfusion is detrimental. This set of experiments will also endeavour to demonstrate that this is the case. Noradrenaline will be used to stimulate the β_1-adrenoceptor, it should be selective at the concentrations used. However, in order to overcome any problems of receptor selectivity and to eliminate any secondary signalling pathways emanating from the β_1-adrenoceptor, forskolin, a direct activator of adenylyl cyclase will be additionally employed.

If β_1-adrenoceptor activation at reperfusion is detrimental and β_2-adrenoceptor or β_3-adrenoceptor activation is beneficial, it is unclear how non-selective agents will act when applied at reperfusion. One study, conducted in anesthetised dogs exposed to regional ischaemia, showed that isoprenaline, provided inotropic support but does not alter infarct size (LaBruno *et al.*, 1998). Therefore, the effects of the non-selective agent isoprenaline applied at reperfusion will be examined in this rat isolated heart model. The effects of administration of the non-selective α/β agonist, adrenaline, were described previously in Chapter 4. Adrenaline had no effect on infarct size at any of the concentrations employed.

If these agonists exert salutary effects by activation of RISK pathways, it should be possible to detect activation of these kinases using Western blotting techniques after treatment with the agonist. This will be an additional aim of this chapter.

6.2 Hypotheses

- Activation of β_1-adrenoceptors at reperfusion will increase infarct size in rat Langendorff heart preparations exposed to regional ischaemia and reperfusion.

- Direct activation of adenylyl cyclase using forskolin will increase infarct size. in Langendorff perfused rat hearts.

- The infarct-sparing effect of postconditioning will be mimicked by β_2 and β_3 adrenoceptor agonists in Langendorff perfused rat hearts.

- Protective effects are mediated via phosphorylation of the RISK pathway kinase Akt.

6.3 Methods

6.3.1 Infarct size study

Male Sprague-Dawley rats 250-350g were obtained from B&K international (Grimston, UK). Langendorff perfused rat hearts were set up as described previously (Chapter 2). After 20 minutes stabilisation, hearts were exposed to 35 minutes regional ischaemia and 2 hours reperfusion.

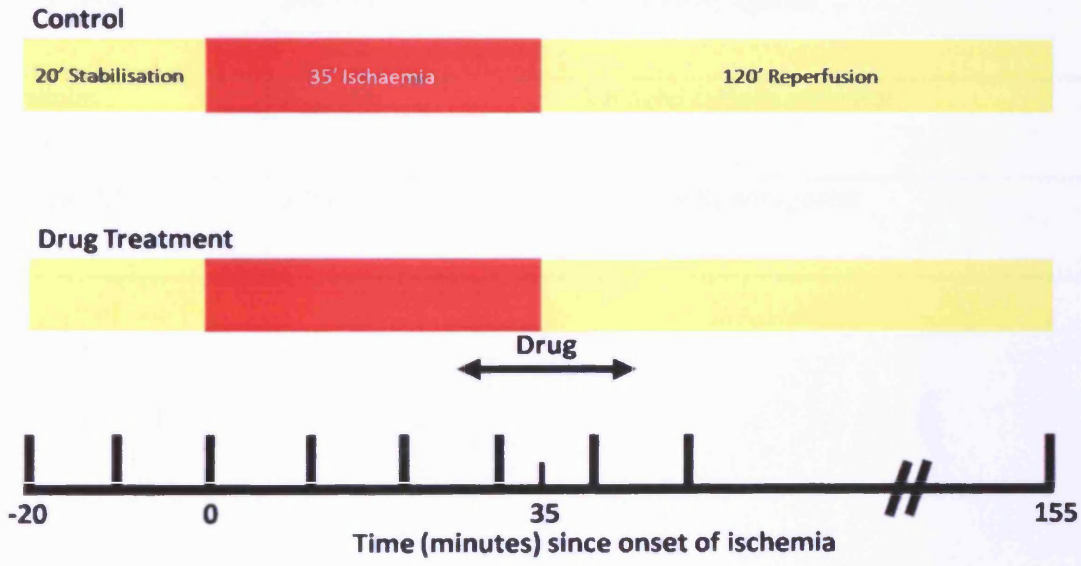

Figure 6-1 Experimental protocols used in this study. Time is shown along the abscissa. Yellow areas denote normal perfusion and red areas indicate ischaemia. Table 6-1 gives details of drugs and concentrations used

A range of selective and non-selective β-adrenoceptor agonists, and the adenylyl cyclase activator, forskolin (Table 6-1) were administered for the final 10 minutes of ischaemia and the first ten minutes of reperfusion (Figure 6-1).

Table 6-1 Pharmacological agents and their concentrations used in this study. All agents were administered for the final 10 minutes of ischaemia and the first ten minutes of reperfusion (Figure 6-1)

Drug	Concentration	Pharmacological action
Isoprenaline	0.1 μM	Non-selective β-adrenoceptor agonist
Noradrenaline	0.1 μM	Selective β_1 agonist
Salbutamol	1μM	Selective β_2 agonist (short acting)
Formoterol	10 nM	Selective β_2 agonist (long acting)
BRL-37344	300 nM	Selective β_3 agonist
Forskolin	250 nM	Adenylyl cyclase activator
ICI-118,551	10nM	Selective β_2 antagonist

Table 6-2 Justifications for the concentrations of drugs used in this study taken from the literature and from preliminary experiments in naïve tissues, the results of which are reported as follows NE, no effect; + small positive inotropic effect; ++ moderate positive inotropic effect; +++ large positive inotropic effect

Drug	Concentration	Literature reference	Inotropic effect
Isoprenaline	100 nM	Similar concentrations have been shown to elicit preconditioning against stunning in guinea-pig isolated atria (Yates *et al.*, 2003).	+++
Noradrenaline	100 nM	This concentration elicited preconditioning against post-ischaemic contractile dysfunction in isolated perfused rat hearts (Asimakis *et al.*, 1994).	+++
Forskolin	250 nM	This concentration causes sub-maximal cardiostimulant effects in isolated perfused rat hearts (England & Shahid, 1987).	+++
Salbutamol	1 μM	A slightly lower dose causes large changes in coronary flow and left ventricular developed pressure in isolated rat heart (Vleeming *et al.*, 1993)	++
Formoterol	10 nM	Submaximal chronotropic effect on guinea pig atria (Freyss Beguin *et al.*, 1983)	++
BRL-37344	300 nM	Submaximal negative inotropic concentration in perfused rat heart (Barbier *et al.*, 2007)	NE

6.3.2 Western blotting

In a further series of experiments, left apex tissue was harvested for western blotting for phosphorylated and total Akt. Samples were taken at the end of ischaemia and three minutes after reperfusion in control tissues and in tissues that had been subjected to ischaemic postconditioning and formoterol treatment at reperfusion (Figure 6-2).

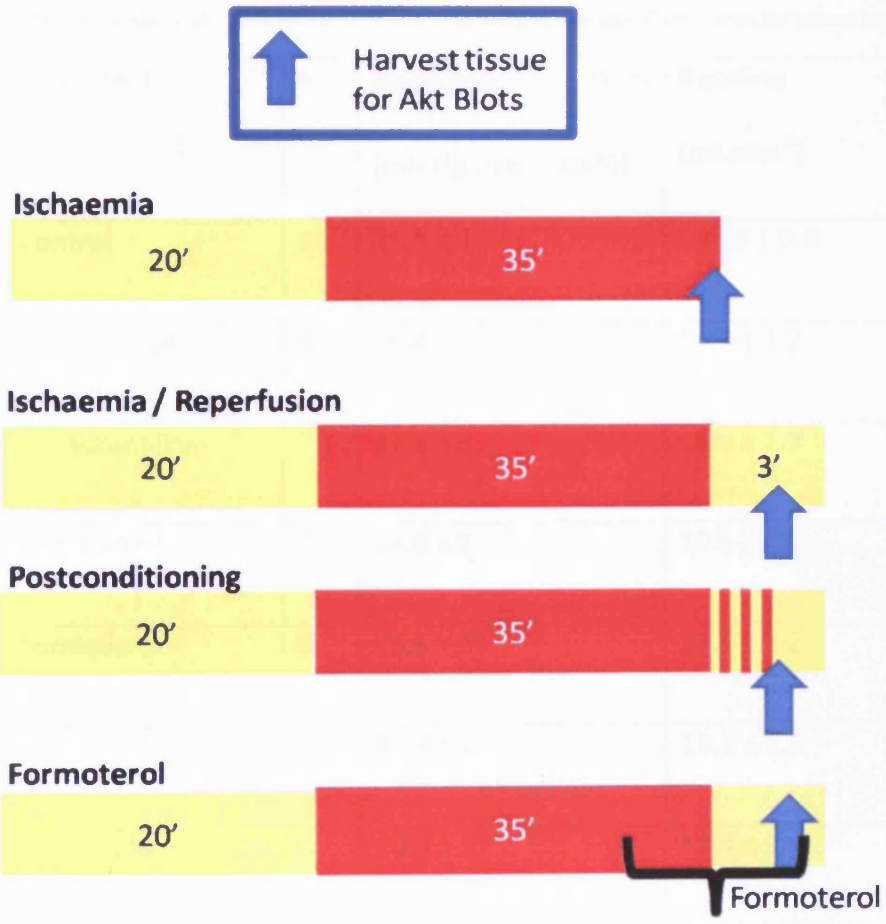

Figure 6-2 Protocol for tissue sampling in Western blotting study. n=4 for all groups except formoterol treatment (n=3).

6.4 Results

6.4.1 Baseline cardiodynamics - infarct size study

The baseline cardiodynamics for hearts used in this study are shown below (Table 6-3). There were no statistically significant differences between any of the treatment groups.

Table 6-3 Baseline cardiodynamics and Area at risk as a percentage of total area for hearts used in these studies

Treatment	n	Baseline RPP (mmHg.min^{-1}/1000)	Baseline CF (ml.min^{-1})	AAR (%Total area)
Control	19	26.3 ± 3	14.18 ± 0.8	30.9 ± 3.4
Isoprenaline	8	20.4 ± 2	14.5 ± 1.2	22.0 ± 2.8
Noradrenaline	7	27.5 ± 2	15.9 ± 1.7	23.6 ± 2.5
Salbutamol	7	17.4 ± 2	10.5 ± 0.8	28.7 ± 4.8
Formoterol	6	25.6 ± 3	17.5 ± 1.2	28.7 ± 4.9
Formoterol + ICI	5	28.3 ± 2	15.7 ± 2.3	25.6 ± 3
BRL-37344	6	20.9 ± 5	12.4 ± 0.4	27.3 ± 4.5
Forskolin	6	30.8 ± 2	15.7 ± 2.1	27.4 ± 3.9

6.4.2 Infarct size

In control tissues, the infarct was 38.8 ± 3.4 % of the area at risk. This was not significantly affected by administration of the non-selective β-adrenoceptor antagonist, isoprenaline at reperfusion which produced an infarct size of 30.8 ± 8.9. Noradrenaline which predominantly activates β_1-adrenoceptors also had no significant effect, the infarct size was 24.8 ± 5.4 (Figure 6-3).

The short-acting β_2-adrenoceptor agonist, salbutamol, did not have any effect on infarct size. The infarct size was 30.6 ± 6.6. However formoterol, a long-acting β_2-adrenoceptor agonist, significantly ($p<0.05$) reduced infarct size to 15.0 ± 3.1 %, representing a 61 % relative reduction in infarct size. No protection by formoterol was seen in the presence of the β_2-adrenoceptor antagonist, ICI-118,111, indeed, the infarct size was 57.3 ± 9.8, significantly ($p< 0.05$) greater than controls (Figure 6-3).

The β_3-adrenoceptor agonist, BRL-37344, reduced infarct size to 17.2 ± 4.4 % of the area at risk when administered at reperfusion, a statistically significant ($p<0.05$) 56 % relative reduction in infarct size compared to controls (Figure 6-3).

The adenylyl cyclase activator, forskolin, led to an increase in infarct size which was significantly ($p<0.05$) greater than controls encompassing 59.0 ± 2.6 % of the area at risk and representing a 52 % relative increase in infarct size (Figure 6-3).

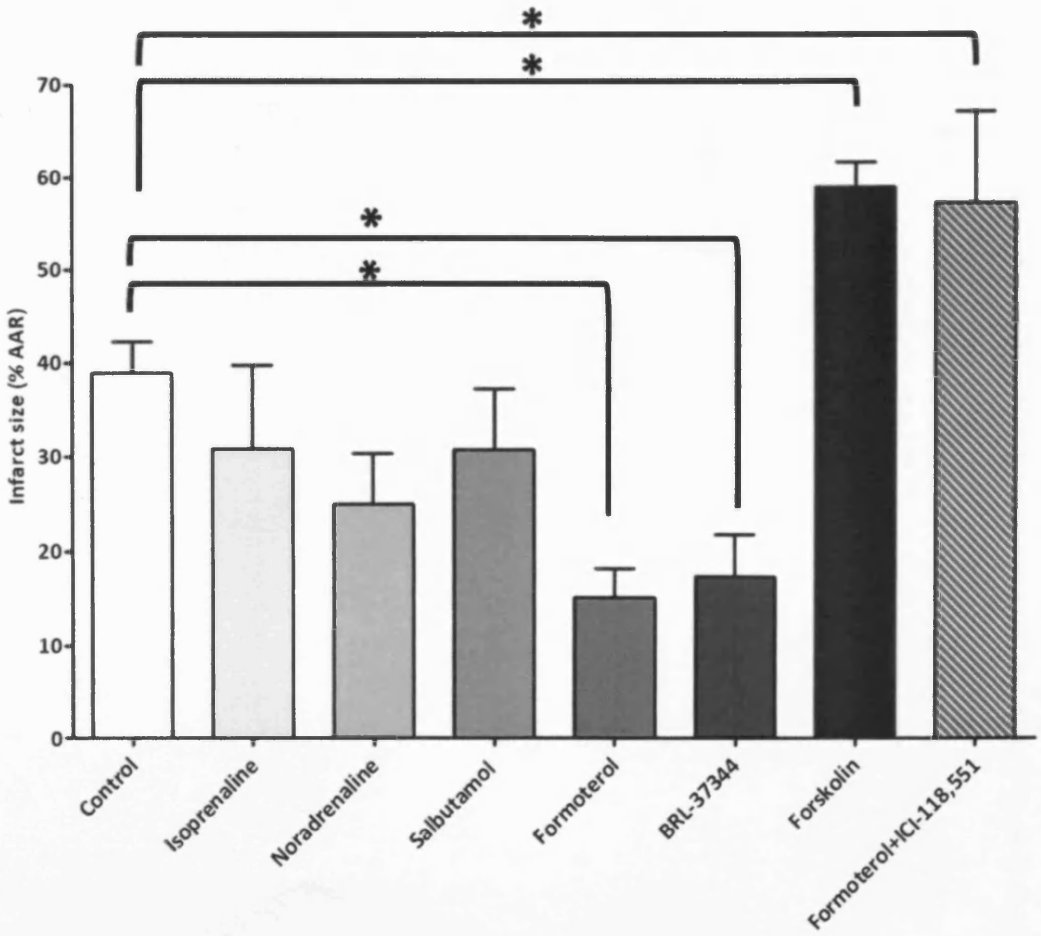

Figure 6-3 Infarct Size expressed as a percentage of the area at risk (%AAR) for the experimental groups in this study * indicates p<0.05..

6.4.3 Functional parameters

Regional ischaemia was confirmed by a drop in coronary flow of at least 30% in all preparations. Rate pressure product also fell rapidly during ischaemia. Recovery of both parameters was seen at reperfusion. No significant differences were seen between experimental groups in the timecourse of coronary flow (Figure 6-4), rate pressure product (Figure 6-5) or left ventricular developed pressure (Figure 6-6) throughout the experiment.

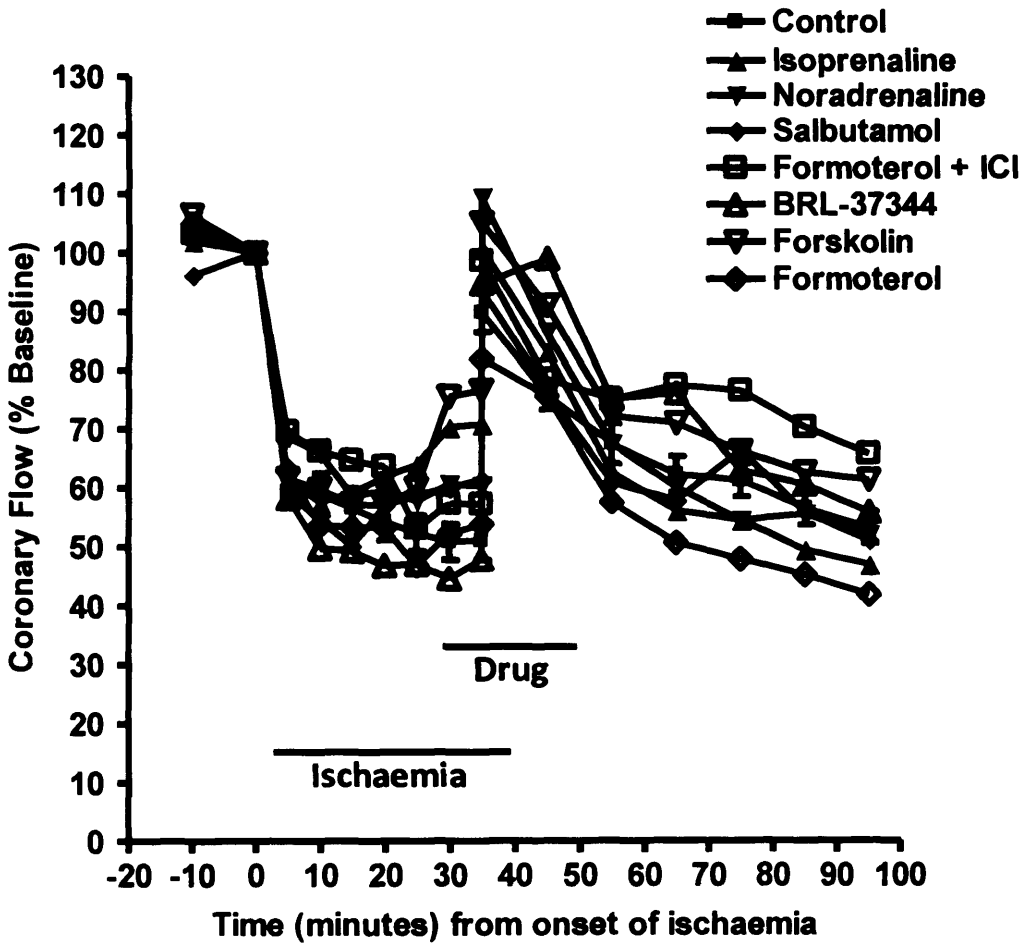

Figure 6-4 Timecourse of coronary flow throughout the experiment. There were no significant differences between the groups. Error bars have been removed from all groups except controls to improve clarity.

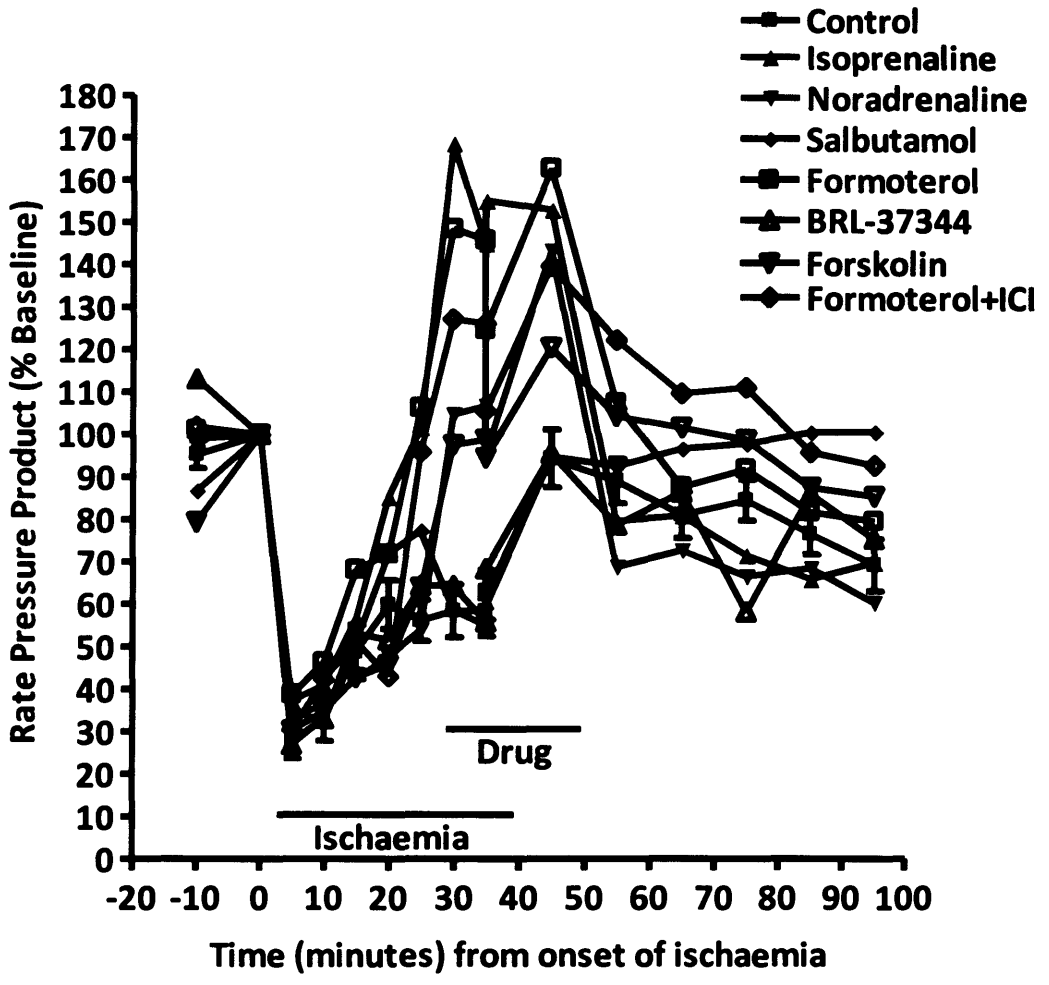

Figure 6-5 Timecourse of rate pressure product throughout the experiment. There were no significant differences between the groups. Error bars have been removed from all groups except controls to improve clarity.

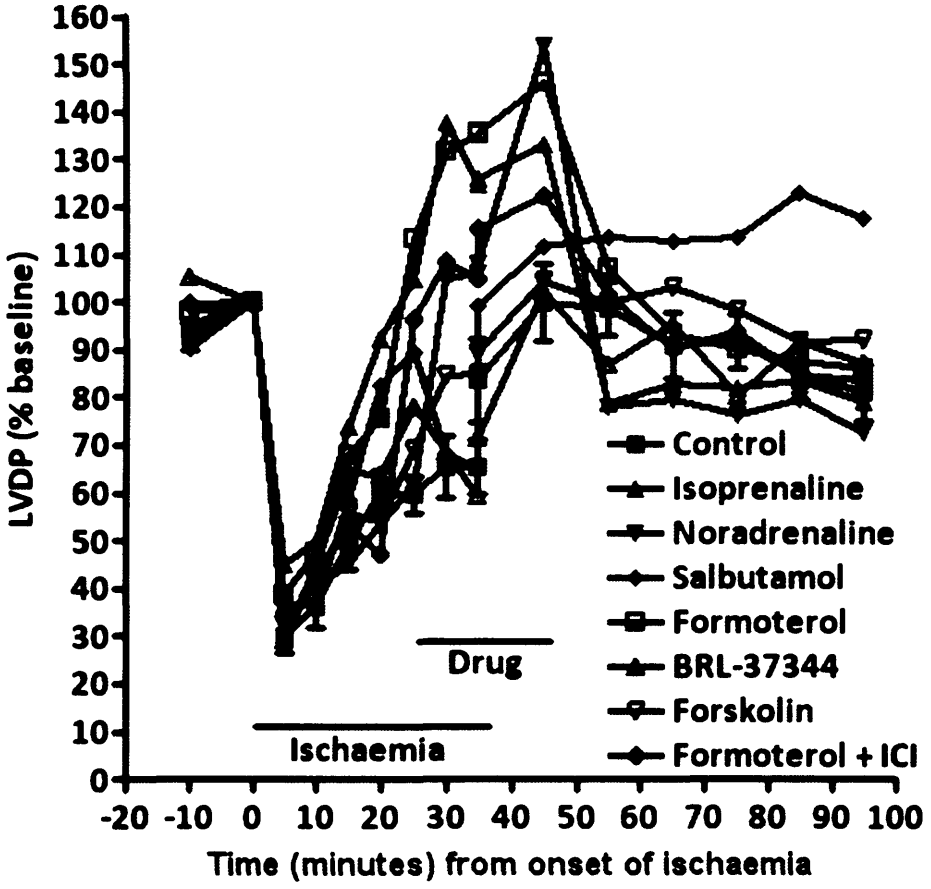

Figure 6-6 Timecourse of LVDP throughout the experiment. There were no significant differences between the groups. Error bars have been removed from all groups except controls to improve clarity.

6.4.4 Baseline cardiodynamics – hearts used for Western blots

There were no significant differences between the experimental groups in terms of the baseline parameters (Table 6-4).

Table 6-4 Baseline cardiodynamics for hearts used in the Western blotting study

Treatment	n	Baseline RPP ($mmHg.min^{-1}/1000$)	Baseline CF ($ml.min^{-1}$)
Stabilisation	4	21.9 ± 2	12.3 ± 0.7
Ischaemia	4	21.6 ± 5	12.0 ± 1.7
Reperfusion	4	22.9 ± 3	12.3 ± 1.3
Ischaemic Postconditioning	4	19.0 ± 3	11.3 ± 0.8
Formoterol Postconditioning	3	18.9 ± 8	14.0 ± 2.0

6.4.5 Akt phosphorylation

There were no significant differences between the experimental groups in terms of the extent of Akt phosphorylation at three minutes after reperfusion (Figure 6-7).

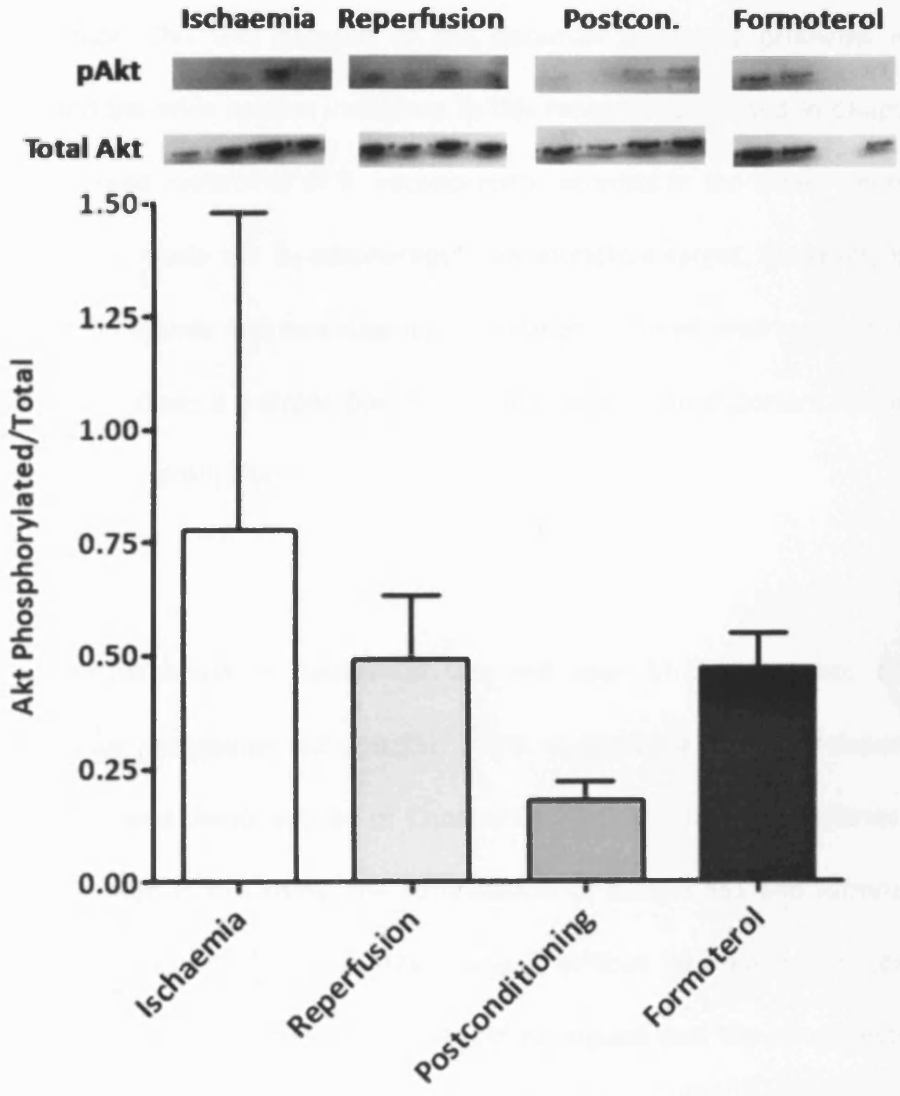

Figure 6-7 Ratios of phosphorylated/total Akt and Western blots for hearts at the end of ischaemia (n=4), at early reperfusion in control (n=4) , postconditioned (n=4) and formoterol (n=3) treated hearts. The western blots from which the data were calculated are shown above the bars. Only one band was visible per blot, this was seen between 50 and 75 Kilo Daltons and is thus characteristic of Akt. Each lane on the gel corresponds to one heart.

6.5 Discussion

The most important novel finding from this study is that postconditioning can be mimicked by application of formoterol, a β_2-adrenoceptor agonist at reperfusion. Similarly, the β_3-adrenoceptor agonist, BRL-37344 reduced infarct size when given just prior to reperfusion. It was decided to concentrate further investigation on the protective effect of the β_2-adrenoceptor. This was because of the potential selectivity problems of β_3-adrenoceptor ligands and the wide species variations in this receptor (discussed in Chapter 5). Additionally, the widespread availability of β_2-adrenoceptor agonists in the clinic, where they are used to treat asthma, made the β_2-adrenoceptor an attractive target. However, because both a β_3-adrenoceptor agonist and an antagonist have been demonstrated to affect infarct development in this model, there is a strong possibility that they play an important role in postconditioning. This certainly warrants further investigation.

The protective effect of formoterol was not seen in the presence of the selective β_2-adrenoceptor antagonist ICI-118,551. This suggests a receptor-dependent mechanism. However, as was demonstrated in Chapter 5, ICI-118,551 alone increases infarct size above controls. Indeed in this study, the combination of ICI-118,551 and formoterol resulted in an infarct size larger than control. This makes it difficult to draw a firm conclusion about the receptor-dependence of the result. It could be argued that the non-selective β-adrenoceptor antagonist, timolol should have been used, as this alone does not appear to have any effect on infarct size (Chapter 5). However, because a β_1-adrenoceptor antagonist decreases infarct size at reperfusion, and β_2-adrenoceptor and β_3-adrenoceptor antagonists increase infarct size, the

effects of a non-selective agent are likely to be a complicated summation of a number of effects. For this reason, the use of ICI-118,551 was preferable. Ultimately, β_2-adrenoceptor knockout strains should be used to investigate these effects, although, such experiments would be complicated by the fact that the animal would be lacking the β_2-adrenoceptor throughout ischaemia and reperfusion.

The mechanism of this protection has only begun to be elaborated by this study. This protection is unlikely to be mediated by a classic β-adrenoceptor signalling pathway via G_s, adenylyl cyclase, cAMP and protein kinase A, because neither isoprenaline (a non selective β-adrenoceptor agonist) nor forskolin (a direct activator of adenylyl cyclase) mimicked this protective effect. Indeed forskolin exerted a pro-infarct effect in this model. Furthermore, noradrenaline which predominately activates β_1-adrenoceptors and thereby signals downstream via protein kinase A, also failed to protect at reperfusion. This detrimental effect of PKA activation at reperfusion may be due entirely to a worsening of ischaemia caused by the positive inotropy and chronotropy elicited by this signalling pathway, however, it is interesting to note that studies have demonstrated pro-apoptotic actions of β_1-adrenoceptor signalling (Xiao et al., 2004)

In an attempt to investigate the effects by which this protection is mediated, samples of apex tissue were taken from hearts which had been treated with formoterol at reperfusion. Western blot analysis did not demonstrate activation of Akt after three minutes of reperfusion, in hearts treated with formoterol at reperfusion. However, Akt phosphorylation was not increased by

postconditioning, in which it is known to play an important role (Schwartz *et al.*, 2006; Sivaraman *et al.*, 2007; Tsang *et al.*, 2004; Zhu *et al.*, 2006). Thus this part of the study lacked a positive control. It is likely therefore that the fact that Akt phosphorylation was not observed in this study was due to a feature of the methodology employed. In particular, the time at which samples were taken is likely to be important. It has been demonstrated that when the adipocytokine, apelin-13 is applied to the heart at reperfusion, Akt phosphorylation increases with time, although the activity of Akt does not follow the same timecourse (Smith *et al.*, 2007). There is not yet any consensus as to the time of maximal Akt phosphorylation after ischaemic postconditioning. Furthermore, when the heart was dissected, it was impossible to be certain whether the sample of tissue taken was from the centre of the risk zone, the edge of the risk zone or from tissue which was normally perfused throughout the experiment. This is likely to have consequences for the levels of tissue phosphorylation. If further time and resources were available, it would be interesting to sample heart tissues at a number of time-points after reperfusion and also in non-ischaemic tissues exposed to formoterol. It would also be interesting to sample also for phosphorylation of another important RISK pathway mediator Erk 1/2 which may also be activated by β-adrenoceptors (Galandrin *et al.*, 2006; Tutor *et al.*, 2007) and is therefore a potential mediator of protection at reperfusion.

This study is the first to demonstrate pharmacological postconditioning by a β_2-adrenoceptor agonist. However, a recent study employing a model of hypoxia and reoxygenation in cultured human umbilical vein endothelial cells demonstrated that addition of formoterol to the medium, throughout hypoxia and reoxygenation led to reduced cell death. The effect was blocked by ICI-118,551 and by a nitric oxide synthase inhibitor (Pottecher *et al.*, 2006). This is

interesting because of the central role of nitric oxide in RISK pathways. However, because the time of administration of formoterol was different to that used in our protocol, it cannot be concluded whether or not the mechanism is the same as in our study. In human umbilical vein endothelial cells, reduction of reactive oxygen species by formoterol administration was an important part of the mechanism (Pottecher *et al.*, 2006) .

It was interesting that an alternative β_2-adrenoceptor agonist, salbutamol did not elicit protection. This may be a simple question of selectivity. Salbutamol is less selective, potent and has a lower affinity for the β_2-adrenoceptor than formoterol (Linden *et al.*, 1996). Any β_1-adrenoceptor activity in this setting might abrogate protective signalling via β_2-adrenoceptors. Thus, it may be that the concentration of salbutamol used in this study was too high. Formoterol is highly selective for β_2-adrenoceptors (Anderson, 1993). Another, major difference between these two molecules is that salbutamol is a 'short acting' β-adrenoceptor and formoterol is a 'long acting' agent. The difference between these is not merely pharmacokinetic, it is thought that long-acting β_2-adrenoceptor agonists may bind to an additional site on the β_2-adrenoceptor (Anderson, 1993) . Whether this has the potential to alter signalling is not certain. Finally, studies have demonstrated that drugs which act as agonists for classic β-adrenoceptor signalling do not necessarily act as activators of secondary signalling pathways such as Erk 1/2, which are likely to lead to cardioprotection. In fact they may possess properties of a neutral antagonist or an inverse agonist. However, only a very small number of β-adrenoceptor agonists have been investigated for their effects on Erk 1/2 signalling and it is not yet clear whether the additional binding site is responsible for this effect. Based on the results described here, this appears to be the case, as formoterol, a long acting agent which binds to both sites was protective whereas salbutamol, which binds to a single site, did not. Thus, this work

highlights the need for a systematic study of a range of β_2-adrenoceptor antagonists to screen them for cardioprotective properties.

It has been demonstrated in rat internal anal sphincter muscle that β_2-adrenoceptor agonists can lead to increased activity of protein kinase G, through a G_i mediated mechanisms (Galandrin *et al.*, 2006; Li *et al.*, 2004). If the same were true in the heart, this mechanism could explain the powerful cardioprotection elicited via β_2-adrenoceptor at reperfusion. Protein kinase G is one of the final stages in the RISK pathways, required to prevent cell death at reperfusion.

6.5.1 Clinical implications

If these findings could be replicated in man and *in-vivo*, they would be of considerable clinical interest. A wide range of β_2 agonists including formoterol, are already available in the clinic for the treatment of asthma. These drugs could potentially be administered immediately prior to reperfusion for their infarct-sparing effects. However, there are many reasons why the same mechanisms may not be important *in vivo* where maximal β_2-adrenoceptor stimulation may already occur. These effects may also be altered by peripheral vasodilator (hypotensive) actions of β_2-adrenoceptor agonists which would be undesirable in conditions of poor cardiac output.

6.6 Conclusions

- Direct activation of adenylyl cyclase at reperfusion is detrimental and leads to increased infarct development.

- Cardioprotection can be elicited by β_2 and β_3 adrenoceptor agonists applied at reperfusion.

- Cardioprotection elicited by the β_2-adrenoceptor agonist formoterol is not seen in the presence of the β_2-adrenoceptor antagonist, ICI-118,551.

- Increased levels of phosphorylated Akt are not detectable after ischaemic or pharmacological postconditioning at 3 minutes after reperfusion.

Chapter 7 - General discussion

7.1 Summary of novel findings

- Ischaemic preconditioning can protect against stunning in isolated rat atria and ventricles. This protection is dependent on β-adrenoceptor activation.

- β-adrenoceptor preconditioning can protect against stunning in isolated rat atria.

- Endogenous and exogenous adrenaline may worsen stunning when applied at reperfusion.

- Adrenaline does not affect infarct size when applied at reperfusion but increases reperfusion contracture in the Langendorff heart preparation.

- The non-selective β-adrenoceptor antagonist, timolol, has no overall effect on infarct size when given at reperfusion after regional ischaemia but blocks the protection elicited by postconditioning in the Langendorff heart preparation.

- Infarct size is greatly increased by antagonists of the β_2- and β_3-adrenoceptor given at reperfusion. This damage cannot be overcome by postconditioning.

- Infarct size is increased by activation of adenylyl cyclase at reperfusion.

- Antagonists of the β_1-adrenoceptor receptor reduce infarct size when applied at reperfusion.

- Agonists of the β_2- and β_3-adrenoceptor reduce infarct size when applied at reperfusion

7.2 Relevance of findings

Catecholamines and adrenoceptors have long been known to have important roles during ischaemia and reperfusion. The inspiration for this investigation resulted from a desire to re-evaluate these roles in the light of the recent discovery of ischaemic postconditioning. Experiments designed to test the hypotheses that postconditioning could be mimicked by β-adrenoceptor activation and that the mechanism of ischaemic postconditioning involved β-adrenoceptor activation formed the most important part of this investigation, both in terms of scientific novelty and because of the potential clinical implications of these hypotheses.

Activation of β-adrenoceptors had already been demonstrated to mimic preconditioning against infarct size. However, experiments designed to demonstrate whether ischaemic preconditioning was dependent on β-adrenoceptor activation had provided conflicting results suggesting that the result obtained was largely dependent on the experimental model employed. Rather than wade into this already complicated and controversial field with little possibility of providing a conclusive answer, it was decided to investigate a lesser studied

possibility, that β-adrenoceptor activation could cause preconditioning against stunning. It was discovered that in the isolated cardiac tissues used, that this was the case and that ischaemic preconditioning also relied upon β-adrenoceptor activation to ameliorate stunning.

Another aspect of interest was the fact that adrenaline is administered to patients in cardiac arrest as part of international resuscitation protocols. This is essentially a mimic and an extension of the central and local catecholamine release which occurs in the body during this condition. Adrenaline is administered for its vascular rather than its cardiac effects in this setting and there is little or no evidence for its effectiveness. It was hypothesised that the reason for the ineffectiveness of adrenaline was that it elicited detrimental cardiac effects. The most important of these is that that adrenaline increases the energy requirements of both the fibrillating and the beating heart and thus worsens ischaemia. It was hypothesised that adverse cardiac effects might offset the benefit achieved by peripheral vasoconstriction and that this could explain the very poor survival of patients undergoing this treatment.

Despite indications that adrenaline may worsen stunning and reperfusion contracture, it did not have any effect on infarct size at any of the concentrations employed. This was initially a surprise but later experiments demonstrated the remarkable infarct-sparing effects of β_2-adrenoceptor activation at reperfusion. Adrenaline has greater efficacy at β_2-adrenoceptors than β_1-adrenoceptor and therefore, this β_2-adrenoceptor activation may be sufficient to offset the damage which would be expected from giving this inotropic agent at reperfusion.

It was demonstrated that in the Langendorff preparation of the rat heart, activation of β-adrenoceptors is required for postconditioning and that postconditioning can be mimicked by agonists of β_2-adrenoceptor and β_3-adrenoceptors. These are the most important results described in this thesis because of their clinical relevance. However these experiments revealed an interesting profile of the effects of β-adrenoceptor agonists and antagonists given at reperfusion (Figure 7-1).

The β_1-adrenoceptor antagonist, CGP-20712A was protective at reperfusion. Noradrenaline, a β_1-adrenoceptor agonist had no significant effect, interestingly though, there was a trend towards smaller infarct sizes in tissues treated with noradrenaline. This is in contrast with the results seen when tissues were treated with forskolin, a direct activator of adenylyl cyclase, which led to a larger area of infarction. Forskolin and β_1-adrenoceptor agonists would be expected to act in a similar manner. This result may be because of a non-selective action of noradrenaline on the β_2-adrenoceptor.

Neither a non-selective β-adrenoceptor agonist (isoprenaline) nor a non-selective antagonist (timolol) had any effect on infarct size. This probably reflects the opposing actions of these receptors on the different subsets of β-adrenoceptors.

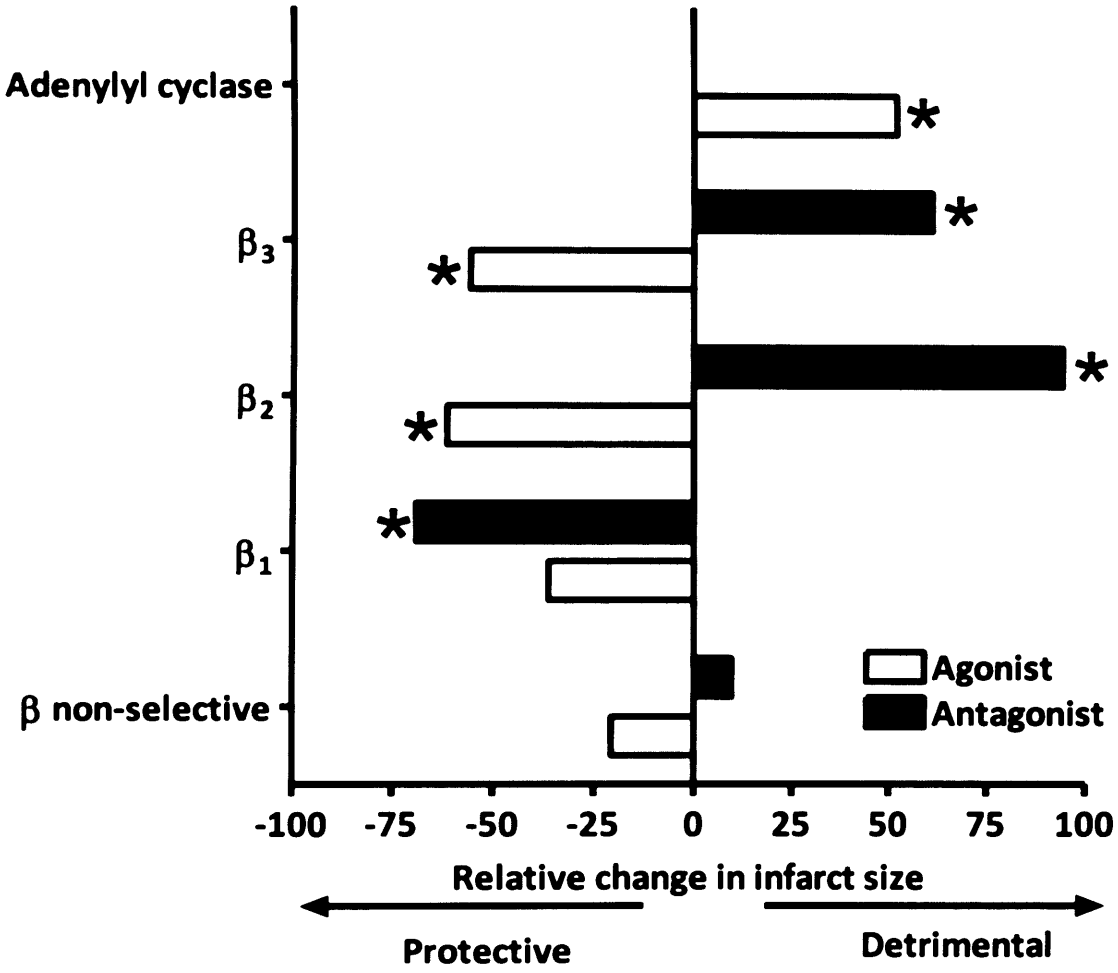

Figure 7-1 Summary of data regarding effects of administering β-adrenoceptor ligands and the direct adenylyl cyclase activator, forskolin at reperfusion. Data are presented as relative reductions or increases in infarct size compared to controls. *indicates a statistically significant change. Data is taken from chapters 5 and 6.

7.3 How does β-adrenoceptor activation confer cardioprotection?

Apart from a short Western blotting study which was inconclusive, this study has been focussed upon β-adrenoceptor subtypes and has not probed the signalling pathways and mechanisms leading to protection. Many other research groups are currently investigating the mechanisms of cardioprotection and hence from the literature some probable downstream targets of β-adrenoceptor signalling can be identified. Activation of RISK pathways at reperfusion and prevention of MPTP opening has been shown to be a common mechanism in ischaemic and pharmacological preconditioning and postconditioning. Thus, although it was not possible to detect enhanced levels of Akt at reperfusion, it remains likely that β_2-adrenoceptor activation exerts cardioprotection by activating RISK pathways at reperfusion. There are potentially two mechanisms by which β-adrenoceptors may cause preconditioning, either by inducing demand ischaemia by positive inotropic and chronotropic effects (a predominantly β_1-adrenoceptor mediated effect) or by receptor-mediated activation of RISK pathways (a β_2-adrenoceptor or β_3-adrenoceptor effect). Different mechanisms may mediate preconditioning against stunning and cell death.

7.4 Limitations of this study

The isolated atrial and ventricular tissue preparations provide a convenient model of post ischaemic stunning, however they are limited by the fact that diffusion cannot supply the whole tissue with oxygen and nutrients and so cell death occurs. The Langendorff buffer perfused heart used in these experiments is a rapid, reliable and reproducible model for studying the effects of pharmacological interventions upon infarct size after regional ischaemia and reperfusion. These models are by necessity reductionist and differ from the physiological condition in a number of respects which have been discussed extensively elsewhere (Doring *et al.*, 1987 ; Skrzypiec-Spring *et al.*, 2007; Sutherland *et al.*, 2000). In particular, high glucose concentrations are required to compensate for the absence of fatty acids, which under physiological conditions are the major energy source of the heart. Oxygen delivery to the tissues is also suboptimal in the absence of erythrocytes and buffers such as that used in these experiments can only provide sufficient oxygen to the heart when coronary flow is higher than the physiological norm (Sutherland *et al.*, 2000). However, perhaps more important in this study is the fact that these preparations were not subjected to circulating catecholamines or sympathetic neuronal transmission. Thus any effects of endogenous catecholamines acting in these preparations would be those stored of synthesised in the heart, and depletion would be expected as the experiment progressed. Furthermore, catecholamines modulate vascular reactivity, thereby affecting peripheral resistance *in vivo*. Thus further study in an *in vivo* model is required.

The duration of ischaemia to which the tissues in these experiments were exposed (30-35 minutes) is short compared to the likely duration of ischaemia experienced by patients

suffering acute myocardial infarction. However species differ in the time over which infarct development takes place, even allowing for different degrees of collateral anastamoses (Philipp *et al.*, 2005). Recent evidence suggests that postconditioning in rats is ineffective after 45 minutes ischaemia (Tang *et al.*, 2006), whereas in humans protection is still possible after several hours of ischaemia (Thibault *et al.*, 2007a; Thibault *et al.*, 2007b; Thibault *et al.*, 2008). Thus, whilst this model is a well-validated experimental tool, it is important to remember that it is far removed from physiological reality in man

7.5 Clinical applicability of results

The demonstration of infarct-potentiating effects of β_2 adrenoceptor and β_3-adrenoceptor antagonists when given at reperfusion suggests that activation of these receptors by endogenous catecholamines exerts a protective effect. If the same is true in man *in-vivo* there would be important clinical consequences. Whilst β-adrenoceptor antagonists undoubtedly save many lives when prescribed for heart failure and hypertension, it is possible that non-selective agents may have detrimental effects acutely at reperfusion after myocardial ischaemia. Patients prescribed β-adrenoceptor antagonists are likely to be at high risk of myocardial ischaemia

When considering experimental results such as these, it is interesting to consider how the results relate to clinical observations. In this study, a detrimental effect of β_2-adrenoceptor antagonism has been demonstrated. There is no clinical use for a β_2-adrenoceptor antagonist (this is probably why there is only one selective compound available for experimental use),

however some clinically used agents such as carvedilol are antagonists of β_2-adrenoceptors in addition to β_1-adrenoceptors. Results from experimental models demonstrated a superiority of carvedilol over metoprolol (a β_1-adrenoceptor selective antagonist) at reducing infarct size when given just before reperfusion in the rabbit (Feuerstein *et al.*, 1998). However, carvedilol has complicated effects because it is additionally a powerful antioxidant and has other atypical pharmacology (Carreira *et al.*, 2006) and thus its effects cannot be attributed solely to its actions on β-adrenoceptors. Interestingly, the experimental benefit of carvedilol over metoprolol did not translate to a clinical benefit (Toelg *et al.*, 2006) thus there is scope for a study of the effects of β_2-adrenoceptor blockade at reperfusion in different species..

The results presented in this thesis confirm the results of other groups which have shown benefits of β_1-adrenoceptor adrenoceptor antagonists at reperfusion and they suggest that exogenously applied β_2-adrenoceptor agonists may be protective when given at reperfusion. If this is the case *in vivo* it would offer a potential new therapeutic strategy in the treatment of myocardial ischaemia – a treatment which would be particularly useful because it could be administered at the time of the clinical reperfusion intervention.

7.6 Future Work

The experiments using isolated atria and ventricles have proven successful in demonstrating that β-adrenoceptor preconditioning can protect against stunning in the rat heart and that in this model ischaemic preconditioning is dependent on β-adrenoceptor activation. However, the mechanisms of protecting against stunning and cell death are not necessarily the same, and

thus it would be useful to carry out a series of similar experiments investigating the roles of β-adrenoceptors in preconditioning against cell death in this model. This could be achieved by increasing the duration of ischaemia. The experiments investigating the effects of β-adrenoceptor ligands at reperfusion could also be repeated in this model.

The study of the effects of β-adrenoceptor activation at reperfusion in the Langendorff preparation leaves open a number of questions which can be divided into two categories. I) A mechanistic study of how β-adrenoceptors elicit cardioprotection. II) A study to determine whether β_2-adrenoceptor activation is cardioprotective in vivo.

The first objective could be achieved in a number of ways. Pharmacological inhibitors exist for most components of RISK pathways and these could be use to determine which kinases were involved in eliciting β-adrenoceptor mediated cardioprotection. These could be used to determine which kinases are essential for β-adrenoceptor postconditioning. Hearts could also be sampled for Western blot analysis of relevant kinases such as Erk 1/2 and PI3K. Additionally, it would be of great interest to know how each of the available β-adrenoceptor ligands affects Erk 1/2 activation. This would involve very many experiments and might be more readily achieved using isolated cardiomyocytes than isolated hearts. Cells in culture could be exposed to β-adrenoceptor ligands and then lysed and Western blotting could be carried out to measure out in order to measure total and phosphorylated proteins of interest.

The *in vivo* study would determine whether β-adrenoceptor agonists at reperfusion would provide a realistic clinical target. Coronary ligation techniques in anesthetised animals are well established and infarct size can be measured post-mortem with similar techniques to those described in this thesis.

Medical research progresses through laboratory experiments of increasing complexity before promising drugs and techniques are tested in the clinic. This work has demonstrated important roles of β-adrenoceptors (both protective and detrimental) at reperfusion after ischaemia in a very basic model. The most important work arising from this study will be aimed at moving these findings along the tortuous pathway from bench to bedside, initially by investigating whether the same mechanisms are important in more complex experimental models *in vivo*.

"Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning"

Winston Leonard Spencer Churchill

Speech at the Mansion House, London, 10th November 1942

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