ADENOSINE A\textsubscript{1} RECEPTOR INDUCED DELAYED PRECONDITIONING: INVESTIGATION OF THE TIME COURSE AND CELLULAR MECHANISMS

A thesis presented by

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INTRODUCTION: Ischaemic heart disease is the leading cause of mortality and morbidity in the Western world. The possibility of exploiting an innate adaptive mechanism to protect ischaemic myocardium has generated considerable excitement and enthusiastic research. Brief periods of ischaemia induce subacute myocardial protection against subsequent ischaemic injury, a phenomenon termed delayed preconditioning which appears to be mediated by adenosine. The temporal profile and the cellular mechanisms underlying this delayed adaptation in experimental animals and in patients with coronary artery disease were the subject of this thesis.

METHODS AND RESULTS: In experimental studies, delayed preconditioning was induced in in vivo rabbit and in vitro rat models of regional myocardial ischaemia-reperfusion with the selective adenosine A₁ receptor (A₁R) agonist 2-chloro-N⁶-cyclopentyladenosine (CCPA). Infarct size expressed as a percentage of risk zone was nearly halved by prior intermittent A₁R activation with CCPA (5 doses at 48h intervals) implying that animals had been maintained in a preconditioned state over 10 days. Using selective inhibitors, it was demonstrated that A₁R induced delayed preconditioning is dependent on a signalling mechanism involving protein kinase C (PKC) and tyrosine kinases (TKs). Downstream of these kinases, A₁R activation induced subacute activation of p38 MAPK, phosphorylation of Hsp27 and enhanced expression of Mn-SOD, implying involvement of these cytoprotective proteins in mediating delayed protection. No role was demonstrated for a nitric oxide-dependent pathway in induction of protection. Delayed myocardial protection, in terms of enhanced tolerance to ischaemia during exercise was demonstrated in patients with coronary artery disease. This adaptation was independent of adenosine.

CONCLUSIONS: These results suggest that the cellular events downstream of A₁R involve both PKC and TKs which in turn, results in phosphorylation/activation of Hsp27, and enhanced activity of Mn-SOD, two potential end-effectors of delayed cardioprotection. The exercise study is the first to demonstrate delayed preconditioning in man. The ability to maintain myocardium in a protected state over several days suggests that A₁R activation may hold promise as a new approach to long-term cardioprotection in patients at increased risk of myocardial infarction.
ACKNOWLEDGEMENTS

During the course of this research, I was supported by a junior research fellowship from British Heart Foundation. I am very grateful to BHF for making this thesis possible.

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Finally, I would like to thank Negin for her love, understanding and continued support, which made the rocky road of the past year seem almost easy.
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LIST OF PUBLICATIONS

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Original Articles

Dana A, Baxter GF, Walker JM, Yellon DM. Prolonging the delayed phase of myocardial protection: repetitive adenosine $A_1$ receptor activation maintains rabbit myocardium in a preconditioned state. *J Am Coll Cardiol* 1998; 31: 1142-1149.


Dana A, Baxter GF, Yellon DM. Delayed preconditioning induced by an adenosine $A_1$ receptor agonist in rabbits is independent of early generation of nitric oxide or late induction of NOS. *J Cardiovasc Pharmacol* 2001; In press.

Reviews


Editorials


**Book Chapters**


**Abstracts**

**Dana A, Baxter GF, Walker JM, Yellon DM.** Prolonging the delayed phase of myocardial protection: anti-infarct effects of adenosine A₁ receptor activation are maintained despite repetitive dosing. *Heart* 1997; 77: 23.

**Dana A, Baxter GF, Walker JM, Yellon DM.** The delayed phase of myocardial protection can be extended by intermittent adenosine A₁ receptor activation in the rabbit. *Eur Heart J* 1997; 18: 566.

**Dana A, Baxter GF, Walker JM, Yellon DM.** Prolonging the delayed phase of myocardial protection: Repetitive adenosine A₁ receptor activation maintains rabbit myocardium in a preconditioned state. *Circulation* 1997; 96: 1-257.


**Dana A, Yellon DM.** Adenosine induced delayed cardioprotection: Investigation into time-course and cellular mechanisms. *Heart* 1999; 81: P14 (Young Research Workers’ Prize Section).

**Dana A, Yellon DM.** Adenosine A₁ receptor induced delayed cardioprotection: Investigation into time-course and cellular mechanisms. *J Mol Cell Cardiol* 1999; 31: A101 (Young Investigator Prize Section).


# LIST OF ABBREVIATIONS

The following is a list of abbreviations used in this thesis:

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<th>Abbreviation</th>
<th>Description</th>
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<td>%</td>
<td>percentage</td>
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<tr>
<td>°C</td>
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</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µl</td>
<td>microlitre</td>
</tr>
<tr>
<td>µM</td>
<td>micromolar</td>
</tr>
<tr>
<td>As0.005nm</td>
<td>absorbance measured at 560 nm</td>
</tr>
<tr>
<td>A1R</td>
<td>adenosine A₁ receptor</td>
</tr>
<tr>
<td>A2R</td>
<td>adenosine A₂ receptor</td>
</tr>
<tr>
<td>A3R</td>
<td>adenosine A₃ receptor</td>
</tr>
<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
</tr>
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<td>ADP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AG</td>
<td>aminoguanidine</td>
</tr>
<tr>
<td>AMI</td>
<td>acute myocardial infarction</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulphate</td>
</tr>
<tr>
<td>AS-ODN</td>
<td>antisense oligodeoxyynucleotide</td>
</tr>
<tr>
<td>ATF-2</td>
<td>activating transcription factor-2</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CABG</td>
<td>coronary artery bypass graft surgery</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CCPA</td>
<td>2-chloro-N⁶-cyclopentyladenosine</td>
</tr>
<tr>
<td>CF</td>
<td>coronary flow</td>
</tr>
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<td>CHE</td>
<td>chelerythrine chloride</td>
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<tr>
<td>CK</td>
<td>creatine kinase</td>
</tr>
<tr>
<td>Cu/Zn-SOD</td>
<td>copper/zinc superoxide dismutase</td>
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<tr>
<td>DAG</td>
<td>diacylglycerol</td>
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<td>ECG</td>
<td>electrocardiogram</td>
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<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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<td>-----------</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid disodium salt</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular-signal-related kinase</td>
</tr>
<tr>
<td>ET-1</td>
<td>endothelin-1</td>
</tr>
<tr>
<td>Gi</td>
<td>pertussis-sensitive inhibitory G-protein</td>
</tr>
<tr>
<td>Gs</td>
<td>pertussis-sensitive stimulatory G-protein</td>
</tr>
<tr>
<td>GSH</td>
<td>reduced glutathione</td>
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<tr>
<td>5HD</td>
<td>sodium 5-hydroxydecanoate</td>
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<td>H2O2</td>
<td>hydrogen peroxide</td>
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<td>HR</td>
<td>heart rate</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
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<td>heat stress</td>
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<td>HSP</td>
<td>heat stress protein</td>
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<tr>
<td>I</td>
<td>infarct volume</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focussing</td>
</tr>
<tr>
<td>i.m.</td>
<td>intra-muscular</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
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<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol triphosphate</td>
</tr>
<tr>
<td>I-R</td>
<td>ischaemia-reperfusion</td>
</tr>
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<td>ISEL</td>
<td>in situ end-labelling</td>
</tr>
<tr>
<td>i.v.</td>
<td>intra-venous</td>
</tr>
<tr>
<td>JNK</td>
<td>c-jun N-terminal kinase</td>
</tr>
<tr>
<td>K(_{\text{ATP}}) channel</td>
<td>ATP-sensitive potassium channel</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>KH</td>
<td>Krebs-Henseleit</td>
</tr>
<tr>
<td>LAD</td>
<td>left anterior descending coronary artery</td>
</tr>
<tr>
<td>LDA</td>
<td>lavendustin A</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>L-NA</td>
<td>N(^{\text{V}})-nitro-L-arginine</td>
</tr>
<tr>
<td>L-NAME</td>
<td>N(^{\text{V}})-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>1-NIL</td>
<td>L-N(^{\text{V}})-L-lysine</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LV</td>
<td>left ventricle</td>
</tr>
<tr>
<td>LVDP</td>
<td>left ventricular developed pressure</td>
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<td>LVEDP</td>
<td>left ventricular end-diastolic pressure</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------------------------------------------</td>
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<td>MAPKAPK2</td>
<td>MAPK-activated protein kinase 2</td>
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<td>MAPK kinase</td>
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<td>MAPK kinase kinase</td>
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<td>mitoK\textsubscript{ATP} channel</td>
<td>mitochondrial K\textsubscript{ATP} channel</td>
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<td>ml</td>
<td>millilitre</td>
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<tr>
<td>MLA</td>
<td>monophosphoryl lipid A</td>
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<td>mm Hg</td>
<td>millimetres of mercury</td>
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<tr>
<td>mm</td>
<td>millimetre</td>
</tr>
<tr>
<td>Mn-SOD</td>
<td>manganese superoxide dismutase</td>
</tr>
<tr>
<td>MPG</td>
<td>N-2-mercaptopropionylglycine</td>
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<tr>
<td>Mph</td>
<td>miles per hour</td>
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<tr>
<td>NA</td>
<td>noradrenaline</td>
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<td>NBT</td>
<td>nitroblue tetrazolium</td>
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<tr>
<td>ODN</td>
<td>oligodeoxynucleotide</td>
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<tr>
<td>NF-\textkappa B</td>
<td>nuclear factor-\kappa B</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
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<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
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<tr>
<td>NS</td>
<td>not significant</td>
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<td>5' NT</td>
<td>5' nucleotidase</td>
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<td>-O</td>
<td>single oxygen</td>
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<tr>
<td>-O\textsubscript{2}^-</td>
<td>superoxide anion</td>
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<td>-OH</td>
<td>hydroxyl radical</td>
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<tr>
<td>ONOO\textsuperscript{-}</td>
<td>peroxynitrite anion</td>
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<td>PaO\textsubscript{2}</td>
<td>arterial partial pressure of oxygen</td>
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<td>arterial partial pressure of carbon dioxide</td>
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<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PIP\textsubscript{2}</td>
<td>phosphatidylinositol 4,5-biphosphate</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<td>PLA\textsubscript{2}</td>
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<td>PLC</td>
<td>phospholipase C</td>
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<tr>
<td>PLD</td>
<td>phospholipase D</td>
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</table>
PLSD protected least significant difference
PMA phorbol 12-myristate 13-acetate
PMSF phenylmethylsulphonyl fluoride
PP2A protein phosphatase 2A
PTCA percutaneous transluminal coronary angioplasty
PtdCho phosphatidylcholine
PtdOH phosphatidic acid
R risk volume
$R^2$ coefficient of determination
RACK receptor of activated C-kinase
ROS reactive oxygen species
R-PIA $N^6$-(phenyl-2R-isopropyl)-adenosine
RPP rate-pressure product
SAPK stress-activated protein kinase
SBP systolic blood pressure
s.c. sub-cutaneous
SDS sodium dodecyl sulphate
SEK SAPK/ERK activating kinase
SEM standard error of mean
SOD superoxide dismutase
SPECT single-photon emission computed tomography
8-SPT 8-(p-sulphophenyl) theophylline
SWOP Second Window of Protection
TBS Tris-buffered Saline
TEMED N, N, N', N'-tetramethylethylenediamine
TK tyrosine kinase
TNF-α tumour necrosis factor-alpha
Tris Tris (hydroxymethyl) methylamine
TTC 2,3,5-triphenyltetrazolium chloride
Tween 20 polyoxyethylene sorbitan monolaurate
U.V. ultraviolet
VF ventricular fibrillation
VT ventricular tachycardia
v/v volume / volume
w/v weight / volume
Chapter One

*General Introduction*
1.1 Coronary Artery Disease

In recent years there has been considerable interest in the pathophysiology of myocardial ischaemia-reperfusion injury. This has partly been driven by epidemiological data confirming the place of coronary artery disease as the leading cause of death in the Western world. Coronary artery disease accounts for 1 in 8 deaths world-wide and for 1 in 4 deaths in the UK (1, 2). Over the past decade, the widespread adoption of thrombolytic therapy and revascularisation procedures, and adjunctive treatment with agents such as aspirin, β-blockers and angiotensin converting enzyme (ACE) inhibitors have revolutionised the management, and improved the outcome for those patients suffering acute myocardial infarction (AMI) who reach hospital care. However, other than rapid reperfusion of the infarct related artery, there have been no therapeutic interventions available to enhance myocardial tolerance to ischaemia and counter the threat of myocardial necrosis. In this context, interventions aimed at modifying the symptoms of angina, such as calcium antagonists and nitrates, have met with little success in altering the prognosis of AMI. Currently, of the estimated 180,000 patients hospitalised annually in UK with AMI, 10-15% die during hospitalisation and another 15-20% die during the following year (2). These figures suggest that for a patient suffering AMI, the outcome remains relatively poor and clearly indicate the need for improved therapeutic strategies in this field. In particular, the time delays inherent in establishing reperfusion in an occluded coronary artery, whether by thrombolytic therapy or emergency coronary angioplasty, further highlight the need for adjunctive therapies which could either slow ischaemic metabolism and cellular injury pending successful reperfusion, or protect myocytes against the undesired and potentially lethal effects of reperfusion.

Several endogenous mechanisms or exogenous interventions are known to both slow the rate of ischaemic metabolism and delay the onset of lethal myocyte injury. One such endogenous adaptive mechanisms is the phenomenon of ischaemic preconditioning, which may be capable of providing additional protection to myocardium under threat of an ischaemic insult. This chapter provides a brief description of the key cellular events that occur during ischaemia and reperfusion,
followed by description of the characteristics and the known cellular mechanisms of ischaemic preconditioning. This introduction would provide a background for the work presented in the remaining chapters of this thesis.

1.2 Myocardial Ischaemia and its Consequences

Ischaemic Syndromes

Although there is still some controversy about the definition of myocardial ischaemia (3) the underlying concept is that ischaemia develops when coronary blood supply to a region of the myocardium is reduced. This results in a reduction in fractional uptake of oxygen and/or substrate in the heart which becomes insufficient to maintain the rate of cellular oxidation (4). The result is an imbalance between myocardial oxygen demand and coronary oxygen supply or "... a condition in which coronary blood flow is inadequate to permit the maintenance of a steady-state metabolism" (5).

The extent of myocardial injury caused by ischaemia depends on a number of factors including the duration of ischaemia, the volume of myocardium subjected to ischaemia, the depth of myocardial ischaemia, which is determined by the amount of blood flow reaching the myocardium through a combination of antegrade flow via the diseased coronary artery and retrograde flow via collaterals, and the endogenous resistance of the myocardium to ischaemia (6). Clinically, myocardial ischaemia can manifest in a number of different ways depending on these factors. First, chronic stable angina, most commonly arises in the face of a fixed reduction in coronary flow resulting from atherosclerotic coronary artery disease, and brought on by periods of increased myocardial oxygen demand such as during exercise or emotional upset. Although frequently accompanied by typical anginal chest pain, these episodes of myocardial ischaemia may be asymptomatic, so called silent ischaemia. In stable angina, the periods of myocardial ischaemia are usually transient and are relieved by manoeuvres that improve the myocardial oxygen supply/demand ratio, such as by cessation of physical activity or by antianginal therapy. Second, are the acute coronary syndromes, which comprise a spectrum of pathophysiological conditions spanning unstable angina, non-ST-elevation myocardial infarction (non-Q-wave or
subendocardial MI) and acute ST-elevation MI. As compared to stable angina, in most cases, these conditions result from a sudden reduction or cessation of blood flow to a region of the heart, caused by rupture of an atherosclerotic plaque in an epicardial coronary artery and formation of a platelet thrombus (reviewed in reference 7). The result, is at least a transient total or subtotal coronary artery occlusion in all cases of acute coronary syndromes. If left untreated, the severity and duration of ischaemia are sufficient to cause the condition to progress into ultimate irreversible cell injury. In patients with acute coronary syndromes, therapeutic strategies to re-establish epicardial blood flow are now well established as the standard of care, be it with antiplatelet agents, antithrombotic agents, fibrinolytic therapy, and where the facilities and expertise are available, coronary angioplasty (8-10).

Acute myocardial ischaemia and reperfusion also results in a number of other syndromes which may occur in isolation or on a background of the ischaemic syndromes discussed above. These include ischaemic myocardial dysfunction that may result in ventricular impairment and the syndrome of heart failure, arrhythmias, myocardial stunning and hibernation. Some of these syndromes are further discussed below.

**Cellular Consequences of Myocardial Ischaemia**

A pivotal factor in ischaemia is that oxygen supply to the mitochondria is inadequate to support oxidative phosphorylation. Uncoupling of oxidative phosphorylation rapidly produces profound biochemical and morphological changes within myocardium, the severity of which are determined by the degree and duration of oxygen deprivation (11).

Ischaemia results in the rapid inhibition of β-oxidation of fatty acids, with a switch to glycolytic metabolism of glucose and glycogen. This anaerobic metabolism is an inefficient source of adenosine triphosphate (ATP) and leads to the intracellular accumulation of anaerobic metabolites such as protons (H⁺) and lactate, which inhibit ATP synthesis and cause cytosolic accumulation of acyl CoA, free fatty acids and triglycerides. An alternative source of energy which is available for a few minutes
after the onset of ischaemia, the creatine phosphate pool, is rapidly depleted, and the generation of ATP from creatine phosphate is accompanied by further accumulation of inorganic phosphate. The fall in ATP is also associated with an increase in hypoxanthene and xanthene levels, which are produced as a result of the breakdown of adenosine (11). Thus during ischaemia, there is a depletion of high energy phosphates and the purine precursor of ATP.

Since the regulation of various cation channels, especially Ca\(^{2+}\), K\(^+\) and Na\(^+\) is ATP-dependent, ionic homeostasis is severely disrupted within a few minutes of the onset of ischaemia. These changes have been reviewed in detail by Petrich et al (12). An early ionic change after the onset of ischaemia is a rapid efflux of K\(^+\) from the cell leading to extracellular accumulation of K\(^+\) and membrane depolarisation. This early phase of K\(^+\) efflux is thought to be mediated through opening of the ATP-sensitive K\(^+\) channel (K\(_{\text{ATP}}\)), in response to a fall in ATP and/or a rise in ADP. Other mechanisms proposed to explain the efflux of K\(^+\) ions include anion coupled efflux, the Na\(^+\)/K\(^+\) ATPase pump and outward movement through ligand operated K\(^+\) channels. Intracellular Na\(^+\) also rises progressively during ischaemia. The mechanisms accounting for the early influx of Na\(^+\) are not clear but may involve the activation of the Na\(^+\)/H\(^+\) exchanger. When ATP levels have declined significantly, the Na\(^+\)/K\(^+\) ATPase activity will decline thereby accentuating Na\(^+\) influx. The associated accumulation of H\(^+\) results in intracellular acidosis, with a fall in intracellular pH to less than 6.8 within minutes of the onset of ischaemia. The combined accumulations of Na\(^+\), K\(^+\), lactate and phosphate during ischaemia lead to a marked rise in cytosolic osmolarity, which in turn accounts for cell swelling and predisposes to loss of sarcolemmal integrity. The early minutes following the onset of myocardial ischaemia are also characterised by a rapid rise in the cytoplasmic Ca\(^{2+}\)-concentration. The major source of this early Ca\(^{2+}\) overload is the sarcoplasmic reticulum; both through reduction of Ca\(^{2+}\) uptake into the sarcoplasmic reticulum by the Ca\(^{2+}\)/Mg\(^{2+}\) ATPase pump, and also through the release of sarcoplasmic reticulum Ca\(^{2+}\) stores via the ryanodine sensitive Ca\(^{2+}\) release channels. As ischaemia is prolonged, the influx of Na\(^+\) triggers the Na\(^+\)/Ca\(^{2+}\) exchanger which extrudes Na\(^+\) in exchange for Ca\(^{2+}\). Intracellular Ca\(^{2+}\) rises further with the activation of several deleterious Ca\(^{2+}\)-dependent enzymes including Ca\(^{2+}\)-ATPases, which hydrolyse ATP.
causing further energy depletion. Proteolysis and membrane disruption begins as Ca\(^{2+}\)-dependent proteases and phospholipases are activated. Ca\(^{2+}\) overloading of mitochondria is apparent as dense areas on electron microscopy and effectively prevents mitochondrial respiration.

The profound metabolic and ionic perturbations that result from myocardial ischaemia may affect the development and propagation of cardiac action potentials and predispose to arrhythmias. Electrophysiological changes related to disturbances of conduction, refactoriness and automaticity occur within a few minutes of the onset of ischaemia and account for early arrhythmias following coronary artery occlusion (13). Several factors may augment and reinforce the electrophysiological changes that predispose to arrhythmias, including local release of catecholamines, the generation of reactive oxygen species, perturbances of the ionic milieu, especially efflux of intracellular K\(^+\), and the generation of inflammatory mediators such as thromboxanes and prostanoids (14). These ischaemic arrhythmias are thought to account for the majority of sudden cardiac deaths resulting from coronary artery disease.

As a result of imbalances in ionic and metabolic homeostasis, ischaemia causes extensive damage to the cytoskeleton, largely brought about by the effects of osmotic swelling. These changes include collapse of the intermediate filament network, re-organisation of the cytoplasmic network, re-localisation of actin fibres around the nucleus, disruption of the microtubules and mitotic spindle, mitochondrial swelling and uncoupling of oxidative phosphorylation (15). It is postulated that the lesions affecting the cytoskeleton play an important role in the development of irreversible myocardial injury during ischaemia or anoxia (15).

**Reperfusion Injury**

Although reperfusion is the prerequisite for salvage of myocardium that has been rendered ischaemic, the readmission of oxygenated blood to the ischaemic myocardium is thought to precipitate a sequence of events that may further exacerbate cellular injury (reviewed in references 16 and 17). The return of blood flow allows washout of harmful metabolites accumulated during ischaemia and
restoration of intracellular pH. However, the early moments of reperfusion may also be associated with a catastrophic Ca\textsuperscript{2+} overload in cells and accentuate the effects of ischaemia-induced Ca\textsuperscript{2+} overload. The return of oxygen to the myocardium is also capable of inducing the production of highly reactive oxygen species (ROS) such as the superoxide anion (O\textsuperscript{2−}), singlet oxygen (O), hydroxyl radicals (OH), and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}). ROS react avidly with many biological molecules, especially with hydrogen in unsaturated fatty acid side chains and in protein thiol groups, leading to lipid peroxidation and protein denaturation (reviewed in reference 18). ROS are generated continuously under normal conditions and detoxified by intracellular and extracellular free radical scavengers and antioxidant enzymes. The most important of the intracellular antioxidants are catalase, superoxide dismutase (SOD; cytosolic Cu/Zn and mitochondrial Mn isoforms), and reduced glutathione. During ischaemia, and to a greater extent during reperfusion the production of ROS increases through several biochemical pathways and may overwhelm the normal antioxidant and scavenging activity of the cell (18).

**Apoptotic versus Necrotic Cell Death**

Distinct from cellular necrosis, which commonly arises from an externally derived insult (death by murder), and is characterised by loss of cytoplasmic membrane integrity and spillage of cellular DNA and actin, and provokes local inflammation, apoptosis is a highly regulated, autonomous form of cell death (programmed cell death or suicide) involving an ATP-dependent process, to eliminate injured or unwanted cells without inducing an inflammatory response (reviewed in reference 19). Apoptosis is characterised by distinct alterations in cell morphology including chromatin condensation and margination, cytoskeletal alterations and membrane blebbing. In later stages of apoptosis, nuclear fragmentation becomes evident, the cytoplasm shrinks progressively, and one or more apoptotic bodies are formed from each dying cell. One of the most important aspects of apoptosis is the preservation of an intact plasma membrane until the cell can be ingested by a neighbouring cell or by phagocytic cells of the macrophage/monocyte lineage, thereby limiting inflammation (20).
Apoptosis is essential for normal development as well as physiological cell turnover, and results from a shift in balance between anti-apoptotic and pro-apoptotic forces within a cell. However, in the past few years, compelling evidence has accumulated indicating that apoptotic cell death may also play a critical role in a variety of cardiovascular diseases including myocardial infarction, heart failure, myocarditis and atherosclerosis (19). Gottlieb et al. (21), were the first to show that ischaemic injury induces myocyte apoptosis and that reperfusion of previously ischaemic myocardium accelerates the apoptotic process. Since then, apoptosis has been demonstrated in various models of myocardial ischaemia-reperfusion injury (22-25). The most commonly used techniques to detect apoptosis are based on the fact that during apoptosis, the genomic DNA is cleaved within internucleosomal DNA segments by an endonuclease selectively activated during apoptosis. These include demonstration of a characteristic ladder-like pattern of DNA fragments following separation of cellular DNA on agarose gels, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) technique, and in situ end-labelling (ISEL) of the 3’ end of genomic DNA (19). Another technique involves the binding of annexin V to phosphatidylserine exposed on the outer leaflet of the apoptotic cell membrane (26). However, using these techniques, in different models of ischaemia-reperfusion injury, the reported values of apoptotic cell death seems to vary from 0.1 to 40% of myocytes, indicating the need for improvement, or development of detection techniques that allow for more accurate quantitative detection of apoptosis in cardiovascular tissue. Moreover, although apoptosis has been verified histologically in acute myocardial infarction, its role in the pathogenesis of ischaemic injury remains unknown (19).

**Myocardial Stunning**

Myocardial stunning describes the prolonged but reversible contractile dysfunction that follows single or multiple brief periods of regional or global ischaemia, which persists after reperfusion despite the absence of irreversible damage, and return of normal or near-normal perfusion (27). In accordance with this definition, stunning is a reversible process which is not caused by a primary deficit of myocardial perfusion, and is therefore quite distinct from myocardial infarction.
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The phenomenon of post-ischaemic contractile dysfunction was first described by Heyndrickx and colleagues in 1975 in conscious dogs undergoing brief coronary occlusions (28), but the term "stunned myocardium" was coined by Braunwald and Kloner in 1982 (27). Since this original description, and with increasing knowledge of the clinical situations in which the human myocardium is exposed to transient ischaemia and subsequently develops stunning, extensive experimental and clinical research has been aimed at elucidating the pathophysiological mechanisms underlying this phenomenon, and means of alleviating its consequences (reviewed in references 29 and 30). It is now recognised that stunning is at least in part responsible for the myocardial dysfunction observed in patients with unstable angina, acute myocardial infarction with early reperfusion, coronary artery bypass graft surgery (CABG), and cardiac transplantation, and may contribute significantly to the morbidity associated with these conditions (29). Myocardial stunning has also been observed experimentally in the setting of exercise induced ischaemia (31-34), and following brief coronary artery occlusions induced at the time of coronary angioplasty (PTCA) (35, 36).

With regards to the pathogenetic mechanisms of myocardial stunning, a number of hypotheses have been proposed of which two are currently most favoured; the oxyradical hypothesis and the calcium hypothesis (30). The oxyradical hypothesis postulates that myocardial stunning results from oxidant stress secondary to the generation of ROS in the initial moments of reperfusion. This hypothesis is based on the one hand, on studies that have directly demonstrated generation of ROS in stunned myocardium by both spin trapping and aromatic hydroxylation techniques, and on the other hand, the observation that antioxidants reproducibly protect against myocardial stunning in a number of animal models and species (37). The calcium hypothesis of myocardial stunning postulates that the contractile dysfunction observed following myocardial ischaemia is partly due to the transient Ca\(^{2+}\) overload during the early phase of reperfusion, and also an alteration of the contractile proteins resulting in decreased sensitivity of myofilaments to Ca\(^{2+}\), so that for any given Ca\(^{2+}\) transient, the myocardium generates less force (reviewed in reference 30). It must be noted that these theories are not mutually exclusive and may represent different facets of the same pathophysiological process.
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Myocardial Hibernation

The concept of myocardial ‘hibernation’ was introduced more than two decades ago to describe impaired left ventricular function that improved following revascularisation (38). This original description did not specify whether this impairment of contractile function was associated with an abnormality in resting myocardial blood flow. However, it became widely accepted that this is the case, and Rahimtoola (39, 40) referred to hibernating myocardium as the persistently impaired function of viable myocardium at rest due to reduced coronary blood flow that can be partially or completely restored to normal, if the myocardial oxygen supply-demand relationship is favourably altered. Hibernation is thought to represent a form of myocardial adaptation to hypoperfusion; as the blood flow to an area of myocardium is reduced, there is a corresponding down-regulation of contractile function, so called ‘perfusion-contraction matching’ (41, 42). As the duration of reduced blood flow extends, the myocardium may be able to reduce its metabolic requirements still further by undergoing a more chronic form of adaptation involving alterations in its morphology and protein content (43, 44). This includes a substantial loss of sarcoplasmic reticulum and contractile proteins, and their replacement with collagen, and nuclear changes with heterochromatin distributed evenly over the nucleoplasm (45). There is debate as to whether these structural changes truly represent an adaptation to substrate deprivation or whether they are degenerative changes as a result of chronic hypoperfusion of the myocardium.

Animal models of long-term coronary stenosis have produced results that suggest a fundamentally different mechanism for myocardial hibernation (46). These studies show that in the presence of chronic coronary artery stenosis, absolute resting coronary blood flow may in fact be normal and it is the coronary flow reserve, defined as the ratio of maximum to basal coronary flow, that is reduced. Evidence from patients with coronary artery disease suggests similar findings in hibernating segments of the myocardium (47, 48). Under these circumstances, episodes of spontaneous excitement and exercise can lead to repetitive cycles of ischaemia precipitated by increased myocardial oxygen demand in the setting of limited blood flow reserve, and when the ischaemia is relieved, the resulting myocardial dysfunction persists despite having normal basal perfusion. These observations have
led to the proposal that hibernation results from repetitive cycles of ischaemia and reperfusion induced myocardial stunning, and not an adaptive downregulation of function in the face of chronic low grade ischaemia (reviewed in reference 45). There is continuing debate as to the exact mechanism underlying myocardial hibernation, but increasing evidence points to the fact that myocardial stunning contributes in one way or another to the chronic dysfunction of the hibernating myocardium. Furthermore, as a result of transmural variations in myocardial blood flow, it is possible that areas of dysfunction secondary to stunning and hypoperfusion may coexist in myocardial segments with impaired systolic function.

1.3 Ischaemic Preconditioning

Until fifteen years ago, it had been generally assumed that repetitive short periods of myocardial ischaemia would have a cumulative deleterious effect resulting in progressive cell necrosis. In 1986 Reimer and colleagues (49), during the course of experiments designed to explore the relative contribution of high energy phosphate depletion and catabolite accumulation to lethal cell injury in the canine myocardium, found unexpected results which formed the basis for the concept of endogenous myocardial adaptation to sublethal ischaemia. Their experimental model involved repetitive brief episodes of regional myocardial ischaemia in the anaesthetised dog, and to their surprise they found that following the initial period of ischaemia there was no further reduction in the ATP levels during subsequent similar ischaemic challenges. They also found that this preservation of ATP was associated with an absence of myocardial infarction in six of the seven dogs studied. These investigators went on to examine the possible protective effects of brief sublethal ischaemia in the same model (50). They found that four 5 minute coronary occlusions separated by 5 minutes of reperfusion immediately prior to 40 minutes of sustained ischaemia resulted in a significant reduction in infarct size to 7% of the myocardium at risk, compared to about 30% in the control group. Thus, despite a longer cumulative duration of coronary occlusion, infarct size in pretreated dogs averaged only about 75% of that seen in controls. Importantly, this protection was lost when the duration of sustained ischaemia was extended to three hours, suggesting that this form of myocardial adaptation delays rather than prevents cell death (50). They termed this
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phenomenon “ischaemic preconditioning” and postulated that the multiple anginal episodes that often precede myocardial infarction in man, may result in a similar delay in cell death following coronary occlusion, and thereby allow for greater salvage of myocardium through reperfusion therapy (50).

These original findings have stimulated a vast amount of research over the past fifteen years aimed at identification of the underlying mechanisms and the characteristics of the protective effects of ischaemic preconditioning. It is now known that apart from timely reperfusion, ischaemic preconditioning is the most powerful and reproducible experimental means of myocardial protection, and it has been demonstrated in most animal species studied including not only dogs (51), but also rabbits (52), rats (53), mice (54), guinea pigs (55), pigs (56), chicken (57), and sheep (58). There is also evidence that the human myocardium can be preconditioned and that ischaemic preconditioning may occur as part of some naturally occurring ischaemic syndromes (59).

1.3.a Characteristics of Ischaemic Preconditioning

The original description of ischaemic preconditioning was in the in vivo canine myocardium and evaluated a reduction in infarct size (50), which has become the ‘gold standard’ for studies evaluating the cardioprotective effects of preconditioning. However, many investigators have sought to expand on the concept of reduction of infarct size in vivo with ischaemic preconditioning, to establish whether preconditioning is also manifest in models other than those involving ischaemia in the intact heart, and to identify surrogate end-points or indices of preconditioning that might be assessed in protocols or models, including the clinical setting, not amenable to measurement of infarct size by standard histopathological methods. Parallel research has examined the characteristics of the varying components of the “typical” preconditioning protocol; specifically, the brief antecedent ischaemic stimulus, and the duration of the intervening reperfusion between the brief and sustained ischaemia, to assess their importance in achieving cardioprotection.
The Preconditioning Stimulus

It has become apparent that the multiple 5 minute bouts of brief coronary artery occlusion used in the study by Murry et al. (50) are not required to elicit a reduction in infarct size. The minimum duration of ischaemia required to precondition the myocardium seems to vary between different species. Evidence from the rabbit and dog models suggests that as little as approximately 2 minutes of brief ischaemia is sufficient to provide an effective preconditioning stimulus (60). Some early studies also suggested that brief bouts of ischaemia do not yield a cumulative “dose-dependent” protective effect, and that ischaemic preconditioning is an all or none phenomenon (61, 62). However, more recent work in the rabbit and pig models, has concluded that the protection conferred by ischaemic preconditioning occurs in a graded pattern, and that increasing the number of the brief preconditioning cycles produces a progressive reduction in infarct size (63-65).

Although brief occlusion of the culprit coronary artery was the original stimulus for infarct size reduction with preconditioning, it has been demonstrated that other strategies may be equally as effective in protecting the ischaemic myocardium. For instance, short (15 minute) periods of coronary artery stenosis (66), and transient imbalances in the ratio of myocardial oxygen supply/demand induced by rapid ventricular pacing (67-69) have been shown to protect the dog and rabbit heart against a subsequent prolonged ischaemic insult, demonstrating that complete coronary occlusion during the preconditioning stimulus is not a prerequisite for initiating protection. Other stressful stimuli have also been shown to precondition against myocardial ischaemia; these include brief episodes of hypoxia (70), brief episodes of volume overload resulting in myocardial dilatation (71), transient whole body hyperthermia (heat stress) (72), exercise (73), and the endotoxin lipopolysaccharide (LPS) or its non-toxic derivative monophosphoryl lipid A (MLA) (74).

Remote ischaemic preconditioning has also been shown to protect the myocardium against lethal ischaemic injury. This form of protection can be achieved with brief ischaemia of myocardial regions separate from the region to undergo a sustained ischaemic insult (intra-organ preconditioning) (75), or following transient ischaemia-
reperfusion of remote non-cardiac tissue (inter-organ preconditioning) such as the intestine, kidney, and skeletal muscle (76-79).

**Temporal Profile of Ischaemic Preconditioning**

In the original preconditioning protocol, the prolonged ischaemic insult was imposed within minutes of the brief ischaemic stimulus (50). Although flow must be re-established between a preconditioning coronary occlusion and the subsequent prolonged ischaemic insult, studies in models of coronary stenosis or subtotal occlusion have suggested that intervening reperfusion is not required to achieve cardioprotection (80, 81). Conversely, if the duration of reperfusion between the last episode of brief ischaemia and the prolonged coronary occlusion is increased, the beneficial effects of preconditioning wane. In rats, rabbits, dogs and pigs, separation of the brief preconditioning ischaemic episodes from the long occlusion by 60-120 minutes results in nearly complete loss of preconditioning's infarct-limiting effect (60, 82-84). This protection can however, be re-established if a further preconditioning stimulus is instituted immediately before the sustained occlusion (85, 86). This transient early phase of protection against myocardial ischaemia has been termed *classic* preconditioning.

If the period of intervening reperfusion between the preconditioning stimulus and the test ischaemic insult is further extended to 12-24 hours, a delayed phase of myocardial protection is observed, which results in a significant reduction in infarct size. This delayed preconditioning or “Second Window of Protection” was originally described in 1993 in the rabbit (87) and canine (88) models of myocardial infarction 24 hours following brief cycles of preconditioning ischaemia. Delayed preconditioning has since been described in a number of species including conscious rabbits (89), rats (90), mice (91) and pigs (92). As compared to the transient nature of classic preconditioning, the duration of the second window of protection is more prolonged and extends between 12-72 hours following the preconditioning stimulus (93-95).
1.3.b Preconditioning Against Other Indices of Ischaemic Injury

**Recovery of Contractile Function**

One of the most commonly studied surrogate end-points of cardioprotection with preconditioning, particularly in isolated perfused heart models, has been the recovery of left ventricular (LV) contractile function following the relief of sustained regional or global ischaemia. These studies have mostly reported that recovery of LV-developed pressure is enhanced in isolated rat and rabbit hearts preconditioned with brief bouts of global ischaemia when compared with controls (96, 97). Conversely, studies in *in vivo* dog and pig models have failed to demonstrate improved recovery of regional contractile function of viable but stunned myocardium during the initial hours after the relief of sustained ischaemic insult (51, 98-100). The general consensus is therefore, that *classic* preconditioning does not attenuate post-ischaemic myocardial stunning, and that the improved recovery of LV function observed in some studies is a secondary manifestation of the ability of preconditioning to reduce lethal cell injury.

In contrast, delayed ischaemic preconditioning has been reproducibly shown to attenuate myocardial stunning following repeated cycles of regional myocardial ischaemia-reperfusion *in vivo* in pigs (101), rabbits (102), and mice (91). This late preconditioning against stunning is independent of a reduction in myocyte death since the ischaemic protocols used to induce myocardial stunning were not sufficient to cause myocardial infarction.

**Ischaemia- and Reperfusion-induced Arrhythmias**

Examination of the effects of *classic* preconditioning on ischaemia- and reperfusion-induced arrhythmias has yielded similar controversy. Studies of preconditioning in the rat model have consistently shown a reduction in the incidence of extrasystoles, ventricular tachycardia (VT) and reversible ventricular fibrillation (VF) (103-106). In dogs, the results have been conflicting, with some studies showing a reduction in the incidence of ischaemia-induced VT and VF in preconditioned hearts compared to controls (105), while others failed to demonstrate a favourable effect of ischaemic preconditioning on arrhythmias (107). There are even reports in the pig model of a
proarrhythmic effect of ischaemic preconditioning (108, 109). It seems therefore that the effects of classic preconditioning on ischaemia- and reperfusion-induced arrhythmias are model and species dependent. There is some evidence for a delayed antiarrhythmic effect of preconditioning 24 hours following brief periods of regional myocardial ischaemia in the conscious rabbit model (89), and rapid ventricular pacing in dogs (110).

**Apoptosis**

Recent evidence from studies of in vivo rat hearts suggests that distinct from a reduction in myocardial necrosis, ischaemic preconditioning may also attenuate the degree of myocyte apoptosis following ischaemia-reperfusion. Piot et al. (111), using the rat model, were the first to report that genomic DNA fragmentation (a hallmark of apoptosis) was markedly attenuated in animals that received five 5 minute bouts of preconditioning ischaemia before a 30 minute test occlusion when compared with time-matched controls subjected to sustained occlusion alone. These original findings have been confirmed by other groups in isolated myocytes and rat hearts, and using other markers of apoptosis such as TUNEL staining, and quantification of pro-apoptotic proteins (112-116).

**1.3.c Surrogate Models of Preconditioning**

A number of investigators have extrapolated the concept of ischaemic preconditioning-induced reduction of infarct size in the intact (be it in vivo or isolated buffer-perfused) heart to the tissue and the cellular level. This allows for studies aimed at identification of intracellular signalling pathways with small quantities of expensive pharmacological agents owing to the small volume of distribution, and also in cell models, to manipulate signalling proteins by the introduction of cDNAs, antisense RNA, recombinant protein and interfering peptides (117). However, these advantages are at the expense of cell phenotypes that differ from the intact heart and cannot be subjected to true ischaemia/reperfusion. These surrogate models of preconditioning include the isolated superfused cardiac tissue samples (118), cultured neonatal or adult cardiomyocytes (112, 119, 120), and isolated myocyte suspensions (121, 122).
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Walker et al. (118) demonstrated "ischaemic" preconditioning of superfused papillary muscle isolated from rabbit right ventricle, using rapid pacing together with hypoxic and substrate-free buffer in lieu of coronary occlusion, and showed an improvement in recovery of contractile force after relief of sustained "ischaemia" as the index of cardioprotection. In the cultured cardiomyocyte model, preconditioning with a brief period of hypoxia, or metabolic inhibition with cyanide in lieu of hypoxia, has been shown to protect against cell death following a prolonged hypoxic insult in a number of species including neonatal and adult rat, rabbit and chick embryos (112, 119, 120). In these models, different end-points are used to evaluate membrane integrity and cell death including leakage of myocardial enzymes such as creatine kinase (CK) and lactate dehydrogenase, permeability of trypan blue, and conversion of contracting, striated and rod shaped cells to an irregular shape with loss of striations. Finally, a modification of the isolated cell concept of preconditioning was developed by Armstrong and Ganote (121, 122), in which freshly isolated and suspended adult rabbit myocytes were rendered ischaemic by centrifugation into a pellet and layering with mineral oil. In this model, the rate of cell death following a prolonged ischaemic insult, quantified by evolution of changes in cell shape and exclusion of trypan blue, evaluated from timed collection of cell aliquots, was significantly reduced in cells that were initially preconditioned by 10 minutes of pelleting followed by resuspension when compared with time-matched control suspensions subjected to sustained pelleting alone.

1.4 Mechanisms of Ischaemic Preconditioning

The mechanisms underlying ischaemic preconditioning are not completely understood. A number of possible mechanisms initially suggested to contribute to the protective effects of preconditioning have however been ruled out. It is known for instance, that the enhanced tolerance conferred by preconditioning ischaemia is not explained by simple recruitment of collateral vessels as the protection is observed irrespective of the degree of collateral flow in dogs (50). Furthermore, preconditioning has been shown to be protective in animal models with little or no collateral flow such as the rat (53), rabbit (52) and pig heart (56), and is also evident in isolated hearts subjected to global ischaemia (96). This latter model of
preconditioning, the isolated buffer perfused heart, also excludes the necessity of blood borne factors for preconditioning. Another early suggestion, that the myocardial stunning induced by the brief periods of preconditioning ischaemia-reperfusion reduces the metabolic demands of the myocardium during the sustained ischaemia, resulting in a smaller infarct size, has also been dismissed (123).

On the other hand, a vast amount of research strongly suggests that preconditioning-induced cardioprotection is a receptor mediated phenomenon. In other words, it is believed that the brief episodes of ischaemia result in the release of a number of substances or “triggers” that act on one or more receptors on the myocyte surface. Activation of these receptors appears to initiate intracellular signalling pathways which in turn act on one or more target or “effector” proteins which ultimately render the myocytes resistant to subsequent lethal ischaemic injury. The current knowledge on the respective triggers, mediators and potential end-effectors of acute and delayed preconditioning is briefly outlined in the following sections.

1.4.a Mechanisms of Classic Preconditioning

Triggers

There are several lines of evidence suggesting a role for a number of endogenous autocrine and paracrine mediators released during the brief period of ischaemia, and acting on local receptors to trigger classic preconditioning. Various models of preconditioning in different species have implicated the involvement of substances such as adenosine (52), acetylcholine (124), catecholamines (125), angiotensin II (126), bradykinin (127), endothelin-1 (128) and opioids (129). The common theme amongst these so called ‘triggers’ of ischaemic preconditioning is that they all act on G protein-coupled transmembrane receptors on the surface of myocytes. The receptors so far identified include adenosine A₁ and A₃ receptors, α₁-adrenoceptors, muscarinic M₂ receptors, bradykinin B₂ receptors, endothelin-1 receptors, angiotensin II receptors and δ-opioid receptors (reviewed in reference 130). Activation of these G protein-coupled receptors seem to form a critical step in initiating acute preconditioning, and in fact, altered sensitivity of Gi proteins after an
initial brief ischaemic stimulus has in itself been postulated as a mechanism for ischaemic preconditioning in the canine heart (131).

**Adenosine.** Adenosine is a purine nucleoside that is produced by catabolism of ATP by many different cell types. The cardiovascular actions of adenosine have been appreciated for many years (reviewed in reference 132). It is known that various forms of myocardial stress, including ischaemia, hypoxia or catecholamines, result in a rapid increase in extracellular levels of adenosine. This in turn results in activation of local adenosine receptors on cardiomyocytes and vascular endothelial cells, which mediate a reduction in metabolic demand of the heart and an increase in myocardial oxygen supply by coronary vasodilatation. These observations have led to the concept that adenosine is a “retaliatory metabolite” with potential cardioprotective effects (133). Due to the efficient transport mechanisms and metabolising enzymes, the effects of adenosine are localised to the site where it is formed, and the duration of its effects are very transient with a half-life in human blood of less than one second (134). Thus adenosine is considered a short-term mediator that maintains the metabolic equilibrium of the heart within a time frame of seconds to minutes.

There are currently 4 recognised subtypes of adenosine receptors: A\(_1\), A\(_2A\), A\(_2B\) and A\(_3\) (132). Adenosine receptors of the cell surface membrane are members of the G-protein coupled receptor family, have 7 putative trans-membrane spanning domains and are glycoproteins. Adenosine A\(_1\) receptors (A\(_1\)R) are coupled to pertussis toxin-sensitive inhibitory G-proteins (G\(_i\)) and mediate the actions of adenosine to reduce the activity of adenyl cyclase and to activate an outward potassium current, I\(_{K,Ado^+}\) which explain the negative chronotropic, dromotropic and ionotropic effects of adenosine in the heart. This mechanism also mediates the anti-\(\beta\)-adrenergic effects of adenosine (135). In addition, activation of A\(_1\)R results in improved glucose utilisation by stimulation of glycolysis (136).

Conversely, adenosine A\(_2\) receptors (A\(_2\)R) are coupled to stimulatory G-proteins (G\(_s\)) which mediate stimulation of adenyl cyclase by adenosine, and a subsequent increase in intracellular cAMP levels (137). Activation of adenosine A\(_2\)R exerts potent vasodilatory effects on coronary and systemic vasculature. The subtype of adenosine
A2R that mediates vasodilatation is not certain, and it is possible that adenosine acts on both A2A and A2B receptors on endothelium and vascular smooth muscle to cause vasorelaxation. Some evidence suggests that endothelium-dependent vasodilatory effects of adenosine A2A agonists may also involve stimulation of nitric oxide formation in endothelium (138). It has been reported that activation of A2R is involved in the stimulation of gluconeogenesis, and reduction in local tissue damage through inhibition of both neutrophil endothelial adherence and plugging, and platelet aggregation and plugging (reviewed in reference 139).

The adenosine A3 receptor (A3R) was cloned from rat testis and from rat and human brains (140, 141). Some evidence suggests that A3R inhibit adenyl cyclase activity and/or stimulate production of inositol 1,4,5-triphosphate (IP3) and increase activity of phospholipase C (142, 143). Adenosine A3R are also postulated to mediate an increase in activity of antioxidant enzymes in several types of cells (144). These receptors may also mediate mast cell degranulation and release of vasoconstrictor substances including histamine (143, 145). The effects of adenosine on cardiovascular system are summarised in table 1.1.

Table 1.1 Physiological Effects of Adenosine on Cardiovascular System

<table>
<thead>
<tr>
<th>Effects of Adenosine</th>
<th>Adenosine Receptor Subtype</th>
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<tbody>
<tr>
<td>Vasodilation</td>
<td>A2A (A2B)</td>
</tr>
<tr>
<td>Bradycardia (negative chronotropy)</td>
<td>A1</td>
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It has long been appreciated that brief periods of myocardial ischaemia result in the release of adenosine via the breakdown of ATP. In 1991, Downey’s group (52) were the first to postulate that this liberation of adenosine during the brief preconditioning ischaemia and resultant stimulation of adenosine receptors on the myocyte membranes, may be the trigger for the reduction in infarct size seen with preconditioning. They demonstrated that non-selective adenosine receptor blockade with 8-p-sulphophenyl theophylline (SPT) and PD 115,199 before the brief ischaemic stimulus (52), or during the interval between the brief and sustained ischaemia (146) abolished the protection of ischaemic preconditioning in an in vivo rabbit model, implying that adenosine receptor occupancy was involved as both a trigger and a mediator of infarct size reduction with preconditioning. Conversely, pretreatment with a selective adenosine A_1R agonist in lieu of the preconditioning ischaemia, mimicked the protective effects of ischaemic preconditioning (147). This group, using the same agonist/antagonist approach in rabbits, further identified adenosine A_3R as being involved in initiating cardioprotection (148). The role of adenosine A_2A in triggering ischaemic preconditioning has been confirmed in isolated rabbit cardiomyocytes (121, 149), and in human atrial trabeculae (150). These original studies did not implicate a role for adenosine A_2A in mediating ischaemic preconditioning. In fact, in cultured chick ventricular myocytes, activation of A_2A receptors was shown to abolish the protective effects mediated by A_1 and A_3 receptors during preconditioning (120). On the other hand, in coronary vascular endothelial cells, in which adenosine A_2A rather than A_1 or A_3 receptors predominate, A_2A have been implicated in the protection against anoxia-reoxygenation injury by ischaemic preconditioning (151).

Since the original studies of Downey and colleagues, a number of investigators have demonstrated the crucial role of adenosine A_1R in triggering the cellular events that culminate in infarct limitation following ischaemic preconditioning in the rabbit (152), dog (153), and pig myocardium (154). In the rat heart on the other hand, the role of adenosine in mediating cardioprotection remains controversial (53, 155). This discrepancy may have resulted from the fact that following a period of ischaemia, the interstitial concentrations of adenosine in the rat heart is 3-4 fold higher than that in the rabbit myocardium and higher concentrations of selective competitive antagonists
are required to abolish the protective effects of preconditioning (156). More recently, it has been demonstrated that overexpression of adenosine A₁R renders the myocytes more resistant to ischaemic injury (157) and mimics the protective effects of ischaemic preconditioning in transgenic mice (158).

**Free Radicals.** In addition to the receptor-mediated mechanisms discussed above, oxygen radicals appear to act as a non-receptor trigger of acute ischaemic preconditioning in some species. For example, in rabbit hearts, the cell permeable free radical scavenger N-2-mercaptopropionyl glycine (MPG), completely abolished protection by ischaemic preconditioning (159). Conversely, infusion of hypoxanthine and xanthine oxidase, to form an oxygen radical generating system, has been shown to mimic ischaemic preconditioning and reduce infarct size (159, 160). However, the ability of free radicals to trigger preconditioning remains controversial as a number of other studies using anti-oxidant enzymes failed to abolish the protective effects of ischaemic preconditioning (reviewed in reference 18). One reason for this discrepancy was proposed by Baines et al. (159) who showed that one, but not four, cycles of preconditioning was blocked MPG. They suggested that free radicals act in concert with other triggers to induce preconditioning. The source of these radicals has been suggested to be the mitochondria (161).

**Mediators**

**Phospholipases.** Signalling mechanisms activated immediately downstream following the binding of ligands to G protein-coupled receptors, seem to involve phospholipases. Phospholipase C (PLC) catalyses the hydrolysis of inositol-containing phospholipids such as phosphatidyl inositol 4,5-biphosphate (PIP₂) found in the plasma membrane. The latter is hydrolysed, by PLC, to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP₃). DAG alters the sensitivity of protein kinase C (PKC) to Ca²⁺, allowing its activation at physiological levels of Ca²⁺. A number of G protein-coupled receptor ligands, such as α₁-adrenoceptor agonists (162), angiotensin II (163), bradykinin (127), and ET-1 (164), have all been shown to activate PKC via a mechanism involving PLC.
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Phospholipase D (PLD) modifies the polar region of phosphatidylcholine and other phospholipids by catalysing the hydrolytic formation of phosphatidic acid (PtdOH), with the concomitant release of the non-phosphorylated base, choline. PtdOH is in turn metabolised by PtdOH phosphohydrolase to DAG resulting in the activation of PKC. Ischaemic preconditioning has been shown to increase the activity of PLD in rabbit (165) and rat (166) myocardium. Furthermore, the adenosine A₁R agonist (R)-N²-(2-phenylisopropyl)-adenosine (165), noradrenaline (167), and ET-1 (167) can stimulate PLD, as well as PLC in the rabbit. The M₂-muscarinic receptor agonist, carbacol, has similarly been shown to stimulate PLD in the chicken heart (168).

Taken together, these studies suggest an important role for phospholipases in the proximal signalling mechanisms involved in mediating ischaemic preconditioning. It is likely that the specific phospholipase involved in this mechanism, is governed by the nature of the preconditioning stimulus. In this regard, Parsons et al. (169) have recently demonstrated differential coupling of adenosine A₁ and A₃ receptors to PLC and PLD respectively, in a chick embryo ventricular myocyte culture model of cardioprotection against ischaemia.

Protein Kinase C. PKC is a family of at least 12 related serine/threonine isoenzymes known to participate in the regulation of ionic homeostasis, vascular tone, myocyte contractility and many other cellular processes (170, 171), many of which are present in the rabbit heart (172). The PKC isoforms can be split into three broad categories: conventional (cPKC), novel (nPKC), and atypical (aPKC) (173,174). The cPKCs (α, β₁, β₂, γ) require Ca²⁺, DAG, and phospholipid for activation. The nPKC isoforms (δ, ε, η, θ) lack the calcium binding region so that these subtypes are not dependent on Ca²⁺ for activation. Finally, isoforms of the aPKC group (ζ, τ, λ, μ) are also independent of Ca²⁺. But in addition, they also lack the Zn²⁺-finger region required for binding of DAG or phorbol ester. Instead, 3′-phospho-inositides may be the activators of aPKCs. Some PKC isoforms may have detrimental effects and others beneficial effects on the myocardium. Many proteins have been proposed as substrates for phosphorylation by PKC which may lead to either positive or negative feedback responses. The individual PKC isoforms in the myocardium are likely to
function in a distinct manner to transduce signals from the cell surface to intracellular response elements (reviewed in reference 175).

It has been proposed that activation of G protein-coupled receptors by various ligands during the brief periods of ischaemia, in turn results in activation of PKC which is thought to be an important intermediate in the signal transduction pathway of ischaemic preconditioning (176). A number of studies in various models have supported this hypothesis originally proposed by Downey (177); ischaemic preconditioning has been blocked by pretreatment with specific PKC inhibitors (178-180), and the substitution of preconditioning ischaemia with various PKC activators such as phorbol 12-myristate 13-acetate (PMA) or DAG mimics the infarct limiting effects of ischaemic preconditioning. However, studies of the role of PKC in dogs have produced conflicting results (181, 182). Initial reports also showed that inhibition of PKC in pigs failed to block ischaemic preconditioning (183). However, a recent study found that while inhibition of PKC alone did not prevent protection, blockade of both PKC and tyrosine kinase simultaneously (see below) was needed to reverse protection (184). This would suggest that in some species, a pathway containing at least one tyrosine kinase acts in parallel to PKC.

Activation of PKC has also been suggested to be linked to the so-called “memory of preconditioning” (177). One of the prerequisites for PKC’s activation is its translocation from the cytosolic to the particulate fraction which includes membranes and cytoskeletal structures. Recent evidence indicates that translocation is dependent on PKC binding to a family of proteins called receptors of activated C-kinase (RACKs) (185, 186). Liu et al. (187) proposed that preconditioning ischaemia induces translocation of PKC to the target site, and while PKC remains translocated the heart is in a preconditioned state, but when the enzyme reverts back to the cytosol, protection is lost. However, the data supporting this hypothesis have been controversial. Although disruption of microtubules with colchicine, which inhibits intracellular translocation, blocked ischaemic preconditioning in rabbits (187), a study in rabbit myocytes was unable to document translocation of PKC-α, ε, or ζ following either ischaemic preconditioning or adenosine treatment (188). Similarly, no differences in subcellular distribution of total PKC was found between
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preconditioned and non-preconditioned canine myocardium (181). More recently, Ping et al. (172) demonstrated that ischaemic preconditioning induces selective translocation of the PKC-ε and η isoforms to the particulate fraction in rabbit heart. Gray et al. (185) have implicated PKC-ε in hypoxic preconditioning of cardiac myocytes using a PKC-ε selective inhibitor peptide that inhibits PKC-ε translocation and function in cardiac myocytes. Using the same isoform-selective peptide inhibitors, Liu et al. (189) have shown, in isolated rabbit cardiomyocytes, that the protection of preconditioning is abolished by the peptide inhibitor of PKC-ε but not by the peptide inhibitors selective for PKC-β, PKC-δ, or PKC-η. This group have subsequently shown that expression of a peptide homologous to PKC-ε RACK, that facilitates isoform selective translocation of PKC-ε, in cardiac myocytes in vitro, or in transgenic mice in vivo, confers protection against ischaemic injury akin to that due to ischaemic preconditioning (190). On the other hand, Kawamura et al. (191) have suggested that in addition to PKC-ε, the δ isoform is also translocated to the membrane fraction following ischaemic preconditioning in isolated rat hearts and is involved in the development of protection against post-ischaemic LV dysfunction. Mitchell et al. (179) have reported similar results in the rat heart. Moreover, Zhao et al. (192) found that expression of constitutively active PKC-δ isoform conferred protection against simulated ischaemia to isolated rat cardiomyocytes. Yoshida et al. (193) reported that PKC-α, δ and ε were translocated to the membrane fraction in rat hearts following ischaemic preconditioning, and that this movement was completely blocked by the PKC inhibitor chelerythrine.

Taken together, these results strongly suggest the role of some PKC isoforms in the mechanism of ischaemic preconditioning, although the nature of the exact isoform(s) involved remains elusive. Moreover, translocation of PKC has not directly been correlated with the presence of a protected state.

Tyrosine Kinase. Protein tyrosine kinases (TK) are a family of enzymes, which unlike PKC which is a serine threonine kinase, phosphorylate proteins on tyrosine residues. TKs play pivotal roles in many signal transduction events and can be broadly categorised as belonging to one of two major groups by virtue of whether they possess or lack the receptor-like features of extracellular ligand-binding and
transmembrane domains: receptor tyrosine kinases and the non-receptor/cytosolic tyrosine kinases (reviewed in reference 194). Receptor TKs are activated by ligand binding to the extracellular region of the receptor, thereby inducing receptor dimerisation, trans-autophosphorylation within the intracellular domain, and activation of TK activity towards intracellular substrates (195). Although non-receptor TKs can also function in receptor-mediated transmembrane signalling by acting as subunits of receptors that lack intrinsic TK activity, most non-receptor TKs are located in the cytosol, associate with the inner face of cytoplasmic membranes, or partition between different subcellular compartments, and their function depends on specific subcellular localisation which is dictated by intrinsic localisation signals (194). Both receptor and non-receptor protein TKs can be further classified into families based on the criteria of amino acid similarity within the catalytic domain and the presence of common structural domains. There are currently over 50 known vertebrate genes that encode receptor TKs and at least 33 that encode non-receptor TKs (194, 195).

In noncardiac cells, evidence has been reported that TKs can act upstream and therefore activate PKC (196, 197), act in parallel pathways (198), or downstream of PKC (199, 200). Maulik et al. (201) were the first to suggest a role for TKs in mediating ischaemic preconditioning by demonstrating that genistein, a relatively selective TK inhibitor, blocks enhanced postischaemic functional recovery in isolated rat hearts. In this study, genistein also blocked preconditioning-induced activation of PLD, suggesting that TK activation is an early step in classic ischaemic preconditioning. A role for TKs has also been demonstrated in mediating protection against infarction in rats (202). Similarly, Baines et al. (203) were able to block protection against infarction in rabbits with genistein, although their data suggested that TK acts downstream of PKC. Recent evidence suggests that ischaemic preconditioning protocols in rabbits activate two specific members of the Src family of protein TKs, Src and Lck, by a mechanism which is dependent on PKC-mediated signalling (204). Despite the apparent involvement of TKs in rats and rabbits, inhibition of TKs failed to block ischaemic preconditioning in dogs (205). Vahlhaus et al. (184) found that genistein alone could not abolish the reduction in infarct size by ischaemic preconditioning in pigs. Yet combined inhibition of both PKC and TKs
completely blocked protection, implying that the two kinases act in parallel in the pig. Although these results suggest the role of protein TKs in mediating ischaemic preconditioning in some species, the nature of the TK involved and its position in the signalling pathways remains elusive.

Mitogen-Activated Protein Kinases. Another family of protein kinases in the mammalian heart that has recently been the subject of much attention, as putative mediators of ischaemic preconditioning, is the mitogen-activated protein kinase (MAPK) superfamily. The best characterised MAPK pathways identified in the heart consist of the 42/44 kDa extracellularly responsive kinases (ERKs) and the two “stress-responsive” MAPK subfamilies, namely, the 46/54 kDa c-Jun N-terminal kinases (JNKs) and the 38 kDa p38 MAPK (reviewed in reference 206). Each pathway follows the same conserved three-tier module (Figure 1.1). A MAP kinase kinase kinase (MAPKKK or MEKK) is activated and in turn phosphorylates a MAP kinase kinase kinase (MAPKK or MEK) on serine/threonine residues (207). The MEK is a dual-specificity kinase that phosphorylates a threonine and a tyrosine within the motif Thr-X-Tyr on the MAPK. Phosphorylation of these residues is essential for the activation of the MAPK, and membership of a given MAPK subfamily can be assigned on the basis of the identity of the X residue (206). The MAPKs themselves are proline directed serine/threonine kinases, preferentially phosphorylating serine and threonine residues within a Pro-X-Ser/Thr-Pro sequence.

Cellular stresses such as hyperosmotic shock, hypoxia/reoxygenation and ROS have been shown to activate the JNKs in cultured cardiomyocytes, as have the proinflammatory cytokines, interleukin-1β and tumour necrosis factor-α (208). The activation of p38 MAPKs in myocytes is less well characterised, but cellular stresses such as ROS, hypoxia/reoxygenation, hyperosmotic shock and proinflammatory cytokines have been similarly shown to activate p38 MAPK (206). Recent evidence suggests that G protein-coupled receptor agonists may also activate p38 MAPK and JNK (198, 209, 210). Activation of ERKs on the other hand, is principally stimulated by a variety of growth factors and G protein-coupled receptors agonists (211). Importantly, several recent studies in isolated hearts and in vivo, have documented
that brief myocardial ischaemia/reperfusion is associated with activation of all of the three MAPK subfamilies (212-218).

Figure 1.1. Scheme for the Activation of Stress-responsive MAPK Cascades. Cellular stresses induce activation of small G proteins, Rac and Cdc42. G protein-coupled receptor agonists activate the small G protein Ras through a PKC-dependent mechanism, whereas receptor tyrosine kinases may directly activate Ras. Ras is recognised to participate in the activation of the ERK cascade and, either directly or indirectly, may activate the stress responsive MAPKs (JNKs and p38 MAPKs).
Of all the MAPK subfamilies, the role of p38 MAPK signalling pathway in mediating classic preconditioning has been most extensively investigated. Six p38 MAPK isoforms have been identified: the alternatively spliced p38 MAPKα₁/α₂ and p38 MAPKβ₁/β₂ isoforms, p38 MAPKγ, and p38 MAPKδ, although it would appear that only the α and β isoforms are expressed to any degree within the heart (206). Of the upstream kinases, MKK4 can phosphorylate JNK and p38 MAPK in vitro (Figure 1.1). Two other kinases, MKK3 and MKK6, selectively activate p38 MAPK in several cell types and exhibit a degree of isoform specificity; MKK3 will only activate p38 MAPKα and γ isoforms, whereas MKK6 can activate the α, β, and γ subtypes (206). The primary substrate of p38 MAPK are two homologous protein kinases, MAPK-activated protein kinase 2 and 3 (MAPKAPK2 and MAPKAPK3) (219). These enzymes have overlapping substrate specificities and both phosphorylate the small heat shock protein Hsp27, an important regulator of actin dynamics (220).

The studies evaluating the role of p38 MAPK in classic preconditioning have mainly examined activation of this enzyme following ischaemia/reperfusion, and whether inhibition of p38 MAPK abrogates the cardioprotective effect. However, the published studies are inconsistent, and the role of p38 MAPK in early preconditioning seems to be controversial (221). While most studies have demonstrated activation of p38 MAPK following ischaemic preconditioning, and some have shown that the downstream substrate, MAPKAPK2, is also activated, studies that have used specific p38 MAPK inhibitors have produced conflicting results. In several studies, specific inhibition of p38 MAPK with SB 203580 or SB 202190, resulted in complete abrogation of the cardioprotective effects, indicating that activation of p38 MAPK is an essential signalling event in early preconditioning (213, 217, 222). In apparent contradiction to these findings, other studies have reported that SB 203580 or SB 202190 themselves can function as cardioprotective compounds resulting in improved post-ischaemic functional recovery (223, 224), reduced myocardial cell death (225, 226) and preconditioning of cardiac myocytes against simulated ischaemia (227). These studies suggest that activation of p38 MAPK may be detrimental to the heart and its inhibition will protect the myocardium against ischaemic injury.
Recent studies in cultured cells have shown that isoforms of the p38 MAPK may possess distinct biological functions (228). The apparent discrepancies observed in the studies evaluating the role of p38 MAPK in ischaemic preconditioning may have arisen as a result of the fact that ischaemia may differentially regulate the different isoforms of p38 MAPK family. For example, Saurin et al. (227) have recently demonstrated in neonatal cardiac myocytes, that sustained simulated ischaemia results in increased phosphorylation of p38 MAPKα but decreased phosphorylation of p38 MAPKβ. The enhanced activity of p38 MAPKα was found to be detrimental in this model, and inhibited by brief periods of ischaemic preconditioning. Another contributing factor that may explain the discrepant results of the studies evaluating the role of p38 MAPK in ischaemic preconditioning, is the efficacy and selectivity of SB 203580. Although, this agent has a potent effect on the activity of p38α and p38β MAPKs, its efficacy for the δ and γ isoforms is low (229). Furthermore, at higher concentrations, SB203580 has been shown to inhibit the JNK family of MAPKs (229). It seems therefore that targeted activation or inhibition of individual p38 MAPK isoforms will be essential to provide conclusive evidence to elucidate the role of p38 MAPKs in ischaemic preconditioning.

Downstream of p38 MAPK, activation of MAPKAPK2 (201, 213, 217), and translocation and phosphorylation of Hsp27 (230, 231) has been reported following ischaemic preconditioning, although the contribution of these events to the cardioprotective effects of ischaemic preconditioning remains unknown.

**Nitric Oxide.** The free radical gas, nitric oxide (NO), is a physiological autocoid formed in cells during the conversion of L-arginine to L-citruline in the presence of O₂ by NADPH-dependent NO synthase (NOS) (232). To date, three isoforms of NOS have been described; neuronal NOS (nNOS or NOS 1), inducible NOS (iNOS or NOS 2), and endothelial cell NOS (ecNOS or NOS 3). NO has multiple physiological actions (reviewed in reference 233). It maintains vascular and thus coronary vasodilatory tone, preserves physiological vascular impermeability, interferes with platelet aggregation, and inhibits adhesion of polymorphonuclear leucocytes and platelets to vascular endothelium. It also exerts negative inotrope and chronotropic effects on cardiomyocytes. Increased availability of NO might protect
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by promoting blood flow, preventing an increase in vascular permeability, and increasing white cell and platelet antiadhesion properties to prevent vascular plugging (233). Conversely, increased NOS activity could also be detrimental to reperfused tissue. In the presence of superoxide anion (\(O_2^-\)) which is abundant during early reperfusion, NO forms peroxynitrite anion (ONOO\(^-\)) and hydroxyl radical (OH) which may initiate lipid peroxidation, inactivate iron-sulphur-centred enzymes involved in mitochondrial respiration, and have other cytotoxic properties (233).

The role of NO in mediating classic preconditioning has been controversial since many different models have been used with a variety of end-points. Some studies, using NOS antagonists, have reported blockade of the cardioprotective effects of ischaemic preconditioning against infarction (234), post-ischaemic depression of mechanical function (235), and reperfusion arrhythmias (236). However, a number of other studies have failed to demonstrate a role for NO in mediating early protection (237-240). Recently, Nakano et al. (241), reported that although exogenous NO can trigger a preconditioned state in isolated rabbit hearts, through a mechanism involving ROS and PKC, endogenous NO is not involved in mediating the protective effects of ischaemic preconditioning.

Others. A number of other mediators and cellular processes have been proposed to play a role in mediating ischaemic preconditioning. These include 5' nucleotidase (5'NT), calcium, and depletion of myocardial energy stores. However, the evidence supporting a role for these mediators remains controversial.

Kitakaze et al. (242) postulated that stimulation of \(\alpha_1\)-adrenoceptors during brief preconditioning ischaemia activates PKC and results in the upregulation of ectosolic or membrane-associated 5'NT, the enzyme responsible for the dephosphorylation of adenosine monophosphate to form adenosine. The resultant increase in myocardial adenosine levels then amplifies PKC activation by a G protein-coupled pathway and culminates in cardioprotection. In support of this hypothesis, this group have demonstrated increased activity of ectosolic 5'NT in response to brief preconditioning ischaemia, together with increased adenosine concentrations in
coronary venous blood during sustained coronary occlusion, in preconditioned dogs versus controls. Moreover, in the canine model, treatment with α,β-methylene adenosine 5' diphosphate, an inhibitor of ectosolic 5'NT, rendered preconditioning ineffective in limiting infarct size (182, 243-245). However, other groups have found that myocardial adenosine levels are significantly depressed, rather than increased, during the sustained ischaemic insult in preconditioned hearts (246-248). Furthermore, Przyklenk et al. (249, 250) did not find a correlation between myocardial 5'NT activity and infarct size, or evidence of augmented 5'NT activity during the sustained coronary occlusion, in preconditioned canine hearts versus time-matched controls.

Based on the known regulatory effects of Ca^{2+} and its participation as a second messenger in many signalling pathways, it has also been suggested that a brief, nonlethal increase in intracellular Ca^{2+} concentration during brief preconditioning ischaemia/reflow may be a component of the second messenger pathway responsible for infarct size reduction with preconditioning (251-253). The proposed sources for this transient Ca^{2+} influx include the Na^+/H^+ and Na^+/Ca^{2+} exchange, release of Ca^{2+} from intracellular stores via activation of IP_3, and changes in the rates of ryanodine-sensitive sarcoplasmic reticular Ca^{2+} uptake and release, and Ca^{2+} induced Ca^{2+} release from the sarcoplasmic reticulum (reviewed in reference 254). However, other studies have refuted the concept that Ca^{2+} influx via Na^+/H^+ and Na^+/Ca^{2+} exchange mediates preconditioning, and no difference has been found in the rate of sarcoplasmic reticular Ca^{2+} reuptake during sustained coronary artery occlusion in preconditioned canine myocardium versus controls (254).

Similarly, with regard to a role for glycogen depletion, the evidence is contradictory. For example, although Wolfe et al. (255, 256) suggested that glycogen depletion and attenuation of intracellular acidosis occurring in response to brief antecedent ischaemia might contribute to infarct size reduction with preconditioning, this has been refuted by observations of persistent preconditioning-induced cardioprotection in the absence of glycogen depletion (257, 258).
**End-effectors**

**ATP-Sensitive Potassium Channels.** Among the candidates for an end-effector or target protein activated by upstream intracellular signalling and conferring protection to the myocyte is the ATP-sensitive potassium channel ($K_{\text{ATP}}$). This channel is normally inhibited by intracellular ATP and opens during periods of energy depletion (reviewed in reference 259). $K_{\text{ATP}}$ channels are found on a wide variety of tissues, and one of their most prominent functions is to modulate insulin release from pancreatic β cells by setting the resting membrane potential ($E_r$) of the cell. An effect on $E_r$ also underlies the mechanism of $K_{\text{ATP}}$ action in vascular smooth muscle (260). In the heart, $K_{\text{ATP}}$ channels are present on the sarcolemma of cardiac myocytes but their function remains unclear. The opening of these channels in cardiomyocytes has little effect on $E_r$, because it is already close to the equilibrium potential for $K^+$, but the outward current carried by $K_{\text{ATP}}$ shortens the action potential duration and if large enough, can render the cell inexcitable. Thus, it has been suggested that suppression of excitability spares energy by reducing that required for active ion cycling because of membrane depolarisation and $Ca^{2+}$ handling (261).

The evidence supporting a role for $K_{\text{ATP}}$ channels in ischaemic preconditioning comes from studies in rats (129), rabbits (262), dogs (263), pigs (264) and man (265) where the blockade of these channels with sulphonylurea receptor antagonists was shown to abolish the protective effects of classic preconditioning. Conversely, pharmacological activation of $K_{\text{ATP}}$ channels with bimakalim, cromokalin, or pinacidil produced cardioprotective effects comparable to that of ischaemic preconditioning (266). Furthermore, PKC has been shown to activate $K_{\text{ATP}}$ channels in human and rabbit ventricular myocytes (267) and a synergistic action of adenosine and PKC on $K_{\text{ATP}}$ channels and shortening of action potential duration has been reported (268). On the basis of these studies, it was hypothesised that the opening of sarcolemmal $K_{\text{ATP}}$ channels during ischaemia is facilitated by the activation of ischaemic preconditioning signalling pathways, resulting in a repolarising current that enhances the cardiac action potential shortening that occurs in the early phase of the long ischaemic insult (269). The result would be better preservation of cellular energy stores and suppression of deleterious downstream events, such as cellular...
Ca^{2+} overload, which in turn reduces cardiac workload and enhances myocardial viability.

More recent work however, indicates that abbreviation of action potential duration may not be necessary for the protection from preconditioning and K_{ATP} channel openers (270, 271). Furthermore, preventing ischaemic action potential shortening by concomitant treatment with dofetilide did not eliminate protection (272). Moreover, K_{ATP} channel openers and ischaemic preconditioning have been shown to be protective in models using unstimulated cardiac myocytes in which action potential duration should not be a factor (57).

These findings have prompted investigation into the role of K_{ATP} channels in other cell membranes, such as that on the inner mitochondrial membrane (mitoK_{ATP}), in mediating ischaemic preconditioning. Garlid et al. (273) showed that diazoxide, a potent opener of mitoK_{ATP} with weak actions on sarcolemmal K_{ATP}, protected isolated rat hearts against ischaemic contracture and improved post-ischaemic functional recovery. These effects were shown to be independent of action potential duration and were reversed by K_{ATP} blockers glibenclamide and 5-hydroxydecanoate (5-HD). Similarly, in a model of simulated ischaemia in intact rabbit ventricular myocytes, Liu et al. (274), using the native autofluorescence of mitochondrial flavoproteins as a marker of opening of mitoK_{ATP}, showed that diazoxide selectively opened the mitoK_{ATP}, and reduced the rate of cell death to about half of that in controls. This protection was abolished by 5-HD, a selective blocker of these channels. Using these and other selective activators and blockers of mitoK_{ATP}, further studies in isolated myocytes (275, 276) and intact hearts (277-280), have supported the role of mitoK_{ATP} in mediating the protective effects of ischaemic preconditioning. Moreover, mitoK_{ATP} channels seem to be modulated by PKC, since direct activation of this enzyme with the phorbol ester PMA both accelerates and augments the mitochondrial oxidation induced by diazoxide (281), and pretreatment with 5-HD abolishes the protective effects of pharmacological preconditioning with adenosine or PMA (282). Further support for the involvement of mitochondrial rather than sarcolemmal K_{ATP} channels in ischaemic preconditioning has come from studies demonstrating that HMR1883, an antagonist selective for surface K_{ATP}, is ineffective in blocking protection against ischaemic injury (278-280, 283).
Although this large body of evidence points to the role of mitoK\textsubscript{ATP} as distal effector proteins mediating the protective effects of ischaemic preconditioning, very recent evidence has suggested an alternative role for mitoK\textsubscript{ATP} as a trigger rather than an effector during the prolonged ischaemic insult. Pain et al. (284) showed that 5-HD administered during a 5 minute preconditioning ischaemia or a short diazoxide pretreatment could block the infarct limiting effect of preconditioning in perfused rabbit hearts, but was ineffective when given after the preconditioning period. Furthermore, they showed that TK inhibition abrogated diazoxide-induced protection, whereas PKC inhibition did not. In addition, application of free radical scavengers during diazoxide pretreatment abolished protection, suggesting that opening of mitoK\textsubscript{ATP} channels is an upstream trigger of ischaemic preconditioning, resulting in the production of ROS and activation of TKs (284). However, other studies have not demonstrated this critical timing for mitoK\textsubscript{ATP} activation and further studies are needed to explain these differences (285).

Taken together, these results strongly suggest the involvement of mitoK\textsubscript{ATP} channels in mediating protection following ischaemic preconditioning. However, the mechanisms by which opening of these channels may protect the ischaemic myocardium has not been elucidated. A number of mechanistic hypotheses have been proposed, which are not mutually exclusive and may all contribute to protection (reviewed in reference 286). These include the salutary effect of opening of mitoK\textsubscript{ATP} channels on regulation of mitochondrial matrix volume and ATP production, regulation of mitochondrial Ca\textsuperscript{2+} handling, and mitochondrial redox state and the production of ROS (286). Further studies are needed to characterise the precise role of the mitoK\textsubscript{ATP} channel and the mechanisms by which opening of these channels may protect the ischaemic myocardium.

**Anti-Oxidant Enzymes.** The effect of preconditioning on anti-oxidant defences is currently equivocal. In the rabbit, \textit{classic} preconditioning did not alter the activities of a range of anti-oxidant enzymes including catalase, Mn-SOD, Cu/Zn-SOD, GSH peroxidase and GSH reductase (287). In the dog (288), however, ischaemic preconditioning increased the activity of Mn-SOD within five minutes with a concomitant decline in Mn-SOD protein, increase in GSH peroxidase activity and
decrease in GSH reductase activity. Preconditioning of the rat heart induced catalase mRNA, Mn-SOD mRNA, and proto-oncogenes (c-fos and c-myc) with an increase in the activities of Mn-SOD, peroxismal catalase and GSH peroxidase (289). More recently, Yamashita et al. have demonstrated a correlation between early protection against infarction and enhanced Mn-SOD activity, following exercise (73) or whole body hyperthermia (72) in rats. The significance of these alterations in the activity of various anti-oxidants remains unknown.

1.4.b Mechanisms of Delayed Preconditioning

The mechanisms underlying the delayed phase of myocardial protection are less well understood than those mediating classic preconditioning. It is generally believed that the subacute adaptation that confers an ischaemia-tolerant phenotype to the myocardium results from the synthesis of, as yet unidentified, cytoprotective proteins and/or post-translational modification of such proteins. Unlike the early phase, delayed ischaemic preconditioning protects not only against myocardial infarction but also against myocardial stunning, and most of the current knowledge regarding the mechanisms of this delayed adaptive phenomenon arises from studies evaluating these two end-points of ischaemic injury. There is also limited evidence for delayed protection against ischaemia and reperfusion induced arrhythmias. However, there is increasing evidence suggesting mechanistic differences between these forms of delayed preconditioning and it is therefore important to differentiate between the various preconditioning stimuli and end-points of myocardial injury. Overall, the cellular mechanisms underlying delayed cardioprotection, parallel those of classic preconditioning, i.e. triggers initiate an intracellular signalling cascade with the ultimate induction/activation of one or more cytoprotective end-effector proteins.

**Triggers**

There is some evidence to suggest involvement of a number of metabolites and ligands including adenosine, NO, ROS and opioid receptor agonists, as the chemical signals that initiate the delayed phase of ischaemic preconditioning.
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**Adenosine.** Baxter et al. (290) were the first to propose that adenosine released during the preconditioning stimulus, triggers the development of delayed protection. They showed that in an *in vivo* rabbit model of regional myocardial ischaemia-reperfusion, delayed protection against infarction induced by brief periods of ischaemic preconditioning 24 hours earlier, was abolished by non-selective inhibition of adenosine receptors with 8-p-sulfophenyl theophylline (SPT) during the preconditioning protocol. Conversely, treatment with a selective adenosine A₁R agonist, 2-chloro-N⁶-cyclopentyladenosine (CCPA), in lieu of ischaemic preconditioning, mimicked infarct-limitation at 24 hours, suggesting the importance of these receptors (290). These findings were confirmed in an isolated rabbit heart model of global ischaemia by the same group (291). Another group have suggested the importance of both A₁ and A₃ but not the A₂A adenosine receptors in triggering delayed protection against infarction in the rabbit (292). However, as opposed to late preconditioning against infarction, adenosine does not seem to play a role in initiating the protective effects of delayed preconditioning against myocardial stunning. In conscious rabbits, late preconditioning against myocardial stunning is not abolished by pretreatment with non-selective adenosine receptor antagonists SPT or PD115199 (293). Furthermore, transient adenosine A₁R activation with CCPA fails to elicit delayed preconditioning against stunning in this model. Similar results have been obtained in the pig myocardium (101). Selective adenosine A₃R activation also fails to elicit delayed protection against myocardial stunning (294). These results suggest the existence of important differences in the mechanisms underlying these two phenomena.

**Nitric Oxide.** Increasing recent evidence from Bolli’s group has implicated NO, generated during the brief periods of preconditioning ischaemia, as an important trigger of delayed preconditioning against both myocardial infarction and stunning (295). Non-selective inhibition of all NOS isoforms before the preconditioning ischaemic stimulus, blocks the development of delayed protection against myocardial stunning and infarction (102, 296). The source of NO formation during the preconditioning ischaemia is likely to be eNOS since selective iNOS inhibition with aminoguanidine or S-methylisothiourea failed to abolish delayed preconditioning (297). Furthermore, exposure to exogenous NO seems to mimic late preconditioning,
since pretreatment with NO donors in the absence of ischaemia induces delayed protective effects against both myocardial infarction and stunning (298).

ROS. Evidence from in vivo pig, rat and rabbit models of regional myocardial ischaemia has implicated the generation of ROS during preconditioning ischaemia, as essential triggers of delayed cardioprotection. Pretreatment with the antioxidant MPG during ischaemic preconditioning, completely prevented the development of delayed preconditioning against myocardial stunning (299), infarction and arrhythmias (90). MPG has also been found to prevent delayed protection against infarction induced by heat stress (72) and exercise (73). Taken together these results suggest that sublethal oxidative stress plays an important role in triggering delayed cardioprotection, although the sources and the identity of these ROS remains elusive.

Opioids. Recent data in rats (300) and mice (301) indicate that pharmacological activation of δ-opioid receptors induces a delayed infarct-limiting effect 24-48 hours later. Whether δ-opioid receptors are involved in triggering the late phase of ischaemic preconditioning is currently unknown.

Mediators
There is increasing evidence for an important role of a number of protein kinases in mediating delayed protection following ischaemic preconditioning.

Protein Kinase C. A number of studies using various pharmacological inhibitors, have implicated PKC in mediating delayed cardioprotection following ischaemic preconditioning (302) or heat stress (303, 304). Conversely, administration of the PKC activator dioctanoyl-sn-glycerol in lieu of ischaemic preconditioning, induced infarct-limitation in the rabbit 24 hours later (305). Similarly, a role for PKC has been demonstrated in delayed protection against infarction in rats 24 hours after heat stress (303, 304), and exercise (306). More recently, ischaemic preconditioning has been shown to cause selective translocation and activation of PKC-ε and PKC-η isoforms, and isoform-selective inhibition of the former seems to abolish delayed protection against myocardial stunning in the rabbit model (172, 307). Similarly, Wilson et al. (308) reported an association between development of delayed
cardioprotection against ventricular arrhythmias, 24 hours after rapid cardiac pacing in dogs, and sustained translocation of PKC-ε to the membrane fraction. Pharmacological preconditioning with NO donors in the absence of ischaemia, also induces, and is dependent on translocation and activation of PKC-ε (309). It is currently not known whether PKC is involved in the development of adenosine A1R induced delayed preconditioning.

**Protein Tyrosine Kinases.** There is some evidence to suggest involvement of protein TKs in mediating some forms of delayed preconditioning. Imagawa et al. (310) were able to block late ischaemic preconditioning against infarction in a rabbit model using genistein, a broad inhibitor of most protein tyrosine kinases. In contrast, genistein failed to abolish heat stress induced delayed preconditioning in rats (303). Subsequent studies have demonstrated selective activation of two members of the Src family of protein tyrosine kinases (Src and Lck) following ischaemic preconditioning, and that inhibition of these isoforms with lavendustin A abolishes delayed protection against myocardial stunning (204, 311). This evidence suggests that protein TKs may act downstream of PKC in mediating delayed protection. The role of tyrosine kinases in mediating adenosine A1R mediated late preconditioning has not been evaluated.

**Mitogen-Activated Protein Kinases-** Ischaemic preconditioning protocols known to induce delayed cardioprotection have been shown to activate all of the 3 MAPK subfamilies, namely, the p44/p42 MAPKs (ERKs), the p38 MAPKs, and the p46/p54 MAPKs (JNKs) (215, 216). Interestingly, the activation of p42/p44 MAPKs and JNKs seems to be PKC dependent and is abolished by treatment with chelerythrine. Conversely, selective overexpression of PKC-ε in adult rabbit myocytes induces activation of p42/p44 MAPKs and protects against simulated ischaemia, an effect that can be abolished by p44/p42 MAPK inhibitors (216). Punn et al. (312), found that simulated ischaemia followed by reoxygenation in neonatal rat ventricular myocytes induced phosphorylation of p42/p44 MAPK, and that inhibition of this phosphorylation abolished delayed protection against prolonged simulated ischaemia. However, it is as yet unknown whether the activation of MAPKs contributes to the development of delayed preconditioning in *in vivo* models.
Transcription Factors. It seems likely that recruitment of the signalling mechanisms outlined above by the preconditioning stimulus, in turn results in activation of transcription factors that control the expression of cardioprotective genes responsible for delayed preconditioning. One such transcription-regulatory element to have been studied in this context is nuclear factor-κB (NF-κB), which is known to modulate gene expression of iNOS (see below). There is increasing evidence to suggest that short term protocols of ischaemia-reperfusion in vivo and in vitro induce activation of NF-κB (313-315), and inhibition of this activation with diethyldithiocarbamate, has been shown to abolish delayed cardioprotection at 24 hours (315). Furthermore, activation of NF-κB seems to depend on a signalling mechanism involving ROS, PKC and protein TKs, suggesting that NF-κB may be a downstream pathway through which multiple signals elicited by ischaemic preconditioning act to induce gene expression (315).

End-effectors
The final effectors of the delayed protection conferred by preconditioning are not known. Sublethal ischaemia results in the production of many new gene-products such as protooncogenes, heat shock proteins, intracellular anti-oxidant enzymes and other regulatory proteins, as well as post-translational modification of such proteins, as part of the cell-stress response to ischaemia. Furthermore, the delayed appearance and the subsequent duration of the second window of protection is consistent with involvement of new protein synthesis and degradation.

Heat Stress Proteins. Heat stress proteins (Hsp) are a family of proteins that are expressed in all living cells, their expression can be induced by a variety of stresses including hyperthermia, oxidative stress, ischaemia, heavy metals and inflammatory mediators, and have been associated with an inducible and transient cellular protection in various cells and organs (316). These proteins are often described according to their molecular weight; e.g. 70 kDa Hsp (Hsp70), 60 kDa Hsp (Hsp60), 27 kDa Hsp (Hsp27). Some of these proteins are expressed constitutively and others are specifically stress inducible. These proteins have many diverse functions in the general maintenance of cellular integrity, including molecular chaperone activity in the control of protein synthesis, folding and degradation, and some Hsps are
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associated with specific organelles (e.g. Hsp60 and the mitochondrion, Hsp27 and the cytoskeleton). The cardiovascular properties of heat stress proteins have recently been reviewed in detail (317).

The involvement of this family of “house keeping” proteins in mediating delayed preconditioning was suggested by an early study which showed an elevation in the myocardial content of the principal inducible heat shock protein, Hsp70i, 24 hours after ischaemic preconditioning in the rabbit (87). Moreover, protein levels of Hsp70 have been directly correlated with the amount of protection (318, 319). Further evidence in support of a role for this group of cytoprotective proteins has come from gene transfection studies (320, 321) and studies with transgenic mice constitutively over-expressing human Hsp70 (322, 323). A number of other studies have, however, questioned the role of Hsp70 in delayed cardioprotection. For example, subsequent studies have found no evidence of Hsp70 induction in rabbits following pharmacological preconditioning with CCPA (324, 325), or MLA (326, 327). Other studies have found a temporal dissociation between the expression of Hsp70 and tolerance to ischaemia following heat stress in rats (328-330), while induction of Hsp70 by heat stress failed to confer delayed cardioprotection in mice (331). Taken together, these results suggest that despite the well documented cardioprotective effects of Hsp70, it is unlikely that its enhanced expression is responsible for mediating delayed preconditioning, and may simply represent an epiphenomenon. It is however still possible that other factors such as post-translational modification or compartmental distribution, rather than the cellular content of Hsps might be important in inducing cardioprotection, and this should be the next focus of investigation.

Anti-Oxidant Enzymes. Kuzuya and colleagues reported an increase in the protein content and activity of the mitochondrial antioxidant Mn-SOD 24 hours after ischaemic preconditioning in dogs, and found that the temporal profile of Mn-SOD induction paralleled that of delayed protection against infarction (88, 288). These authors did not find an increase in the activity or content of any other antioxidant enzymes. The same group have found a similar association between induction of Mn-SOD and delayed cardioprotection in rats following heat stress (72), or exercise (73).
Similar findings have been reported in neonatal rat cardiomyocytes 24 hours after hypoxia (332). Importantly, in vivo administration of antisense oligonucleotides to Mn-SOD has been shown to abolish late preconditioning against infarction induced by heat stress (333), and exercise (73), suggesting that Mn-SOD upregulation is essential for these forms of delayed cardioprotection. Other studies, however, have not found upregulation of antioxidant defences during the late phase of preconditioning. Xi et al. (331) did not find enhanced expression of Mn-SOD 24 hours following heat stress in the mouse heart. Similarly, Tang et al. did not detect an increase in the activity of a number of antioxidant enzymes, including Mn-SOD, Cu,Zn-SOD, catalase, glutathione peroxidase, or glutathione reductase, 24 hours after ischaemic preconditioning in conscious pigs (334) and rabbits (335). The precise role of Mn-SOD in mediating delayed cardioprotection remains unknown.

Nitric Oxide Synthase. Increasing recent evidence from Bolli's laboratory points to an important role for iNOS in mediating delayed ischaemic preconditioning against myocardial infarction and stunning (295). These investigators have shown that selective inhibition of iNOS 24 hours after ischaemic preconditioning, completely abrogates delayed protection against myocardial stunning (297), and infarction (336). These findings were confirmed by studies in transgenic mice, with targeted disruption of the iNOS gene. In these animals, compared to control mice, lack of induction of iNOS protein 24 hours after ischaemic preconditioning, was associated with complete abrogation of the protective effects against infarction (337). Recent evidence suggests that other forms of delayed preconditioning, including that induced by MLA (338, 339), diazoxide (340), and NO donors (341), are also dependent on upregulation of iNOS. The role of iNOS in mediating adenosine A_1 receptor induced late cardioprotection is not known, and recent reports have arrived at conflicting conclusions (342, 343). Furthermore, the mechanisms by which enhanced production of NO, as a result of iNOS upregulation, may protect the ischaemic myocardium remain elusive.

K\textsubscript{ATP} Channels. A number of recent studies in rabbits, using an antagonist approach, have suggested that opening of K\textsubscript{ATP} channels during the prolonged ischaemic insult may be necessary for induction of delayed protection against infarction following
preconditioning with ischaemia (344, 345), MLA (346), heat stress (347), adenosine A₁R agonists (325, 348), and δ₁-opioid receptor agonists (300). In contrast, delayed ischaemic preconditioning against myocardial stunning does not seem to require $K_{ATP}$ channel activity (345), therefore suggesting mechanistic differences between delayed preconditioning against infarction and stunning. Moreover, as with classic preconditioning, the mechanisms whereby opening of $K_{ATP}$ channels confers protection to the ischaemic myocardium remain illusive.

Others. Recent studies in rabbits have implicated the involvement of two other proteins as potential effectors of delayed ischaemic preconditioning. The first of these, cyclooxygenase-2, was found to be upregulated 24 hours after ischaemic preconditioning in rabbit myocardium (349), and its pharmacological inhibition abrogated protection against infarction and stunning at this time point. The same group have also suggested a role for aldose reductase, an enzyme that catalyses the metabolism of glucose to sorbitol and detoxification of ROS-derived lipid aldehydes, in mediating delayed cardioprotection (350). Further studies are needed to confirm the role of these proteins in the late phase of ischaemic preconditioning, and to elucidate the mechanisms by which they may protect the ischaemic myocardium.

1.5 Preconditioning the Human Myocardium

The potential for clinical application of such a powerful protective phenomenon has generated enormous interest in whether the human myocardium behaves in a similar way to that of animals in the experimental laboratory, and is amenable to protection by ischaemic/pharmacological preconditioning, and if so, what are the underlying mechanisms? The driving force behind the extensive research that followed, was 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.

*In vitro Studies*

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 (351), and isolated atrial trabeculae obtained at the time of cardiac surgery (352) both suggest that protection can be induced in vitro using metabolic and functional end-points respectively. Moreover, using the same in vitro models, it has been demonstrated that the mechanisms of protection in human tissue closely resemble that observed in many animal species; namely the involvement of adenosine as an important trigger, PKC as an intermediate intracellular messenger, and the K_ATP channel as a potential end effector protein (57, 265, 353, 354). Recent work by Arstall et al. (355) provides direct evidence that in addition to classic preconditioning, human ventricular myocytes in vitro exhibit delayed cardioprotection 24 hours following a short period of simulated ischaemia. Similar findings have been reported in an adult human cardiac myoblast cell line (356), and in isolated human atrial appendage (357). It must be pointed out however, that the use of simulated rather than true ischaemia in these in vitro models, along with assessment of injury using surrogate metabolic and functional end-points rather than necrosis, does not allow extrapolation of these findings to reflect the response of in vivo human ventricular myocardium to sustained ischaemia.

Preinfarction Angina

In the clinical setting, there is some evidence to suggest that preconditioning may occur naturally in patients with coronary artery disease. Many patients experience brief episodes of ischaemia before an acute myocardial infarction. It is theoretically possible that this preinfarct angina has the potential to precondition the myocardium, thereby reducing infarct size and improving survival. A number of studies have evaluated the outcome of patients suffering an acute myocardial infarction in relation to the presence of preinfarction angina. These studies have demonstrated that patients suffering angina prior to a myocardial infarction 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 (358-362). Follow up studies have suggested that in patients with preinfarct angina, long term survival is also improved compared with patients who are asymptomatic prior to their infarction (363, 364).
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. Preconditioning, by virtue of delaying myocardial necrosis and improving post-ischaemic functional recovery, as seen in laboratory animals, may contribute to the improved outcome in patients with preinfarct angina. Interestingly, recent evidence suggests that the time interval between the last episode of angina and the index myocardial infarction is very important. Reports from the TIMI-9B investigators (364), and studies by Ishihara et al. (363) and Yamagishi et al. (365) indicate that prodromal angina is only protective if it occurs within 24-48 hours of myocardial infarction; a time-course that closely resembles that of the delayed phase of myocardial protection following ischaemic preconditioning in animal models.

However, in addition to the possible protection conferred by ischaemic preconditioning, infarct size and the degree of preservation of post ischaemic left ventricular function are determined by a number of other factors including the extent of collateral circulation to the ischaemic myocardium, time from onset of infarction to reperfusion of the infarct related artery and residual coronary stenosis after reperfusion. Studies that have analysed the degree of collateralisation to the ischaemic zone after an infarction have found no increase in angiographically visible collateral vessels in patients with preinfarct angina (360, 361). It must be noted, however, that coronary angiography at 90 minutes following thrombolysis is unlikely to provide information about the degree of collateral recruitment to the ischaemic zone during coronary occlusion and it is therefore difficult to rule out a contribution by collateral circulation in this setting. Interestingly, in a very recent study, resting myocardial-dual isotope SPECT using $^{123}$I-15-(p-iodophenyl)-3-(R,S)-methylpentadecanoic acid ($^{123}$I-BMIPP) and thallium ($^{201}$TI) in the subacute phase of MI was employed to assess areas at risk and necrotic myocardium respectively. These authors found that patients with preinfarct angina had significantly smaller infarcts compared to those with no preceding symptoms, in the face of no significant difference in areas of myocardium at risk, suggesting that the presence of preinfarction angina did not predict improved collateral recruitment to the ischaemic zone (365).
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 following thrombolysis in patients with preinfarct angina (363, 366). This hypothesis is based on the known inhibitory effects of adenosine, released during the brief periods of preinfarct ischaemia, on platelet aggregation following activation of A2R on platelet membranes, which has been suggested to modify thrombus formation and thereby promote earlier reperfusion after thrombolysis (367). In this regard, Przyklenk and colleagues have recently demonstrated that in anaesthetised open-chest dogs, brief periods of ischaemia prior to a long ischaemic insult attenuates platelet-mediated thrombosis and improves vessel patency, and that this effect is abolished by inhibition of adenosine receptors (368, 369).

**Warm-up Angina**

Some patients are able to exercise to the point that they develop angina, rest, and then continue exercising with minimal or no further development of symptoms. This phenomenon, variably termed warm-up or first-effort angina, was for many years thought to be mediated by coronary vasodilation and recruitment of collateral vessels resulting in improved blood supply to the ischaemic myocardium during the second period of exertion (370). Recent investigations however, suggest that other mechanisms might be involved in warm-up angina. Studies have examined haemodynamic and metabolic characteristics during consecutive exercise testing (371), or consecutive periods of myocardial ischaemia resulting from pacing-induced tachycardia (372). This work has provided evidence for increased efficiency of myocardial metabolism, in terms of reduced oxygen consumption at a given work load and a reduction in anginal symptoms and ST-segment changes, during a second ischaemic period. These favourable changes were not accompanied by recruitment of collateral vessels as evidenced by similar coronary and great cardiac vein blood flow measurements. A recent study suggests that the degree of myocardial stunning following exercise induced myocardial ischaemia may also be attenuated if a preceding period of exercise had been performed 30 minutes earlier (373). Studies investigating the temporal profile of warm-up angina have demonstrated that the duration of this phenomenon is 1-2 hours following the first period of exercise, a
time course that closely parallels that of classic ischaemic preconditioning (374, 375).

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 prior to exercise fails to abolish the warm-up phenomenon (376, 377). Furthermore, investigation into the role of $K_{ATP}$ channels in mediating this form of myocardial adaptation has provided conflicting results (378, 379). It is therefore not clear at this point whether the adaptation observed during repeated exercise is a representation of the preconditioning phenomenon, or if 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.

**Studies of Myocardial Preconditioning During PTCA**

Coronary angioplasty (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 studies have addressed this issue using various indices of myocardial ischaemia including clinical, electrocardiographic, metabolic and haemodynamic measurements. Most of these studies, but not all (380), have shown that if the duration of the first balloon inflation is longer than a “threshold” of about 60-90 seconds, all indicators of myocardial ischaemia, including chest pain severity, abnormalities of LV 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, providing evidence for myocardial adaptation induced by the first period of ischaemia (381-385). As with many studies of ischaemic preconditioning in man, 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 (382), measurement of cardiac vein flow (381), changes in blood flow velocity in the contralateral coronary artery (386) and more accurately, by assessment of intracoronary pressure derived-collateral flow index during successive balloon inflations (387), have shown that although collateral recruitment occurs in some patients, it can not 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. Tomai et al. (388) reported that blockade of K$_{ATP}$ channels with oral glibenclamide prior to angioplasty abolishes the reduction in ischaemic indices observed during subsequent balloon inflations, implying a role for these channels in mediating this form of adaptation. This finding is supported by the observation that opening of these channels with nicorandil reduces the electrocardiographic indices of ischaemia during coronary angioplasty (389). Furthermore, an important role has been demonstrated for adenosine in mediating myocardial adaptation during coronary angioplasty. Inhibition of adenosine receptors by bamiphylline (376) or aminophylline (390) abolishes myocardial adaptation during the second balloon inflation. Conversely, intracoronary infusion of adenosine prior to PTCA, independent of its vasodilatory effect, attenuates ischaemic indices during the first balloon inflation (391). Two other recent reports have suggested a role for both opioid (392) and bradykinin (393) 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 employed ST-segment elevation on the surface or intracoronary 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 (394, 395), a recent study by Downey’s group clearly indicates a dissociation between ST-segment changes on the electrocardiogram and myocardial protection in terms of infarct limitation (278). Their finding, 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 $K_{\text{ATP}}$ channels, thought to modulate ECG voltages, is clearly distinguished from their effect on the mitochondrial $K_{\text{ATP}}$ channels which have been proposed as a mediator of cardioprotection (275).

**Studies of Myocardial Preconditioning During CABG**

Possibly the most direct evidence for preconditioning in man 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, Yellon et al. (396) reported a prospective study examining the effects of a preconditioning protocol of two cycles of 3 minute of global ischaemia (induced by intermittent cross-clamping the aorta and pacing the heart at 90 beats /min) followed by 2 minutes of reperfusion prior to a 10 minute period of global ischaemia and ventricular fibrillation. 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 Jennings’ group (50). 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 (397). Of considerable interest, however, was the finding that the ATP
levels did not differ between preconditioned and control groups. This emphasises the need for multiple end-points to be used, especially in studies where 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, Perrault and colleagues (398), using a similar preconditioning protocol of one 3 min episode of aortic cross clamping before the onset of cardioplegic arrest, failed to show any beneficial effects compared to the control group; in fact the preconditioned group of patients had more CK release compared to case-matched controls. Similarly negative results have been reported by another group (399). 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 where a potential for suboptimal myocardial protection increases the risk of perioperative infarction (400). 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 (401, 402). Resolution of these discrepancies requires further research in the field.

1.6 Aims of the Study

It can be deduced from the evidence outlined above that the human myocardium is amenable to preconditioning and also that preconditioning occurs as a natural feature of some ischaemic syndromes. The prolonged time-course of the delayed phase of myocardial protection following ischaemic preconditioning or treatment with pharmacological preconditioning mimetic agents, makes this potentially of greater clinical relevance. However, the use of brief antecedent ischaemia as a means of inducing cardioprotection, except in a few circumstances such as the setting of cardiac surgery, is not desirable. On the other hand, the use of pharmacological agents to stimulate the receptors implicated in triggering preconditioning, in lieu of brief ischaemia, may provide a more benign approach for eliciting cardioprotection.
However, many agents evaluated in the experimental laboratory are limited in terms of their therapeutic potential because of profound toxicity when administered in vivo. One potential candidate is adenosine; currently in clinical use for treatment of some forms of supraventricular arrhythmia and as a diagnostic tool used in conjunction with certain myocardial imaging modalities. The safety profile of adenosine, along with the demonstration that adenosine or its analogues, when used in vivo, result in myocardial adaptation and enhanced tolerance to ischaemia both in the experimental laboratory and in man, make this agent of great potential value for use in the clinical setting for induction of myocardial protection.

However, virtually nothing is known about the mechanisms responsible for the protection of the ischaemic myocardium many hours following transient activation of adenosine receptors. Only after elucidation of the characteristics and the cellular mechanisms underlying this potent mode of cardioprotection, will it be possible to fully exploit this phenomenon to protect against an ischaemic insult in patients with coronary artery disease, with the ultimate aim of maintaining the heart in a sustained or chronic preconditioned state. This thesis describes work aimed at identification of the cellular mechanisms underlying the delayed phase of myocardial protection following pharmacological preconditioning with a selective adenosine A1R agonist (Chapters 4-6). The other aims of this thesis were to evaluate the possibility of prolonging the duration of protection against infarction, and maintenance of myocardium in a preconditioned state over a period of several days (Chapter 3), and in the last section (Chapter 7), to demonstrate a 'second window of protection' in patients with coronary artery disease, with investigation into the underlying mechanisms, and in particular, the role of adenosine.
Chapter Two

General Methods
2.1 Animals

Most of the experimental part of the work presented in this thesis was performed in an in vivo rabbit model of regional myocardial ischaemia and reperfusion (Chapters 3, 4, and 6). Chapter 5 describes experiments performed in isolated rat hearts perfused by the Langendorff technique and subjected to regional myocardial ischaemia-reperfusion. For studies performed in the in vivo rabbit model, male New Zealand White rabbits were used throughout. All rabbits were obtained from the commercial breeder Froxfield Farms UK Limited (Froxfield) and later Highgate Farm (Market Rasen), as the genetic stock was sold onto this breeder. For studies in rats, male Wistar rats were used throughout, obtained from Froxfield Farms. All procedures and care of animals were in accordance with UK Home Office guidelines set out in the Animals (Scientific Procedures) Act 1986, published by Her Majesty’s stationery office. Animals were acclimatised in the institutional animal house for 5-7 days after delivery and had free access to a standard pelleted diet and water.

In all animal experiments, pharmacological preconditioning was carried out using the highly selective adenosine A₁R agonist 2-chloro-N⁶-cyclopentyladenosine (CCPA) at a dose of 100 μg/kg. In various tissues, CCPA has been shown to have high affinity and an almost 1000-fold selectivity for A₁R compared to other adenosine receptor subtypes (292, 403). In rabbit experiments, CCPA 100 μg/kg was administered to conscious animals via a marginal ear vein. In rat experiments, the same dose of CCPA was administered intravenously via the internal jugular vein to anaesthetised animals. Following CCPA treatment, animals were allowed to recover for various periods (24-48 hours) and then subjected to myocardial infarction. In the following sections, the methods for the in vivo and in vitro infarction procedures, and for determination of infarct size are outlined.
2.2 **In vivo Infarct Model in the Rabbit**

Male New Zealand White rabbits (Body weight 2.2-3.5 kg) were used in these experiments. Twenty four to 48 hours following various pre-treatments (detailed in chapters 3, 4 and 6), animals were subjected to regional myocardial ischaemia-reperfusion *in vivo*, under general anaesthesia. Rabbits were pre-medicated by intramuscular (i.m.) injection with the narcotic analgesic ‘Hypnorm’ 0.15 ml/kg (obtained from Janssen Pharmaceuticals, Pettridge, containing fentanyl citrate 315 μg/ml and fluanisone 10 mg/ml). Once the animal had become ataxic, developed pupillary constriction and dilatation of ear veins, the marginal ear vein was cannulated, and anaesthesia was induced with an intravenous (i.v.) injection of sodium pentobarbitone 30-40 mg/kg (‘Sagatal’; obtained from Rhône Mérieux, Harlow). Thereafter, hourly supplements of ‘Hypnorm’ 0.1 ml/kg i.m. and sodium pentobarbitone 5-10 mg/kg i.v. were administered as required to maintain surgical anaesthesia.

The neck area was shaved and 1 ml 2% v/v lignocaine hydrochloride (Antigen Pharmaceuticals Limited, Roscrea, Ireland) was injected subcutaneously (s.c.) to provide local anaesthesia. A midline incision was made in the neck and a tracheostomy performed. The animals were intubated with a 3.5 mm tube (Portx Limited, Hythe) and mechanically ventilated with a positive pressure respirator and room air supplemented with oxygen at a rate of 1 Hz (small animal ventilator, Harvard Apparatus Limited, Edenbridge). The right common carotid artery was cannulated with a rigid polyethylene cannula (size five; Portex Limited, Hythe), filled with 0.9% w/v saline containing 10-20 U/ml heparin sodium (Multiparin; CP Pharmaceuticals Limited, Clwyd), and connected via a three-way luer-lock tap to a pressure transducer (Lectromed UK Limited) for periodic haemodynamic measurements. The other arm of the three-way tap was used to withdraw blood for periodic measurement of arterial blood pH and gas parameters, including partial pressures of oxygen (PaO₂) and carbon dioxide (PaCO₂) using an automated blood gas analyser (AVL993; AVL Medical Instruments UK Limited, Stone). Tidal volume was adjusted as necessary to maintain arterial pH between 7.35-7.50.
The animal core temperature was measured intermittently by a rectal thermometer and maintained at 38.5±0.5°C using a heating pad.

The surface electrocardiogram (ECG) was recorded from four leads, one attached to each limb, using self adhesive electrodes (Blue Sensor N-10-A; Medicotest UK Limited, St. Ives). A single channel ECG recorder (Lectromed UK Limited) was used for continuous monitoring of heart rate, arrhythmias and ST-segment changes.

The chest area was shaved. A median sternotomy was performed and the pericardium incised to expose the heart. A 3/0 silk suture on an atraumatic needle (Mersilk W546; ETHICON®, Edinburgh) was passed around a prominent anterolateral branch of the left coronary artery approximately halfway between the left atrial appendage and the apex. The ends of the suture were threaded through a polypropylene tube to form a snare. After stabilisation and baseline measurements, regional myocardial ischaemia was induced by pulling the snare taut against the myocardium and clamping it in position. Ischaemia was confirmed by the presence of regional LV hypokinesia and epicardial cyanosis associated with ST-segment elevation on ECG. After 30 minutes of ischaemia, the snare was released and reperfusion confirmed by conspicuous reactive hyperaemia of the risk zone, gradual resolution of ST-segment elevation, and the occurrence of reperfusion-induced ventricular premature beats. In animals that developed ischaemia- or reperfusion-induced VF, attempts were made to restore sinus rhythm by gentle flicking the apex of the heart with a cotton bud. The myocardium was reperfused for 120 minutes after which infarct size was assessed as detailed below.

2.3 In vitro Infarct Model in the Rat

Male Wistar rats (body weight 250-350 g) were used in these studies. Twenty four hours after various treatments (detailed in chapter 6), rats were deeply anaesthetised with an intraperitoneal (i.p.) injection of sodium pentobarbitone 60 mg/kg and anticoagulated with heparin 1000 U/kg i.p. A median sternotomy was performed, the heart rapidly excised and immediately immersed in 4°C Krebs-Henseleit (KH) buffer solution containing in mMol: NaCl 118, KCl 4.7, CaCl₂ 1.8, KH₂PO₄ 1.2, MgSO₄.
The aortic root was cannulated and the heart perfused retrogradely by the Langendorff technique at constant pressure (100 cm H$_2$O). The KH buffer had been previously equilibrated with 95% O$_2$/5% CO$_2$ at 37.5°C to maintain pH at 7.4±0.05 (pO$_2$=60-75 kPa). A water-filled latex balloon, coupled to a pressure transducer (Lectromed UK Limited), was inserted into the LV cavity via the left atrial appendage for periodic pressure recordings. Left ventricular end-diastolic pressure (LVEDP) was adjusted to between 8-12 mmHg and maintained throughout the experiment. Myocardial temperature was measured by a thermoprobe inserted into the right ventricle via the pulmonary artery. The hearts were surrounded by a water jacketed chamber to maintain the myocardial temperature constant at 37.5±0.5°C. A 3/0 silk suture on a round bodied needle (Mersilk W546; ETHICON®, Edinburgh) was placed around the left coronary artery a few millimetres distal to the aortic root. The suture was threaded through a 10 mm polypropylene tube to form a snare (Figure 2.1). After 20 minutes of stabilisation, regional myocardial ischaemia was induced by tightening the snare and clamping it onto the epicardial surface with a haemostat clamp. Following 35 minutes regional myocardial ischaemia the hearts were reperfused for 120 minutes by releasing the snare. Coronary flow (CF) was measured periodically throughout the ischaemia-reperfusion protocol by timed collection of the coronary effluent. Heart rate (HR) and left ventricular developed pressure (LVDP = left ventricular systolic pressure - LVEDP) were continuously recorded. Rate pressure product (RPP) was calculated as the product of HR and LVDP.
Figure 2.1. Schematic of Ischaemia-Reperfusion Procedures in the Isolated Lagendorff Perfused Rat Heart

Hearts were subjected to 35 min regional myocardial ischaemia by occluding the coronary artery followed by 2 hours reperfusion. Zinc-cadmium (Zn-Cd) sulphide fluorescent microspheres were infused via the aortic root at the end of reperfusion, and following ligation of the silk suture, to delineate the myocardial area at risk. KH = Krebs-Henseleit
2.4 Assessment of Risk Zone and Infarct Size

Rabbit Experiments
At the end of 120 minutes reperfusion, the rabbits were anticoagulated with heparin 500 U i.v. The animals were sacrificed with pentobarbitone overdose, the heart excised and immediately attached to a Langendorff apparatus via the aortic root and retrogradely perfused with cold saline to remove blood. The coronary suture was ligated and the aortic root perfused with 2-4 ml of a 5 mg/ml suspension of 1-10 μm zinc cadmium sulphide fluorescent microspheres (Duke Scientific, Palo Alto, California) to define the risk zone. Under ultraviolet (UV) light the tissue supplied by the occluded branch appears non-fluorescent (Figure 2.2, Panel I). The hearts were then weighed, frozen at -18°C for 2-18 hours, and then cut into 2 mm slices from apex to base perpendicular to the long axis of the heart. After defrosting, the slices were incubated at 37°C in a 1% w/v solution of triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4) for 15-20 minutes and fixed for 24-48 hours in 4% v/v formalin solution. TTC stains non-infarcted myocardium a brick-red colour, based on the formation of the red formazan pigment that results from the reduction of TTC by lactate dehydrogenase or NADH (404), whereas infarcted tissue appears pale or grey indicating a loss of these constituents from necrotic tissue (Figure 2.2, Panel II). The slices were traced on acetate sheets and under UV light fluorescent and non-fluorescent areas distinguished. The areas of infarcted tissue (I) and myocardium at risk (R) were determined, in a ‘blinded’ fashion, by computerised planimetry (Summa Sketch II, Summa Graphics) and the corresponding volumes (cm³) calculated by multiplication of each area (cm²) by slice thickness (0.2 cm). The slice infarct and risk volumes were summed for each heart. Left ventricular infarct size was expressed as the percentage infarction of the risk zone (I/R).

Rat Experiments
At the end of the reperfusion period, the coronary artery ligature was retied and a 5mg/ml suspension of zinc-cadmium sulphide fluorescent microspheres was infused slowly through the aorta to delineate the myocardial risk zone under UV light (Figure 2.1) Thereafter these hearts were treated in the same manner as the rabbit hearts as described above.
Figure 2.2. Method of Infarct Size Determination. Panel I) visualised under UV light, depicts non-perfused risk zone versus perfused zone which contains yellow fluorescent microspheres; Panel II) illustrates red (TTC positive) viable tissue versus grey/white (TTC negative) necrotic tissue. The silk coronary artery ligature can also be seen.
In models of regional myocardial ischaemia and reperfusion, the principal determinants of myocardial infarct size are the degree of collateral circulation to the ischaemic region, the size of the risk zone and myocardial temperature. In these experiments, the following steps were taken to minimise variations in these parameters so as to allow comparisons between various treatment groups: (i) Rabbits and rats were used for infarct procedures; these species have minimal myocardial collateral circulation and a very limited ability to form collaterals during ischaemia (405); (ii) In both the rabbit and rat models of regional myocardial ischaemia/reperfusion, there is a significant correlation between infarct and risk zone sizes (406). In all experiments, infarct size is expressed as a percentage of risk zone to allow for differences in vascular anatomy or occlusion site. In the rabbit heart, the regression line relating infarct size to volume of risk region has a non-zero intercept of ~ 0.3 cm³ (406, 407). In rabbit experiments, therefore, hearts with risk zone volumes less than 0.4 cm³ were prospectively excluded from further analysis. In rat models, the infarct/risk regression line has a zero intercept (406), and therefore no hearts were excluded on the basis of risk zone volume. (iii) In animal models of regional myocardial ischaemia/reperfusion, differences in temperature as little as 1°C can significantly affect infarct size (408). In rabbit experiments, the core body temperature was maintained at the physiological range for the species at 38.0-39.0°C. In the isolated rat heart experiments, myocardial temperature was maintained at 37.0-38.0°C.
Chapter Three

Prolonging Delayed Preconditioning by Intermittent Application of the Preconditioning Stimulus
Chapter Three

3.1 Introduction

Endogenous adenosine, released by myocytes and vascular endothelium during periods of ischaemia, by activation of adenosine $A_1$ and $A_3$ receptors, has been implicated as an important trigger of both phases of myocardial protection following ischaemic preconditioning. Blockade of adenosine receptors during preconditioning has been reported to abolish both the early (52) and the late (290) cardioprotective effects of ischaemic preconditioning. Furthermore, substitution of the preconditioning ischaemia with intravenous administration of selective adenosine $A_1$R agonists, has been shown to induce both early (147, 409) and delayed (290) protection against infarction.

These findings point to a therapeutic potential for adenosine $A_1$R agonists in ischaemic heart disease. However, the main shortcoming of such therapy is that it would have to be given as a pretreatment to patients at risk of coronary thrombosis. Such pretreatment could be achieved however, if the duration of the protection afforded by preconditioning was extended, thereby maintaining the myocardium in a “preconditioned” state over a long period of time. A study by Tsuchida et al. (410) has addressed the possibility of maintaining “classic” preconditioning by using a continuous i.v. infusion of the selective $A_1$ agonist 2-chloro-$N^6$-cyclopentyladenosine (CCPA) in a rabbit model of infarction induced by 30 minutes of regional ischaemia followed by 3 hours of reperfusion. Rabbits subjected to a 6 hour infusion of CCPA showed a 59% reduction in infarct size compared to the saline treated group. Infarction in a group receiving a 72 hour infusion of CCPA however, was the same as in the 72 hour vehicle group. Furthermore, the protective effects of a 5 minute ischaemic preconditioning stimulus were attenuated following a 72 hour infusion of CCPA. The group that received a 72 hour infusion of saline however, was preconditioned with 5 minutes of ischaemia. Theses authors concluded that myocytes become desensitised to the protective effects of CCPA with prolonged exposure, and that such tachyphylaxis also abolishes the beneficial effects of classic ischaemic preconditioning. In separate experiments, the same group examined the effect of multiple 5 minute episodes of regional ischaemia in a conscious, chronically instrumented rabbit model (411). Animals subjected to 40 to 65 five-minute coronary
occlusions over a 3 to 4 day period showed a marked attenuation in infarct size limitation compared to a group of animals that had been preconditioned with a single 5 minute occlusion.

Although the above studies have partially addressed the question of prolonging the early phase of myocardial protection, there are as yet no reports of experiments exploring the possible extension of the “second window of protection”. The aims of the current study were twofold; (i) to confirm previous observations from this laboratory indicating that delayed protection against myocardial infarction is induced following transient activation of adenosine A1R; and (ii) to examined if this delayed phase of myocardial protection can be maintained over a long period of time by chronic intermittent adenosine A1R activation. In particular, these studies were undertaken to establish if a schedule of chronic dosing with an adenosine A1 agonist would maintain or increase the degree of myocardial protection, or if tachyphylaxis would result from cumulative dosing.

3.2 Materials and Methods

Male New Zealand White rabbits weighing 2.2-3.5 kg were used in these experiments. Animals were housed in individual cages and had free access to food and water throughout the preparation period.

3.2.a Single Dose Studies

Preliminary studies were performed to confirm previous observations that a delayed phase of myocardial protection is induced after transient adenosine A1R activation (290). CCPA (Research Biochemicals Inc through Semat, St Albans, UK) was dissolved in sterile 0.9% sodium chloride at a concentration of 500 µg/ml. Conscious rabbits were randomised to receive a single i.v. bolus of CCPA 100 µg/kg or saline vehicle (0.5 ml) via a marginal ear vein. Twenty four or 48 hours later, the animals were subjected to 30 minutes regional myocardial ischaemia and 120 minutes reperfusion in vivo and the resultant infarct size was determined, as described in Chapter 2 (Fig. 3.1).
3.2.b Chronic Intermittent Dosing Study

On the basis of these preliminary studies and previous work from this laboratory characterising the time course of the delayed protection conferred by $A_1R$ activation (324), the schedule for intermittent dosing of animals was determined. Conscious rabbits were treated with repeated i.v. boluses of CCPA 100 $\mu$g/kg or saline vehicle (0.5 ml) at 48 hour intervals. The treatments were administered on a random basis and the animals were returned to their cages with no further manipulation between the treatments. Approximately 48 hours after the fifth dose (day 10) the animals were subjected to an infarction procedure *in vivo* and the infarct size measured (Fig. 3.1).

(A. Single Dose Studies)

- 24 hours
- 48 hours

(B. Chronic Intermittent Dosing Study)

- DAY 0 2 4 6 8 10

Figure 3.1 Experimental Protocol for Pharmacological Preconditioning

A. In single dose studies the animals were treated with a single bolus of CCPA 100 $\mu$g/kg 24 or 48 hours prior to the infarct procedure. B. In the chronic intermittent dosing study animals were treated at 48 hourly intervals over a period of 10 days with repeated boluses of CCPA and subjected to an infarction procedure on day 10. Arrows represent a single intravenous bolus of CCPA 100 $\mu$g/kg.
3.2.c Acute Haemodynamic Effects of CCPA

The acute haemodynamic effects of an i.v. bolus of CCPA 100 μg/kg in anaesthetised rabbits have been described previously (290). In order to further explore if rabbits pretreated with CCPA for ten days had developed tolerance to the effects of adenosine A<sub>1</sub>R activation, a subgroup of animals (n=6 per group) were treated with a further bolus of CCPA 100 μg/kg at the end of the reperfusion period and the haemodynamic response was monitored for 10 minutes prior to excision of the heart. This involved measurement of systolic blood pressure (SBP) and heart rate (HR) at baseline (at the end of 120 minute reperfusion), and at 1, 2, 3, 5 and 10 minutes following the CCPA bolus.

3.2.d Statistical Analysis

The data are presented throughout as mean±SEM values. The significance of differences in mean values of I, R and I/R between the two treatment groups was evaluated by student’s unpaired t-test. Any differences between haemodynamic parameters at different time points was assessed by two-way analysis of variance (ANOVA) followed by Fisher’s least significant difference test used post hoc for individual differences. The null hypothesis was rejected when p≤0.05.

3.3 Results

3.3.a Exclusions

A total of 54 rabbits was used for these studies. Thirty were used for the single dose studies and 24 for the chronic intermittent dosing study. In the single dose studies, 2 rabbits were excluded due to intractable VF during the infarct protocol (1 in 48 h vehicle group and 1 in 24 h CCPA group); 1 was excluded due to failure of the TTC stain (24 h CCPA group); and 1 heart was excluded due to absence of a risk zone (48 h CCPA group). In the chronic intermittent dosing study 4 hearts were excluded; 1 due to severe VF during the infarction protocol, 2 due to embolisation at the time of microsphere infusion for risk zone determination, and 1 due to coronary artery being punctured at the time of insertion of the suture (all in CCPA group). Data is reported on 46 animals which successfully completed the two study groups.
3.3.b Single Dose Studies

Table 3.1 represents the infarct size and risk volume data for rabbits pharmacologically preconditioned with CCPA at 24 and 48 hours prior to the infarct procedure. Figure 3.2 represents the I/R ratios graphically. CCPA administration 24 hours prior to infarction resulted in a significant reduction in I/R ratio from 41.3±4.4% in the vehicle group to 23.0±3.9% (p<0.05). The corresponding values for the 48 hour CCPA/vehicle groups were 37.7±2.9% and 26.1±3.0% respectively (p<0.05). There were no differences in haemodynamic parameters, body temperature or arterial pH at any time points, between the various groups. These data confirm previous results from this laboratory indicating that a delayed phase of myocardial protection is induced 24 and 48 hours following transient A1R activation (324).

Table 3.1. Infarct Size Data for Single Dose Studies

<table>
<thead>
<tr>
<th>Group</th>
<th>No of Animals</th>
<th>Risk Volume (cm^3)</th>
<th>Infarct Volume (cm^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>6</td>
<td>0.94±0.19</td>
<td>0.40±0.09</td>
</tr>
<tr>
<td>48 h</td>
<td>6</td>
<td>0.95±0.15</td>
<td>0.36±0.07</td>
</tr>
<tr>
<td>CCPA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>7</td>
<td>1.07±0.08</td>
<td>0.25±0.05</td>
</tr>
<tr>
<td>48 h</td>
<td>7</td>
<td>1.06±0.08</td>
<td>0.29±0.05</td>
</tr>
</tbody>
</table>

Values are mean±SEM.
3.3.c Chronic Intermittent Dosing Study

**Systemic Haemodynamic Changes during Ischaemia-Reperfusion**

Table 3.2 summarises the changes in HR and RPP during the infarction protocol in animals pretreated for 10 days with intermittent (48 hourly) CCPA/vehicle administration. There were no differences in baseline RPP values between the two groups. There was a small decline in RPP during the 30 minute regional ischaemia with no recovery during reperfusion. This was mainly due to a reduction in SBP as HR remained relatively unchanged throughout the infarct protocol. The haemodynamic changes with time however were very similar in the two groups and are therefore unlikely to have contributed to the differences in infarct size.

---

**Figure 3.2 Infarct Size in Single Dosing Studies.**

The figure represents the reduction in infarct size 24 or 48 hours following a single i.v. bolus of CCPA compared to time matched vehicle (VEH) treated animals. *p<0.05 compared to time matched vehicle treated group (unpaired Student’s t-test).
Table 3.2. Haemodynamic Parameters During Ischaemia-Reperfusion

<table>
<thead>
<tr>
<th></th>
<th>Pre-ischaemia (29 min)</th>
<th>Ischaemia (29 min)</th>
<th>Reperfusion (60 min)</th>
<th>Reperfusion (120 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HR (beats/min)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>230.0±6.2</td>
<td>251.0±8.6</td>
<td>224.0±7.2</td>
<td>225.0±8.3</td>
</tr>
<tr>
<td>CCPA</td>
<td>227.3±5.2</td>
<td>233.6±5.8</td>
<td>220.9±7.9</td>
<td>223.6±8.1</td>
</tr>
<tr>
<td><strong>SBP (mmHg)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>95.0±2.5</td>
<td>82.9±1.9</td>
<td>75.5±2.2</td>
<td>73.4±2.7</td>
</tr>
<tr>
<td>CCPA</td>
<td>90.6±3.3</td>
<td>78.8±2.8</td>
<td>75.5±2.5</td>
<td>73.5±2.5</td>
</tr>
<tr>
<td><strong>RPP (mmHg/min x 10³)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>21.9±1.0</td>
<td>20.8±0.9</td>
<td>16.9±0.8</td>
<td>16.5±0.7</td>
</tr>
<tr>
<td>CCPA</td>
<td>20.5±0.8</td>
<td>18.5±0.9</td>
<td>16.8±0.7</td>
<td>16.3±0.6</td>
</tr>
</tbody>
</table>

HR = heart rate; SBP = systolic blood pressure; RPP = rate pressure product.

Infarct Size Data

Absolute infarct size and risk zone volumes are presented in Table 3.3 for saline and CCPA pretreated animals. Figure 3.3 represents infarct size as a percentage of area at risk. Intermittent CCPA pretreatment caused a marked resistance to myocardial infarction compared to vehicle pretreated controls indicating that the delayed phase of cardioprotection was present at 10 days of repeated adenosine A₁R activation. The I/R ratio in CCPA pretreated animals was 26.6±3.7% compared with 45.9±5.5% in controls (p<0.01). Risk zone volume, a major determinant of infarct size, was similar between the two groups and averaged at 1.1-1.2 cm³. Arterial pH and body temperature were also similar in the two groups at all time points.

Table 3.3. Infarct Size Data for Chronic Intermittent Dosing Study

<table>
<thead>
<tr>
<th>Group</th>
<th>No.of Animals</th>
<th>Risk Volume (cm³)</th>
<th>Infarct Volume (cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>10</td>
<td>1.19±0.10</td>
<td>0.58±0.10</td>
</tr>
<tr>
<td>CCPA</td>
<td>10</td>
<td>1.12±0.08</td>
<td>0.31±0.05 *</td>
</tr>
</tbody>
</table>

Values are mean±SEM. *p<0.05 compared with corresponding saline treated group (student’s unpaired t-test).
Figure 3.3 Infarct Size in Chronic Intermittent Dosing Studies.

Percentage infarction of the risk zone in chronic intermittent dosing study. 10 days of intermittent treatment with CCPA resulted in marked protection against infarction compared to the vehicle pretreated group. Data are presented as mean±SEM. **p<0.01 compared to vehicle treated group (unpaired Student’s t-test).

Acute Haemodynamic Effects of CCPA Following Chronic Treatment

Figure 3.4 illustrates the acute haemodynamic effects of a bolus of CCPA 100 μg/kg administered at the end of the reperfusion period in animals pretreated for 10 days with intermittent CCPA or vehicle. Baseline parameters were similar in both groups; (CCPA pretreated group: HR: 227±9 beats per minute, systolic BP: 75±3 mmHg; vehicle pretreated group: HR: 237±10 beats per minute, systolic BP 73±4 mmHg). There was a rapid decline in heart rate and blood pressure which was very similar in the two groups. By 10 min following CCPA administration, there was a 20.0±2.4% reduction in SBP and a 28.3±2.8% reduction in HR in the saline pretreated group compared with 18.9±1.8% and 29.1±3.4% respectively in the CCPA pretreated group (p=NS). This similarity in the haemodynamic response following a bolus of CCPA in the two groups, provides strong evidence that rabbits exposed to 10 days of repeated administration had not developed tolerance to the effects of the adenosine A1R agonist.
Figure 3.4. Acute Haemodynamic Effects of CCPA

In rabbits pretreated for 10 days with intermittent CCPA (●) or saline vehicle (○), animals received a further bolus of CCPA 100 μg/kg at the end of reperfusion. A, changes in heart rate, B, changes in systolic blood pressure. Data are presented as percentage changes (±SEM) from baseline values.
3.4 Discussion

These studies provide further evidence of a delayed phase of myocardial protection against infarction, induced 24 and 48 hours following transient adenosine $A_1$R activation and support previous results from this laboratory (324). Furthermore, the results of the present study show for the first time, that this ‘second window of protection’ can be maintained over a 10 day period by intermittent pharmacological preconditioning with an $A_1$R agonist, with no evidence of tachyphylaxis to the infarct-limiting or haemodynamic effects of CCPA. The resilience against myocardial infarction seen in rabbits chronically preconditioned with intermittent CCPA was comparable to that seen at 24 and 48 hours following a single dose of CCPA. Moreover, following 10 days of pretreatment, the acute haemodynamic response to a bolus of CCPA was no different in animals that had received either CCPA or saline pretreatment, providing further evidence that myocytes had not developed tolerance to the effects of the $A_1$-selective agonist.

3.4.a No Development of Tachyphylaxis

In the present study the haemodynamic effects of CCPA 100$\mu$g/kg in conscious rabbits during the 10 day preconditioning protocol were not measured, although it is likely that the acute haemodynamic response is less pronounced in the conscious state than in anaesthetised animals. Furthermore, in a previous study describing the acute haemodynamic response to a bolus of CCPA 100$\mu$g/kg in anaesthetised rabbits (290), the bradycardia and hypotension induced by the $A_1$R agonist had completely resolved by 90 minutes following administration, implying that the compound is eliminated within a few hours. It is therefore unlikely that the marked anti-infarct effects observed after 10 days of pretreatment with intermittent CCPA had resulted from the transient bradycardia and hypotension induced following the administration of the agonist. In addition, since the haemodynamic parameters at the start of the infarction procedure were similar between the two groups, there is no evidence to suggest cumulative haemodynamic effects of the $A_1$R agonist over the 10 day treatment period.
Previous studies have reported desensitisation of adenosine A<sub>1</sub>R following chronic exposure to agonists. These include studies examining regulation of adenosine receptors in brain (412, 413), kidney (414) and adipocytes (415-419) in the rat, chick embryonic myocytes (420, 421) and hamster smooth muscle DDT1 MF-2 cells (422, 423). Desensitisation occurred in a time- and dose-dependent and reversible fashion; thus chronic exposure to adenosine A<sub>1</sub>R agonists resulted in tolerance after 2-7 days in rat adipocytes, 24-44 hours in chick embryo myocytes and 18-44 hours in hamster DDT1 MF-2 cells. Few studies have explored the regulation of adenosine A<sub>1</sub>R in the mammalian myocardium. Lee et al. (424) reported desensitisation of rat atrial adenosine A<sub>1</sub>R following a 7 day i.v. infusion of N<sup>6</sup>-(phenyl-2R-isopropyl)-adenosine (R-PIA), a selective A<sub>1</sub> agonist. In the study by Tsuchida et al. (410), the time course of the desensitisation was not investigated, but tolerance to the cardioprotective effects of CCPA developed in the rabbit myocardium between 6-72 hours of a continuous infusion. Furthermore, in that study the 72 hour CCPA group received a total in excess of 3 mg/kg of CCPA over a 3 day period, a thirty-fold higher dose than that shown to precondition the rabbit myocardium (410), providing further evidence for a dose-dependent influence on tachyphylaxis. All the cited studies however, were undertaken either in cell culture models continuously exposed to adenosine analogues, or studies that were performed in vivo used a continuous i.v. infusion for administration of the agonists. The time- and dose-dependence of A<sub>1</sub>R desensitisation would imply that reducing the dose-frequency of administering the agonist might delay the development of tolerance to its effects. Interestingly in a study by Casati et al. (425), the time-course of desensitisation to the haemodynamic effects of CCPA was investigated following twice daily i.p administration of the agonist to spontaneously hypertensive rats. Tolerance to the bradycardic effect of CCPA, the main A<sub>1</sub>R mediated action, did not develop for 21 days. These results further support the findings of this study that reduced frequency of exposure to adenosine agonists delays or may even prevent tachyphylaxis.

3.4.b Time Course of Delayed Preconditioning

The temporal profile of the delayed phase of myocardial protection following ischaemic or pharmacological preconditioning has been described for various endpoints of ischaemia-reperfusion injury. The “second window of protection” against
myocardial necrosis in the rabbit, extends between 24-72 hours following preconditioning ischaemia (93), a time course identical to that observed following pharmacological preconditioning with CCPA in the same species (324). Meng et al. have reported delayed cardioprotection against post-ischaemic myocardial dysfunction in the rat 4-72 hours following transient $\alpha_1$-adrenoceptor activation with norepinephrine (426). Furthermore, delayed protection against ischaemia-reperfusion induced ventricular arrhythmias extends between 24-72 hours following brief periods of cardiac pacing in the canine myocardium (95). Late preconditioning against myocardial stunning, described by Bolli’s group seems to have a similar time course and is protective 12-72 hours after brief periods of myocardial ischaemia-reperfusion in the conscious pig (94). The prolonged nature of these protective effects, as opposed to the short-lived “classic” preconditioning which only lasts for 1-2 hours following the preconditioning stimulus, potentially allows “re-preconditioning” at 48-72 hour intervals, a dosing schedule that maintains the myocardium in a protected state, as shown in the present study, without development of tolerance. The protective effects of intermittent CCPA administration beyond 10 days was not examined in this study, but as with the bradycardic effect reported by Casati et al. (425), it is likely to be extended to at least 21 days. Furthermore, since the dosing schedule in the current study was even less frequent than that used by Casati et al. (425), it is possible that desensitisation of $A_1$R may be even further delayed or may not occur altogether. Indeed, as the findings of the present study suggest, it is possible that there is some optimal dosing regimen that affords maximal chronic protection against myocardial ischaemia without development of tolerance.

3.4.c Similar Studies

Since the publication of this study, there have been two preliminary reports supporting the findings of this study, and suggesting that exploitation of the relatively prolonged nature of the delayed protection observed following pharmacological preconditioning, may allow repeated dosing with a preconditioning-mimetic agent in order to maintain the heart in a chronic state of protection. Using a structurally different selective adenosine $A_1$ agonist, GR79236, Travers et al. (427) confirmed that transient $A_1$R activation induces delayed myocardial protection against infarction in rabbits. These investigators also showed that following 7 days of
daily i.v. bolus treatment with the $\alpha_1$ agonist, rabbits were maintained in a preconditioned state as evidenced by significantly attenuated infarct size compared to placebo treated controls (427). More recently, Hill et al. (428) reported that despite development of tolerance to the haemodynamic effects of nitrates, rabbits treated with daily transdermal nitroglycerin patches for 28 days, remained chronically preconditioned with diminished infarct size comparable to that in ischaemically preconditioned rabbits.

3.4.d Clinical Relevance

The issue of whether the heart can be maintained in a chronic state of preconditioning is important from a therapeutic standpoint. Certain patient groups, such as those with non-ST-elevation acute coronary syndromes, including unstable angina and non-Q-wave MI, are at high risk of progression to acute coronary occlusion, and more than 10% die or suffer a myocardial infarction (or reinfarction) within 6 months with about one half of these events occurring during the acute early phase (9). These patients form a reasonably well defined high risk group who might benefit from treatment with pharmacological agents that trigger or augment myocardial preconditioning over a period of several days or weeks and could therefore effectively maintain the myocardium in a chronically protected or "preconditioned" state. Although the ultimate treatment for myocardial infarction is prompt restoration of blood flow to the ischaemic myocardium, such cardioprotective strategies would enhance the time window during which revascularisation therapies can be effectively instituted, and may improve the outcome in a select group of patients with unstable angina. The results of this study provide some basis for optimism that chronic pharmacological preconditioning may result in long-term cardioprotection.
3.5 Summary

In summary, the results of this study confirm previous findings from this laboratory indicating that transient activation of cardiac adenosine A$_1$R in rabbits, induces delayed protection against infarction at 24-48 hours. Furthermore, these studies show for the first time, that intermittent (every 48 hours) adenosine A$_1$R activation over a 10 day period, with a highly selective agonist, maintains the rabbit myocardium in a protected or “preconditioned” state against ischaemia-reperfusion injury. In contrast to studies exploring maintenance of classic preconditioning, this study does not find any evidence for tolerance to the cardioprotective or haemodynamic effects of CCPA over this time period.
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Adenosine $A_1R$ Induced Delayed Preconditioning:
Role of Protein Kinases & Hsp27
4.1 Introduction

Although increasing evidence points to an important role for activation of A1R in triggering the late phase of ischaemic preconditioning against infarction, the intracellular signalling pathways downstream of A1R mediating this delayed cardioprotection have not been elucidated. Protein kinase C (PKC) has been shown to play an important role in A1R induced signal transduction in myocardial tissue (429-431). Furthermore, several studies indicate the involvement of PKC in mediating both the early phase (reviewed in reference 432) and the second window (302, 307) of ischaemic preconditioning. On the other hand, increasing evidence has recently implicated involvement of tyrosine kinase (TK) activation in the signalling mechanism of ischaemic preconditioning (203, 204, 213, 310, 433). TK signalling is activated by a number of G protein-coupled receptors (198, 434-436), and as suggested by the study of Maulik et al. (213) may form an early step in the mechanism of ischaemic preconditioning. Conversely, studies in non-cardiac tissue suggest that tyrosine phosphorylation may occur downstream of PKC (199, 200), a view that has been supported by recent evidence in rabbit myocardium subjected to classic ischaemic preconditioning protocols (203, 204). On the other hand, Vahlhaus et al. (184) recently reported that blockade of both TK and PKC is necessary to abolish ischaemic preconditioning in pigs, suggesting that these enzymes may act in parallel pathways to mediate preconditioning. However, the involvement of these two families of protein kinases in A1R induced delayed preconditioning has not been evaluated. Therefore, in the first part of the present study, the role of PKC and TK signalling in acquisition of delayed tolerance to myocardial ischaemia 24 hours after A1R activation in the rabbit was examined.

Another important issue regarding the delayed protection against infarction conferred by transient adenosine A1R activation is the nature of the distal effector or target protein(s) mediating this protection. One potential end-effector protein that has been the subject of recent interest is the constitutively expressed 27 kDa heat shock protein (Hsp27). Overexpression of mammalian Hsp27 has been shown to confer significant cellular resistance against heat shock, tumour necrosis factor, oxidative stress and a number of cytotoxic drugs (437-440). Importantly, recent evidence
suggests that overexpression of Hsp27 in adult cardiac myocytes confers enhanced resistance against injury mediated by simulated ischaemia (441). These small Hsps can function in different, seemingly unrelated cytoprotective processes such as RNA stabilisation (438), molecular chaperoning and preventing unfolded proteins from irreversible aggregation (442), regulation of apoptosis (443) and interaction with and stabilisation of the cytoskeleton (444). This latter function of Hsp27 seems to be dependent on its state of phosphorylation. Thus, unphosphorylated Hsp27 behaves as an F-actin capping protein and inhibits actin polymerisation, whereas the phosphorylated Hsp27 isoforms promote polymerisation, which confers resistance against stress-induced microfilament disorganisation (reviewed in reference 444). In this regard, it has been shown that cells overexpressing Hsp27 contain an actin cytoskeleton that is more resistant to disruption due to oxidative stress, than control cells or those overexpressing a non-phosphorylatable mutant of Hsp27 (439, 445). Hsp27 is phosphorylated by the mitogen-activated protein kinase-activated protein kinase-2 (MAPKAPK-2), a stress-sensitive kinase which is sequentially phosphorylated in a cascade of kinases involving p38 mitogen-activated protein kinase (p38 MAPK) (445, 446). The p38 MAPK/MAPKAPK-2 pathway in the myocardium is activated by a number of stressful stimuli (reviewed in reference 206), and several groups have demonstrated its activation by ischaemic preconditioning protocols (212, 217, 447-450). Importantly, recent evidence suggests that p38 MAPK/MAPKAPK-2 pathway is also activated following exposure to adenosine, in both cultured cardiomyocytes (451) and isolated perfused rat hearts (210).

On the basis of the aforementioned, and considering the fact that cytoskeletal disruption is one of the determining events in the cascade of ischaemia-reperfusion injury (15), it was hypothesised that A, R induced delayed cardioprotection may be mediated by activation of the p38 MAPK/MAPKAPK-2 pathway, resulting in enhanced cytoskeletal stabilisation during the index ischaemic insult. In the second part of the present study this hypothesis was investigated by measuring p38 MAPK activity and expression and phosphorylation of Hsp27 in rabbit myocardium 24 hours after transient A, R activation.
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Enhanced expression and activity of the mitochondrial antioxidane, Mn-SOD has also been shown to play an important role in mediating the subacute myocardial protection observed after a number of stressful stimuli, including ischaemia (452), whole body hyperthermia (333), and exercise (73) in rats. Recent evidence suggests that treatment of a variety of tissues, including rat cardiac myocytes, with the adenosine A$_1$R agonist N$^6$-(phenyl-2R-isopropyl)-adenosine (R-PIA) results in upregulation of endogenous antioxidant enzymes including Mn-SOD over a 90-120 minute period (453). However, the delayed effects of adenosine A$_1$R activation on the regulation of Mn-SOD activity, and the signalling mechanisms through which any potential effects may be mediated, remain unknown. In the final part of this study, these effects were investigated.

4.2 Materials and Methods

4.2.a Experimental Protocol

Male New Zealand White rabbits weighing 2.1-2.8 kg were used in these experiments. On day 1, conscious animals were pharmacologically preconditioned and/or received protein kinase inhibitors. Animals were assigned to 6 experimental groups (Figure 4.1). Group I (control) received an i.v. bolus of sterile 0.9% saline 0.5 ml. Group II (CCPA) animals were pharmacologically preconditioned with a single i.v. bolus of CCPA (100 µg/kg). Animals in groups I and II were also treated with the vehicle used for protein kinase inhibitors (4% v/v ethanol in sterile water). To examine the role of PKC in mediating A$_1$R induced delayed preconditioning, groups III (CHE+Sal) and IV (CHE+CCPA) received the same treatment as groups I and II respectively; in addition they were given an i.v. infusion of the selective PKC inhibitor chelerythrine chloride (CHE, 5 mg/kg), 10 minutes prior to the saline/CCPA bolus. Rabbits in groups V (LDA+Sal) and VI (LDA+CCPA) were given the same treatment as in groups I and II. To evaluate the potential role of TK in the signalling pathway downstream of A$_1$R, these animals were also treated with the selective TK inhibitor lavendustin A (LDA, 1.3 mg/kg), 10 minutes before the saline/CCPA bolus. Chelerythrine (CHE) was obtained from Alexis Corporation, UK, and lavendustin A (LDA) from Calbiochem-Novobiochem Ltd., UK. Both CHE
and LDA were dissolved in sterile water containing 4% v/v ethanol at concentrations of 5 mg/ml and 1 mg/ml respectively.

Figure 4.1. Experimental protocol.

On day 1, animals were pharmacologically preconditioned with an i.v. bolus of CCPA or received saline (groups I and II). Animals in these groups were also treated with protein kinase inhibitor vehicle (VEH, 4% v/v ethanol in sterile water). Animals in groups III and IV received an i.v. infusion of chelerythrine 5 mg/kg (CHE) 10 min before the saline/CCPA bolus. Animals in groups V and VI received an i.v. infusion of lavendustin A 1.3 mg/kg (LDA) before the saline/CCPA bolus. On day 2, animals were subjected to 30 min regional myocardial ischaemia and 2 h reperfusion in vivo. The small arrow indicates the timing of myocardial sampling in animals that were not subjected to ischaemia-reperfusion, for analysis of p38 MAPK activity, Hsp27 expression and phosphorylation, Mn-SOD activity.
4.2.b Ischaemia-Reperfusion Protocol in vivo

On day 2, twenty four hours after various treatments, rabbits were anaesthetised and underwent an infarction procedure in vivo, consisting of 30 minutes regional myocardial ischaemia and 2 hours reperfusion (Figure 4.1), as described in section 2.2. At the end of reperfusion, myocardial area at risk was determined with fluorescent microspheres, and infarct size assessed by TTC staining, and areas of infarcted tissue (I) and myocardium at risk (R) quantified by computerised planimetry as detailed in section 2.4. Infarct size was expressed as a percentage of the risk area.

4.2.c Analysis of Myocardial p38 MAPK Activity

In a different group of animals, 24 hours after the various treatments outlined above, rabbits (n=3 per group) were euthanised by an overdose of pentobarbital sodium. A bolus dose of 500 IU i.v. heparin sodium was administered and hearts were immediately excised, and intramyocardial and intracoronary blood was washed out with ice-cold saline. Left ventricular myocardial samples were rapidly frozen by immersion in liquid nitrogen and stored at -80°C for later analysis.

In these myocardial samples, p38 MAPK catalytic activity was determined by an in vitro kinase assay using recombinant activating transcription factor-2 (ATF-2) as a substrate. These assays were performed by Miss Jenny Papakrivopoulou, who was blinded to the treatments received by the animals, using the p38 MAPK assay kit from New England Biolabs® Inc. according to the instructions for the kit. The methods for this assay are briefly described.

**Total Protein Preparation**

About 200 mg crushed, frozen LV tissue was homogenised in 1 ml lysis buffer (Appendix) with 1 mmol/l phenylmethylsulfonyl fluoride (PMSF) added fresh to the buffer. The homogenate was then sonicated for 10 seconds and centrifuged at 12,900 x g for 5 minutes to pellet cell debris. The samples were sonicated and centrifuged a second time and the supernatant removed to a fresh tube on ice. Two 20 μl aliquots of supernatant were taken from each sample for determination of total protein concentration (section 4.2.f).
Selective Immunoprecipitation of Active p38 MAPK

An appropriate volume of the supernatant from each sample, containing 200 μg total protein was removed and phosphorylated p38 MAPK was immunoprecipitated using a monoclonal phospho-specific antibody to p38 MAPK. The activation of p38 MAPK requires phosphorylation of Thr^{180} and Tyr^{182} within a TGY motif (454). Immunoprecipitation was achieved by adding 20 μl of resuspended immobilised phospho-p38 MAPK (Thr^{180}/Tyr^{182}) monoclonal antibody to each tissue lysate which was then incubated with gentle rocking overnight at 4°C. The next day, each sample was microcentrifuged for 30 seconds at 4°C, and the pellet was washed twice with 500 μl of lysis buffer, followed by two washes with 500 μl of kinase buffer (Appendix).

Kinase Assay

In the resulting immunoprecipitate, the p38 MAPK catalytic activity was determined using the in vitro kinase assay to phosphorylate recombinant ATF-2. Thr^{71} of ATF-2 is a major ATF-2 phosphorylation site required for transcriptional activity, and quantification of this phosphorylation provides an index of p38 MAPK activity (455).

Each pellet was suspended in 50 μl of kinase buffer supplemented with 200 μM ATP and 2 μg ATF-2 fusion protein, and incubated for 30 minutes at 30°C. The reaction in the mixture was terminated by adding 25 μl 3x sodium dodecyl sulphate (SDS) sample buffer (Appendix). The sample was then boiled at 100°C for 5 minutes, vortexed and microcentrifuged for 2 minutes.

SDS-PAGE and Western Immunoblotting

The reaction mixture was then separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Phosphorylation of ATF-2 was assessed by Western immunoblotting using a rabbit polyclonal phospho-specific ATF-2 antibody, which detects ATF-2 only when phosphorylated at Thr^{71}, and immunoreactivity was detected by enhanced chemiluminescence (ECL, Amersham, Little Chalfont, UK). In order to correct for differences in protein loading and possible alterations in p38 MAPK protein content in the samples, the blots were then stripped by incubation in 62.5 mM Tris-HCl, pH
6.8, 100 mM β-mercaptoethanol, 2% SDS for 30 minutes at 50°C followed by two washes in Tris-buffered saline (TBS) and 0.05% v/v polyoxyethylenesorbitan monolaurate (Tween 20), then blocked with 5% dried milk powder (Marvel) in TBS/0.05% Tween. The filters were then reprobed with anti-p38 MAPK (Santa Cruz Biotechnology, CA) followed by incubation for 1 hour in 1:1000 swine anti-rabbit HRP conjugated secondary antibody (Dako, Denmark), and immunoreactivity detected with ECL. Immunoreactive bands were quantified by laser densitometry using the Sharp JX-330 scanner. The ratio of phospho-ATF2 to p38 MAPK immunoreactivity was determined for each sample, and the results were expressed as fold activation over control. The methods for SDS-PAGE and Western immunoblotting are detailed in section 4.2.d.

4.2.d Analysis of Hsp27 Expression and Phosphorylation

In myocardial samples obtained 24 hours after various pretreatments, one- and two-dimensional gel electrophoresis was performed to assess expression and post-translational phosphorylation of Hsp27.

**Total Protein Preparation**

Approximately 250 mg frozen tissue was powdered using a stainless steel mortar and pestle precooled in liquid nitrogen, then transferred to a 1.5 ml microcentrifuge tube. Powdered tissue was resuspended in 1.0 ml suspension buffer containing enzyme inhibitors to prevent protein degradation (Appendix). These samples were hand homogenised, sonicated for 10 seconds and centrifuged at 12,900 g for 5 min to pellet cell debris and supernatants transferred to clean microcentrifuge tubes. The samples were sonicated and centrifuged a second time and the supernatant transferred to a fresh tube. Two 20 μl aliquots of supernatant were taken from each sample and stored at -80°C for later analysis of total protein concentration (section 4.2.f). An equal volume of sample buffer (Appendix) was added to the remaining supernatant and the samples boiled at 100°C for 10 minutes. The samples were allowed to cool briefly on ice, before being stored at -80°C.
**Protein Separation (SDS-PAGE)**

Proteins were separated by SDS-PAGE on 12.5% w/v SDS denaturing polyacrylamide gels essentially according to Laemli (456). The running gel was prepared by mixing 9ml running gel base (Appendix), 15 ml 30% w/v acrylamide (Protogel®; National Diagnostics UK Limited, Hull) and 12 ml distilled water. The polymerisation catalysts 15 μl N,N,N′,N′-tetramethylethylenediamine (TEMED) and 150 μl 10% w/v ammonium persulphate (APS) were added and the gel poured. The gel was overlayed with distilled water whilst setting for 15-30 minutes. The stacking gel was subsequently prepared: 3 ml stacking gel base (Appendix), 2 ml 30% w/v acrylamide, 7 ml distilled water, 20 μl 8% w/v bromophenol to help visualise the wells, 10 μl TEMED and 100 μl 10% w/v APS. The stacking gel was poured over the running gel and a well-forming comb inserted before it was allowed to set for 15 minutes. The gel was placed in a vertical electrophoresis tank (V15.17; GibcoBRL Life Technologies, Paisley) containing 1x running buffer (Appendix). Low Mw (2.35-46.0 kD) Rainbow™ markers (RPN 755; Amersham Life Science, Little Chalfont) were loaded onto the gel together with 60 μg of each sample; the volume of sample required was calculated using the protein concentration as described below. Electrophoresis of samples was performed at 120 V for 4-6 hours.

**Two-Dimensional (2D) Gel Electrophoresis**

Sample preparation for 2D gel electrophoresis was carried out as described above. Two-dimensional gel electrophoresis was carried out using isoelectric focussing in the first dimension and SDS-PAGE in the second dimension. Following preparation of samples, these were diluted by addition of an equal volume of sample buffer. Isoelectric focussing was carried out using a Biorad mini-protean II 2D cell (Bio-Rad Laboratories Ltd., Hemel Hempstead) essentially according to the Biorad protocol. First dimension 1mm diameter rod gels were prepared using the following mixture; 5.5 g 9.2 M urea; 1.33 ml 4% acrylamide, 2.0 ml 10% Triton® X-100; 1.6% pH 5-8 Bio-lytes®; 0.4% pH 3-10 Bio-lytes® (Bio-Rad Laboratories Ltd., Hemel Hempstead). After addition of 1.97 ml distilled water, the solution was degassed thoroughly for 15 minutes. Gels were polymerised by adding 10 μl 10% APS (0.01%) and 10 μl TEMED (0.1%). This solution was used to cast a set of 16 rod gels using the casting tubes. Upper chamber (100 mM NaOH), and lower chamber
(10 mM H$_3$PO$_4$) buffers were made fresh and degassed thoroughly for 30 minutes prior to electrophoresis. Rod gels were pre-electrophoresed at 200V/10 minutes; 300V/15 minutes; 400V/20 minutes. Myocardial tissue samples (containing 150 µg total protein) were added to the top of the gel rods and were overlayed with 10µl overlay buffer (Appendix). Isoelectric focussing was carried out by running the samples at 500V for 10 minutes followed by 750V for 3 hours. Biorad 2D-PAGE isoelectric focussing standards were used as markers. The rod gels were then ejected from the glass tubes and placed in 2 ml equilibration buffer for 15 minutes. The rods were then transferred onto pre-set 12.5% SDS-PAGE gels prepared as described above. Second dimension SDS-PAGE was carried out as described above for protein separation.

**Hsp27 Western Blotting**

Proteins separated by 1D or 2D SDS-PAGE were transferred electrophoretically, at 200 mA for 1 hour, onto nitro-cellulose membranes (Hybond-C; Amersham Life Science Ltd, Little Chalfont) using a Trans-Blot™ SD semi-dry Transfer Cell (Bio-Rad Laboratories Ltd., Hemel Hempstead). The membranes were incubated, with rocking, in 40-50 ml block buffer (phosphate buffered saline [PBS] pH 7.2 containing 0.05% v/v Tween 20 and 5% w/v dried milk powder) at room temperature for 1 hour to block non-specific binding sites. The membrane was subsequently incubated for 1 hour in 20 ml of the primary antibody, mouse monoclonal anti-human HSP27 IgG, cross reactive with rabbit Hsp27 (Santa Cruz Biotechnology, CA), at a 1:200 dilution in block buffer. The excess primary antibody was rinsed away by 3x 5 minute washes with block buffer. The membrane was then incubated for 1 hour with the secondary antibody, horseraddish peroxidase- (HRP) conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, CA), at a 1:2000 dilution in block buffer. Excess secondary antibody was removed by washing for 3x 5 minutes with block buffer, and once with PBS (pH 7.2) containing 0.05% v/v Tween 20. The final wash without milk protein, helps to reduce the background signal during detection.

Blots were developed using a proprietary enhanced chemiluminescence (ECL) detection kit (Amersham Life Science). This method relies on the ability of HRP and H$_2$O$_2$ to catalyse the oxidation of luminol in alkaline conditions. Immediately
following oxidation, luminol is in an excited state which subsequently decays to ground state via a light emitting pathway. This light output can be increased 1000-fold by using phenol enhancers which also extend duration of light emission (peak at 5-20 minutes), hence enhanced chemiluminescence. The light emitted (428 nm) can be detected by short exposure to blue-light sensitive auto-radiographic film. In practice, the nitrocellulose membranes were incubated in the ECL kit reagents for one minute. The filter was then exposed to auto-radiographic film (Hyperfilm™ MP; Amersham Life Science), in the dark for 120-150 seconds. The film was placed in developer (Photosol CD18, Photosol Limited, Basildon) for one minute, rinsed in distilled water and finally fixed (Photosol CF40, Photosol Limited) for one minute.

4.2.e Analysis of Myocardial Mn-SOD Activity

Mn-SOD activity in myocardial samples was determined by the nitroblue tetrazolium (NBT) method (73, 288, 332, 333). Approximately 200 mg LV tissue was homogenised in a 2 ml solution of 20 mmol/l PBS containing 1 mmol/l ethylenediaminetetraacetic acid (EDTA) and centrifuged at 900 g at 4°C for 15 minutes. The supernatant was sonicated and then diluted 15 fold in PBS/EDTA solution. Two 20 μl aliquots of the diluted supernatant were taken from each sample and stored on ice for later analysis of total protein concentration (section 4.2.f). Solution A, containing sodium carbonate 50 mmol/l, xanthene 0.1 mmol/l, NBT 0.025 mmol/l and EDTA 0.1 mmol/l, was warmed to 25°C for 20 minutes. Xanthene oxidase was diluted 1:50 in solution B (containing 2 mol/l (NH₄)₂SO₄ and 1 mmol/l EDTA) to a final activity of 20 U/ml. Myocardial samples were incubated at 37°C with the reaction mixture of NBT, xanthine-xanthine oxidase, and 1 mM potassium cyanide to inhibit the activity of the cytosolic Cu,Zn-SOD, as follows:

\[
\begin{align*}
940 \mu l & \quad \text{Solution A} \\
20 \mu l & \quad 1\text{mM potassium cyanide} \\
20 \mu l & \quad 20 \text{U/ml xanthene oxidase} \\
20 \mu l & \quad \text{Tissue sample/Blank sample}
\end{align*}
\]

The reaction of xanthene-xanthene oxidase was used as a source of superoxide radicals, which in the presence of a blank sample, reacted with NBT resulting in the
formation of blue formazan, the rate of which was measured colourimetrically using a spectrophotometer. Rate of increase in $A_{560\text{nm}}$ was measured at 37°C over a period of 3 minutes. These measurements were then repeated in the presence of myocardial tissue samples. The rate of generation of the formazan was reduced in the presence of myocardial Mn-SOD. The activity of Mn-SOD is expressed relative to the protein concentration in the supernatant according to the following formula:

$$\text{Mn-SOD activity} = \frac{(B/T - 1) \times 50}{\text{mg protein in 20} \mu\text{l}} \text{ (in U/mg protein)}$$

where B and T represent the rate of increase in $A_{560\text{nm}}$ for the blank and tissue samples respectively, and 50 is the dilution factor for xanthene oxidase. The measurement of Mn-SOD activity in each sample was performed in duplicate, and mean values taken.

4.2.f Determination of Sample Total Protein Concentration

Total protein concentration of each sample was determined using the bicinchoninic acid (BCA) assay (Pierce Chemical Co.; Rockford, Illinois). This assay combines the biuret reaction, reduction of $\text{Cu}^{2+}$ to $\text{Cu}^{+}$ by protein in an alkaline environment, with the highly sensitive and selective colourimetric detection of copper cations ($\text{Cu}^{+}$) by bicinchoninic acid. A protein standard curve was prepared, in duplicate, using a 2.5 mg/ml solution of bovine serum albumin (BSA) in distilled water. Myocardial samples prepared as described above were similarly diluted with distilled water. BCA working solution (1 ml), 50 parts reagent A (bicinchoninic acid solution) plus one part reagent B (copper sulphate solution), was added to each standard and test sample. The tubes were incubated at 37°C for 30 minutes and then allowed to cool before the $A_{560\text{nm}}$ was measured using a spectrophotometer. $A_{560\text{nm}}$ values were corrected against a blank (0 mg/ml protein standard) and the mean values used to plot a standard curve. A simple curve fit was performed to obtain the equation for the standard curve regression line which was used to calculate the protein concentration in each test sample.

4.2.g Statistical Analysis

The data are presented as mean±SEM. The significance of the differences in mean values of I, R and I/R, and also the mean values of p38 MAPK activity, Mn-SOD activity and Hsp27 protein expression between the experimental groups was
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evaluated by one-way ANOVA followed by Fisher’s protected least significant difference (PLSD). Differences between haemodynamic measurements at different time points were assessed by two-way ANOVA with repeated measures. The null hypothesis was rejected at p<0.05.

4.3 Results

4.3.a Exclusions

A total of 66 rabbits were used in these experiments. Forty eight animals were used for the infarct studies. Of these, six were excluded; 4 due to intractable VF during the infarct protocol (2 in the control group, 1 in the CHE+CCPA group, and 1 in the LDA+CCPA), 1 heart was excluded due to failure of TTC stain (CHE+Saline group) and 1 heart was excluded because the risk zone was less than 0.4 cm$^3$ (CHE+CCPA group). Data on infarct size are therefore presented for 42 animals that successfully completed the infarct protocol. Eighteen animals were used for analysis of myocardial p38 MAPK activity, Hsp27 expression/phosphorylation and Mn-SOD activity (n=3 per group).

4.3.b Haemodynamic Changes During Ischaemia-reperfusion

Table 4.1 summarises the changes in heart rate, systolic blood pressure (SBP) and rate-pressure product (RPP) during the infarction protocol in the six experimental groups. There were no differences in baseline haemodynamic performance between any of the groups. There was a small decline in SBP and RPP during 30 minute ischaemia with no recovery during reperfusion. These haemodynamic changes with time were very similar between all the groups.

4.3.c Myocardial Risk and Infarct Size

Table 4.2 presents the body weights, and volumes of risk and infarct zones in the 6 experimental groups. In these groups, the mean volume of myocardial tissue at risk during coronary artery occlusion was in the range 0.9-1.2 cm$^3$ representing about 40-45% of total left ventricular tissue volume. There were no significant differences in ischaemic risk zone among the experimental groups. The absolute infarct size was
also similar between the groups. Infarct size expressed as a percentage of area at risk (I/R) in the six experimental groups is presented in figure 4.2.

### Table 4.1. Haemodynamic Parameters During Myocardial Ischaemia/Reperfusion

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>Ischaemia</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (beats/min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>241±7</td>
<td>239±8</td>
<td>240±9</td>
</tr>
<tr>
<td>CCPA</td>
<td>236±6</td>
<td>240±7</td>
<td>247±5</td>
</tr>
<tr>
<td>CHE + Saline</td>
<td>240±6</td>
<td>247±9</td>
<td>245±9</td>
</tr>
<tr>
<td>CHE + CCPA</td>
<td>248±12</td>
<td>248±12</td>
<td>238±15</td>
</tr>
<tr>
<td>LDA + Saline</td>
<td>238±3</td>
<td>236±4</td>
<td>233±7</td>
</tr>
<tr>
<td>LDA + CCPA</td>
<td>248±7</td>
<td>240±8</td>
<td>246±6</td>
</tr>
<tr>
<td></td>
<td>SBP (mmHg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>93±4</td>
<td>85±3</td>
<td>79±4</td>
</tr>
<tr>
<td>CCPA</td>
<td>90±3</td>
<td>82±3</td>
<td>72±2</td>
</tr>
<tr>
<td>CHE + Saline</td>
<td>92±3</td>
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<td>CHE + CCPA</td>
<td>93±6</td>
<td>79±6</td>
<td>76±4</td>
</tr>
<tr>
<td>LDA + Saline</td>
<td>90±3</td>
<td>81±3</td>
<td>82±4</td>
</tr>
<tr>
<td>LDA + CCPA</td>
<td>89±2</td>
<td>80±3</td>
<td>72±3</td>
</tr>
<tr>
<td></td>
<td>RPP (mmHg/min x10³)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>22.6±1.6</td>
<td>20.3±1.2</td>
<td>19.1±1.5</td>
</tr>
<tr>
<td>CCPA</td>
<td>21.0±0.5</td>
<td>19.6±0.7</td>
<td>18.9±0.5</td>
</tr>
<tr>
<td>CHE + Saline</td>
<td>22.3±1.2</td>
<td>21.5±1.3</td>
<td>19.1±1.2</td>
</tr>
<tr>
<td>CHE + CCPA</td>
<td>22.3±1.6</td>
<td>19.7±2.0</td>
<td>18.3±1.6</td>
</tr>
<tr>
<td>LDA + Saline</td>
<td>21.4±0.5</td>
<td>19.8±0.9</td>
<td>19.2±1.2</td>
</tr>
<tr>
<td>LDA + CCPA</td>
<td>21.1±0.9</td>
<td>20.4±1.2</td>
<td>18.7±1.0</td>
</tr>
</tbody>
</table>

Values are mean±SEM.
Table 4.2. Body Weight, Myocardial Area at Risk and Infarct Size.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body Weight (kg)</th>
<th>R (cm²)</th>
<th>I (cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>2.49±0.09</td>
<td>1.03±0.09</td>
<td>0.42±0.05</td>
</tr>
<tr>
<td>CCPA</td>
<td>8</td>
<td>2.49±0.05</td>
<td>1.12±0.12</td>
<td>0.28±0.06</td>
</tr>
<tr>
<td>CHE + Saline</td>
<td>7</td>
<td>2.59±0.07</td>
<td>1.17±0.07</td>
<td>0.49±0.07</td>
</tr>
<tr>
<td>CHE + CCPA</td>
<td>6</td>
<td>2.47±0.11</td>
<td>0.99±0.08</td>
<td>0.36±0.05</td>
</tr>
<tr>
<td>LDA + Saline</td>
<td>7</td>
<td>2.32±0.06</td>
<td>0.93±0.08</td>
<td>0.39±0.06</td>
</tr>
<tr>
<td>LDA + CCPA</td>
<td>6</td>
<td>2.37±0.06</td>
<td>1.19±0.08</td>
<td>0.49±0.07</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

Figure 4.2. Myocardial Infarct Size.

Infarct size is expressed as a percentage of the region at risk of infarction. Open circles represent individual animals; solid circles, mean±SEM. *p<0.01 vs control.
Pretreatment with CCPA 24 hours before myocardial infarction resulted in the expected significant 45\% reduction in I/R compared to saline treated controls (23.7±3.1 vs 43.0±4.1\% respectively; p<0.05). Thus, transient activation of A_1R had induced a delayed preconditioning effect against infarction at 24 hours. The effect of inhibition of PKC was evaluated by administering CHE before CCPA or saline injections. Prior treatment with CHE 5 mg/kg completely abolished the infarct-limiting effect of delayed pharmacological preconditioning with CCPA, whereas it did not significantly affect infarct size in saline treated animals (I/R; 37.3±4.1 and 41.1±4.7\% respectively, p=NS vs control). Similarly, inhibition of TK using the selective antagonist LDA 1.3 mg/kg administered prior to the CCPA bolus, abrogated the limitation of infarction in these animals, whereas LDA on its own had no effect on infarct size (I/R; 38.2±4.9 and 42.8±4.8\% respectively, p=NS vs control). These results point to an important role for these two groups of protein kinases in the signalling mechanism downstream of A_1R, and mediating its delayed cardioprotective effects.

4.3.d p38 MAPK Activity

Activation of p38 MAPK in myocardial samples obtained from rabbits pretreated 24 hours earlier was determined by measurement of its catalytic activity using the in vitro kinase assay with recombinant ATF-2 as substrate. As seen in figure 4.3, pretreatment 24 hours earlier with the A_1R agonist, CCPA, resulted in an almost seven fold increase in the activity of p38 MAPK (689±63\%, p<0.01). This increased activity was abolished by prior inhibition of either PKC or TK. Pretreatment with CHE or LDA alone did not significantly affect p38 MAPK activity at 24 hours. None of the above treatments affected total expression of p38 MAPK protein as seen when filters were reprobed with anti-p38 MAPK antibody.
Figure 4.3. Myocardial p38 MAPK Activity.

In myocardial samples obtained 24 h after the various treatments, p38 MAPK catalytic activity was determined using an *in vitro* kinase assay to phosphorylate ATF2, and the reaction mixture separated by SDS-PAGE. The same filter was probed sequentially with anti-phospho-ATF2 and anti-p38 MAPK. The ratio of activation was calculated as phospho-ATF2:p38 MAPK immunoreactivity and was normalised to 1 for the control group. Panel a) Representative Western immunoblots from different experimental groups. Panel b) The ratio of phospho-ATF2:p38 MAPK immunoreactivity. The data represent n=3 animals per group, and are plotted as mean±SEM. *p<0.05 (one-way ANOVA).
4.3.e Hsp27 Expression and Phosphorylation

One dimensional SDS-PAGE was used to assess expression of Hsp27 protein in myocardial samples obtained 24 hours after various treatment protocols. Figure 4.4 shows a representative Western blot. There were no differences in total Hsp27 protein content between the six experimental groups, indicating that transient activation of A,R in rabbit myocardium, does not result in enhanced expression of Hsp27 protein at 24 hours. However, since the stabilising activity of Hsp27 on the actin cytoskeleton seems to depend on its phosphorylation state (439, 457, 458), 2D-PAGE was next used to analyse changes in post-translational phosphorylation of Hsp27 which might be induced by the A,R agonist. The results of 2-D PAGE are presented with the acidic region (anode + charge) on the left and the basic region (cathode - charge) on the right. Phosphorylation of Hsp27 can occur on up to 3 sites (Ser-15, Ser-78 and Ser-82) (459, 460). This increases the total negative charge of the protein, with a resultant decrease in isoelectric point and an increased leftward mobility of Hsp27 into the acidic (anode) region of the IEF gel. The four major phosphorylation isoforms of Hsp27 are the non-phosphorylated, the mono-, the di- and the tri-phosphorylated.

![Figure 4.4. Myocardial Hsp27 Expression.](image)

Figure shows a representative immunoblot probed for Hsp27 protein in myocardial samples from various treatment groups.
| I) Control |  |
| II) CCPA |  |
| III) CHE+CCPA |  |
| IV) CHE+Saline |  |
| V) LDA+CCPA |  |
| VI) LDA+Saline |  |

**Figure 4.5. Myocardial Hsp27 Phosphorylation.**

In myocardial samples obtained 24 h after the various treatments, Hsp27 phosphorylation was determined by 2D-PAGE. Figure shows representative 2D immunoblots from 3 animals per experimental group. The small arrow indicates the non-phosphorylated Hsp27 isoform.
As seen in figure 4.5, in the control hearts Hsp27 was detected primarily in the non-phosphorylated form (the most positively charged) with minor contribution from the mono-phosphorylated isoforms. Prior treatment with CCPA 100 μg/kg 24 hours earlier resulted in an acidic shift in the position of the Hsp27 isoforms corresponding to increased phosphorylation of the protein and probably representing the bi- and tri-phosphorylated isoforms of Hsp27. This phosphorylation pattern was completely inhibited by prior inhibition of either PKC or TK, so that 2-D gels of myocardial samples from these animals were similar to that from controls (figure 4.4). Prior treatment with CHE or LDA alone did not affect Hsp27 phosphorylation. These results indicate that adenosine A₁R activation induces phosphorylation of Hsp27 at a time point that corresponds to the delayed infarct-limiting effects of this treatment, and that Hsp27 phosphorylation is mediated by a PKC and TK-dependent signalling pathway.

### 4.3.f Mn-SOD Activity

Activity of Mn-SOD in myocardial samples obtained from rabbits pretreated 24 hours earlier was determined by the nitroblue tetrazolium method. As shown in figure 4.6, CCPA pretreatment resulted in significantly increased Mn-SOD activity at 24 hours, compared to the control group (200.3±10.2 vs 120.3±5.2 U/mg, p<0.001). Pretreatment with either CHE or LDA attenuated this increase (150.4±10.2 and 134.2±5.3 U/mg respectively) so that myocardial Mn-SOD activity in these experimental groups was not significantly different from that in the control group. These results suggest that transient activation of myocardial A₁R also results in delayed enhanced activity of the mitochondrial antioxidant, at a time-point when the late cardioprotective effects of CCPA are established. The increased activity of Mn-SOD seems to depend on a signalling mechanism involving both PKC and TKs.
Figure 4.6. Myocardial Mn-SOD Activity

In myocardial samples obtained 24 h after the various treatments, Mn-SOD activity was determined by the NBT method. Figure shows Mn-SOD activity corrected for protein content in the experimental groups. The data represent n=3 animals per group, and are plotted as mean±SEM. **p<0.001 vs vehicle treated group.
4.4 Discussion

The present study provides new insight into the cellular mechanisms responsible for conferring increased myocardial tolerance to lethal ischaemic injury 24 hours after transient activation of A1R. The results show that the significant protection against myocardial infarction observed in the rabbit, 24 hours after treatment with the selective A1R agonist CCPA, is completely abolished by prior inhibition of either PKC, or TK with their potent and selective inhibitors, CHE and LDA respectively. These data point to the crucial role of these two families of protein kinases in mediating A1R induced delayed preconditioning. In the second part of the present study, a significant increase in myocardial p38 MAPK activity 24 hours after CCPA treatment is demonstrated. Pharmacological preconditioning with CCPA was also associated with increased phosphorylation of the cytoprotective protein Hsp27 at 24 hours. Both the increase in p38 MAPK activity and Hsp27 phosphorylation were PKC and TK dependent and were inhibited by prior treatment with either CHE or LDA. The activation of p38 MAPK therefore appears to occur distal to PKC and TK. Taken together, these results, for the first time, suggest an important role for the p38 MAPK/Hsp27 pathway in the signalling mechanism underlying A1R induced delayed preconditioning, and that this pathway is downstream of, and dependent on PKC and TK activation. Furthermore, transient adenosine A1R activation resulted in enhanced myocardial Mn-SOD activity at 24 hours. This increase in the activity of Mn-SOD was also abolished by inhibition of either PKC or TK, suggesting that regulation of Mn-SOD is mediated by a signalling mechanism involving both groups of kinases. This finding also points to the possible involvement of Mn-SOD in mediating the delayed cardioprotective effects of CCPA.

4.4.a Role of Protein Kinases in Delayed Preconditioning

Previous studies have implicated PKC in mediating delayed cardioprotection following ischaemic preconditioning (172, 302, 307). In the present study, it is shown that PKC also mediates A1R induced late protection against infarction. CHE, a potent inhibitor of PKC (IC50 ~ 0.7 μmol/l) with very high selectivity for PKC compared to PKA (250:1), or protein TK (150:1) (461), was used in the present study at a dose (5 mg/kg) which has been shown to abolish delayed ischaemic
preconditioning against both infarction (302) and stunning (307) in rabbit myocardium. Furthermore, Ping et al. (172) have reported that a preconditioning protocol of 6x4 minute coronary occlusion/4 minute reperfusion in conscious rabbits induces translocation of PKC isoforms \( \varepsilon \) and \( \eta \) from the cytosolic to the particulate fraction, which is prevented by prior treatment with CHE at the same dose as that used in the present study. Similarly, Wilson et al. (308) reported an association between development of delayed cardioprotection against ventricular arrhythmias, 24 hours after rapid cardiac pacing in dogs, and sustained translocation of PKC-\( \varepsilon \) to the membrane fraction. Gray et al. (185) have implicated PKC-\( \varepsilon \) in hypoxic preconditioning of cardiac myocytes using PKC-\( \varepsilon \) selective inhibitor peptide. Using the same isoform-selective peptide inhibitors, Liu et al. (189) have shown, in isolated rabbit cardiomyocytes, that the protection of preconditioning is abolished by the peptide inhibitor of PKC-\( \varepsilon \) but not by the peptide inhibitors selective for PKC-\( \beta \), PKC-\( \delta \), or PKC-\( \eta \). This group have subsequently shown that expression of a peptide homologous to PKC-\( \varepsilon \) RACK, that facilitates isoform selective translocation of PKC-\( \varepsilon \), in cardiac myocytes \textit{in vitro}, or in transgenic mice \textit{in vivo}, confers protection against ischaemic injury akin to that due to ischaemic preconditioning (190). On the other hand, Kawamura et al. (191) have suggested that in addition to PKC-\( \varepsilon \), the \( \delta \) isoform is also translocated to the membrane fraction following ischaemic preconditioning in isolated rat hearts and is involved in the development of protection against post-ischaemic LV dysfunction. Mitchell et al. (179) have reported similar results in the rat heart. Moreover, Zhao et al. (192) found that expression of constitutively active PKC-\( \delta \) isoform conferred protection against simulated ischaemia to isolated rat cardiomyocytes. Yoshida et al. (193) reported that PKC-\( \alpha \), \( \delta \) and \( \varepsilon \) were translocated to the membrane fraction in rat hearts following ischaemic preconditioning, and that this movement was completely blocked by the PKC inhibitor CHE. These apparent inconsistencies may be due to the different species and models used in the above studies. While some evidence suggests that A\( _1 \)R activation results in transient translocation of PKC-\( \delta \) in rat ventricular myocytes (431), it is currently not known which other PKC isoforms may be activated in the myocardium downstream of A\( _1 \)R, and mediate its delayed preconditioning effect against infarction.
Imagawa et al. (310) first demonstrated the involvement of TK in ischaemic preconditioning induced second window of protection against infarction, using the TK inhibitor genistein. However, genistein, originally considered to be selective for TK, seems to have other non-selective effects such as inhibition of serine/threonine kinases (e.g. PKC and PKA) at higher concentrations (462). Importantly, genistein has also been reported to inhibit A1R in non-cardiac cells (463), although Imagawa et al. (310) showed that it did not abolish the haemodynamic effects of A1R receptor activation in rabbits. A more selective TK inhibitor, LDA, was therefore used in the present study. LDA is a potent inhibitor of protein TK (IC50 = 0.5 μmol/l) and the epidermal growth factor (EGF) receptor kinase (IC50 = 29 nmol/l), with much weaker actions at inhibiting PKC or PKA (IC50 > 200 μmol/l) (464). In the present study, LDA was used at a dose of 1.3 mg/kg, which assuming distribution in total body water, would yield an approximate plasma concentration of 5 μmol/l, 10-fold higher than the IC50 for inhibition of protein TK but well below that for other protein kinases. This dose of LDA completely abrogated the protection induced by pretreatment with CCPA 24 hours earlier, whereas LDA alone had no effect on infarct size (figure 2). A similar dose of LDA (1 mg/kg) has been shown to abolish the late preconditioning effect against stunning in a conscious rabbit model (433). Moreover, Ping et al. (204) have recently demonstrated that a PC protocol of 6x4 minute coronary occlusion/4 minute reperfusion in conscious rabbits induces selective activation of 2 members of the Src family of protein TK (Src and Lck) in the myocardium, with no effect on EGF receptor kinases, and that this activation is abolished by pretreatment with LDA, at a similar dose to that used in the present study. In that study, LDA did not affect the translocation of PKC-ε following ischaemic preconditioning thereby pointing to the selectivity of LDA. It is therefore very unlikely that LDA, at the dose used in the present study, inhibited any other protein kinases other than protein TK.

Taken together, the results of the present study indicate a crucial role for both PKC and TK in mediating delayed preconditioning against infarction following A1R activation in rabbits. The relative positions of PKC and TK in the signalling pathway downstream of A1R was not examined in this study, although the fact that inhibition of either group of enzymes completely abolished CCPA induced late
preconditioning, would suggest that these kinases function in the same, rather than parallel signalling pathways. Some evidence suggests that following ischaemic preconditioning in rabbits, TK activation occurs downstream of and is dependent on PKC activation (203, 204). However, it is also noteworthy that recent evidence in rats and pigs suggests that these two groups of enzymes may act in parallel to mediate early ischaemic preconditioning (184, 202). Further studies are necessary to address the relative positions of these enzymes in the signalling cascade downstream of A1R.

In the present study, it is also shown, in myocardium not subjected to ischaemia-reperfusion, that 24 hours after treatment with CCPA, p38 MAPK activity was significantly increased compared to saline treated controls. Previous studies have demonstrated activation of myocardial p38 MAPK following a number of stresses, including ischaemia-reperfusion (reviewed in reference 206), and also after activation of G protein-coupled receptors including A1R (209, 210, 450). However all these studies have examined the acute profile of p38 MAPK activation. For example, Haq et al. (210) showed, in the isolated perfused rat heart, that infusion of adenosine resulted in rapid activation of p38 MAPK that was maximal at 5 minutes and declined thereafter. The present study is the first to show that transient A1R activation also induces a second phase of enhanced p38 MAPK activity at 24 hours in rabbit myocardium. Moreover, these results suggest that the delayed activation of p38 MAPK is downstream of, and dependent on PKC and TK activation, since pretreatment with either CHE or LDA, at doses that abrogated delayed protection at 24 hours, completely abolished the enhanced p38 MAPK activity. This is consistent with other reports of a role for PKC and TK in activation of p38 MAPK in both non-cardiac tissue and in myocytes (198, 209, 465). Taken together, these results point to a potential role for p38 MAPK in mediating delayed A1R induced preconditioning in the rabbit. In support of this concept, recent results by Carroll et al. (356) have shown, in an adult human cardiac myoblast cell line, that pretreatment with the selective p38 MAPK inhibitor SB203580, completely abolishes delayed protection 24 hours following ischaemic or adenosine induced preconditioning. Similarly, Zhao et al. (466) have recently reported that A1R induced preconditioning against
Chapter Four

infarction in mice, is associated with delayed activation of p38 MAPK via a TK sensitive mechanism.

4.4.b Role of Hsp27 in Delayed Preconditioning

One substrate for p38 MAPK is the protein kinase MAPKAPK-2 which itself phosphorylates Hsp27 on serine residues. Hsp27 is constitutively expressed in virtually all organisms, and in some cell types is present at concentrations close to 1% of total protein (444). Phosphorylation induces dramatic changes in the supramolecular structure of Hsp27. Thus, while non-phosphorylated isoforms of Hsp27 can form oligomeric complexes of up to 1 MDa, phosphorylation brings about a shift towards small molecular species consisting mainly of homodimers and also monomers (458). Some of the biochemical activities of Hsp27, such as its chaperone function, have been reported to be either unrelated to its phosphorylation state (467), or depend on presence of large unphosphorylated oligomers (468). On the other hand, the cytoprotective activity of Hsp27 involving changes in the oligomeric structure of Hsp27 and stabilisation of the actin cytoskeleton seems to depend on its phosphorylation state (reviewed in reference 444). Direct evidence that Hsp27 interacts with actin filaments in vivo, comes from studies showing that cells overexpressing Hsp27 following gene transfection grow and survive better on exposure to cytochalasin D, a disrupter of actin filaments, than control cells (458). Moreover, overexpression of a non-phosphorylatable mutant of Hsp27 which is mostly organised as high molecular mass oligomers, had no effect on actin dynamics, and did not prevent cytochalasin D-induced growth inhibition. Furthermore, treatment of cells with the p38 MAPK inhibitor SB 203580 which resulted in decreased phosphorylation of Hsp27 and a shift towards the high molecular mass oligomers, totally inhibited Hsp27-dependent protection against cytochalasin D-induced filament disruption (458). Conversely, treatment with sodium arsenite, which induces phosphorylation of Hsp27 and increases the proportion of low molecular mass Hsp27, has been shown to potentiate the protective effect of this protein (445).

In the present study, treatment of rabbits 24 hours earlier did not result in enhanced expression of myocardial Hsp27. This is consistent with a preliminary report by
Heads et al. (469) who found no change in Hsp27 expression 24 hours following CCPA or ischaemic preconditioning in rabbit myocardium, but showed in subcellular fractionation studies, that these preconditioning protocols resulted in redistribution of Hsp27 from the membrane to the soluble fraction. In the present study, these findings have been extended to show for the first time, that while Hsp27 from control hearts is mainly non-phosphorylated, 24 hours after pharmacological preconditioning with CCPA, Hsp27 is primarily in a phosphorylated form, and that this pattern of phosphorylation is mediated by a signalling mechanism dependent on both PKC and TK activation. Although these results do not support a direct causal relationship between Hsp27 phosphorylation and delayed A,R induced infarct-limitation, their temporal relationship and the fact that both are blocked by pretreatment with either CHE or LDA are strongly suggestive. In further support of a role for the MAPKAPK-2/Hsp27 pathway as a distal mediator of A,R induced delayed protection, recent preliminary data from Yellon’s laboratory has indicated that in a human cardiac myoblast cell line, delayed protection 24 hours following ischaemic or adenosine induced preconditioning is abolished by prior treatment with a peptide inhibitor of MAPKAPK-2 (unpublished observations, Carroll R and Yellon DM, 2000).

4.4.c Role of Mn-SOD in Delayed Preconditioning

The current results also demonstrate subacute increased activity of the mitochondrial antioxidant Mn-SOD, 24 hours after pharmacological preconditioning with CCPA, and that this enhanced activity is regulated by a signalling mechanism involving both PKC and TK. This finding suggests that Mn-SOD may also participate in conferring delayed protection against infarction in the rabbit myocardium. However, these preliminary results require further confirmation, and to establish a definitive role for Mn-SOD in mediating delayed preconditioning, it is necessary to demonstrate that inhibition of enhanced activity of Mn-SOD is capable of blocking the subacute cardioprotective effects of CCPA (see Chapter 5).
4.5 Summary

In conclusion, the results of the present study show that the delayed myocardial protection 24 hours following transient A₁R activation with CCPA in rabbits is mediated by a signalling mechanism involving both PKC and TK. This signalling cascade in turn results in activation of p38 MAPK, phosphorylation of Hsp27, and enhanced activity of Mn-SOD. These results provide the first direct evidence for activation of the p38 MAPK/Hsp27 pathway 24 hours following A₁R activation, and although correlative, suggest an important role for this pathway as a distal mediator of A₁R-induced delayed preconditioning in rabbits. Further confirmatory studies using specific pharmacological inhibitors of this pathway are needed to establish its role as a distal effector of A₁R-induced delayed cardioprotection. These data also suggest that upregulation of Mn-SOD may play a role in mediating delayed protection following treatment with A₁R agonists.
Chapter Five

Adenosine A$_7$R Induced Delayed Preconditioning:

Role of Mn-SOD
5.1 Introduction

An important issue regarding the delayed protection against infarction conferred by transient adenosine $A_1$R activation is the nature of the distal effector or target protein(s) mediating this protection. Recent evidence suggests that one or more cellular antioxidant enzymes may be upregulated following heat stress or transient sublethal ischaemia and contribute to the cytoprotection observed during subsequent lethal ischaemia-reperfusion. One such enzyme is manganese-superoxide dismutase (Mn-SOD). Mn-SOD is an enzymatic antioxidant that catalyses the dismutation of the superoxide anion ($O_2^-$) to $H_2O_2$ (470). Mn-SOD is located in the mitochondrial matrix near the electron transport chain, which is an important cellular source of ROS production within the cell. A critical role for Mn-SOD in protection against oxidant injury has been demonstrated both in vitro and in vivo (reviewed in reference 18). Importantly, overexpression of Mn-SOD in transgenic mice, has been shown to result in enhanced resistance against myocardial ischaemia-reperfusion injury (471).

Expression of Mn-SOD is transcriptionally increased by exposure to a number of stressful stimuli (18). It has also been reported that treatment of a variety of tissues, including rat cardiac myocytes, with the adenosine $A_1$R agonist $N^\alpha$-(phenyl-2R-isopropyl)-adenosine (R-PIA) results in upregulation of endogenous antioxidant enzymes including Mn-SOD over a 90-120 minute period (453). Moreover, the results presented in chapter 4 suggest that the delayed limitation of infarction observed in rabbits 24 hours following treatment with CCPA is associated with a significant increase in myocardial Mn-SOD activity, and that this increase is attenuated by prior inhibition of either PKC or tyrosine kinases, strategies that were also shown to abolish the cardioprotective effects of delayed preconditioning with CCPA. Although this would suggest a contributory role for upregulation of endogenous myocardial Mn-SOD in mediating the subacute effect of $A_1$R activation on tissue tolerance to prolonged ischaemia, further direct evidence is needed to support this hypothesis. In the present study, this hypothesis was examined by evaluating the effect of suppression of increased myocardial Mn-SOD by intravenous injection with antisense oligodeoxynucleotides (ODNs) against rat Mn-SOD, on
acquisition of delayed tolerance to myocardial ischaemia 24 hours after CCPA treatment in the rat.

5.2 Materials and Methods

Male Wistar rats (body weight 250-350 g) were used in these experiments. Animals were acclimatised in the institutional animal house for 5-7 days after delivery and had free access to a standard pelleted diet and water.

5.2.a Experimental Protocol

On day 1, animals were pharmacologically preconditioned. Rats were lightly anaesthetised with a combination of Hypnorm® and Midazolam (1 part midazolam + 1 part Hypnorm + 2 parts sterile water administered at a dose of 1 ml/kg s.c.). Under sterile conditions, a 2 cm right lateral incision was made in the neck and the right internal jugular vein cannulated with a Y-CAN 0.7 mm paediatric cannula (Simcare Ltd. UK). Rats were assigned to 6 experimental groups (Figure 5.1). Group I (control) received an i.v. bolus of sterile 0.9% saline 0.5 ml. Group II (CCPA) were pharmacologically preconditioned with a single i.v bolus of CCPA at a dose of 100 μg/kg. CCPA was dissolved in sterile 0.9% saline to a concentration of 100 μg/ml (final volume administered 0.25-0.35 ml). To examine the effect of suppression of induction of Mn-SOD, groups III (AS-ODN + CCPA) and IV (AS-ODN + Saline) were also treated with a slow intravenous infusion (over 5 minutes) of a twenty two mer phosphorothioated derivative of the antisense oligodeoxynucleotide (AS-ODN), 5'-CACGCCGCCGACACAACATTG-3', against the initiation site of rat Mn-SOD mRNA (73, 332), 5 minutes prior to the CCPA (group III) or saline (group IV) bolus at a dose of 5 mg/kg (Figure 5.1). The antisense ODN is a synthetic DNA molecule with a sequence complementary to the specific Mn-SOD mRNA initiation sequence. Thus it will bind to the Mn-SOD mRNA in a sequence-specific manner and prevent the expression of the mRNA, which will in turn inhibit or reduce the production of Mn-SOD protein (472). Phosphorothioation of the ODN backbone, in which single oxygen molecules are substituted by sulphur at a non-bridging position with phosphorus, is the modification of choice for ODNs, resulting in prolongation of their activity by making them resistant to nuclease activity (472).
To control for non-specific effects of treatment with AS-ODN, in particular non-specific protein binding, two similar ODNs with the same length as the AS-ODN were used. Group V (S-ODN + CCPA) was treated with the sense ODN, containing the complementary bases, (S-ODN), 5'-CAATGTTGTGTCGGGCGGCGTG-3', and group VI (Scr-ODN + CCPA) with the scrambled ODN (Scr-ODN), 5'-ACGCACAAAGGATAACATTACT-3', at a dose of 5 mg/kg 5 minutes prior to receiving a bolus of CCPA (Figure 5.1). All phosphorothioated oligodeoxynucleotides were purchased from MWG-Biotech Ltd., UK, and dissolved in sterile 0.9% saline at a concentration of 2 mg/ml. Following these treatments, the intravenous cannula was removed, jugular vein tied and the neck wound sutured with 3/0 silk (Mersilk W502, Ethicon). The animals were then allowed to recover from the anaesthesia with postoperative analgesia with bupranorphine HCl 50 μg/kg s.c. (Vetergesic®, Reckitt & Colman Product Ltd, Hull, U.K.), and were returned to their cages with no further manipulation.

5.2.b Ischaemia-Reperfusion Protocol in vitro

Twenty four hours after various treatments, rats were anaesthetised with pentobarbital sodium 60 mg/kg i.p. and anticoagulated with heparin 1000 U/kg i.p. A median sternotomy was performed, the heart was rapidly excised and perfused retrogradely by the Langendorff technique at constant pressure (100 cm H2O), as described in section 2.3. The hearts were subjected to 35 minutes regional myocardial ischaemia followed by 2 hours reperfusion (Figure 5.1) after which myocardial infarct size was assessed as detailed in section 2.4.

5.2.c Determination of Myocardial Mn-SOD Content

In a different group of animals, 24 hours after the same treatments as outlined above, myocardial samples were obtained for assessment of Mn-SOD content and activity. As a positive control for induction of Mn-SOD, group VII animals were treated with an i.v. injection of recombinant murine tumour necrosis factor-α (TNF-α) (R&D Systems Europe Ltd.) at a dose of 10 μg/kg. TNF-α is a known potent inducer of Mn-SOD (73, 473, 474). Twenty four hours after the various treatments, animals were killed by an overdose of pentobarbital sodium.
Figure 5.1. Experimental protocol. On day 1, animals were pharmacologically preconditioned with an i.v. bolus injection of CCPA or received saline vehicle (groups I and II). Animals in groups III and IV were also treated with an i.v. infusion of antisense oligodeoxynucleotide (AS-ODN) to rat Mn-SOD, 5 min before the CCPA/saline bolus. Animals in group V received the sense (S-ODN) and those in group VI, the scrambled (Scr-ODN) oligodeoxynucleotide before the CCPA bolus. On day 2, animals were anaesthetized, hearts isolated and subjected to 35 min regional ischaemia (I) and 2 h reperfusion (R) in vitro. The small arrow indicates the timing of myocardial sampling in animals that were not subjected to ischaemia-reperfusion, for analysis of Mn-SOD content and activity.
Chapter Five

The hearts were immediately excised and myocardial tissue was rinsed in phosphate-buffered saline (PBS), and blood in the coronary arteries was washed out by retrograde perfusion of the ascending aorta with an adequate volume of PBS. Both atria and the right ventricle were dissected. Left ventricular myocardial samples were rapidly frozen by immersion in liquid nitrogen and stored at -80°C.

Myocardial content of Mn-SOD was determined by SDS polyacrylamide gel electrophoresis (PAGE). Myocardial tissue samples were prepared as described in section 4.2. Electrophoresis was performed with 100 μg of protein/sample on 12.5% polyacrylamide SDS-PAGE gels according to Laemmli (456) and as detailed in section 4.2. Proteins were then transferred electrophoretically onto nitrocellulose membrane (hybond-C; Amersham, UK) overnight at 180 mA and 4°C. Following transfer, filters were probed with rabbit polyclonal IgG against Mn-SOD (kind gift from Dr Kuzuya, University of Osaka, Japan) at 1:200 dilution. Following rinsing in wash buffer, the filter was probed with HRP-conjugated swine anti-mouse IgG (Dako Ltd, UK) at 1:2000 dilution. Antibodies were diluted in PBS; 0.05% Tween-20; 5% dried milk powder (Marvel) at room temperature. Blots were developed using an enhanced chemiluminescence detection system (Amersham, UK) and exposed to Kodak X-Omat AR film. Autoradiographs were scanned with a Sharp JX-330 scanner, and the band density was analysed by densitometry.

5.2.d Determination of Myocardial Mn-SOD Activity

Mn-SOD activity in myocardial samples was determined by the nitroblue tetrazolium (NBT) method (73, 288, 332, 333), as described in section 4.2.e.

5.2.e Statistical Analysis

The data are presented throughout as mean values ± SEM. The significance of the differences in mean values of I, R, I/R, and Mn-SOD content and activity between the treatment groups was evaluated by one-way analysis of variance (ANOVA) followed by Fisher’s protected least significant difference (PLSD) test. Any differences between haemodynamic or coronary flow measurements at different time points were assessed by two way analysis of variance with repeated measures.
followed by Fisher’s PLSD test used post hoc for individual differences. The null hypothesis was rejected at p<0.05.

5.3 Results

5.3.a Exclusions
A total of 87 rats were used for these studies: 59 animals were used in the studies of myocardial infarction and 28 (4 per group) in the analysis of myocardial Mn-SOD content and activity. Nine rats were excluded from the final analysis of infarct size for technical reasons: two hearts due to failure of TTC stain (one in the CCPA group, one in the AS-ODN + CCPA group); three hearts developed intractable ventricular fibrillation during ischaemia-reperfusion (one in the CCPA group, one in the AS-ODN + CCPA and one in the Scr-ODN + CCPA group); two hearts developed severe hypotension (LVDP<50mmHg) after insertion of the LV balloon (one in the control group and one in the S-ODN + CCPA group); one heart was excluded due to myocardial rupture at the time of coronary occlusion (Scr-ODN + CCPA group); one heart was excluded due to air embolisation at the start of reperfusion (CCPA group). Data is therefore reported on 50 successfully completed experiments.

5.3.b Coronary Flow and Haemodynamic Measurements
Coronary flow (CF) measurements during the ischaemia-reperfusion protocol are presented in figure 5.2. There were no differences in baseline CF values between the various groups which averaged at 11.5-13.0 ml/min. During regional ischaemia the CF was significantly reduced (p<0.01) in all groups to mean values of 5.5-7.0 ml/min with recovery to pre-ischaemic values on reperfusion, and then a gradual decline during the subsequent 2 hours of reperfusion. There were no significant differences in CF between the various groups at any time point during ischaemia-reperfusion.

Table 5.1 summarises HR, LVDP and RPP data recorded in the six experimental groups at baseline and during ischaemia-reperfusion. There were no significant differences between haemodynamic parameters at baseline between any of the groups. There was a significant reduction in LVDP and RPP during ischaemia in all groups with a return towards pre-ischaemic values in early reperfusion.
Figure 5.2. Coronary Flow Measurements During Ischaemia-reperfusion.
Abbreviations are the same as in the legend to figure 5.1. Data are mean±SEM.
*p<0.01 compared to baseline (two-way ANOVA with repeated measures).
However there were no differences in haemodynamic performance between the different groups at any time point.

Table 5.1 Haemodynamic Parameters During Ischaemia-reperfusion

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>Ischaemia</th>
<th>Reperfusion</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>5 min</td>
<td>30 min</td>
<td>15 min</td>
</tr>
<tr>
<td><strong>HR (beats/min)</strong></td>
<td></td>
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<tr>
<td>Control</td>
<td>302±8</td>
<td>276±13</td>
<td>267±14</td>
</tr>
<tr>
<td>CCPA</td>
<td>304±9</td>
<td>285±7</td>
<td>286±15</td>
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<tr>
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<td>274±7</td>
<td>270±12</td>
</tr>
<tr>
<td>AS-ODN + Saline</td>
<td>286±8</td>
<td>270±10</td>
<td>275±9</td>
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<tr>
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<td>270±13</td>
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<tr>
<td>Scr-ODN + CCPA</td>
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<td>270±13</td>
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<td><strong>LVDP (mmHg)</strong></td>
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<td>67±5</td>
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<td>108±6</td>
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<td>69±6</td>
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<td>68±3</td>
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<td><strong>RPP (mmHg/min x 10³)</strong></td>
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<td>18.1±1.6</td>
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<td>20.4±1.6</td>
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<td>29.9±2.3</td>
<td>11.7±1.5</td>
<td>18.4±1.2</td>
</tr>
</tbody>
</table>

Values are mean±SEM.
5.3.c Myocardial Risk and Infarct Size

Table 5.2 presents the volumes of risk and infarct zones in the 6 experimental groups. In these groups, the mean volume of myocardial tissue at risk during coronary artery occlusion was in the range 450-520 mm$^3$ representing about 45-50% of total left ventricular tissue volume. There were no significant differences in ischaemic risk zone among the experimental groups. The absolute infarct size was significantly smaller in CCPA treated animals and in the S-ODN + CCPA group compared to controls.

Table 5.2. Body Weight, Myocardial Area at Risk and Infarct Size.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body Weight (g)</th>
<th>R (mm$^3$)</th>
<th>I (mm$^3$)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>11</td>
<td>302±7</td>
<td>478±26</td>
<td>203±24</td>
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<tr>
<td>CCPA</td>
<td>8</td>
<td>306±14</td>
<td>509±41</td>
<td>112±18*</td>
</tr>
<tr>
<td>AS-ODN + CCPA</td>
<td>8</td>
<td>317±10</td>
<td>472±19</td>
<td>185±14</td>
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<tr>
<td>AS-ODN + Saline</td>
<td>8</td>
<td>313±10</td>
<td>468±36</td>
<td>194±20</td>
</tr>
<tr>
<td>S-ODN + CCPA</td>
<td>8</td>
<td>320±14</td>
<td>448±24</td>
<td>100±18*</td>
</tr>
<tr>
<td>Scr-ODN + CCPA</td>
<td>7</td>
<td>322±6</td>
<td>523±37</td>
<td>155±17</td>
</tr>
</tbody>
</table>

Values are mean±SEM. *p<0.05 vs control group (one-way ANOVA).

Infarct size expressed as a percentage of area at risk (I/R) in the six experimental groups is presented in figure 5.3. Pretreatment with CCPA 24 hours before myocardial infarction resulted in a significant reduction in I/R compared to saline treated controls (22.3±3.3 vs 42.1±3.8% respectively; p=0.001). The effect of suppression of Mn-SOD was evaluated by using AS-ODN administered i.v. before CCPA or saline injections. Prior treatment with AS-ODN 5 mg/kg completely abolished the infarct-limiting effect of delayed pharmacological preconditioning with CCPA, whereas it did not significantly affect infarct size in saline treated animals (I/R; 39.4±2.8 and 46.5±4.8% respectively, p=NS vs control group). On the other hand, administration of S-ODN or Scr-ODN 5 mg/kg 5 minutes prior to CCPA preconditioning, did not affect the cardioprotection observed at 24 hours (I/R; 24.7±3.9 and 29.3±1.8% respectively, p=0.001 vs control).
Figure 5.3. Myocardial Infarct Size.

Abbreviations are the same as in the legend to figure 5.1. Infarct size is expressed as a percentage of the region at risk of infarction. Open circles represent individual hearts; solid circles, mean±SEM. *p=0.001 vs control and AS-ODN treated groups (one-way ANOVA).

5.3.d Mn-SOD Content and Activity

Figure 5.4 shows myocardial content of Mn-SOD protein. Mn-SOD activity assayed in myocardial samples obtained 24 hours after various treatments is presented in figure 5.5. Prior treatment with CCPA 24 hours earlier enhanced myocardial induction and resulted in a significant 56% increase in myocardial Mn-SOD activity compared to saline treated animals (207±25 vs 132±16 U/mg, p<0.001). This enhanced Mn-SOD content and activity was abolished in animals pretreated with AS-ODN (120±11 U/mg), whereas the S-ODN or Scr-ODN treated groups had significantly increased Mn-SOD protein and activity (200±32 and 205±20 U/mg respectively, p<0.001 vs control).
Figure 5.4. Myocardial Mn-SOD Content.

Panel a): Representative Western blot probed with polyclonal antibody specific for rat Mn-SOD. Panel b): Computerised densitometry analysis of Western blots (n=4 per group). TNF-α: pretreatment with recombinant murine tumour necrosis factor-α 24 hours earlier. Bars represent mean±SEM. *p<0.05 vs control and AS-ODN treated groups (one-way ANOVA).
TNF-α is a known potent inducer of Mn-SOD, and the group that had been treated with this cytokine had the highest level of myocardial Mn-SOD content and activity (243±30 U/mg, p<0.001 vs control), although these values were not statistically significant from those in the CCPA treated group.

**Figure 5.5. Myocardial Mn-SOD Activity.**

Myocardial samples obtained 24 h after various pretreatment were analysed for the activity of the mitochondrial Mn-SOD by the nitroblue tetrazolium method (n=4 per group). The activity of Mn-SOD is expressed relative to the protein concentration of the sample. Bars represent mean±SEM. *p<0.001 vs control and AS-ODN treated groups (one-way ANOVA).
5.4 Discussion

The present study provides new insight into the cellular mechanisms responsible for conferring increased myocardial tolerance to lethal ischaemic injury 24 hours after transient activation of $A_1$R. The results show that pretreatment with the selective adenosine $A_1$ agonist CCPA in the rat, produces significant protection against myocardial infarction 24 hours later, similar to the delayed CCPA induced protection previously reported in the rabbit (290, 324). This delayed infarct-limiting effect of CCPA was associated with a significant increase in myocardial Mn-SOD content and activity. Pretreatment of rats with AS-ODN to Mn-SOD completely abolished the cardioprotective effects of CCPA and the associated induction of Mn-SOD. Importantly, treatment with the S-ODN or the Scr-ODN did not affect either the cardioprotection or the enhanced Mn-SOD expression induced by CCPA, thereby ruling out non-specific effects of treatment with oligodeoxynucleotides in abolishing protection. Taken together, these data point to an important role for induction of Mn-SOD in mediating the delayed infarct-limiting effects observed 24 hours following $A_1$R activation.

5.4.a Adenosine $A_1$ Receptors and Preconditioning

Downey's group (52, 147) were the first to demonstrate a role for adenosine, released during the brief periods of preconditioning ischaemia and acting on adenosine $A_1$R, as an important trigger of the mechanisms mediating early or 'classic' preconditioning in the rabbit myocardium. These original findings were later confirmed in further studies in the rabbit, dog and pig models of ischaemic preconditioning (152, 153, 475). In the rat heart on the other hand, the role of adenosine in mediating cardioprotection has been controversial, with most studies (but not all 476) failing to show a critical role for adenosine in mediating early preconditioning (53, 155, 477, 478). However, this discrepancy could have resulted from the fact that following a brief period of ischaemia, the interstitial concentration of adenosine released in the rat heart is 3-4 fold higher than that in the rabbit myocardium and higher concentrations of selective agonists and antagonists are required to mimic or abolish the protective effects of early preconditioning (156).
Two studies had previously suggested a role for \( \alpha_1 \)R as a trigger of delayed (second window) myocardial protection against infarction in the rabbit (290, 324). Studies reported in Chapters 3 and 4 further support the induction of late preconditioning by transient \( \alpha_1 \)R activation. Similar findings have been reported by another group using a different selective \( \alpha_1 \)R agonist (427). However, all previous studies have been performed in rabbit models. The present study is the first evidence that transient activation of \( \alpha_1 \)R in the rat, induces similar delayed cardioprotective effects against lethal ischaemic injury 24 hours later. The haemodynamic effects of an intravenous bolus of CCPA 100 \( \mu \)g/kg have been reported in the anaesthetised rabbit (290) and in conscious hypertensive rats (425). CCPA administration results in a brief (<90 minute) period of bradycardia and hypotension (~30% reduction in HR and BP from baseline), implying that the compound is eliminated within a few hours. In the present study the haemodynamic effects of CCPA 24 hours prior to the ischaemia-reperfusion protocol were not measured in order to minimise the surgical trauma involved during pharmacological preconditioning and administration of ODNs. However, the haemodynamic variables and CF measurements at the start of the infarction procedure were similar between the experimental groups. It is therefore very unlikely that the marked infarct limiting effects observed 24 hours after treatment with CCPA had resulted from the transient bradycardia and hypotension induced after the administration of the \( \alpha_1 \)R agonist.

5.4.b Cardioprotective Role of Mn-SOD

The mitochondrial Mn-SOD belongs to a class of enzymes that catalyse the dismutation of two superoxide radicals to form hydrogen peroxide and molecular oxygen (470). The demonstration that reactive oxygen species contribute to ischaemia-reperfusion injury suggests that increasing the content or activity of endogenous cellular antioxidant enzymes should protect tissues from the deleterious effects of ischaemia-reperfusion injury. However, the addition of SOD alone or in conjunction with other antioxidants such as catalase to the perfusion solution following myocardial ischaemia, have provided conflicting results (reviewed in reference 479). In addition to the variation in doses and kinetics under different experimental conditions, one of the major problems with these studies has been that the exogenous antioxidant enzymes cannot permeate the cells to the sites where free
radicals are generated. On the other hand, strategies that have resulted in enhanced activity of endogenous Mn-SOD have proven to be consistently protective. For example, it has been shown that pretreatment of rats with cytokines such as interleukin-1α, leukaemia inhibitory factor and TNF-α results in an increase in the activity of endogenous Mn-SOD and protects against subsequent myocardial ischaemia-reperfusion injury (474, 480-482). Moreover, Yamashita et al. have demonstrated that the subacute myocardial adaptation observed 24-48 hours after heat stress (72, 333, 483), sublethal hypoxia/ischaemia (90, 332), exercise (73), or ischaemic preconditioning (452), is mediated by induction and enhanced activity of endogenous Mn-SOD. Similar results have been reported by Zhou et al. in rat myocytes preconditioned with brief periods of anoxia and reoxygenation (484). Further still, it has recently been demonstrated that overexpression of Mn-SOD in transgenic mice results in reduced infarct size and improved functional recovery after ischaemia-reperfusion (471). Taken together, these results indicate an important protective role for Mn-SOD in reducing oxygen derived free radical induced injury during reperfusion of the ischaemic myocardium, and that induction of endogenous Mn-SOD may play an important role in mediating delayed cardioprotection following a number of stressful stimuli such as heat stress, ischaemia or exercise. It must be noted, however, that not all studies have demonstrated subacute induction of antioxidants following sublethal preconditioning ischaemia. For example, Tang et al. (485) did not observe any alteration in the activity of a number of antioxidants including Mn-SOD, Cu, Zn-SOD, catalase, glutathione (GSH) peroxidase or GSH reductase in conscious pigs 24 hours following a preconditioning stimulus consisting of a sequence of ten 2 min coronary occlusion/2 min reperfusion cycles. The reasons for these apparently discrepant results are not clear, although it may be due to species differences, since the same group did not demonstrate a late phase preconditioning effect against infarction using various cycles of preconditioning ischaemia in the conscious pig model (92).

The results of the present study suggest, for the first time, that the delayed cardioprotection induced by transient activation of A₁R in the rat is also associated with significantly enhanced myocardial Mn-SOD content and activity, and that pretreatment with AS-ODN which inhibited the induction and activation of Mn-
SOD, abolished the delayed infarct-limiting effects induced by CCPA. The ODNs used in the present study have previously been shown to abolish the induction of Mn-SOD following sublethal hypoxia in rat cultured cardiomyocytes (332), and following exercise (73), heat stress (333), and ischaemic preconditioning (452) in an in vivo rat model. The suppression of Mn-SOD induction in these studies also abrogated the delayed cardioprotective effects of various preconditioning stimuli. In the present study, the localisation of ODNs following intravenous injection was not assessed by imaging. It is therefore not possible to speculate about the exact site of inhibition of Mn-SOD within the myocardium. Yamashita et al. have recently characterised the time course of accumulation of ODNs within the myocardium following in vivo delivery with intraperitoneal injection in the rat, using 5' FITC-labelled AS-ODN to Mn-SOD (73). They reported significant labelling of myocardial tissues at various times following systemic administration of ODNs; in endothelial cells at 2-4 hours, at vascular smooth muscle at 4 hours, and in cardiac myocytes at 8 hours.

5.4.c Mechanism of Induction of Mn-SOD

The signalling pathways that regulate induction of Mn-SOD many hours following transient activation of A1R are not known. Studies reported in Chapter 4 in rabbits suggest that CCPA induced enhanced Mn-SOD activity at 24 hours is mediated via a PKC and TK dependent pathway, since pharmacological inhibition of either enzyme attenuated the increase in Mn-SOD activity, and abolished the delayed cardioprotective effects of CCPA. This is in concordance with other reports of a role for protein kinases in regulation of induction of Mn-SOD in endothelial cells (486, 487), human lung adenocarcinoma cells (488), and human leukocytes (489). On the other hand, an important role has been demonstrated for reactive oxygen species (ROS), cytokines and the oxidant sensitive transcription factor NF-κB in modulating Mn-SOD gene expression (73, 474, 490-492), all of which have also been implicated in mediating delayed cardioprotective effects following ischaemic preconditioning (73, 90, 298, 315, 328, 484). The interaction between transient activation of A1R and potential generation of ROS, or activation of cytokines or NF-κB was not addressed in the present study and warrants further investigation.
5.5 Summary

In summary, these results show that transient activation of A, R induces delayed myocardial protection in rats, similar to that previously reported in rabbits. This protection is associated with enhanced Mn-SOD expression and activity and is abolished by prior treatment with AS-ODN to rat Mn-SOD. These results provide the first direct evidence that induction and activation of Mn-SOD plays a crucial role in mediating delayed myocardial adaptation following A, R activation. These findings point to a potential therapeutic role for adenosine or its analogues in protecting the myocardium against not only ischaemia-reperfusion injury, but also cardiotoxicity induced by ROS in other circumstances, such as that seen following treatment with anticancer chemotherapeutic agents.
Chapter Six

Adenosine A₁R Induced Delayed Preconditioning:
Role of Nitric Oxide
Chapter Six

6.1 Introduction

A large body of evidence from Bolli’s laboratory points to an important role for early generation of nitric oxide (NO), during the ischaemic preconditioning stimulus, and subacute induction of the inducible isoform of NO synthase (iNOS), in the genesis of delayed preconditioning against infarction (295). This group have shown that non-selective pharmacological inhibition of all NOS isoforms with \( N^\bullet -\text{nitro-L-arginine (L-NA)} \), blocks the development of late preconditioning against infarction in a conscious rabbit model (296), while pretreatment with NO donors mimics protection against infarction in this model (298), thereby implying a role for NO as a trigger of delayed preconditioning against infarction. Furthermore, Imagawa et al. (493) and Takano et al. (336) found that administration of selective iNOS inhibitors prior to the prolonged ischaemic insult, 24-48 hours after ischaemic preconditioning, also abrogated delayed protection against infarction in the rabbit, implying a role for iNOS as a potential mediator of this cardioprotective effect. These data are supported by evidence obtained from genetically modified mice with targeted disruption of the iNOS gene, in which there was loss of delayed infarct-limitation following ischaemic preconditioning (337).

The intracellular signalling elements mediating adenosine A1R-induced and NO-induced delayed preconditioning seem to be similar. Studies presented in Chapter 4 demonstrate that the delayed protection against infarction observed 24 hours following activation of A1R in rabbits is dependent on a signalling mechanism involving both PKC and protein TKs, since pharmacological inhibition of either group of enzymes prior to adenosine A1R induced preconditioning completely abolishes the limitation of infarction observed at 24 hours. Similarly, Ping et al. (309) have recently demonstrated NO-mediated activation of PKC isoforms in the rabbit heart, which in turn results in activation of protein TKs (204), a signalling mechanism that is thought to mediate delayed protection against infarction in this species.

Recent evidence suggests that some of the regulatory functions of adenosine in cardiac tissue are mediated by generation of NO. For example, Martynyuk et al.
(494, 495) have shown that the anti-adrenergic effect of adenosine on calcium current in isolated rabbit atrioventricular nodal cells is dependent on A₁R induced production of intracellular NO. Other reports have suggested induction of NO synthesis by adenosine in rat vascular smooth muscle cells (496, 497), rat cardiac myocytes (498), and pig coronary artery endothelial cells (499).

On the basis of the aforementioned, it was hypothesised that A₁R induced delayed cardioprotection may be mediated by early generation of NO, and/or subacute induction of iNOS. This hypothesis was investigated in the rabbit model of A₁R induced delayed preconditioning against infarction, examining the effect of pharmacological inhibition of NOS prior to A₁R activation, and selective inhibition of iNOS before the onset of ischaemia-reperfusion.

6.2 Materials and Methods

Male New Zealand White rabbits weighing 2.0-3.0 kg were used in these experiments. Animals were housed in individual cages and had free access to food and water throughout the preparation period.

6.2.a Experimental Protocol

On day 1, conscious animals were pharmacologically preconditioned or received saline vehicle. Rabbits were assigned to 8 experimental groups (Figure 6.1). Group I (Control) received an i.v. bolus of sterile 0.9% saline 0.5 ml. Group II (CCPA) animals were pharmacologically preconditioned with a single i.v. bolus of CCPA (100 μg/kg). CCPA was dissolved in 0.9% sterile sodium chloride at a concentration of 500 μg/ml. To examine the potential role of early generation of NO in mediating A₁R induced delayed preconditioning, groups III (L-NAME+Sal) and IV (L-NAME+CCPA) received the same treatment as groups I and II respectively; in addition they were given a slow i.v. infusion of the non-selective NOS inhibitor N^G^-nitro-L-arginine methyl ester (L-NAME, 10 mg/kg), 10 minutes prior to the saline/CCPA bolus.
Figure 6.1. Experimental protocol. On day 1, animals were pharmacologically preconditioned with an i.v. bolus injection of CCPA or received saline vehicle (groups I and II). On day 2, animals were subjected to myocardial ischaemia-reperfusion in vivo. Animals in groups III and IV were also treated with an i.v. infusion of L-NAME 10mg/kg, 10 min before the CCPA/saline bolus. Animals in groups V and VI, were treated with an i.v. bolus of L-NIL 10 mg/kg, 10 min before the onset of myocardial ischaemia. Animals in groups VII and VIII received a s.c. injection of aminoguanidine 300 mg/kg, 60 min before ischaemia-reperfusion.
Next, the role of subacute induction of iNOS at 24 hours following pharmacological preconditioning with CCPA in mediating its delayed cardioprotective effects was examined. Rabbits in groups V-VIII were given the same treatment as in groups I and II. On day 2, however, these animals were also treated with two structurally dissimilar selective iNOS inhibitors, 24 hours following the saline/CCPA bolus and prior to the onset of the ischaemia-reperfusion protocol in vivo (Figure 6.1). Animals in groups V (Sal+L-NIL) and VI (CCPA+L-NIL) were treated with an i.v. bolus of the selective iNOS inhibitor L-N^6-(1-lminoethyl)-lysine (L-NIL, 10 mg/kg) 10 minutes before the onset of ischaemia. Rabbits in groups VII (Sal+AG) and VIII (CCPA+AG) received a s.c. bolus of aminoguanidine (AG, 300 mg/kg) 60 minutes prior to the ischaemia-reperfusion protocol. This dose of AG has previously been shown to abolish infarct-limitation 48 hours following ischaemic preconditioning in this rabbit model (493). L-NAME was obtained from Sigma (Poole, UK) and dissolved in sterile water at a concentration of 10 mg/ml. L-NIL (Alexis Corporation UK Ltd) was dissolved in sterile water at a concentration of 25 mg/ml. AG (Sigma Chemical, Poole, UK) was dissolved in sterile water at a concentration of 300 mg/ml.

6.2.b Ischaemia-Reperfusion Protocol in vivo

On day 2, twenty four hours after various treatments, animals underwent an infarction procedure in vivo, consisting of 30 minutes regional myocardial ischaemia and 2 hours reperfusion (Figure 6.1), as described in section 2.2. At the end of reperfusion, myocardial area at risk and infarct size were assessed by double staining with TTC and fluorescent microspheres as described in section 2.4.

6.2.c Statistical Analysis

The data are presented as mean±SEM. The significance of the differences in mean values of I, R and I/R between the experimental groups was evaluated by one-way ANOVA followed by Fisher’s protected least significant difference (PLSD). Differences between haemodynamic measurements at different time points were assessed by two-way ANOVA with repeated measures. The null hypothesis was rejected at p<0.05.
6.3 Results

6.3.a Exclusions
A total of 57 rabbits were used in these experiments. Three rabbits were excluded due to failure of completion of ischaemia-reperfusion protocol: 1 due to intractable VF during ischaemia (saline+L-NIL group), 1 due to haemorrhage from the coronary artery after insertion of the coronary ligature (CCPA+L-NIL group) and 1 due to failure of reperfusion of the myocardium at risk (CCPA group). Data on infarct size are therefore presented for 54 animals that successfully completed the infarct protocol.

6.3.b Haemodynamic Changes During Ischaemia-reperfusion
Table 6.1 summarises the changes in heart rate, systolic blood pressure (SBP) and rate-pressure product (RPP) during the infarction protocol in the 8 experimental groups. There were no differences in baseline haemodynamic performance between any of the groups. Administration of the selective iNOS inhibitors L-NIL and AG did not result in significant change in haemodynamic parameters. There was a small decline in SBP and RPP during 30 minutes ischaemia with no recovery during reperfusion. These haemodynamic changes with time were very similar between all the groups.

6.3.c Myocardial Risk and Infarct Size
Table 6.2 presents the number of animals, the body weights, and volumes of risk and infarct zones in the 8 experimental groups. In these groups, the mean volume of myocardial tissue at risk during coronary artery occlusion was in the range 0.9-1.0 cm³ representing about 40-45% of total left ventricular tissue volume. There were no significant differences in ischaemic risk zone among the experimental groups. The absolute infarct size was in the range 0.20-0.45 cm³. Compared to the control group, infarct size was significantly smaller in the groups that had been pretreated with CCPA.
### Table 6.1. Haemodynamic Parameters During Ischaemia-Reperfusion

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<th>Group</th>
<th>Pre-drug</th>
<th>Baseline</th>
<th>Ischaemia 5 min</th>
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<td>CCPA + L-NIL</td>
<td>87±5</td>
<td>83±4</td>
<td>73±4</td>
<td>72±3</td>
<td>67±3</td>
<td>63±3</td>
</tr>
<tr>
<td>Saline + AG</td>
<td>93±6</td>
<td>88±3</td>
<td>74±3</td>
<td>74±2</td>
<td>66±4</td>
<td>68±3</td>
</tr>
<tr>
<td>CCPA + AG</td>
<td>91±6</td>
<td>87±4</td>
<td>82±3</td>
<td>73±4</td>
<td>69±3</td>
<td>65±3</td>
</tr>
<tr>
<td><strong>RPP, mmHg/min×10³</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>23.2±1.1</td>
<td>21.8±1.3</td>
<td>21.4±0.6</td>
<td>18.6±0.9</td>
<td>17.0±0.5</td>
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<tr>
<td>CCPA</td>
<td>22.2±1.1</td>
<td>19.8±1.0</td>
<td>19.3±0.9</td>
<td>17.7±1.1</td>
<td>16.6±1.2</td>
<td></td>
</tr>
<tr>
<td>L-NAME + Saline</td>
<td>22.6±1.6</td>
<td>19.5±1.4</td>
<td>18.7±1.5</td>
<td>16.7±0.7</td>
<td>16.2±1.1</td>
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</tr>
<tr>
<td>L-NAME + CCPA</td>
<td>21.5±1.3</td>
<td>18.6±1.3</td>
<td>18.6±1.2</td>
<td>17.6±1.3</td>
<td>16.2±1.4</td>
<td></td>
</tr>
<tr>
<td>Saline + L-NIL</td>
<td>23.4±1.0</td>
<td>23.5±1.5</td>
<td>20.1±1.5</td>
<td>18.5±1.3</td>
<td>17.0±0.2</td>
<td>15.9±0.7</td>
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<tr>
<td>CCPA + L-NIL</td>
<td>22.4±1.0</td>
<td>21.3±0.8</td>
<td>19.1±1.0</td>
<td>18.6±0.9</td>
<td>17.9±0.9</td>
<td>17.8±1.1</td>
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<tr>
<td>Saline + AG</td>
<td>23.4±1.7</td>
<td>23.3±1.6</td>
<td>19.2±1.0</td>
<td>19.2±1.3</td>
<td>17.0±1.4</td>
<td>17.9±1.9</td>
</tr>
<tr>
<td>CCPA + AG</td>
<td>22.9±1.5</td>
<td>22.2±0.5</td>
<td>21.6±0.9</td>
<td>20.0±0.7</td>
<td>17.9±0.8</td>
<td>17.1±0.7</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Predrug values refer to haemodynamic parameters prior to administration of L-NIL or AG.
Chapter Six

Table 6.2. Body Weight, Myocardial Area at Risk and Infarct Size.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body Wt (kg)</th>
<th>R (cm²)</th>
<th>I (cm²)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>2.53±0.10</td>
<td>0.98±0.13</td>
<td>0.45±0.08</td>
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<tr>
<td>CCPA</td>
<td>8</td>
<td>2.47±0.09</td>
<td>0.88±0.11</td>
<td>0.21±0.05*</td>
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<tr>
<td>L-NAME + Saline</td>
<td>6</td>
<td>2.45±0.14</td>
<td>0.94±0.13</td>
<td>0.42±0.08</td>
</tr>
<tr>
<td>L-NAME + CCPA</td>
<td>7</td>
<td>2.59±0.09</td>
<td>0.90±0.07</td>
<td>0.24±0.03*</td>
</tr>
<tr>
<td>Saline + L-NIL</td>
<td>6</td>
<td>2.48±0.12</td>
<td>0.87±0.10</td>
<td>0.34±0.05</td>
</tr>
<tr>
<td>CCPA + L-NIL</td>
<td>8</td>
<td>2.42±0.11</td>
<td>0.93±0.07</td>
<td>0.26±0.03*</td>
</tr>
<tr>
<td>Saline + AG</td>
<td>5</td>
<td>2.57±0.14</td>
<td>0.97±0.12</td>
<td>0.39±0.07</td>
</tr>
<tr>
<td>CCPA + AG</td>
<td>6</td>
<td>2.65±0.09</td>
<td>0.97±0.12</td>
<td>0.26±0.06*</td>
</tr>
</tbody>
</table>

Values are mean±SEM, * p<0.05 vs Control.

Infarct size expressed as a percentage of area at risk (I/R) in the eight experimental groups is presented in figure 6.2. Pretreatment with CCPA 24 hours before myocardial infarction resulted in a significant 41% reduction in I/R compared to saline treated controls (27.3±4.7 vs 46.0±3.7% respectively; p=0.001). Thus, in accordance with previous studies in this model, transient activation of A1R induced a delayed preconditioning effect against infarction at 24 hours. The effect of non-selective inhibition of NOS isoforms was evaluated by administering L-NAME before CCPA or saline injections. Prior treatment with L-NAME 10 mg/kg did not significantly affect infarct size in saline treated animals (I/R; 45.5±3.6%, p=NS vs control), and failed to abrogate the infarct-limiting effect of delayed pharmacological preconditioning with CCPA (I/R; 25.8±2.4%, p<0.005 vs control). These results suggest that early generation of NO does not play a role in mediating A1R induced late preconditioning against infarction. Next, the effect of selective inhibition of iNOS prior to the ischaemia-reperfusion protocol, 24 hours after pharmacological preconditioning with CCPA was examined. Treatment with the two selective iNOS inhibitors L-NIL 10 mg/kg or AG 300 mg/kg did not affect the limitation of infarction induced by CCPA (I/R; 28.8±3.6 and 26.9±3.8% respectively, p=NS vs CCPA group). Administration of these drugs to saline treated animals did not significantly alter infarct size compared to the control group.
Figure 6.2. Myocardial Infarct Size. Infarct size is expressed as a percentage of the region at risk of infarction. Open circles represent individual animals; solid circles, mean±SEM. *p<0.01 vs control (one-way ANOVA).
The regression relationship between absolute infarct size and risk zone volume is shown in figure 6.3. For any given risk zone volume, infarct size was smaller in CCPA treated hearts compared to saline pretreated hearts, irrespective of co-treatment with NOS inhibitors. These results suggest that delayed induction of iNOS, 24 hours after activation of adenosine A1R, is not necessary for the genesis of late preconditioning against infarction.

![Graph showing the relation between infarct size and risk zone volume.](image)

**Figure 6.3. Relation Between Infarct Size and Risk Zone Size.**

Open circles represent CCPA pretreated hearts (groups II, IV, VI and VIII). Solid circles indicate saline pretreated hearts (groups I, III, V and VII). Simple regression lines have been fitted to the unweighted data using the Statview statistical package.

\[
\text{INF} = 0.038 + 0.217 \times \text{RISK}; \quad R^2 = 0.295 \quad (\text{CCPA})
\]

\[
\text{INF} = -0.01 + 0.447 \times \text{RISK}; \quad R^2 = 0.636 \quad (\text{Control})
\]
6.4 Discussion

The principal findings of this study are that the significant protection against myocardial infarction observed in the rabbit, 24 hours after treatment with the selective A₁R agonist CCPA, was not affected by prior non-selective inhibition of NOS isoforms, indicating that A₁R induced delayed preconditioning is not mediated by proximal signalling mechanisms involving the generation of NO. Secondly, selective inhibition of iNOS with two structurally unrelated iNOS inhibitors 24 hours after pharmacological preconditioning with CCPA and prior to ischaemia-reperfusion, did not attenuate the marked limitation of infarction observed in these animals. These results would argue against a role for induction of iNOS as a distal mediator of late preconditioning induced by A₁R activation.

6.4.a NO as a Trigger of Delayed Preconditioning

Increasing recent evidence has pointed to a role for generation of NO, during the brief periods of ischaemia-reperfusion, as an important trigger of delayed preconditioning against infarction in rabbits (295). Specifically, non-selective NOS inhibition with Nω-nitro-L-arginine (L-NA) during preconditioning ischaemia has been shown to abolish delayed protection against infarction (296) while, administration of NO donors in lieu of ischaemia was found to induce a delayed infarct-limiting effect similar to ischaemic preconditioning in rabbits and mice (298, 337). The NO generated during preconditioning ischaemia is thought to result in the formation of NO-derived reactive oxygen species (ROS) such as peroxynitrite anion (ONOO⁻) and/or hydroxyl radical (·OH) which in turn activate PKC (298, 309). PKC is known to play an important role in the signalling mechanism involved in delayed ischaemic preconditioning against infarction and stunning (302, 307), and studies reported in chapter 4 demonstrate its involvement in late protection against infarction following A₁R activation in rabbits. However, activation of PKC by adenosine occurs via its action on adenosine A₁ receptors coupled to pertussis toxin-sensitive G proteins (429, 500), and is not thought to involve ROS or NO-mediated pathways. The results of the present study and those reported in chapter 4 support this notion since non-selective inhibition of NOS isoforms during pharmacological preconditioning with CCPA failed to abrogate its delayed infarct-limiting effects,
whereas inhibiting PKC abolished late cardioprotection, thus suggesting that activation of PKC in the signalling mechanism downstream of A1R is independent of early generation of NO.

In this study, L-NAME was used to inhibit potential generation of NO following administration of CCPA. L-NAME is a potent NOS antagonist effective against all isoforms with some selectivity for ecNOS (501), the proposed isoform responsible for generation of NO during brief preconditioning ischaemia (295). The dose of L-NAME used in the present study has previously been shown to block delayed pharmacological preconditioning with MLA in rats (502), and a similar dose has been reported to abolish delayed pharmacological preconditioning with diazoxide, a potent opener of mitochondrial K_{ATP} channels, in rabbits (340). This dose of L-NAME did not alter infarct size in saline treated animals (group III) compared to controls and failed to abolish CCPA induced delayed protection (group IV). In addition to its effect on NOS, L-NAME has been reported to act as a muscarinic receptor antagonist (503). However, in this rabbit model L-NAME was ineffective at abolishing delayed protection, and therefore, additional pharmacological properties of L-NAME other than NOS inhibition do not detract from the conclusion. Therefore, despite evidence for A1R induced NO generation in some key physiological settings (494-497, 499), NO does not appear to play a role in triggering the signal cascade of A1R induced delayed preconditioning.

6.4.b iNOS as a Mediator of Delayed Preconditioning

Recent evidence using pharmacological inhibitors of iNOS have suggested an important role for subacute induction of this enzyme and generation of NO during the prolonged ischaemic insult, in mediating delayed cardioprotection following ischaemic preconditioning (336, 493). Moreover, ischaemic preconditioning has been shown to result in upregulation of myocardial iNOS transcript levels, protein content and activity (337, 504). Further evidence in support of a role for iNOS has come from a study with iNOS gene knockout mice in which, ischaemic preconditioning failed to induce delayed protection against infarction. Similarly, involvement of iNOS has been demonstrated in mediating delayed pharmacological preconditioning
with MLA (338, 339). On this basis, a role for iNOS in the delayed infarct-limitation 24 hours following A,R activation in the rabbit was examined.

In the present study, inhibition of iNOS with two unrelated selective iNOS inhibitors, L-NIL and AG, failed to abolish the delayed protection against infarction observed in the CCPA treated group. Administration of either of these agents to saline treated animals (groups V and VII) did not significantly alter infarct size compared to the control group. Furthermore, treatment with the iNOS antagonists prior to myocardial ischaemia-reperfusion did not result in significant haemodynamic perturbations. These results suggest that in contrast to delayed cardioprotection following ischaemic preconditioning or pharmacological preconditioning with MLA, iNOS does not play a significant role in limiting infarction 24 hours after pharmacological preconditioning with CCPA. This finding is corroborated by a recent preliminary report from Yellon’s laboratory, indicating that CCPA is equally effective in inducing late preconditioning against infarction in both wild type and iNOS knockout mice (343). Conversely, a recent report by Kukreja’s group suggests that targeted disruption of the iNOS gene abolishes delayed preconditioning against infarction in mice (342). The reasons for the discrepancy between this latter study, and the data presented in this chapter and those of Bell et al. (343) is not immediately obvious and warrants further investigation.

In this study, two unrelated selective iNOS inhibitors were used to examine the role of this enzyme in late preconditioning. The first of these, AG, has an IC\textsubscript{50} of 160.0 \(\mu\)mol/l for constitutive NOS versus 5.4 \(\mu\)mol/l for iNOS (505, 506). AG was administered subcutaneously 60 minutes prior to the onset of myocardial ischaemia. This route of administration of AG was chosen on the basis of the slow onset of its vascular actions and the increase in its potency with time of incubation (501, 506, 507), and also to avoid the acute haemodynamic effects following intravenous injection of AG (297). Furthermore, AG administered subcutaneously at comparable doses to that used in the present study has successfully been used to block iNOS activity (508-510), and importantly, has been shown to abolish delayed protection against infarction following ischaemic (336, 493) or MLA induced preconditioning (338). It is therefore very likely that in the present study, in group VIII, the limitation
of infarction 24 hours after CCPA treatment occurred in the face of iNOS inhibition. Moreover, to confirm these observations with AG, and to exclude the possibility that due to non-specific effects, AG had failed to abolish late preconditioning against infarction in this model, an additional, chemically unrelated selective iNOS inhibitor was used. L-NIL is a potent iNOS inhibitor with an IC\(_{50}\) of 3.3 \(\mu\)mol/l for iNOS compared to an IC\(_{50}\) of 92 \(\mu\)mol/L for constitutive NOS, with similar \textit{in vivo} selectivity to that of AG (511, 512). In the present study, a dose of 10 mg/kg of L-NIL was used, which assuming distribution in total body water, would yield an approximate plasma concentration of 50 \(\mu\)mol/l, in excess of 10-fold higher than the IC\(_{50}\) for inhibition of iNOS but below that for constitutive NOS. This dose of L-NIL failed to abrogate delayed preconditioning against infarction 24 hours after CCPA treatment. Administration of L-NIL had no appreciable haemodynamic effects and did not alter infarct size in saline treated animals (group V) compared to controls. Taken together, the failure of two unrelated selective blockers of iNOS to inhibit the infarct sparing action of delayed CCPA induced preconditioning, strongly suggests that iNOS is not involved in mediating this protection.

It is important to emphasise that although the results of the present study argue against involvement of iNOS as a mediator of delayed pharmacological preconditioning with CCPA, they do not rule out a role for enhanced NO production by sources other than iNOS during the index ischaemia/reperfusion insult. In this regard, Kim et al. (513) have demonstrated delayed upregulation of coronary eNOS in conscious dogs following brief coronary occlusion. Bhagat et al. (514) have recently reported cytokine-induced upregulation of eNOS and enhanced NO production in the absence of iNOS expression in humans in vivo. Importantly, a recent preliminary report suggests that transient activation of adenosine A\(_1\)R is associated with enhanced generation of NO products during ischaemia-reperfusion 24 hours later, in a murine model with targeted disruption of the iNOS gene (343). In the present study, the fact that there was no appreciable change in arterial blood pressure in animals treated with selective iNOS inhibitors (groups V-VIII), would suggest that these agents did not inhibit NO production by vascular eNOS. It is still possible, therefore, that enhanced generation of NO by eNOS may be involved in the delayed protective effects following A\(_1\)R agonists.
6.5 Summary

In summary, these results suggest that the delayed myocardial protection 24 hours following transient A1R activation with CCPA in rabbits is not dependent on early generation of NO since non-selective inhibition of NOS isoforms failed to abolish this protection. Furthermore, the delayed limitation of infarction 24 hours after treatment with the A1R agonist does not involve subacute induction of iNOS since selective inhibition of this enzyme also failed to abrogate cardioprotection. Whether enhanced generation of NO from other sources is important in mediating delayed pharmacological preconditioning against infarction remains to be proven and requires a molecular approach, such as the use of transgenic and gene-targeted murine models.
Chapter Seven

Exercise-induced Ischaemia and Myocardial Adaptation: Role of Adenosine
7.1 Introduction

Warm-up angina is an intriguing phenomenon whereby, in subjects with coronary artery disease, the angina induced by initial exercise is attenuated, or even disappears, if they briefly slacken or interrupt their exertion before resuming it at the same or an even greater level of intensity (374, 375, 515-519). The mechanisms underlying this attenuation of myocardial ischaemia during a second period of exercise are not completely understood, and have been investigated over the past few years in patients with ischaemic heart disease, using models of repeated exercise on treadmill, bicycle exercise tests, and repeated myocardial ischaemia induced by rapid atrial pacing (374, 375, 515-519). On the basis of the findings from these studies, some of the original hypotheses regarding the potential mechanisms mediating warm-up angina have been refuted. For example, the ischaemia-sparing effect of the warm-up phenomenon does not appear to be associated with downregulation or stunning of left ventricular contractile function, variations in adrenergic tone or increased myocardial perfusion through the collateral circulation (371-373, 519, 520). On the basis of these and other observations, it has been speculated that the myocardial adaptation observed during successive periods of exercise-induced ischaemia may be analogous to that seen in experimental ischaemic preconditioning (521). This hypothesis is supported by the demonstration that myocardial oxygen consumption is reduced during a second exercise stress test (371) or pacing induced myocardial ischaemia (372) suggesting increased metabolic efficiency, a feature of preconditioning. Moreover, the time course of the warm-up phenomenon is consistent with that of classic ischaemic preconditioning, lasting no longer than 60-90 minutes (374, 375).

If the warm-up phenomenon does represent a clinical correlate of experimental ischaemic preconditioning, it is unknown whether as in animal models, there is a corresponding delayed phase or second window of protection. Indeed, no studies have been able to directly demonstrate a delayed preconditioning effect in the intact human myocardium. The aim of this study was therefore to investigate whether in patients with coronary artery disease, brief periods of demand myocardial ischaemia induced by physical exercise, result in delayed myocardial adaptation at 24 hours.
Chapter Seven

In addition, the role of adenosine in induction or augmentation of this adaptation was investigated. It was hypothesised that if the warm-up phenomenon is a manifestation of ischaemic preconditioning, prior treatment with adenosine in patients with coronary artery disease, would protect against exercise-induced ischaemia in a similar way to previous exercise. Moreover, sequential exercise following drug administration should provide no additional benefit. However, in this setting of demand ischaemia in stable coronary disease, adenosine would not be therapeutically useful, as any potential $A_1$R-mediated myocardial protection would be offset by the coronary steal effect of $A_2$R-mediated coronary vasodilatation. Indeed adenosine is widely used to induce perfusion defects in radionuclide perfusion imaging. Therefore, in the present study, a highly selective adenosine $A_1$R agonist, GR79236 (N-[1S, trans]-2-hydroxycyclopentyl]-adenosine), developed for use in humans (GlaxoSmithKline) was used to mimic the warm-up effect. The $EC_{50}$ of GR79236 for $A_2$R is approximately 100 times greater than that for $A_1$R in a range of preparations (522). This selectivity avoids $A_2$R-mediated coronary vasodilatation which would mask any protective, and potentially therapeutic effect. In anaesthetised rabbits and conscious pigs, GR79236 has been shown to mimic the effect of ischaemic preconditioning against infarction, with maximal protection occurring at doses of 10 $\mu$g/kg and 3.5 $\mu$g/kg respectively (523, 524). Studies in normal human volunteers have shown that the drug is well tolerated when administered at doses of up to 20 $\mu$g/kg as a slow intravenous bolus, with no adverse haemodynamic effects and a terminal elimination half-life of 1-2 hours (GlaxoSmithKline, unpublished observations).

7.2 Methods

7.2.a Patient Selection

The subjects included in this study were patients with chronic stable angina pectoris who had previously been shown to have a positive exercise test for myocardial ischaemia, with horizontal or downsloping ST-segment depression $\geq$2.0 mm 80 ms after the J point compared with the baseline ST-segment. All patients had significant coronary artery disease documented by coronary angiography ($\geq$70% stenosis in at least one major epicardial coronary artery) performed within 4 months of their participation in the study. In addition, the subjects had to have a normal resting ECG
with no evidence of conduction defects or repolarisation abnormalities which could interfere with interpretation of ST-segment changes during exercise. All patients were normotensive and in sinus rhythm at the time of entry into the study, and without evidence of previous myocardial infarction, heart failure, cardiomyopathy or valvular disease. No patient had ST-segment elevation during dynamic exercise or a history of angina at rest. Also excluded from the study were patients with a history of bronchial asthma, diabetic patients taking sulphonylurea oral hypoglycaemic agents (which are known to block myocardial $K_{ATP}$ channels), and patients on dipyridamole or xanthene derivatives (which are known to interfere with adenosine metabolism and action).

7.2.b Study Design

This was a double-blind, randomised, placebo controlled cross-over study, comparing the cardioprotective effects of GR79236 with placebo during repeated exercise tests. All patients gave written informed consent for participation in the study, which was approved by the University College London Hospitals Committee on the Ethics of Human Research. The study was conducted in two phases, with each phase taking place over two days. On the first day of phase I, each patient underwent two successive treadmill exercise tests, using the Bruce protocol (Ex 1 and Ex 2), with a recovery period of 15 minutes between the tests to re-establish baseline electrocardiographic conditions. To examine the delayed effects of exercise, each patient was asked to perform two further exercise tests 24 hours later (Ex 3 and Ex 4), using the same protocol (Figure 7.1). In order to evaluate the role of adenosine A$_1$R in inducing enhanced myocardial tolerance to exercise, or augmenting exercise-induced adaptation, patients were randomly assigned to the GR or the placebo groups. Patients in the GR group were treated with an intravenous infusion of GR79236 10 $\mu$g/kg, 30 minutes prior to Ex 1. Patients in the placebo group received an equivalent intravenous infusion of placebo (0.9% sodium chloride). GR79236 and matching placebo were kind gifts from GlaxoSmithKline and were supplied in 5 ml ampoules at a concentration of 1 mg/ml. The appropriate volume of GR79236/placebo was added to 100 ml 0.9% sodium chloride and was administered as an intravenous infusion over 10 minutes, via a cannula in the anticubital vein.
Figure 7.1. Study Protocol for Evaluation of Delayed Myocardial Adaptation During Repeated Exercise (Ex).
During the study drug infusion and for 20 minutes thereafter, the patients remained in a recumbent position and underwent regular monitoring of haemodynamic and ECG parameters. After a period of at least 2 weeks, to allow for washout of any carryover effects from the study drug or repeated exercise during phase I, patients were crossed over to the remaining treatment arm of the study and underwent the same protocol of 4 exercise tests over 2 days in phase II (Figure 7.1).

7.2.c Exercise Protocol
All exercise tests were performed between 1:30 p.m. and 5:00 p.m. Beta-adrenergic blocking agents and calcium channel antagonists were stopped 72 hours and long-acting nitrates 48 hours prior to each stage of the study. Only sublingual nitroglycerin spray was used during the latter period. No patient was on treatment with the K<sub>ATP</sub> channel opener nicorandil. On the days of the study, subjects were instructed not to exercise and to avoid any unusual exertion, and they were rested for at least 60 minutes prior to the first exercise test on each day. During each phase of the study, subjects abstained from drinking caffeinated beverages.

Exercise testing was performed according to the standard Bruce protocol. This consisted of walking on the treadmill with the speed and gradient increasing incrementally after each 3 minute stage; stage I: 1.7 mph at 10%, stage II: 2.5 mph at 12%, stage III: 3.4 mph at 14%, stage IV: 4.3 mph at 16%, stage V: 5.0 mph at 18%. No patient was able to exercise beyond stage V of the Bruce protocol. A standard 12-lead ECG and arterial blood pressure (cuff sphygmomanometer) were obtained with the patient in the recumbent position prior to and after infusion of the study drug. These measurements were also obtained with the patient in the standing position at baseline (just before the onset of exercise), at 1-minute intervals during exercise, at peak exercise, and each minute up to 10 minutes after each period of exercise (recovery), as well as at 1.0 mm ST-segment depression, at the onset of angina, and when it was clinically indicated. Three ECG leads (III, V<sub>2</sub> and V<sub>5</sub>) were monitored continuously before, during, and after exercise, and up to date averaged QRS complexes of all ECG leads were displayed continuously on the screen. The level of the ST-segments, 80 ms after the J point, was calculated after signal averaging by a computer-assisted system (CASE Marquette 12, Milwaukee, Wisconsin) in all 12
leads. The position of ECG electrodes were marked on the patients’ skin with a marker pen, so that consistent electrode positions were used in each patient on different days of the study.

Predefined criteria for interrupting the test were (1) ST-segment depression ≥3 mm, (2) maximal age-related heart rate calculated by the formula [220 – age], (3) severe chest pain, (4) physical exhaustion, and (5) occurrence of other harmful conditions, such as hypotension, severe arrhythmia and dyspnoea. Myocardial ischaemia was diagnosed when a horizontal or downsloping ST-segment depression of 1.0 mm at 80 ms after the J point was observed in at least 1 lead. The ECG strips of all tests were evaluated independently by 2 cardiologists experienced at interpretation of exercise tests, who were blind to the randomisation and the order of the tests. In the case of disagreement, the matter was resolved by consensus. For each exercise test, the following parameters were measured: resting (baseline) HR and BP, time to 1.0 mm ST-segment depression and to onset of chest pain, HR and BP at 1.0 mm ST-segment depression, total exercise time, HR and BP at peak exercise, and maximum ST-segment depression noted on averaged tracings obtained just before the end of exercise. Rate-pressure product (HR x systolic BP) was calculated for each time point. Recovery time was the time from the end of exercise to the final appearance of 1.0 mm ST-segment depression.

7.2.d Statistical Analysis

Data are presented as mean ± SD. To examine for any carry over effects of the study drug or repeated exercise from the first to the second phase of the study, mean values of quantitative variables for each group were compared using unpaired Student t test. This did not show significant differences between variables in the two different phases of the study. The data were therefore grouped for the two phases; e.g. GR group in phase I and GR group in phase II. One way analysis of variance for repeated measures was then used to compare haemodynamic and ECG data between sequential exercise tests and between the two study groups. When significant differences were detected, pairwise comparisons were made using the Scheffé F test. A p value <0.05 was considered statistically significant.

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7.3 Results

Sixteen men and one woman were recruited to this study. The one female patient developed chest pain at rest with associated ischaemic changes on ECG on the first day of the study prior to infusion of the study drug, and therefore took no further part in the study. Data is therefore presented on sixteen male subjects aged 64 ± 11 years, who successfully completed the two phases of the study. The duration of the patients' symptoms ranged from 3 to 48 months. Coronary angiography had revealed that eight patients had single vessel disease in the left anterior descending (LAD) coronary artery, three had double-vessel disease and five had triple-vessel disease. There were no significant differences in baseline characteristics between patients randomised to the GR group during phase I of the study, and those in the placebo group (Table 7.1).

Table 7.1. Clinical Characteristics

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<th>Number of Patients (%)</th>
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Risk Factors

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</tr>
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<td>Diabetes</td>
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</tr>
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<td>Family History of IHD</td>
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Drug Therapy

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</tr>
<tr>
<td>β-blocker</td>
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</tr>
<tr>
<td>Nitrate</td>
<td>4 (25)</td>
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<tr>
<td>Calcium Antagonist</td>
<td>7 (44)</td>
</tr>
<tr>
<td>ACE inhibitor</td>
<td>2 (13)</td>
</tr>
</tbody>
</table>
7.3.a Effects of GR79236 Infusion

There were no significant adverse events following injection of either GR79236 or placebo. Prior to infusion of the study drug on day 1, haemodynamic parameters were similar between the two groups; placebo group: HR 69±9 bpm, SBP 127±12 mmHg; GR group: HR 74±10 bpm, SBP 134±15 mmHg. In the GR group, infusion of the study drug resulted in a slight but statistically significant reduction in HR to 67±10 bpm (p<0.05 vs pre-infusion), although SBP did not differ significantly (132±15 mmHg). Infusion of placebo did not significantly alter haemodynamic parameters; HR 68±9 bpm, SBP 126±12 mmHg. Infusion of GR79236 also resulted in prolongation of the PR interval on the ECG (GR: 166±12 to 190±14 ms, p<0.001; placebo: 169±15 to 171±13 ms, p=NS). No patient developed second or third degree heart block following drug administration.

7.3.b Haemodynamic and ECG Parameters During Exercise

The main results of the four exercise tests are summarised in table 7.2. Just before the first exercise on day 1, there were no significant differences in baseline haemodynamic parameters between the two groups. All patients achieved at least 1.0 mm ST-segment depression during each period of exercise. Ischaemic threshold, defined as RPP at 1.0 mm ST-segment depression, for the successive periods of exercise is presented in Figure 7.2. In the placebo group, ischaemic threshold significantly increased during Ex 2 compared with Ex 1. On the second day, ischaemic threshold remained significantly elevated during the first period of exercise (Ex 3) and did not further increase during Ex 4. The enhanced ischaemic threshold observed during Ex 2 to Ex 4 was primarily as a result of increased HR at 1 mm ST-segment depression, since SBP values at corresponding time points during Ex 1 to Ex 4 remained relatively unchanged (Table 7.2). These results indicate that the first period of exercise-induced myocardial ischaemia on day 1, resulted in attenuation of myocardial ischaemia during further exercise, both at 15 minutes and 24 hours following Ex 1.
Table 7.2. Haemodynamic Variables During Repeated Exercise Tests

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>GR79236</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td></td>
<td>Ex 1</td>
<td>Ex 2</td>
</tr>
<tr>
<td><strong>Backbone Values</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR, bpm</td>
<td>69±8</td>
<td>77±12*</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>126±13</td>
<td>123±12</td>
</tr>
<tr>
<td>RPP, mmHg/min x 10^3</td>
<td>8.7±1.5</td>
<td>9.9±2.4*</td>
</tr>
<tr>
<td><strong>1 mm ST depression</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR, bpm</td>
<td>109±15</td>
<td>119±23*</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>153±19</td>
<td>152±22</td>
</tr>
<tr>
<td>RPP, mmHg/min x 10^3</td>
<td>16.6±3.6</td>
<td>19.4±4.6**</td>
</tr>
<tr>
<td>Time, s</td>
<td>244±90</td>
<td>293±121*</td>
</tr>
<tr>
<td><strong>Time to pain onset, s</strong></td>
<td>300±158</td>
<td>310±94*</td>
</tr>
<tr>
<td><strong>Peak Exercise</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR, bpm</td>
<td>130±19</td>
<td>139±21*</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>169±25</td>
<td>172±26</td>
</tr>
<tr>
<td>RPP, mmHg/min x 10^3</td>
<td>22.2±5.4</td>
<td>23.9±6.1**</td>
</tr>
<tr>
<td><strong>Max ST Depression</strong></td>
<td>3.1±0.6</td>
<td>3.0±1.0</td>
</tr>
<tr>
<td><strong>Exercise Time, s</strong></td>
<td>432±130</td>
<td>468±118*</td>
</tr>
<tr>
<td><strong>Recovery Time, s</strong></td>
<td>298±179</td>
<td>228±184*</td>
</tr>
</tbody>
</table>

Values are mean±SD. *p<0.05 vs Ex 1; **p<0.01 vs Ex 1; †p<0.05 vs Ex 3. Time to pain onset refers to patients who developed angina.
In the GR group, ischaemic threshold during Ex 1 was not significantly different to that in the placebo group. In these patients, ischaemic threshold significantly increased during the Ex 2 on day 1, and during both periods of exercise on day 2, similar to that seen in the placebo group (Figure 7.2). In none of the four periods of exercise in the GR group, was ischaemic threshold significantly different to that in the corresponding exercise test in the placebo group. Taken together, these results indicate that prior activation of adenosine A₁R with GR79236 did not attenuate exercise-induced ischaemia during Ex 1. Moreover, the increase in ischaemic threshold observed during Ex 2 to Ex 4 in the control group, was not augmented by prior treatment with GR79236.

Compared to Ex 1, exercise tolerance defined as time to 1 mm ST-segment depression, significantly increased during Ex 2 in both groups, and was even higher during Ex 3 and Ex 4 on day 2, although the values were not significantly different from Ex 2 (Table 7.2). Thirteen patients developed angina during Ex 1 in both the placebo and GR groups. Of these, four patients did not experience chest pain during further exercise. In the remaining nine patients, time to onset of angina progressively increased from Ex 1 to Ex 4 in each group, although only that for Ex 1 was significantly different from the rest.

Total exercise time was also significantly increased during Ex 2 and on day 2 in both groups. Patients also achieved higher HR and RPP during these exercise tests although peak systolic BP remained relatively constant (Table 7.2). Prior treatment with GR79236 did not result in improved haemodynamic parameters at any point. Maximum ST-segment depression at peak exercise averaged at about 3.0 mm. There was a trend toward a reduction in ST depression with successive exercise, although the difference did not achieve statistical significance. After each exercise test, recovery time progressively decreased from Ex 1 to Ex 4, in both groups. Only the recovery time for Ex 1 was significantly different from that for the other three exercise tests.
Figure 7.2. Ischaemic Threshold During Repeated Exercise Testing (Ex).
The left panel represents values for individual patient. The right panel represents the mean values + SD.
RPP; Heart rate x systolic blood pressure. * p<0.01 vs Ex 1.
7.3 Discussion

The main findings of the present study, carried out in patients with stable angina who underwent consecutive exercise tests, can be summarised as follows: 1) Myocardial ischaemia induced by dynamic exercise resulted in development of increased tolerance to a further period of exercise performed within minutes, or at 24 hours following the first exercise test. This adaptation was manifest as an increase in ischaemic threshold, time to onset of angina, total exercise time, maximum workload achieved during exercise, and a reduction in recovery time, 2) Prior activation of adenosine A<sub>1</sub>R with the selective agonist GR79236 did not improve ischaemic parameters during Ex 1, or augment the exercise-induced adaptation observed during successive exercise tests. Taken together, these findings indicate that the warm-up phenomenon is apparent not only immediately after a first exercise test, but also at 24 hours following exercise induced myocardial ischaemia. However, when the intervening period between exercise testing is extended to two weeks, the enhanced resistance to ischaemia is no longer observed. Activation of adenosine A<sub>1</sub>R prior to exercise is unable to mimic the warm-up effect.

7.4.a Assessment of the Warm-up Phenomenon

Previous studies performed in patients with coronary artery disease undergoing consecutive stress tests have suggested that a variable proportion of these patients, ranging from 20 to 80%, tolerate the second period of ischaemia better than the first, i.e. they demonstrate the warm-up phenomenon (371, 372, 375, 516-519). However, the marked variability of results in some of the previous studies is probably due to utilisation of parameters such as time to onset of angina, exercise duration and electrocardiographic variables at peak exercise, which are greatly influenced by the subjective attitude of both the physician and the patient. In the present study, there was an improvement in most ischaemic indices with successive exercise tests. However, the main end-points used to compare the results of serial exercise tests were the heart rate and the rate-pressure product at 1.0 mm ST-segment depression, which represent valid and objective non-invasive indices of the ischaemic threshold, itself a measure of myocardial oxygen consumption at the onset of ischaemia (525, 526). Conversely, parameters such as the time to 1.0 mm ST-segment depression and
to pain onset are considered as global indices of exercise tolerance, which also take into account peripheral mechanisms responsible for enhanced exercise performance including a training effect (525-527).

Thus, in agreement with recent studies (375, 518, 519), the results of the present study indicate an improvement of about 15% in ischaemic threshold during a second exercise test, when this is performed within 15 minutes of the first test. In addition, a novel finding of this study is that the enhanced ischaemic threshold observed during Ex 2, is still present when a third exercise test is performed 24 hours after the first test, with no further improvement in ischaemic threshold during a fourth exercise test performed within minutes of Ex 3. Taken together, these results suggest that repetitive periods of myocardial ischaemia resulting from dynamic exercise induce a delayed adaptive phenomenon manifest as improved ischaemic indices during further exercise testing at 24 hours. This late adaptation may represent a form of delayed ischaemic preconditioning.

7.4.b Mechanisms of the Warm-up Phenomenon

The improvement in ischaemic threshold observed during a second exercise test, performed within minutes of a first test, may be explained by 1) an improvement in myocardial oxygen supply, which in turn may result from stenosis dilatation, collateral recruitment or myocardial perfusion redistribution; 2) a form of myocardial adaptation such as that caused by ischaemic preconditioning; 3) a slower rate of increase in cardiac workload, similar to that resulting from exercise training; and 4) an adaptive downregulation of regional myocardial contractile function in the ischaemic region, which diminishes ischaemia by decreasing oxygen demand.

Okazaki et al. (371) studied patients with isolated LAD coronary artery stenosis during two successive periods of supine ergometric exercise tests. These investigators cannulated the great cardiac vein in their patients to measure regional coronary flow using the thermodilution technique, and also measured oxygen consumption during sequential exercise testing. They found a reduction in electrocardiographic indices of myocardial ischaemia during the second exercise test. Based on measurements of great cardiac vein flow during the two successive periods
of exercise, the authors concluded that the attenuation in myocardial ischaemia during the second exercise was not due to enhanced coronary flow to the ischaemic zone. Conversely, they demonstrated a reduction in myocardial oxygen consumption in the ischaemic zone during the second exercise test (371). Similarly, Williams et al. (372) subjected patients with isolated LAD artery stenosis to two sequential periods of myocardial ischaemia induced by rapid atrial pacing. The reduction in ischaemic indices observed during the second pacing period, assessed by severity of angina, ST-segment depression and lactate extraction was accompanied by a reduction in myocardial oxygen consumption, and occurred in the face of comparable coronary flow in the ischaemic myocardium measured using great cardiac vein cannulation and thermodilution (372). Despite the limitations of the techniques used in these studies to evaluate regional myocardial blood flow, their findings suggest that the warm-up phenomenon is not due to improved collateral blood supply to the ischaemic zone. More recently, Bogaty et al. (520) investigated the role of enhanced myocardial perfusion in warm-up angina using single-photon emission computed tomography (SPECT) with thallium-201, during successive exercise testing. They found no difference in the extent of myocardial perfusion deficit between the two periods of exercise, although ischaemic threshold significantly increased during the second exercise test (520). Taken together, these studies strongly argue against a role for improved collateral supply to the ischaemic myocardium as the mechanism of warm-up angina. Theses studies do not, however, rule out the possibility of redistribution of transmural myocardial blood flow from epicardium to endocardium contributing to the attenuation of ischaemia during a second exercise test. However, even if enhanced oxygen supply to the ischaemic myocardium may contribute to the improved tolerance during a second exercise test performed within minutes of an earlier period of exercise, considering the prolonged time interval between Ex 2 and Ex 3 in the present study, improved collateral perfusion of the ischaemic region or redistributive transmural myocardial flow is unlikely to account for the attenuation of ischaemia observed during exercise tests performed on day 2.

It has also been suggested that the warm-up phenomenon may be a result of a training effect (526, 528). Tomai et al. (375) have investigated this possibility in patients with coronary artery disease undergoing three successive exercise tests.
They found that if the third period of exercise is performed within minutes of the first two, the increase in ischaemic threshold is still observed during Ex 3. Conversely, if the intervening period between Ex 2 and Ex 3 is extended to 90 minutes, the attenuation in ischaemic indices is abolished and the ischaemic threshold in Ex 3 is comparable to that in Ex 1. Similar results have been reported by another group (374). Using a different approach, Rinaldi et al. (373) evaluated the effects of repeated episodes of exercise-induced ischaemia on LV function assessed by two-dimensional echocardiography. They also found a time-dependent effect of exercise-induced ischaemia on the degree and duration of LV dysfunction. When the second exercise test was performed 30 minutes after Ex 1, despite the greater workload achieved, there was a reduction in the degree of LV dysfunction compared to that induced by Ex 1. Extending the intervening period to 60 minutes resulted in a more severe and prolonged impairment of LV function after Ex 2, whereas at 4 hours, the two periods of exercise induced a comparable degree of LV dysfunction. In the present study, the increase in ischaemic threshold observed in Ex 2, was maintained 24 hours later during Ex 3 and Ex 4. However, during the first exercise in phase II of the study, which was performed two weeks after phase I, ischaemic threshold had returned to baseline values which were not significantly different from that observed in Ex 1 in phase I. Taken together, these results suggest that warm-up angina is not a manifestation of exercise training, but represents a form of myocardial adaptation which is apparent within minutes of the first exercise and lasts for only 60-90 minutes. The protection induced by the first exercise seems to “reappear” at 24 hours, and is not augmented by further periods of exercise-induced ischaemia on day 2. This temporal profile closely resembles that of early and delayed ischaemic preconditioning in laboratory animals and supports the hypothesis that warm-up angina may indeed represent a human model of ischaemic preconditioning.

**Role of Adenosine**

Studies in a number of human models of ischaemic preconditioning have implicated adenosine as an important factor in induction of early protection against ischaemic injury (265, 354, 391, 529). In the present study, a selective A1R agonist was used to mimic the protection observed during the warm-up effect. Prior treatment with GR79236 did not improve haemodynamic performance during Ex 1 compared to the
placebo treated group. Furthermore, there was evidence of warm-up protection against exercise-induced ischaemia in the presence of GR79236. These findings suggest that prior activation of adenosine A$_1$R is unable to mimic the protection induced by sequential exercise. The lack of benefit from GR79236 observed in this study can be interpreted in three ways: 1) the dose of GR79236 used in the present study was insufficient to precondition human myocardium, 2) although a model of ischaemic preconditioning, the warm-up phenomenon is mediated by mechanisms that are not dependent on adenosine, or 3) warm-up angina is not a manifestation of ischaemic preconditioning and is mediated by other unknown mechanisms.

In anaesthetised rabbit and conscious pig models of myocardial ischaemia-reperfusion, GR79236 has been shown to mimic the protective effect of ischaemic preconditioning against infarction, with maximal protection occurring at doses of 10 \( \mu g/kg \) and 3.5 \( \mu g/kg \) respectively (523, 524). At a dose of 10 \( \mu g/kg \), GR79236 also induces delayed protection against infarction in rabbits (427). More importantly, in patients with coronary artery disease undergoing CABG, infusion of GR79236 prior to cardiopulmonary bypass at the same dose as that used in the present study, resulted in reduced leakage of troponin-T, a sensitive marker of myocardial injury, although this difference just failed to reach statistical significance (529). These results suggest that the dose of GR79236 used in this study is sufficient to induce pharmacological preconditioning in a number of different models, and that the lack of a protective effect observed during sequential exercise cannot be explained on the basis of insufficient dose.

It appears therefore, that activation of adenosine A$_1$R is not an essential event in triggering the warm-up effect during sequential exercise testing. The lack of involvement of adenosine in mediating warm-up angina has also been suggested by previous studies that have used an antagonist approach. Bamiphylline, a selective adenosine A$_1$R antagonist, at a dose previously shown to block adaptation to ischaemia during PTCA (376), failed to abolish the warm-up effect (530). Similarly, studies with theophylline or aminophylline, non-selective adenosine antagonists, suggest that the adaptation observed during serial exercise testing is not adenosine-
dependent (377, 520). Taken together, the results of these studies strongly argue against a role for adenosine in mediating warm-up angina.

A number of studies have also evaluated the role of $K_{\text{ATP}}$ channels in mediating warm-up angina (378, 379, 519, 531). However these studies have produced conflicting results which may be explained by the differing end-points evaluated. For example, Tomai et al. (379) found that administration of oral glibenclamide 10mg, a selective $K_{\text{ATP}}$ channel blocker, 90 minutes before two sequential treadmill exercise tests, abolished the increase in ischaemic threshold observed during the second exercise test in the control group. This dose of glibenclamide had been previously shown by the same group to abolish the myocardial adaptation with successive balloon occlusions during PTCA (388). Similar findings were recently reported by Ovunc (531) in a study of 18 patients with type II diabetes. These studies suggest that $K_{\text{ATP}}$ channels are involved in the mechanism of warm-up angina. Conversely, Correa and Schaeffer found that 10 mg oral glibenclamide did not attenuate the enhanced exercise tolerance during the second of two sequential exercise tests, although ischaemic threshold was not assessed in this study. Bogaty et al. (519) reported a reduction in electrocardiographic indices of ischaemia and exercise-induced regional LV dysfunction during a second exercise tests. When the same protocol of sequential exercise was performed by diabetic patients on chronic oral dosing with glibenclamide, a similar attenuation in ischaemic indices was observed with re-exercise. These latter studies argue against a role for $K_{\text{ATP}}$ channels in mediating the protective effects of exercise-induced myocardial ischaemia. Considering the discrepancies in the results of studies evaluating the effects of $K_{\text{ATP}}$ channel blockade with glibenclamide on indices of ischaemia during sequential exercise, it is difficult to draw any firm conclusions about the involvement of these channels in the mechanism of warm-up angina.

It seems therefore that exercise-induced myocardial ischaemia is capable of eliciting an adaptive response that results in attenuated ischaemic indices during further exercise. The biphasic temporal profile of this form of myocardial adaptation, along with evidence for increased myocardial metabolic efficiency and the absence of increased perfusion of the ischaemic myocardium during the second period of
exercise, suggest a mechanism akin to ischaemic preconditioning. However, the reproducible evidence against involvement of adenosine in mediating this protection argues against a preconditioning-like effect. The evidence for the role of $K_{ATP}$ channels in warm-up angina remains inconclusive.

### 7.4.c Similar Studies

During the course of the present study, two reports were presented of similar work aimed at demonstration of delayed myocardial adaptation following exercise-induced ischaemia. Tomai et al. (532), in a study with a protocol similar to that used in the present study, found that during a treadmill exercise test performed 24 hours after two sequential exercise tests, ischaemic threshold was comparable to that during Ex 1, although exercise tolerance remained significantly improved. They concluded that a first exercise-induced ischaemic challenge induces the early phase of preconditioning but not the late phase. Bilinska et al. (533) subjected patients with angina, who had suffered an acute MI within 8 weeks of entry into the study, to two bicycle ergometer exercise tests. The time interval between the two periods of exercise ranged from 24-96 hours. Interestingly, they found that all ischaemic indices significantly improved during the second exercise test when this was performed at 24, 48 or 72 hours after the first ischaemic challenge. However, if the recovery period between Ex 1 and Ex 2 was extended to 96 hours, all electrocardiographic indices of ischaemia including ischaemic threshold and exercise tolerance returned to values that were not significantly different to those during Ex 1. These results would suggest that the time course of myocardial adaptation resulting from a first exercise-induced ischaemic challenge is identical to that of delayed ischaemic preconditioning against infarction (93) and stunning (94) reported in experimental animals, and strongly suggest a mechanistic link. The reasons behind the discrepancies between the results of the present study and the Bilinska study (533), and those reported by Tomai et al. (532) are not immediately obvious. The only difference in the end-points evaluated in the studies is the definition of ischaemic threshold; defined as RPP at 1.0 mm ST-segment depression in the present study and the study by the Polish group, as compared to RPP at 1.5 mm ST depression in the Tomai study. However, this is unlikely to explain the conflicting results obtained in the studies. Further studies in the field are required to resolve these discrepancies.
7.4 Summary

In summary, the results of the present study performed in patients with stable angina suggest that myocardial ischaemia resulting from the stress of exercise, induces enhanced tolerance to a subsequent period of exercise performed either within minutes or 24 hours after Ex 1. The biphasic pattern of this adaptation suggests that the warm-up phenomenon may represent a human model of ischaemic preconditioning. The selective A_1R agonist GR79236, was unable to mimic the warm-up protection, or augment the attenuation in ischaemic indices induced by the first period of exercise, suggesting that adenosine A_1R do not play an important role in induction of warm-up angina.
Chapter Eight

General Discussion
8.1 Summary and Discussion

Major advances in our knowledge of the pathophysiology of acute coronary syndromes and their management have resulted in a marked reduction in the morbidity and mortality associated with these conditions. Nonetheless, the outcome of these conditions remains poor, with one month mortality rates of up to 10% reported for patients suffering AMI and other unstable coronary syndromes in major clinical trials. In the “real world”, outside the carefully controlled setting of clinical trials, the prognosis of these patients is likely to be even worse (534). These figures point to the need for novel therapeutic modalities which may provide additional protection to the ischaemic myocardium. Ischaemic preconditioning may potentially be a strategy for delaying myocardial necrosis in the event of acute ischaemia, thereby increasing the time window for effective institution of reperfusion therapy. In particular, the prolonged time-course of the delayed phase of myocardial protection following ischaemic preconditioning makes it potentially of greater clinical relevance. However, the use of brief antecedent ischaemia as a means of inducing cardioprotection is not desirable. On the other hand, the use of pharmacological agents to stimulate the receptors implicated in triggering preconditioning, in lieu of brief ischaemia, may provide a more benign approach for eliciting cardioprotection. Some evidence points to the important role of adenosine as a trigger of delayed ischaemic preconditioning. However, at the start of this thesis, virtually nothing was known about the mechanisms responsible for the protection of the ischaemic myocardium many hours following transient activation of adenosine receptors. Only after elucidation of the characteristics and the cellular mechanisms underlying this potent mode of cardioprotection, will it be possible to fully exploit this phenomenon to protect the ischaemic myocardium in patients with coronary artery disease.

The aims of this thesis were to identify the temporal characteristics and the intracellular mechanisms underlying the delayed phase of myocardial protection following selective activation of adenosine A₁R. The choice of a selective adenosine A₁R agonist rather than adenosine as a means of induction of delayed preconditioning was based on the fact that in patients with coronary disease,
adenosine may not be therapeutically useful as any potential A\textsubscript{1}R-mediated myocardial protection may be offset by the coronary steal effect of A\textsubscript{2}R-mediated coronary vasodilatation.

Preliminary studies were performed in an in \textit{vivo} rabbit model of regional myocardial ischaemia-reperfusion, which confirmed that transient activation of adenosine A\textsubscript{1}R with the selective agonist CCPA, induces robust and sustained protection against myocardial infarction at 24 and 48 hours (Chapter 3). Although this delayed protection is more long lasting than the transient limitation of infarction observed immediately following ischaemic preconditioning, it is itself short lived and protects the myocardium against infarction for only 2-3 days. However, considering the need for pretreatment in clinical situations where pharmacological preconditioning may be a viable means of protecting the myocardium against the potential threat of lethal ischaemic injury, it is desirable to extend the duration of protection beyond this timeframe and maintain the myocardium in a chronic preconditioned state for periods of several days to weeks. However, studies that aimed to extend the duration of protection induced by classic preconditioning, either by subjecting rabbits to repeated brief episodes of ischaemia or a continuous infusion of pharmacological preconditioning agent (CCPA), had failed to demonstrate long lasting protection mainly due to development of tachyphylaxis (410, 411). It was hypothesised that exploiting the relatively prolonged nature of the second window of protection, would allow a dosing regimen with the pharmacological preconditioning agent (adenosine A\textsubscript{1}R agonist) that may avoid the development of tolerance, while maintaining the myocardium in a preconditioned state. Studies in the conscious rabbit model, using intermittent dosing with CCPA at 48 hourly intervals, provided the first proof of this concept [Dana et al. \textit{J Am Coll Cardiol} 1998; 31: 1142-1149]. When rabbits were subjected to 30 minutes regional myocardial ischaemia and 2 hours reperfusion in \textit{vivo} after 10 days of intermittent treatment with CCPA, the hearts had significantly reduced infarct size compared with those of placebo treated animals, suggesting that the duration of protection had been extended to 10 days with no evidence of downregulation of A\textsubscript{1}R function (Chapter 3). Moreover, the haemodynamic response to a bolus of CCPA on day 10 was similar in the two groups of animals, whether they had received intermittent treatment with CCPA or saline, further confirming that
animals had not developed tolerance to the effects of the A₁R agonist. One important point that was not evaluated in this study is whether during the prolonged protection conferred by intermittent dosing with CCPA, transient episodes of sublethal myocardial ischaemia, as may occur in patients with unstable angina, would have an additive protective effect on the ultimate infarct size as a result of a lethal ischaemic challenge. In other words, does classic preconditioning with brief episodes of myocardial ischaemia provide additional resistance against ischaemic injury in the setting of delayed pharmacological preconditioning with CCPA? Although not strictly addressing the same question, a study by Stambaugh et al. (535) conducted in cultured chick ventricular myocytes, suggests that the protection against myocyte death induced by delayed pharmacological preconditioning with MLA has an additive effect to that of early preconditioning with ischaemia, adenosine or pinacidil, a K<sub>ATP</sub> channel opener. These findings raise the possibility that in the clinical setting, a similar additive effect may be observed between pharmacological preconditioning agents and antianginal drugs such as nicorandil, which themselves may be capable of inducing classic preconditioning. It can also be speculated that in the setting of unstable angina before myocardial infarction, intermittent pharmacological treatment with an adenosine A₁R agonist may provide protection against myocardial infarction over and above that conferred by preinfarct angina.

Since the publication of the studies outlined in Chapter 3, there have been two preliminary reports supporting the findings of this study, and suggesting that repeated dosing with a preconditioning-mimetic agent maintains the heart in a chronic state of protection. Travers et al. (427) showed that following 7 days of daily i.v. bolus treatment with the selective A₁R agonist GR79236, rabbits were maintained in a preconditioned state as evidenced by significantly attenuated infarct size compared to placebo treated controls. More recently, Hill et al. (428) reported that despite development of tolerance to the haemodynamic effects of nitrates, rabbits treated with daily transdermal nitroglycerin patches for 28 days, remained chronically preconditioned and had diminished infarct size comparable to that in rabbits that underwent ischaemic preconditioning prior to infarction.
Next, the intracellular signalling events downstream of adenosine A1R involved in the induction of delayed tolerance to myocardial ischaemia were investigated. Using an antagonist approach, two family of intracellular enzymes were identified that mediated delayed protection against infarction induced by CCPA in the in vivo rabbit model; PKC and TKs [Dana et al. Circ Res 2000; 86: 989-997]. Inhibition of either family of enzymes with the respective antagonists CHE and LDA, completely abrogated the limitation of infarction observed 24 hours after pharmacological preconditioning with CCPA (Chapter 4). These results suggest that PKC and TKs act in the same signalling pathway downstream of adenosine A1R, which ultimately results in myocardial protection at 24 hours. One limitation of this study was that the results rely on manipulation of the activity of PKC and TKs with pharmacological agents which have been reported to selectively inhibit each enzyme. Whilst it is believed that the actions of these agents are specific, in the setting of the whole animal there is the potential for non-specific interactions. In particular the activities of PKC and TKs were not assessed in myocardial tissue pretreated with CCPA, or in the groups that had been treated with the enzyme inhibitors. Furthermore, the nature of the specific isoforms of these enzymes that are activated following treatment with CCPA remains unknown. However, a report from Bolli's laboratory (172) indicates that in a conscious rabbit model, cycles of brief myocardial ischaemia with intervening reperfusion, induce selective translocation of PKC isoforms ε and η from the cytosolic to the particulate fraction without subcellular redistribution of total PKC activity, and that CHE at the same dose as that used in the study in Chapter 3 completely abolishes this translocation. Similarly, LDA at a dose comparable to that used in this study has been shown to selectively inhibit protein TKs without an effect on receptor TKs or PKC (204). Further work in this field is required to characterise the specific members of the PKC and TK families which are activated downstream of adenosine A1R, and contribute to the genesis of delayed resistance against infarction. This will be best achieved by studies in mice with targeted disruption of the genes encoding specific enzyme isoforms, or in transgenic animals overexpressing such isoforms. A preliminary report of one such study has recently been presented, which implicates PKCe as the isoform involved in the genesis of late ischaemic preconditioning (536).
The same antagonist approach was also used to examine a role for early generation of NO as an upstream event in the signalling mechanism mediating A1R-induced delayed preconditioning against infarction in the *in vivo* rabbit model (Chapter 6). A non-selective inhibitor of all NOS isoforms, L-NAME, was used to inhibit NO generation following pharmacological preconditioning with CCPA, 24 hours prior to myocardial infarction. Despite the evidence for the role of NO in mediating some of the regulatory functions of adenosine in cardiac tissue, and its involvement in induction of delayed protection against infarction following ischaemic preconditioning, this study surprisingly did not implicate NO in development of tolerance to ischaemic injury 24 hours after treatment with CCPA. Although inhibition of NOS activity with L-NAME was not verified in this *in vivo* model, this has been demonstrated in other models, and importantly, L-NAME at the dose used in this study has been shown to block delayed pharmacological preconditioning with MLA or diazoxide, in rat and rabbit models (340, 502).

Another important issue regarding the delayed protection against infarction conferred by transient adenosine A1R activation is the nature of the distal effector or target protein(s) mediating this protection. Studies in Chapters 4, 5 and 6 examined the role of two cytoprotective proteins that may potentially contribute to the development of delayed protection against infarction. Analysis of myocardial tissue from rabbits pharmacologically preconditioned with CCPA 24 hours earlier, showed a significant increase in the activity of p38 MAPK and phosphorylation of a downstream target protein Hsp27. Similarly, Mn-SOD activity in these myocardial samples was significantly enhanced compared to that in saline treated controls. Importantly, these changes appeared to be dependent on a signalling mechanism involving PKC and TK, and occurred at a time point when the myocardium was in a preconditioned state. These results strongly suggest the involvement of these cytoprotective proteins in induction of delayed cardioprotection by CCPA.

The results obtained from the studies in Chapter 4 were the first evidence that transient activation of adenosine A1R induces subacute changes in p38 MAPK activity and Hsp27 phosphorylation [Dana et al. *Circ Res* 2000; 86: 989-997]. This study did not however evaluate a cause and effect relationship between induction of
p38 MAPK activity and Hsp27 phosphorylation, and development of an ischaemia tolerant phenotype. However, since the publication of this work, two studies using an antagonist approach, have provided confirmatory evidence for the importance of the p38 MAPK/MAPKAPK-2 pathway in mediating A₁R-induced delayed preconditioning (356, 466). Furthermore, recent preliminary data from Yellon's laboratory has indicated that in a human cardiac myoblast cell line, delayed protection 24 hours following ischaemic or adenosine induced preconditioning is abolished by prior treatment with a peptide inhibitor of MAPKAPK-2 (unpublished observations, Carroll R and Yellon DM, 2000).

The only other study to evaluate the subacute effects of a preconditioning stimulus on the phosphorylation pattern of Hsp27 has been performed in cultured endothelial cells from the human aorta or umbilical vein. In these cells, Loktionova et al. (537) demonstrated that heat stress induced delayed cytoprotection against simulated ischaemia, which was associated with marked suppression of dephosphorylation of Hsp27 during simulated ischaemia. This is thought to result from the action of protein phosphatase 2A (PP2A), which itself is activated during ischaemia (538). Interestingly, the activity of PP2A is negatively regulated by phosphorylation by protein TKs. Considering that the phosphorylation of cellular proteins is maintained in homeostasis by the protein phosphatases actively opposing kinases, it seems that both p38 MAPK/MAPKAPK-2 pathway and PPA2 are closely linked in regulation of the phosphorylation of Hsp27. On the basis of the results of Loktionova et al. (537) and those of the studies in Chapter 4, it is plausible to speculate that various preconditioning stimuli may exert their cytoprotective effects by maintaining a cytosolic pool of phosphorylated Hsp27 above a certain threshold level during an ischaemic insult, which in turn protects F-actin from disruption. Accordingly, exogenous inhibitors of Hsp27 dephosphorylation have been shown to stabilise microfilaments during simulated ischaemia, and provide a comparable cytoprotective effect (537, 539). Future studies in this area may focus on evaluating the temporal profile of subacute activation of p38 MAPK and phosphorylation of Hsp27 following CCPA, and comparison with that of delayed protection against infarction. It would also be intriguing to know whether intermittent treatment with PP2A inhibitors can maintain Hsp27 in a hyperphosphorylated form and thereby confer a state of chronic
preconditioning. In this regard studies in the newly developed transgenic mice overexpressing hyperphosphorylated Hsp27 or non-phosphorylatable mutants of Hsp27 may provide invaluable insight into the role of this protein and its phosphorylation state in the genesis of delayed preconditioning.

The preliminary results obtained in rabbit myocardium regarding induction of Mn-SOD 24 hours following treatment with CCPA, were further expanded in studies in an in vivo rat model [Dana et al. Circulation 2000; 101: 2841-2848]. In this species, transient adenosine A₁R activation resulted in enhanced myocardial Mn-SOD protein expression and activity 24 hours later, at a time point when there was significant protection against infarction. Importantly, in vivo administration of antisense ODN to Mn-SOD abolished both the increased protein expression and the enhanced tolerance to myocardial ischaemia (Chapter 5). It seems therefore that in common with other stimuli for delayed preconditioning such as heat stress (333), exercise (73) and ischaemia (452), induction of Mn-SOD protein and activity plays a pivotal role in development of subacute protection against infarction following CCPA.

The role of iNOS as a potential distal effector protein involved in induction of delayed CCPA induced preconditioning against infarction was investigated using pharmacological inhibitors of iNOS [Dana et al. J Cardiovasc Pharmacol 2001; In press]. The results suggested that in the in vivo rabbit model of regional myocardial ischaemia-reperfusion, induction of iNOS is not crucial to the development of delayed preconditioning by CCPA since selective inhibition of iNOS with two structurally distinct selective inhibitors, L-NIL and AG, failed to abrogate protection (Chapter 6). The main limitation of this study is that the results rely on manipulation of the activity of iNOS with pharmacological agents reported to selectively inhibit its activity. Whilst it is believed that the actions of these agents are specific, in the setting of the whole animal there is the potential for non-specific interactions. Demonstration of selective action of L-NIL and AG in this model by measuring the activity of the various NOS isoforms in myocardial tissue after treatment with the inhibitors would have greatly complimented the results, and provided more direct evidence against the involvement of iNOS in the genesis of delayed preconditioning this model. These additional experiments were not performed due to lack of time.
Another approach to investigation of the role of iNOS, is the use of transgenic mice with targeted disruption of the iNOS gene. However, two studies using this approach have provided conflicting results (342, 343). The exact role of iNOS in mediating delayed protection following A₁R activation remains unknown and requires further investigation.

Taken together, the results of the studies detailed in Chapters 4, 5 and 6, provide strong evidence that two seemingly unrelated stress responsive inducible elements (ie, p38 MAPK/Hsp27 pathway and Mn-SOD) are required to mediate the protection conferred by delayed preconditioning by CCPA. The regulation of both these end-effectors following A₁R activation is dependent on a signalling mechanism involving PKC and TKs. The involvement of yet another potential effector protein, the mitochondrial K\text{ATP} channel has been suggested by other studies (325, 348). It is likely that other as yet unidentified signalling pathways/mediators may also be involved in the induction of delayed protection. It seems therefore that the shift of the heart to an ischaemia-resistant phenotype represents a complex response requiring the co-ordinated activation of multiple genes. This is not dissimilar from other conditions in which the heart changes its phenotype (eg. Hypertrophy). How these potential effectors of delayed protection may interact to produce the final response to an ischaemic challenge remains unknown. It is possible that these cytoprotective proteins act independently to provide protection against the differing deleterious facets of ischaemia-reperfusion injury. For example, stabilisation of the cellular cytoskeleton and a chaperoning effect by Hsp27, reduction of ROS-mediated cellular injury by Mn-SOD and other antioxidants, and improvement of cellular energetics and prevention of mitochondrial Ca\textsuperscript{2+} overloading by opening of mitochondrial K\text{ATP} channels. However, this is likely to be a simplistic and naïve proposal, and it is more likely that these seemingly unrelated proteins function in a co-ordinated fashion to produce the myocardial preconditioned phenotype. For example, some evidence has suggested that the integrity of the actin cytoskeleton may have a regulatory function in gating of cardiac K\text{ATP} channels (540-542). More importantly, Baines et al. (543) have recently demonstrated that pharmacological disruption of the cytoskeleton by cytochalasin D, completely abolishes the protection conferred to cardiomyocytes by direct opening of the mitochondrial K\text{ATP} channels with diazoxide. It is therefore
conceivable that A1R induced delayed preconditioning, by activation of the p38 MAPK/Hsp27 pathway, results in preservation of actin microfilaments during the sustained ischaemic insult, which in turn may maintain opening of mitochondrial $K_{\text{ATP}}$ channels with ultimate reduction in ischaemia-reperfusion induced myocardial injury. On the other hand, recent evidence from two laboratories suggests that pharmacological opening of mitochondrial $K_{\text{ATP}}$ channels may merely result in enhanced production of ROS, and function as a trigger of cardioprotection (284, 544). The ROS generated by opening of mitochondrial $K_{\text{ATP}}$ channels may in turn, through a signalling mechanism involving PKC and TKs, induce activation of the p38 MAPK/Hsp27 pathway and enhanced expression of Mn-SOD. These proposals are purely speculative and require further investigation to elucidate the interactions between the potential end-effectors of delayed preconditioning.

Deciphering the mechanisms of delayed preconditioning induced by activation of adenosine A1R is important not only for our understanding of how the heart adapts to stress but also for its clinical implication. The ultimate aim of the extensive research in the field of preconditioning is to provide a conceptual framework for developing novel therapeutic strategies that can be used in patients with coronary artery disease. However, no studies have been able to directly demonstrate a delayed preconditioning effect in the intact human myocardium. The study detailed in Chapter 7 investigated whether brief periods of demand myocardial ischaemia induced by physical exercise, result in delayed myocardial adaptation at 24 hours in patients with coronary artery disease. The results of this study suggest that warm-up angina is indeed a biphasic phenomenon, with attenuation of ischaemic indices observed during a second period of exercise performed either within minutes or 24 hours after a first exercise. These findings may represent the first direct demonstration of a delayed phase of myocardial preconditioning in man. However, the contribution of other factors such as collateral recruitment or redistributive transmural myocardial flow to this adaptive response can not be excluded. Moreover, the results of this study, in agreement with those of others (377, 520, 530), argue against a role for adenosine A1R activation in induction of the warm-up phenomenon. Further work in this field is required to clarify whether the intact human myocardium is amenable to protection by delayed ischaemic or
pharmacological preconditioning, and to evaluate the role of $A_{1}R$ in induction of such protection. Studies in which an ischaemic preconditioning stimulus (e.g. exercise), or pharmacological preconditioning with adenosine or its analogues are applied 24-48 hours prior to planned revascularisation procedures (such as PTCA or CABG) may shed further light on this issue.

8.2 Conclusions and Future Directions

There can be little doubt that the elucidation of the pathophysiology and the cellular mechanisms of ischaemic preconditioning has 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 similar way to that seen in the experimental laboratory and be amenable to protection by ischaemic preconditioning. This evolving field however, has so far failed to provide any direct evidence that this plethora of experimental and clinical research may one day translate into a clinical reality which would ultimately benefit patients with coronary artery disease. Despite this, the knowledge gained as a result of this research has enabled us to identify several classes of pharmacological agent that may be able to mimic the protection conferred by ischaemic preconditioning. One such agent is adenosine or its more receptor type-specific analogues. Others include bradykinin/ACE inhibitors, opioids, NO donors and the $K_{\text{ATP}}$ channel opener nicorandil. A number of studies in routine (low risk) patients have been performed with the aim of proving the concept of pharmacological preconditioning in man, and to establish the safety and tolerability of these agents using indirect end-points to detect myocardial ischaemia and small differences in myocardial viability and extent of micro-necrosis. These findings provide some basis for optimism that a beneficial and clinically detectable improvement in myocardial protection may yet 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.

Further research in the basic laboratory is crucial to verify and expand our knowledge regarding the intracellular signalling mechanisms mediating delayed
myocardial protection following pharmacological preconditioning with adenosine or its analogues. Some future directions for this work have been alluded to in section 8.1. However, it is also timely that large scale clinical trials involving 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 (545). The cohort of patients randomised in these studies may include those with non-ST elevation acute coronary syndromes presenting with persistent ST segment depression on ECG, elevated serum troponin levels, impaired LV function, and diabetics, whether treated medically or with early revascularisation. These patients must be randomised to known preconditioning-mimetic agents (such as adenosine) versus placebo, in addition to standard therapy, and evaluated in terms of robust end-points of clinical outcome. Similarly, high risk patients undergoing elective revascularisation 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 vast amount of research in the field of preconditioning may translate into a clinical reality.
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APPENDIX

Protein Preparation, SDS-PAGE and Western Blotting Buffers

Suspension Buffer
0.1 M NaCl, 10 mM Tris (hydroxymethyl) methylamine (Tris; pH 7.6), 1 mM ethylenediamine tetraacetic acid disodium salt (EDTA; pH 8.0), 2 mM sodium pyrophosphate, 2 mM NaF, 2 mM β-glycerophosphate, 0.1 mg/ml phenylmethyl-sulphonylfluoride (PMSF), 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml trypsin inhibitor, 1 µg/ml Sigma protease inhibitor, 80 µg/ml bestatin. A stock solution of suspension buffer was prepared and stored at −20°C; PMSF was added just prior to use since it is very labile in aqueous solution.

Sample Buffer
100 mM Tris (pH 6.8), 200 mM dithiothreitol (DTT), 2% w/v sodium dodecyl sulphate (SDS), 0.2% w/v bromophenol blue in ethanol, 20% v/v glycerol. A stock solution of sample buffer was prepared and stored at −20°C; DTT was added just prior to use as it is very labile.

Running Gel Base
18.16 g Tris, 0.4 g SDS, 60 ml distilled water; pH adjusted to 8.8 with HCl. Volume was made up to 100 ml with distilled water.

Stacking Gel Base
6.05 g Tris, 0.4 g SDS, 60 ml distilled water; pH adjusted to 6.8 with HCl. Volume was made up to 100 ml with distilled water.

10x Running Buffer
144.2 glycine, 10.0 g SDS, 30.3 g Tris, 1 L distilled water. 1x running buffer was prepared by diluting 10x stock with distilled water.
Appendix

P38 MAPK Assay Buffers

**Lysis Buffer**
20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-Glycerolphosphate, 1 mM Na₃VO₄, 1 μg/ml leupeptin, 1 mM PMSF. The latter was added fresh before use.

**Kinase Buffer**
25 mM Tris (pH 7.5), 5 mM β-Glycerolphosphate, 2 mM DTT, 0.1 mM Na₃VO₄, 10 mM MgCl₂.

**Transfer Buffer**
25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5).

**3x SDS Sample Buffer**
187.5 mM Tris-HCl (pH 6.8 @ 25°C), 6% w/v SDS, 30% glycerol, 150 mM DTT, 0.03% w/v bromophenol blue. 1x sample buffer was prepared by diluting 3x stock with distilled water.

**Blocking Buffer**
1x TBS, 0.1% Twee-20 with 5% w/v non-fat dry milk; to make up a 150 ml solution, 15 ml 10x TBS was added to 135 ml distilled water and mixed. 7.5 g non-fat dry milk was added and at the end, 0.15 ml Tween-20 was added.
2D Electrophoresis Gel and Buffers

First Dimension Tube gels
9.2 M urea, 4% acrylamide, 20% Triton X-100, 1.6% Bio-Lyte 5/7 ampholyte, 0.4% Bio-Lyte 3/10 ampholyte, 0.01% ammonium persulphate (APS), 0.1% TEMED. The last two ingredients (polymerisation catalysts) were added after degassing of the solution, and tube gels were then cast.

Upper Chamber Buffer (100 mM NaOH)
0.2 g NaOH in 250 ml distilled water; degassed for 30 minutes.

Lower Chamber Buffer (10 mM H₃PO₄)
1.3 ml concentrated H₃PO₄ diluted in 2 L distilled water; degassed for 30 minutes.

First Dimension Sample Buffer
9.5 M urea, 2.0% Triton X-100, 5% β-mercaptoethanol, 1.6% Bio-Lyte 5/7 ampholyte, 0.4% Bio-Lyte 3/10 ampholyte, diluted with distilled water. This buffer was stored in 0.5 ml aliquots at –80°C.

First Dimension Sample Overlay Buffer
9.0 M urea, 0.8% Bio-Lyte 5/7 ampholyte, 0.2% Bio-Lyte 3/10 ampholyte, 500 μl 0.05% w/v bromophenol blue stock solution, diluted with distilled water. This buffer was stored in 0.5 ml aliquots at –80°C.