Ischaemia/reperfusion injury and preconditioning in the human myocardium: The role of alpha 1 adrenoceptors and disease states

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By

Mahmoud Loubani

University of Leicester

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

ABSTRACT

The present studies have demonstrated that α_1-adrenoceptors play an important role in the ischaemia/reoxygenation-induced injury of the human atrial myocardium. They have shown that stimulation of α_1-adrenoceptors with phenylephrine protects against injury whereas their blockade with prazosin is detrimental, both effects obtained in a dose-dependent manner. They have also shown that the effect of the stimulation or blockade of α_1-adrenoceptors depends on the time of administration so that α_1-adrenoceptors' stimulation is protective when given prior to ischaemia but detrimental when given during ischaemia. On the contrary, α_1-adrenoceptors' blockade is beneficial during ischaemia, detrimental during reoxygenation but has no significant effect prior to ischaemia. It appears that similar maximal protection can be obtained with α_1-stimulation prior to ischaemia and with α_1-blockade during ischaemia although the combination of the two does not induce additional protection. Furthermore, the protective effect of α_1-stimulation prior to ischaemia is as potent as ischaemic preconditioning. In this thesis, I have also demonstrated that protection with pharmacological preconditioning by activation of α_1-adrenoreceptors or adenosine receptors is identical to that of ischaemic preconditioning (IP) in the human myocardium.

These studies have provided novel information to understand the underlying mechanism of protection by preconditioning of the human myocardium. They have shown that mitoK_{ATP} channels, PKC and p38MAPK are an integral part of the cellular signal transduction involved in this cardioprotection in which mitoK_{ATP} channels are placed upstream and p38MAPK is placed downstream of PKC.

The abolition of the ability of the human myocardium to be protected by ischaemic and pharmacological preconditioning without exacerbating the susceptibility to ischaemic injury when nicorandil, a mitoK_{ATP} channel opener and nitric oxide donor, was administered clinically was unexpected. I demonstrated that the likely cause of the failure to precondition the myocardium of patients on nicorandil is the unresponsiveness of the mitoK_{ATP} channels since protection cannot be obtained with diazoxide, a specific mitoK_{ATP} channel opener, but can be elicited by activation of PKC and p38MAPK that are downstream of mitoK_{ATP} channels in the signalling transduction cascade of preconditioning.

The sulfonylureas glibenclamide and gliclazide, that block K_{ATP} channels, have distinctive effects on IP. Thus, although glibenclamide abolished the protective effect of preconditioning even at 0.1 μM, gliclazide did at a higher concentration 30μM. The cardioprotection induced by diazoxide, which open mitoK_{ATP} channels was also abrogated by glibenclamide. However glibenclamide did not block the protective effect of activation of PKC and p38MAPK.

In the final studies I demonstrated that ischaemic and pharmacological preconditioning equally elicit a delayed or second window of protection in the human myocardium that lasts between 24 and 72 hours following the preconditioning stimulus. The occurrence of angina also mimicked the delayed protection conferred by ischaemic and pharmacological preconditioning. In addition, I showed that as in the first window of protection, mitoK_{ATP} channels, PKC and p38MAPK are essential components of the signal transduction mechanism of the delayed protection.

PUBLICATIONS

Published articles arising from this thesis:

1. **M Loubani**, M Galiñanes. Alpha 1 adrenoceptors during simulated ischaemia and reoxygenation of the human myocardium: effect of the dose and time of administration. **Journal of Thoracic and Cardiovascular Surgery 2001;122:103-112.**

2. **M Loubani**, M Galiñanes. Pharmacological and ischaemic preconditioning of the human myocardium: mitoK$_{ATP}$ channels are upstream and p38MAPK is downstream of PKC. **BMC Physiology 2002;2:10.**

3. **M Loubani**, M Galiñanes. Long-term administration of nicorandil abolishes ischaemic and pharmacological preconditioning of the human myocardium: role of MitoK$_{ATP}$ channels. **Journal of Thoracic and Cardiovascular Surgery 2002;124:750-757.**

4. **M Loubani**, A Hassouna, M Galiñanes. Delayed preconditioning of the human myocardium: signal transduction and implications for the timing of surgery. **Cardiovascular Research 2004;61:600-609.**

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2. A Hassouna, **M Loubani**, AG Fowler, NB Standen, M Galiñanes. Can the diabetic heart be Ppreconditioned? Role of mitoK_{ATP} channels, PKC and p38MAPK. **Circulation 2002;106:245.**

3. **M Loubani**, M Galiñanes. Chronic treatment with the mitoK_{ATP} channel opener nicorandil prevents preconditioning of the human myocardium. **Circulation 2001;104:II-226.**

4. **M Loubani**, M Galiñanes. Pharmacological and ischaemic preconditioning of the human myocardium: MitoK_{ATP} channels are upstream and p38MAPK is downstream of PKC. **Circulation 2001;104:II-101.**

5. **M Loubani**, M Galiñanes. MitoK_{ATP} channels are upstream of PKC and p38MAPK activation in the preconditioning of the human myocardium by alpha 1 adrenoceptors. **European Heart Journal 2001;22:384.**

6. **M Loubani**, M Galiñanes. Pretreatment with nicorandil prevents ischaemic and alpha 1 preconditioning of the human myocardium due to defective mitoK_{ATP} channels. **European Heart Journal 2001;22:688.**

7. **M Loubani** and M Galiñanes. The activation of α_1-adrenoceptors prior to ischaemia is as potent as ischaemic preconditioning in the human myocardium: Role of p38MAPK and MitoK_{ATP} channels. **Circulation 2000;102:II464.**

8. **M Loubani** and M Galiñanes. The influence of alpha 1 adrenoceptors in the ischaemic human myocardium: beneficial, detrimental or both? **European Heart Journal 2000;21:510.**

9. **M Loubani** and M Galiñanes. Alpha 1 adrenoceptors mediate the protection of ischaemic preconditioning of the human myocardium through PKC and mitoK_{ATP} channels. **European Heart Journal 2000;21:512.**

PRESENTATIONS

International Meetings:

1. **M Loubani**, AG Fowler, NB Standen, M Galiñanes. Blockade of preconditioning by sulfonylureas can be overcome by activation of PKC and p38MAPK. **Annual Scientific Sessions of American College of Cardiology, Chicago, Illinois, 30th March-2nd April 2003.**

2. A Hassouna, **M Loubani**, AG Fowler, NB Standen, M Galiñanes. Can the diabetic heart be preconditioned? Role of mitoK_{ATP} channels, PKC and p38MAPK. **American Heart Association Scientific Sessions 2002, Chicago, Illinois, 17th-20th November**.

3. **M Loubani**, M Galiñanes. Chronic treatment with the mitoK_{ATP} channel opener nicorandil prevents preconditioning of the human myocardium. Presented at the **American Heart Association Scientific Sessions 2001, Anaheim, California, 11th-14th November**.

4. **M Loubani**, M Galiñanes. Pharmacological and ischaemic preconditioning of the human myocardium: MitoK_{ATP} channels are upstream and p38MAPK is downstream of PKC. Presented at the **American Heart Association Scientific Sessions 2001, Anaheim, California, 11th-14th November**.

5. **M Loubani**, M Galiñanes. MitoK_{ATP} channels are upstream of PKC and p38MAPK activation in the preconditioning of the human myocardium by alpha 1 adrenoceptors. Presented at the **XXIII Annual Congress of the European Society of Cardiology in Stockholm, 1st - 5th of September 2001.**

6. **M Loubani**, M Galiñanes. Pretreatment with nicorandil prevents ischaemic and alpha 1 preconditioning of the human myocardium due to defective mitoK_{ATP} channels. Presented at the **XXIII Annual Congress of the European Society of Cardiology in Stockholm, 1st - 5th of September 2001.**

7. **M Loubani**, M Galiñanes. The activation of α_1-adrenoceptors prior to ischaemia is as potent as ischaemic preconditioning in the human myocardium: Role of p38MAPK and mitoK_{ATP} channels. Presented at **73rd Scientific Sessions of the American Heart Association, New Orleans, LA, 12th – 15th November 2000.**

8. **M Loubani**, M Galiñanes. The influence of alpha 1 adrenoceptors in the ischaemic human myocardium: beneficial, detrimental or both? Presented at the **XXII Annual Congress of the European Society of Cardiology in Amsterdam, 26th - 30th of August 2000.**

9. **M Loubani**, M Galiñanes. Alpha 1 adrenoceptors mediate the protection of ischaemic preconditioning of the human myocardium through PKC and mitoK_{ATP} channels. Presented at the **XXII Annual Congress of the European Society of Cardiology in Amsterdam, 26th - 30th of August 2000.**

National Meetings:

1. **M Loubani**, M Galiñanes. Alpha 1 adrenoceptors mediate ischaemic preconditioning in the human myocardium. Presentation at the **Society of Cardiothoracic Surgeons in Great Britain and Ireland Annual Meeting in London, 12th - 15th March 2000.**

Local Meetings:

1. **M Loubani**, M Galiñanes. Delayed preconditioning of the human myocardium by angina: implications for the timing of surgery. **Midlands Cardiothoracic Surgical Meeting in Birmingham, 7th March 2003.**

2. **M Loubani**, A Fowler, NB Standen, M Galiñanes. Differential effect of sulfonylureas on preconditioning of the human myocardium. **Midlands Cardiothoracic Surgical Meeting in Birmingham, 1st March 2002.**

3. **M Loubani**, M Galiñanes. MitoK_{ATP} channels are upstream of PKC and p38 MAPK activation in the preconditioning of the human myocardium by alpha 1 adrenoceptors. Presented at the **Midlands Cardiothoracic Surgical Meeting in Keele University, Staffordshire on 2nd March 2001.**

4. **M Loubani**, M Galiñanes. Chronic administration of nicorandil abolishes the protection of preconditioning of the human myocardium: role of mitoK_{ATP} channels. Presented at the **Midlands Cardiothoracic Surgical Meeting in Sheffield, 15th September 2000.**

5. **M Loubani**, M Galiñanes. Alpha 1 adrenoceptors mediate the protection of ischaemic preconditioning of the human myocardium through PKC and mitoK_{ATP} channels. Presented at **the Leicestershire Research Prize Day, 12th June 2000**.

6. **M Loubani**, M Galiñanes. The influence of alpha 1 receptors on the ischaemic human myocardium: Beneficial, detrimental or both? Presented at the **Midlands Cardiothoracic Surgical Meeting in Birmingham, February 2000.**

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DEDICATION

To my father and my mother who helped me to start on this road and to my dear wife for her support to stay on it

CONTENTS

ABBREVIATIONS USED IN TEXT

IP	Ischaemic preconditioning
PMA	Phorbol-12-myristate-13-acetate
p38MAPK	p38 Mitogen activated protein kinase
$MitoK_{ATP}$	Mitochondrial ATP dependent potassium channels
SI/R	Simulated ischaemia/reoxygenation
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide
CK	Creatine kinase
8-SPT	8-p-sulphophenyltheophyline
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
Pi	Phosphate
ECG	Electrocardiogram
MI	Myocardial infarction
UA	Unstable angina
MVO_2	Myocardial ventilation oxygen consumption
LV	Left ventricle
ATPase	Adenosine triphosphatase
NO	Nitric oxide
TNF	Tumour necrosis factor
rTPA	Recombinant tissue plasminogen activator
CsA	Cyclosporin A
Ki	Concentration for half inhibition
CyP	Cyclophilins

PPIase	Peptidyl-prolyl cis-trans isomerase
cAMP	Cyclic adenosine monophosphate
SOD	Superoxide dismutase
PTCA	Percutaneous transluminal coronary angioplasty
ACS	Acute coronary syndrome
CABG	Coronary artery bypass grafts
PIP_2	Phosphatidylinositol biphosphate
PKC	Protein kinase C
DAG	1,2 diacylglycerol
IP3	Inositol 1,4,5 triphosphate
DMSO	Dimethyl sulfoxide
SI	Simulated ischaemia
R	Reoxygenation

Chapter 1

Introduction

1.1 ISCHAEMIA

1.1.1 Definition

Both hypoxia and ischaemia are situations in which a tissue is subject to oxygen deprivation, but in ischaemia the blood flow is also disrupted which prevents the wash-out of lactic acid and other waste products from the cell.[1,2] Oxygen deprivation causes inhibition of oxidative phosphorylation which results in the loss of ATP with a concomitant rise in ADP, AMP and Pi concentrations and activation of anaerobic glycolysis as an attempt to maintain tissue ATP. Anaerobic glycolysis is incapable of producing sufficient ATP to keep normal cardiac contraction and if this situation is maintained, then lactic acid accumulates, intracellular pH drops, glycolysis is inhibited and the tissue ATP levels drop precipitously leading ultimately to cell death.[3] It is a combination of high lactic acid,[4] low pH[5] and elevated Pi and ADP[6] that is responsible for the inhibition of contraction. Global ischaemia is an essential feature of cardiac surgery whereas regional ischaemia is a more usual clinical situation caused by coronary thrombosis and complete arterial occlusion or by severe reduction in coronary blood flow.

1.1.2 Pathophysiology

Myocardial ischaemia can occur as a result of increased myocardial oxygen demand, reduced myocardial oxygen supply, or both.[7,8] Clinically ischaemia can manifest as angina and with ST-segment deviation on ECG,[9] and it may be detected by reduced uptake of thallium 201 or technetium 99 in the myocardium,[10] and by regional or global impairment of ventricular function.[11,12]

In the presence of coronary obstruction, an increase of myocardial oxygen requirements caused by exercise, tachycardia, or emotion leads to a transitory imbalance.[13] This condition is frequently termed "demand" ischaemia and is responsible for most episodes of chronic stable angina. In other situations, the imbalance is caused by acute reduction of oxygen supply secondary to increased coronary vascular tone (i.e., coronary vasospasm) or by marked reduction or cessation of coronary flow as a result of platelet aggregates or thrombi.[14,15] This condition, termed "supply" ischaemia, is responsible for myocardial infarction (MI) and most episodes of unstable angina (UA). In many circumstances, ischaemia results from both an increase in oxygen demand and a reduction in supply.

The heart is an aerobic organ and therefore relies almost exclusively on the oxidation of substrates for the generation of energy. It can develop only a small oxygen debt and still have enough energy to function normally. Thus, in a steady state, determination of the rate of myocardial oxygen consumption (i.e., rate of myocardial ventilation oxygen consumption (MVO_2)) provides an accurate measure of its total metabolism.[16] The major determinant of MVO_2 is cardiac contractility.[7,8] It has been known for many years that the total metabolism of the arrested, quiescent heart is only a small fraction of that of the working organ.[17,18] The small fraction of MVO_2 in the non-contracting heart is required for the physiologic processes not directly associated with contraction. Increases in the frequency of depolarisation of the non-contracting heart are accompanied by only small increases in MVO_2.

During ischaemia, the drop in pH that occurs due to the build up of lactic acid leads to activation of the Na^+/H^+ antiporter to try and restore intracellular pH (pHi). Since ATP concentrations are greatly reduced, the Na^+/K^+ ATPase is inhibited and the Na^+

that enters the cell cannot be pumped out again, which results in increased intracellular Na^+. This in turn causes Ca^{2+} to rise because the Na^+/Ca^{2+} antiporter that usually pumps Ca^{2+} out of the cell, is inhibited or reversed. The conversion of ATP to ADP and AMP is rapid and reversible. AMP is slowly converted into adenosine and then inosine and xanthine through a purine degradation pathway. These nucleosides leak out of the cell (and may have vasodilator effects through purinergic receptors) and lead to a gradual depletion of adenine nucleotide, which may contribute to the complications of ischaemia. If sufficient oxygen is available, xanthine may be further oxidised by xanthine oxidase, which produces oxygen free radicals that are very damaging to the tissue. The depletion of ATP and elevated Ca^{2+} that occurs in ischaemia leads to a gradual decline in cellular integrity as degradative enzymes are activated and ATP-dependent repair processes are unable to operate. If the tissue remains ischaemic for prolonged periods, this deterioration leads to necrotic cell death. However, shorter periods of ischaemia are accompanied by less damage and this can be reversed by ATP-dependent processes provided the mitochondria remain sufficiently intact to generate the ATP upon reoxygenation.

1.1.3 Clinical consequences

Coronary artery disease is the single most common cause of death in the USA, so that approximately 13 million people (6.9% of population) had ischaemic heart disease and its various complications in 2002.[19] It accounted for 53% of all cardiovascular deaths in the period 1999-2002. Congestive heart failure, as a result of ischaemic cardiomyopathy, has become the most common discharge diagnosis in USA hospitals.[20] Approximately 865,000 Americans have acute new or recurrent MI annually, of whom 179,514 (20%) died.[19] More than half of men and 64% of women

who die suddenly from coronary artery disease have no previous symptoms of the disease.[19] Despite the fact that about 335,000 people died of coronary heart disease in an emergency department caused in the majority by cardiac arrest usually resulting from ventricular fibrillation,[19] the in hospital mortality of anerior MI has been declining from 11.2% to 9.4%.[21] In the United States in 2002, an estimated 1.5 million patients underwent diagnostic cardiac catheterisation with 1.2 million angioplasty procedures, and another 515,000 individuals underwent coronary artery bypass surgery.[19] Survivors of MI exhibit a poorer prognosis as well. They have a 1.5- to 15-times higher risk of mortality and morbidity than the rest of the population without prior MI and are at higher risk for subsequent MI, as well as for fatal and near-fatal arrhythmias as a result of myocardial ischaemia. Within a year of MI, 25% of men and 38% of women die. Within 6 years, 18% of men and 34% of women have a second MI, 7% of men and 6% of women experience sudden death, 22% of men and 46% of women are disabled with congestive heart failure, and 8% of men and 11% of women have a stroke.[19]

1.2 REPERFUSION

1.2.1 Definition

Following a short ischaemic episode (<10-15 minutes), the performance of the heart is impaired as a result of some damage but given time it would fully recover. However if the period of ischaemia is prolonged, the tissue becomes irreversibly damaged exhibiting blebbing of the plasma membrane, loss of ionic homeostasis, swelling and de-energisation of mitochondria and release of intracellular enzymes reflecting breakdown of membrane integrity. Once the integrity of the plasma membrane is

destroyed the cell cannot recover. It should be noted that after a prolonged period of hypoxia/ ischaemia tissue damage could be exacerbated by reperfusion. This phenomenon known as reperfusion injury is of considerable clinical relevance and understanding its causes may have important consequences.

1.2.2 Causes of reperfusion injury

a) Calcium overload

The precise mechanisms leading to Ca^{2+} overload is still unclear.[23,23] However, it has been proposed that during ischaemia H^+ are produced in excess and accumulate.[23] These are then exchanged for extracellular Na^+, slowly during ischaemia and rapidly during early reperfusion, by H^+-Na^+ exchange.[23] The increased intracellular Na^+ is in turn exchanged for Ca^{2+} by the Na^+-Ca^{2+} exchanger, which causes Ca^{2+} overload.[23]

b) Oxygen free radical generation

It is now recognised that reperfusion is associated with a burst of oxygen free radicals production,[24] but the source of these radicals is still debatable. *In vivo*, oxygen free radicals are produced both enzymatically and non-enzymatically. Enzymatic sources include NADPH oxidases located on the cell membrane of polymorphonuclear cells, macrophages and endothelial cells[25,26,27] and cytochrome P_{450}-dependent oxygenases.[28] The proteolytic conversion of xanthine dehydrogenase to xanthine oxidase provides another enzymatic source of oxygen free radicals that may mediate deleterious processes *in vivo*.[29] However, the formation of xanthine oxidase activity varies between species and yet reperfusion injury does not correlate with its activity.[30]

A probably more important source of free radicals is the mitochondrial respiratory chain.[31] In particular, when the respiratory chain is inhibited by lack of oxygen and

then re-exposed to oxygen,[32] ubiquinone can become partially reduced to ubisemiquinone, which can then react with the oxygen to produce superoxide and consequently other oxygen free radicals.[33] Another site of oxygen free radical production within the mitochondrial electron transport chain *in vitro* have been localized to complexes I and III.[34] Complex I produces superoxide to the matrix side of the mitochondrial membrane exclusively, whereas complex III appears to produce superoxide to both the matrix and intermembrane space in roughly equal amounts.[35,36,37]

Whilst small fluctuations in the steady-state concentration of these oxidants may actually play a role in intracellular signalling,[38] uncontrolled increases in the steady-state concentrations of these oxidants lead to free radical-mediated cell damage. These effects of oxygen free radicals are probably related to oxidation of protein thiol groups[39] that have been shown to be responsible for the impaired respiratory chain activity of mitochondria isolated from ischaemic hearts. Oxygen free radicals also cause oxidation of glutathione, which then forms mixed disulphides with proteins. Such protein modification is thought to have inhibitory effects on ion pumps and therefore exacerbate the effects of ATP deprivation on ionic homeostasis. Furthermore, oxygen free radicals can cause peroxidation of the unsaturated fatty acid components of the phospholipids[40] rendering them more susceptible to attack by phospholipase A2 whose activity may already be increased by elevated Ca^{2+}. The combination of oxygen free radicals and elevated Ca^{2+} leads to damage to mitochondria, which may represent a critical event in the transition from reversible to irreversible reperfusion injury. Oxygen free radicals may also cause direct damage to polysaccharides[41] and DNA in the cells.[42,43]

c) Neutrophil and inflammatory factors

Neutrophils, as a first-line defence of the organism against invading pathogens, have an integral part in the acute inflammatory response to tissue injury.[44] They accumulate in ischaemic and reperfused myocardium under the influence of chemoattractants, and there is overwhelming evidence demonstrating that they participate in myocardial injury after ischaemia and reperfusion.[45,46,47,48,49,50]

i. Neutrophil adhesion and migration

Extravasation of neutrophils in postcapillary venules is mediated by at least three sequential steps: (1) initial rolling of neutrophils along the endothelium; (2) neutrophil activation, strengthening of neutrophil adhesion, and cessation of rolling; and (3) transendothelial migration.[51,52,53] Neutrophil rolling is mediated by the selectin family of adhesion molecules: E-selectin, L-selectin, and P-selectin.[54] The selectins initiate rolling and tethering of circulating neutrophils to the endothelial surface and facilitate exposure to various neutrophil activators.[55,56,57,58,59,60,61] At physiological flow rates, these events promote neutrophil recruitment by the local microenvironment and provide the basis of activation-induced adhesion strengthening through ß2 integrins.[51,52,53,62,63,64]

Firm attachment of neutrophils to the endothelial cell and direction of neutrophil transendothelial migration are mediated by the neutrophil ß2 integrins. These glycoproteins possess a common ß2 chain (CD18) and one of three separate αchains (CD11a, CD11b, or CD11c).[65] Neutrophils express ß2 integrins, and chemotactic stimulation results in a rapid but transient upregulation of CD11b/CD18, which is a

prerequisite for firm neutrophil attachment to the endothelium and subsequent diapedesis.[50,51,52,53] Integrins bind to endothelial cell immunoglobulin-like counterreceptors, namely, intercellular adhesion molecule 1 (ICAM-1), which constitutes the principal ligand for neutrophil CD11b/CD18 (Mac-1 or Mo1).[66] CD11b/CD18 is also the receptor for C3bi (CR3), one of the breakdown components of the third component of complement C3.[65] ICAM-1 is upregulated by cytokine stimulation,[67] and the increased neutrophil adherence to endothelial cells after endothelial exposure to oxygen free radicals[68] or anoxia-reoxygenation[69] is dependent on ICAM-1. In addition, interaction of CD11b/CD18 with biological surfaces mediates the massive and prolonged oxygen free radicals production by adherent neutrophils in response to physiological concentrations of chemotactic ligands, which are very weak agonists when tested with neutrophils in suspension.[70,71,72,73] Interestingly, soluble isoforms of cell adhesion molecules (e.g., ICAM-1, L-selectin, and E-selectin) have been detected in blood and tissue fluids, and by retaining biological activity, these molecules may potentially modulate inflammatory reactions.[74,75]

Isolated adult cardiac myocytes express ICAM-1 after stimulation with cytokines[76] or postischaemic cardiac lymph,[77] and the adhesion of neutrophils to these cells is dependent on ICAM-1 and CD11b/CD18.[72,76,77] Adherence of neutrophils to cytokine-stimulated cardiac myocytes activates the respiratory burst, resulting in highly compartmentalized oxidative myocyte injury,[78] and in anoxia-reoxygenated isolated myocytes, neutrophil-mediated augmentation of cellular damage also appears to be dependent on ICAM-1.[79] Therefore ICAM-1–dependent neutrophil adherence may be an important therapeutical target.[71,72,73,78] Induction of ICAM-1 mRNA has recently

been found in the postischaemic heart during early reperfusion,[80] and rapid reperfusion-induced expression of ICAM-1 mRNA in the border zone of viable myocytes surrounding necrotic myocardial regions (in association with intense neutrophils infiltration) which strongly indicates that inflammatory tissue injury by these mechanisms plays an important role in myocardial reperfusion injury *in vivo*.[81]

Diminished basal NO release from coronary endothelial cells after myocardial ischaemia and reperfusion promotes adherence of neutrophils in vitro through a CD11b/CD18-dependent mechanism.[82] In addition, in the feline mesenterial preparation, ischaemia-reperfusion and pharmacological NO synthesis inhibition increase neutrophil adherence and microvascular albumin leakage by mechanisms dependent on ICAM-1 and CD11b/CD18 respectively.[83,84] NO may also attenuate thrombin-induced platelet activating factor synthesis in endothelial cells,[85] and recent data have suggested that inhibition of NO synthesis in cultured endothelial cells increases intracellular oxidative stress and is associated with ICAM-1–mediated neutrophils adherence.[86] It is therefore conceivable that NO from endothelial cells may act in an autocrine fashion to regulate various endothelial cell adhesive mechanisms. Constitutive[87] and cytokine-inducible[88] NO synthases are present in cardiac myocytes, and it is tempting to speculate that NO can also regulate expression of adhesion molecules in these cells.

ii. Cardiotoxic potential of neutrophils

1. Release of oxygen free radicals

Neutrophils contain an extensive cytotoxic armamentarium, and their potential to destroy tissue is mediated by concerted and synergistic effects of exocytosed granule constituents and generation of oxygen free radicals.[89,90,91] Activation of the neutrophil membrane–associated NADPH oxidase system by various soluble and particulate stimuli initiates a respiratory burst characterized by a marked increase in cellular oxygen consumption and generation of superoxide anions.[90] The superoxide anions apparently dismutate quantitatively to hydrogen peroxide, although it is possible that hydrogen peroxide and superoxide anions may react in the metal-catalysed modified Haber-Weiss reaction to form highly reactive hydroxyl radicals. However, most stimuli that induce superoxide generation by neutrophils also cause the release of myeloperoxidase from the granules, and this enzyme efficiently removes hydrogen peroxide by catalysing the interaction of hydrogen peroxide with Cl^- to form hypochlorous acid. Hypochlorous acid is a powerful oxidant that may chlorinate or oxidize a variety of target molecules, and reactions of hypochlorous acid with primary amines or ammonia can give rise to chloramines, which are also energetic oxidants. By these mechanisms, hypochlorous acid is considered to be primarily responsible for the oxygen free radical-dependent cytotoxicity of neutrophils.[89,90]

The experimental cardiotoxicity of chemically and photochemically generated oxygen free radicals is extensively documented,[92,93,94,95] and the cytotoxicity of neutrophil-derived oxygen free radicals has been amply demonstrated in cultured endothelial cells.[96,97,98,99,100] Furthermore, neutrophils exacerbate cellular damage in anoxia-

reoxygenated cultured myocytes by mechanisms that depend, in part, on oxygen free radicals.[79,101] In isolated arteries, neutrophil-derived oxygen free radicals can induce vascular contraction, and in isolated perfused hearts, they aggravate postischaemic coronary endothelial dysfunction[102] and decrease left ventricular mechanical performance.[103,104] One mechanism behind the vasoconstricting properties of activated neutrophil may be inactivation of endothelial nitric oxide (NO) by neutrophil-derived superoxide.[105,106] However, neutrophil also can release NO,[107,108] although evidence indicates that neutrophil stimulation results in progressive inactivation of neutrophil-derived NO by concomitant release of oxygen free radicals.[108] NO may directly inhibit neutrophil NADPH oxidase activity,[109] and the mechanisms underlying modulation of vasomotor tone by neutrophil are therefore likely to be complex. Oxidants from activated neutrophil can also cause depression of calcium transport in isolated cardiac myocyte sarcoplasmic reticulum,[110] and neutrophil-derived hydrogen peroxide may promote release of proinflammatory arachidonic acid metabolites (i.e., prostacyclin) from cultured endothelial cells.[111] Furthermore, chemically generated superoxide anions can generate potent chemotactic activity in plasma[112] or after incubation with arachidonic acid,[113] and it is possible that neutrophil-derived superoxide anions can have similar effects. Interestingly, neutrophil-derived oxygen free radicals can oxidize LDL *in vitro*,[114] and the multiple proatherogenic properties of oxidized LDL[115] may therefore provide an additional link between neutrophils and human ischaemic heart disease, although neutrophils are usually not thought to play a role in atherogenesis.[116]

2. Neutrophil derived proteinases

Although interest has focused on the cytotoxic potential of neutrophil oxidants, neutrophil degranulation also releases several proteolytic enzymes into the extracellular space.[89,91] The serine proteinase elastase has been implicated most consistently in neutrophil-mediated tissue damage.[89,117] Several neutrophil-derived proteinases (including elastase) are highly positively charged, and their cationic nature may contribute to tissue damage by direct alterations in target cell surface charge or by enhancing binding to cell membranes and extracellular matrix components.[91] Elastase can hydrolyse a host of proteins in the extracellular matrix (e.g., elastin, fibronectin, and collagen types III and IV) and plasma (e.g., complement proteins and clotting factors),[117,118] and although they are generally resistant to proteinases, most tissue collagens are readily cleaved by neutrophil collagenase (albeit with different substrate specificity for individual collagen types).[119] In addition, elastase may inhibit platelet function by proteolysis of platelet membrane glycoproteins.[120] A synergism is thought to exist between neutrophil elastase and neutrophil-generated oxygen free radicals *in vivo*, since neutrophil oxidants (i.e., hypochlorous acid) can inactivate the powerful antiproteinases present in plasma and extracellular fluid (e.g., α_1-proteinase inhibitor).[89,91,121] Furthermore, neutrophil-generated oxygen free radicals can promote activation of latent metalloproteinases (e.g., collagenase and gelatinase) released by neutrophils.[89,119]

In cultured endothelial cells, elastase can enhance cell detachment and destruction of monolayer integrity without evidence of cytolysis,[122,123,124] and postischaemic migration of neutrophil through the vascular endothelium may be dependent on

elastase.[125] Evidence also indicates that neutrophil-mediated damage to cultured endothelium is dependent on a synergistic interaction of proteases and oxygen free radicals,[100,126] and elastase plays a role in neutrophil-dependent increased anoxia-reoxygenation injury in cultured endothelial cells[123] and cardiac myocytes.[79,101] Cleavage of interstitial matrix molecules by collagenase and elastase may generate peptide fragments that are chemotactic for monocytes,[127,128] and it is possible that this mechanism can promote recruitment of monocytes to the postischaemic myocardial inflammatory zone.

3. Arachidonic acid metabolites and platelet activating factor

In addition to oxygen free radicals and proteinases, activated neutrophils release several other proinflammatory mediators with a wide range of biological activities. Stimulation of phospholipase A_2 after neutrophil activation mobilizes membrane lipids and results in generation of 5-lipoxygenase products (e.g., leukotriene B_4[129]) and the phospholipid platelet activating factor.[130] Arachidonic acid metabolism may proceed through differing pathways in neutrophils from different species,[131] and cyclooxygenase (present in small amounts in human neutrophils) may convert arachidonic acid to cyclic endoperoxides, for example, thromboxane A_2.[132] Furthermore, activated neutrophil can release phospholipase A_2 into the external environment,[133] thereby enhancing production of eicosanoids and platelet activating factor by other cells, and neutrophil can transfer eicosanoids for processing in endothelial cells[134] and platelets.[135] Thromboxane-B_2 concentration in cardiac lymph is elevated after reperfusion subsequent to 1 hour of myocardial ischaemia,[136] and enhanced neutrophil-dependent myocardial generation of eicosanoid metabolites (e.g.,

leukotriene B_4 and thromboxane B_2) has been demonstrated *ex vivo* after experimental myocardial infarction.[137] In addition, leukotrienes[138] and platelet activating factor[139] can be generated in buffer-perfused hearts after ischaemia and reperfusion, and cultured human endothelial cells produce platelet activating factor upon stimulation with inflammatory cytokines (e.g., TNF),[140] hydrogen peroxide,[55] or thrombin.[56] Interestingly, endothelial platelet activating factor synthesis is also increased by plasmin, streptokinase, or recombinant tissue-plasminogen activator (rTPA).[141,142]

Leukotriene B_4 and platelet activating factor are potent stimulants of neutrophil chemotaxis, adhesion to endothelial cells, and oxidative metabolism and degranulation[143,144]and may serve to amplify neutrophil-mediated tissue injury and vascular permeability.[145] Leukotrienes C_4, D_4, and E_4 and thromboxane A_2 and platelet activating factor can cause coronary vasoconstriction and depression of left ventricular function.[146,147] In addition to stimulation of neutrophil, platelet activating factor produces aggregation and degranulation of platelets, and the decrease in coronary flow and cardiac function by platelet activating factor is likely to be dependent on platelet products.[139]

iii. Myocardial neutrophil accumulation

In experimental models, neutrophil accumulation is accelerated by reperfusion,[148,149] and in dogs, the greatest rate of neutrophil localization after 1 hour of myocardial ischaemia is observed in the first hour of reperfusion.[150] During reperfusion after sustained myocardial ischaemia, neutrophil accumulation occurs preferentially in the

subendocardial region[150,151] and may correlate with infarct size.[152] Interestingly, reperfusion after brief (e.g., 12-minute) periods of myocardial ischaemia apparently is not associated with neutrophil accumulation.[151]

iv. Microvascular neutrophil plugging

Neutrophils are larger and much stiffer than erythrocytes, and the cytoskeletal assembly after neutrophil activation is associated with additional decreases in cellular deformability.[153] These haemorheological properties can promote physical trapping of neutrophils in myocardial capillaries after ischaemia and reperfusion, and thus neutrophils may contribute to the no-reflow phenomenon.[149,154] In addition, regional plugging of the myocardial microvasculature by neutrophil is likely to be enhanced by various other postischaemic microvascular alterations, e.g., neutrophil aggregation, reduced myocardial perfusion pressure, and upregulation of neutrophil–endothelial cell adhesive activities (e.g., in association with reduced endothelial NO production).[45,51,83,155]

v. Complement, chemokines, and other chemotactic factors

During myocardial ischaemia and reperfusion, the complement cascade may be activated after complement proteolysis by myocardial proteases[117,156]or by interaction between complement component C1 and heart mitochondrial membranes released from disrupted myocytes.[157] In addition, neutrophils can directly activate complement by action of proteases[158] or oxygen free radicals.[159] Complement fixation has been demonstrated in ischaemic myocardium[160] and appears to correlate with the

localization of neutrophil accumulation.[161] Evidence indicates that experimental myocardial ischaemia rapidly induces complement activation,[157,162,163] and the ability of postischaemic cardiac lymph to stimulate isolated neutrophils is neutralized by anti-C5a antiserum.[162,163]

The role of the complement system in myocardial ischaemia and reperfusion has been well described.[164] C5a is a strong neutrophil chemoattractant, and generation of C3bi on the endothelial cell surface *in vitro* elicits rapid CD11b/CD18-dependent neutrophil adhesion.[165] In pigs, intracoronary administration of C5a reduces coronary blood flow and myocardial contractile function by mechanisms dependent on myocardial neutrophil accumulation and production of thromboxane A_2 and leukotrienes.[166] The canine coronary vasculature may be less responsive to thromboxanes, and C5a appears to dilate canine coronary arteries *in vivo* and *in vitro*.[167] In addition to recruitment and activation of neutrophil within the ischaemia-reperfused myocardium, complement-derived products can directly contribute to myocardial injury by neutrophil-independent mechanisms. C3a can decrease left ventricular contraction and coronary flow in isolated guinea pig hearts,[168] and similar alterations, myocardial oedema, and release of creatine kinase, have been observed in isolated rabbit hearts perfused with human plasma (a situation eliciting complement activation).[169] In a recent study, reperfusion with neutrophil and plasma or neutrophils and C5a reduced ventricular function and coronary flow after global ischaemia in an isolated rat heart model, whereas reperfusion with only plasma, neutrophil, complement-activated plasma, or C5a failed to induce significant alterations.[170] In addition, electron paramagnetic resonance spectroscopy measurements indicated that

reperfusion with neutrophil and plasma resulted in marked prolongation in the duration of oxygen free radical generation.[170]

Although the complement system is believed to be one of the most important sources of inflammatory mediators after myocardial ischaemia and reperfusion, a novel superfamily of low-molecular-weight chemotactic cytokines known as chemokines has recently been defined; chemokines are secreted by several types of cells in response to inflammatory stimuli *in vitro*.[171,172] Chemokines are subdivided into α and ß subfamilies on the basis of the presence or absence of an intervening amino acid between the first two of four conserved cysteines, and the two subfamilies differ in their target cell selectivity, i.e., α or C-X-C chemokines primarily stimulate neutrophil, whereas ß or C-C chemokines predominantly act on monocytes, basophils, eosinophils, and T cells.[171,172,173] Specifically, IL-8 synthesized by endothelial cells after stimulation with TNF or IL-1 is a strong neutrophil chemoattractant.[174] Chemokines possess proteoglycan-binding sites, and IL-8 can induce transendothelial neutrophil migration, rapid shedding of L-selectin, and upregulation of neutrophil integrins, possibly by generation of a chemotactic gradient of immobilized matrix-associated IL-8.[60.61] Various other neutrophil chemotactic agents are released from the postischaemic myocardium, e.g., leukotrienes[137,138] and platelet activating factor,[90,139] and neutrophil chemoattraction and activation after myocardial ischaemia and reperfusion are therefore likely to be the result of amplification by numerous interacting proinflammatory mechanisms, with several of the involved mediators playing the role of initiator and product.[50]

d) Mitochondrial pore opening

Upon reoxygenation following a period of ischaemia the mitochondria become energised and accumulate the excess Ca^{2+} that has increased in the cytosol. This high matrix Ca^{2+} coupled with oxidative stress induce the opening of a non-specific pore in the mitochondrial inner membrane and this process is further sensitised by the depletion of mitochondrial adenine nucleotides and the elevated Pi, both of which are a consequence of prolonged ischaemia.[175,176,177,178] Opening of a single pore immediately depolarises the mitochondria and this further activates pore opening that leads to mitochondrial swelling and releases all their nucleotides and other cofactors. ATP production is now no longer possible and if the pores remain open for any length of time, the cell is doomed to die.[179,180,181]

The opening of the pore upon reperfusion has been observed in isolated heart cells using confocal microscopy;[182,183] a mitochondrial impermeant fluorescent dye enters the mitochondria only upon reoxygenation whilst a fluorescent dye (Rhodamine 123), which is accumulated by energised mitochondria is lost from the mitochondria under the same conditions. In the perfused heart, 2-deoxyglucose uptake into the mitochondria, which is normally impermeant, only occurs during reperfusion and not during ischaemia alone.[182,183]

The mechanism of pore opening has been extensively studied in isolated mitochondria and shown to allow passage of molecules up to about 1500Da.[180] The pore is triggered by high concentrations of matrix Ca^{2+} but the sensitivity to Ca^{2+} is greatly increased by the presence of Pi, oxidative stress, depolarisation, adenine nucleotide depletion and any factor that induces the adenine nucleotide translocase to take up the "c" conformation. In contrast any factor that induces the "m" conformation inhibits pore

formation as does decreasing the matrix pH and the immunosuppressant drug cyclosporin A (CsA).[184] The pore can be closed by removing Ca^{2+} with a chelating agent such as EGTA. Many of the inducing factors come into play during ischaemia, but the acid pH will inhibit pore opening and the de-energised mitochondria will not accumulate the Ca^{2+} to trigger the pore. On reperfusion the energised mitochondria accumulate the Ca^{2+} and the lactic acid is lost from the cells, restoring the pH to a value that allows pore opening.[176,179]

The action of CsA to inhibit pore formation requires only nM concentrations and the Ki values of CsA[184] and its analogues for inhibition of pore opening correspond to their Ki values for inhibition of mitochondrial peptidyl-prolyl cis-trans isomerase (PPIase). PPIase activity is a well-documented property of the cellular proteins binding CsA, known as cyclophilins (CyP), and involves the isomerisation of the peptide bond adjacent to a proline residue in a protein. The unique mitochondrial isoform of PPIase is thought to bind to a matrix facing proline of the adenine nucleotide translocase and this binding of matrix CyP has been shown to be increased under conditions of oxidative stress as a result of a disulphide cross-link between 2 matrix facing cysteine residues on the ANT. Adenine nucleotides decrease the sensitivity of the pore to Ca^{2+} probably through their internal binding site on the carrier, and oxidative stress reduces the affinity of the ADP binding site in addition to its effect on CyP binding.[176] Direct demonstration that the proposed components of the pore act in the manner suggested has been achieved by reconstitution of the pure components.

1.2.3 Clinical consequences of reperfusion injury

a) Arrhythmias

Reperfusion arrhythmias were recognised by Tennant and Wiggers in 1935 to follow the reintroduction of blood flow to the ischaemic myocardium.[185] These arrhythmias, in addition to their importance as a marker of successful reperfusion of an occluded coronary artery,[186] require special attention because haemodynamics may rapidly deteriorate during ventricular tachycardia or ventricular fibrillation. It is therefore important clinically to establish the mechanism and treatment of such reperfusion arrhythmias. In animal experiments arrhythmias may occur within seconds of the onset of reperfusion.[187] In humans reperfusion arrhythmias are commonly associated with intracoronary thrombolytic treatment[186] and primary coronary angioplasty.[188,189] They may be less common after intravenous thrombolytic treatment,[190] and in these circumstances it has been proposed that they pose no additional threat to life.[191] The disparity may, however, be related to the rate of recanalisation. Yamazaki et al showed that in dogs sudden reperfusion was more likely to be associated with a high frequency of arrhythmias than was staged reperfusion.[192] In a randomised clinical study comparing intravenous thrombolytic treatment with primary coronary angioplasty, ventricular fibrillation was significantly more common in the angioplasty group (6.7% v 2.0%).[188] These studies provided angiographic proof of reperfusion, which was not available in several of the multicentre trials of thrombolysis.[190,193,194]

The duration of the preceding ischaemia is an important determinant of vulnerability to arrhythmias after reperfusion. Balke et al observed a peak of 67% in the frequency of ventricular fibrillation when reperfusion was achieved after 20-30 minutes of ischaemia, as opposed to 22% after 60 minutes.[195] Very early thrombolytic treatment may be expected to cause more prominent reperfusion arrhythmias. The European

Myocardial Infarction Project Group randomised 5469 patients to thrombolytic treatment before admission or to later treatment in hospital. Although mortality did not differ in the two groups, prehospital ventricular fibrillation occurred significantly more often in patients treated out of hospital.[196] The incidence of ventricular fibrillation in clinical settings has been reported to be low.[197,198,199,200] In the study by Yoshida et al,[201] ventricular fibrillation occurred in only 5.3% in the control group following angioplasty for acute anterior wall myocardial infarction. In these patients, acceleration of ventricular rate occurred before ventricular fibrillation.

Electrophysiological mechanisms of reperfusion arrhythmias are still being disputed. Arrhythmias in experimental models of ischaemia and reperfusion were previously believed to be re-entrant arrhythmias that resulted from heterogeneous recovery of the refractory period and conductivity.[202,203] However, Kaplinsky et al[204] found that reperfusion arrhythmias after 30 minutes of ischaemia consist of 2 types: an instantaneous ventricular arrhythmia (onset at 0 to 1 minute) and a delayed ventricular arrhythmia (onset at 2 to 7 minutes). They demonstrated that the former was a re-entrant arrhythmia caused by electrical heterogeneity and associated with a high frequency of ventricular fibrillation. In contrast, the latter was caused by increased ventricular automaticity and associated with a low frequency of ventricular fibrillation.[204] This led them to speculate that reperfusion ventricular tachycardia and ventricular fibrillation are not associated with a single mechanism.[204] Murdock et al[205] demonstrated that the conduction delay resulting from myocardial ischaemia rapidly returned to control times with reperfusion providing evidence against reentry. Vera et al[206] recorded early afterdepolarizations both during ischaemia and during reperfusion and postulated that afterdepolarizations participated in the genesis of reperfusion ventricular tachycardia and ventricular fibrillation. Using three-dimensional mapping,

Pogwizd and Corr found that 75% of reperfusion ventricular complexes originated from endocardial foci without enough evidence of reentry.[207] Other studies have clinically demonstrated that accelerated idioventricular rhythm and ventricular tachycardia that occur after reperfusion are probably cAMP-mediated arrhythmias and are therefore likely to be triggered arrhythmias.[201] More recently, the demonstration that increased extracellular and intracellular resistances contribute to slowing and failure of impulse propagation in ischaemic myocardium, has led to the investigation of cell-to-cell electrical uncoupling. This is measured by a sudden increase of tissue resistance after 10 to 20 minutes of ischaemia and is associated with the onset of ventricular fibrillation.[208] Furthermore reduced expression of Cx43 accelerates the onset and increases the incidence, frequency, and duration of ventricular tachyarrhythmias after coronary artery occlusion. Thus diminished electrical coupling per se plays a critical role in arrhythmogenesis induced by acute ischaemia.[209] This work has been further supported by more evidence from Cascio et al[210] examining the cell to cell uncoupling in reperfused papillary muscle.

A great deal has been detected from animal studies regarding the mechanism of accelerated idioventricular rhythm and ventricular tachycardia when occluded vessels were reperfused. However, among clinical studies, varied incidence of reperfusion arrhythmias has been demonstrated which may suggest that mechanisms responsible for accelerated idioventricular rhythm and ventricular tachycardia in clinical cases might be somewhat different from those observed in experimental models.

b) *Myocardial stunning*

i. Definition

This phenomenon was first described by Heyndrickx et al[198] in conscious dogs undergoing brief coronary occlusions followed by reperfusion. They reported that regional contraction remained depressed for more than 3 hours after a 5 minute coronary occlusion and for more than 6 hours after a 15 minute occlusion. Thus, it became clear that after a brief episode of severe ischaemia, prolonged myocardial dysfunction with gradual return of contractile activity occurs and the name myocardial stunning was coined in 1982.[211] This has been defined as the mechanical dysfunction that persists after reperfusion despite the absence of irreversible damage and despite restoration of normal or near-normal coronary flow.[22] The two essential points of this definition are that postischaemic dysfunction, no matter how severe or prolonged, is a fully reversible abnormality and that the dysfunction is not caused by a primary deficit of myocardial perfusion.[22] Subsequently, it has become increasingly evident that postischaemic myocardial stunning is part of the natural history of coronary artery disease and may contribute significantly to the morbidity associated with this disorder.[199]

Myocardial stunning, as defined above, is not a single entity but rather a syndrome that has been observed in a wide variety of experimental settings with major pathophysiological differences. The heterogeneity of myocardial stunning is confirmed by the fact that it has been observed under a variety of conditions, including after a single completely reversible ischaemic episode, multiple brief completely reversible episodes of ischaemia, single, partly irreversible ischaemic

episode such as in subendocardial infarction resulting in an admixture of infarction and stunning of adjacent viable myocardium, global ischaemia in vitro, global myocardial ischaemia as observed after cardioplegia arrest in vivo, and after exercise-induced ischaemia.[212]

Clinically, global myocardial stunning has been observed most frequently in patients who have undergone ischaemic cardiac arrest during cardiopulmonary bypass,[213,214] despite modern cardioplegia techniques. Such hearts may not recover for days, and many of these patients require inotropic support. In patients who have had MI, both with and without administration of thrombolytic therapy, stunned myocardium lies adjacent to infarcted myocardium. Improvement in ventricular function occurs gradually over the course of days to weeks.[215] Myocardial stunning is also a common feature of UA[216] and exercise induced angina.[217] Persistent wall-motion abnormalities can be observed by echocardiography at a time when chest pain, ST-segment deviation, and regional perfusion have recovered. It affects both systolic and diastolic function.[200,211,216,217]

ii. Mechanisms of myocardial stunning

1. Insufficient energy production by mitochondria

It was observed in the early 1980s, that a coronary occlusion for 15 minutes caused ATP concentration to fall in the stunned myocardium and then to recover later together with cardiac function.[211] It was shown, that after the creatine phosphate stores are exhausted, myocardial ATP concentration declines with the accumulation of its metabolites such as adenosine, inosine and hypoxanthine.[211,218] These

accumulated metabolites capable of being used as precursors to resynthesise ATP through the salvage pathway, are washed away during reperfusion.[211,218,219] Thus the "backbone" of ATP will now be resynthesised by the slow de novo pathway.[218,219] This process may take several days to occur.[218,219] Therefore, it was speculated that there is a causal relationship between depletion of adenine nucleotides and myocardial function.[211]

However, this hypothesis is now refuted since a correlation between myocardial ATP levels and recovery of myocardial contractility was not observed in certain models of myocardial stunning.[220] Furthermore, it was also shown that by administering a nucleotide precursor (adenosine) during postischaemic reperfusion, causes an increase in ADP and ATP levels, but with no improvement in cardiac contractility.[220] This finding also suggests that a decreased availability of nucleotide precursors is not the primary cause for stunned myocardium.[220] An increased creatine phosphate content found in stunned myocardium further confirms that the phosphorylating capability of mitochondria is not lost in this phenomenon.[221]

2. Impaired energy use by myofibrils

Greenfield and Swain[222] have shown that myofibrillar creatine kinase activity is decreased in stunned myocardium. A reduction of free ADP, that is used to produce ATP at the contractile site by the myofibrillar creatine kinase was also observed in the stunned myocardium.[220] Thus, a disruption of the myofibrillar end of the creatine phosphate shuttle was proposed as a possible mechanism of stunning.[222] However, this hypothesis is unlikely since inotropic stimulation causes an immediate and sustained increase in performance in the stunned myocardium.[220] This increase in

myocardial performance suggests that the residual activity of the enzymes are still able to run the myofibrillar ATPase reaction at increased rates.[220]

3. Reduced myofilament responsiveness to calcium

It has been found that both myofilament Ca^{2+} sensitivity and maximal Ca^{2+} - activated force is reduced in the stunned myocardium.[223,224] Thus, it has been hypothesized that a reduced responsiveness of myofilaments to Ca^{2+} is a mechanism for myocardial stunning. Either a decreased intracellular free Ca^{2+} concentration ([Ca]i transient) or a decreased sensitivity of myofilaments to Ca^{2+}, could cause a reduced myofilament responsiveness to extracellular Ca^{2+} in the stunned heart.[223] Since Marban[223] and Kusuoka et al[224] have observed an increase in Ca^{2+} transients in the stunned heart, it is proposed that a decreased sensitivity of myofilaments to Ca^{2+} as the mechanism for myocardial stunning. This decreased sensitivity could be a result of a shift in myofilament Ca^{2+} sensitivity and/or a reduction in the maximal Ca^{2+}-activated force.[223,224,225]

Murphy et al[226] found that intracellular magnesium $[Mg^{2+}]i$ rises during ischaemia and remains elevated during early reflow. It has been observed that as $[Mg^{2+}]i$ rises the Ca^{2+}- activated force relation is shifted to the right.[224] However, these changes in $[Mg^{2+}]i$ transients do not explain the reduced maximal Ca^{2+} - activated force. Furthermore, a reduced sensitivity of myofilaments to calcium mediated by increased $[Mg^{2+}]i$, does not explain the ability of the stunned myocardium to respond to inotropic stimuli with a normal contractile reserve.[220,227]

Studies by Gao et al[228] have found that troponin I is partially degraded in the stunned heart. Usually, the troponin-tropomyosin complex inhibits the binding of myosin and actin. A structural alteration occurs within tropomyosin when Ca^{2+} binds to troponin C. [229] This exposes the myosin binding site to actin.[225] The free energy needed to cause this structural change in the troponin-tropomyosin complex is obtained from the energy supplied by the Ca^{2+} binding to troponin C.[225] This energy is then transduced by troponin T and troponin I.[64] Since troponin I is partially degraded in the stunned myocardium by Ca^{2+}-activated protease the energy obtain from Ca^{2+} binding to troponin C cannot be effectively transduced to cause the necessary structural change in the troponin-tropomyosin complex.[225,228] Thus, a greater $[Ca^{2+}]i$ is required to bring about muscle contraction, or in other words myofilament responsiveness to Ca^{2+} is reduced. The same studies have also found a reduction of both myofilament Ca^{2+} sensitivity and maximal Ca^{2+}-activated force.[225,228] Since only limited proteolysis of troponin I occurs (by calpain I) this would not affect the upstream mechanisms controlling $[Ca^{2+}]i$.[226] Thus, the ability of the stunned myocardium to respond to inotropic stimuli with a normal contractile reserve is explained.[225] Turnover of troponin I takes several days and this could provide an explanation for time dependent recovery of the stunned myocardium.[211,228]

4. Calcium Overload

Though intracellular Ca^{2+} is vital for excitation-contraction coupling, it has been suggested that an increase in transient $[Ca^{2+}]i$ during ischaemia and early reperfusion could be a mechanism for myocardial stunning.[225] It has been observed that the intracellular Ca^{2+} concentration increases during the first 10 - 15 minutes of total

ischaemia and remains elevated (for at least 5 minutes) during early reperfusion. A similar period of ischaemia is required for myocardial stunning to occur.[211] Bolli[22] and Opie[230] found that reperfusion with solutions containing low Ca^{2+} concentrations after 15 minutes of ischaemia, would significantly attenuate postischaemic dysfunction. Furthermore, it has been shown that exposure of isolated ferret hearts to a transient Ca^{2+} overload mimics several features of stunning, even in the absence of prior ischaemia.[231] Features such as decreased maximal Ca^{2+}-activated force and sensitivity to Ca^{2+}, ATP depletion, and absence of histological evidence of irreversible injury were observed.[232]

Based on previous findings, Kusuoka et al[233] proposed that an increase in $[Ca^{2+}]i$ during ischaemia and early reperfusion could activate Ca^{2+}-dependent protein kinases, which could then cause changes in myofilaments to decrease Ca^{2+} sensitivity and / or maximal Ca^{2+}-activated force through phosphorylation of contractile proteins. Recent studies by Gao et al[228] have concluded that an increase in $[Ca^{2+}]i$ during ischaemia and early reperfusion causes Ca^{2+}-activated protease to partially degrade troponin I in the stunned myocardium. This in turn decreases the responsiveness of myofilaments to calcium. An increase in $[Ca^{2+}]i$ could generate oxygen radicals via xanthine oxidase, which could also contribute to stunning.[229]

5. Reduced contractile protein activation due to sarcoplasmic reticulum dysfunction

The normal myocardial contraction - relaxation cycle depends on a proper functioning Ca^{2+} release - uptake cycle.[234,235] Release of Ca^{2+} from the sarcoplasmic reticulum

stores causes the intracellular free Ca^{2+} concentration to rise. As mentioned earlier Ca^{2+} binding to troponin C initiates contraction. Relaxation is then achieved by sequestration of Ca^{2+} by the sarcoplasmic reticulum through Ca^{2+}-ATPase activity.[234,236] Krause et al[234] found that the sarcoplasmic reticulum isolated from stunned myocardium had decreased Ca^{2+} uptake ability and Ca^{2+}, Mg^{2+}-ATPase activity. This decrease in calcium uptake ability would result in less sequestration of Ca^{2+} and in turn less subsequent release from the sarcoplasmic reticulum stores[234] and reduced contractile protein activation.[234] Thus, a dysfunction of the sarcoplasmic reticulum uptake ability could be a possible mechanism of myocardial stunning.

The changes in $[Ca^{2+}]i$ may explain the ability of the stunned myocardium to achieve contractile function comparable to preischaemic levels with the addition of the exogenous Ca^{2+} or other inotropic agents.[22,221,227] The addition of inotropic agents would increase intracellular Ca^{2+} concentration and contractile protein activation. However, this hypothesis cannot explain the increase in $[Ca^{2+}]i$ transients observed in the stunned myocardium.[225]

6. Generation of oxygen free radicals

In the early 1980s, it was postulated that the generation of oxygen-derived free radicals could be a mechanism of myocardial stunning.[22,237] These free radicals are unstable, cytotoxic and highly reactive variations of the oxygen molecule.[229] It has been possible to attenuate myocardial stunning in open-chest dogs[229] by administration of free radical scavengers such as superoxide dismutase (SOD) and

catalase before and during ischaemia, and throughout reperfusion. SOD acts by catalysing the dismutation of superoxide ions ($\cdot O_2^-$) to hydrogen peroxide (H_2O_2) and O_2, while catalase acts by reducing H_2O_2 to O_2 and H_2O.[22,229] It has been found that administering SOD or catalase alone does not significantly attenuate myocardial stunning.[231] This suggests that both $\cdot O_2^-$ and H_2O_2 are important contributors to stunning. Through the administration of dimethylthiourea (effective $\cdot OH$ scavenger) Bolli et al[231] were able to attenuate stunning in open-chest dogs and also implicate $\cdot OH$ in the pathogenesis of myocardial stunning. Thus, it appears that $\cdot O_2^-$ and $\cdot OH$ are contributors to stunning by direct cytotoxicity and H_2O_2 as a precursor of $\cdot OH$.

In spite of the results discussed above, the evidence to implicate oxygen-derived free radicals in the pathogenesis of stunning is inconclusive,[22,237] because these studies did not measure directly free radical generation in the presence and absence of myocardial stunning. However, using spin trap a-phenyl N-tert-butyl nitrone and electron paramagnetic resonance spectroscopy it has been possible to measure the free radical generation in the stunned heart.[22,237] It has been shown that free radicals are generated during coronary occlusion and dramatically increase during early reperfusion with a peak production within 2-4 minutes of reperfusion.[22,237] A linear positive relationship between free radical generation and the severity of ischaemia has also been found.[22]

Free radicals can be generated through xanthine oxidase and activated neutrophils,[22,229] and the administration of allopurinol, a xanthine oxidase inhibitor has been shown to markedly improve contractile recovery.[238] However, experiments by Eddy et al[239] have shown that in the human and rabbit hearts there are undetectable

levels of xanthine oxidase and xanthine dehydrogenase activity which could question the role of this mechanism in myocardial stunning in these species. Whether neutrophils are a source of free radical generation causing myocardial stunning still remains uncertain.[240] Studies which relied on neutrophil depletion through the use of leukocyte filters and neutrophil antisera have provided inconsistent results as to whether a beneficial effect on postischaemic function is achieved.[237,240] It is also suggested that the ischaemic period required to cause myocardial stunning is not adequate to cause neutrophil activation. However, there are many other processes in which oxygen-derived free radicals could be formed. These include activation of the arachidonate cascade, autoxidation of catecholamines and damage to the electron transport chain in the mitochondria.[22]

The mechanism by which oxygen-derived free radicals may cause stunning is poorly understood.[241] Oxygen-derived free radicals may bring about multiple changes to the cellular structure and function.[22,237] They may cause structural injury in enzymes, proteins and nucleic acids.[237] In addition, free radicals may alter the fluidity and permeability of the cell membrane through lipid peroxidation.[237] However, this latter mechanism is considered unlikely by Hearse[237] since lipid peroxidation is a relatively slow process and cannot occur in a short space of time (period in which free radicals impose their effect) after a brief period of ischaemia. It has also been suggested that oxygen-derived free radicals could cause an increase in permeability to Ca^{2+} in sarcolemma and rapidly release Ca^{2+} from the sarcoplasmic reticulum that would be the direct cause of damage.[230,242] The Ca^{2+}, Mg^{2+}-ATPase activity could be impaired by free radicals as a decrease in Ca^{2+} uptake ability is observed in the isolated

sarcoplasmic reticulum exposed to oxygen radicals.[22] Thus, oxygen-derived free radical may also cause stunning through Ca^{2+} overload.

1.2.4 Methods of reperfusion

The best method to combat ischaemia and its consequences is the reestablishment of perfusion to the myocardial tissue. This can be achieved in a number of ways.

a) Thrombolysis

This is the therapeutic use of thrombolytic agents to dissolve clots in coronary arteries in the acute stage of myocardial infarction. This however has a number of contraindications and side effects and the ideal thrombolytic agent has not yet been developed.[243,244]

b) Percutaneous coronary interventions

This normally refers to percutaneous transluminal coronary angioplasty (PTCA), with or without stenting.[245,246] However other modalities have been developed that include brachytherapy which is intracoronary radiation therapy with either gamma- or beta-ray devices, coronary atherectomy, ablative laser-assisted angioplasty, catheter-based thrombolysis and mechanical thrombectomy.

c) Coronary artery bypass graft surgery

Coronary artery bypass graft surgery (CABG) using cardiopulmonary bypass or on the beating heart without cardiopulmonary bypass and with or without minimal access are the main surgical options for the treatment of coronary artery

disease.[247,248,249,250,251,252] However other options include transmyocardial laser revascularization or percutaneous transmyocardial laser revascularization that have been used when bypass graft surgery is not feasible.[253,254]

d) Intra-aortic balloon counterpulsation

Since the 1980s, intra-aortic balloon counterpulsation (IABCP) has been increasingly used in various clinical situations as a lifesaving intervention to attain haemodynamic stabilization prior to definite therapy.[255] Diastolic augmentation enhances perfusion of the coronary circulation and carotid arteries. The reduction in end-diastolic pressure decreases aortic impedance (afterload) and augments systole. IABCP reduces aortic impedance and systolic pressure, leading to a 15-25% reduction in LV wall stress. The reduction in afterload improves LV volume, LV emptying, and myocardial oxygen consumption. Diastolic aortic pressure augmentation increases myocardial perfusion and coronary blood flow. The effects on coronary blood flow are variable, but the boost generally ranges from 10-20% in ischaemic territories. IABCP can decrease LV filling pressures by 20-25% and can improve cardiac output by 20% in patients with cardiogenic shock; therefore, IABCP reduces myocardial oxygen demand significantly.

1.3 MYOCARDIAL PROTECTION

Before describing the different modalities of myocardial protection it is necessary to understand the concept of myocardial oxygen consumption and its changes in the various clinical situations.

The heart represents less than 0.5 percent of body weight, yet it accounts for over 7 percent of resting oxygen consumption. Myocardial oxygen consumption (MVO_2) can be readily calculated using the Fick equation if coronary blood flow (CBF) and arterial (CaO_2) and coronary sinus ($CcsO_2$) oxygen contents are known [$MVO_2 = CBF \times (CaO_2 - CcsO_2)$]. Cardiac muscle extracts much more oxygen in the normal state than other organs, and thus increased myocardial oxygen consumption is achieved primarily by increases in coronary blood flow. The left ventricle consumes approximately 8 ml of O_2 per 100 g of myocardium per minute in a normal human subject at rest. During potassium-induced arrest, O_2 consumption falls to 1.5 ml per 100 g of myocardium per minute, a decrease of over 81 percent. The three major determinants of MVO_2 are heart rate, stroke work (the area within the pressure-volume loop, which incorporates afterload), and inotropic state. The relationship between MVO_2 and heart rate, stroke work, and inotropic state is almost linear.[256,267] MVO_2 is greater when a low stroke volume is ejected against high pressures than when large stroke volumes are ejected against low aortic pressures.

During cardiac surgery, MVO_2 varies widely. The lowest MVO_2 occurs when the heart is arrested. Maximum MVO_2 occurs shortly after weaning from cardiopulmonary bypass when the heart is repaying the oxygen debt incurred during the aortic cross-clamp period. In a series of classic experiments, Buckberg et al[258] examined MVO_2 during different conditions of myocardial activity: the empty beating heart, the fibrillating heart, and the arrested heart. MVO_2 was greatest during normothermic (37°C) fibrillation and least during hypothermic (22°C) arrest. Hyperkalemic arrest achieved a reduction in MVO_2 from 5.6 ± 1.95 ml/100 g per minute to 1.1 ± 0.4 ml/100 g per minute. Hypothermia reduced MVO_2 to 2.9 ± 0.9

mL/100 g per minute, while the combination of potassium-induced arrest and moderate hypothermia reduced MVO_2 to 0.3 ± 0.1 ml/100 g per minute.

The main methods of myocardial protection are described below:

1.3.1 Cardioplegia

In 1955, Melrose et al[259] first described the use of hyperkalaemic blood cardioplegia to electively arrest the heart in diastole to reduce metabolic demand, improve visualization, and facilitate the execution of surgery. The "cardioplegic" solution was given via the aortic root shortly after a clamp was placed across the aorta. However, Melrose abandoned cardioplegia because of the cardiac injury caused by this hyperkalaemic solution. Other investigators questioned hyperkalaemic solutions because of focal inflammatory lesions in the myocardium.[260] Subsequently, these lesions were thought to be due to abnormal calcium flux, but injury was eliminated by reducing the potassium concentration in the cardioplegic solution.[261]

Following the important discovery of Melrose et al,[259] several improvements were made in the composition and delivery of cardioplegia. The fundamental precept of any cardioplegic technique involves protection against ischaemic injury during the aortic cross-clamp period when normal antegrade coronary perfusion is absent. An optimal metabolic supply/demand ratio requires both a reduction in high-energy phosphate utilization and an increase in the delivery of oxygen and metabolic substrates. The previous practice of intermittent aortic cross-clamping was abandoned

by most surgeons when it was realized that the ensuing ventricular fibrillation greatly increased cardiac energy requirements. Hypothermic hyperkalaemic cardioplegic solutions were introduced to induce asystolic arrest to minimize cardiac energy requirements. Intermittent doses of cardioplegia were administered every 15 to 20 minutes to provide oxygen and metabolic substrates for the basal requirements of the arrested hypothermic heart and also to washout of accumulated toxic products. These multidose cardioplegic infusions also were needed to prevent rises in myocardial temperatures and displacement of intracoronary cardioplegic solution by noncoronary collateral flow.

Newer technologies involving both the composition and delivery of cardioplegic solutions continue in an effort to improve the critical ratio between myocardial supply and demand.

a) Mechanisms of cardioplegic protection

Potassium-induced mechanical arrest will reduce oxygen consumption by 80%. This combined with hypothermia will reduce consumption by another 10-15%. Aerobic metabolism can be maintained with oxygenated cardioplegia. Hypothermic arrest is sustained with readministration every 15-30 minutes. During cardioplegic arrest myocardial rewarming is prevented with systemic hypothermia and the additional application of aortic and ventricular vents, and caval occlusion.

b) Composition of cardioplegia

St Thomas's crystalloid cardioplegic solution was the most commonly used for myocardial protection. However, blood cardioplegia is becoming increasingly used as blood has the advantage of oxygen carrying capacity, histidine and haemoglobin buffers, free radical scavengers in red blood cells and metabolic substrates. Blood also has improved rheologic and oncotic properties, which may lessen myocardial oedema. Buffers such as THAM, histidine, and $NaHCO_3$ form a slightly alkaline solution for reperfusion that can counteract intracellular acidosis. Small amounts of Ca^{2+} (0.1-0.5 mM/L) restore Ca^{2+} that has been chelated by citrate. Potassium concentrations range from 10-25 mM/L, with the first dose being the highest. Other substrates are being evaluated, including allopurinol, SOD, deferoxamine, adenosine, nucleoside transport inhibitors, and potassium-channel openers.

1.3.2 Preconditioning

a) Definition

The observation that serial brief episodes of ischaemia with intervening reperfusion did not lead to progressive depletion of high-energy phosphates in the canine myocardium[262] led the same group of investigators to examine the response of hearts "preconditioned" with short bursts of sublethal ischaemia prior to a sustained ischaemic insult.[263] Their finding that the onset of infarction was delayed in pretreated hearts, with a significant reduction in ultimate myocardial infarct size, resulted in recognition of the concept of ischaemic preconditioning, with a proposal that the mechanism responsible involved a slowing of consumption of high-energy phosphates during the prolonged ischaemic insult. Since then, this marked limitation of infarction

induced by antecedent brief periods of ischaemia has been demonstrated in every animal species studied.[264] Subsequent studies have suggested that protection against other end points of injury such as myocardial stunning and reperfusion arrhythmias may also be possible.[265,266,267] Characterization of the time frame of protection has demonstrated a biphasic pattern with an initial ("classical" or "early") powerful phase that lasts 1 to 2 hours after the preconditioning stimulus and a subsequent "second window" 12 to 72 hours later.[268,269] It is also important to note that in experimental studies ischaemic preconditioning has been found to limit infarction when the duration of the sustained ischaemic insult is ≈ 30 to 90 minutes, but is ineffective when this period is extended to 3 hours.[264] This temporal limitation of ischaemic preconditioning implies that the protection is only observed when prolonged ischaemia is followed by timely reperfusion.

The potential for clinical application of such a powerful protective phenomenon has generated enormous interest in identification of the underlying intracellular signalling pathways, with the ultimate aim of pharmacologically exploiting these mechanisms to develop therapeutic strategies that can enhance myocardial tolerance to ischaemia-reperfusion injury in patients with coronary artery disease. Extensive research over the past 15 years has gone a long way in elucidating a number of membrane receptor–linked cellular triggers, intracellular signalling cascades, and potential cytoprotective end-effector proteins that may be involved in mediating the protective effects of ischaemic preconditioning.[264] However, the application of these findings to the clinical setting depends primarily on proof of safety and efficacy when compared with other strategies of myocardial protection and secondarily on identification of well-

defined cohorts of patients who stand to benefit from pretreatment with such cardioprotective agents.

b) Preconditioning in the human heart

Ethical considerations restrict the nature of experimental work on the human heart and thereby render the evidence indirect. Numerous approaches have, to some extent, circumvented this problem. Studies in cells derived from isolated human ventricular myocytes[270,271] and in isolated atrial trabeculae obtained at the time of cardiac surgery[272] suggest that protection can be induced in vitro using metabolic and functional end points. Moreover, using the same in vitro models, it has been demonstrated that the mechanisms of protection in human tissue closely resemble those observed in many animal species, namely, the involvement of adenosine as an important trigger, protein kinase C as an intermediate intracellular messenger, and the ATP-dependent K^+ (K_{ATP}) channel as a potential end-effector protein.[273,274,275,276]

In the clinical setting, there is some evidence to suggest that preconditioning may occur naturally in patients with coronary artery disease. Patients suffering angina before MI have a better in-hospital prognosis; a reduced incidence of cardiogenic shock, congestive cardiac failure, and life-threatening ventricular arrhythmias associated with reperfusion; and smaller infarcts as assessed by release of cardiac enzymes.[277,278,279,280] Follow-up studies have suggested that in patients with preinfarction angina, long-term survival is also improved as compared with patients who are asymptomatic before infarction.[281,282] Whether the protection conferred to

these patients as a result of their preceding ischaemic symptoms represents a form of myocardial adaptation similar to ischaemic preconditioning remains a subject of debate.[283] On the one hand, the issue of enhanced collateral development in patients with preceding angina symptoms remains unresolved. Another equally attractive hypothesis, although not mutually exclusive from the mechanisms underlying ischaemic preconditioning, is facilitation of more rapid reperfusion of the infarct-related artery after thrombolysis in patients with preinfarction angina.[281,284] This hypothesis is based on the known inhibitory effects of adenosine, released during the brief periods of preinfarction ischaemia, on platelet aggregation after activation of A_2 receptors on platelet membranes, which has been suggested to modify thrombus formation and thereby promote earlier reperfusion after thrombolysis.[285] Indeed, in anaesthetised open-chest dogs, brief periods of ischaemia before a long ischaemic insult attenuates platelet-mediated thrombosis and improves vessel patency, and this effect is abolished by inhibition of adenosine receptors.[286]

The phenomenon of "warm-up angina," in which patients complain that their angina symptoms are worse in the morning but improve during the course of the day, has been the subject of research over the past few years.[287,288] This work has provided evidence for increased efficiency of myocardial metabolism, in terms of reduced oxygen consumption at a given workload and a reduction in angina symptoms and ST-segment changes, during a second period of either exercise or angina resulting from pacing-induced tachycardia. These favourable changes were not accompanied by recruitment of collateral vessels, as evidenced by similar coronary and great cardiac vein blood flow measurements. Similarly, a reduction in electrocardiographic evidence of silent ischaemia during successive periods of exercise has been

demonstrated.[289] A recent study suggests that the degree of myocardial stunning after exercise-induced myocardial ischaemia may also be attenuated if the patient had performed a preceding period of exercise 30 minutes earlier.[290] Studies investigating the temporal profile of warm-up angina have demonstrated that the duration of this phenomenon is 1 to 2 hours after the first period of exercise, a time course that closely parallels that of classic ischaemic preconditioning.[291,292] Moreover, we have recently shown that in addition to immediate protection, patients with stable angina have improved exercise tolerance 24 hours after a period of exercise-induced myocardial ischaemia, a finding that may represent delayed preconditioning.[293] However, a recent study using a similar study protocol failed to show enhanced exercise tolerance 24 hours after a period of exercise, thereby arguing against delayed protection in this model.[294] The reasons for the differences between these studies is not immediately obvious and requires further investigation.

These findings suggest that the warm-up phenomenon is at least partly due to metabolic adaptation of myocardium, which induces tolerance to subsequent ischaemia, a process that closely resembles ischaemic preconditioning. However, studies that have examined the cellular mechanisms mediating warm-up angina do not fully support this hypothesis. For instance, inhibition of adenosine receptors before exercise fails to abolish the warm-up phenomenon.[295,296] Furthermore, investigation into the role of K_{ATP} channels in mediating this form of myocardial adaptation has provided conflicting results.[297,298] It is therefore not clear at this point whether the adaptation observed during repeated exercise is a representation of the preconditioning phenomenon or whether other mechanisms are involved.

Furthermore, despite attempts by some investigators, a major role for recruitment of collateral vessels contributing to this phenomenon has not been ruled out.

c) Myocardial adaptation during revascularisation procedures

PTCA provides a unique opportunity to study the response of the human myocardium to brief periods of controlled ischaemia and reperfusion. The procedure usually involves repeated intracoronary balloon inflations with intervening periods of perfusion, and in theory the first period of ischaemia may enhance the myocardial tolerance to subsequent balloon inflations via classic ischaemic preconditioning. Several recent studies have addressed this issue using various indices of myocardial ischaemia including clinical, electrocardiographic, metabolic, and haemodynamic measurements. Most of these studies have shown that if the duration of the first balloon inflation is longer than a "threshold" of ≈ 60 to 90 seconds, all indicators of myocardial ischaemia, including chest pain severity, abnormalities of left ventricular regional wall motion, ST-segment elevation, QT dispersion, ventricular ectopic activity, lactate production, and release of myocardial markers such as CKMB, are attenuated during subsequent balloon inflations, which provides evidence for myocardial adaptation induced by the first period of ischaemia.[299,300,301,302,303,304] As with many studies of ischaemic preconditioning in humans, a major confounding factor during successive balloon inflations in PTCA studies is the acute recruitment of collateral vessels. However, studies that have controlled for this effect by angiographic grading of the collateral vessels[300] measurement of cardiac vein flow[299]; changes in blood flow velocity in the contralateral coronary artery[305]; and, more accurately, by assessment of intracoronary pressure-derived collateral flow index

during successive balloon inflations[306] have shown that although collateral recruitment occurs in some patients, it cannot fully explain the myocardial adaptation observed during repeated balloon inflations.

Investigation into the mechanisms underlying this rapid protection of the myocardium during PTCA has provided further support for a preconditioning-like effect. Blockade of K_{ATP} channels with oral glibenclamide before angioplasty abolishes the reduction in ischaemic indices observed during subsequent balloon inflations, which implies a role for these channels in mediating this form of adaptation.[307] This finding is supported by the observation that opening of these channels with nicorandil reduces the electrocardiographic indices of ischaemia during coronary angioplasty.[308] Furthermore, an important role has been demonstrated for adenosine in mediating myocardial adaptation during coronary angioplasty. Inhibition of adenosine receptors by bamiphylline[309] or aminophylline[310] abolishes myocardial adaptation during the second balloon inflation. Conversely, intracoronary infusion of adenosine before PTCA, independent of its vasodilatory effect, attenuates ischaemic indices during the first balloon inflation.[311] Two other recent reports have suggested a role for both opioid[312] and bradykinin[313] receptors in mediating myocardial adaptation during PTCA. These studies provide further evidence that myocardial tolerance to further ischaemic episodes can be induced by preceding brief periods of ischaemia and that this tolerance may be mediated by the same mechanisms as those involved in ischaemic preconditioning in animal models.

However, recent experimental evidence has provided grounds for caution when interpreting the results of these PTCA studies, which have mostly used ST-segment elevation on ECG as an end point reflecting the degree of myocardial ischaemia, and its attenuation during successive balloon inflations as an indicator of enhanced myocardial resistance to ischaemia. Although this assumption was supported by earlier experimental studies of repeated coronary artery occlusion in collateral-deficient pig and rabbit hearts,[314,315] a recent study clearly indicates a dissociation between ST-segment changes on the ECG and myocardial protection in terms of infarct limitation.[316] The finding of these authors, that the changes in ST-segment voltage during coronary artery occlusion may merely represent an epiphenomenon distinct from the cardioprotective effect of ischaemic preconditioning, is particularly pertinent when evaluating or designing mechanistic studies using pharmacological agents to mimic or abolish the cellular signalling mechanisms of ischaemic preconditioning. It is imperative that the influence of these pharmacological tools on the sarcolemmal K_{ATP} channels, which are thought to modulate ECG voltages, is clearly distinguished from their effect on the mitochondrial K_{ATP} channels, which have been proposed as a mediator of cardioprotection.[317]

Possibly the most direct evidence for preconditioning in humans comes from studies that have examined the effect of preconditioning protocols in patients undergoing cardiac surgery in which resistance to global ischaemia is assessed, a setting that is not confounded by changes in collateral recruitment. In this respect, it has been reported in a prospective study examining the effects of a preconditioning protocol of 2 cycles of 3 minutes of global ischaemia (induced by intermittently cross-clamping the aorta and pacing the heart at 90 bpm) followed by 2 minutes of reperfusion before a 10-

minute period of global ischaemia and ventricular fibrillation.[318] Patients subjected to this protocol had better preservation of ATP levels in myocardial biopsies during a subsequent 10-minute global ischaemic period. These metabolic changes were almost identical to those seen in dogs by Reimer et al[262] However, total myocardial ATP content may not reflect local turnover within subcellular compartments and certainly does not provide information about the efficiency of cellular metabolism in terms of ATP requirements. In a more recent study involving a larger group of patients, serum levels of troponin T were used as an indicator of myocardial cell necrosis. Using this end point, patients subjected to the same preconditioning protocol suffered less necrosis as determined by release of troponin T.[319] Of considerable interest, however, was the finding that the ATP levels did not differ between preconditioned and control groups. This emphasizes the need for multiple end points to be used, especially in studies in which small differences in myocardial viability without overt clinical effects are expected.

On the other hand, studies that have used other cardioprotective strategies during the prolonged period of ischaemia, such as hypothermia or cardioplegia, have not consistently demonstrated additional protection by ischaemic preconditioning. For instance, the use of similar preconditioning protocols of one 3-minute episode of aortic cross-clamping before the onset of cardioplegic arrest failed to show any beneficial effects compared with the control group; in fact, the preconditioned group of patients had more creatine kinase release compared with case-matched controls.[320] Similarly negative results have been reported by another group.[321] These divergent results have led to the hypothesis that in the setting of coronary artery bypass surgery, the additional protection conferred by ischaemic preconditioning may only be

demonstrable in cases in which a potential for suboptimal myocardial protection increases the risk of perioperative infarction.[322] However, this hypothesis is not supported by recent studies that indicate improved myocardial preservation by ischaemic preconditioning during coronary bypass or valve surgery despite optimal protection with hypothermia and cardioplegia.[323,324] Resolution of these discrepancies is obviously required before ischaemic preconditioning can be routinely used in clinical settings.

d) Benefit to patients

Although it would appear from the evidence outlined above that the human myocardium is amenable to preconditioning, this does not imply that clinical benefit will automatically follow. Prompt reperfusion will always remain the most effective method of infarct size limitation and is therefore the most important determinant of prognosis. Preconditioning, by virtue of delaying myocardial necrosis, prolongs the time window during which revascularization therapies can be effectively instituted. However, the use of brief antecedent ischaemia as a means of prophylactic induction of this protection is not desirable or feasible in most circumstances. On the other hand, the use of pharmacological agents capable of mimicking the protective effects of preconditioning, in lieu of brief ischaemia, may provide a more benign approach for eliciting cardioprotection. However, even with the development of pharmacological agents that may be capable of mimicking the protection, timing of administration remains a critical limiting factor.

First, deployment of pharmacological preconditioning strategies necessitates pretreatment; the pathophysiology of the preconditioning phenomenon dictates that the myocardium must be preconditioned before the onset of a potentially lethal ischaemic insult. This depends on identification of a relatively well-defined cohort of patients who are at high risk of acute coronary occlusion and stand to benefit from preconditioning or from pretreatment with agents that trigger or augment myocardial preconditioning.

The acute coronary syndromes (ACSs) comprise a spectrum of pathophysiological conditions spanning unstable angina, non–ST-elevation MI, and acute ST-elevation MI. In patients with acute MI with persistent ST elevation, early reperfusion to re-establish epicardial blood flow is well established as the standard of care, be it with early fibrinolytic therapy or, where the facilities and expertise are available, with primary angioplasty.[325] As far as pharmacological preconditioning strategies are concerned, these patients are unlikely to benefit from such treatment, and their management should focus on early restoration of coronary artery patency and potential strategies to minimize reperfusion injury. On the other hand, non–ST-elevation ACSs, including unstable angina and non–Q-wave MI, mark the transition from stable coronary artery disease to an unstable state and constitute the leading cause of hospital admission in patients with coronary artery disease. This group of patients is at a high risk of progression to acute coronary occlusion, and >10% die or suffer a MI (or reinfarction) within 6 months, with about one half of these events occurring during the acute early phase.[326]

This cohort of patients with non–ST-elevation ACS forms a reasonably well-defined high-risk group that might benefit from pretreatment with agents that trigger or augment myocardial preconditioning over a period of several days or weeks and could therefore effectively maintain the myocardium in a protected or "preconditioned" state. A number of these patients who suffer a MI after unstable symptoms may be "naturally" preconditioned by their preceding ischaemic episodes. Recent evidence, however, suggests that this natural protection is limited to those patients in whom the episodes of preinfarction angina occur during a narrow time window in relation to the infarct.[251,252]

It is worth noting that even when prior treatment with the pharmacological preconditioning agent is feasible, the duration of the protection afforded is limited. The temporal profile of the protective effects of preconditioning in humans is unknown but, according to experimental evidence in laboratory animals, it is unlikely to exceed 48 to 72 hours.[327,328] Therefore, unless the onset of an ischaemic event can be predicted with accuracy, repeated dosing with the potential preconditioning drug will be necessary in these high-risk patients to maintain the preconditioned state. Early experimental evidence suggested that the protective effects of classic ischaemic preconditioning are lost after prolonged periods of repetitive ischaemia[329] or chronic pharmacological preconditioning with selective adenosine A_1 agonists.[330] However, recent encouraging evidence indicates that tachyphylaxis could be overcome by exploiting the prolonged time course of the second window of protection. Intermittent treatment of conscious rabbits with an optimal dosing regimen of pharmacological preconditioning with selective adenosine A_1 receptor agonists maintains the animals

in a preconditioned state over a period of several days and results in a significant reduction in infarct size.[331,332]

Very few studies have evaluated a protective role for pharmacological preconditioning strategies in patients with non–ST-elevation ACS. In this regard, a recent report suggests that opening of K_{ATP} channels with nicorandil, in addition to standard aggressive medical therapy for unstable angina, results in a significant reduction in the incidence of myocardial ischaemic episodes and tachyarrhythmias.[333] This may purely represent an anti-ischaemic effect due to the vasodilatory properties of nicorandil. However, because the patients in this study were already on maximal antianginal therapy, and in particular a significant proportion were treated with intravenous or oral nitrates, it is possible that the protection observed in the nicorandil group, be it only using soft end points of myocardial injury, may at least partially be due to a preconditioning-like effect.[334] These encouraging findings, coupled with very recent experimental evidence indicating that nicorandil specifically activates the mitochondrial rather than the sarcolemmal K_{ATP} channels in rabbit ventricular myocytes,[335] provide a promising new approach to myocardial protection in patients with unstable angina.

Although the conditions of the majority of patients with non–ST-elevation ACS will stabilize with effective anti-ischaemic medications, ≈ 50% of such patients will require coronary angiography and revascularization because of failure of medical therapy assessed by recurrence of ischaemic symptoms at rest or demonstration of provokable ischaemia during stress testing.[326] The optimal timing of revascularization

procedures in patients with ACS is under debate, although recent evidence points to the benefit of early intervention.[336] However, the complication rate associated with revascularization procedures in unstable patients is appreciably higher than that in patients with stable coronary artery disease. For example, emergency PTCA in patients with refractory unstable angina is associated with a periprocedural mortality rate of 1% to 3% and nonfatal infarction occurs in a further 6% to 10%, with a need for emergency surgery in up to 12%.[326,337] The potential for and the time course of any protection conferred by preceding angina episodes in this situation is not known, although some evidence suggests that unstable symptoms occurring in the 6 to 12 hours before PTCA may have a preconditioning-like effect.[338] Conversely, the Thrombolysis in Myocardial Ischaemia (TIMI) IIIB study suggested that emergency PTCA performed within 24 hours of enrolment was the most powerful predictor of periprocedural death and MI.[337] Although the risk associated with the procedure diminishes if a patient is allowed to "cool off" and the plaque is at least partly healed, this longer waiting period carries the risk of progression to MI and death. These patients may therefore have the most to benefit from pretreatment with agents that mimic preconditioning or augment the protection afforded by naturally occurring ischaemic preconditioning, thereby reducing the degree of myocardial injury in the event of periprocedural complications associated with PTCA. At the other end of the spectrum are patients with stable angina undergoing elective PTCA, who have a relatively low risk of complete coronary artery occlusion and MI (<5%). However, as more high-risk procedures are performed, and considering the potential benefits associated with this potent mode of cardioprotection, it is possible that application of pharmacological preconditioning agents may find a place routinely before elective angioplasty.

Similar complications may arise during cardiac surgery. In patients with unstable angina undergoing CABG, perioperative mortality rates of 3.7% and infarction rates of 9.9% have been reported,[339] which are considerably higher than those associated with elective surgery. Even in patients with stable coronary artery disease, despite carefully controlled intraoperative ischaemic periods and hypothermia, sensitive markers of tissue injury such as troponin T indicate that discrete necrosis occurs.[340,341] Moreover, as surgeons undertake more complex and higher-risk operations, the need for better preservation methods increases. In a situation such as CABG, the administration of an agent before surgery that could enhance myocardial defences would reduce susceptibility to focal necrosis during surgery and permit the extension of the intraoperative ischaemic period. High-risk patients with poor preoperative left ventricular function, extensive coronary artery disease, or severe left ventricular hypertrophy could certainly benefit if the degree of protection were improved by invoking endogenous cellular adaptive mechanisms. The possibility that organ preservation before transplantation might be amenable to the same improved protection, as suggested by some experimental evidence,[342,343] is also of significant interest. This might allow an extension of the "cold ischaemic time" between harvesting and implantation, facilitating optimal matching of recipient to donor, as well as affording a potential improvement in early myocardial function.

e) Signal transduction of preconditioning

The understanding of the precise mechanism of preconditioning is vital to be able to harness all its beneficial effects. However, despite more than a decade of research trying to determine the mechanisms of preconditioning, they still remain unclear. Signalling pathways orchestrating cardioprotection are conceptually classified into

triggers, mediators and end effectors of preconditioning with multiple distinct signalling pathways appearing to converge towards an end effector that initially was putatively identified as the mitochondrial ATP-sensitive potassium channel (mitoK_{ATP}).

The initial phase of preconditioning may start by release of a trigger substance during the brief episodes of ischaemia and reperfusion that may bind to surface receptors coupled to G_i proteins.[344,345,346,347] Among these substances are adenosine, bradykinin, cathecholamines, opioids, and acetylcholine.[344,345,346,347] Other trigger substances have been identified as prostanoids, nitric oxide, and low doses of reactive oxygen intermediates.[344,345,346,347] Recently a possible role for innate immunity in triggering preconditioning has been suggested where specially tumour necrosis factor alpha is indicated as a trigger of the preconditioning response.[348] The trigger substances may cause activation of kinase cascades where translocation of protein kinase C, especially the ε isoform, from the cytosolic to the particulate fraction may be crucial to the response.[349,350] Tyrosine kinases as well as mitogen activated protein kinases appear involved in the signalling cascade in several species, although which kinase cascade is upstream or downstream continues to be an issue of debate.[351,352] Adenosine signalling has been linked to protein kinase C-dependent opening of mitochondrial ATP-sensitive potassium channels (K_{ATP}).[353] The K_{ATP} channels are suggested to be the end-effectors of myocardial protection in a number of models; however, their role remains controversial as most data in the field are discrepant.[352,354] Recent data indicate that activation of the K_{ATP} channel causes release of reactive oxygen species[347] and one may speculate that their role in preconditioning is linked to this. The next step in the signalling pathway is activation of transcription factors by protein

kinases, reactive oxygen species, and nitric oxide, of which nuclear factor κ-B (NFκB) in particular has been investigated in both classic and delayed models.[355,356] NFκB is a redox sensitive transcription factor that regulates transcription of a battery of genes most of which are associated with proinflammatory effects such as cytokines, chemokines, and leukocyte adhesion molecules.[357] Some candidate genes for organ protection in preconditioning are also regulated by NFκB. Pharmacologic blocking of NFκB translocation inhibits preconditioning in both classic and delayed models.[355,356]

1.4 ALPHA 1 ADRENOCEPTORS

1.4.1 Structure and distribution

Adrenergic receptors (α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2, β_3) are members of the G-protein-coupled receptor superfamily of membrane proteins that mediate the actions of the endogenous catecholamines, noradrenaline and adrenaline. They are involved in a wide spectrum of physiological functions and are the site of action of a considerable percentage of currently prescribed therapeutic agents. These proteins are proposed to traverse the membrane in seven transmembrane spanning α-helical domains linked by three intracellular and three extracellular loops.[358,359] The original classification of adrenoceptors on functional basis has been superseded with radioligand, and biochemical studies accumulating evidence indicating that the adrenoceptors are a heterogeneous group of distinct but related proteins. This conclusion has been confirmed with molecular cloning.[360] Initially the major adrenoceptor classes were further subdivided on functional and anatomical grounds: α-adrenoceptor mediated effects, such as vasoconstriction, were considered α_1-adrenoceptor effects, in part

based on actions of agonists and antagonists that could differentiate such responses from α_2-adrenoceptor effects, which mediate feedback inhibition by noradrenaline on its release from presynaptic terminals.[361] Similarly, the β_1-adrenoceptor mediated effects on the force and rate of contraction in the heart were differentiated from β_2-adrenoceptor mediated effects, such as promotion of smooth muscle relaxation in the bronchi and vessels. Subsequent research showed that this classification scheme based on anatomic distribution is overly simplistic: many, probably most, organs have β_1- and β_2-adrenoceptor s as well as α_1- and α_2-adrenoceptors.[358,359,360] The genomic structure of the α_1-adrenoceptors has been reported, and all three subtypes have a large intron after the TM6 domain. With the exception of α_{1D} all the adrenoceptor subtypes are polymorphic with genetic variation in the coding and noncoding regions.[359,362]

The α_1-adrenoceptors are important mediators of sympathetic nervous system responses, particularly those involved in cardiovascular homeostasis, such as arteriolar smooth muscle constriction and cardiac contraction.[363] α_1-adrenoceptors regulate many physiological processes, including smooth muscle contraction and hence vascular tone, myocardial inotropy, and hepatic glucose metabolism.[358] Each of the α_1-adrenoceptor subtypes shows linkage to G_q and activate phospholipase C, but differences have been noted in signaling capacities.[364] The α_{1A}-adrenoceptor subtype is the predominant α_1-adrenoceptor in the heart and in certain parts of the vasculature such as arteries.[361] α_{1B}-adrenoceptors are implicated in blood pressure regulation since stimulation of the α_{1B}-adrenoceptor results in vasoconstriction and blood pressure elevation.[365] Contraction of large caliber arteries have been found to be controlled by the α_{1D}-adrenoceptors.[366] The α_2-adrenoceptors are of predominant

importance for the mediation of the regulatory effects of catecholamines on several renal functions, including renin release, glomerular filtration, and Na^+ and water excretion.[367,368,369]

The α_1-adrenoceptors utilize a variety of second messenger pathways to modulate cellular function.[370] Studies with many cell types demonstrate that all α_1-adrenoceptors activate phospholipases C and A_2.[371] In addition to mobilizing intracellular calcium, the α_1-adrenoceptors have also been shown to activate calcium influx via voltage-dependent and independent calcium channels.[360] Additionally, these receptors signal through both pertussis toxin-sensitive G-proteins[371] and G proteins of the G_q family.[372] Minneman et al[360] studied the coupling of the α_1-adrenoceptor subtypes and noted that there were marked differences in the ability of α_1-adrenoceptors to generate intracellular second messengers. In particular, they noted that the α_1-adrenoceptor was the most efficiently coupled to calcium release and inositol phosphate production whereas the α_{1D}-adrenoceptor was poorly coupled to intracellular signaling cascades,[364] suggesting potential differences in the functional outcomes of α_1-adrenoceptor activation.

One of the principle adrenergic drugs in clinical use is phenylephrine and it is a selective α_1-adrenoceptor agonist.[373] This drug has been used extensively in the literature for the investigation of α_1-adrenoceptor effects. Prazosin on the other hand is a selective α_1-adrenoceptor[374] and these two drugs will be used in the experiments in this thesis as the pharmacological tools in the investigation of α_1-adrenoceptors involvement in ischaemia, reperfusion and preconditioning.

1.4.2 Role of alpha 1adrenoceptors in ischaemia, reperfusion and preconditioning

Generally, during sustained myocardial ischaemia, noradrenaline accumulation within ischaemic myocardium is mainly caused by a locally induced nonexocytotic release of noradrenaline.[375,376] Kurz et al[377] findings indicated that nonexocytotic release is also the underlying mechanism of ischaemia-evoked noradrenaline release in human myocardium. Nonexocytotic release of noradrenaline during ischaemia, which is a consequence of energy starvation of the sympathetic nerve terminal, contributes to the genesis of malignant arrhythmias[378,379] and accelerates the development of myocardial injury[380] in experimental ischaemia.

Studies using acute and chronic sympathetic denervation and antiadrenergic agents demonstrate that this local metabolic, rather than being centrally induced, noradrenaline release is critically involved in the progression of ischaemic cell damage and the occurrence of ventricullar fibrillation in early ischaemia. As a consequence of local metabolic catecholamine release, extracellular noradrenaline reaches 1,000 times the normal plasma concentration within 20 minutes of ischaemia. Myocardial ischaemia results in a temporary supersensitivity to catecholamines of the myocytes. This is due to a twofold increase in α_1-adrenoceptor and a 30% increase in β-adrenoceptor number at the cell surface. The sensitization of adenylate cyclase during the first 20 minutes of total ischaemia is followed by a rapid inactivation of the enzyme that also includes the coupling protein Gs. The deleterious combination of extremely high noradrenaline concentrations with an at least temporarily enhanced responsiveness of the tissue to catecholamines is thought to accelerate the propagation of the wavefront of irreversible cell damage in the ischaemic myocardium. Moreover,

the inhomogenous distribution of catecholamine excess within the heart is considered to promote malignant arrhythmias by unmasking and enhancing electrophysiological disturbances in early ischaemia.[381]

This is confirmed by further research by Seyfarth et al[382] confirming that sustained ischaemia for 20 minutes induced an endogenous release of noradrenaline, which amounted to 239±26 pmol/g. However, one cycle of 5 minutes transient ischaemia followed by 5 minutes of reperfusion reduced ischaemia induced noradrenaline release to 107±17 pmol/g (55% reduction); two cycles resulted in a release of 71±6 pmol/g (70% reduction). Three and four cycles did not further reduce ischaemia-induced noradrenaline release (79±8 pmol/g (67% reduction) and 102±21 pmol/g (57% reduction), respectively). Noradrenaline release in all groups with transient ischaemia was statistically different from the release in the group without transient ischaemia.[382]

This and other research has raised the possibility of involvement of α_1-adrenoceptors in the phenomenon of ischaemic preconditioning. Short administration of catecholamines prior to the onset of prolonged ischaemia has been found to precondition the rat heart against post ischaemic myocardial stunning,[383,384] to reduce infarct size in rabbits[385] and to reduce ischaemia induced arrhythmias in dogs[386] and rats.[387] This further supported by work from Ravingerova et al[388] demonstrating that the sensitivity of the rat heart to ischaemic arrhythmias can be modulated by ischaemic preconditioning and that the protection is mediated via stimulation of α_1-adrenoceptors coupled with Gi-proteins. Furthermore reduction in ST-segment changes and cardiac pain severity during ischaemia observed in humans after two sequential coronary balloon inflations have been shown to be abolished by

pretreatment with phentolamine, an α-antagonist, suggesting that ischaemic preconditioning is mediated by α-adrenoceptors in human cardiomyocytes.[305] Moreover, human atrial trabeculae obtained during coronary bypass surgery and subjected to ischaemia *in vitro* demonstrate the development of ischaemic preconditioning, which is specifically mediated by α_1-adrenoceptors.[389] Despite this compelling evidence there remains a number of questions unanswered regarding the role of α_1-adrenoceptors[390] in preconditioning and this is further compounded by the fact that the underlying mechanism by which α_1-adrenoceptors mediate ischaemic preconditioning remains unknown. [391]

1.5 AIMS OF THESIS

In this thesis I will investigate: (i) the role of alpha 1 adrenoceptors in ischaemia and reperfusion injury in the human myocardial tissue, (ii) the role of alpha 1 adrenoceptors in ischaemic preconditioning and compare their efficacy to adenosine receptors, (iii) the role and order of the mediators involved in the signal transduction mechanism of preconditioning namely the mitochondrial potassium dependent adenosine triphosphate channels (MitoK_{ATP}), protein kinase C and p38mitogen activated protein kinase (p38MAPK), (iv) the clinical implications of the use of K_{ATP} channels openers such as nicorandil and the use of blockers such as sulfonylureas in relation to the ability to precondition human myocardium and (v) finally, the characterization of the delayed window of pharmacological and ischaemic preconditioning both occurring in vitro and in vivo in the human myocardium and also the investigation of the signal transduction mechanism involved.

Chapter 2

Methods

2.1 INTRODUCTION

Ischaemic heart disease is the single most common cause of mortality in the western world. Over the last two decades, a great deal has been learned about the pathophysiology of myocardial ischaemia, the consequences of reperfusion and how the adverse effects may be combated. Most of our knowledge has been gained by using *in vivo* and *in vitro* experimental animal models, and the extrapolation of this information to the human heart has resulted in the implementation of novel therapeutic approaches and in a progressive decrease in the death rate attributed to cardiac ischaemic events.

Studies on cardiac ischaemia and reperfusion in man are difficult because of the presence and potential influence of a whole host of clinical factors. The utilization of human isolated myocytes,[270,392] papillary muscle[393,394] and atrial myocardium[275,395,396,397]has provided a means to investigate directly the effects and mechanisms of ischaemia and reperfusion in man, without the need to resort to assumptions made from animal studies, and to safely test interventions intended to be used clinically. Thus the use of human cultured myocytes[270,398]and right atrial tissue,[273,275] for example, has served to identify some of the mechanisms involved in ischaemic preconditioning in the human myocardium, which, compared with those found in other animal species,[399,400,401,402,403] has opened the door for its clinical application.[404]

The right atrial preparation is of particular interest, because the tissue is easily obtainable from patients undergoing open-heart surgery, it is simple to prepare and the procedure is inexpensive. The preparation has been fully characterised and the

stability of the human right atrium when incubated in a buffered medium, its response to various degrees of ischaemic insult and the short and prolonged effects of reperfusion described.[405]

2.2 METHODS

2.2.1 Preparation of atrial slices

Specimens of human right atrium appendage were obtained from patients undergoing elective heart surgery. Local ethical committee approval was obtained for the harvesting technique. During surgery, the right atrial tissue is routinely removed for venous cannulation and establishment of cardiopulmonary bypass. Samples were quickly immersed in cold (4°C) Krebs/Henseleit/Hepes medium (in mM): NaCl (118), KCl (4.8), $NaHCO_3$ (27.2), KH_2PO_4 (1), $MgCl_2$ (1.2), $CaCl_2$ (1.25), glucose (10), Hepes (20). The medium had been pre-bubbled with 95% O_2/5% CO_2 to attain PO_2 of 25–30 kPa and pH 7.4. The atrial appendage was immediately sliced manually with skin-graft blades (Swann-Morton Ltd, Sheffield, UK), each slice to a thickness of 300-500μm and a weight of 30-50 mg, as originally described for the preparation of rat renal slices.[406] Briefly, the tissue was placed with the epicardial surface face down on filter paper fixed to a rectangular glass base (5×25 cm). A ground-glass slide (2.5×7.5 cm) was then pressed against the tissue and the blade was drawn between the slide and the tissue. The slicing apparatus and the tissue was kept wet at all times with ice-cold medium (4–10°C).

2.2.2 Experimental time course

After preparation, the slices (2–9 slices per specimen) were blotted with wet filter paper, loaded into glass 25 ml Erlenmeyer flasks containing 10 ml of preoxygenated

medium at 37°C. The flasks were then placed in a shaking water bath (100 cycles/min) at 37°C for 30 min to allow equilibration. The slices were then rinsed with the medium, blotted and placed in clean flasks containing 10 ml of medium according to the various study protocols. The water bath used for the experiments is shown in the Figure 2-I.

For the induction of simulated ischaemia, the slices were washed with one rinse of medium bubbled with 95% N_2/5% CO_2 at pH 6.8. The glucose in the medium was replaced with 10 mM 2-deoxy-glucose to maintain constant osmolarity of the medium. The removal of the substrate glucose and altering the pH of the incubation medium are designed to simulate ischaemic conditions. The atrial tissue is unable to use 2-deoxy-glucose as a substrate. The slices were transferred to clean flasks containing 10 ml of the same medium, which was continuously bubbled with 95% N_2/5% CO_2 and maintained at 37°C during the entire ischaemic period. Monitoring of PO_2 with an oxygen detector electrode (Oxylite™; Optronix Ltd, Oxford, UK) revealed that the PO_2 in the medium was 0 kPa. At the end of each ischaemic period, the non-oxygenated medium was removed; the slices were rinsed with oxygenated medium 95% O_2/5% CO_2 and incubated in 10 ml of oxygenated medium containing 10 mM glucose at 37°C for a further 120 min. For the experiments on the second window of ischaemic preconditioning foetal calf serum was added to the Krebs Hensleit solution as well as gentamicin.

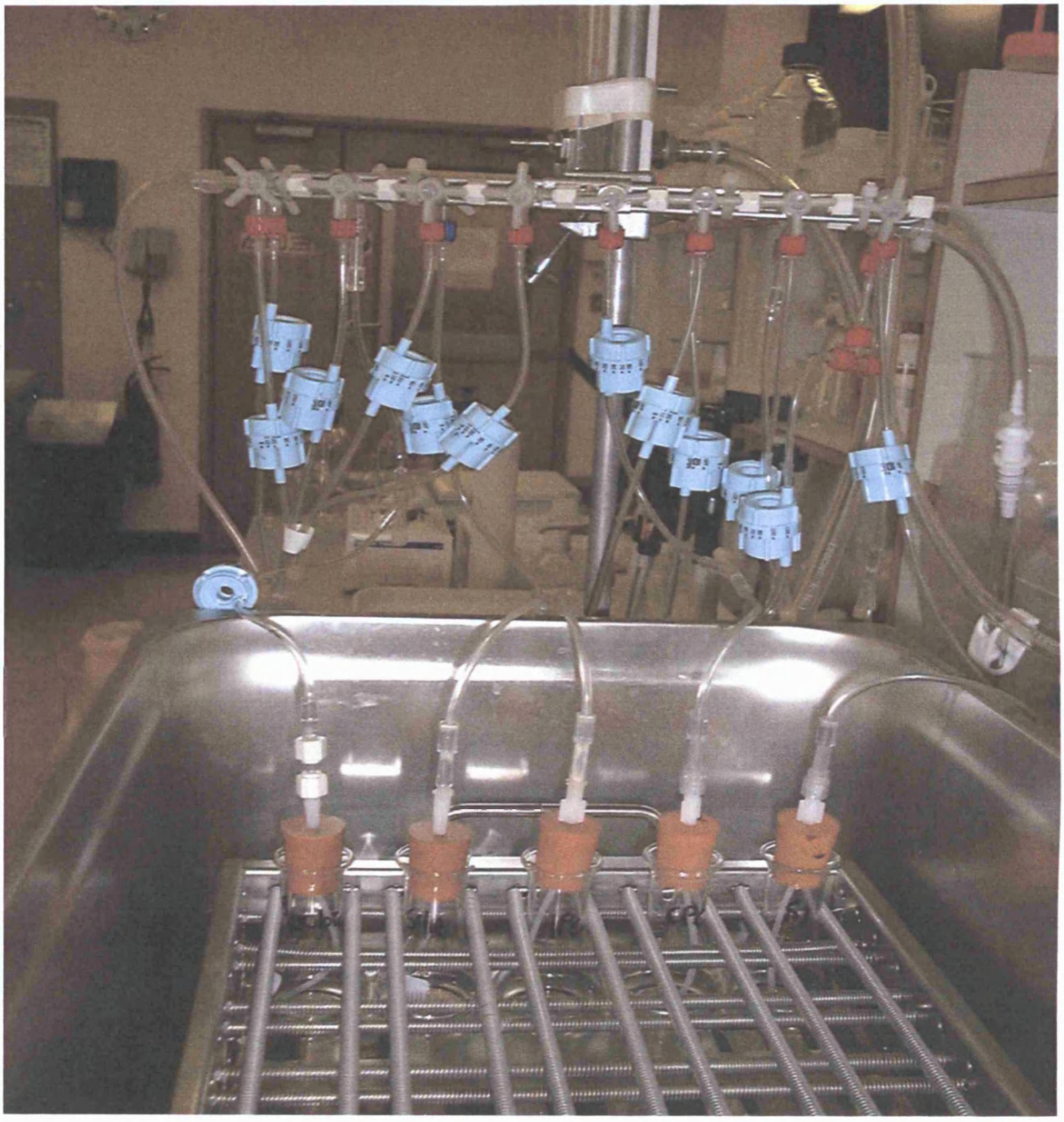

Figure 2-I: This photograph shows the water bath used for all the experiments. The various gases are bubbled into the incubation fluid via tubes. The bath temperature is maintained at 37°C.

2.2.3 Assessment of myocardial injury

Tissue injury was determined by measuring the leakage of creatine kinase (CK) into the incubation medium during the 120min reoxygenation period. This was assayed by a kinetic ultraviolet method based on the formation of NADPH (Sigma Diagnostics Catalogue No. DG147-K) and the results were expressed as U/g wet weight. The CK reagents measure CK activity based on the methods recommended by the German Society for Clinical Chemistry.[407] The enzymatic reactions involved in the assay are as follows:

$$\text{Creatine Phosphate} + \text{ADP} \xleftrightarrow{\text{CK}} \text{Creatine} + \text{ATP}$$

$$\text{ATP} + \text{Glucose} \xleftrightarrow{\text{HK}} \text{ADP} + \text{G-6-P}$$

$$\text{Glusose-6-Phosphate} + \text{NADP} \xleftrightarrow{\text{G-6-PDH}} \text{6-PG} + \text{NADPH}$$

CK catalyses the reaction between creatine phosphate and adenosine diphosphate (ADP) forming creatine and adenosine triphosphate (ATP). The ATP formed is utilized to phosphorylate glucose producing glucose-6-phosphate (G-6-P) in the reaction catalysed by hexokinaze (HK). Subsequently G-6-P is oxidised to 6-phosphogluconate (6-PG) in the presence of nicotinamide adenine dinucleotide (NADP). This reaction is catalysed by glucose-6-phosphate dehydrogenase (G-6PDH). During this oxidatio an equimolar amount of NADP is reduced to NADPH resulting in an increase in absorbance at 340 nm. The rate of change in absorbance is directly proportional to CK activity.

The two reagents for this assay are reconstituted with distilled water, mixed together and dispensed into a 98-well flat-bottom microtiter plate (Nunc Brand Products, Denmark). The incubation medium is then added to the wells, allowed to icubate for 1

minute at 37°C. The initial absorbance is measured at 340 nm using a spectrophotometer (Benchmark, Bio-Rad laboratories, California, USA) and then at exactly 1, 2 and 3 minutes follwing the initial absorbance reading. The mean absorbance change per minute (ΔA/min) is determinted. CK activity in units per litre is calculated using the formula:

$$\text{CK activity (U/L)} = \frac{\Delta A/\text{min} \times TV \times 1000}{6.22 \times LP \times SV}$$

TV: Total volume (ml)

SV: Sample volume (ml)

6.22: Millimolar absorptivity of NADPH at 340 nm

LP: Lightpath 1 cm

1000: Conversion of units per mlto units per litre

Finally the number of units of CK calculated is divided by the weight of the original specimen used in the study protocol and the results expressed as Uof CK/g of wet weight.

Tissue viability was assessed by the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) to a blue formazan product at the end of the experimental time. The tissue was loaded into a Falcon conical tube (15 ml, Becton Dickinson Labware, New Jersey, USA) into which 2 ml of phosphate buffer solution (0.05 M), containing MTT (1.25mg/ml, 3Mm at final concentration), was added and then incubated for 30min at 37°C. Following this, the tissue was homogenized in 2 ml

of dimethylsulfoxide (Homogenizer Ultra-Turrax T25, dispersing tool G8, IKA-Labortechnic, Staufen, Germany) at 9500 rpm for 1min. The homogenate was then centrifuged at 1000g for 10min and 0.2ml of the supernatant was dispensed into a 98-well flat-bottom microtiter plate (Nunc Brand Products, Denmark). After this, the absorbance of the blue formazan formed was measured on a plate reader (Benchmark, Bio-Rad laboratories, California, USA) at 550nm and the results expressed as mmol /g wet wt.

2.2.4 Randomisation of specimens into study protocols

Following the preparation of atrial appendages obtained from patients undergoing either coronary artery bypass surgery or aortic valve surgery as described above, the resulting specimens were weighed and randomised into various study protocols. The specimens required to complete a study protocol were allocated numbers prior to starting a study. Numbers were drawn out randomly to determine the order of entry of specimens into a study protocol. The specimens from an atrial appendage were then entered into the study protocol according to that order. The number of atrial slices obtained from each appendage varied between 2-9 and therefore specimens from one appendage may be entered into different studies to avoid duplication within a particular study group.

Chapter 3

Alpha 1 adrenoceptors and simulated ischaemia/reoxygenation injury

3.1 INTRODUCTION

Activation of α_1-adrenoceptors induces positive inotropic, chronotropic and dromotropic actions in the heart and vascular effects[408,409,410,411] that are clinically exploited to optimize haemodynamic conditions. However, it is generally believed that activation of α_1-adrenoceptors is detrimental to the ischaemic heart, a thesis that would be supported by the increased release of cardiac and plasma catecholamines[412] and the enhanced density of cardiac α_1-adrenoceptors during ischaemia.[413,414,415] However, it has recently been shown that α_1-adrenoceptors mediate the protection induced by ischaemic preconditioning in animals and in the human myocardium[383,389,393,416,417,418] via phospholipase C and protein kinase C activation,[389] and this has given rise to the possibility that the effect of α_1-adrenoceptors in ischaemic injury may depend on the dose and the time of their activation (i.e., before, during or after ischaemia). To investigate this, right atrial appendages were obtained from patients undergoing elective coronary bypass surgery and the muscles subjected to various protocols in an *in vitro* model of simulated ischaemia and reoxygenation characterized in our laboratory[405] and described in Chapter 2.

3.2 METHODS

3.2.1 Experimental preparation

Experiments were performed on myocardium obtained from the right atrial appendage of patients undergoing elective coronary artery surgery or aortic valve replacement. Patients were excluded if they had enlarged atriums, atrial arrhythmias, poor left ventricular function (ejection fractions <30%), and right ventricular failure or were taking oral hypoglycaemic agents, opioid analgesia, K_{ATP} channel openers or

catecholamines. The right atrial specimens were prepared for study as described in Chapter 2.

3.2.2 Solutions and Drugs

The incubation medium was prepared daily with de-ionized distilled water as described in Chapter 2. The α_1-adrenoceptor agonist, phenylephrine and α_1-adrenoceptor antagonist, prazosin were used dissolved in de-ionized distilled water immediately before their use. All reagents were obtained from Sigma.

3.2.3 Experimental protocols

After sectioning the atrium, the preparations were allowed to stabilise for 30min and then randomly allocated to various protocols. In all the studies simulated ischaemia was induced for a period of 90min followed by 120min of reoxygenation. Some of the preparations were not made ischaemic and instead were aerobically perfused for 240min to serve as aerobic matched controls.

Study 1: Dose-response to phenylephrine and prazosin:

In this study, various concentrations of the α_1-adrenoceptor agonist phenylephrine (0.01, 0.1, 1, 10 and 100μM) and the α_1-adrenoceptor antagonist prazosin (0.1, 1, 10, and 100μM) were added to the incubation media for 10min prior to ischaemia, during ischaemia and during reoxygenation (n=6 preparations/group) as shown in Figure 3-I.

Study 2: Influence of the time of administration of phenylephrine and prazosin:

This was investigated by the exposure of the myocardial tissue to the optimal concentrations of phenylephrine (0.1μM) and prazosin (10μM) shown in study 1 for

10min prior to ischaemia, during ischaemia and during reoxygenation alone and in combination (n=6 preparations/group) as shown in Figure 3-II.

Study 3: Protective potency of phenylephrine and prazosin alone and in combination:
To study this, the most beneficial dose and time of administration for phenylephrine and prazosin seen in the two previous studies were used alone and in combination (n=6 preparations/group) as shown in Figure 3-III.

Study 4: Phenylephrine and ischaemic preconditioning:
To investigate the potency of protection of the α_1-agonist phenylephrine as compared to that of ischaemic preconditioning, right atrial specimens (n=6/group) were subjected to a protocol of ischaemia/reoxygenation identical to the one used in the previous studies. Phenylephrine at a concentration of 0.1μM was used prior to ischaemia for 10min or for 5min followed by 5min washout. Ischaemic preconditioning was induced by 5min ischaemia/5min reoxygenation immediately before the 90min ischaemia, a protocol shown to afford maximal protection in this preparation.[419] The protocol for this study is demonstrated in Figure3-IV.

3.2.4 Statistical analysis

All data are presented as mean±SEM. ANOVA was used for multiple comparisons with application of a post hoc Tukey's test. Statistical significance was taken as $p<0.05$.

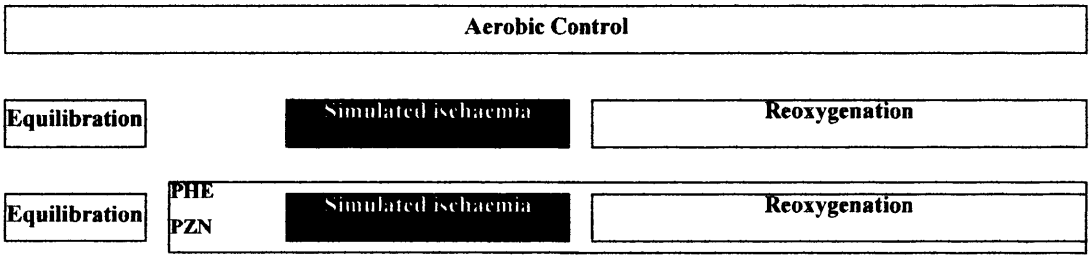

Figure 3-I: Schematic representation of the protocol for study 1. Phenylephrine (PHE) or Prazosin (PZN) were added for 10 minutes prior to simulated ischaemia/reoxygenation, during simulated ischaemia and reoxygenation at doses 0.01, 0.1, 1, 10 and 100μM.

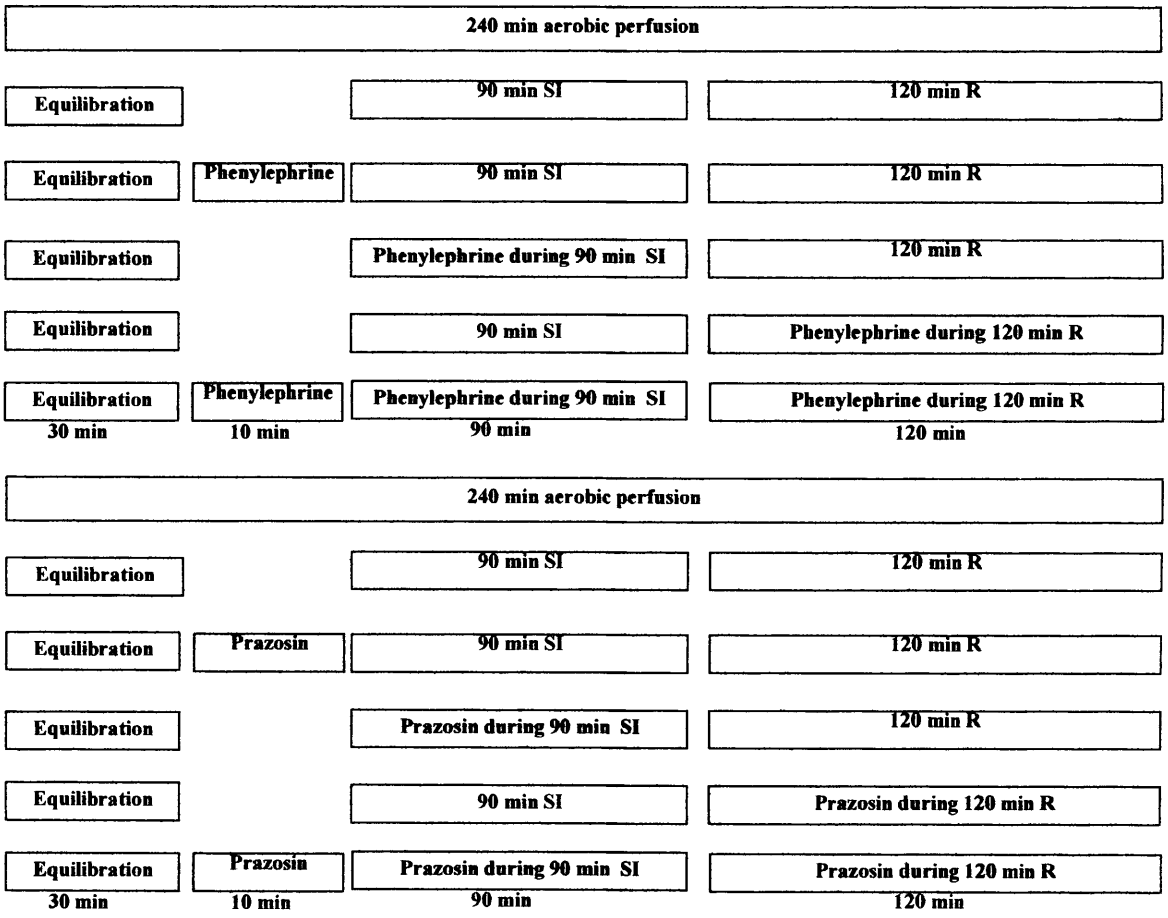

Figure 3-II: Schematic representation of protocol for Study 2 to study the influence of time of administration of phenylephrine and prazosin. SI: simulated ischaemia, R: reoxygenation.

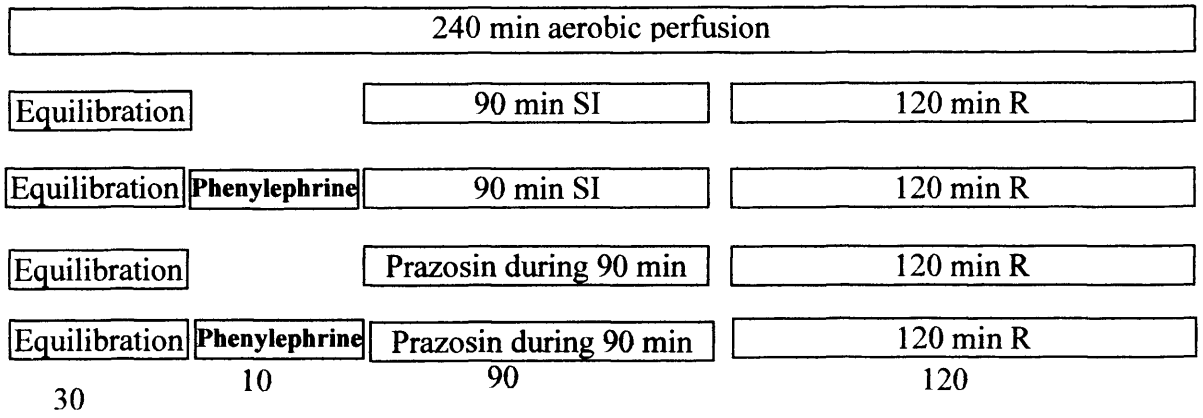

Figure 3-III: Schematic representation of protocol for Study 2 to study the influence of time of administration of phenylephrine and prazosin. SI: simulated ischaemia, R: reoxygenation.

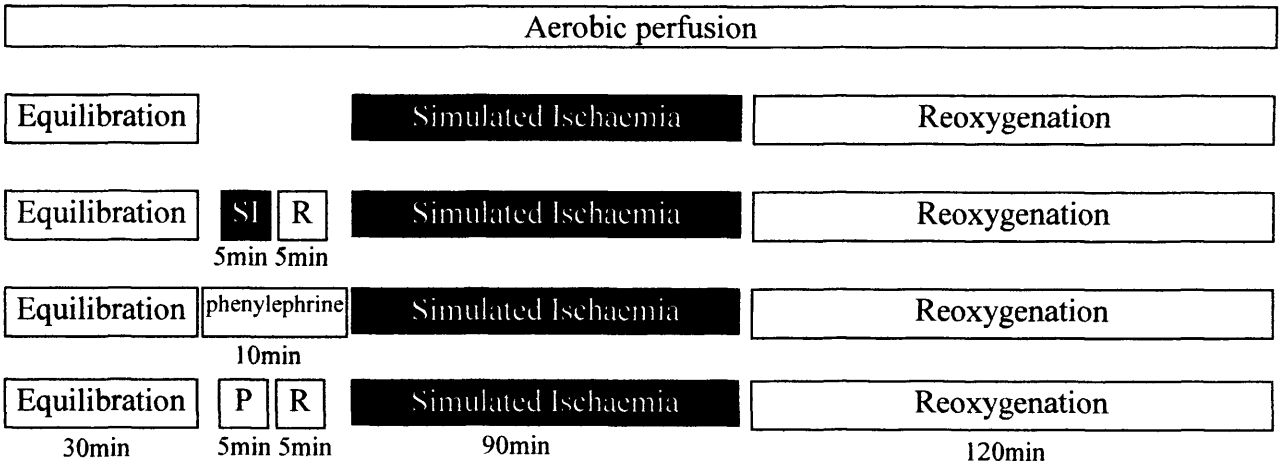

Figure 4-IV: Schematic representation of the protocol for Study 4 to investigate the potency of α_1 sdrenoceptor activation versus preconditioning. SI; simulated ischaemia, P: phenylephrine, R: reoxygenation..

3.3 RESULTS

Study 1: Dose-response to phenylephrine and prazosin:

The results shown in Figures 3-VA and 3-VB on CK leakage and MTT reduction demonstrate that phenylephrine induces a dose-response curve with maximal protection at 0.1 and 1μM and loss of protection at doses of 10μM and above. It is important to note that although protection was lost at the highest concentrations of phenylephrine they did not exert a detrimental effect, so that CK leakage and MTT mean values were similar to those seen in the phenylephrine-free group.

As seen in Figures 3-VIA and 3-VIB, CK leakage and MTT reduction were not significantly affected by prazosin when present in the incubation media throughout the experiment at the lowest concentrations of 0.1 and 1μM; however, in contrast with the results of phenylephrine, prazosin was detrimental at the highest concentrations (10 and 100μM) as shown by significant increase in CK leakage and the decrease in MTT reduction.

Study 2: Influence of the time of administration of phenylephrine and prazosin:

Figure 3-VIIA and 3-VIIB show the results of phenylephrine when given at different times at a chosen concentration of 0.1μM, shown to be the most effective dose in study 1. They demonstrate, by both CK leakage and MTT reduction, that maximal protection was obtained when phenylephrine was administered prior to ischaemia alone, an effect that was equivalent to the one obtained with phenylephrine throughout the entire experimental time. Phenylephrine when given during the reoxygenation period alone was less protective than when given prior to ischaemia and this was reflected by a modest but significant decrease in CK leakage without

affecting MTT reduction. Interestingly, the administration of phenylephrine during ischaemia alone was detrimental as seen by the significant decrease in MTT reduction. It is worth noting that the adverse effect of phenylephrine during ischaemia was completely counteracted when the drug was also present prior to ischaemia and during reoxygenation.

Figures 3-VIIIA and 3-VIIIB show the results on CK leakage and MTT reduction for prazosin, given at various times, at a chosen concentration of 10μM. These results show that prazosin did not have a significant effect when given prior to ischaemia alone, that it was detrimental when given during reoxygenation alone, to a degree similar to the administration of prazosin during the entire experimental time, and that it was protective when given during ischaemia alone. Importantly, the beneficial action of prazosin during ischaemia was abolished when the drug was present prior to ischaemia.

Study 3: Protective potency of phenylephrine and prazosin alone and in combination:
As indicated by the results on CK leakage and MTT reduction shown in Figures 3-IXA and 3-IXB, phenylephrine given prior to ischaemia alone and prazosin given during ischaemia alone afforded similar protection and the combination of the two did not result in greater protection than that obtained with each of them.

Study 4: Phenylephrine and ischaemic preconditioning:
Figures 3-XA and 3-XB show that ischaemic preconditioning induces a significant decrease in CK leakage and better preserves MTT reduction when compared to the muscles subjected to ischaemia/reoxygenation alone. The results confirm previous

findings from our laboratory using an identical preparation and protocol.[419] They also show that the α_1-agonist phenylephrine given for 10min prior to ischaemia or for 5min with 5min washout prior to ischaemia, thus mimicking the ischaemic preconditioning protocol, results in similar protection to that seen with ischaemic preconditioning.

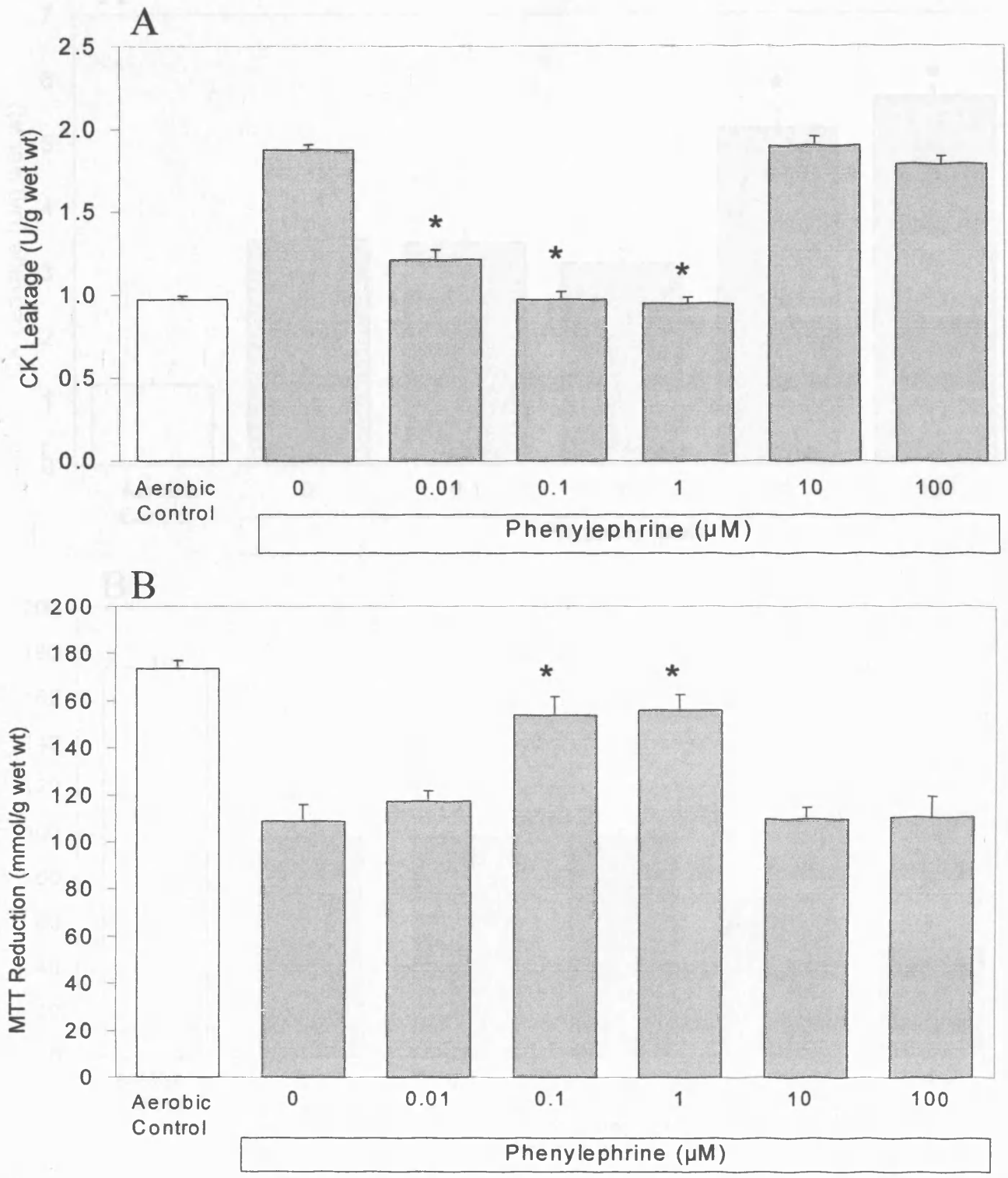

Figure 3-V: CK leakage (A) and MTT reduction (B) of human right atrial myocardium subjected to 90min ischaemia/120min reoxygenation and incubated with various concentrations of phenylephrine. The drug was present in the incubation media for 10min before the induction of ischaemia, during ischaemia and during reoxygenation. Columns represent the mean of 6 experiments and the bars represent the SEM. *P<0.05 vs. phenylephrine-free group.

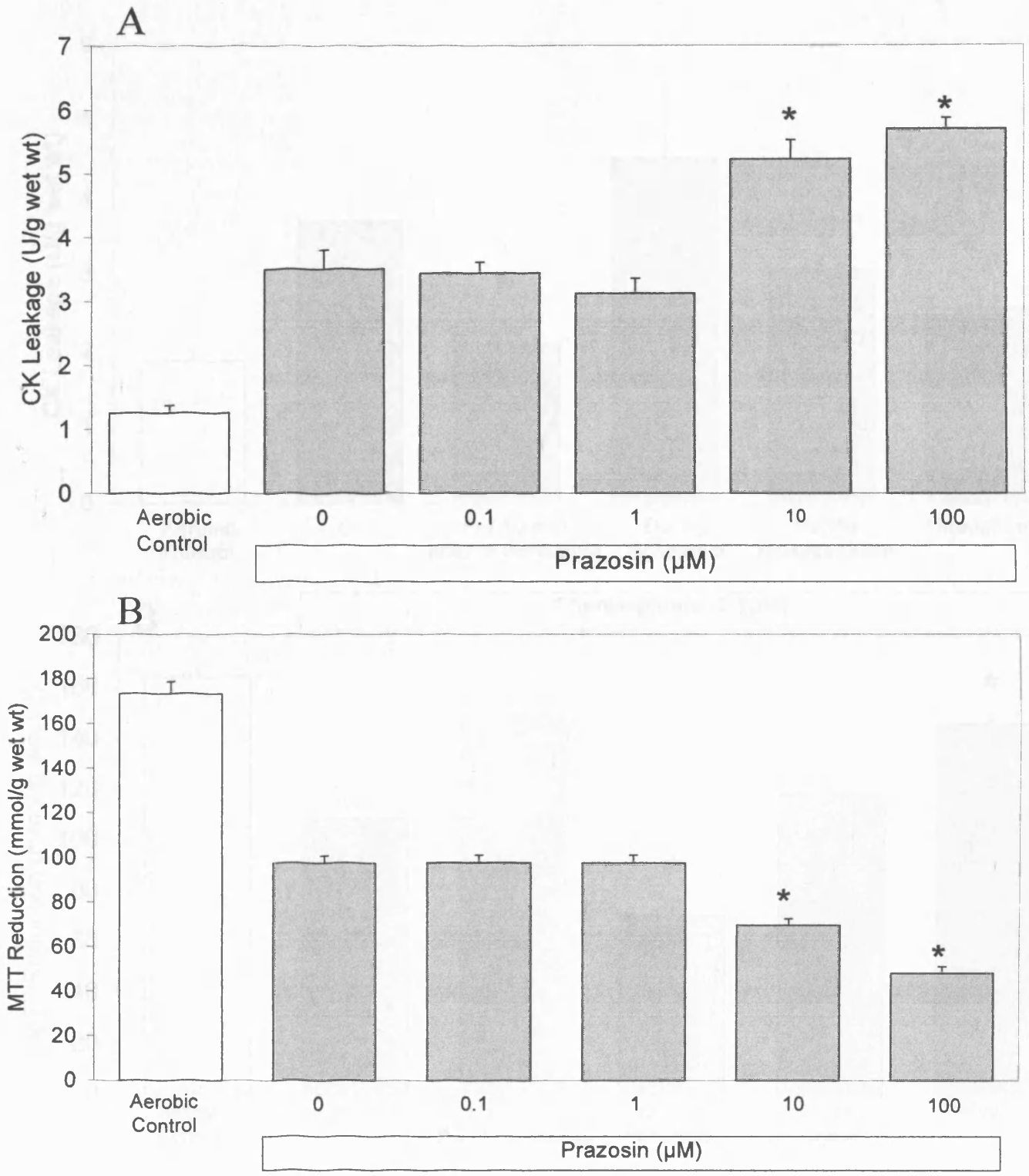

Figure 3-VI: CK leakage (A) and MTT reduction (B) of human right atrial myocardium subjected to 90min ischaemia/120min reoxygenation and incubated with various concentrations of prazosin. The drug was present in the incubation media for 10min before the induction of ischaemia, during ischaemia and during reoxygenation. Columns represent the mean of 6 experiments and the bars represent the SEM. *$p<0.05$ vs. prazosin-free group.

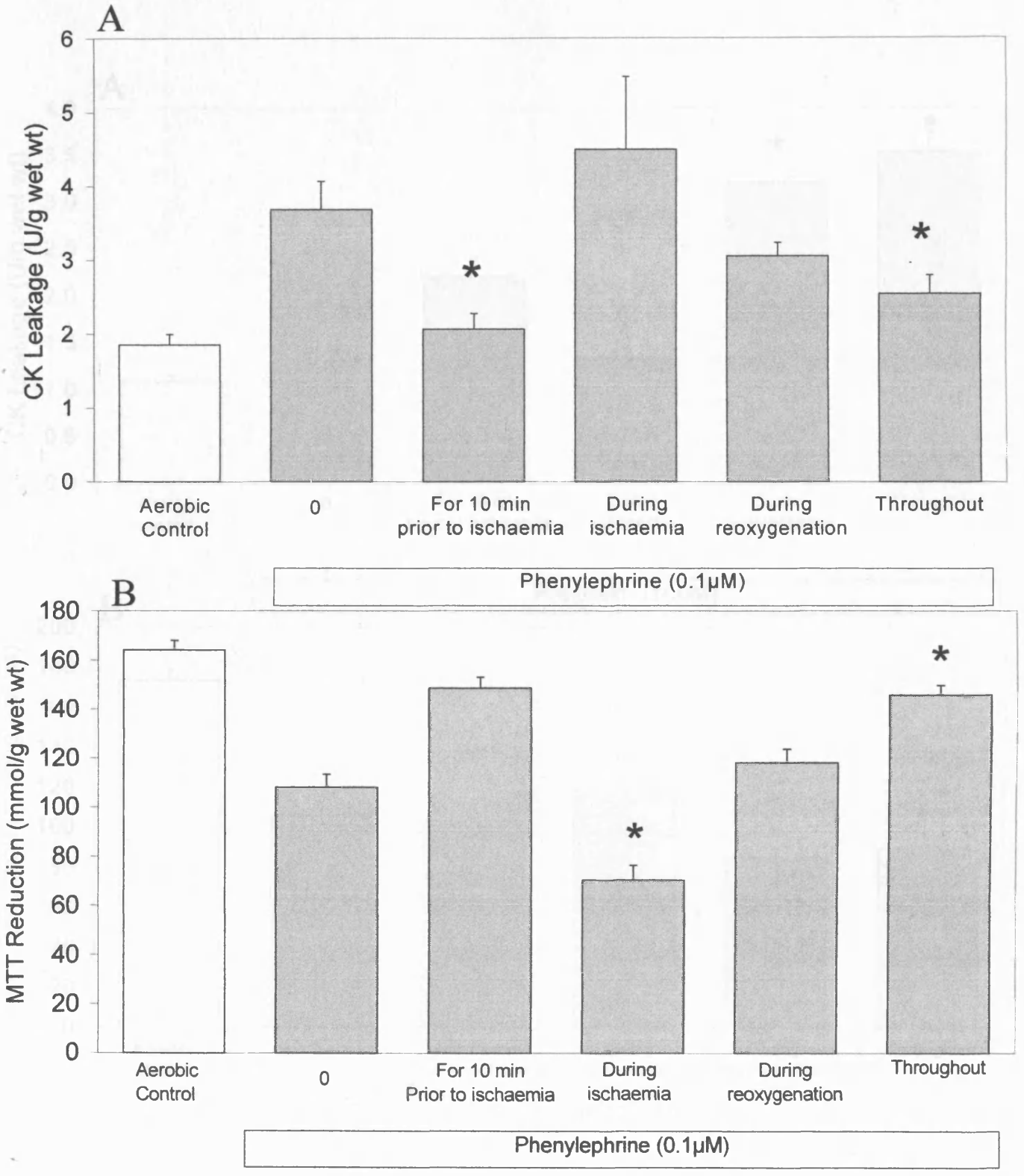

Figure 3-VII: CK leakage (A) and MTT reduction (B) of human right atrial myocardium subjected to 90min ischaemia/120min reoxygenation and incubated with 0.1μM phenylephrine for different times. Columns represent the mean of 6 experiments and the bars represent the SEM. *$p<0.05$ vs. phenylephrine-free group.

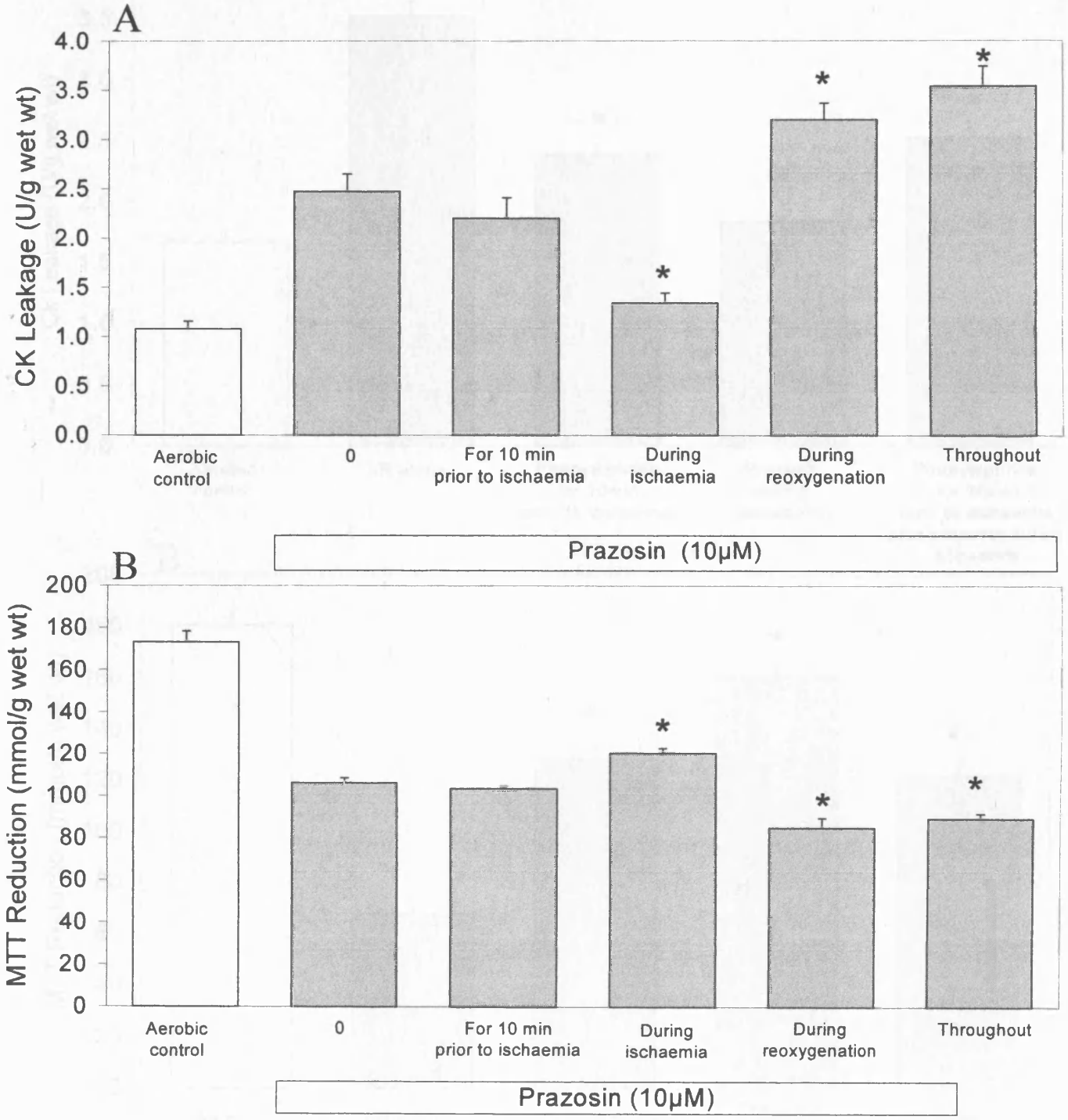

Figure 3-VIII: CK leakage (A) and MTT reduction (B) of human right atrial myocardium subjected to 90min ischaemia/120min reoxygenation and incubated with 10μM prazosin for different times. Columns represent the mean of 6 experiments and the bars represent the SEM. *$p<0.05$ vs. prazosin-free group.

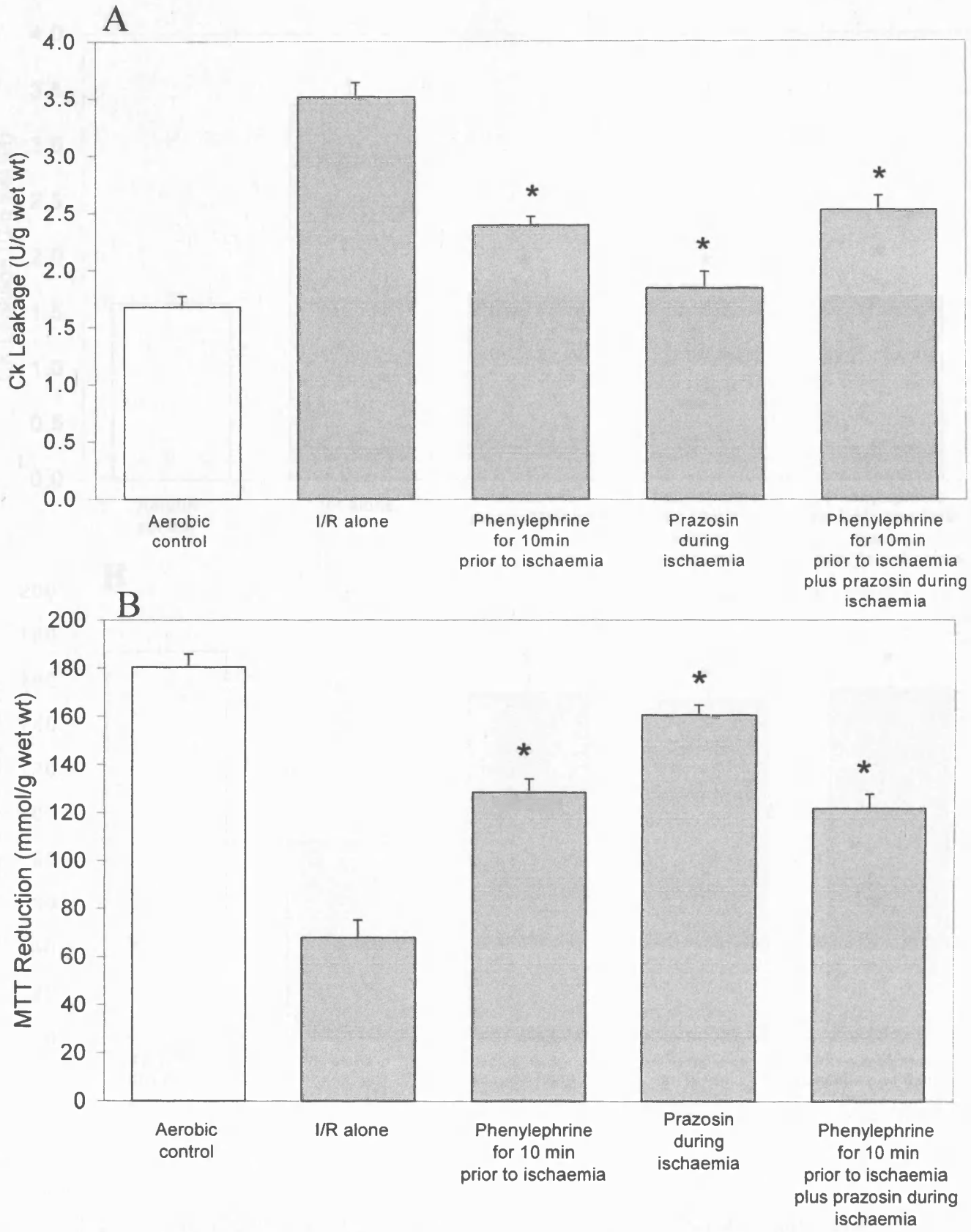

Figure 3-IX: CK leakage (A) and MTT reduction (B) of human right atrial myocardium subjected to 90min ischaemia/120min reoxygenation and incubated with phenylephrine (0.1μM) for 10min prior to ischaemia and with prazosin (10μM) during ischaemia alone and in combination. Columns represent the mean of 6 experiments and the bars represent the SEM. *$p<0.05$ vs. ischaemia/reoxygenation (I/R) alone group.

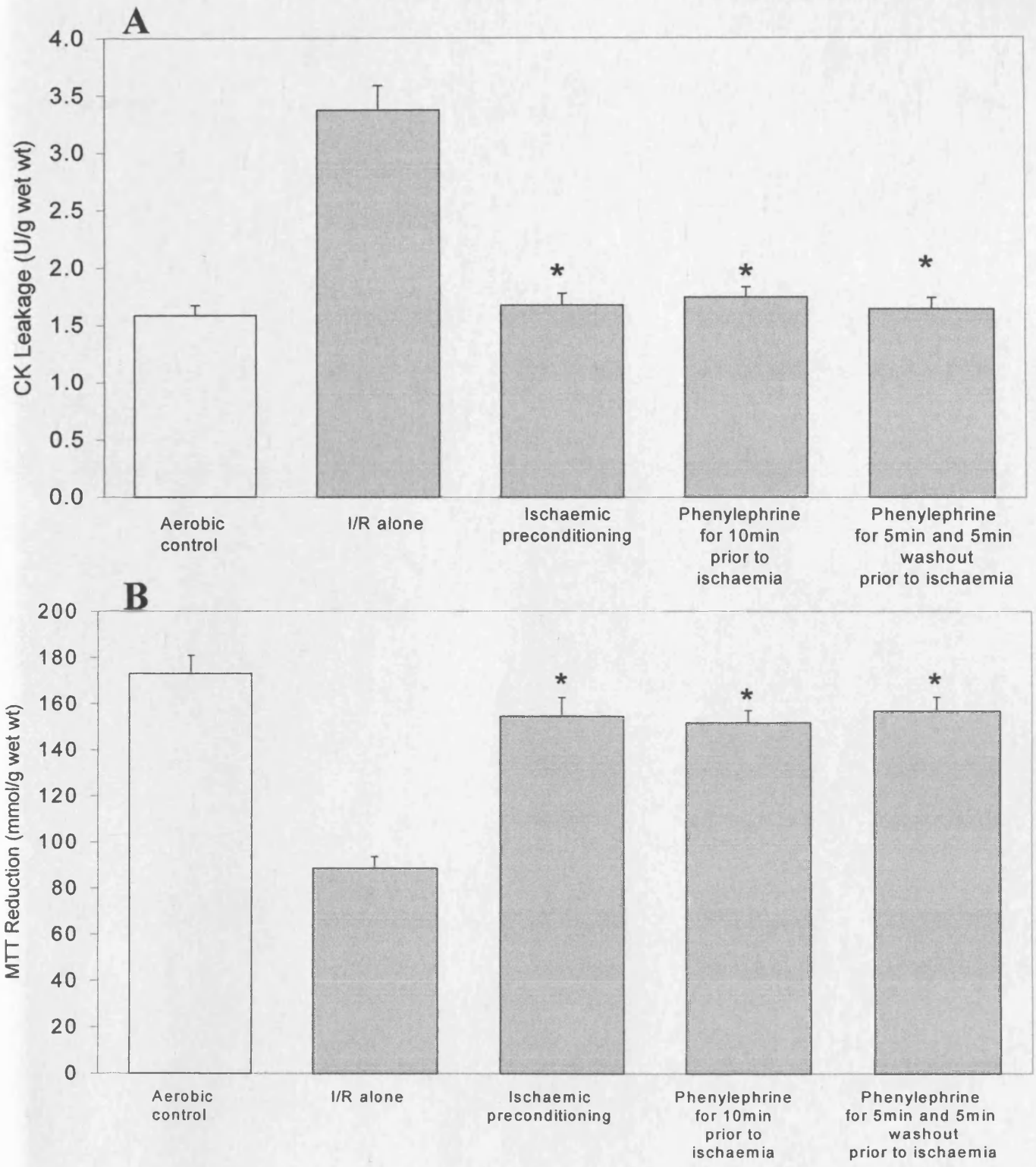

Figure 3-X: CK leakage (A) and MTT reduction (B) of human right atrial myocardium subjected to 90min ischaemia/120min reoxygenation following ischaemic preconditioning (5min ischaemia/5min reoxygenation) or incubation with phenylephrine (0.1μM) for 10min or for 5min then 5min washout. Columns represent the mean of 6 experiments and the bars represent the SEM. *$p<0.05$ vs. ischaemia/reoxygenation (I/R) alone group.

3.4 DISCUSSION

The present studies have demonstrated that α_1-adrenoceptors play an important role in the ischaemia/reoxygenation-induced injury of the human atrial myocardium. Thus, they show that stimulation of α_1-adrenoceptors with phenylephrine protects against injury whereas their blockade with prazosin is detrimental, both effects obtained in a dose-dependent manner. They have also shown that the effect of the stimulation or blockade of α_1-adrenoceptors depends on the time of administration so that α_1-adrenoceptors' stimulation is protective when given prior to ischaemia but detrimental when given during ischaemia, and on the contrary, α_1-adrenoceptors' blockade is beneficial during ischaemia, detrimental during reoxygenation and has no significant effect prior to ischaemia. It appears that similar maximal protection can be obtained with α_1-stimulation prior to ischaemia and with α_1-blockade during ischaemia although the combination of the two does not induce additional protection. Furthermore, the protective effect of α_1-stimulation prior to ischaemia is as potent as ischaemic preconditioning. These studies are the first in dissecting the role of α_1-adrenoceptors during ischaemia and reoxygenation of the human myocardium and the results have important mechanistic and clinical implications that warrant further discussion.

The results that α_1-adrenoceptor activation prior to ischaemia is cardioprotective is in agreement with the observation of other investigators that α_1-adrenoceptors participate in the protection induced by ischaemic preconditioning in the rat heart[383,420] and in the human myocardium.[389] However, it is worth noting that while α_1-adrenoceptor stimulation mimicked the protection of ischaemic preconditioning in our studies, protection was less and did not replicate the one obtained with ischaemic

preconditioning in the study of Cleveland et al[389] also using the human myocardium. Certainly, there are differences in the experimental model and the doses of the α_1-adrenoceptor agonist phenylephrine used in the two studies that may explain the differing results. Thus, our studies were carried out in a model of necrosis in which muscles were not electrically stimulated and subjected to 90min of simulated ischaemia whereas in the study of Cleveland et al[389] the muscles were stimulated and functional recovery, as opposed to necrosis, was assessed after only 45min of ischaemia. Furthermore, our dose-response study with phenylephrine showed that there is a bell-shaped response with maximal protection at concentrations of 0.1 and 1μM and loss of protection at concentrations ≥10μM, doses that were used in their study.[389] It is of interest that similar protection was obtained with phenylephrine administered for 10min immediately prior to ischaemia or for only 5min with 5min washout before ischaemia, thus suggesting that a short period of stimulation of α_1-adrenoceptors is sufficient to attain maximal benefit.

The harmful effect seen when α_1-adrenoceptors were activated only during ischaemia and the protection obtained with their blockade were not unexpected and are supported by the reported literature.[421,422] However, an important contribution of the present studies is that the activation of α_1-adrenoceptors during ischaemia does not diminish the protection induced by the activation of these receptors prior to ischaemia. It was also important that the protection seen with α_1-adrenoceptor blockade during ischaemia is lost when the blockade is continued during reoxygenation. These results contradict the never confirmed assumption that activation of α_1-adrenoceptors during reoxygenation may extend reperfusion injury.[423] In fact, they show that activation of α_1-adrenoceptors during reoxygenation does not

influence significantly myocardial injury and that, on the contrary, α_1-adrenoceptor blockade augments injury.

Although the signal transduction pathways that follow the activation of α_1-adrenoceptors are well described,[273,424,425,426,427,428,429,430,431,432,433,434,435] the precise mechanisms responsible for their opposing actions in ischaemia/reoxygenation remain unclear. It is possible that the increase in cytosolic calcium induced by α_1-adrenoceptor agonists via Camp[422] may mediate the effects discussed above. Indeed, calcium overload can be harmful when happening during ischaemia[436] and it may precondition the heart and be protective[437,438] when it occurs prior to ischaemia. The demonstration by Miyawaki and Ashraf[439] that a transient increase in cytosolic calcium during ischaemic preconditioning is an important trigger for the activation and translocation of the protein kinase C isoforms α and δ further support this hypothesis.

The binding of agonists to α_1-adrenoceptors also causes the activation of phospholipase C and this hydrolyzes phosphatidylinositol 4,5 biphosphate (PIP_2) resulting in the production of inositol~1,4,5-triphosphate (IP3) and 1,2 diacylyglycerol (DAG).[424,425] IP3 acts on the sarcoplasmic reticulum increasing intracellular Ca^{2+} [426,427] while DAG activates protein kinase C,[428] which in turn activates the trans-sarcolemmal voltage dependent Ca^{2+} channels,[429,430,431,432] the Na^+/H^+ channels[433] and the opening of mitochondrial K_{ATP} channels.[273] There is evidence that stimulation of α_1-adrenoceptors via activation of protein kinase C also enhances 5'-nucleotidase activity and hence adenosine formation[434] that has been shown to influence the outcome of myocardial ischaemia/reperfusion in a number of

experimental models and species.[435,440,441] To which extent each of these mechanisms are participating in the action of α_1-adrenoceptors during ischaemia and reoxygenation is unclear; however from our studies it is evident that the result of the use of agents that activate or blockade these receptors may vary widely depending on time of initiation and termination of their administration and it is possible that more than one mechanism may be involved. Clearly, more studies are needed to elucidate the underlying mechanism of these actions.

A possible limitation of the present studies is the use of atrial tissue as opposed to ventricular tissue so that any extrapolation should be made with caution and this is valid for all the experiments performed in this thesis. Another possible limitation might be that right atrial appendages were obtained from patients with antianginal medication and this potentially may have had some influence on the ischaemia/reoxygenation injury. Furthermore, my studies were performed in an *in vitro* preparation and therefore the results should be validated in *in vivo* studies before these interventions are considered for clinical application.

In spite of the above potential shortcomings, the findings of my studies may have important therapeutic implications for myocardial protection during cardiac surgery, cardiac transplantation, and angioplasty and in acute myocardial infarction where agents acting on α_1-adrenoceptors are frequently used. Particular attention must be paid during cardiac surgery where phenylephrine is used routinely to elevate the mean arterial blood pressure during cardiopulmonary bypass, and in doing so one may be unwittingly exacerbating myocardial injury during cardiac ischaemia when collateral blood flow enters the ischaemic myocardium. However, these studies have shown that

such undesirable effects can be fully counteracted by the administration of phenylephrine prior to the induction of cardiac ischaemia.

Having established the involvement of α_1-adrenoceptors in ischaemia/reperfusion injury and their involvement in preconditioning of the human myocardium, I turned my attention to unravel their precise role in preconditioning and their interaction with other triggers of preconditioning.

Chapter 4

Ischaemic and pharmacological preconditioning

4.1 INTRODUCTION

IP is a powerful protective endogenous adaptive response of the heart and other organs that has been described as the most potent intervention against a prolonged ischaemic insult.[263,419,442,443,444,445] However, the application of IP requires a physical cut of the blood supply and its use can be difficult or impractical in many clinical situations. In addition, the benefit of IP may be limited to the area of the heart supplied by the temporarily occluded artery, although some investigators have reported protection of the whole heart by preconditioning a selected myocardial area[446] and by distal preconditioning of other organs such as the kidneys.[447,448] A way to circumvent the above potential problems associated with the clinical application of IP may be preconditioning by pharmacological means or the manipulation of the signalling pathway involved in the protection. A number of membrane receptors have been advocated to be involved in the phenomenon of IP which includes α_1 [353,356,391] and β-adrenoceptors,[450] opioid[451] and adenosine A1 and A3 receptors.[440,452] Other factors such as heat shock proteins,[453,454] bradykinin,[455] calcium[437] and nitric oxide synthase activity[456,457]have also been shown to participate in the protection of ischaemic preconditioning, however, whether the various forms of pharmacological preconditioning share the same molecular mechanism with IP is not fully elucidated.

Following the results of the previous chapter, the aims of the series of studies in this chapter were to investigate the efficacy of pharmacological preconditioning of the human myocardium with α_1-adrenoceptor and adenosine receptor agonists as compared to IP.

4.2 METHODS

4.2.1 Experimental Preparation

Experiments were performed on muscle obtained from the right atrial appendage of patients undergoing elective coronary artery bypass graft surgery or aortic valve replacement in the cell necrosis model described in Chapter 2.[405] Identical exclusion criteria to those discussed in Chapter 3 were adopted.

4.2.2 Solutions and Chemicals

The incubation medium was prepared daily with de-ionized distilled water as described in Chapter 2. The drugs used in the experiments in this chapter included the α_1-adrenoceptor agonist phenylephrine, α_1-adrenoceptor antagonist prazosin, adenosine to activate the adenosine recptors and the adenosine receptor antagonist 8-p-sulphophenyltheophyline. All the drugs used were dissolved in de-ionized distilled water immediately before their use. All the chemicals were purchased from Sigma Chemicals.

4.2.3 Experimental Time Course

All the muscles (between three and five per specimen) were equilibrated at 37°C for a 30min period. Then some of the preparations were added to new flasks, which also contained 10ml of oxygenated medium, for another 210min (240min total), to serve as time-matched aerobic controls. The rest of the preparations were subjected to a 90min period of simulated ischaemia (SI) at 37°C. Following this the muscles were reoxygenated for another 120min by incubation in 10ml of oxygenated medium at 37°C with added glucose. At the end of the experimental protocols, samples from the incubation media used during the reoxygenation period were collected for the

assessment of CK leakage and the tissue was taken for the assessment of viability by reduction of MTT. All agents tested were added for 5 or 10min at the end of the equilibration period and before the induction of SI. The doses of the agent used in the present studies were selected following preliminary dose-response studies for each of the drugs.

4.2.4 Study groups

The studies were performed in different phases to investigate the efficacy of pharmacological preconditioning with α_1-adrenoceptors and adenosine receptors activation as compared to IP. In all the studies 6 preparations, each from the right atrium of equal numbers of patients, were used per group.

In Study 1, the efficacy of preconditioning via α_1-adrenoceptors was investigated using the following protocol as represented in Figure 4-I: (i) time-matched aerobic control, (ii) SI/R alone, (iii) IP induced with 5min SI/ 5min R before SI, (iv) phenylephrine (0.1μM) for 10min before SI, (v) phenylephrine (0.1μM) for 5min and 5min washout before SI and (vi) prazosin (10μM) for 10min prior to IP.

In Study 2, the efficacy of preconditioning via α_1-adrenoceptors versus adenosine receptors was investigated using the following protocol: (i) time-matched aerobic control, (ii) SI/R alone, (iii) IP induced with 5min SI/ 5min SI before SI, (iv) phenylephrine (0.1μM) for 5min and 5min washout before SI, (v) adenosine (100μM) for 5min and 5min washout before SI, (vi) 8-p-sulphophenyltheophyline (8-SPT, 100μM) for 10min before SI/R, (vii) prazosin (10μM) for 10min before adenosine (100μM) for 5min and 5min washout and (viii) 8-SPT (100μM) for 10min before

phenylephrine (0.1μM) for 5min and 5min washout. The protocol of this study is demonstrated in Figure 4-II.

In Study 3, the efficacy of preconditioning via α_1-adrenoceptors or adenosine receptors alone and in combination with IP was investigated using the following protocol as shown in Figure 4-III: (i) time-matched aerobic control, (ii) SI/R alone, (iii) IP induced with 5min of SI/ 5min R before SI, (iv) phenylephrine (0.1μM) for 5min and 5min washout before SI, (v) adenosine (100μM) for 5min and 5min washout before SI, (vi) phenylephrine (0.1μM) for 5min and 5min washout before IP, (vii) adenosine (100μM) for 5min and 5min washout before IP and (viii) adenosine (100μM) for 5min and 5min washout followed by phenylephrine (0.1μM) for 5min and 5min washout prior to SI.

4.2.5 Assessment of tissue injury and viability

Tissue injury was determined by measuring the leakage of CK into the incubation medium during the 120min reoxygenation period and tissue viability was assessed by the reduction of MTT to a blue formazan product at the end of the experimental time as described in Chapter 2.

4.2.6 Statistical analysis

All data are presented as mean±SEM. All values were compared by ANOVA with application of a post hoc Tukey's test. Statistical significance was taken as $p<0.05$.

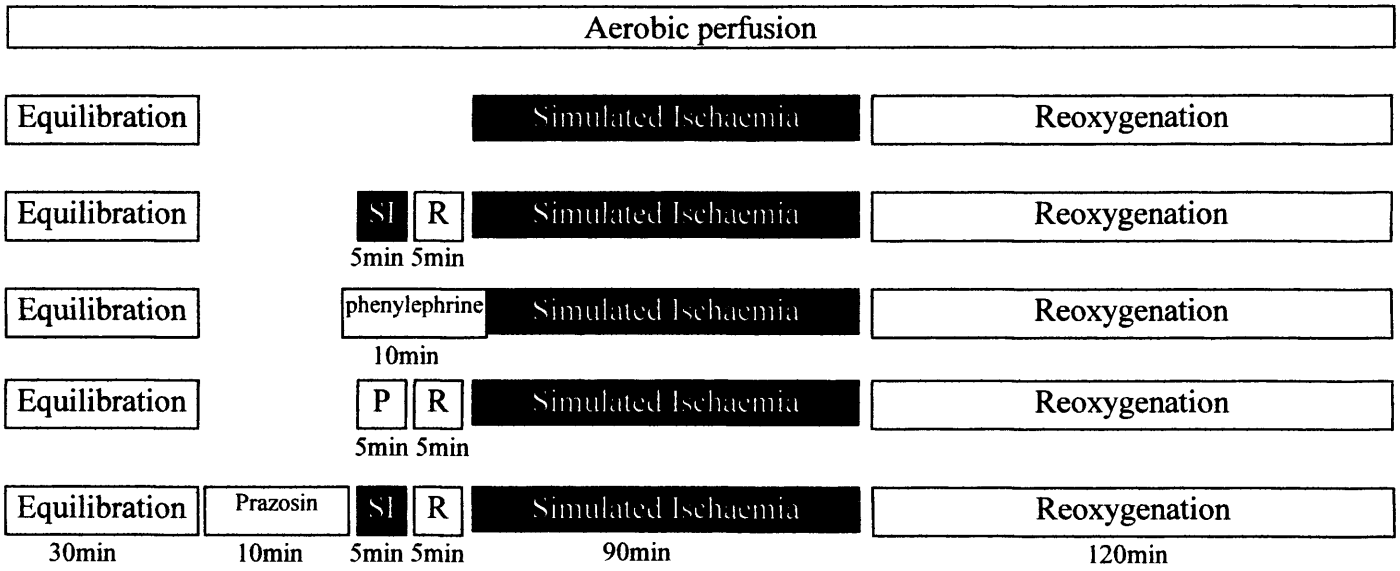

Figure 4-I: Schematic representation of the protocol for Study 1 to investigate the efficacy of preconditioning via α_1 sdrenoceptor activation. SI; simulated ischaemia, P: phenylephrine, R: reoxygenation..

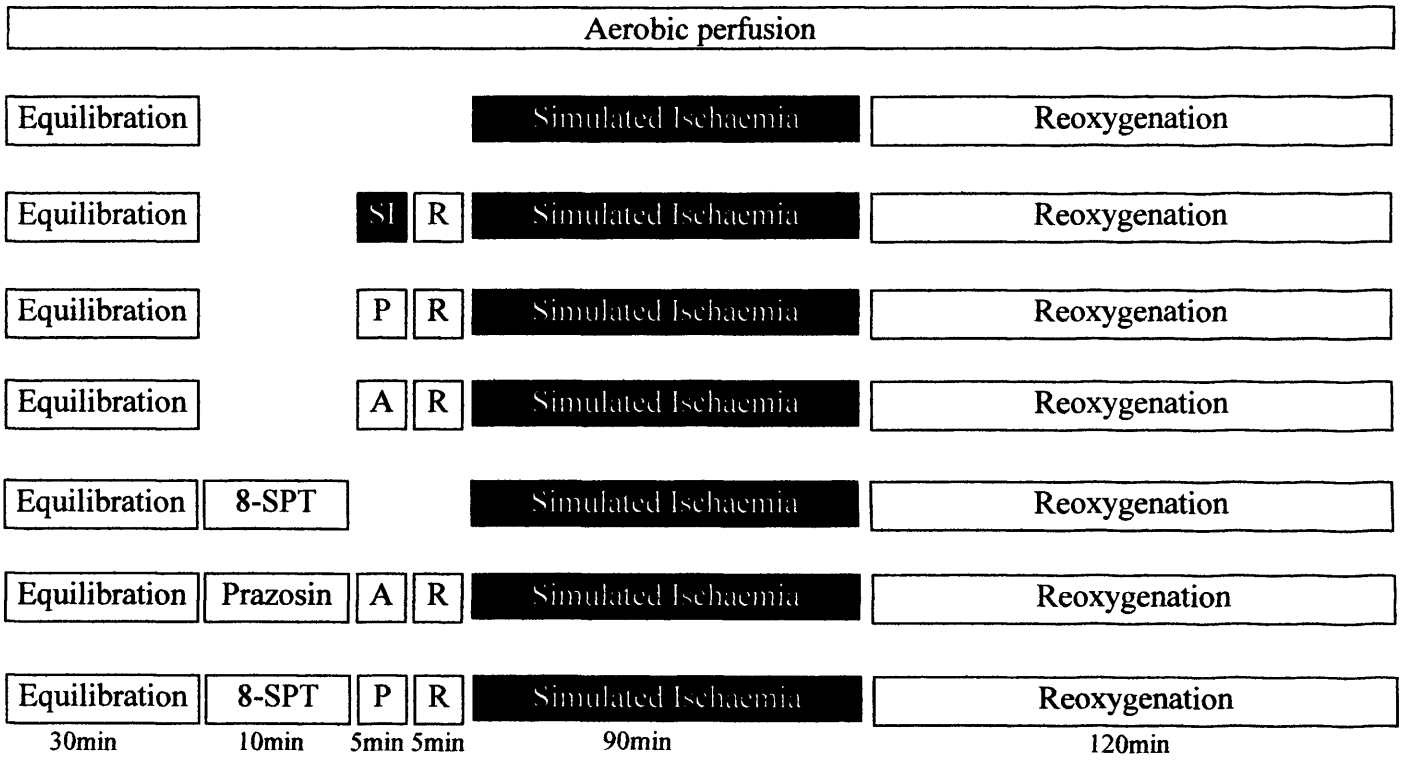

Figure 4-II: Schematic representation of the protocol for Study 2 to investigate the efficacy of of α_1-adrenoceptors versus adenosine receptors for preconditioning. activation. SI: simulated ischaemia, R: reoxygenation, P: phenylephrine, A: Adenosine, 8-SPT: 8-p-sulphophenyltheophyline.

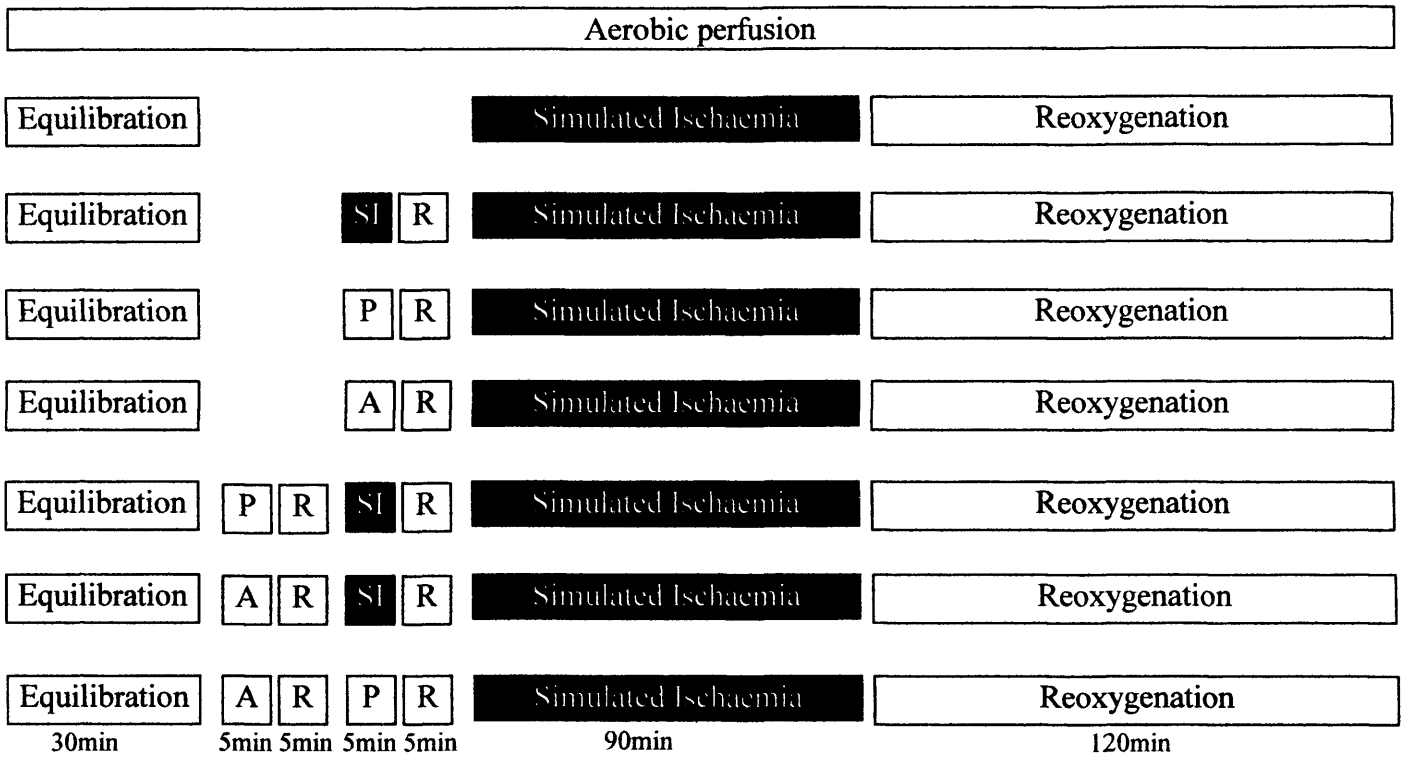

Figure 4-III: Schematic representation of the protocol for Study 3 to investigate the efficacy of preconditioning via α_1- adrenoceptors and adenosine receptors alone and in combination with ischaemic preconditioning. SI: simulated ischaemia, R: reoxygenation, P: phenylephrine, A: Adenosine.

4.3 RESULTS

All samples entering the studies completed the applied protocol and were included in the analysis.

Preconditioning via α_1-adrenoceptors (Study 1):

As shown in figures 4-IVA and 4-IVB, SI/R alone resulted in a significant increase in CK leakage and decrease in MTT reduction when compared to the aerobic controls. As expected, IP caused a significant protection with CK leakage and MTT reduction mean values similar to those seen in the aerobic controls. Almost identical CK leakage and MTT values to those seen with IP were obtained when the α_1-adrenoreceptor agonist phenylephrine was administered before ischaemia, which confirms the results in the previous chapter. It is worth noting that phenylephrine was equally effective when given for 10min or for only 5min followed by 5min washout prior to ischaemia (i.e., resembling the protocol of IP). Importantly, this study also demonstrates that the favourable effect of IP on CK leakage and MTT reduction can be completely abolished by the α_1-adrenoceptors antagonist prazosin. Overall, this study suggests that the cardioprotective effect of α_1-adrenoceptors activation is as potent as IP in the human myocardium and that in fact IP is mainly mediated via activation of α_1-adrenoceptors.

α_1-adrenoceptors versus adenosine receptors for preconditioning (Study 2):

The results shown in Figures 4-VA and 4-VB demonstrate that the administration of phenylephrine prior to ischaemia conferred a similar protection to that of adenosine and of IP. They also show that the protection of phenylephrine cannot be reversed by

the adenosine receptor antagonist 8-SPT and that the protective effect of adenosine cannot be reversed either by prior blockade of α_1-adrenoceptors with prazosin.

Preconditioning via α_1-adrenoceptors and adenosine receptors alone and in combination with IP (Study 3):

Figures 4-VIA and 4-VIB confirm the results of studies 1 and 2 in that phenylephrine or adenosine are as protective as IP. They also demonstrate that their use in combination does not result in additive protection.

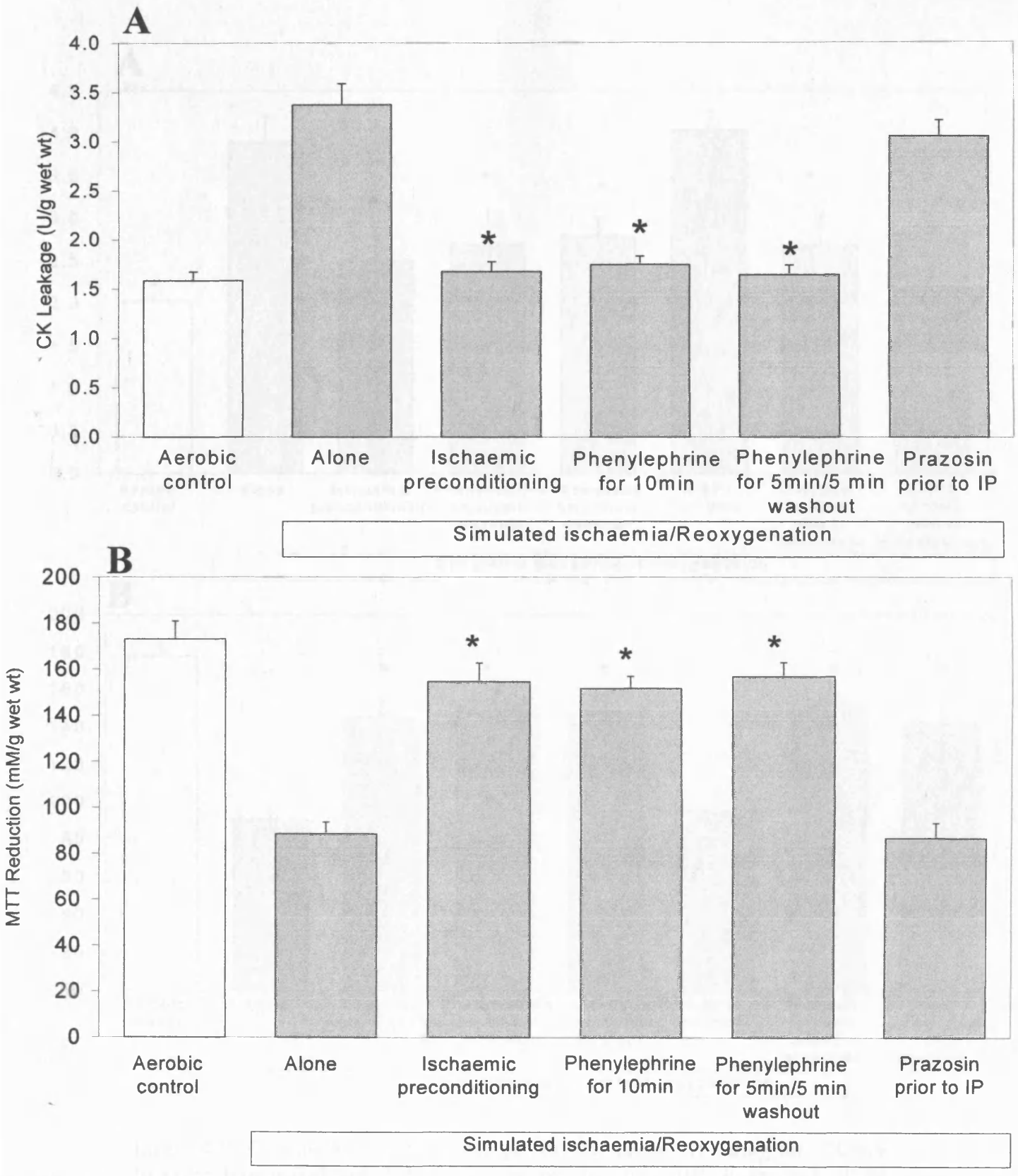

Figure 4-IV: Creatine Kinase (CK) leakage into the media (A) during the 120 min reoxygenation period and MTT reduction by the slices (B) at the end of the reoxygenation period in human atrial myocardium subjected to various protocols (see text for details) to investigate the efficacy of α_1-adrenoreceptors for preconditioning (Study 1). Data are expressed as mean ±SEM of six experiments. *$p<0.05$ vs SI/R alone group.

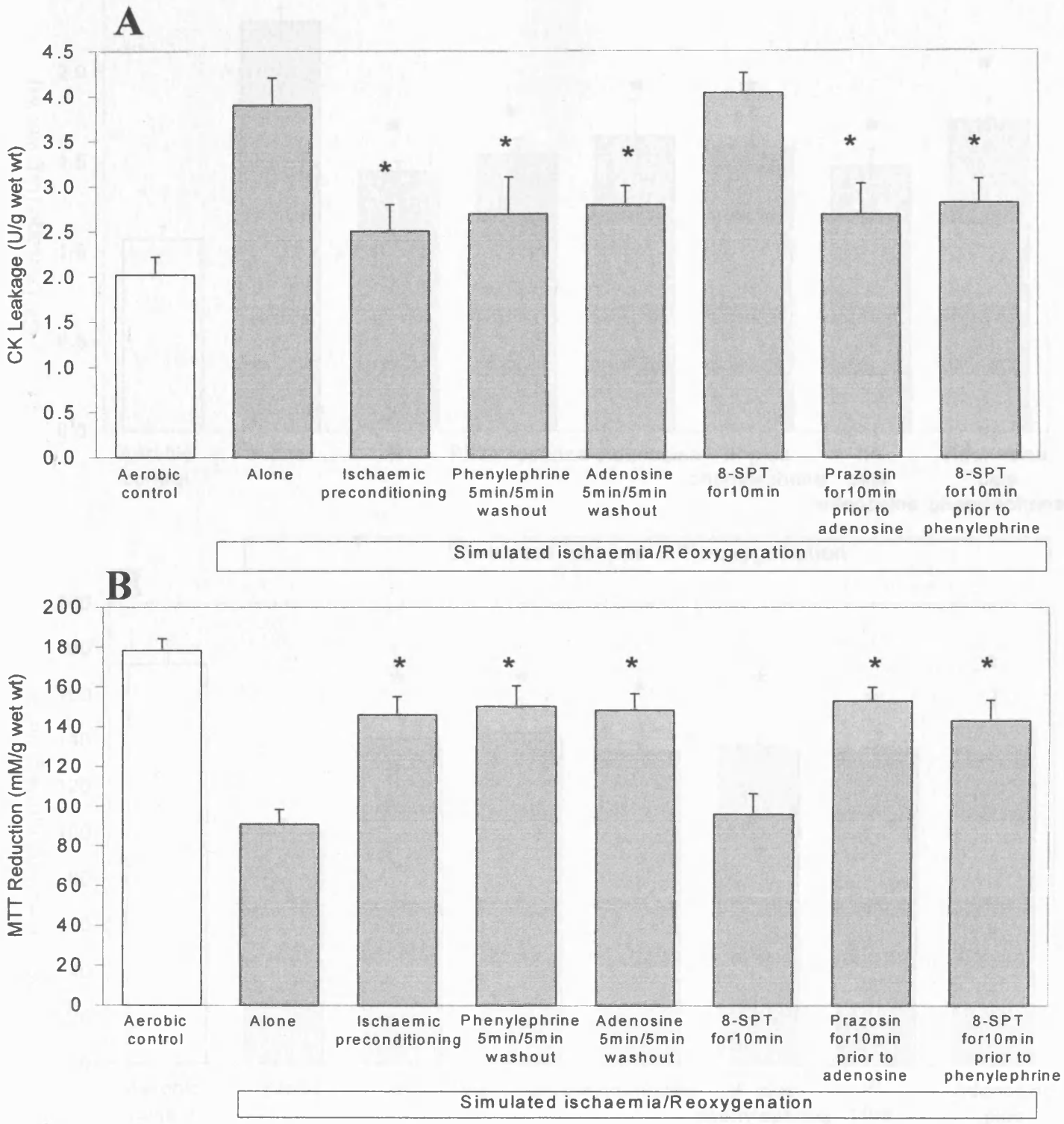

Figure 4-V: Creatine Kinase (CK) leakage into the media (A) during the 120 min reoxygenation period and MTT reduction by the slices (B) at the end of the reoxygenation period in human atrial myocardium subjected to various protocols (see text for details) to investigate the efficacy of α_1-adrenoreceptors versus adenosine receptors for preconditioning (Study 2). Data are expressed as mean ±SEM of six experiments. *$p<0.05$ vs SI/R alone group. (8-SPT: 8-p-sulphophenyltheophyline).

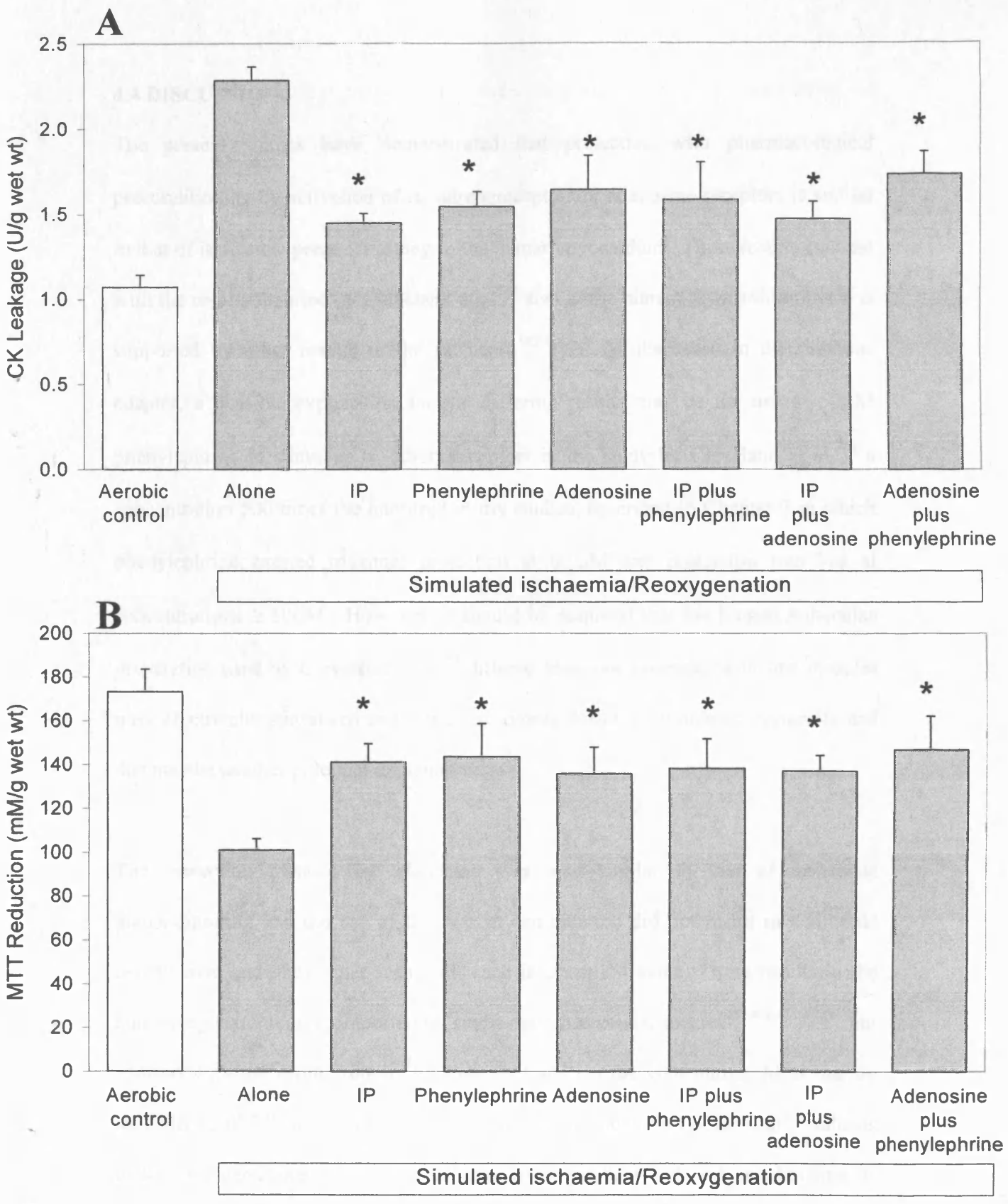

Figure 4-VI: Creatine Kinase (CK) leakage into the media (A) during the 120 min reoxygenation period and MTT reduction by the slices (B) at the end of the reoxygenation period in human atrial myocardium subjected to various protocols (see text for details) to investigate the efficacy of preconditioning via α_1-adrenoreceptors and adenosine receptors alone and in combination with IP (Study 3). Data are expressed as mean ±SEM of six experiments. *$p<0.05$ vs SI/R alone group.

4.4 DISCUSSION

The present studies have demonstrated that protection with pharmacological preconditioning by activation of α_1-adrenoreceptors or adenosine receptors is similar to that of ischaemic preconditioning in the human myocardium. These results contrast with the results reported by Cleveland et al[389] also in the human myocardium but it is supported by other results in the rat heart.[383,420,458] As discussed in the previous chapter, a possible explanation for the differing results may be the use of 50μM phenylephrine to stimulate α_1-adrenoreceptors in the study by Cleveland et al,[389] a concentration 500 times the one used in my studies, described in Chapter 3 in which phenylephrine exerted maximal protection at 0.1μM and protection was lost at concentrations ≥ 10μM. However, it should be admitted that the human trabeculae preparation used by Cleveland et al[389] differed from our preparation in that muscles were electrically stimulated and subjected to only 45min of simulated ischaemia and this may be another potential explanation.

The protection induced by adenosine was also similar to that of ischaemic preconditioning and the use of the two in combination did not result in additional benefit over and above that seen with each intervention alone. These results in the human myocardium are supported by studies in other animal species[459,460,461,462,463]but contrast with the results reports by Leesar et al[311] in the vivo human heart and by McCully et al[464,465]in the isolated rabbit heart. In the study by Leesar et al[311] patients undergoing percutaneous transluminal coronary angioplasty were subjected to three 2-minute balloon inflations 5 minutes apart. Under these conditions the administration of adenosine was more effective in limiting ST-segment shift than the balloon inflation protocol. Although we have previously shown[419] that 4 to 5 minutes of

ischaemia are sufficient to precondition the human myocardium, independent of whether this time is attained with one or two cycles of ischaemia, it is conceivable that in their study[311] the balloon inflation protocol may not have been sufficient to induce enough ischaemia to trigger preconditioning. This is a strong possibility under clinical conditions where collateral flow may lessen the severity of ischaemia. Furthermore, in that study[311] changes in ST-segment, that are modulated by sarcolemmal K_{ATP} channels,[466] were used as the main end-point and is now well recognized, as described below, that the protection of preconditioning may be mediated by mitochondrial rather than by sarcolemmal K_{ATP} channels.[467,468,469,470,471,472] The results reported by McCully et al[464,465] that adenosine is more potent than and extends the cardioprotection of ischaemic preconditioning in the rabbit heart are difficult to explain because they used a protocol of 5 minutes ischaemia/5 minutes reperfusion for preconditioning which is identical to the one used in our studies and shown to afford optimal protection in other preparations.[440,449] However, since adenosine and ischaemic preconditioning use an identical cellular signal transduction mechanism to induce protection, the most likely explanation may be the presence of some unknown factor that may have influenced the severity of the ischaemic preconditioning insult and therefore its protective efficacy.

It is worth noting that in our studies blockade of α_1-adrenoreceptors did not mitigate the protection induced by adenosine and that blockade of adenosine receptors did not prevent the protection induced by stimulation of α_1-adrenoreceptors. These results suggest that in the human myocardium the sarcolemmal receptors participating in preconditioning are independently connected to the downstream molecular transduction cascade. This concept is also supported by Tsuchida et al[473] that showed

that protection can be restored when adenosine receptor-blocking agents are co-infused with the α_1-adrenergic agonist phenylephrine. The finding that the α_1-adrenoreceptor antagonist prazosin blocked the protection of ischaemic preconditioning in the present studies and that adenosine receptor blockades can also block this protection in other models of the human myocardium[272,276]does not contradict the above thesis and it is compatible with the suggestion that a threshold must be reached before ischaemic preconditioning can protect the heart.[474]

On completion of these studies that defined the role of α_1-adrenoreceptors in preconditioning I wanted to investigate the underlying intracellular signal transduction mechanism of preconditioning and this is explored in the next chapter.

Chapter 5

Signal transduction mechanism of preconditioning

5.1 INTRODUCTION

Having investigated the efficacy of ischaemic and pharmacological preconditioning in the human myocardium, I planned in this chapter to elucidate the intracellular signalling pathways underlying the cardioprotection. The intracellular sequence of events that translate the binding of the various agonists to their membrane receptors into the protection of preconditioning remains under intense investigation as the knowledge of the signal transduction mechanism offers the best opportunity for manipulation and exploitation of this phenomenon. It has been reported that α_1-adrenoceptors are coupled with protein kinase C (PKC) through phospholipase activity,[475,476] that in turn activates p38 Mitogen Activated Protein Kinase (p38MAPK) in some cardiac preparations.[373,477,478,479] ATP sensitive potassium channels have also been implicated in the signal transduction mechanism of IP,[373,400,480] and recent evidence from several investigators[468,469,470]including ourselves[471] has shown that the mitochondrial and not the sarcolemmal K_{ATP} channels are involved. The order of involvement of the above mediators remains controversial although recently it has been suggested that mitochondrial K_{ATP} channels are the triggers in the signal transduction mechanism rather than the end effectors. [467]

The aim of these studies was to elucidate the contribution and sequence of activation of PKC, p38MAPK and mitoK_{ATP} channels.

5.2 METHODS

5.2.1 Experimental Preparation

Experiments were performed on muscle obtained from the right atrial appendage of patients undergoing elective coronary artery bypass graft surgery or aortic valve replacement in the cell necrosis model described in chapter 2.[405] An identical exclusion criteria to those discussed in Chapter 3 were applied in this chapter.

5.2.2 Solutions and Chemicals

The incubation medium was prepared daily with de-ionised distilled water as described in Chapter 2. The α_1-adrenoceptor agonist phenylephrine, mitoK_{ATP} channel blocker 5-hydroxydecanoate, PKC inhibitor chelerythrine and p38MAPK activator anisomycin were used dissolved in de-ionised distilled water, while mitoK_{ATP} opener diazoxide, PKC activator phorbol 12-myristate 13-acetate (PMA) and p38MAPK specific inhibitor SB203580 were dissolved in DMSO. All the chemicals were purchased from Sigma Chemicals.

5.2.3 Experimental Time Course

All the muscles were equilibrated at 37°C for a 30min period. Then some of the preparations were added to new flasks, which also contained 10ml of oxygenated medium, for another 210min (240min total), to serve as time-matched aerobic controls. The rest of the preparations were subjected to a 90min period of simulated ischaemia (SI) at 37°C as described in Chapter 2. Following this the muscles were reoxygenated (R) for another 120min by incubation in 10ml of oxygenated medium at 37°C with added glucose. At the end of the experimental protocols, samples from the incubation media used during the reoxygenation period were collected for the

assessment of CK leakage and the tissue was taken for the assessment of viability (reduction of MTT). All agents tested were added for 5 or 10min at the end of the equilibration period and before the induction of SI. The doses of the agent used in the present studies were selected following preliminary dose-response studies for each of the drugs.

5.2.4 Study groups

The studies were performed in different phases to investigate the signal transduction cascade underlying the protection of preconditioning. In all the studies 6 preparations, each from the right atrium of equal numbers of patients, were used per group.

In Study 1, the role of PKC on the cardioprotection of α_1-adrenoceptors activation was investigated using the following groups as shown in Figure 5-I: (i) time-matched aerobic control, (ii) SI/R alone, (iii) preconditioning with phenylephrine (0.1μM) before SI, (iv) phorbol 12 myristate 13-acetate (PMA, 1μM) alone for 10min prior to SI, (v) PMA (1μm) for 10min prior to phenylephrine, (vi) chelerythrine (10μM) alone for 10min prior to SI and (vii) chelerythrine (10μm) for 10min before phenylephrine.

In Study 2, the role of the p38MAPK pathway on the cardioprotection of α_1-adrenoceptors activation was examined using the following groups as shown in Figure 5-II: (i) time-matched aerobic control, (ii) SI/R alone, (iii) preconditioning with phenylephrine (0.1μM) before SI, (iv) anisomycin (1nM) for 10min prior to SI, (v) anisomycin (1nM) for 10min prior to phenylephrine, (vi) SB203580 (10μM) for 10min prior to SI and (vii) SB203580 (10μm) prior to phenylephrine preconditioning.

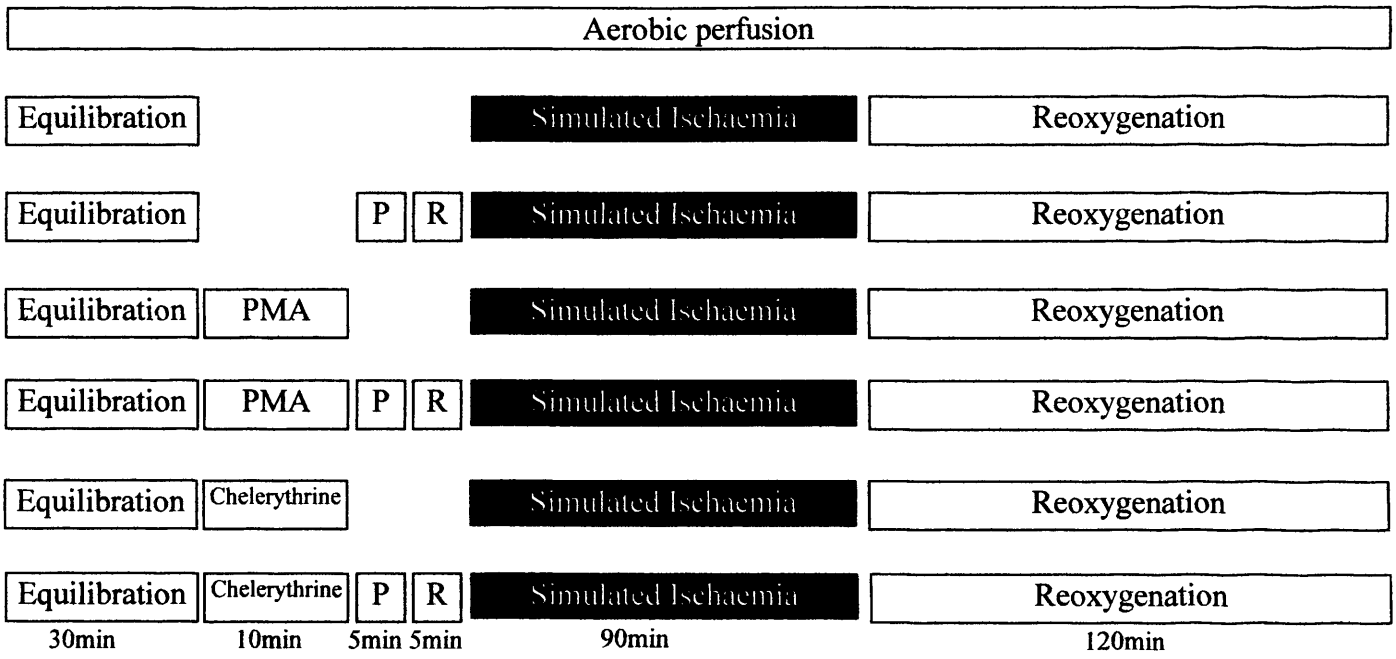

Figure 5-I: Schematic representation of the protocol for Study 1 to investigate the role of protein kinase C on the cardioprotection of α_1 receptor activation. P: phenylephrine, R: reoxygenation, PMA: phorbol 12-myristate 13-acetate.

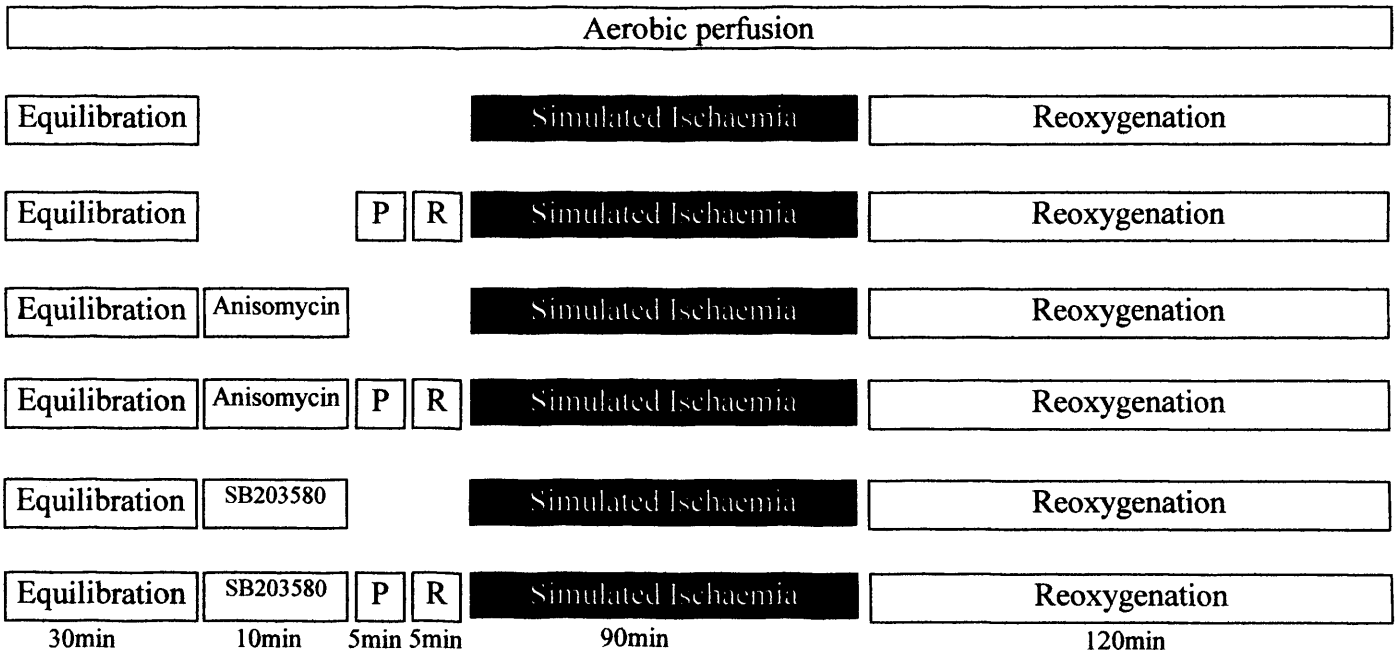

Figure 5-II: Schematic representation of the protocol for Study 2 to investigate the role of p38MAPK on the cardioprotection of α_1 receptor activation. P: phenylephrine, R: reoxygenation.

In Study 3, the role of mitoK$_{ATP}$ channels on the cardioprotection of α_1-adrenoreceptors activation was investigated. For this, the following groups were studied as shown in Figure 5-III: (i) time-matched aerobic control, (ii) SI/R alone, (iii) preconditioning with phenylephrine (0.1μM) before SI, (iv) diazoxide (100μM) alone for 10min prior to SI, (v) diazoxide (100μm) for 10min before phenylephrine, (vi) 5-hydroxydecanoate (1mM) alone for 10min prior to SI, and (vii) 5-hydroxydecanoate (1mM) for 10min prior to phenylephrine.

In Study 4, the role of PKC, p38MAPK and mitoK$_{ATP}$ channels in the cardioprotective effect of IP and pharmacological preconditioning with α_1-adrenoceptor and adenosine receptor activation was investigated using the following groups as described in Figure 5-IV: (i) time-matched aerobic control, (ii) SI/R alone, (iii) IP alone prior to SI, (iv) chelerythrine (10μM) for 10min prior to IP, (v) SB203580 (10μM) for 10min prior to IP, (vi) 5-hydroxydecanoate (1mM) for 10min prior to IP, (vii) phenylephrine (0.1μM) for 5min and 5min washout before SI, (viii) chelerythrine (10μM) for 10min prior to phenylephrine (0.1μM) for 5min and 5min washout, (ix) SB203580 (10μM) for 10min prior to phenylephrine (0.1μM) for 5min and 5min washout, (x) 5-hydroxydecanoate (1mM) for 10min prior to phenylephrine (0.1μM) for 5min and 5min washout, (xi) adenosine (100μM) for 5min and 5min washout before SI, (xii) chelerythrine (10μM) for 10min prior to adenosine (100μM) for 5min and 5min washout, (xiii) SB203580 (10μM) for 10min prior to adenosine (100μM) for 5min and 5min washout and (xiv) 5-hydroxydecanoate (1mM) for 10min prior to adenosine (100μM) for 5min and 5min washout.

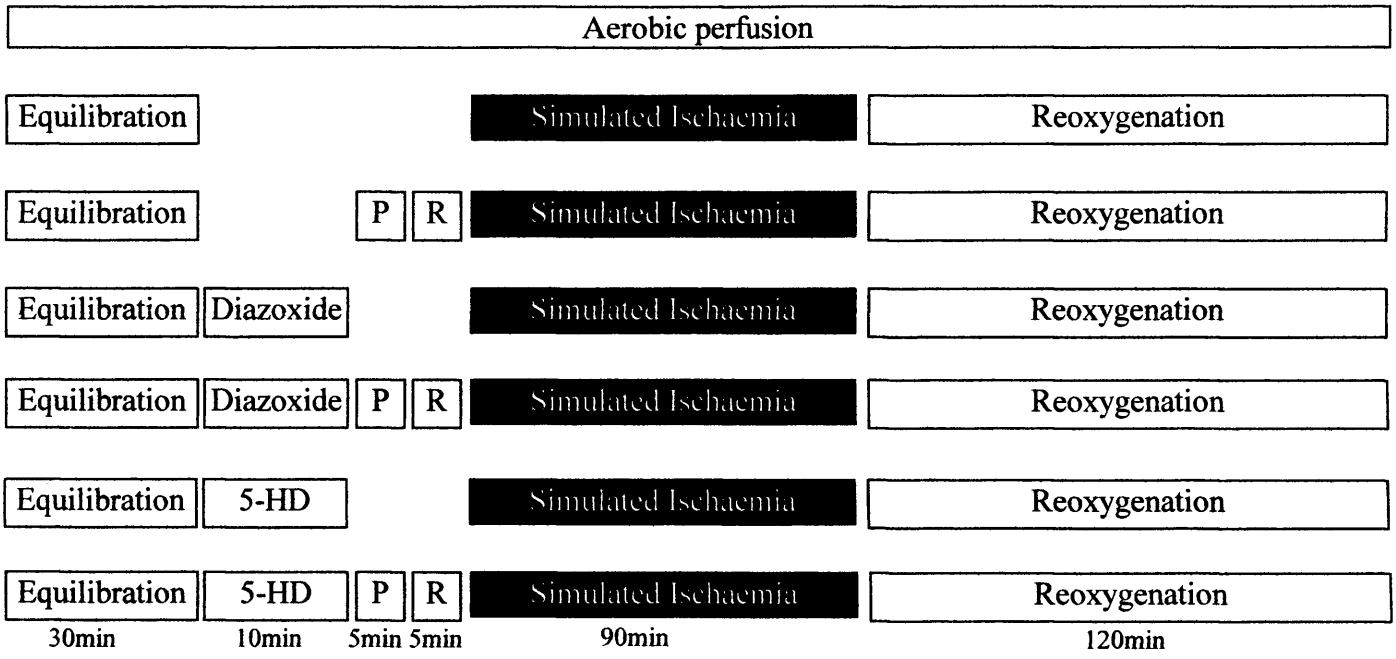

Figure 5-III: Schematic representation of the protocol for Study 3 to investigate the role of mitoK$_{ATP}$ channels on the cardioprotection of α_1 receptor activation. P: phenylephrine, R: reoxygenation, 5-HD: 5-Hydroxydecanoate.

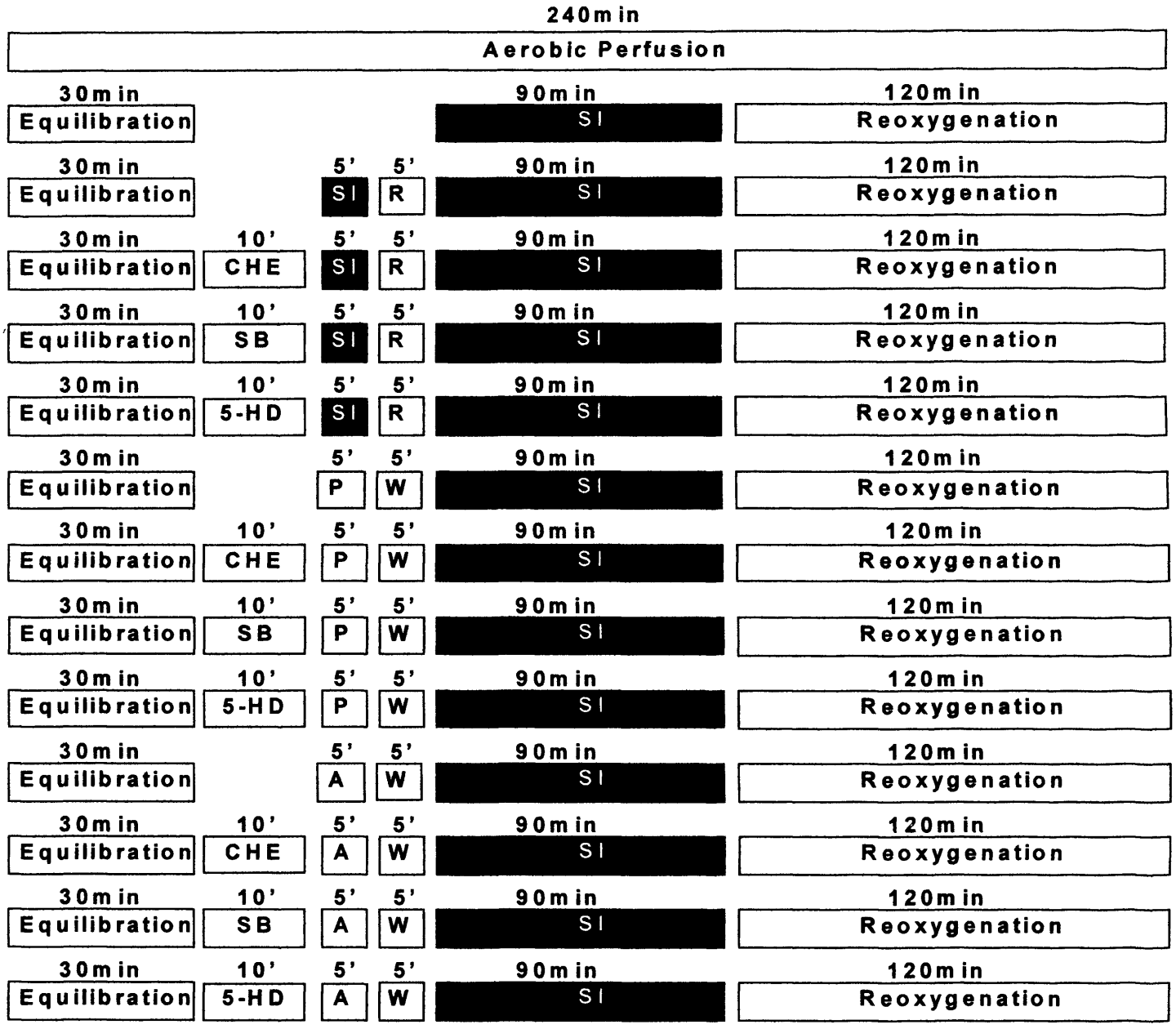

Figure 5-IV: Protocol for Study 4 to investigate the role of PKC, p38MAPK and mitoK$_{ATP}$ channels in the cardioprotective effect of IP and PP with phenylephrine (P) or adenosine (A) (n=6 specimens/group): (i) time-matched aerobic control, (ii) SI/R alone, (iii) IP alone prior to SI, (iv) chelerythrine (CHE) for 10min prior to IP, (v) SB203580 (SB) for 10min prior to IP, (vi) 5-hydroxydecanoate (5-HD) for 10min prior to IP, (vii) phenylephrine (P) for 5min and 5min washout (W) before SI, (viii) chelerythrine (CHE) for 10min prior to phenylephrine (P) for 5min and 5min washout (W), (ix) SB203580 (SB) for 10min prior to phenylephrine (P) for 5min and 5min washout (W), (x) 5-hydroxydecanoate (5-HD) for 10min prior to phenylephrine (P) for 5min and 5min washout (W), (xi) adenosine (A) for 5min and 5min washout (W) before SI, (xii) chelerythrine (CHE) for 10min prior to adenosine (A) for 5min and 5min washout (W), (xiii) SB203580 (SB) for 10min prior to adenosine (A) for 5min and 5min washout (W) and (xiv) 5-hydroxydecanoate (5-HD) for 10min prior to adenosine (A) for 5min and 5min washout (W).

Study 5 was designed to elucidate the sequence of the participation of involvement of mitoK_{ATP} channels, PKC and p38MAPK in the signal transduction cascade of cardioprotection. For this purpose, in addition to the aerobic time-matched control and SI/R alone the following groups were studied as described in Figure 5-II: (i) diazoxide (100μM) alone for 10min prior to SI, (ii) chelerythrine (10μM) for 20min with diazoxide (100μM) added for the last 10min prior to SI, (iii) SB203580 (10μM) for 20min with diazoxide (100μM) added for the last 10min prior to SI, (iv) PMA (1μM) alone for 10min prior to SI, (v) SB203580 (10μM) for 20min with PMA (1μM) added for the last 10min before SI, (vi) 5-hydroxydecanoate (1mM) for 20min with PMA (1μM) added in the last 10min before SI, (vii) anisomycin (1nM) alone for 10min prior to SI, (vii) chelerythrine (10μM) for 20min with anisomycin (1nM) added for the last 10min prior to SI and (viii) 5-hydroxydecanoate (1mM) for 20min with anisomycin (1nM) added for the last10min prior to SI.

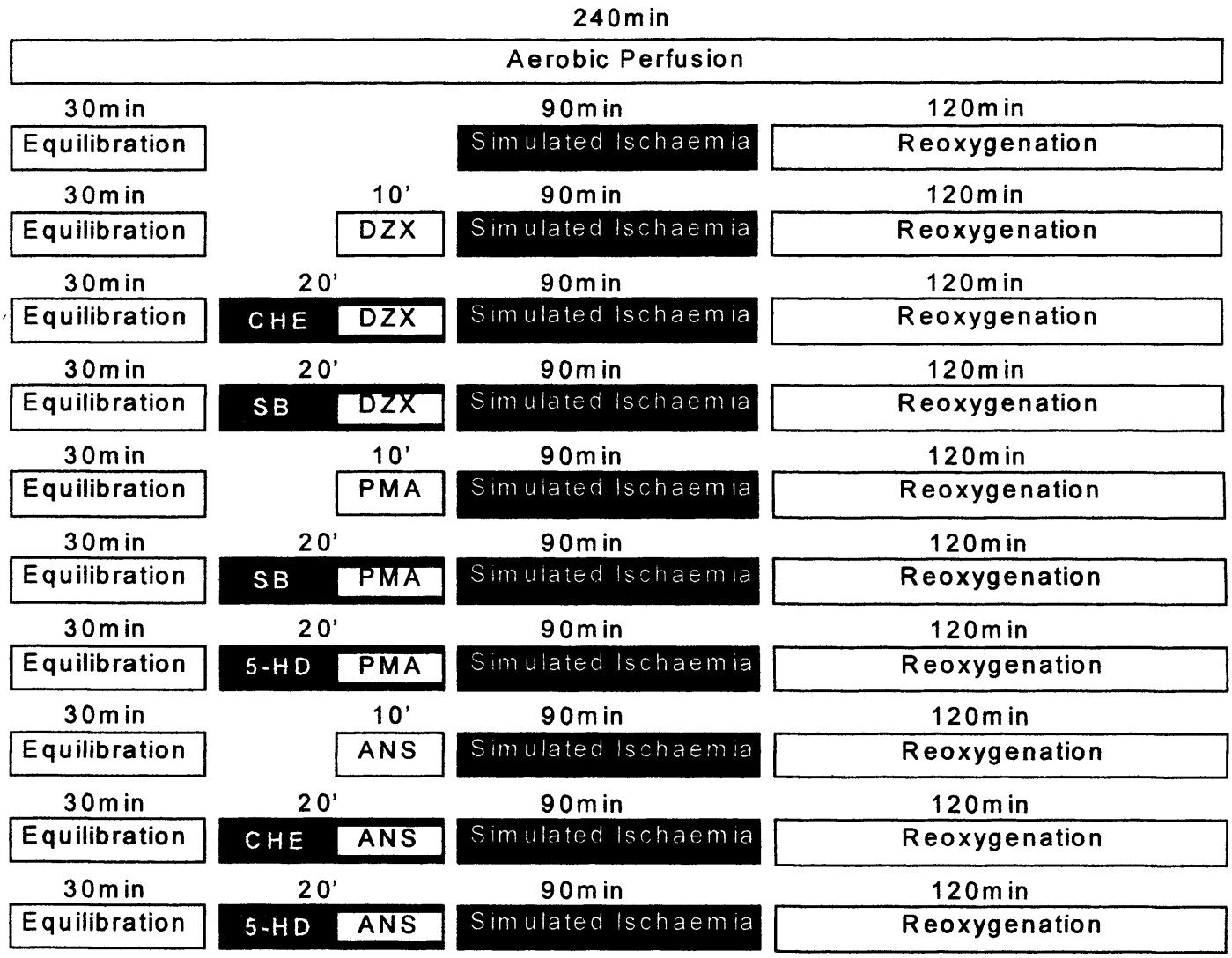

Figure 5-V: Study 5 protocol designed to elucidate the sequence of the participation of involvement of mitoK$_{ATP}$ channels, PKC and p38MAPK in the signal transduction cascade of cardioprotection. For this purpose, in addition to the (i) aerobic time-matched control and (ii) SI/R alone the following groups were studied (n=6 specimens/group): (iii) diazoxide alone for 10min prior to SI, (iv) chelerythrine (CHE) for 20min with diazoxide added for the last 10min prior to SI, (v) SB203580 (SB) for 20min with diazoxide added for the last 10min prior to SI, (vi) PMA alone for 10min prior to SI, (vii) SB203580 (SB) for 20min with PMA added for the last 10min before SI, (viii) 5-hydroxydecanoate (5-HD) for 20min with PMA added in the last 10min before SI, (ix) anisomycin (ANS) alone for 10min prior to SI, (x) chelerythrine (CHE) for 20min with anisomycin (ANS) added for the last 10min prior to SI and (xi) 5-hydroxydecanoate (5-HD) for 20min with anisomycin (ANS) added for the last 10min prior to SI.

5.2.5 Assessment of tissue injury and viability

Tissue injury was determined by measuring the leakage of CK into the incubation medium during the 120min reoxygenation period and tissue viability was assessed by the reduction of MTT to a blue formazan product at the end of the experimental time as described in Chapter 2.

5.2.6 Statistical analysis

All data are presented as mean±SEM. All values were compared by ANOVA with application of a post hoc Tukey's test. Statistical significance was taken as $p<0.05$.

5.3 RESULTS

All samples entering the studies completed the applied protocol and were included in the analysis.

Role of PKC on preconditioning via α_1-adrenoreceptor activation (Study 1):

Figures 5-VIA and 5-VIB show that the PKC activator PMA mimics the protective effect of phenylephrine and that the use of PMA and phenylephrine in combination does not afford additional protection to the one seen with each of these agents alone. In addition, the PKC inhibitor chelerythrine, which did not have a significant effect on CK leakage and MTT reduction as compared to the SI/R group when given alone, completely abolished the protection obtained with phenylephrine.

Role of p38MAPK on preconditioning via α_1-adrenoreceptor activation (Study 2):

The results of this study shown in Figures 5-VIIA and 5-VIIB reveal that the p38MAPK activator anisomycin when given alone reduces CK leakage and improves

MTT reduction to a degree similar to phenylephrine and that the use of anisomycin and phenylephrine in combination does not result in greater protection to that seen with each of these agents alone. They also show that the p38MAPK inhibitor SB203580 does not modify the extent of injury sustained during SI/R when given alone but that it abolishes the protection of phenylephrine.

Role of mitoK$_{ATP}$ channels on preconditioning via α_1-adrenoreceptor activation (Study 3):

The results of this study are shown in Figures 5-VIIIA and 5-VIIIB. They demonstrate that the protection of diazoxide on CK leakage and MTT reduction when given alone was almost identical to phenylephrine and that no additional protection was observed when diazoxide and phenylephrine were used in combination. The lack of a significant effect when 5-hydroxydecanoate was given alone and the abolition of the protection induced by phenylephrine when this and 5-hydroxydecanoate were given in combination further support the participation of mitoK$_{ATP}$ channels in the protection induced by α_1-adrenoceptor agonists.

Role of PKC, p38MAPK and mitoK$_{ATP}$ channels on IP and preconditioning via adenosine receptor activation (Study 4):

Figures 5-IXA and 5-IXB show that, as expected, IP was abolished by chelerythrine, SB203580 or 5-hydroxydecaonate. They also show that adenosine elicited similar protection to that of IP and that both are equally abolished by inhibition of PKC and p38MAPK and the blockade of the mitoK$_{ATP}$ channels.

Sequence of activation of the mediators of pharmacological and ischaemic preconditioning (Study 5):

The results on CK leakage and MTT reduction shown in Figures 5-XA and 5-XB demonstrate that, as expected, identical protection is obtained with diazoxide (mitoK_{ATP} channel opener), PMA (PKC activator) and anisomycin (p38 MAPK activator). Importantly, they also show that whilst the protection of diazoxide is abolished by the PKC antagonist chelerythrine and the p38MAPK antagonist SB203580, the protective effect of PMA is abolished by SB203580 but not by the mitoK_{ATP} channel blocker 5-hydroxydecanoate, and the protective action of anisomycin is unaffected by chelerythrine and 5-hydroxydecanoate.

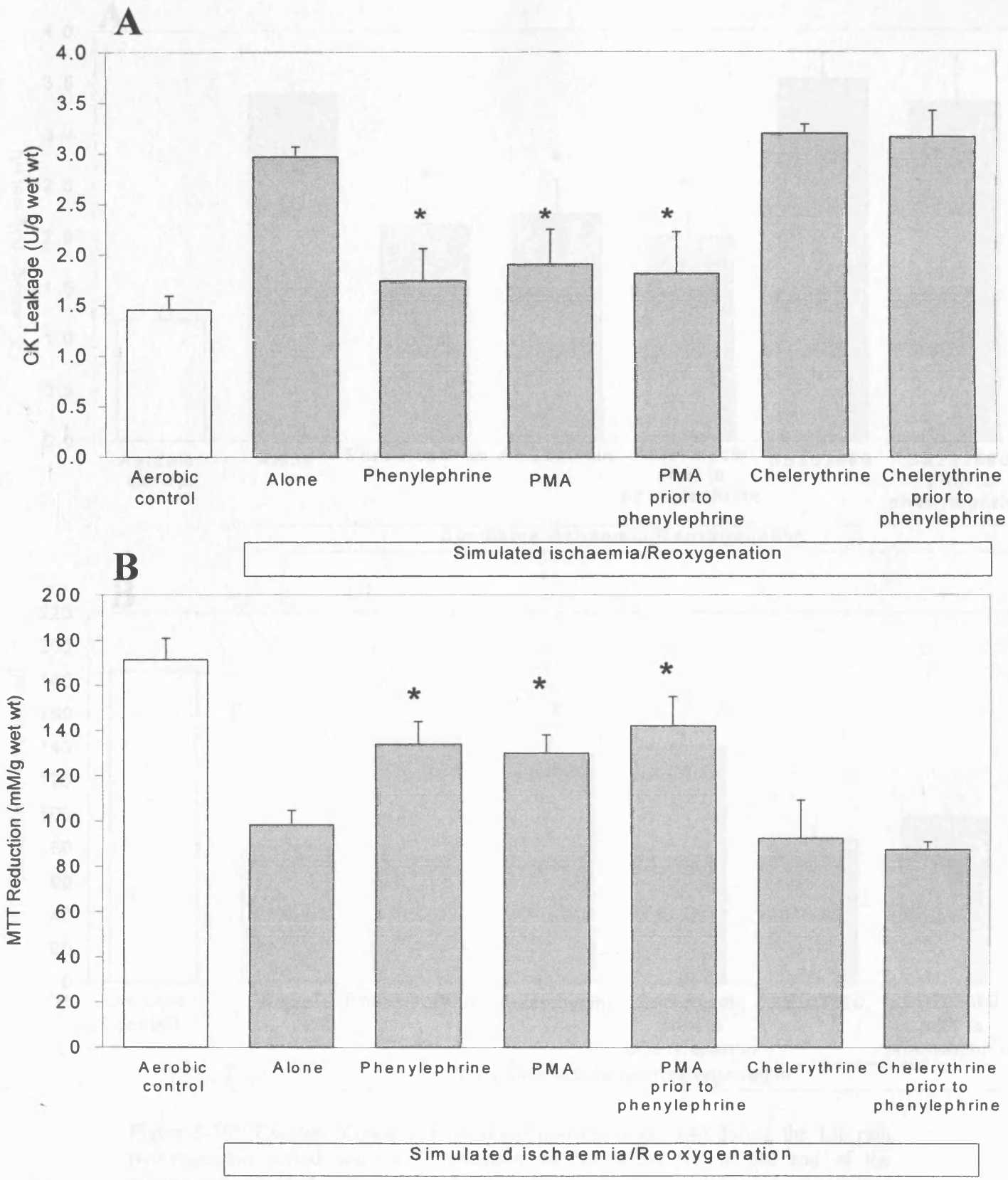

Figure 5-VI: Creatine Kinase (CK) leakage into the media (A) during the 120 min reoxygenation period and MTT reduction by the slices (B) at the end of the reoxygenation period in human atrial myocardium subjected to various protocols (see text for details) to investigate the role of PKC on preconditioning via α_1-adrenoreceptor activation (Study 1). Data are expressed as mean ±SEM of six experiments. *$p<0.05$ vs SI/R alone group. (PMA: phorbol 12 myristate 13-acetate).

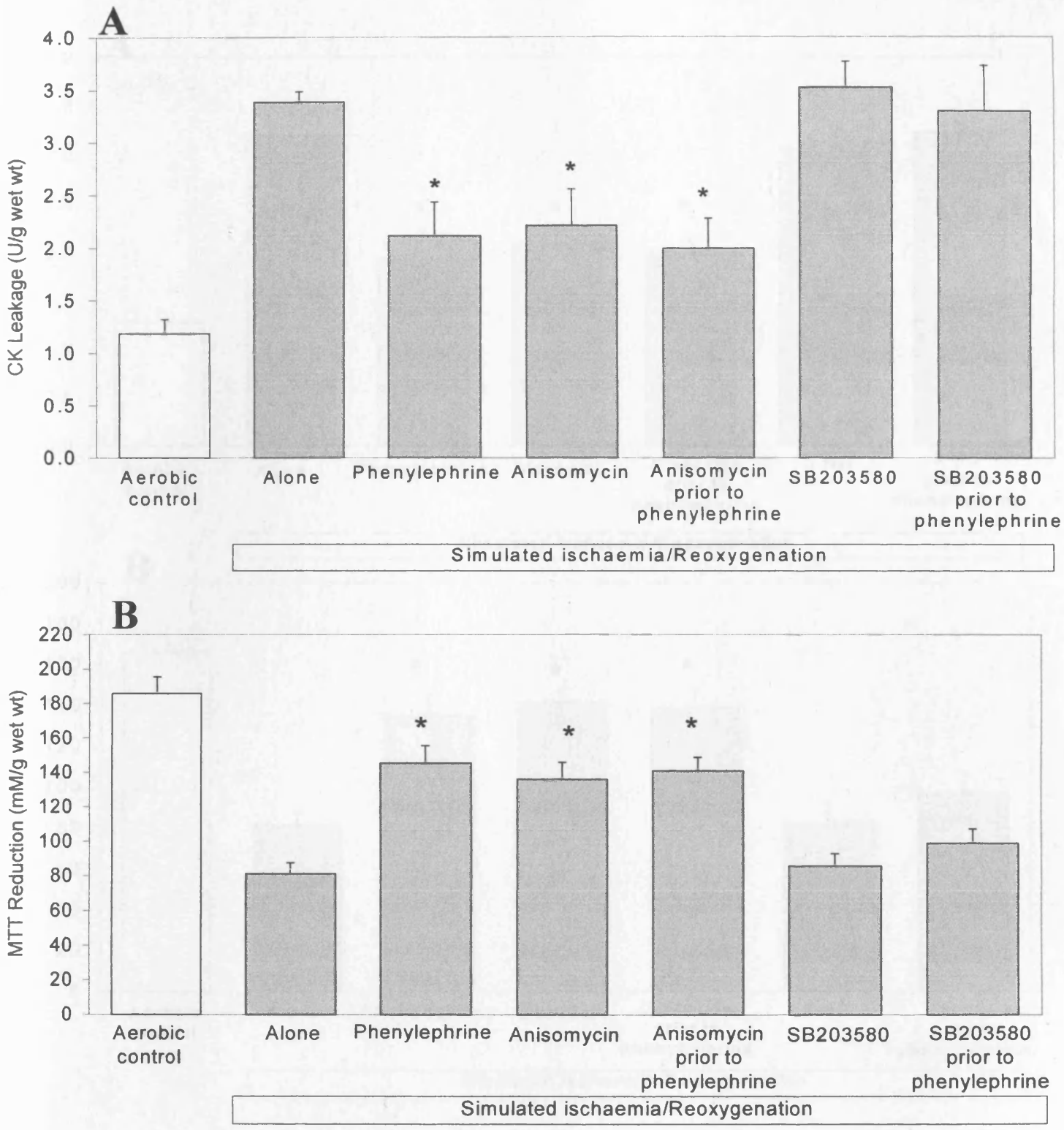

Figure 5-VII: Creatine Kinase (CK) leakage into the media (A) during the 120 min reoxygenation period and MTT reduction by the slices (B) at the end of the reoxygenation period in human atrial myocardium subjected to various protocols (see text for details) to investigate the role of p38MAPK on preconditioning via α_1-adrenoreceptor activation (Study 2). Data are expressed as mean ±SEM of six experiments. *$p<0.05$ vs SI/R alone group.

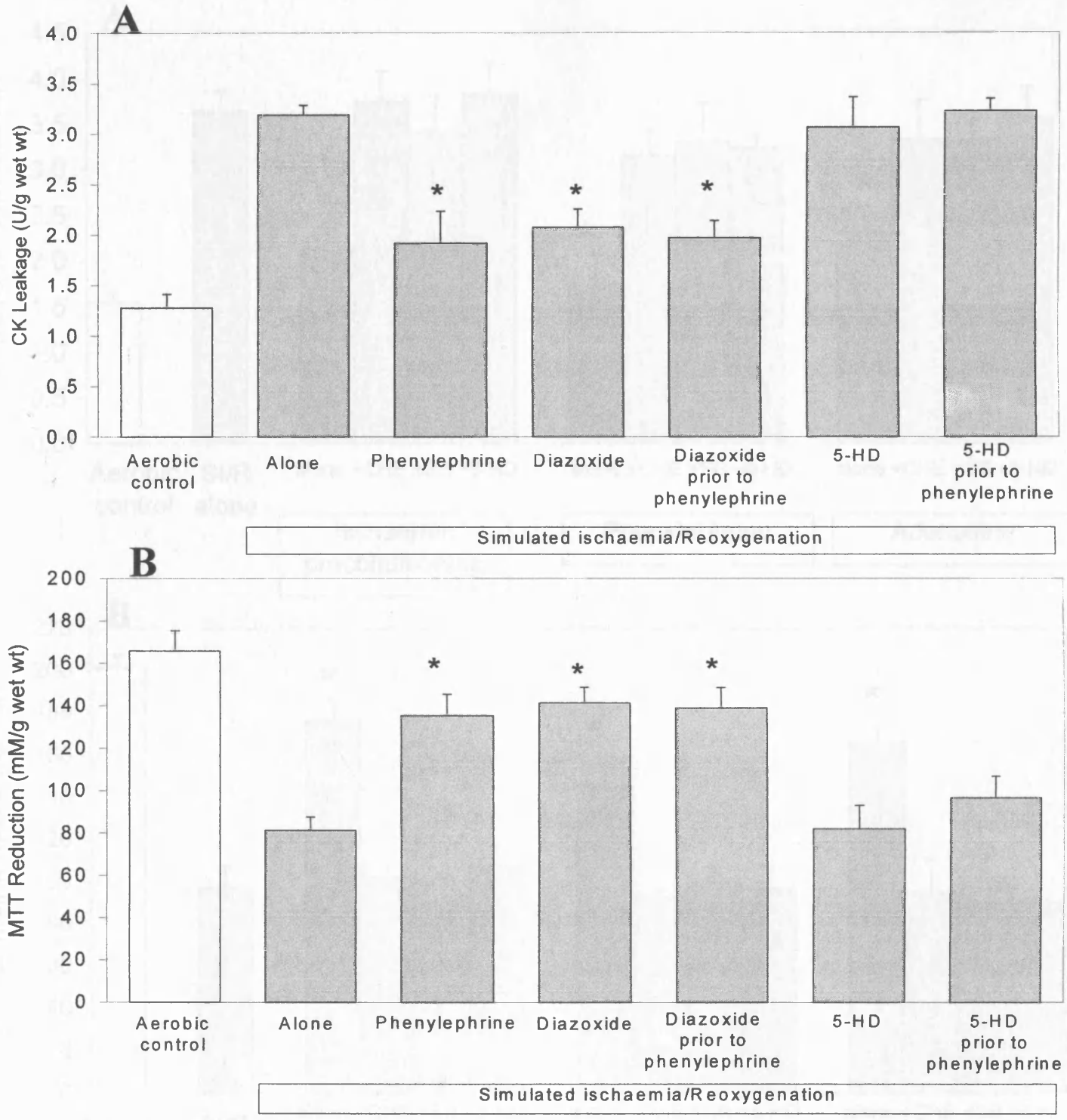

Figure 5-VIII: Creatine Kinase (CK) leakage into the media (A) during the 120 min reoxygenation period and MTT reduction by the slices (B) at the end of the reoxygenation period in human atrial myocardium subjected to various protocols (see text for details) to investigate the role of mitoK$_{ATP}$ channels on preconditioning via α_1-adrenoreceptor activation (Study 3). Data are expressed as mean ±SEM of six experiments. *p<0.05 vs SI/R alone group. (5-HD: 5-hydroxydecanoate).

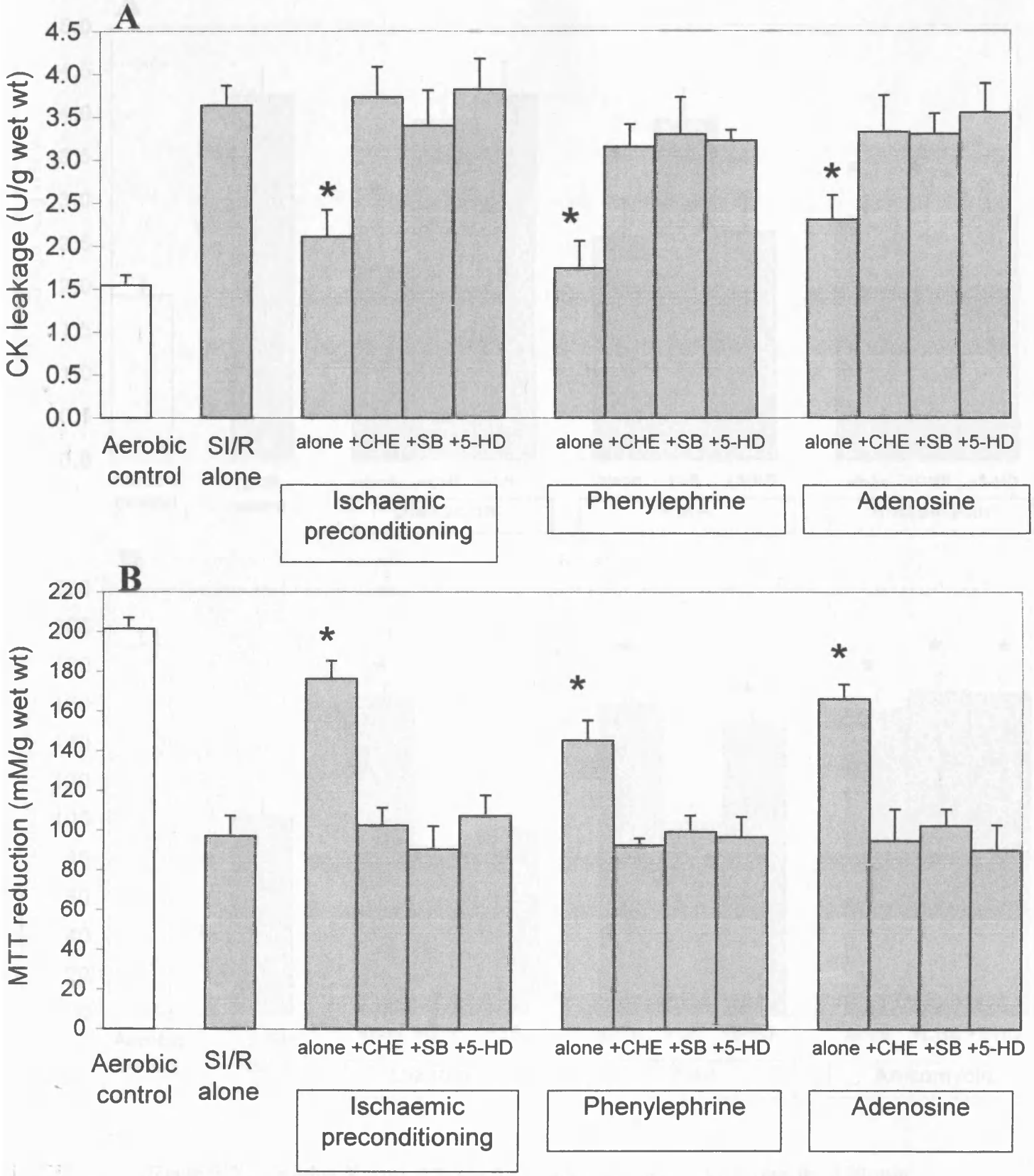

Figure 5-IX: Creatine Kinase (CK) leakage into the media (A) during the 120 min reoxygenation period and MTT reduction by the slices (B) at the end of the reoxygenation period in human atrial myocardium subjected to various protocols (see text for details) to investigate the role of PKC, p38MAPK and mitoK_{ATP} channels on ischaemic and phramcological preconditioning via alpha 1adrenoceptor and adenosine receptor activation (Study 4). Data are expressed as mean±SEM of six experiments. *$p<0.05$ vs SI/R alone group. (5-HD: 5-hydroxydecanoate, SB: SB203580).

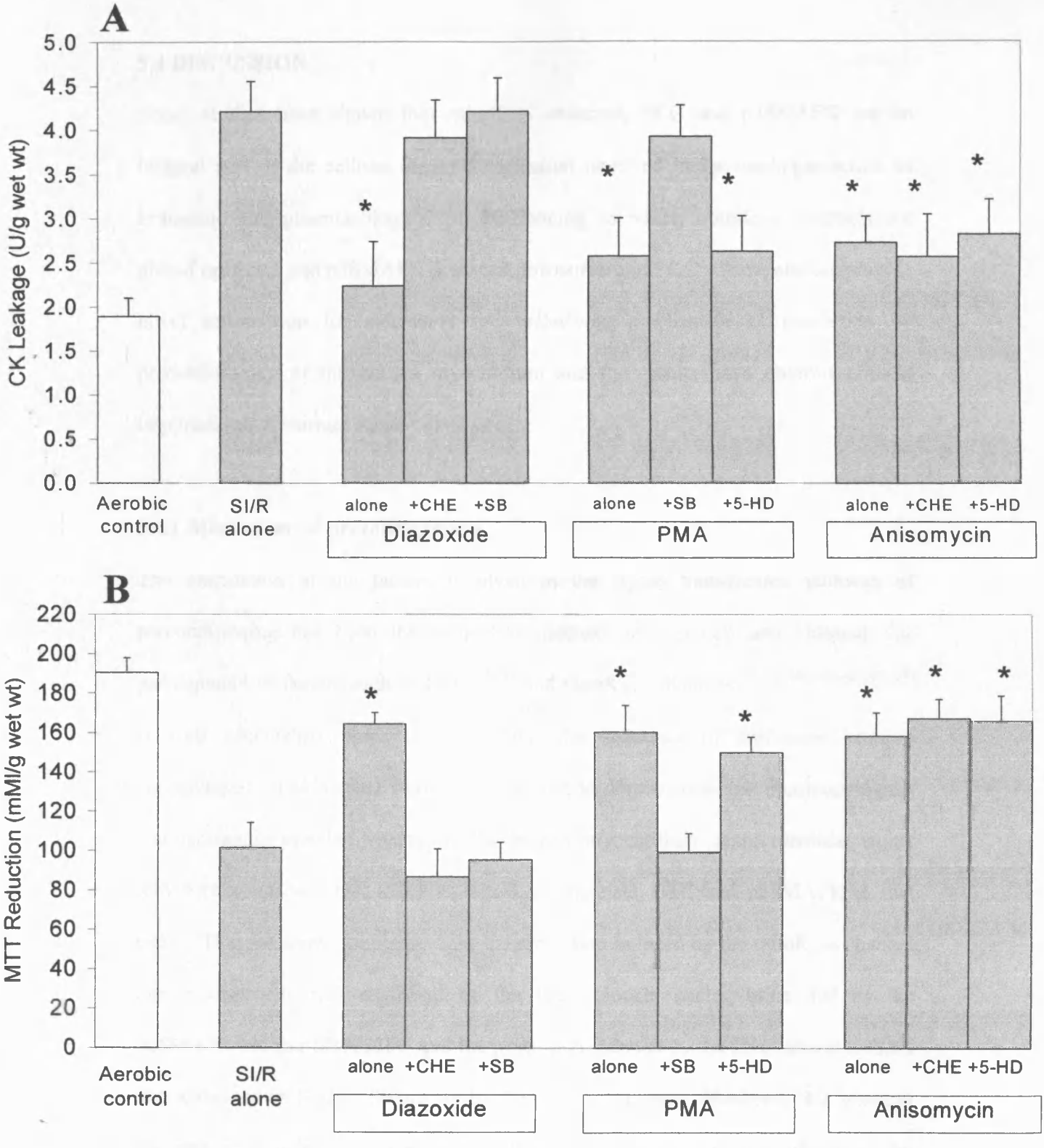

Figure 5-X: Creatine Kinase (CK) leakage into the media (A) during the 120 min reoxygenation period and MTT reduction by the slices (B) at the end of the reoxygenation period in human atrial myocardium subjected to various protocols (see text for details) to investigate the sequence of activation of the mediators of pharmacological and ischaemic preconditioning (Study 5). Data are expressed as mean ±SEM of six experiments. *$p<0.05$ vs SI/R alone group. (CHE: chelerythrine, 5-HD: 5-hydroxydecanoate, SB: SB203580, PMA: phorbol-12-myristate-13-acetate).

5.4 DISCUSSION

These studies have shown that mitoK$_{ATP}$ channels, PKC and p38MAPK are an integral part of the cellular signal transduction involved in the cardioprotection of ischaemic and pharmacological preconditioning in which mitoK$_{ATP}$ channels are placed upstream and p38MAPK is placed downstream of PKC. These studies provide novel information to understand the underlying mechanism of protection by preconditioning of the human myocardium and the results have obvious clinical importance that warrant further discussion.

5.4.1 Mechanism of preconditioning

The elucidation of the factors involved in the signal transduction pathway of preconditioning has been the subject of intense investigation and although the participation of factors such as PKC[481,482] and mitoK$_{ATP}$ channels[273,400,461,462,463,471,480] is well established, their relevance and the sequence of activation remains controversial. The present studies are the first to demonstrate that pharmacological and ischaemic preconditioning of the human myocardium share identical signal transduction cascade that involves mitoK$_{ATP}$ channels, PKC and p38MAPK in that order. Thus, as shown in Figure 5-X, the protection induced by the mitoK$_{ATP}$ channel opener diazoxide was abolished by the PKC blocker chelerythrine and by the p38MAPK blocker SB203580, and the protection induced by the PKC activator PMA was abolished by SB203580 but not by the mitoK$_{ATP}$ channel blocker S-HD, whereas the protection induced by the p38MAPK activator anisomycin was unaffected by chelerythrine or 5-HD. Wang et al[483] also showed that PKC inhibition with chelerythrine or calphostin C completely abolished the beneficial effects of diazoxide in the isolated rat heart, thus providing further support that mitoK$_{ATP}$ channels are

upstream of PKC. However, Pain et al[467] and Miura et al[484] using chelerythrine and calphostin C to block PKC were unable to confirm this observation in the rabbit, suggesting that these differences could arise as a result of different animal species. In spite of this, it is interesting to note that Pain et al[467] reported that genistein, a tyrosine kinase antagonist, blocked the protection induced by diazoxide which indicates that activation of kinases also lies downstream of mitoK$_{ATP}$ channels in the rabbit heart.

My demonstration that activation of any one of the components of the transduction cascade investigated in our studies (mitoK$_{ATP}$ channels, PKC, p38MAPK) can provide identical protection and that blockade of any of them individually completely abolishes protection indicates that in the human myocardium there is only one pathway of protection by preconditioning. The failure to obtain additional protection when more than one agent was used to induce pharmacological preconditioning or when these agents were used in combination with ischaemic preconditioning further support this thesis. But again, the mechanism of preconditioning the human myocardium may not be applicable to all species as suggested by the need to combine the inhibition of PKC and tyrosine kinase to abort the protection of preconditioning in pigs.[485]

The molecular interactions between the various components of the preconditioning pathway are not well understood. There is evidence that mitoK$_{ATP}$ channel opening increases radical oxygen species production which in turn may activate PKC,[467] but it is well known that mitoK$_{ATP}$ channels are also modulated by PKC.[469] The above and the realization of selective translocation of PKC with preconditioning,[481,483,486] suggest that PKC through different isoforms may play a dual role upstream and

downstream of mitoK_{ATP} channels. Our observation that blockade of PKC abolishes the protection by diazoxide does not eliminate that possibility.

My results have shown that activation of p38MAPK is a crucial step in the transduction pathway of preconditioning in the human myocardium. The activation of p38MAPK requires phosphorylation of Thr180 and Tyr182 within a TGY motif[487] by the MAP kinases MKK3 and MKK6.[488] It has been reported that the PKC activator PMA activates p38MAPK[477] but the exact mechanism remains unclear. Although activation of p38MAPK has been connected to preconditioning in the rabbit heart,[489,490] the relationship could not be established in rat[491] and pig[492] hearts. Therefore, it seems that once more the components of the signal transduction pathway of preconditioning are species-dependent.

It is still unknown whether the activation of p38MAPK is the last step of the transduction cascade that phosphorylates the end-effector and whether there is a simple or multiple effectors and their location. p38MAPK can phosphorylate a wide range of proteins some of which may be potential candidates for end-effectors of preconditioning. Thus, for example, the low molecular weight heat shock protein HSP27 may be phosphorylated by p38MAPK via the intermediate MAPKAPK2[493] and this may lead to polymerisation of actin filaments[494] and to increase tolerance of the cytoskeleton to stress.[495] Translocation of PKC isoforms to mitochondrial sites, intercalated discs and nucleus may suggest that p38MAPK activation in these places may activate enzymes involved with energy production, intercellular communication through cell junctions or gene transcriptions.

My results suggest that mitoK$_{ATP}$ channels are not the end-effectors of cardioprotection by preconditioning of the human myocardium. The concept that mitoK$_{ATP}$ channels may be the end-effectors of preconditioning has been based on the efficacy of diazoxide, a highly selective mitoK$_{ATP}$ channel opener, to mimic cardioprotection by preconditioning[470,471] and the blockade of this protection by 5-hydroxydecanoate,[470,471] a specific mitoK$_{ATP}$ channel blocker.[496] However, it was never clear whether the actions of these channels were limited to the mitochondria or were part of a more complex signal transduction cascade with effects on other cellular structures. The effect of opening the mitoK$_{ATP}$ channels is still a matter of controversy and whereas some investigators have reported large decreases in mitochondrial membrane potential affecting respiration and resulting in a reduction in Ca^{2+} uptake into mitochondria,[497,498] others have observed little effect on membrane potential, bioenergetics or Ca^{2+} uptake but important changes on matrix and intermembrane space volumes.[499] The reason given for these discrepancies has been the use of different doses of mitoK$_{ATP}$ channel openers in the former studies[499] but this does not clarify how changes in mitochondrial volume are connected to PKC activation, that is downstream in the signal transduction cascade.

The diagram in Figure 5-XI describes my proposal of the signal transduction cascade of preconditioning in the human myocardium. It is suggested that upon activation of sarcolemmal receptors (e.g. adenosine receptors, α_1-adrenoreceptors) mitoK$_{ATP}$ channels are opened via Gi proteins and PKC, possibly PKC-δ.[483] The opening of mitoK$_{ATP}$ channels will activate possibly a different PKC isoform probably via the production of radical oxygen species.[467] PKC may then translocate to various cellular sites including the mitochondria, sarcolemma and intercalated discs, and nucleus, a

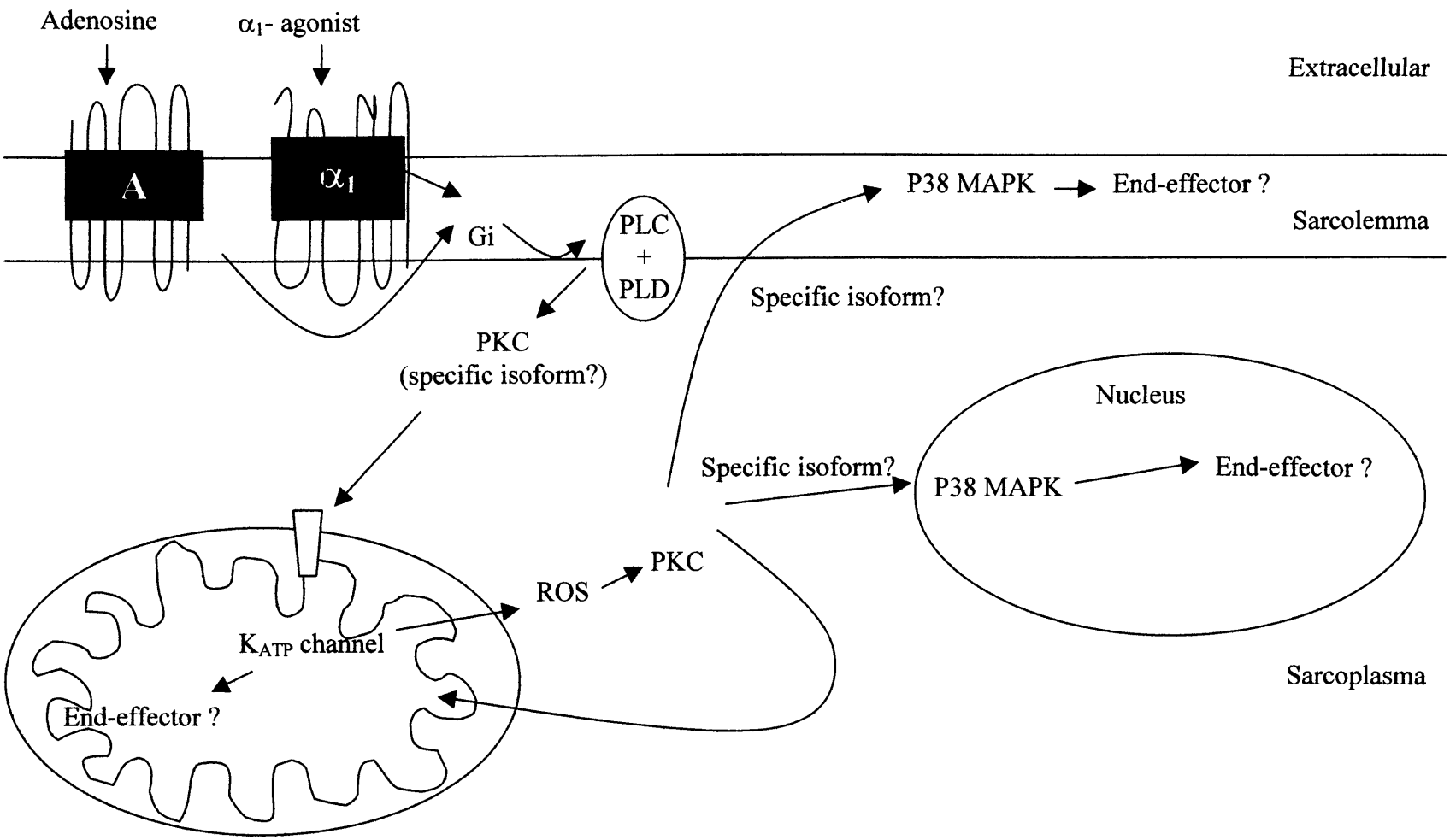

Figure 5-XI: Proposed schematic representation of the signal transduction mechanism leading to cardioprotection by pharmacological and ischaemic preconditioning of the human myocardium. Upon activation of sarcolemmal receptors mitoK_{ATP} channels are opened via G proteins and PKC, possibly PKC-δ or ε. The opening of mitoK_{ATP} channels will activate PKC possibly via the production of radical oxygen species (ROS). PKC may then translocate to various cellular sites including the mitochondria, sarcolemma and intercalated discs, and nucleus, a phenomenon that may involve specific PKC isoforms, where p38MAPK will be activated. In turn, p38MAPK may activate a single or multiple end-effectors directly or via MAPK intermediates.

phenomena that may involve specific PKC isoforms, where p38MAPK will be activated. In turn, p38MAPK may activate a single or multiple end-effectors directly or via MAPK intermediates. It is clear that more studies are required to fully elucidate the signal transduction pathway of preconditioning and the coupling between its components.

5.4.2 Clinical implications

The finding that the cardioprotection achieved by activation of α_1-adrenoreceptors and adenosine receptors is as potent as the one obtained with ischaemic preconditioning and that their use in combination does not result in additional benefit has obvious major clinical implications because maximal protection can be attained without the need to occlude the coronary arteries to induce ischaemia. The procurement of cardioprotection with stimuli of receptors as diverse as α_1-adrenoreceptors and adenosine receptors can be clinically advantageous because some of these agents may be contraindicated in certain conditions (e.g. α_1-adrenoreceptor agonists in hypertension, adenosine in the presence of alterations of the cardiac conduction system). Furthermore, blockade of one of these sarcolemmal receptors does not preclude cardioprotection by activation of the other receptors. These interventions can be useful to combat ischaemic injury in different clinical conditions such as coronary angioplasty, cardiac surgery and heart transplantation; however, it is necessary to mention that our studies were performed in an *in-vitro* preparation and therefore any extrapolation to the clinical setting should be made with caution.

The realization that cardioprotection by pharmacological and ischaemic preconditioning of the human myocardium is mediated by one obligatory signal

transduction pathway also opens the therapeutical window for direct manipulation of its components. Recently, we have demonstrated that although the myocardium from patients with poor left ventricular function (ejection fraction <30%) or with diabetes cannot be protected with ischaemic preconditioning, the mitoK_{ATP} channel opener diazoxide elicited protection in the former but not in the latter.[500] This suggests that if part of the signal transduction cascade is affected by disease states, cardioprotection can still be obtained by bypassing the defective components. It is also of clinical relevance that blockade at any stage of the signal transduction involved in preconditioning does not seem to exacerbate injury suggesting that this pathway is solely used for cardioprotection and not in tissue injury.

The studies in this chapter have established the signal transduction mechanism and the order of the various components involved in preconditioning of the human myocardium. In the next two chapters I will investigate the impact of nicorandil and sulfonylureas, which are drugs commonly used in cardiac patients, on preconditioning and the signal transduction mechanism.

Chapter 6

The influence of nicorandil on preconditioning

6.1 INTRODUCTION

Nicorandil was first introduced in clinical practice in 1984 as the first in a new class of anti-angina drugs and since then it has become widely used for the control of angina as part of combination therapy and more recently it is being increasingly used as the first line and the sole treatment in both stable[501,502] and unstable angina.[503] Nicorandil is a nicotinamide nitrate ester that has been shown to have a comparable anti-angina effect to beta-blockers and calcium antagonists. It has a bimodal mechanism of action combining two vasodilator mechanisms, it increases potassium conductance in the cell membrane resulting in potassium outflow from the cell causing membrane hyperpolarization[504] and also increases cellular levels of cGMP,[505] both actions causing vasorelaxation. The nitrate like action of nicorandil dilates epicardial coronary arteries[506] that results in an increase in the blood supply to the ischaemic region of the myocardium. In addition, nicorandil has been shown to open K_{ATP} channels in ischaemic cardiomyocytes[507] and there is strong evidence that the mitoK_{ATP} rather than the sarcolemmal K_{ATP} channels are involved in the protection of ischaemic preconditioning,[273,419,467,471] probably by decreasing the mitochondrial membrane potential.[495,508] Yellon's laboratory has reported that nicorandil can mimic the protection of ischaemic preconditioning,[509] which has led to the suggestion that patients receiving nicorandil for the control of angina may be permanently protected.[510]

The aims of this study were to investigate the effect of long-term administration of nicorandil: (i) on the tolerance of the human myocardium to ischaemia and (ii) on the protection of ischaemic and pharmacological preconditioning and (iii) on its signal transduction mechanism.

6.2 METHODS

6.2.1 Patient Selection and Experimental Preparation

Experiments were performed on muscle obtained from the right atrial appendage of patients undergoing elective coronary artery surgery in the cell necrosis model described in Chapter 2.[405] As before patients were excluded if they had large atriums, atrial arrhythmias, poor left ventricular function (ejection fractions <30%), diabetes and right ventricular failure or were taking oral hypoglycaemic agents, opioid analgesia, or catecholamines. Patients on nicorandil had their last dose on the morning of surgery 2.5±0.4 hours prior to harvesting of the atrial appendage. These patients were on nicorandil for a mean of 18.6±2.5 months and a mean dose of 20mg/day. Table 6-I contains the demographic and clinical data of all the patients included in these studies. The specimens were collected in oxygenated HEPES buffered solution at 4-5°C and immediately sectioned and prepared for study as described in Chapter 2.

Table 6-I: Demographic and clinical data of patients included in the studies.

Study	Treatment	Number of patients	Number of specimens	Age (Years)	Male:Female	Period of exposure to Nicorandil (Months)
1	No Nicorandil	7	24	67.1±6.3	5:2	0
	On Nicorandil	6	24	65.3±6.6	5:1	20.1±2.1
2	No Nicorandil	9	30	66.3±4.8	5:4	0
	On Nicorandil	8	30	66.8±7.2	6:2	16.7±2.1
3	No Nicorandil	8	30	67.6±3.6	7:1	0
	On Nicorandil	9	30	65.3±5.8	6:3	19.5±2.7

6.2.2 Assessment of tissue injury and viability

Tissue injury was determined by measuring the leakage of CK into the incubation medium during the 120min reoxygenation period and tissue viability was assessed by the reduction of MTT to a blue formazan product at the end of the experimental time as described in Chapter 2

6.2.3 Solutions and Drugs

The incubation medium was prepared daily with de-ionised distilled water as described in Chapter 2. Phenylephrine, prazosin, 5-hydroxydecanoate and anisomycin were used dissolved in de-ionised distilled water, while diazoxide and phorbol 12-myristate 13-acetate (PMA) were dissolved in DMSO. Anisomycin is an antibiotic that inhibits protein synthesis and has been demonstrated to activate p38MAPK while PMA is a phorbol ester that is widely used to activate PKC. All the drugs doses were chosen following extensive preliminary dose response experiments. All reagents were obtained from Sigma.

6.2.4 Experimental Protocols

All atrial muscles were allowed to equilibrate under aerobic conditions for 30 minutes prior to 90 minutes of simulated ischaemia and 120 minutes of reoxygenation. There were 6 specimens in each group from 6 different patients with a total of 6 to 9 patients being enrolled in each study.

Study 1: To investigate the effect of the long-term administration of nicorandil on the myocardial tolerance to ischaemia and on the protection of ischaemic and pharmacological preconditioning with phenylephrine as shown in Figure 6-I:

Atrial muscles obtained from appendages of patients treated or not treated with nicorandil were randomly assigned to one of the following groups: (i) aerobic control, (ii) simulated ischaemia/reoxygenation (SI/R) alone, (iii) ischaemic preconditioning (IP) with 5 minutes of SI and 5 minutes R and (iv) phenylephrine (0.1μM) for 5 minutes and 5 minutes washout prior to SI/R.

Study 2: To investigate the effect of the long-term administration of nicorandil on the responsiveness of mitoK_{ATP} channels during preconditioning as shown in Figure 6-I:

Atrial slices obtained from appendages of patients treated or not treated with nicorandil were randomly assigned to one of the following groups: (i) aerobic control, (ii) SI/R alone, (iii) IP with 5 minutes of SI and 5 minutes R, (iv) diazoxide (100μM), a Mito K_{ATP} channel opener, for 10 minutes prior to SI/R and (V) 5-hydroxydecanoate (1mM), a Mito K_{ATP} channel blocker, for 10 minutes prior to SI/R.

Study 3: To investigate the effect of the long-term administration of nicorandil on the kinases pathway involved in preconditioning as shown in Figure 6-III:

Atrial slices obtained from appendages of patients treated with or not treated with nicorandil were randomly assigned to one of the following groups: (i) aerobic control, (ii) SI/R alone, (iii) IP with 5 minutes of SI and 5 minutes R, (iv) phorbol 12-myristate 13-acetate (PMA) (1μM), a PKC activator, for 10min prior to SI/R and (v) anisomycin (1nM), a p38MAPK activator, for 10min prior to SI/R.

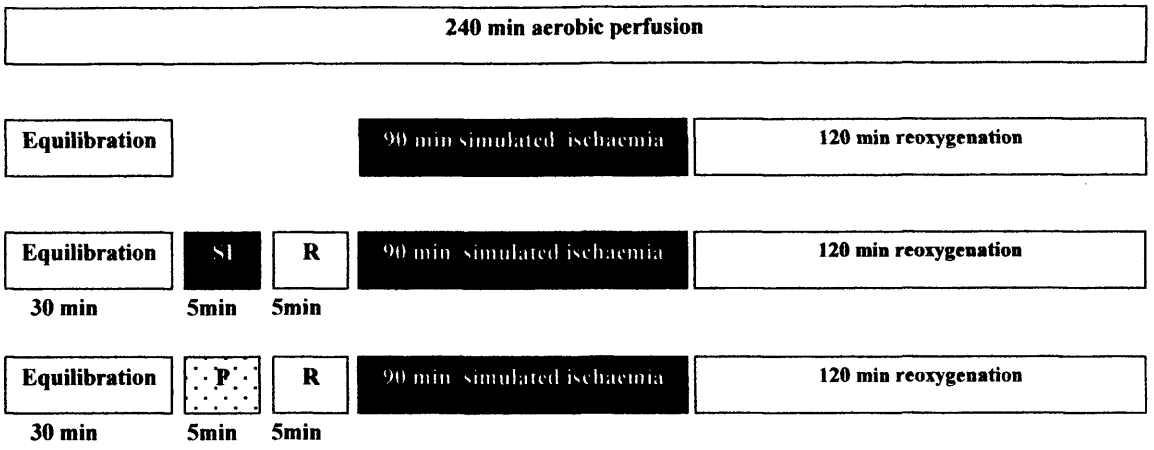

Figure 6-I: Schematic representation of the protocol for study 1. The same protocol was applied on atrial tissue obtained from patients treated and not treated with nicorandil. SI: simulated iscahemia, R: reoxygenation, P: phenylephrine.

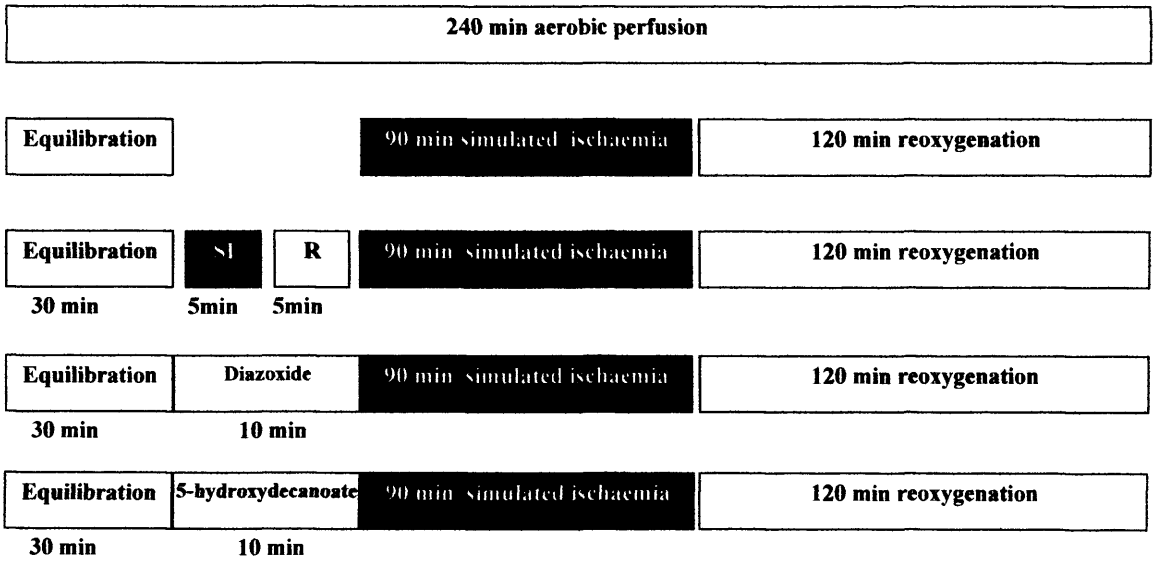

Figure 6-II: Schematic representation of the protocol for study 2. The same protocol was applied on atrial tissue obtained from patients treated and not treated with nicorandil. SI: simulated iscahemia, R: reoxygenation.

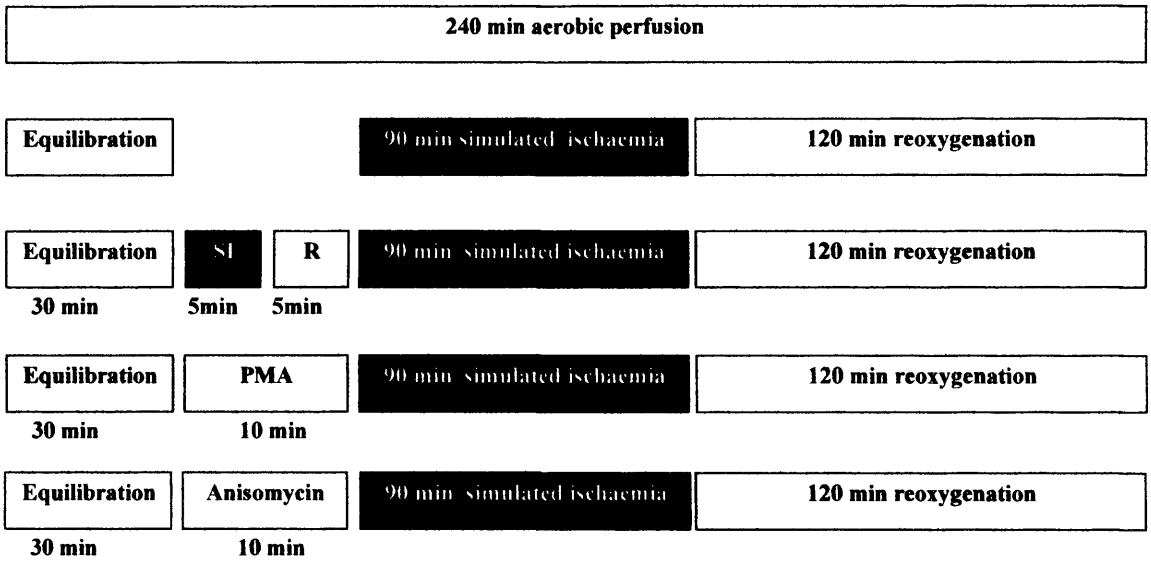

Figure 6-III: Schematic representation of the protocol for study 2. The same protocol was applied on atrial tissue obtained from patients treated and not treated with nicorandil. SI: simulated iscahemia, R: reoxygenation, PMA: phorbol 12-myristate 13-acetate.

6.2.5 Statistical analysis

All data are presented as mean±standard error of the mean (SEM). Mean values were compared by ANOVA with application of a post hoc Tukey's test. Statistical significance was taken as $p<0.05$.

6.3 RESULTS

The effect of the long-term administration of nicorandil on the myocardial tolerance to ischaemia and on the protection of ischaemic and pharmacological preconditioning with phenylephrine (Study 1): As shown in Figures 6-IVA and 6-IVB, the increased CK leakage and decrease in MTT reduction induced by ischaemia/reoxygenation were similar in the muscles from patients with and without long-term nicorandil treatment. They also show that ischaemic and pharmacological preconditioning with phenylephrine resulted in identical protection in the nicorandil-free group but they failed to protect the myocardium in the nicorandil-treated group.

The effect of the long-term administration of nicorandil on the responsiveness of mitoK_{ATP} channels during preconditioning (Study 2): As expected, Figures 6-VA and 6-VB show that the selective opening of mitoK_{ATP} channels with diazoxide resulted in a protection (i.e. reduced CK leakage and increased MTT reduction) similar to that of ischaemic preconditioning with no detrimental effect beyond that of ischaemia/reoxygenation alone when the channels were blocked with 5-hydroxydecanoate. In contrast with these results, diazoxide did not protect the myocardium in the nicorandil-treated group.

The effect of the long-term administration of nicorandil on the kinases pathway involved in preconditioning (Study 3): The results shown in Figures 6-VIA and 6-VIB demonstrate that activation of PKC and p38MAP kinase resulted in a similar protection to that of IP as shown by the CK leakage and MTT reduction values in both nicorandil-free and nicorandil-treated groups.

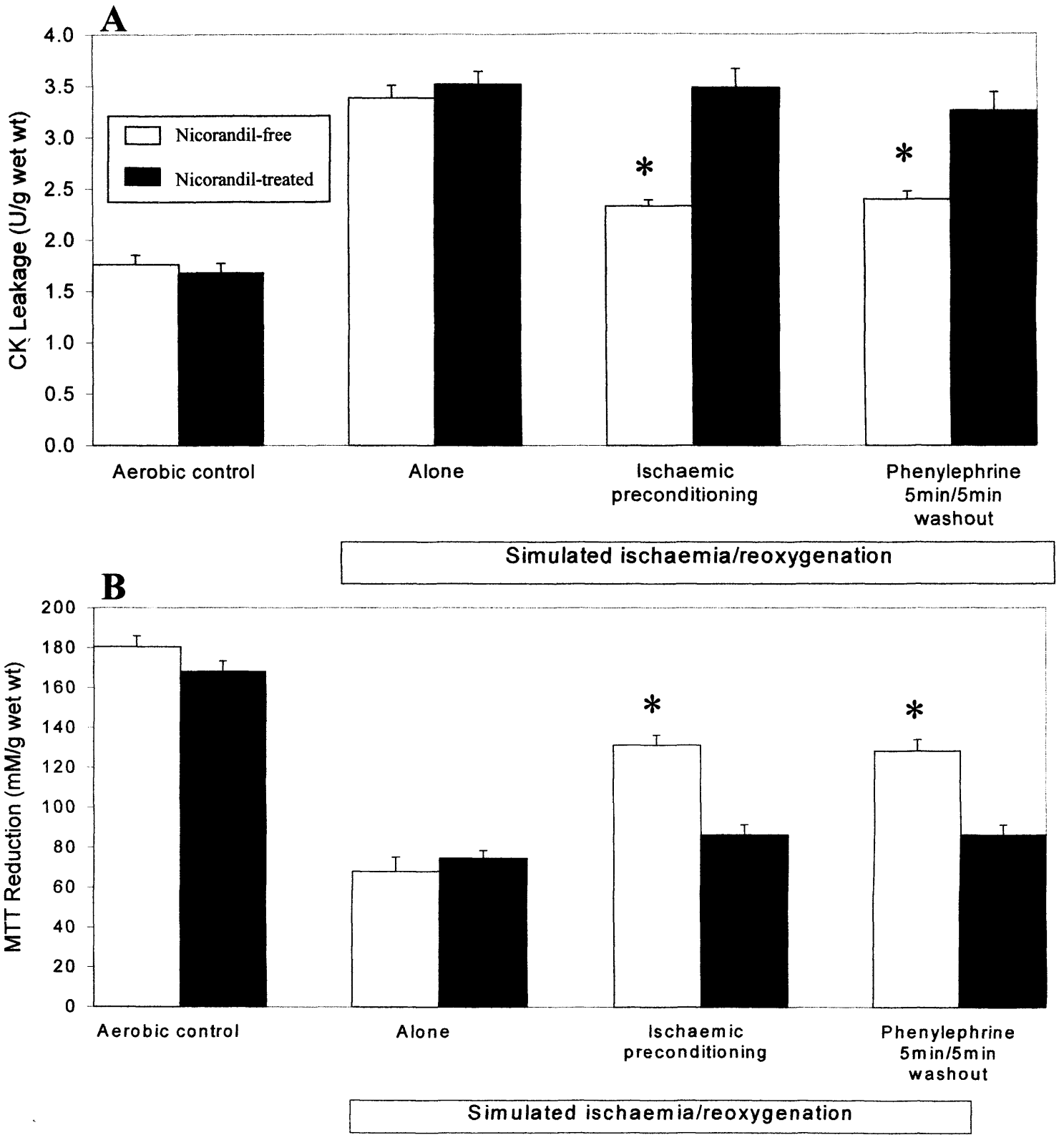

Figure 6-IV: Creatine Kinase (CK) leakage into the media (A) during the 120 min reoxygenation period and MTT reduction by the slices (B) at the end of the reoxygenation period in human atrial myocardium subjected to various protocols (see text for details) to investigate the effect of the long-term administration of nicorandil on the myocardial tolerance to ischaemia and on the protection of ischaemic and pharmacological preconditioning with phenylephrine (Study 1). Data are expressed as mean ±SEM of six experiments. *$p<0.05$ vs. simulated ischaemia/reoxygenation alone group.

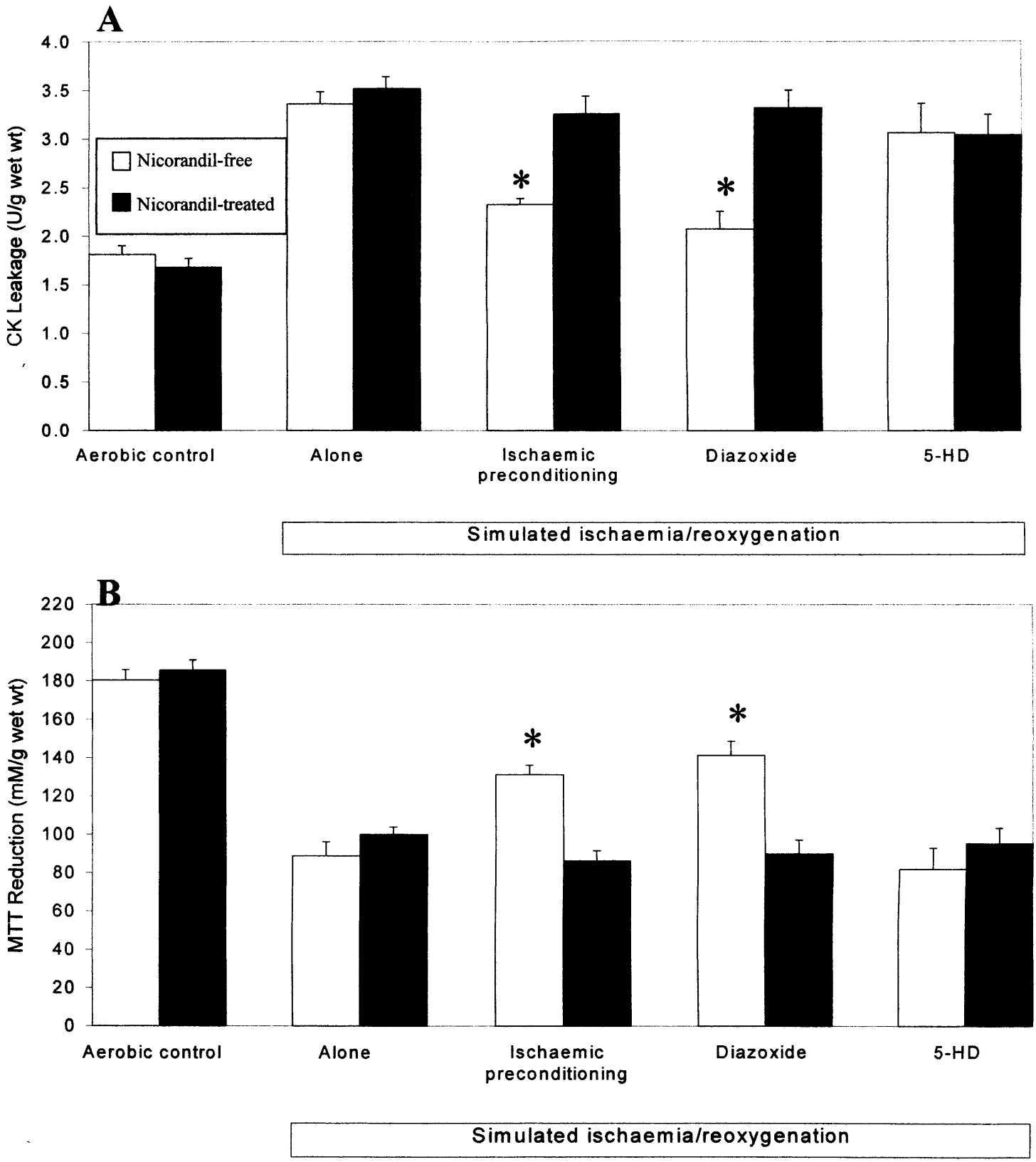

Figure 6-V: Creatine Kinase (CK) leakage into the media (A) during the 120 min reoxygenation period and MTT reduction by the slices (B) at the end of the reoxygenation period in human atrial myocardium subjected to various protocols (see text for details) to investigate the effect of the long-term administration of nicorandil on the responsiveness of mitoK$_{ATP}$ channels during preconditioning (Study 2). Data are expressed as mean ±SEM of six experiments. *$p<0.05$ vs. simulated ischaemia/reoxygenation alone group. (5-HD: 5-hydroxydecanoate).

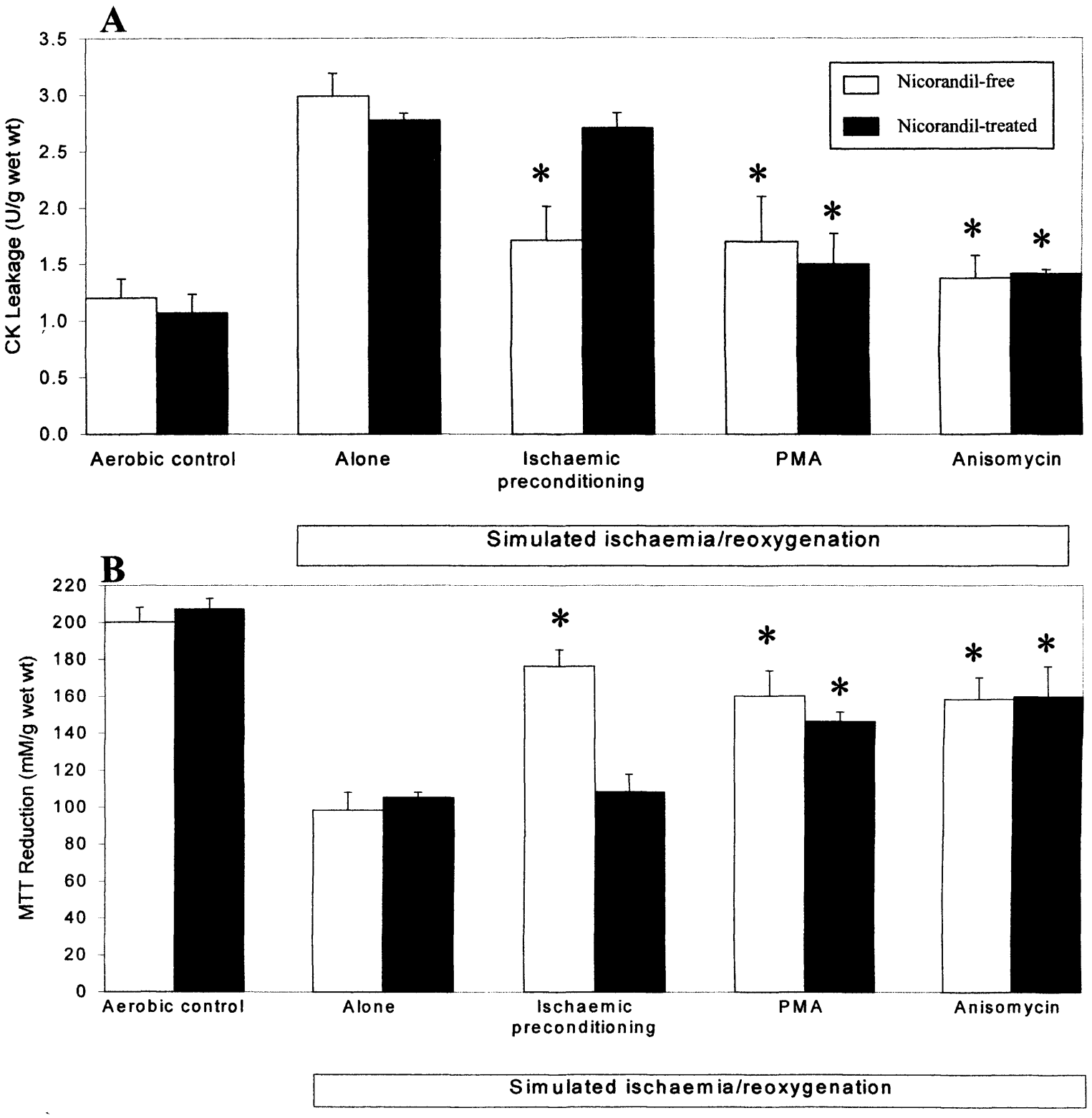

Figure 6-VI: Creatine Kinase (CK) leakage into the media (A) during the 120 min reoxygenation period and MTT reduction by the slices (B) at the end of the reoxygenation period in human atrial myocardium subjected to various protocols (see text for details) to investigate the effect of the long-term administration of nicorandil on the kinases pathway involved in preconditioning (Study 3). Data are expressed as mean ±SEM of six experiments. *$p<0.05$ vs. simulated ischaemia/reoxygenation alone group. (PMA: phorbol 12 myristate 13-acetate).

6.4 DISCUSSION

The present studies have demonstrated that the long-term administration of nicorandil, a mitoK$_{ATP}$ channel opener[509] and nitric oxide donor,[511] abolishes the ability of the human myocardium to be protected by ischaemic and pharmacological preconditioning without exacerbating the susceptibility to ischaemic injury. In addition, they have shown that the likely cause of the failure to precondition the myocardium of patients on nicorandil is the unresponsiveness of the mitoK$_{ATP}$ channels since protection cannot be obtained with diazoxide, a specific mitoK$_{ATP}$ channel opener, but can be elicited by activation of PKC and p38MAPK that are downstream of mitoK$_{ATP}$ channels in the signalling transduction cascade of preconditioning.[512] These results have important clinical implications and shed light into the mechanism of protection by preconditioning which are discussed below.

Opening of the mitoK$_{ATP}$ channels has been demonstrated to be an obligatory step in the signal transduction mechanism of preconditioning.[273,467,471] Thus, nicorandil and other mitoK$_{ATP}$ channel openers have been shown to mimic the cardioprotection of ischaemic preconditioning when given acutely (i.e. immediately before the ischaemic insult) in both animal[467,507,510] and human studies.[273,471] As a result, and as suggested by Carr and Yellon,[510] one might be tempted to hypothesize that the long-term administration of mitoK$_{ATP}$ channel openers induces a permanent state of protection against ischaemic injury. However, the present studies are the first to report that when mitoK$_{ATP}$ channel openers are given on a long-term basis, as it occurs clinically, these channels become unresponsive with loss of the cardioprotection of preconditioning.

My findings contrast with those reported by Carr and Yellon[510] showing that the long-term administration of nicorandil is actually protective. Their and our studies used human atrial myocardium and the only difference between the two studies is that they assessed recovery of contractile function in their study as opposed to tissue viability in ours, which makes it difficult to find an obvious explanation. Further fuel is added to the controversy when the same authors observed that the protection of the myocardium of patients receiving long-term nicorandil is in fact abolished by the application of ischaemic preconditioning.[510] These results require clarification because it is difficult to understand how two protective interventions using identical signal transduction mechanism can annul each other.

The mechanism by which the long-term administration of nicorandil renders the mitoK$_{ATP}$ channels unresponsive to precondition with ischaemia and with diazoxide is not completely elucidated by the present studies but they have shown that the activation of kinases, that I have previously shown to be downstream of mitoK$_{ATP}$ channels in Chapter 5, is unaffected because their activation can still elicit protection. It has been suggested that the generation of free radical species is the link between mitoK$_{ATP}$ channels and activation of PKC.[467] If this is the case, then it may be speculated that a permanent opening state of mitoK$_{ATP}$ channels results in a reduction in the formation of free radicals, a thesis that gains support by the demonstration that nicorandil posses antioxidant properties.[512]

The present studies raise fundamental questions on the clinical utility and safety of nicorandil and other mitoK$_{ATP}$ channel openers for the control of angina symptoms. The permanent opening of the mitoK$_{ATP}$ channels appears to deprive the heart from

the intrinsic protective mechanism of preconditioning that may be a risk factor in the presence of ischaemic heart disease. Therefore if the beneficial action of nicorandil is solely due to its nitrate effect, then the use of this compound may not be fully justified. However if, as discussed earlier, the maintenance of mitoK_{ATP} channels in an open state may reduce the generation of free radicals and oxidative stress then these beneficial effects of nicorandil may counterbalance the loss of preconditioning. Indeed oxidative stress has been suggested as an important mechanism of many disease states including the inflammatory response in diabetes,[513] atherosclerosis,[514] cardiac hypertrophy[515] and heart failure.[516] It is clear that further experimental and clinical studies are required to fully elucidate the mechanism and the clinical repercussions of nicorandil and similar agents on ischaemic heart disease and ischaemic syndromes. This question in fact has partly been answered by the IONA study group.[517] This study compared nicorandil (20mg twice daily) with placebo in 5,126 high-risk patients with stable angina. All patients required further antianginal treatment at recruitment and took the study drug in addition to optimised standard antianginal therapy. The IONA study revealed that fewer patients in the nicorandil group experienced the combined primary end-point of coronary heart disease death, non-fatal MI or unplanned admission to hospital with cardiac chest pain (13.1% vs.15.5%; relative risk 0.83, 95% CI 0.72-0.97; mean follow-up 1.6 years). However, the main apparent benefit for the group treated with nicorandil was a reduction in unplanned admission, as there was no difference between nicorandil and placebo in the combined secondary endpoint of coronary heart disease death or nonfatal MI. Furthermore, the IONA study only assessed the role of nicorandil as an 'add-on' therapy. However, data from the IONA study would suggest that nicorandil may be a worthwhile add-on therapy for high risk patients of a cardiovascular event as 66% of

patients in IONA had a previous MI. Although, as nicorandil was used with various combinations of antianginals including beta-blockers, calcium channel blockers and long-acting nitrates, this study does not provide information as to when to add in nicorandil.

It is necessary to mention that I performed these studies in an *in vitro* preparation that was not electrically stimulated (i.e. non-beating) and it was not possible to obtain functional data and therefore any extrapolation to the clinical setting should be made with caution. Another potential limitation of my studies was the use of atrial as opposed to ventricular myocardial tissue and again any extrapolation of the conclusions from these studies to the ventricular myocardium should also be made with caution.

Although these studies have demonstrated the unresponsiveness of the mitoK_{ATP} channels as the cause of failure to precondition the myocardium of nicorandil- treated patients, they show that protection can still be obtained by direct activation of PKC or p38MAPK that are downstream of mitoK_{ATP} channels in the signal transduction pathway of ischaemic preconditioning. Therefore protection can be elicited by bypassing the steps of the transduction cascade that may be affected by disease states such as diabetes or heart failure[500] or by medication such as sulfonylureas and anti-angina treatment such as nicorandil as shown from the current studies. Undoubtedly greater understanding of the various elements participating in the signal transduction pathway and identification of agents with selective activity on those factors to avoid unwanted side effects will be required before its exploitation may be considered in the clinical setting.

Chapter 7

The influence of sulfonylureas on preconditioning

7.1 INTRODUCTION

Sulfonylureas are widely used in the treatment of type 2 diabetes. They stimulate insulin secretion from pancreatic β-cells by closing their principal target in the cell membrane, the ATP-sensitive potassium (K_{ATP}) channel. Blocking of K_{ATP} channels results in the depolarisation of the cell membrane, thereby triggering the opening of voltage-gated Ca^{2+} channels, leading to the elevation of intracellular Ca^{2+} and the stimulation of insulin secretion.[518] However, K_{ATP} channels of differing subtypes are also expressed in both cardiac and vascular smooth muscle cells, and inhibition of these channels by sulfonylureas may be related to certain cardiovascular side effects of the drugs.[519,520]In the heart, there is extensive evidence that K_{ATP} channels are involved in the cardioprotection induced by IP,[419] and the sulfonylurea glibenclamide is known to inhibit such protection.[298,307,500]

The exact identity of the channels involved in cardioprotection and the mechanism by which this occurs has been the subject of much recent controversy. A considerable body of pharmacological evidence, based on the selectivity of the K_{ATP} channel opener diazoxide and the blocker 5-hydroxydecanoate (5-HD), led to suggestions that a mitochondrial K_{ATP} channel is involved in IP.[470,471,521] However the molecular composition of any such mitochondrial K_{ATP} channel remains unconfirmed, while the selectivity of these compounds has also been questioned.[522] In particular, diazoxide and 5-HD have recently been shown to exert effects on mitochondrial metabolism that appear unrelated to K_{ATP} channels, but which might account for their effects on cardioprotection.[523] Furthermore, in mice, knockout experiments suggest that the Kir6.2 subunit of cardiac sarcolemmal K_{ATP} is required for protection by IP.

Despite these considerations, the known specificity of sulfonylureas for their respective receptors[524] may explain their differential effects on IP. This view is consistent with recent evidence demonstrating that, unlike glibenclamide, the sulfonylurea glimepiride does not abolish the protection of ischaemic preconditioning.[525,526] Another sulfonylurea in wide clinical use, gliclazide, shows high selectivity for pancreatic over cardiac K_{ATP} channels.[527] In the present study, I have therefore compared the dose-dependent effects of gliclazide with those of glibenclamide on IP in a human tissue model.

Diabetes mellitus is a common disease in the general population and particularly in patients with ischaemic heart disease. It has been associated with increased morbidity and mortality in cardiac surgery.[528] This may be due to the effects of diabetes on the various organs and vasculature. However, K_{ATP} channel blockade by sulfonylureas may also contribute to the poor outcome of diabetic patients subjected to myocardial ischaemia.[518,529] We hypothesized that these clinical observations might correlate with the loss of cardioprotection in diabetic tissue *per se*, since recent findings in the human atrial appendage model are consistent with the protective mechanism activated by diazoxide or IP, possibly the mitoK_{ATP} channel or another mitochondrial mechanism, lying upstream of PKC and p38MAPK in the IP signal transduction pathway. Accordingly, the negative effect of sulfonylureas on protection by preconditioning might be offset by stimulation of factors downstream of mitochondria and K_{ATP} channels. Here we have therefore investigated whether the block by glibenclamide of cardioprotection by IP can be bypassed following stimulation of downstream signal transduction cascades.

7.2 METHODS

7.2.1 Experimental Preparation

Experiments were performed on trabeculae muscle sections obtained from the right atrial appendage of patients undergoing elective coronary artery bypass graft surgery or aortic valve replacement. We employed the cell necrosis model described in Chapter 2.[405] Donor patients were excluded if they had enlarged atria, diabetes mellitus, atrial arrhythmias, poor left ventricular function (ejection fraction <30%), right ventricular failure or were receiving oral hypoglycaemic agents, opioid analgesia, K_{ATP} channel openers or catecholamines. The demographic data of all the patients included in these studies is included in Table 7-I. The specimens were collected in oxygenated Krebs Henseleit HEPES buffer (KHH) and sliced immediately at 4-5°C as described in Chapter 2.

Table 7-I: Demographic data of all the patients included in the studies.

Study	Number of patients	Number of specimens	Male:Female	Age
Study 1	17	88	11:6	62.4±6.8
Study 2	14	72	10:4	63.3±7.1

7.2.2 Solutions and Chemicals

The incubation medium was prepared daily with de-ionised distilled water as described in Chapter 2. All the chemicals were purchased from Sigma Chemicals. Gliclazide was provided by Technologie Servier (Orleans, France).

7.2.3 Experimental Time Course

All the muscle sections (between three and five per specimen) were equilibrated at 37°C for a 30min period. Some of the preparations were added to new flasks, which also contained 10ml of oxygenated medium, for another 210min (240min total), to serve as time-matched aerobic controls. The remaining preparations were subjected to a 90min period of SI at 37°C as described above. Following this the muscle sections were R for a further 120min by incubation in 10ml of oxygenated medium at 37°C with added glucose. At the end of the experimental protocols, aliquots of the incubation media used during the 120min reoxygenation period were collected for the assessment of CK leakage while the tissue was taken for the assessment of cell viability by reduction of MTT. The drugs under test were added for 10min at the end of the equilibration period and before the induction of SI.

7.2.4 Study groups

There were 8 specimens, each from a different patient group, for each protocol.

Study 1: To investigate whether the effect of glibenclamide and gliclazide on ischaemic preconditioning is dose dependent. The following groups were studied as shown in Figure 7-I: (i) time matched aerobic control, (ii) 90min simulated ischaemia/120min reoxygenation (SI/R) alone, (iii) IP with 5min simulated ischaemia

and 5min reoxygenation prior to SI/R, (iv to vii) glibenclamide at various concentrations (0.1, 1, 3 or 10μM) for 10min prior to IP and (viii to xi) gliclazide at various concentrations (1, 10, 30 or 100μM) for 10min prior to IP.

Study 2: To investigate whether the abolition of preconditioning-induced protection by glibenclamide can be offset by stimulation of the signal transduction cascade downstream to mitoK_{ATP} channels the following groups were studied as shown in Figure 7-II: (i) time matched aerobic control, (ii) 90min SI/120min R alone, (iii) IP with 5min simulated ischaemia and 5min reoxygenation prior to SI/R, (iv) diazoxide (100μM) for 10min prior to SI/R, (v) phorbol 12-myristate-13-acetate (PMA) (1μM) for 10min prior to SI/R, (vi) anisomycin (1nM) for 10min prior to SI/R, (vii) glibenclamide (1μM) for 10 min prior to diazoxide (100μM) administered for another 10min and then followed by SI/R, (viii) glibenclamide (1μM) for 10 min prior to PMA (1μM) administered for another 10min and then followed by SI/R, (ix) glibenclamide (1μM) for 10 min prior to anisomycin (1nM) administered for another 10min and then followed by SI/R. The concentration of each of the agents used was chosen from previous studies from our laboratory.

7.2.5 Assessment of tissue injury and viability

Tissue injury was determined by measuring the leakage of CK into the incubation medium during the 120min reoxygenation period and tissue viability was assessed by the reduction of MTT to an insoluble blue formazan product at the end of the experimental period as described in Chapter 2.

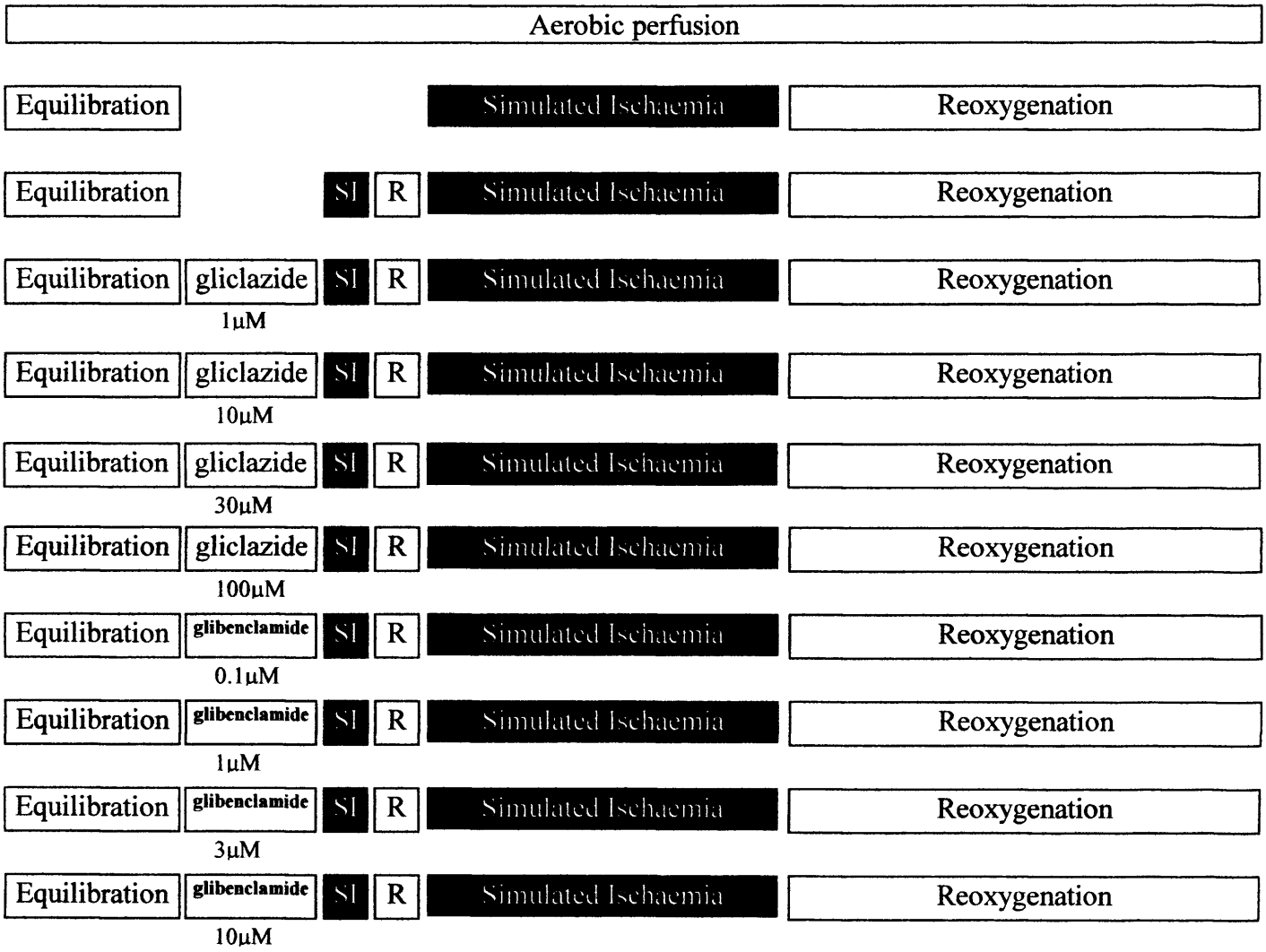

Figure 7-I: Schematic representation of the protocol for Study 1. SI: simulated ischaemia, R: reoxygenation.

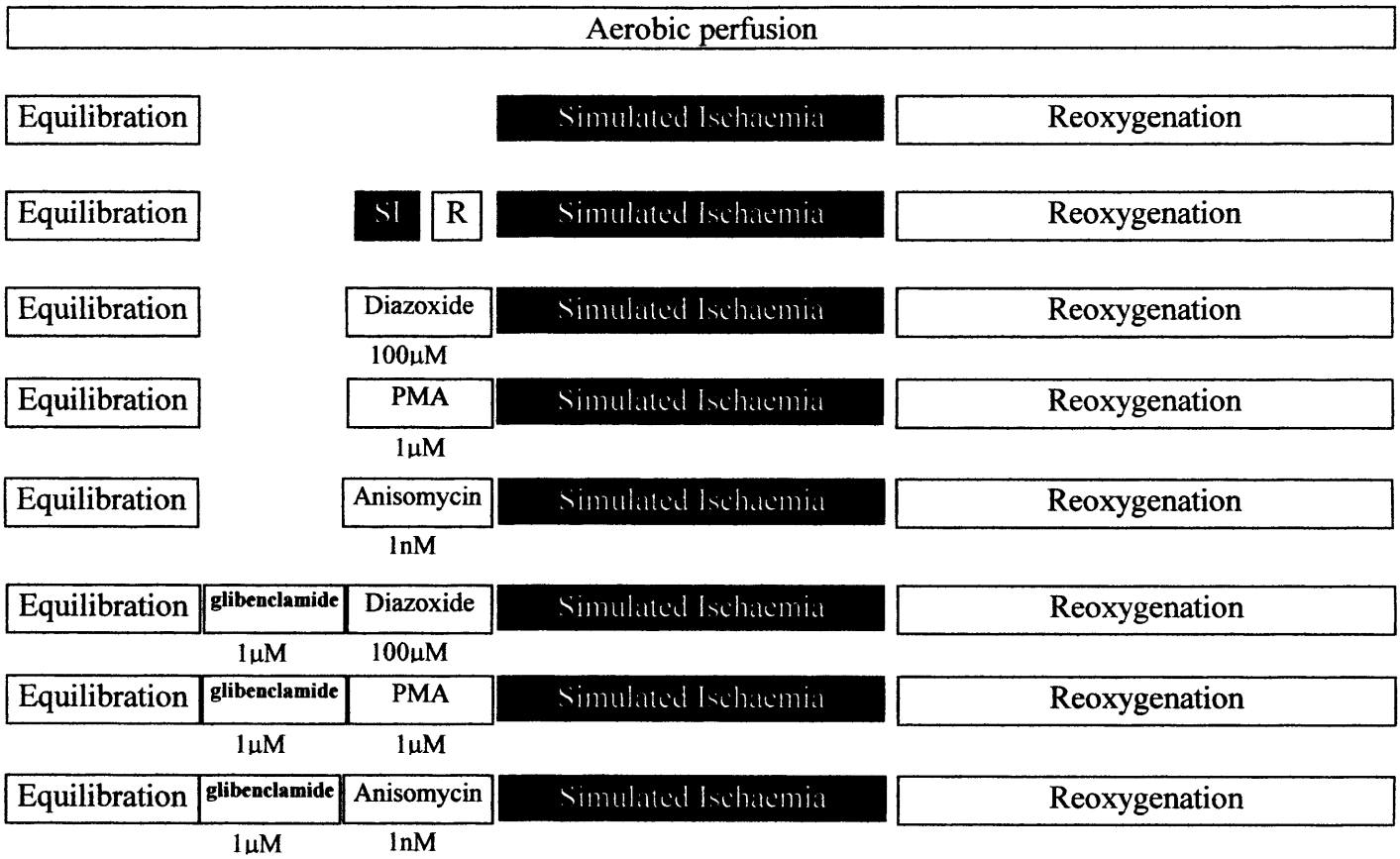

Figure 7-II: Schematic representation of the protocol for Study 2. SI: simulated ischaemia, R: reoxygenation, PMA: phorbol 12-myristate-13-acetate.

7.2.6 Data Analysis

Results were expressed as mean values ± SEM. Statistical significance was tested using analysis of variance (ANOVA) followed by the the application of post-hoc Tukey's test, and $p<0.05$ was considered statistically significant. Dose-response relations were fitted to the following equation using the least squares algorithm in Sigmaplot (Jandel Scientific).

$$y = 1 - \left[1 + \left(x / K_i\right)^H\right]^{-1} \qquad \text{Equation (1)}$$

Where y is the fractional block of IPC protection, x is the sulfonylurea concentration, K_i is the concentration for half inhibition, and H is the Hill coefficient.

Dose-response curves for gliclazide inhibition of protection as manifest by the increase in CK release following IP were constructed by calculating y, the fractional inhibition of IP in the presence of gliclazide as:

$$y = \left\{\left(CK_{Glic} - CK_{IPC}\right)/\left(CK_{SIR} - CK_{IPC}\right)\right\} \qquad \text{Equation (2)}$$

Where CK_{SIR} , CK_{IP} , and CK_{Glic} are the levels of MTT reduction with SI/R alone, in the presence of IP, and with ischaemic preconditioning plus gliclazide at the given concentration, respectively. Similar curves were constructed for MTT reduction.

7.3 RESULTS

All the specimens entering into the studies were included in the analysis.

Effects of glibenclamide and gliclazide on ischaemic preconditioning

Figures 7-IIIA and 7-IIIB show the effects of the sulfonylureas glibenclamide and gliclazide on IP as assessed by CK release and MTT reduction, respectively. SI/R increased CK release substantially (4.4-fold) over that observed in aerobic control

slices, indicative of increased tissue damage (Figure 7-IIIA). Preconditioning with 5 minutes simulated ischaemia followed by five minutes reoxygenation had a protective effect, so that SI/R increased CK release by only 2.2-fold over the aerobic control under these conditions ($p<0.05$ compared to SI/R alone). Glibenclamide abolished protection by IP at all concentrations used (0.1, 1, 3, and 10 μM).

In contrast, gliclazide at 1 μM did not significantly reduce protection by IP. At 10 μM protection was reduced, but not abolished ($p<0.05$ compared to SI/R alone). However, the protective effect of IP was lost at the higher concentrations of gliclazide tested (30 and 100 μM). Figure 7-IIIC shows the dose-response curve for the inhibition of IP protection by gliclazide calculated according to equation (2). The fitted line gives a gliclazide concentration for half-inhibition of 4.5 μM.

Figure 7-IIIB shows that the results obtained from measurements of MTT reduction were essentially similar to those described above for CK release. SI/R reduced MTT reduction from that observed in aerobic controls, consistent with reduced tissue viability, and IP had a protective effect, increasing MTT reduction above the level seen with SI/R alone. As seen for CK release, glibenclamide abolished protection by IP at all concentrations tested, while gliclazide at 1 μM did not significantly reduce protection. At 10 μM protection was reduced, but not abolished, and the protective effect of IP was lost at 30 and 100 μM gliclazide (Figure 7-IIIC). Fitting the dose-response curve (not shown) gave a gliclazide concentration for half-inhibition of 4.8 μM, very close to the value obtained for CK release.

Effect of stimulation of the downstream transduction cascade of preconditioning in the presence of glibenclamide

Figure 7-IVA and 7-IVB show the effect of glibenclamide on protection induced by stimulation of the preconditioning pathway at various stages. As previously reported in Chapter 5, diazoxide (100μM), PMA (1μM) or anisomycin (1nM) resulted in an equivalent reduction in CK leakage and preservation of MTT to that induced by IP itself. Interestingly, however, whilst the protection induced by diazoxide was abolished in the presence of glibenclamide, the protection obtained by PKC activation with PMA or by p38MAPK activation with anisomycin remained unaffected.

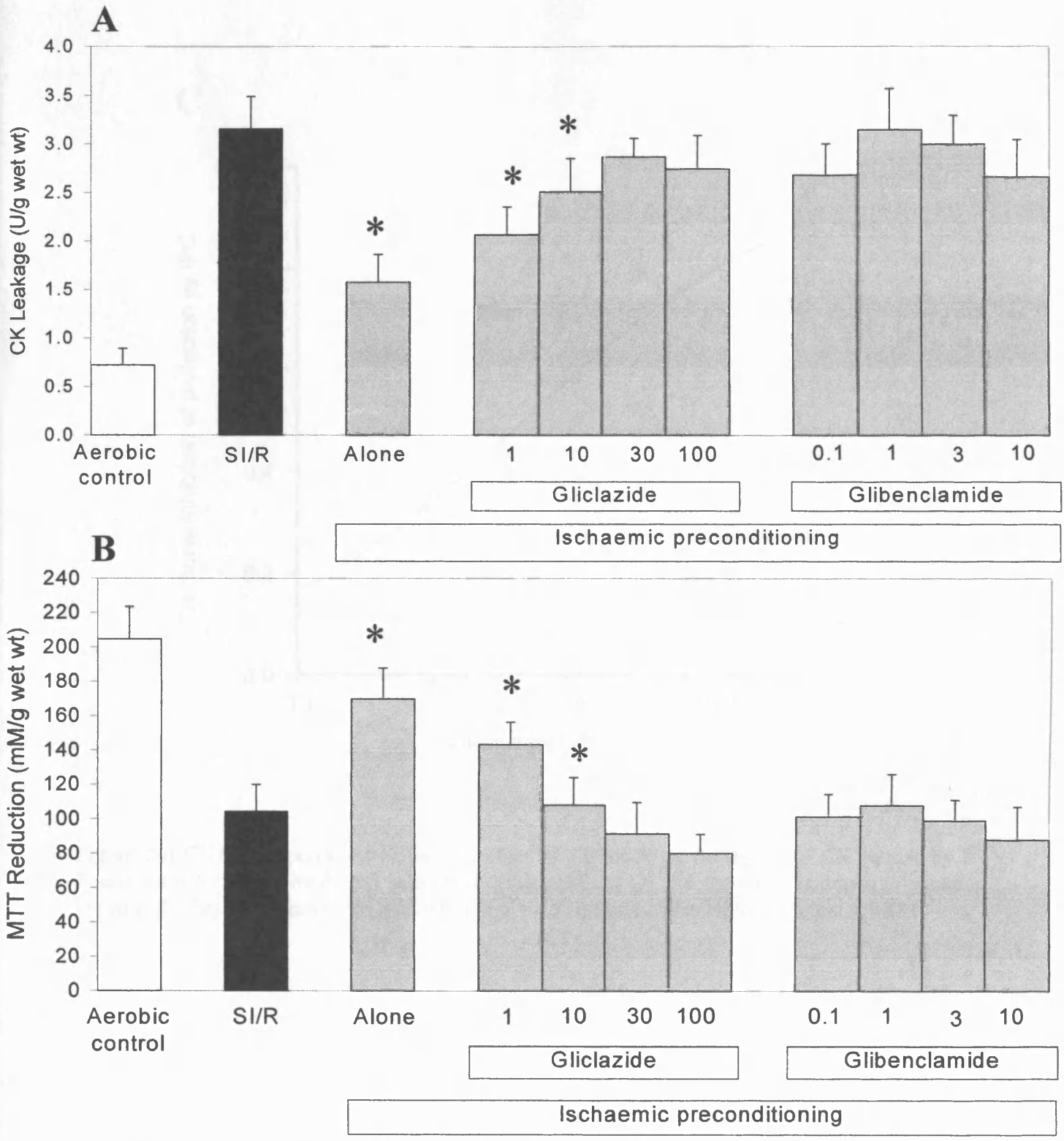

Figure 7-III: (A) Creatine Kinase (CK) leakage into the media during the 120 min reoxygenation period, (B) MTT reduction by the slices at the end of the reoxygenation period. Human atrial myocardium was subjected to the protocols in Study 1 to investigate the dose-response of glibenclamide and gliclazide in μM on ischaemic preconditioning. Data are expressed as mean (±SEM) for n=8 (*$p<0.05$ vs SI/R alone).

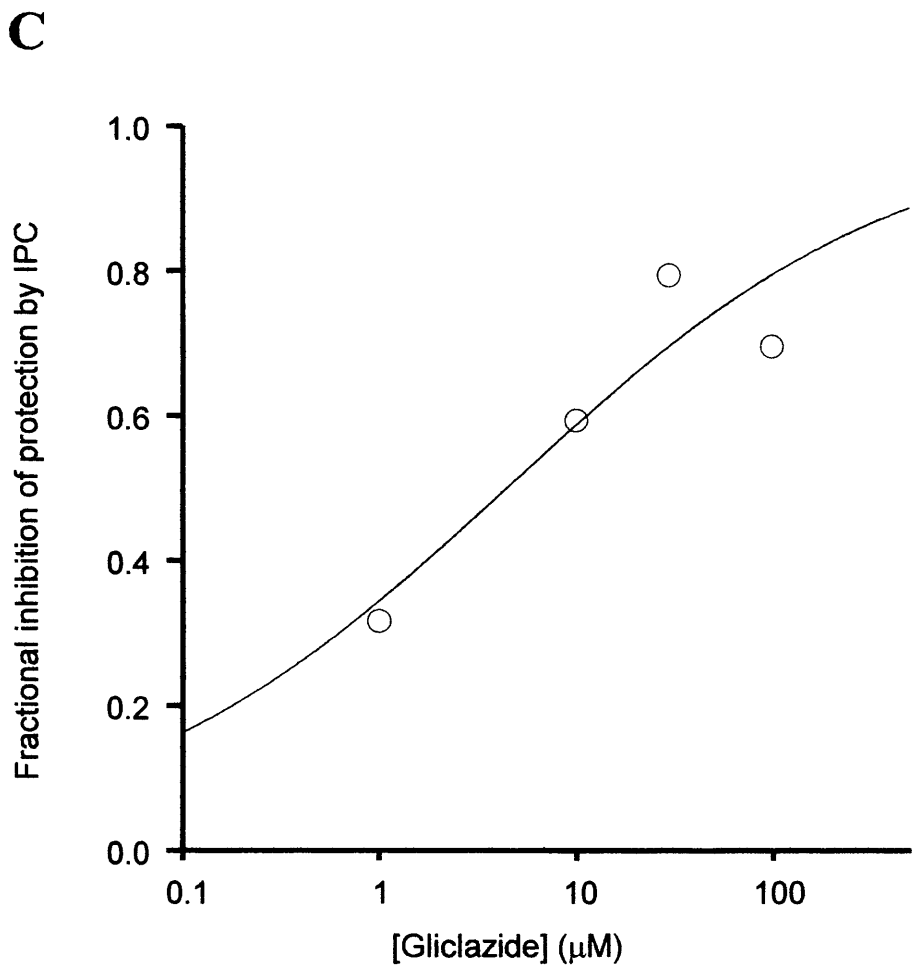

Figure 7-IIIC Dose-response curve for the effect of gliclazide on protection of CK release by IP. Points show fractional inhibition calculated using equation (2) and the curve is drawn to equation (1) with K_i, the concentration for half-inhibition = 4.5 μM and H, the Hill coefficient = 0.431.

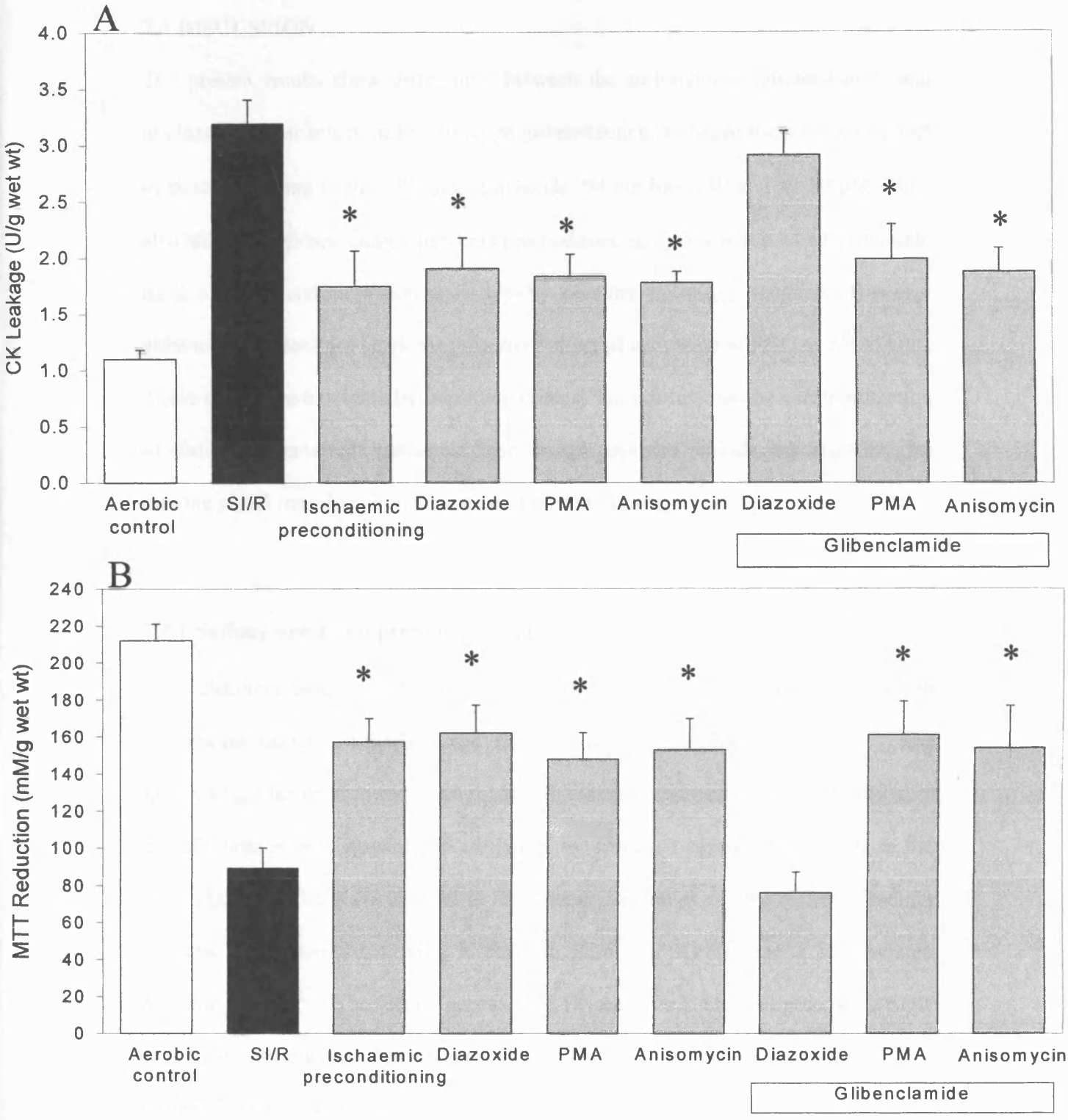

Figure 7-IV: (A) Creatine Kinase (CK) leakage into the media during the 120 min reoxygenation period, (B) MTT reduction by the slices at the end of the reoxygenation period in human atrial myocardium subjected to various protocols in Study 2 to investigate the effect of glibenclamide on the signal transduction mechanism of preconditioning. Data are expressed as mean (±SEM) for n=8. (*$p<0.05$ vs SI/R alone).

7.4 DISCUSSION

The present results show differences between the sulfonylureas glibenclamide and gliclazide in their effect on IP. Although glibenclamide abolished the protective effect of preconditioning even at 0.1 μM, gliclazide did not block IP at 1 or 10 μM. They also show that glibenclamide prevents preconditioning by diazoxide which is thought have a mitochondrial action, possibly by opening mitoK_{ATP} channels. However glibenclamide does not block the protective effect of activation of PKC or p38MAPK. These results have potentially important clinical implications for the cardioprotection of diabetic patients with ischaemic heart disease and also provide important insights into the signal transduction mechanism of preconditioning.

7.4.1 Sulfonylureas and preconditioning

K_{ATP} channels have been demonstrated to be involved in the signal transduction mechanism of both ischaemic and pharmacological preconditioning.[352,471,521] The failure of gliclazide at lower doses to abolish the cardioprotective effect of ischaemic preconditioning may possibly be explained by sequence specific differences in the K_{ATP} channels. The K_{ATP} channel is an octameric complex of two different protein subunits: an inwardly-rectifying K-channel, Kir6.2 or Kir6.1, and a sulfonylurea receptor, SUR.[530,531] The former acts as an ATP-sensitive K-channel pore while SUR is a channel regulator that endows Kir6.2 with sensitivity to drugs such as the inhibitory sulfonylureas and to K-channel openers. K_{ATP} channels in different tissues are composed of different Kir and SUR subunits. The different types of SUR subunit endow the K_{ATP} channels with different sensitivities to various drugs.[524] Thus cloned K_{ATP} channels containing SUR1, the isoform expressed in the pancreas, are blocked by gliclazide with high affinity, whereas channels with the cardiac isoform SUR2A

are not.[532] Similarly, in native tissues gliclazide shows >100-fold selectivity for K_{ATP} channels of native β-cells over sarcolemmal K_{ATP} channels of rat cardiac ventricular myocytes, while in contrast glibenclamide shows similar potency in both tissues.[527] The present results suggest a similar difference between pancreatic and cardiac effects for these two sulfonylureas in human tissue. The doses of glibenclamide and gliclazide were selected to be equipotent in stimulation of insulin secretion, but only supratherapeutic doses of gliclazide blocked preconditioning while all doses of glibenclamide abolished the cardioprotection.

7.4.2 Sequence of the signal transduction of preconditioning

The elucidation of the factors involved in the signal transduction pathway of preconditioning has been the subject of intense investigation and although the participation of factors such as PKC[273,352,481] and K_{ATP} channels[400,521,522] is well established, their relevant sequence of activation remains controversial. Evidence from our laboratory using human tissue shown in Chapter 5 as well as in the rabbit[467] and rat[483] suggests that PKC is in fact downstream of K_{ATP} channels, or at any rate of the stage in the protective pathway which is triggered by diazoxide, while PKC also appears to be upstream of p38MAPK in human and rabbit.[467] The present findings are consistent with this sequence, since the K_{ATP} channel blocker glibenclamide did not prevent cardioprotection by direct pharmacological activation of PKC or p38MAPK.

7.4.3 Clinical implications

The findings from my studies obviously have implications for diabetic patients on sulfonylureas since the results imply that their myocardium can still benefit from the

cardioprotective effect of preconditioning provided that a sulfonylurea that does not block IP at therapeutic doses is used. In terms of gliclazide, plasma levels have been reported to vary between 2.6 and 8 μg/ml. or (8 to 23μM).[533,534,535] Not all the drug is free in solution however and an estimated 95% of the drug will bind to proteins.[536] From these data we estimate peak free gliclazide to be no higher than 0.4 to 1.15μM. If the isolated atrial tissue model we have used corresponds to events that occur in the intact heart in vivo, such concentrations should have minimal effects on preconditioning, and of course the average concentration will in any case be considerably lower than the peak values. Overall, this suggests that gliclazide can be used in patients with ischaemic heart disease without abolishing cardioprotection. It should be emphasized, however, that diabetes *per se* may be an additional cause for the failure to precondition the myocardium, as previously shown by our laboratory,[500] and therefore under some circumstances both diabetes and sulfonylureas could contribute to the abolition of cardioprotection.

Another important implication of the current studies is that it may still be possible to induce the cardioprotective effect of ischaemic preconditioning in the presence of drug-induced mitochondrion-based dysfunction, such as blockade of K_{ATP} channels, by manipulation of the signal transduction pathway further downstream. PMA and anisomycin cannot be used *in vivo*, but it is possible that further research in this area could lead to improved agents that target downstream pathways for cardioprotection.

Having investigated the mechanism of cardioprotection by the first window of preconditioning in the human myocardium and whether this may be affected by agents on clinical use, I turned my attention in the next chapter to the characterization

of the delayed or second window of protection of ischaemic and pharmacological preconditioning and to the investigation of the signal transduction mechanism involved.

Chapter 8

Delayed preconditioning of the human myocardium

8.1 INTRODUCTION

As explained before in this thesis, IP is an inherent protective mechanism that, induced by brief periods of ischaemia and reperfusion, protects the heart against prolonged ischaemic damage.[263] This protection manifests itself in increased resistance to infarction,[263,537,538] decreased reperfusion induced arrythmias[187,539]and contractile dysfunction[540,541] and slowing of adenosine triphosphate decline[262] during ischaemia. This endogenous protective mechanism has been shown to exist in all animal species studied including man.

The cardioprotective effect of ischaemic preconditioning occurs in two phases, the first is immediate and lasts for two to four hours[479] while the delayed or second window of protection occurs at least 24 hours following the initial sub-lethal ischaemic insult and has been shown to last up to 72 hours in certain species.[542] The early or first window of protection has been extensively investigated and there is evidence that the beneficial effect is mediated by Protein Kinase C (PKC),[543] p38 mitogen activated protein kinase (p38MAPK) and ATP-sensitive potassium (K_{ATP}) channels.[544,545] The existence of delayed cardioprotection in the human myocardium has been previously demonstrated in this laboratory,[419] however the second window has not been fully characterised in man and the underlying signal transduction mechanism remains unclear.

The aims of these studies were: (i) to characterise the second window of ischaemic and pharmacological preconditioning using the *in vitro* model of simulated ischaemia and reoxygenation of human atrial myocardium described in Chapter 2, (ii) to examine whether the delayed cardioprotection is elicited in vivo by angina, and (iii) to

investigate the role of mitoK$_{ATP}$ channels, PKC and P38MAPK in the signal transduction mechanism of protection.

8.2 METHODS

8.2.1 Patient Selection and Experimental Preparation

Experiments were performed on muscle obtained from the right atrial appendage of patients undergoing elective coronary artery surgery in the cell necrosis model of simulated ischaemia and reoxygenation described in Chapter 2. Identical exclusion criteria to those used in the previous chapters were used here and the demographic data of the patients included in these studies is shown in Table 8-I

Table 8-I: Demographic data of all the patients included in the studies.

Study	Number of patients	Number of specimens	Male:Female	Age(years)
1	17	64	11:6	65±2.3
2	51	240	38:13	63±5.1
3	15	75	13:2	61±3.8
4	23	104	17:6	64±4.4

8.2.2 Assessment of tissue injury and viability

Tissue viability was assessed by the reduction of MTT to a blue formazan product at the end of the experimental time and tissue injury was determined by measuring the leakage of CK into the incubation medium during the 120min reoxygenation period as described in Chapter 2.

8.2.3 Solutions and Chemicals

The incubation medium was prepared daily with de-ionised distilled water and contained (in mmol/l): $NaCl_2$ (118), KCl (4.8), $NaHCO_3$ (27.2), $MgCl_2$ (1.2), KH_2PO_4 (1.0) $CaCl_2$ (1.25), D-glucose (10), HEPES (20), 10% foetal calf serum and 100μl of gentamicin per 10 mls of solution. Phenylephrine, 5-hydroxydecanoate and anisomycin were used dissolved in de-ionised distilled water, while adenosine, diazoxide and phorbol 12-myristate 13-acetate (PMA) were dissolved in DMSO. Anisomycin is an antibiotic that inhibits protein synthesis and has been demonstrated to activate p38MAPK while PMA is a phorbol ester that is widely used to activate PKC. All the drugs doses were chosen following extensive preliminary dose response experiments. All reagents were obtained from Sigma Chemicals.

8.2.4 Study Protocols

All atrial muscles were allowed to equilibrate under aerobic conditions for 30 minutes prior to being included in a study protocol.

Study 1: To assess the durability and viability of the preparation

Atrial muscles (n=8/group) were aerobically perfused for various periods ranging from 0 to 480 hours.

Study 2: To define the second window of ischaemic and pharmacological preconditioning

The muscles were randomised into one of the following groups (n=8/group) as shown in Figure8-I: (i) aerobic time-matched control, (ii) 90 minutes of simulated ischaemic /120 minutes reoxygenation (SI/R), (iii) IP with 5 minutes of ischaemia and 5 minutes

reoxygenation prior to SI/R, (iv) phenylephrine (0.1μm) for 5 minutes/5 minute washout prior to SI/R and (v) adenosine (100μm) for 5 minutes/ 5 minutes washout prior to SI/R. Samples were aerobically perfused for varying periods of time (0, 12, 24, 48, 72, or 96 hours) following the preconditioning protocol and prior to the 90 minutes of SI /120 minutes R.

Study 3: To study the in vivo effect of angina on delayed preconditioning

In this study atrial appendages were taken from 15 patients undergoing coronary artery bypass surgery who have had a single episode of angina of between 5 and 10 minutes duration prior to surgery. All the patients were in hospital and accurate time of the episode of angina and duration were documented by medical and nursing staff. ECG and cardiac enzymes were taken at the time of angina and patients with evidence of myocardial infarction were excluded. These were taken from patients who had their angina at varying times from 5 hours to 81 hours prior surgery. The atrial appendages from each patient were then sliced and randomly assigned to one of the following groups as shown in Figure 8-II: (i) aerobic perfusion, (ii) 90 minutes SI followed by 120 minutes R, (iii) ischaemic preconditioning with 5 minutes ischaemia/5 minutes reoxygenation prior to SI/R (iv) phenylephrine (0.1μm) for 5 minutes and 5 minutes washout prior to SI/R and (v) adenosine (100μm) for 5 minutes and 5 minutes washout prior to SI/R.

Study 4: To investigate the role of mitoK_{ATP} channels, PKC and p38MAPK in the signal transduction pathway of cardioprotection by delayed preconditioning

To achieve this, the following groups, also shown in Figure 8-III, were studied (n=8/group): (i) 24 hours aerobic perfusion, (ii) 90 minutes SI/120 minutes R, (iii) ischaemic preconditioning with 5 minutes ischaemia/5 minutes reoxygenation, (iv) phenylephrine (0.1μm) for 5 minutes/washout for 5 minutes, (v) diazoxide (100μm) for 10 minutes, (vi) PMA (1μm) for 10 minutes, (vii) anisomycin (1nm) for 10 minutes (viii) 5-hydroxydecanoate (100 μm) for 10 minutes prior to ischaemic preconditioning, (ix) chelerythrine (10μm) for 10 minutes prior to ischaemic preconditioning, (x) SB 203508 (10μm) for 10 minutes prior to ischaemic preconditioning, (xi) 5-hydroxydecanoate (100μm) for 10 minutes prior to preconditioning with phenylephrine,(xii) chelerytherine (10μm) for 10 minutes prior to preconditioning with phenylephrine, (xiii) SB 203508 (10μm) for 10 minutes prior to preconditioning with phenylephrine. In groups (ii) to (xiii) each intervention was followed by 24 hours of aerobic perfusion prior to 90 minutes SI/120 minutes R.

8.2.5 Statistical analysis

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

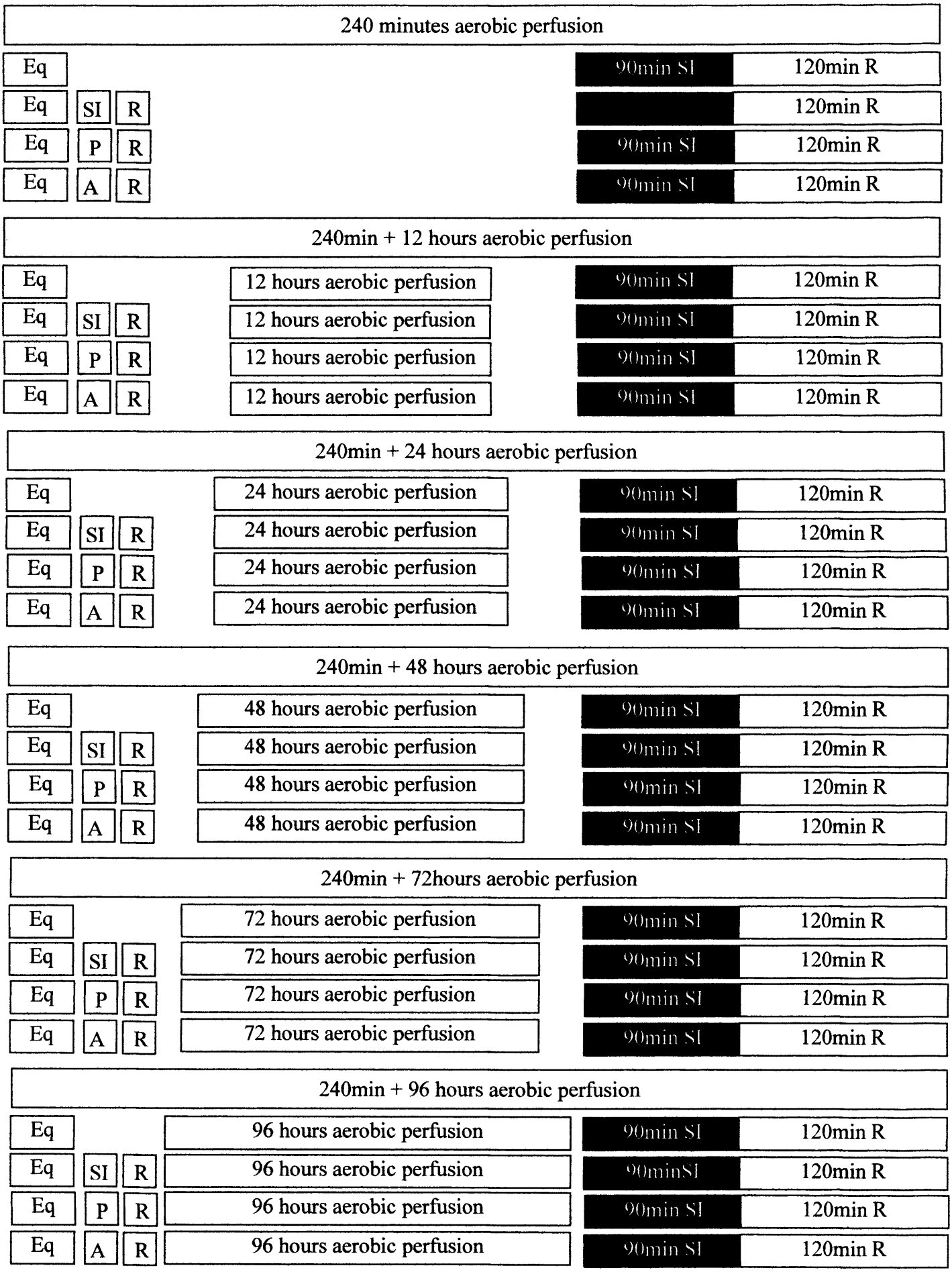

Figure 8-I: Schematic representation of the protocol for study 2. Eq: 30 minutes equilibration, SI: simulated ischaemia, R: reoxygenation, P: phenylephrine, A: adenosine.

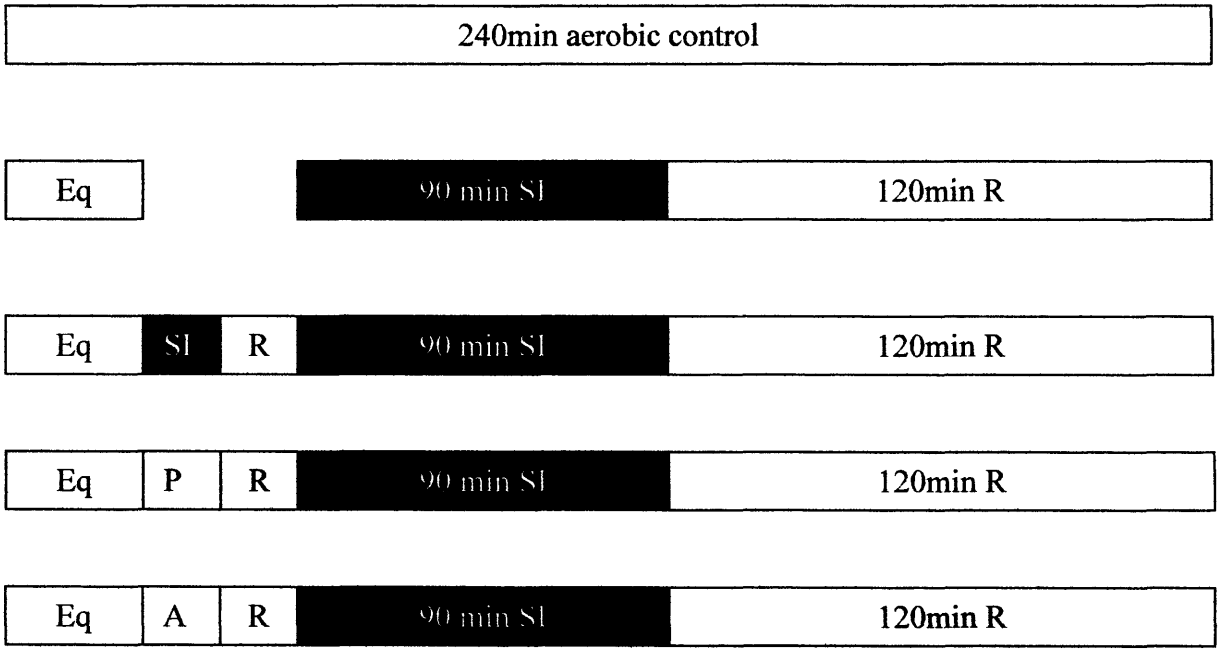

Figure 8-II Schematic representation of the protocol for study 3. Eq: 30 minutes equilibration, SI: simulated ishaemia, R: reoxygenation, P: phenyephrine, A: adenosine.

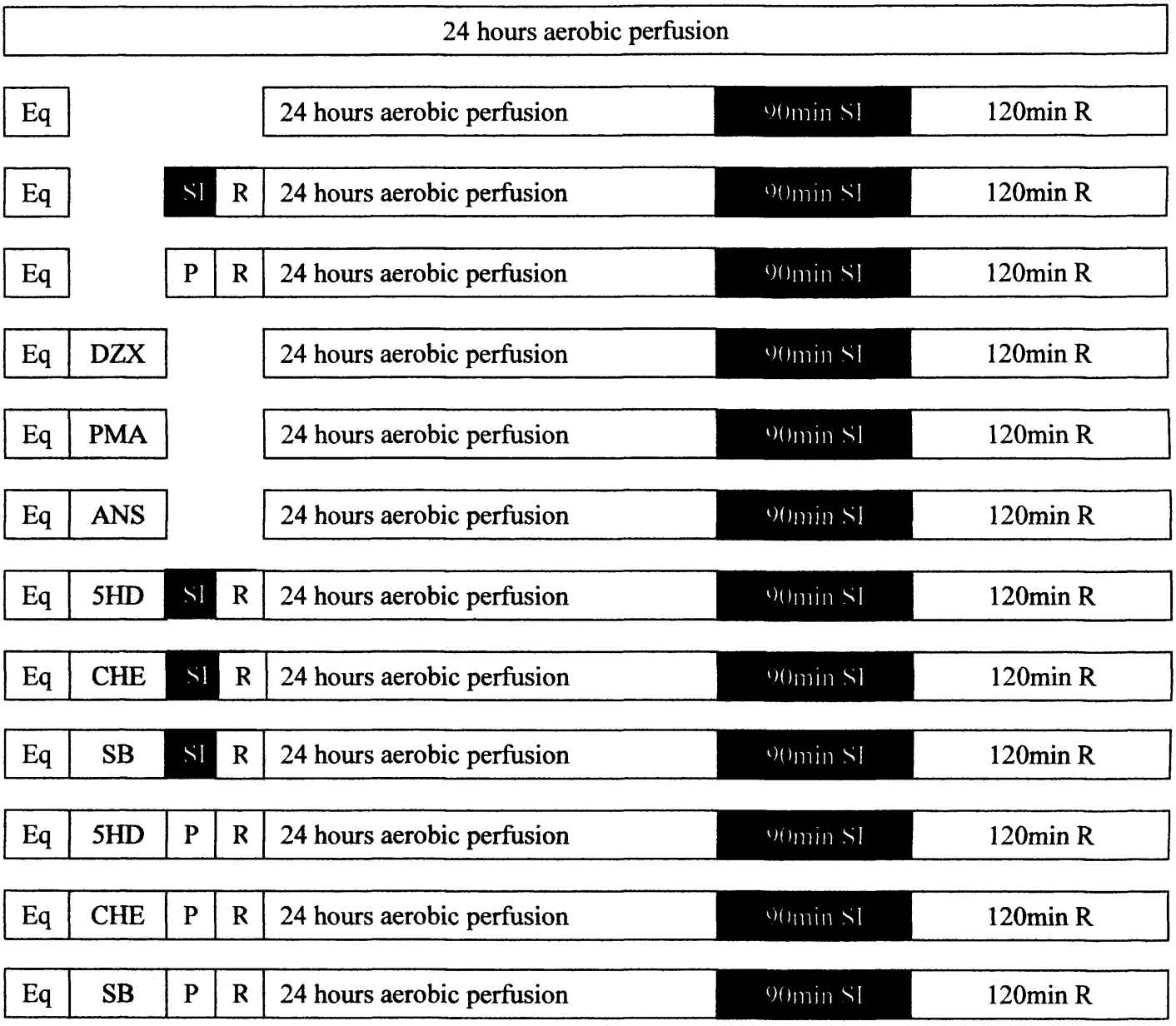

Figure 8-III: Schematic representation of the protocol for study 4. Eq: 30 minutes equilibration, SI: simulated ischaemia, R: reoxygenation, P: phenylephrine, DZX: diazoxide, PMA: phorbol 12 myristate 13-acetate, ANS: anisomycin, 5HD: 5-hydroxydecanoate, CHE: chelerythrine, SB: SB203580.

8.3 RESULTS

All specimens that were randomised and entered the study were included in the analysis.

Study 1; Durability and viability of the preparation:

As shown in Figure 8-IV, the mean MTT values for the first 12 days were similar to those observed in fresh muscle and in the muscle aerobically incubated for only 30 min; however, MTT was significantly reduced by more than half of the fresh muscles by 21 days of aerobic incubation. These results suggest that in this preparation the myocardial tissue remains viable for at least 12 days

Study 2; Characterization of the second window of protection:

Figure 8-VA shows that SI/R resulted in a significant decrease in MTT mean values and that both ischaemic and pharmacological preconditioning with phenylephrine or adenosine resulted in significant early protection (0 hours between preconditioning and SI/R – first window). Protection was lost 12 hours after the preconditioning intervention but this was regained by 24 hours, it was maintained up to 72 hours and then it was lost beyond this period. A mirror image of the MTT results were observed with the CK leakage values that are shown in Figure 8-VB, for the first 24 hours; however beyond this period CK release fell sharply in all study groups including the aerobically incubated group. Our laboratory has previously demonstrated[405] that in this in vitro atrial muscle preparation CK is continuously released during aerobic incubation of the tissue and that therefore measurements of CK leakage into the tissue may not be an appropriate index of tissue injury beyond 24 hours of incubation.

Study 3; Delayed preconditioning by angina in vivo:

Figures 8-VIA and 8-VIB show the individual MTT and CK leakage values of atrial muscles from patients having angina prior to surgery and subjected to various protocols of ischaemic and pharmacological preconditioning. The results demonstrate that the muscles from patients presenting with angina ≤ 20 hours of surgery were not preconditioned and that preconditioning with ischaemia and with phenylephrine and adenosine elicited similar cardioprotection. They also show that between 29 and 70 hours of the episode of angina the muscles are preconditioned and that preconditioning either with ischaemia or pharmacologically with phenylephrine or adenosine does not confer additional protection as reflected by the MTT and CK values. However, protection was lost beyond 70 hours of the angina episode and the tissue could be preconditioned again with ischaemia or pharmacologically.

Study 4; The role of mitoK_{ATP} channels, PKC and P38MAPK in delayed preconditioning:

Figures 8-VIIA and 8-VIIB show the results for the MTT reduction and the CK leakage and demonstrate that preconditioning with ischaemia and pharmacologically with phenylephrine have a similar protection of the myocardial tissue at 24 hours. This protective effect was matched by opening of mitoK_{ATP} channels with diazoxide and by activation of PKC with PMA or P38MAPK with anisomycin and blockade of each of these three factors resulted in the loss of the cardioprotective effect.

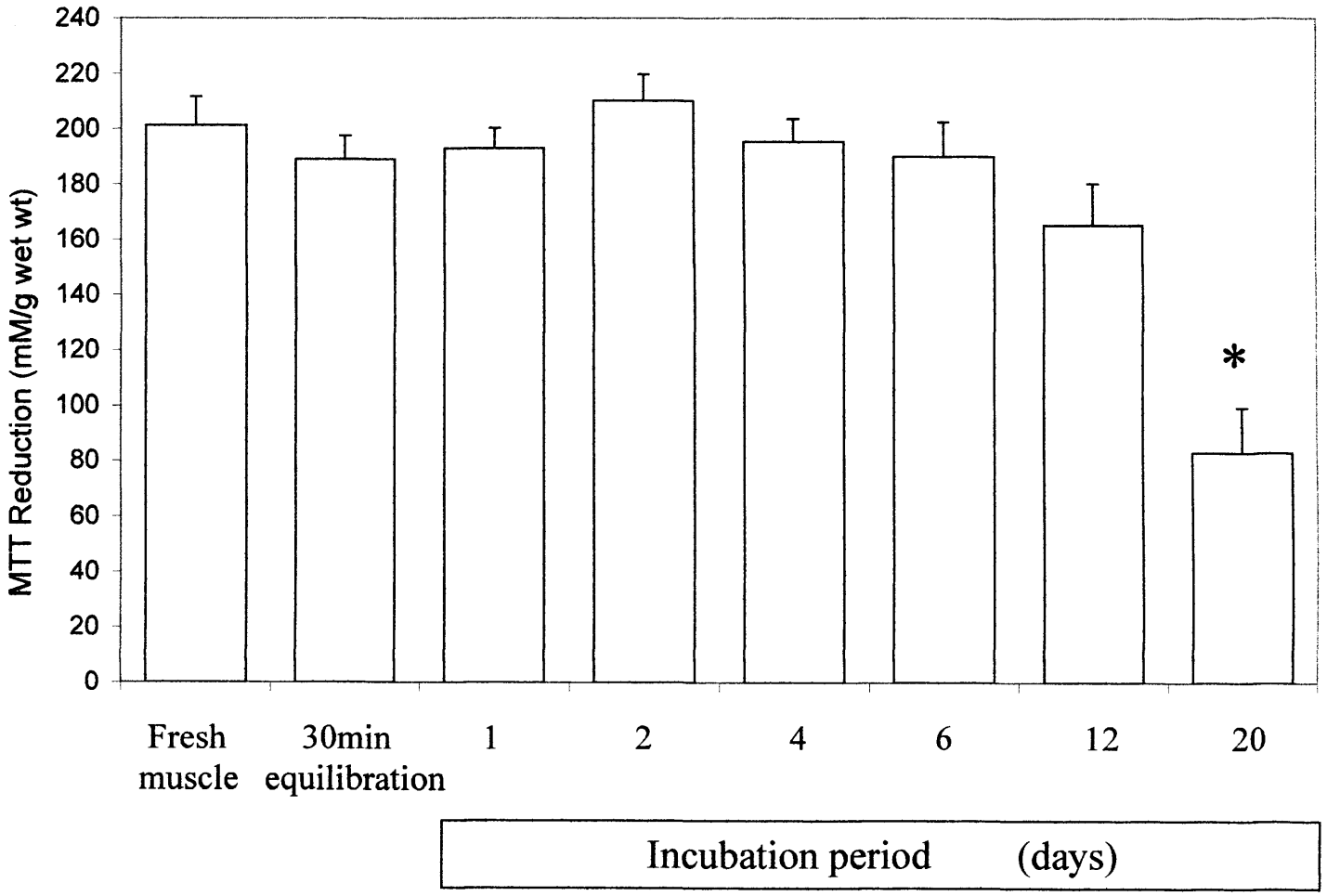

Figure 8-IV: MTT reduction in atrial myocardium at the end of increasing periods of aerobic incubation.

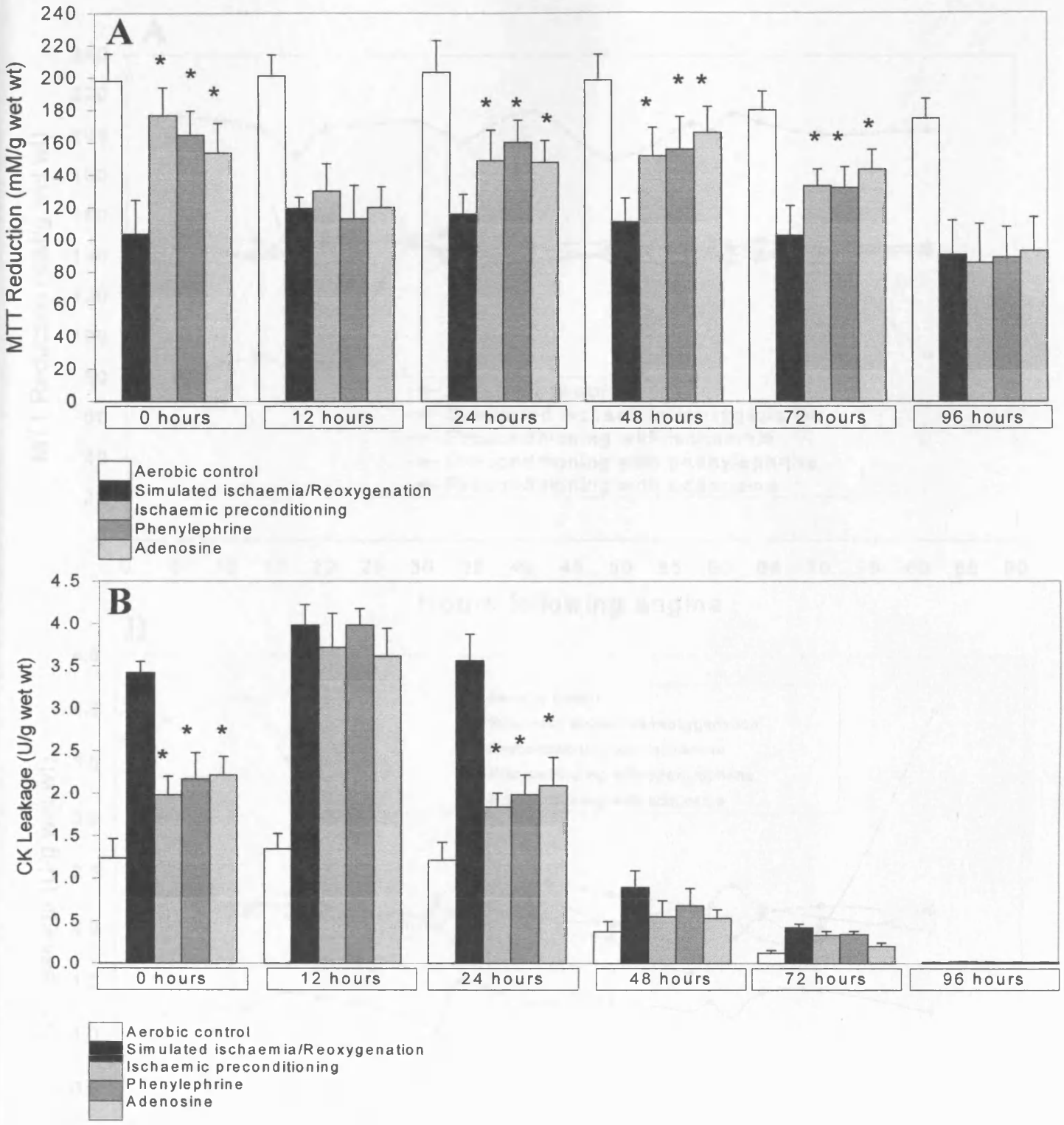

Figure 8-V: MTT reduction at the end of the reoxygenation period (A) and creatine kinase (CK) leakage into the media (B) during the 120 min reoxygenation period in human atrial myocardium subjected to various protocols (see text for details) to define the second window of ischaemic and pharmacological preconditioning. Data are expressed as mean ±SEM of eight experiments. *$p<0.05$ vs. corresponding simulated ischaemia/reoxygenation alone group.

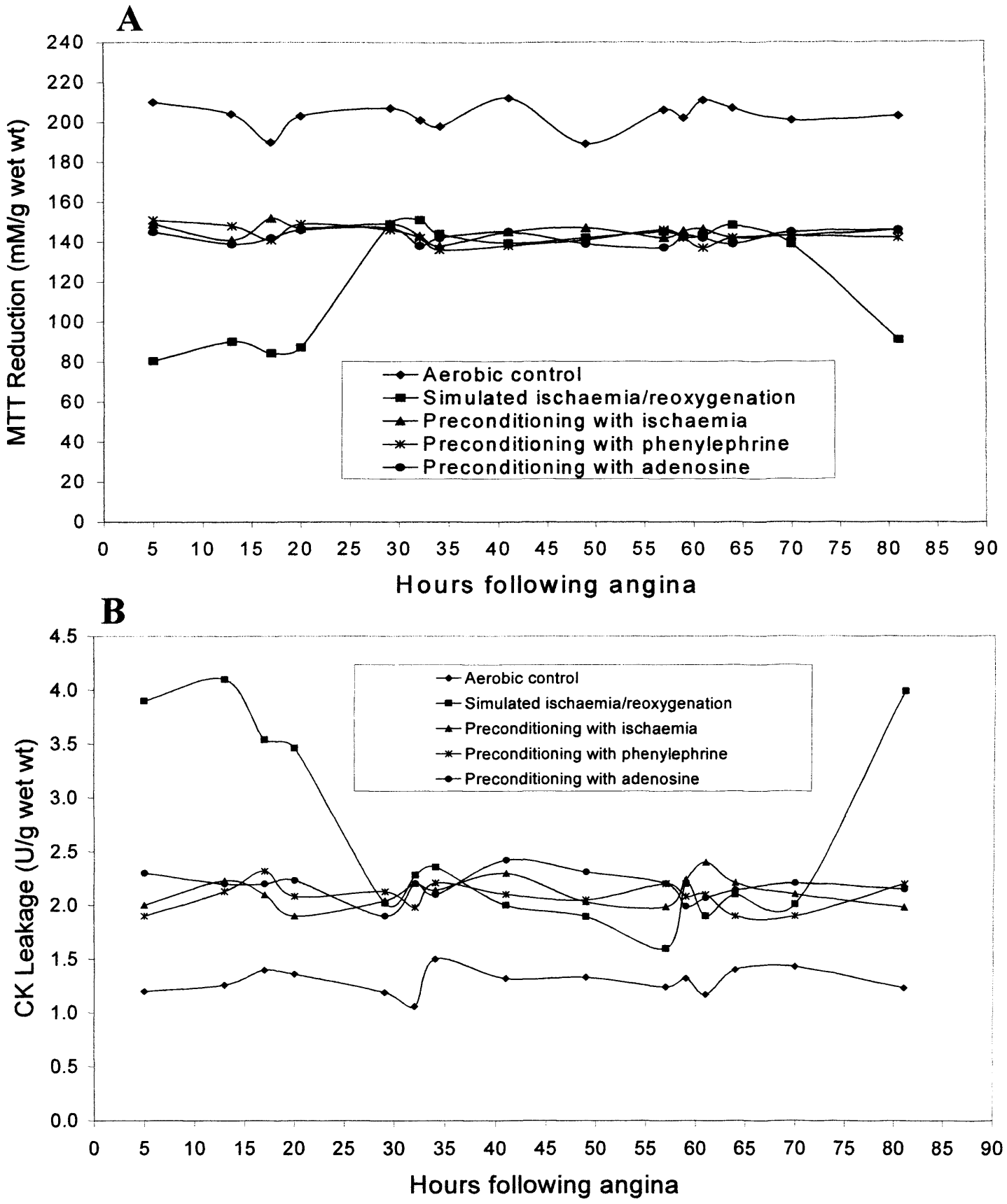

Figure 8-VI: MTT reduction by the slices at the end of the reoxygenation period (A) and creatine kinase (CK) leakage into the media (B) during the 120 min reoxygenation period by human atrial myocardium subjected to various protocols (see text for details) to study the effect of angina on preconditioning). Every time point represents the muscle from one patient subjected to the various protocols.

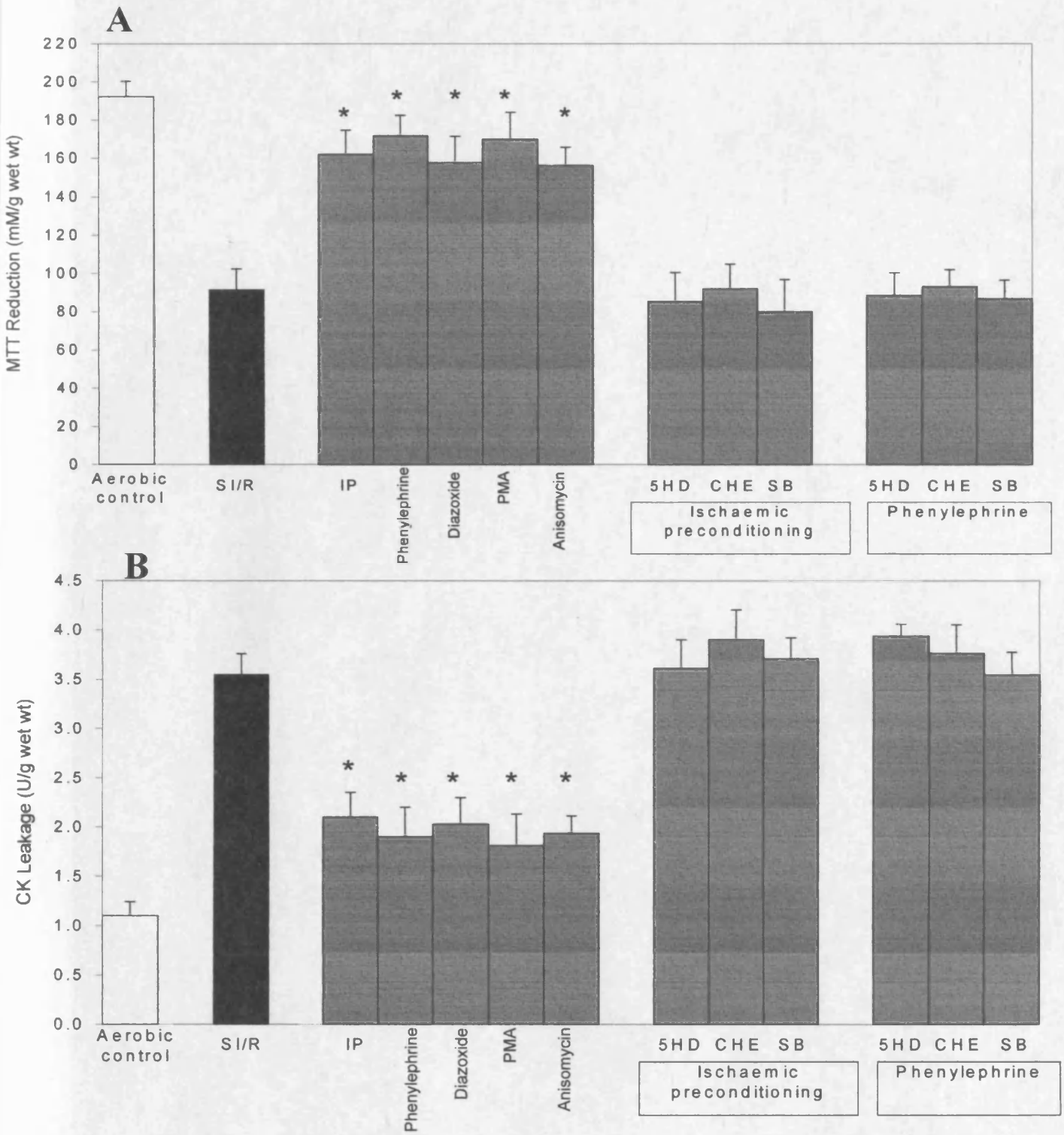

Figure 8-VII: MTT reduction at the end of the reoxygenation period (A) and creatine kinase (CK) leakage into the media (B) during the 120 min reoxygenation period by human atrial myocardium subjected to various protocols (see text for details) to investigate the signal transduction of preconditioning. Data are expressed as mean ±SEM of eight experiments. *$p<0.05$ vs. simulated ischaemia/reoxygenation alone group. SI/R: simulated ischaemia/reoxygenation, IP: ischaemic preconditioning, 5HD: 5-hydroxydecanoate, CHE: chelerythrine, SB: SB203580.

8.4 DISCUSSION

The present studies have demonstrated that ischaemic and pharmacological preconditioning equally elicit a delayed or second window of protection in the human myocardium that lasts between 24 and 72 hours following the preconditioning stimulus. They have also shown that the occurrence of angina mimics the delayed protection conferred by ischaemic and pharmacological preconditioning and that mitoK_{ATP} channels, PKC and p38MAPK are essential components of the signal transduction mechanism of this delayed protection. The clinical importance and the contribution of these results to the understanding of the mechanism underlying the delayed protection of preconditioning warrant further discussion.

8.4.1 The delayed phase of preconditioning

A previous report from our laboratory[419] using a similar but not identical *in vitro* preparation to the one used in the present studies and another study from Arstall et al[271] using foetal cardiomyocytes have shown evidence of delayed cardioprotection in the human myocardium. Here I have now fully characterized for the first time the phenomenon of delayed protection in man and shown that this is confined to a period between 24 and 72 hours following the preconditioning stimulus. The previous findings in this laboratory[419] showed that the beneficial effect of the second window of preconditioning was not as potent as the protection of the first window and this contrast with the present studies demonstrating that the early and delayed protections of preconditioning are equipotent as suggested by the results on MTT and CK leakage. A possible explanation for the difference between the two studies may be that in the current investigations the incubation medium was changed every twelve hours and it was supplemented with foetal calf serum, which may have made the

preparation more stable. Furthermore the addition of antibiotics to the medium may have prevented the growth of bacteria and this also may have contributed to a more stable preparation. However, the controversy is further fuelled by the observation that in the infarct size model the delayed protection is less effective than the early window in the rabbit[327] and in the dog.[546] Indeed additional studies may be required to elucidate this issue.

It is worth noting that the delayed cardioprotection elicited in the human myocardium by pharmacological preconditioning with adenosine and phenylephrine exhibited a similar potency and identical window of protection to that of preconditioning with ischaemia. Experimental evidence for a role of adenosine and phenylephrine in delayed cardioprotection has been found in the rabbit[546] and in the mouse.[547] In the present studies we only investigated the role of adenosine and phenylephrine, however it is likely that other triggers such as reactive oxygen species,[548] nitric oxide,[549] bradykinin,[550] opioids[551] and prostanoids,[552] which have been shown to play a role in the delayed protection in animal studies, would also be operative in the human myocardium.

It should be clarified that although the benefit on the MTT results for the entire second window were similar to those seen in the first window, the CK leakage values declined when muscles were incubated for periods longer than 24 hours. This pattern of CK release is probably a consequence of the constant enzyme leakage into the incubation media[405] and the resultant gradual lower tissue content. Therefore, CK values beyond the 24 hours incubation period may not represent the degree of the ischaemic insult to which the muscle is subjected in this preparation.

8.4.2 Preconditioning with angina

My finding that an episode of angina results in delayed preconditioning of the atrial myocardium against an ischaemic insult as denoted by the assessment of CK leakage and MTT reduction is supported by the demonstration that angina preceding myocardial infarction by 24 hours results in limitation of the infarct size.[553] Cardioprotection was absent when the ischaemic insult was induced between 5 and 20 hours of the episode of angina, it was present between 29 and 70 hours after the angina and it was again lost beyond this period. In spite of the failure of angina to precondition outside this well-defined time period, the muscles maintained the potential to become protected by the acute application of ischaemic preconditioning and by the administration of adenosine and phenylephrine. The results also show that once the protection is obtained by one of the preconditioning stimuli the application of additional preconditioning triggers does not lead to an increased level of protection. It has been reported, however, that the protection conferred by ischaemic preconditioning may be enhanced when combined with adenosine in sheep hearts.[554] If ischaemic and pharmacological preconditioning are using identical transduction pathway, it is difficult to accept that combination of the two treatments results in additional cardioprotection. Therefore the most probable explanation for the results of the latter study is that the IP protocol was insufficient to elicit maximal protection and that this was only obtained when the two interventions, IP and adenosine, were applied in combination. The results of this study are however, limited by the small number of patients included in the study and that only one patient was used for each time point. Furthermore only one patient was included beyond the 74 hours.

8.4.3 Signal transduction mechanism

My demonstration that similar cardioprotection to the one obtained with ischaemic and pharmacological preconditioning can be achieved by opening the mitoK$_{ATP}$ channels and by activating PKC and p38MAPK and that blockade of any of these three factors abrogates protection suggests that all three are essential components of the signal transduction pathway of the delayed or second window preconditioning in the human myocardium. A role for the mitoK$_{ATP}$ channels in the delayed protection of preconditioning has also been shown in rabbits[555,556] and a participation of PKC and p38MAPK has also been suggested in the rabbit[557,558] and in the dog.[559,560] I have previously shown in this thesis that the three factors are also essential components of the early or first window of preconditioning thus suggesting that the signal transduction pathways of the first and second window of preconditioning may be identical in man. What remains to be explained is the mechanism by which protection is lost between the first and second windows of preconditioning while still maintaining the potential for preconditioning with a new ischaemic or pharmacological stimulus. The fact that delayed preconditioning can be abolished by blockade of the signal transduction pathway before the start of the prolonged ischaemic insult suggests that the end effector(s) of cardioprotection is(are) activated at some stage after the initiation of ischaemia which challenges the view that production of new proteins is the cause for the delayed protection.[561,562] A possible explanation for the loss of cardioprotection between the two windows of preconditioning may be the production of some factor(s), also triggered by the preconditioning stimulus, that would counteract transiently the action of some of the components of the signal transduction pathway and this could include the end-effector(s). However, any potential mechanism for this loss of cardioprotection can be

overcome by the application of a new preconditioning stimulus. The elucidation of the cause of this temporal loss of cardioprotection has important clinical implications and would require further investigation.

Chapter 9

Conclusions and future direction

9.1 Conclusions

The present studies have demonstrated that α_1-adrenoceptors play an important role in the ischaemia/reoxygenation-induced injury of the human atrial myocardium. Thus, they show that stimulation of α_1-adrenoceptors with phenylephrine protects against injury whereas their blockade with prazosin is detrimental, both effects obtained in a dose-dependent manner. They have also shown that the effect of the stimulation or blockade of α_1-adrenoceptors depends on the time of administration so that α_1-adrenoceptors' stimulation is protective when given prior to ischaemia but detrimental when given during ischaemia, and on the contrary, α_1-adrenoceptors' blockade is beneficial during ischaemia, detrimental during reoxygenation and has no significant effect prior to ischaemia. It appears that similar maximal protection can be obtained with α_1-stimulation prior to ischaemia and with α_1-blockade during ischaemia although the combination of the two does not induce additional protection. Furthermore, the protective effect of α_1-stimulation prior to ischaemia is as potent as ischaemic preconditioning. These studies are the first in dissecting the role of α_1-adrenoceptors during ischaemia and reoxygenation of the human myocardium.

The present studies have also shown that protection with pharmacological preconditioning by activation of α_1-adrenoreceptors or adenosine receptors is identical to that of ischaemic preconditioning in the human myocardium. These studies demonstrated that mitoK_{ATP} channels, PKC and p38MAPK are an integral part of the cellular signal transduction involved in this cardioprotection in which mitoK_{ATP} channels are placed upstream and p38MAPK is placed downstream of PKC. This

provides novel information to understand the underlying mechanism of protection by preconditioning of the human myocardium.

The long-term administration of nicorandil, a mitoK$_{ATP}$ channel opener and nitric oxide donor, abolishes the ability of the human myocardium to be protected by ischaemic and pharmacological preconditioning without exacerbating the susceptibility to ischaemic injury. In addition, I have shown in this thesis that the likely cause of the failure to precondition the myocardium of patients on nicorandil is the unresponsiveness of the mitoK$_{ATP}$ channels since protection cannot be obtained with diazoxide, a specific mitoK$_{ATP}$ channel opener, but can be elicited by activation of PKC and p38MAPK that are downstream of mitoK$_{ATP}$ channels in the signalling transduction cascade of preconditioning.

My results show differences between the sulfonylureas glibenclamide and gliclazide in their effect on IP. Although glibenclamide abolished the protective effect of preconditioning even at 0.1 μM, gliclazide did not block ischaemic preconditioning at 1 μM. I have also shown that glibenclamide prevents preconditioning by diazoxide which is thought have a mitochondrial action, possibly by opening mitoK$_{ATP}$ channels. However glibenclamide does not block the protective effect of activation of PKC or p38MAPK. These results have potentially important clinical implications for the cardioprotection of diabetic patients with ischaemic heart disease and also provide important insights into the signal transduction mechanism of preconditioning.

The present studies have demonstrated that ischaemic and pharmacological preconditioning equally elicit a delayed or second window of protection in the human myocardium that lasts between 24 and 72 hours following the preconditioning stimulus. They have also shown that the occurrence of angina mimics the delayed protection conferred by ischaemic and pharmacological preconditioning and that mitoK$_{ATP}$ channels, PKC and p38MAPK are essential components of the signal transduction mechanism of this delayed protection.

9.2 Future direction

Fifteen years of extensive research and publication of in excess of 1500 papers in the field of ischaemic preconditioning have vastly extended our understanding of the mechanisms underlying the pathogenesis of ischaemia-reperfusion injury. There can be little doubt that the elucidation of the pathophysiology and the cellular mechanisms of the phenomenon of ischaemic preconditioning have taught us the means of protecting the myocardium in the experimental setting. Clinical studies in this field, while fraught with limitations, have pointed to the fact that the human myocardium may respond in a way similar to that seen in the experimental laboratory and may be amenable to protection by ischaemic preconditioning. It is expected that this evidence will translate into clinical reality to benefit patients with coronary artery disease.

A number of studies in routine (low-risk) patients have been performed with the aim of proving the concept of pharmacological preconditioning in humans and to establish the safety and tolerability of these agents using indirect end points to detect myocardial ischaemia, small differences in myocardial viability, and extent of micronecrosis. These findings provide some basis for optimism that a beneficial and

clinically detectable improvement in myocardial protection may be possible. However, this goal can only be achieved when carefully designed clinical studies using hard end points of clinical outcome have been undertaken in appropriate subsets of patients at short-term risk of coronary artery occlusion. These studies have as yet not been undertaken. In my opinion, whereas further research in the basic laboratory continues to identify the next steps in the signalling cascade mediating myocardial preconditioning, it is timely that large-scale trials of high-risk patients at multiple centres were performed with the currently available preconditioning-mimetic agents, with comparisons against pre-existing myocardial protective strategies. Such studies need to focus on the high-risk groups of patients with particular emphasis on those subsets with features predictive of a worse outcome, who stand to gain the most benefit from additional cardioprotective strategies. The cohorts randomised in these studies may include patients with non-ST-elevation ACS presenting with persistent ST-segment depression on ECG, elevated serum troponin levels, or impaired left ventricular function, whether treated medically or with early revascularization. These patients must be randomised to preconditioning-mimetic agents versus placebo, in addition to standard therapy, and evaluated in terms of robust end points of clinical outcome. Similarly, high-risk patients undergoing elective revascularization procedures need to be included in studies evaluating the clinical efficacy of preconditioning-mimetic treatments in terms of reduction in periprocedural infarct size, heart failure, and mortality. It is only with demonstration of improved outcome in such large-scale studies that the past 15 years of research may translate into a clinical reality. To this end, it is also important that the role of the kinases shown to participate in cardioprotection by preconditioning are fully elucidated and that the end-effector(s) or protection identified.

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