APOPTOSIS AND NECROSIS IN ISCHAEMIA/REOXYGENATION INJURY OF THE HUMAN MYOCARDIUM: MECHANISM OF PROTECTION BY ISCHAEMIC PRECONDITIONING

HUNAID AHMED VOHRA MB BS MRCS(Ed)

Cardiac Surgery/Department of Cardiovascular Sciences
University of Leicester
Glenfield Hospital
Leicester, United Kingdom.

A thesis presented to the University of Leicester for the degree of Doctor of Medicine
2005
Dedicated to my parents and to my wife and son.
ABSTRACT OF THESIS

Although considerable work has been carried out in the literature in the context of reperfusion injury in relation to necrosis, there is very little data in humans on the role of apoptosis. Moreover, the contribution and mechanisms relating to apoptosis in the context of ischaemic preconditioning remain undefined. My experiments were performed on free-hand sections of right atrial appendage with simulated ischaemia and reoxygenation. Cell damage was measured following release of creatine kinase and cell viability measured using the vital stain MTT (3-[4,5 Dimethylthiazol-2-y1]-2,5 diphenyltetrazolium bromide). Cell apoptosis and necrosis were visualised in tissue sections by means of fluorescent dyes using TUNEL assay and Propidium Iodide, respectively. Quantification was done by laser fluorescence confocal microscopy and NIH-image software. Caspase activation was measured by fluorometric assay. Gene Chip® microarrays were used to analyse mRNA isolated from right atrial appendages (n=3/group) subjected to SI/R, IPC and caspase-3 inhibition. The initial studies were carried out with specific ischaemia/reoxygenation time-points. Apoptosis was shown to be greater than necrosis after 90min simulated ischaemia (SI) and 8hr reoxygenation (R) but necrosis was greater than apoptosis by 24hr R. Inhibition of caspase-8+9 by z.IETD.fmkkz.LEHD.fmkk (70nM) significantly reduced apoptosis following 90min SI and 2hr R and inhibition of caspase-3 by z.DEVD.fmkk (70nM) almost completely abolished apoptosis, both without effecting necrosis. I have also shown that ischaemic preconditioning (IPC) is more efficacious in reducing apoptosis than necrosis. IPC inhibits necrosis in the human myocardium by signal transduction pathways that involve mitoK<sub>ATP</sub> channels, PKC and p38MAPK. However, apoptosis that is inhibited by activation of mitoK<sub>ATP</sub> channels and PKC is p38MAPK-independent. The observed changes in gene expression may help to understand the pathophysiology of ischaemic/reoxygenation injury and the mechanism of cardioprotection. With the information obtained in this thesis we have gained more information on the role of apoptosis in ischaemia/reperfusion injury of the human myocardium and a greater understanding of the underlying mechanisms and the signal transduction of cardioprotection by IPC. It is hoped that this knowledge will contribute to the design of therapeutical strategies that may reduce myocardial ischaemia/reperfusion injury in man.
## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title page</td>
<td>i</td>
</tr>
<tr>
<td>Abstract</td>
<td>iii</td>
</tr>
<tr>
<td>Index</td>
<td>iv</td>
</tr>
<tr>
<td>Figures</td>
<td>xi</td>
</tr>
<tr>
<td>Tables</td>
<td>xiii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>xiv</td>
</tr>
<tr>
<td>Publications</td>
<td>xvi</td>
</tr>
<tr>
<td>Presentations</td>
<td>xvii</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>xix</td>
</tr>
<tr>
<td>CHAPTER I: INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>1.1 Introduction</td>
<td>2</td>
</tr>
<tr>
<td>1.2 Ischaemia in the heart: stunning, hibernation, angina and infarction</td>
<td>3</td>
</tr>
<tr>
<td>1.3 Mechanisms of ischaemic injury</td>
<td></td>
</tr>
<tr>
<td>1.3.1 Reversible ischaemia</td>
<td>5</td>
</tr>
<tr>
<td>1.3.2 Irreversible ischaemia</td>
<td>6</td>
</tr>
<tr>
<td>1.4 Reperfusion injury</td>
<td>6</td>
</tr>
<tr>
<td>1.4.1 Mechanisms of reperfusion injury</td>
<td>7</td>
</tr>
<tr>
<td>1.5 Modes of myocardial cell death</td>
<td></td>
</tr>
<tr>
<td>1.5.1 Necrosis</td>
<td>8</td>
</tr>
<tr>
<td>1.5.2 Apoptosis</td>
<td>9</td>
</tr>
<tr>
<td>1.6 The phenomenon of apoptosis or 'programmed cell death'</td>
<td>10</td>
</tr>
<tr>
<td>1.6.1 Caspases</td>
<td>11</td>
</tr>
</tbody>
</table>
CHAPTER II: EXPERIMENTAL MATERIALS AND METHODS

2.1 Introduction

2.2 Patients

2.3 The Human Right Atrial Trabeculae Model
   2.3.1 Preparation of atrial tissue and solutions

2.4 Experimental techniques
   2.4.1 Collection of human right atrial appendage, preparation of sections and ischaemia-reoxygenation protocols
   2.4.2 Measurement of tissue injury by creatine kinase assay
   2.4.3 Measurement of tissue viability by the MTT assay
   2.4.4 Assessment of apoptosis and necrosis
   2.4.5 Quantitation of caspase activity by fluometric assay
   2.4.6 cDNA microarrays

2.5 Discussion
   2.5.1 Patients
      Diabetes Mellitus
      Poor left ventricular function
      "K\text{ATP} channel openers"
      Chronic atrial fibrillation
   2.5.2 The Human Right Atrial Trabeculae model
      Advantages
      Comparison with other models
      Limitations
   2.5.3 CK and MTT assays
CHAPTER III: EFFECT OF THE DEGREE OF ISCHAEMIC INJURY AND REOXYGENATION TIME ON THE TYPE OF MYOCARDIAL CELL DEATH IN MAN: ROLE OF CASPASES AND CHANGES IN GENE EXPRESSION.

3.1 Introduction 60

3.2 Material and Methods 61

3.2.1 Patients 61

3.2.2 Collection of atrial samples and solutions 62

3.2.3 Experimental protocols 62

3.2.4 Assessment of tissue injury and viability 63

3.2.5 Assessment of apoptosis and necrosis 63

3.2.6 Quantitation of caspase activity by fluorometric assay 64

3.2.7 cDNA microarrays 64

3.3 Statistical analysis 64

3.4 Results 64

3.4.1 Study 1 64

Apoptosis and necrosis 64

MTT reduction 65

CK release 65

Caspase activity 66
CHAPTER IV: EFFECT OF CASPASE INHIBITION ON APOPTOSIS AND NECROSIS IN ISCHAEMIA AND REOXYGENATION OF THE HUMAN MYOCARDIUM

4.1 Introduction

4.2 Aims of the study

4.3 Material and Methods
  4.3.1 Patients
  4.3.2 Collection of atrial samples and solutions
  4.3.3 Experimental protocols
  4.3.4 Assessment of apoptosis and necrosis
  4.3.5 Quantitation of caspase activity by fluorometric assay

4.4 Statistical analysis
4.5 Results

4.5.1 Study 1 92

4.5.2 Study 2 93
    Apoptosis and necrosis 93
    Caspase levels 94

4.6 Discussion

4.6.1 Caspase-8/9 are upstream of caspase-3 in the apoptotic cascade 95
4.6.2 Attenuation of apoptosis by caspase inhibition 96
4.6.3 Effect of caspase inhibitors on necrosis 98
4.6.4 Advantages of irreversible inhibitors 98
4.6.5 Limitations of the study 99

4.7 Conclusion 99

CHAPTER V: PRECONDITIONING OF THE HUMAN MYOCARDIUM AGAINST APOPTOSIS AND NECROSIS INDUCED BY ISCHAEMIA/REOXYGENATION

5.1 Introduction 108

5.2 Material and Methods

5.2.1 Patients 108
5.2.2 Preparation of atrial tissue and solutions 109
5.2.3 Experimental protocols 109
5.2.4 Measurement of tissue injury by CK assay 109
5.2.5 Assessment of apoptosis and necrosis 109

5.3 Statistical analysis 110
5.4 Results

Creatine kinase (CK) release 110
Apoptosis and necrosis 110

5.5 Discussion 111

5.6 Conclusion 113

CHAPTER VI: PRECONDITIONING OF THE HUMAN MYOCARDIUM AGAINST APOPTOSIS AND NECROSIS INDUCED BY ISCHAEMIA/REOXYGENATION: ROLE OF MITOKATP CHANNELS, PROTEIN KINASE C AND P38MAPK

6.1 Introduction 120

6.2 Material and Methods

6.2.1 Patients 121
6.2.2 Collection of atrial samples and solutions 121
6.2.3 Experimental protocols 121
6.2.4 Measurement of tissue injury by CK assay 122
6.2.5 Assessment of apoptosis and necrosis 122
6.2.6 cDNA microarrays 122

6.3 Results

6.3.1 Study 1

Creatine kinase (CK) release 123
Tissue apoptosis and necrosis 123

6.3.2 Study 2 124
6.4 Discussion

6.4.1 MitoK_{ATP} channels and IPC 125
6.4.2 PKC and IPC 126
6.4.3 p38MAP Kinase and IPC 128
6.4.4 Changes in gene expression and IPC 130
6.4.5 Clinical implications and study implications 133

6.5 Conclusion 133

CHAPTER VII: CONCLUSIONS 143

BIBLIOGRAPHY 149
Figures

CHAPTER I
1.1 Apoptosis and necrosis cell death pathways 19

CHAPTER II
2.1 Shaking water bath with atrial slices in flasks 38
2.2 TUNEL staining after 100µM hydrogen peroxide treatment 54
2.3 TUNEL staining after DNase-I digestion of nuclei 54

CHAPTER III
3.1 Experimental protocols for the study 76
3.2 TUNEL and Propidium Iodide staining at 2hr, 8hr and 24hr reoxygenation 77
3.3 MTT reduction at 2hr, 8hr and 24hr reoxygenation 78
3.4 Total caspase activity in fresh tissue and at 2hr, 8hr and 24hr reoxygenation 80
3.5 Caspase-3 like activity in fresh tissue and at 2hr, 8hr and 24hr reoxygenation 81
3.6 Effect of caspase-3 inhibition on apoptosis and necrosis 82
3.7 Representative TUNEL images 86
3.8 Representative Propidium Iodide images 87

CHAPTER IV
4.1 Experimental protocols for study 1 100
4.2 Dose response effect of caspase-3 inhibitor on total caspase, caspase-3 and caspase-8+9 activities in SI/R 101
4.3 Dose response effect of caspase-8+9 inhibitors on total caspase, caspase-3 and caspase-8+9 activities in SI/R 102
4.4 Experimental protocols for study 2 103
4.5 Effect of caspase-3 and caspase-8+9 inhibitors on TUNEL and Propidium Iodide staining in SI/R 104
4.6 Representative TUNEL images 105
4.7 Representative Propidium Iodide images 105
4.8 Effect of caspase-3 and caspase-8+9 inhibitors on total caspase, caspase-3 and caspase-8+9 activities in SI/R 106

CHAPTER V
5.1 Experimental protocols for the study 114
5.2 Effect of IPC on CK leakage in SI/R 115
5.3 Effect of IPC on TUNEL and Propidium Iodide staining in SI/R 116
5.4 Representative TUNEL images 117
5.5 Representative Propidium Iodide images 118

CHAPTER VI
6.1 Experimental protocols for the study 1 134
6.2 Experimental protocols for the study 2 135
6.3 Effect of 5-HD, chelerythrine and SB203580 on CK leakage with SI/R and IPC 136
6.4 Effect of diazoxide, anisomycin and PMA on CK leakage with IPC 137
6.5 Effect of 5-HD, chelerythrine and SB203580 on TUNEL and Propidium Iodide staining with SI/R and IPC 138
6.6 Effect of diazoxide, anisomycin and PMA on TUNEL and Propidium Iodide staining with IPC 139
6.7 Representative TUNEL images 140
6.8 Representative Propidium Iodide images 141
Tables

CHAPTER III
1. CK release at 2,8 and 24 hours reoxygenation 79
2. CK release and MTT reduction with caspase-3 inhibition 83
3. Effect of SI/R on gene expression 84

CHAPTER VI
1. Effect of IPC and caspase-3 inhibition on gene expression 142
   induced by SI/R
Acknowledgements

I would like to take this opportunity to thank the following people for their contribution in making this thesis possible and with whom it has been a privilege to work.

Professor Manuel Galiñanes — Head of Cardiac Surgery — whose constant support, encouragement and influence has been invaluable during my period as a clinical research fellow.

Professor Sir Peter Bell — Head of the Department of Surgery — who always believed that it was possible for me to complete the work in two years.

Dr Alan Fowler — Research Associate Cardiac Surgery — I would like to thank Alan for his technical assistance and frequent discussions which has made this thesis possible.

I am grateful to the Consultant Cardiothoracic Surgeons at the Glenfield Hospital, namely Mr R Firmin, Mr L Hadjinikolaou, Mr M Hickey, Mr J Leverment, Mr A Sosnowski, Mr T Spyt, and Mr J Szostek for providing human atrial samples.

This work was made possible with the help of a BUPA Research Fellowship and a personal contribution from Professor M Galiñanes.

I am grateful to the patients who consented to take part in the research.
I would like to acknowledge my colleagues at the University Clinical Sciences for their patience and help. They include Ms Lizelle Bernhardt, Mr TMF Chowdhry, Mr M Elahi, Mr L Hadjinikolaou, Mr Ashraf Hassouna, Mr Kostas Kotidis, Dr Bashir Matata, Dr Lincoln Shenje and Ms Christina Smart.

Last, but not the least, I would like to thank my mum and dad for their prayers and intellectual upbringing and my wife, Huma, my son Zainulabideen and my in-laws for their love and support.

All the studies included in this thesis were designed by myself and Prof. M Galiñanes. I was responsible for consent of study patients on a rota basis. The collection of atrial samples from theatre, transfer to the laboratory, free-hand sectioning of atrial appendages, carrying out of experimental protocols, processing of atrial muscles for cryosectioning, all staining procedures for all sections, preparation of the slides for microscopy, capture of confocal microscopy images at the Wellcome confocal microscopy unit at University College London, computer software image analysis and the performance of all assays for all the experiments included in this thesis was done by myself. Microarrays were carried out by the Department of Biochemistry at the University of Leicester. The analysis of all results including micrarrays, drawing of graphs and tables was performed by me.
Publications originating from my research

Full Papers


A Fowler, H Vohra, P Wu, M Galinanes: High levels of apoptosis and necrosis precede the loss of heart function. Submitted.


H Vohra, A Fowler, M Galiñanes: Preconditioning of the human myocardium against apoptosis and necrosis induced by ischemia/reoxygenation: role of mitoK<sub>ATP</sub> channels, protein kinase C and p38MAPK. Submitted.

Scientific Abstracts


Presentations

International

Sep '04: Vohra H, Fowler A, Galiñanes M: p38 MAPK is at the cross-road of protection by ischemic preconditioning of the human myocardium against cell death. 18th Annual Meeting of the European Association of Cardiothoracic Surgery, Leipzig, Germany.

Dec '03: Vohra H, Fowler A, Galiñanes M: p38 MAPK is at the cross-road of protection by ischemic preconditioning of the human myocardium against cell death. American Society of Cell Biology, San Francisco, USA.


Dec '03: Vohra H, Fowler A, Galiñanes M: Apoptosis is the dominant mode of cell death in the human myocardium in the early reperfusion period. American Society of Cell Biology, San Francisco, USA.


Feb '03: Vohra H, Fowler A, Galiñanes M: Apoptosis and necrosis are temporally different following ischaemia and reoxygenation of the human myocardium. Apoptosis 2003, Luxembourg.


xviii
National


Jul '03: Vohra H, Fowler A, Galiñanes M. p38MAPK is at the cross-road of cardioprotection in preconditioning with ischaemia and with cardioplegia in the human myocardium. Cardiothoracic Research Club Meeting, Leicester.


Regional

Mar '04: Vohra H, Fowler A, Galiñanes M. p38MAPK is at the cross-road of cardioprotection by ischaemic preconditioning in the human myocardium. Midlands Cardio-thoracic meeting, Walsgrave Hospital, Coventry.


Mar '03: Vohra H, Fowler A, Galiñanes M: Apoptosis and necrosis are temporally different following ischaemia and reoxygenation of the human myocardium. Midlands Cardio thoracic meeting, Heartlands Hospital, Birmingham.


Feb '02: Vohra H, Fowler A, Galiñanes M: ‘Apoptosis in the ischaemic and reoxygenated human myocardium. Midlands Cardio-thoracic meeting, Queen Elizabeth Hospital, Birmingham.
Abbreviations used:

ADP: adenosine diphosphate
AFC: amino-fluoro-methylcoumarin
AIF: apoptosis inducing factor
ANOVA: Analysis of variance
APAF-1: apoptosis protease activating factor-1
ATP: adenosine triphosphate
CABG: coronary artery bypass grafting
CAD: caspase-activated DNase
cDNA: complementary deoxyribonucleic acid
CIFA: cytochrome-c interacting factor of apoptosis
CK: creatine kinase
CMK: chloromethyl ketone
CPC: preconditioning with cardioplegia
DGK: diacylglycerol kinase
DMSO: dimethylsulfoxide
DNA: deoxyribonucleic acid
dUTP: 2'-deoxyuridine 5'-triphosphate
EF: ejection fraction
eNOS: endothelial nitric oxide synthase
ELISA: enzyme-linked immunosorbent assay
FADD: Fas-associated death domain
FCS: foetal calf serum
FITC: fluorescein isothiocyanate
FMK: fluoromethyl ketone
G-6-P: glucose-6-phosphate
GTN: glyceryl trinitrate
HIF: hypoxia-inducible factor
5-HD: 5-hydroxydecanoate
HSP: heat shock protein
ICE: interleukin converting enzyme
IGF: insulin-like growth factor
IL: interleukin
IPC: ischaemic preconditioning
I/R: ischaemia/reperfusion
KHH: Krebs Henseleit Hepes
LDH: lactate dehydrogenase
LV: left ventricle
MAPK: mitogen activated protein kinase
mito $K_{\text{ATP}}$ channels: mitochondrial ATP-sensitive potassium channels
mRNA: messenger ribonucleic acid
MTT (3-[4,5 Dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide
NADP: nicotinamide adenine dinucleotide phosphate
NO: nitric oxide
NS: not significant
PARP: poly(ADP-ribose)polymerase
PBS: phosphate buffered saline
6-PG: 6-phosphoglycerate
Pi: inorganic phosphate
PI: propidium iodide
PKC: protein kinase C
PS: phosphatidylserine
PTCA: percutaneous transluminal coronary angioplasty
R: reoxygenation
ROS: reactive oxygen species
SARP: secreted apoptosis related protein
SEM: standard error of the mean
SI: simulated ischaemia
SI/R: simulated ischaemia and reoxygenation
TdT: terminal deoxynucleotidyl transferase
TNF: tumour necrosis factor
TNFAIP: TNF-α induced protein
TRADD: TNFR-associated death domain protein
TRAIL: TNF-related apoptosis-inducing ligand
TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling
VEGF: vascular endothelial growth factor
CHAPTER I

INTRODUCTION
1.1 Introduction

Coronary artery disease remains the most common cause of death in Western countries. Prolonged periods of myocardial ischaemia can cause myocyte death. While reperfusion is essential, it is also associated to oxidative stress and calcium overload that may cause cardiomyocyte loss. This is a clinical problem associated with procedures such as thrombolysis, angioplasty and coronary bypass surgery that are commonly used to establish the blood flow and minimise the damage of the heart due to severe myocardial ischaemia. The clinical consequences of this reperfusion may lead to myocardial stunning, reperfusion arrythmias and lethal reperfusion injury. Lethal reperfusion injury is defined as injury caused by restoration of blood flow after an ischaemic episode leading to death of cells that were reversibly injured during a preceding ischaemic episode. While cell death contributing to infarction usually displays the pathological features of necrosis (section 1.5.1), there is increasing evidence that another pathway leading to cell death, apoptosis, may also contribute to the total cell loss during ischaemia and reperfusion. However, the extent of lethal reperfusion injury has been debated by scientists and physicians alike for years. One of the problems is that the development of necrosis and apoptosis cannot be accurately followed in time, both in experimental animal preparations and in man. It is also not possible to see the effect of ischaemia and that of reperfusion in the same tissue.

The role of necrotic cell death in ischaemic heart disease in animal and human models has been studied in great detail [115,122,124,240]. Total proximal coronary occlusions of >15 minutes results in irreversible cell injury in the canine heart [124]. In humans,
necrosis is a well-known pathway to cardiomyocyte death in the pathogenesis of myocardial infarction. However, the exact role of apoptosis and necrosis in ischaemia-reperfusion (I/R) injury in humans is unknown. Using the atrial trabeculae model [309] for human myocardium, I aimed to determine the time-course of apoptosis and necrosis in ischaemia/reoxygenation injury, the comparative roles of initiator and effector enzymes (caspases, section 1.6.1) that are associated with ischaemia/reoxygenation-induced apoptosis and the protective mechanisms that are currently available which may be used to limit reoxygenation-induced injury in the human myocardium. With this information it is hoped that strategies may be developed to reduce reperfusion-induced cardiomyocyte loss in the human heart.

In the remainder of this chapter, I will discuss the processes of ischaemia and reperfusion in more detail followed by an overview of the phenomenon and control of apoptosis or 'programmed cell death' in the light of the present literature. I conclude this chapter with an evaluation of the human models of reperfusion including the right atrial trabeculae model and a presentation of the specific aims of my thesis.

1.2 Ischaemia in the heart: stunning, hibernation, angina and infarction

Ischaemia occurs when the blood flow to an organ is insufficient to meet its metabolic demands. Myocardial ischaemia is defined as an imbalance between fractional uptake of oxygen and the rate of cellular oxidation in the heart either due to a reduction in the
myocardial oxygen supply or an increase in oxygen demand. Recent advances in cardiovascular research have increased our understanding of the complex continuum of events resulting in end-stage coronary artery disease [105].

Ischaemia may have several potential outcomes: (1) when ischaemia is brief (<15 min) [124,142], a transient (minutes-weeks) [142] post-ischaemic ventricular dysfunction occurs on reperfusion, a condition named **stunned myocardium**, (2) when it is severe and prolonged (>15 min) [124], irreversible damage occurs, with no recovery in contractile function upon reperfusion, **myocardial infarction** (3) when ischaemia is less severe, but still prolonged, the myocytes may remain viable but exhibit depressed contractile function. This is called **hibernating myocardium** and reperfusion restores contractility [68].

Clinically, if the coronary atherosclerotic plaque is stable and occlusive enough (50% or more stenosis) [127], it may cause symptoms of **angina pectoris**. Rupture of the plaque with resultant thrombosis results in an acute condition which leads to the loss of myocytes called **myocardial infarction**. If medical care is not provided quickly, this may lead to an irreversible injury leading to loss of cells that can be lethal to the patient. In the event of survival of the patient, an impairment of left ventricular systolic function along with myocardial transformation from a normal elliptical shape to a mechanically disadvantaged and enlarged profile may lead to heart failure. This adaptive phenomenon is termed **remodelling** [295].
1.3 Mechanisms of ischaemic injury

1.3.1 Reversible ischaemia

The mitochondria play an important role in cellular energy production and through ATP production maintain intracellular ionic homeostasis. Within seconds of the onset of ischaemia, intracellular acidosis develops as a result of the shortage of oxygen at the mitochondrial level. Acidosis reduces calcium movements within the sarcolemma, sarcoplasmic reticulum and myofilaments [68]. Shortly after, the energy charge of the myocyte is reduced, creatine phosphate declines faster and to a greater extent than ATP. This results in a rapid increase in the intracellular concentration of inorganic phosphate (Pi). Anaerobic metabolism then develops and contributes to the formation of limited amounts of ATP. Concomitantly, there is a decline in the consumption of ATP due to decreased contractility. Reperfusion at this stage results in recovery of high energy phosphate production, signifying that the mitochondria are functionally intact and can carry on aerobic metabolism. All the inhibitory biochemical events, including the high levels of Pi, are reversed. However the functional recovery is not immediate due to the phenomenon of 'myocardial stunning'. This is the prolonged contractile dysfunction that occurs during reperfusion despite the absence of irreversible injury [27]. The duration of myocardial stunning is longer than that of the preceding ischaemia. However, by definition, this form of injury is fully reversible. A number of studies have suggested that oxygen-derived free radicals (ROS) contribute to this post-ischaemic ventricular dysfunction. The release of free radicals has been directly demonstrated with electron paramagnetic resonance spectroscopy and has been shown to be related to contractile dysfunction [23]. Further, there is evidence that the phenomenon of stunning is also
related to abnormalities of calcium homeostasis [22]. However, the above two plausible mechanisms are not mutually exclusive. Thus, oxygen free radicals may cause changes in calcium distribution secondary to sarcolemmal and sarcoplasmic reticulum disturbances which in turn could lead to further free radical production [7].

1.3.2 Irreversible ischaemia
If the coronary flow remains severely reduced, as may occur with an occlusive thrombus superimposed on a discrete and fissured atherosclerotic plaque [266], biochemical ischaemia intensifies and will ultimately lead to irreversible myocardial damage. Prolongation of ischaemia leads to a further decrease in intracellular pH. The early increase in lactate is followed by a decline with a further decrease in tissue content of ATP and creatine phosphate. Profound ionic changes [66] take place with reduction of intracellular K⁺ and Mg⁺, and an increase of Na⁺ and cytosolic Ca²⁺. It is worth noting that the total tissue Ca²⁺ concentration remains unchanged [66] but there is redistribution of the ion within the cell.

1.4 Reperfusion injury
After ischaemia, reperfusion may not lead to the recovery of the myocardium. Reperfusion may lead to a further increase in myocardial stiffness without increasing ATP production. During reperfusion there is sustained release of lactate, ions and creatine kinase from the cardiomyocytes along with massive influx of calcium and severe mitochondrial damage suggesting that reperfusion causes a wash-out of these substances
and also exacerbation of their release [22]. Eventually, there is cell membrane damage leading to increased permeability to Ca$^{2+}$ and Mg$^{+}$ ions as well as larger molecules. In this way, the major role of the mitochondria is switched from producing ATP to sequestering cytosolic Ca$^{2+}$ and other ions. This leads to anaerobic metabolism which is unable to cope with the existing myocardial requirements. A vicious cycle is thus generated which may result in the ultimate disruption of the sarcolemma and cardiomyocyte necrosis. Thus, *mitochondria play a pivotal role in reperfusion damage.*

Reperfusion is an absolute prerequisite for the salvage of the myocardium. Nevertheless, reperfusion may cause an injury more than that achieved during ischaemia [218]. There is convincing evidence that reperfusion is able to precipitate a number of adverse cellular changes and the term *reperfusion injury* is now widely used.

### 1.4.1 Mechanisms of reperfusion injury

The following factors may contribute to reperfusion injury:

1. **Calcium overload hypothesis**: Recovery of energy re-activates the calcium pump of the sarcoplasmic reticulum which sequesters Ca$^{2+}$, sometimes exceeding its capacity leading to leakage within the cytosol. This excess Ca$^{2+}$ is taken up by the mitochondria which affects ATP production [69]. The normalisation of interstitial pH due to washout of protons on reperfusion results in a gradient to be created between the cytosol and the interstitium which, in turn, causes the activation of Na/H exchanger [229]. This activation causes a net influx of Na into the cytosol, which in turn results in activation of Na$^+$/Ca$^{2+}$ exchange mechanism leading to efflux of Na$^+$ and influx of Ca$^{2+}$ that contributes to tissue injury [149]. The excess
in intracellular Na\(^+\) will also lead to the osmotic cellular uptake of water, which causes stretching of the cell membrane and further ionic disturbances [242].

(2) Oxidative stress hypothesis: Readmission of oxygen might paradoxically contribute to ischaemic damage by the formation of oxygen free radicals [3,22-25,89,289,301]. Normally, the anti-oxidant defence systems in the aerobic myocardium are able to get rid of the oxygen free radicals. However, ischaemia reduces this ability, especially in the mitochondrial compartment, making the tissue more susceptible to injury [67,68].

1.5 Modes of myocardial cell death

1.5.1 Necrosis

When subjected to episodes of ischaemia and reperfusion, the heart manifests forms of injury that may include post-ischaemic ventricular dysfunction or stunning, arrhythmias or in the severest form, myocardial infarction. The characteristic pattern of ischaemic cell injury involves fluid and electrolyte imbalance leading to accumulation of water and swelling of the cell and organelles together with margination and clumping of nuclear chromatin. Subsequently, the development of a severe membrane permeability defect [33] and the unregulated influx of calcium results in irreversible cardiomyocyte injury. Ultimately, the cells develop physical defects (holes) in cell membranes and rupture. This encompasses the phenomenon of necrosis. Thus, cellular necrosis has its origins in an environmental perturbation [298], such as may occur during a non-lethal heart attack or chronic angina.
1.5.2 Apoptosis

Recently, considerable attention has been directed to apoptosis, which is another pathway to cell death [33,174]. The pioneering work of Kerr and colleagues [137] has established apoptosis as a physiological form of cell deletion through factors intrinsic to the cell, by which a growing multicellular organism can maintain cellular homeostasis and sculpt cellular structures without initiating an inflammatory response. Cardiomyocyte apoptosis has been implicated in diseases of the human heart like heart failure [213], cardiomyopathies [17] and arrhythmias [118]. It has been shown to occur in animal models of ischaemia and reperfusion [74,89,131] and in isolated cell lines exposed to oxidative stress [155]. However, the slow response of isolated animal cells to oxidative stress has made it difficult to establish the relationship in time between apoptosis [193] and necrosis [209]. Consequently, the role and pathophysiological relevance of apoptosis to survival of the myocardium in the humans has yet to be demonstrated.

Although necrosis and apoptosis have generally been regarded as the two fundamental forms of cell death, controversy still exists amongst investigators in this regard. Recently, necrosis has been more precisely defined as the sum of degradative changes that follow any type of cell death [152]. The term Oncosis has been introduced to refer to the pathway to cell death associated with cell swelling in contrast to ‘apoptosis’ which is the route to cardiomyocyte death that is associated with cell shrinkage [175]. On the basis of this nomenclature, necrosis follows the onset of apoptotic or oncocytic cell death. My definition of necrosis is based on the staining of the cell with propidium iodide subsequent to the rupture of the sarcolemmal membrane.
1.6 The phenomenon of apoptosis or ‘programmed cell death’

Cells undergoing apoptosis are characterised by a series of stereotypic morphological changes both in the nucleus and in the cytoplasm. A group of enzymes called caspases (derived from cysteinyl-aspartate proteases) are responsible for the majority of these changes. These include shrinkage of the cell, blebbing of the cell membrane, nuclear condensation and fragmentation. The biochemical alterations comprise specific DNA strand breaks into nucleosomal lengths of 160-200 base pairs. This is due to the activity of a family of calcium dependent endogenous endonucleases [50]. Others include the hydrolysis of several intracellular proteins, the appearance of integrins [27] and the nucleosome octomer core [308] (involved in immune-mediated processes) and the flipping of the cell membrane phosphatidylserine from the normal inner side to the outer surface [310]. Although not clearly understood, phagocytosis of apoptotic cells involves many cell surface receptors including phosphatidylserine which forms the basis of annexin V staining [278] of apoptotic cells. The genes regulating the process of apoptosis have been classified into three categories:

(a) *suppressor genes*: some members of bcl protein family

(b) *intermediate genes*: c-myc, p53 and Fas/Fas-ligand and

(c) *effector genes*: cysteinyl-aspartate proteases (caspases) and interleukin converting enzymes (ICE) family.
1.6.1 Caspases

Most of the biochemical features that were observed by Kerr et al [137] are caused by a set of cysteine proteases that are activated specifically in apoptotic cells. These proteases are homologous to each other and are part of a large protein family known as caspases [8]. The studies in the nematode Caenorhabditis Elegans discovered the connection between a family of caspases and apoptosis with the discovery that nematode cell death abnormal gene CED-3 is related to caspase-1 [308]. Over a dozen caspases have been identified in humans; about two-thirds of these have been suggested to function in apoptosis [63,274].

The family of caspases can be grouped into three classes:

- group 1- *inflammatory caspases*
- group 2- *effector caspase* (demolition caspases) and
- group 3- *initiator caspases*.

The group 2 caspases or the effector caspases which include caspases 3, 6 and 7 are believed to play a central role in the execution of apoptosis. It has been shown recently that caspase-3, a pivotal regulator of apoptosis has numerous substrates which include caspase-6, caspase-10, fodrin, gelsolin, Fas, the 3 nuclear lamins, Ras related G proteins, members of the PKC and MAP kinase families, the catalytic sub-unit of DNA dependent protein kinase and poly(ADP-ribose)polymerase (PARP) [204]. Nagata [198] has shown that caspase-activated DNAse (CAD) pre-exists in humans as an inactive complex with an inhibitory sub-unit, ICAD. Activation of CAD, which leads to breakdown of DNA and is responsible for the nucleosomal ladder seen by DNA gel electrophoresis, occurs by
means of caspase-3 mediated cleavage of the inhibitory sub-unit by diverse pathways in humans TF-1 cells [189].

Caspase-3 is inhibited reversibly by aldehydes of the tetra-peptide substrate, DEVD (Ac.DEVD.CHO) [205] and irreversibly by the membrane permeant inhibitor benzylxycarbonyl-V-A-D(OMe)-fluoromethylketone (zVAD.fmk). Use of zVAD.fmk, a non-specific inhibitor of effector caspases, is associated with a reduction in myocardial infarction and reduced apoptosis associated with ischaemia-reperfusion injury in rat myocytes [301]. However, the precise role of caspase-3 activation and the effect of caspase-3 inhibition in ischaemia-reperfusion injury in the human myocardium remains to be elucidated.

Human diseases in which caspase-3 activation has been implicated include oesophageal cancer [300], Legionella Pneumophilia-induced apoptosis [76], Parkinson’s disease [99], Alzheimer’s disease [140] and Huntington’s disease [86].
1.6.2 Mechanisms leading to apoptosis

Two main pathways have been identified in the activation of caspase-mediated apoptosis:

(a) **extrinsic (death receptor-mediated)** and (b) **intrinsic (mitochondrial)**

**Extrinsic pathway:** This is the activation of caspases via receptor-mediated mechanisms, especially Fas and tumour necrosis factor (TNFR) of two members of the family, caspase-2 [283] and -8 [41,42,165]. There are a number of characteristic death receptors, including Fas, TNFR-1, death receptor(DR)-3 (Apo-3) and TNF-related receptor, DR-4 and DR-5. Binding of the respective ligands to these external receptors results in intracellular recruitment of dedicated adaptor proteins, TNFR-associated death domain protein (TRADD)[42,132] for TNFR-1 and DR-3. A trimeric ligand aggregates Fas cell surface membrane receptors, also known as Apo-1 or CD95. Fas/CD95 death receptor assemblies then recruit Fas-associated death domain proteins (FADD)[41,141,223]. TRADD and FADD then interact with domains on procaspases (procaspase-8 or procaspase-10), thus activating them and precipitating cellular proteolytic damage.

**Intrinsic pathway:** This pathway involves the participation of the principal regulator of cell metabolism, the mitochondria. The universal triggering event in apoptosis is the dissociation of cytochrome-c from the electron transport chain [143]. Mitochondrial membrane depolarisation and poration may be preceded by penetration of the outer membrane by the ‘cytochrome-c interacting factor of apoptosis’ (CIFA) [147]. Pores then form due to conformational changes in the outer mitochondrial membrane channel proteins like ‘mitochondrial permeability transition’ leading to two caspase-activating
pathways [97]. The first involves the apoptosis inducing factor (AIF) [166] and the second consists of cytochrome-c and pro-caspase-9 which recruit an apoptosis protease activating factor (APAF-1) [315] from the cytoplasm to form a complex within the cytoplasm called the ‘apoptosome’. The pro-caspase-9 then activates to caspase-9 within the apoptosome which through the sequential downstream activation of caspase-3 executes its lethal effects.

The Bcl-2 family.
The discovery of bcl-2 proteins has increased our understanding of the regulatory factors that are involved in the mechanism of apoptosis. Bcl-2 gene expression in humans correlates with resistance to apoptotic cell death. In coronary artery ligation models and in vivo models of heart failure, bcl-2 expression is markedly up-regulated [213], which is thought to be compensatory to the increased cell death. Bcl-2 blocks the release of cytochrome-c and AIF from the mitochondrial membrane thus suggesting a potential site for its anti-apoptotic activity [143,269]. Furthermore, bcl-2 is thought to control apoptosis through regulation of an anti-oxidant pathway [110] that may control redox potential via mitochondrial thiols. It is thought that the co-localisation of bcl-2 with bax, which is pro-apoptotic, with the formation of heterodimers on the mitochondrial membrane may be a key site [302] of its action. Thus if the bax/bcl-2 ratio is altered, this will allow the dominant pro- or anti-apoptotic influences to prevail [269]. Another member of the family, bcl-xL offers protection from apoptosis by binding to bax, thus preventing the formation of bax homodimers in the outer mitochondrial membrane which accelerate the process [216].
1.7 Apoptosis in heart disease

Cell death via apoptosis has been implicated in many diseases of the heart including conduction defects [119], myocardial infarction [246], ischaemic [215] and dilated cardiomyopathy [213], cardiac allograft rejection [271], ischaemia/reperfusion injuries [311] and arrhythmogenic right ventricular dysplasia [206]. Cardiovascular remodelling may involve cell proliferation, apoptosis and fibrosis. In a rat model of cardiac hypertrophy induced by pressure overload, Teiger et al. [273] demonstrated that the highest levels of apoptosis were observed just before the development of cardiac hypertrophy and the levels decreased as the hypertrophy progressed. It has been shown that after a myocardial infarction, apoptosis is increased in comparison to necrosis [131] and left ventricular remodelling late after the infarction is associated with increased apoptosis in areas remote from the ischaemic injury [245]. Hence, apoptotic cell death may contribute to the progression from good (>50%) to moderate (30-50%) or poor (<30%) left ventricular ejection fraction after an acute myocardial infarction. Furthermore, the significance of apoptotic cell death and its contribution to heart function in pathologies such as aortic valve disease has not been demonstrated.

1.8 Apoptosis in ischaemia/reperfusion injury of the heart

Ischaemia/reperfusion injury in cardiomyocytes via the generation of oxidative stress, leading to the production of reactive oxygen species (ROS) has been shown to induce apoptosis [67,287]. In the context of cardiac surgery, the myocardium is subjected to a period of global ischaemia while on cardiopulmonary bypass. Cardiopulmonary bypass
will by itself, lead to the activation of neutrophils leading to the release of ROS like superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (OH)\cite{25,238} and the activation of cytokines and complement \cite{138}. Reperfusion of the ischaemic myocardium may lead to damage to the endothelium leading to inhibition of nitric oxide (NO) production by endothelial nitric oxide synthase (eNOS) \cite{151}. The damage is further exacerbated by the relatively low anti-oxidant status of the myocardium as a result of preceding ischaemia \cite{24}. NO synthesis has been shown to prevent apoptosis in ischaemia/reperfusion injury in the myocardium through inhibition of caspase-3 activity \cite{294}. Furthermore, adhesion of neutrophils to the endothelium during reperfusion also leads to release of ROS from these cells as described above \cite{25,238}. The resultant severe oxidative stress can target lipids and lipid-soluble anti-oxidants in patients \cite{45} with the formation of lipid hydroxperoxides \cite{182}. It has also been shown that these free radicals can also bind to sulphhydril groups on membrane proteins \cite{73}. Other investigators in our laboratory have demonstrated that in diabetic patients there is a rapid increase in nitrotyrosine, an index of peroxynitrite generation before the initiation of cardiopulmonary bypass \cite{182}. The same study also reported a steady increase in protein carbonyls in the plasma before and during cardiopulmonary bypass further supporting the concept that oxidative stress is exacerbated in diabetics as compared to non-diabetics during cardiac surgery. In the same study \cite{182}, glycercyl trinitrate (GTN), a nitric oxide donor, decreased the formation of carbonyls and lipid hydroxyperoxides in patients with diabetes and the production of nitrotyrosines in both diabetic and non-diabetic patients.
Unpublished results in our laboratory have shown that hydrogen peroxide, a strong inducer of oxidative stress, results in increased apoptosis in the human myocardium. Incubation in 100μM hydrogen peroxide (H₂O₂) followed by 2 hours of reoxygenation at 37°C in normoxic Krebs Henseleit Hepes (KHH) buffer at a pH of 7.4 resulted in a significant production of DNA strand breaks as measured by terminal deoxynucleotidyl transferase (Tdt)-mediated dUTP nick end labelling (TUNEL) technique. Also, DNA gel electrophoresis was performed on DNA extracted from tissue after similar treatment which showed a typical ladder pattern of apoptosis [50]. Other investigators have shown that reactive oxygen species derived from hydrogen peroxide result in apoptosis in cultured neonatal rat cardiac ventricular myocytes [3]. Oxidative stress has been implicated in apoptosis of several types including rabbit cardiomyocytes [89], rat kidney cells [258] and rat brain [287].

Experiments in transgenic mice have shown that overexpression of Bcl-2 renders the heart more resistant to apoptosis and I/R injury [40]. A mitochondrial pathway that is resistant to oxidative stress induced apoptosis involves activation of mitochondrial ATP-sensitive potassium (mito $K_{ATP}$) channels in cultured neonatal rat myocytes [3]. Furthermore, overexpression of heat shock proteins (HSP), HSP60 and HSP10, individually and in combination result in resistance to apoptosis induced by simulated ischaemia and reoxygenation [160]. There is evidence that the inhibition of p38 mitogen-activated protein (MAP) kinase results in a decrease in apoptotic cell death and improved cardiac function in isolated rabbit hearts [172].
Thus, there are several reports of the interaction of different components of the apoptotic pathway and ischaemia-reperfusion injury that provide evidence that oxidative stress induces apoptosis in animal models and that this injury can be ameliorated by antioxidant therapy.

However, the full significance of apoptotic cell death in ischaemia-reperfusion injury in the human myocardium remains to be determined. It is, therefore, important to establish the temporal relationship between ischaemia/reoxygenation induced apoptosis and necrosis in the human model. This will pave the way to distinguish the pathways involved in these pathophysiological processes.
DNA damage
Mild
Irreversible
Excessive
PARP activation
overactivation of PARP
DNA repair
SURVIVAL OF CELL
procaspase-3, 8, 9
nuclear membrane

APOPTOSIS < p53 mediated
mitochondrion intermembrane space
Caspase-9
procaspase-9
AIF
ROS
Cyt c
Apaf-1
t-Bid

Death receptor complex

Death receptor complex
Cell Exterior

antagonists to PARP and caspase independent / dependent pathways are shown encircled.

With thanks to: Alan Fowler
1.9 Synthetic caspase inhibitors

The use of synthetic caspase inhibitors facilitates the analysis of caspase activity in \textit{in-vitro} research experiments. The hope is that development of these pharmacological applications will result in a reduction of harmful apoptosis in several disease states.

1.9.1 Mechanism of action

Synthetic caspase inhibitors are peptides that act as competitive substrates for the true substrates for cleavage. They range from a single amino-acid residue (e.g. Boc.Asp.FMK) to trimers (e.g. z-VAD.FMK) or tetramers (e.g. YVAD.FMK). The mechanism of action of these inhibitors relies on the chemical groups attached to them. Irreversible, competitive inhibitors are produced by linking the peptide to fluor- or chloro-methyl ketones (.FMK, .CMK). The linking of an aldehyde group (.CHO) leads to the formation of a reversible inhibiton complex that does not chemically alter the caspase. Although peptide inhibitors are potent agents, their activity may be limited as a consequence of their membrane permeability in intact cells. The .FMK inhibitors are more membrane permeable than the .CHO inhibitors which function in intact cells at a concentration of less than 1 µM. In general, the shorter the peptides, the greater the permeability.

1.9.2 Specificity

The efficacy of these inhibitors may, however, be limited as a result of inhibition of other proteases for example cathepsin, both in vitro and in cultured cell lines at concentrations that inhibit caspases [256]. YVAD, although less membrane permeable, is the more
specific. Others agents like DEVD.CHO and DEVD.FMK, although potent inhibitors of caspase-3, also partially inhibit caspase-7, 8 and 10 as well [77,178].

1.9.3 In vivo use of synthetic inhibitors

Caspase inhibitors were first used in models in which liver damage is induced by antibodies to CD95. It was shown that mice pretreated with z.VAD.FMK were resistant to liver injury induced by up-regulation of CD95 or TNFα [148]. Apoptosis induced in an hepatic cell line using similar agents can be blocked by YVAD.CMK [239]. It has also been shown that caspase inhibition is effective at reducing reperfusion injury in the myocardium following attenuation of apoptosis [299] in an animal model. Also, Ruetten et al [241] showed that DEVD.CHO inhibition of I/R-induced caspase-3 activation resulted in a substantial improvement of post-ischaemic contractile recovery, whilst not affecting apoptosis.

On the basis of the current literature on animal models, caspase inhibitors may seem highly attractive therapeutic tools. However, issues relating to permeability, specificity, potency, toxicity and delivery need to be addressed. Further research to determine their efficacy in humans is vital before these drugs can be tested for the management of cardiac and other human diseases.
1.10 Cardioprotection

Early reperfusion is the primary goal in any strategy for maintaining viability and protecting the heart from the harmful effects of ischaemia. However, early reperfusion is not always possible or practical, especially in an evolving myocardial infarction. This conundrum has given rise to the concept of cardio-protection in which a variety of interventions are used to slow the rate of progression of ischaemic injury to contain irreversible injury such that more tissue is available for salvage when reperfusion is finally attempted. It has been suggested that tolerance of the heart to ischaemia may be enhanced in many ways including

1. promotion of anaerobic metabolism with exogenous glucose
2. the reduction of intracellular Ca$^{2+}$ overload with drugs like calcium antagonists or
3. limiting cell swelling with osmotic agents.

Many studies have reported the success of various interventions in improving function upon reperfusion, reducing infarct size or a reduction in markers of cardiac injury. This concept may ultimately be translated from the laboratory to the clinical arena.

1.10.1 Methods of cardio-protection during cardiac surgery

Three different approaches have been shown to reduce injury, namely:

1. The rapid and complete chemical induction of cardiac arrest with various cardioplegic solutions based on concentrations of potassium [105,130], acetylcholine [211] or tetrodotoxin [39] or the depletion of extracellular calcium [28]. Several additives have been incorporated into cardioplegic solutions in order to stabilise the cell membranes
[105,109], to supply energy [221,231,288], to improve the redox state [38], to reduce calcium overload [107,297] and so on and so forth.

(2) The reduction of cardiac metabolism by the use of hypothermia. It has been shown that there is increased protection as the temperature of the heart is progressively reduced from 37°C to 4°C [104,106,130].

(3) The use of ‘intermittent ischaemia’ which uses short periods of ischaemia with adequate periods of reperfusion [279].

(i) Cardioplegia

Cardioplegia employs the infusion of a protective solution through the coronary vasculature, resulting in rapid diastolic arrest and the slowing of degradative cellular reactions [29]. Myocardial protection by cardioplegia solutions is based on the principle that arresting the heart in diastole reduces the energy requirements by the heart by 90%. The distribution of cardioplegia may, however, not be homogenous in severe coronary artery disease thus leaving areas vulnerable to ischaemic damage.

(ii) Hypothermia

Hypothermia works by reducing myocardial oxygen consumption. It can be classified as mild (34 degrees C), moderate (28-32 degrees C) or deep (15-22 degrees C). Deep hypothermia is a recognised method of achieving neuroprotection during total circulatory arrest, but is not without serious implications and risks to the patient. Furthermore, a reduction of only an additional 7% is reported in myocardial oxygen consumption with a
combination of $28^\circ C$ and electromechanical arrest of the heart (which on its own leads to a 90% decrease) [30,104]. Therefore, the extra benefit obtained from hypothermia is low.

Randomised controlled trials (RCTs) have been conducted to study the effects of hypothermia in cardiac surgery. A recent review [235] of seventeen RCTs could not find a definite advantage of hypothermia over normothermia in the incidence of clinical events. Hypothermia was associated with a reduced stroke rate, but this was off set by a trend towards an increase in non-stroke related perioperative mortality and myocardial damage. Further trials are needed to comment on the effect of temperature during cardiopulmonary bypass on subtle neurological deficits.

The adverse effects of hypothermia include the production of metabolic acidosis, a shift of the oxyhaemoglobin dissociation curve to the left (corresponding to a high affinity), an increase in plasma viscosity and a decrease in erythrocyte deformability (reducing flow through the micro-capillaries). Hypothermia also causes vasoconstriction which may hinder the blood supply. Despite a reduction in oxygen consumption, cold protection does not completely stop the cellular energy consumption [193], and does not prevent the impairment of enzymatic activity and myocardial contracture [19]. As a result, hypothermia is at present less frequently used.

(iii) Intermittent ischaemia

Intermittent cross-clamping is a well-recognised method of avoiding ischaemic injury during cardiac surgery. Studies have shown that the clinical outcome in terms of
incidence of perioperative infarction, survival and event-free follow-up is not different between intermittent cross-clamping and cardioplegia [50,52,70]. However, it has been reported that cardiac function immediately after cessation of cardiopulmonary bypass is better with cardioplegia than with intermittent cross-clamping [70] and that cardioplegia results in a better preservation of high-energy phosphates, glycogen, and ultrastructure than intermittent cross-clamping [70].

1.10.2 Effect of cardioplegia on apoptosis

A recent comparative study of the cardioprotective role of hypothermic cardioplegic arrest versus ischaemia/reoxygenation on the regulation of mRNA gene expression in isolated rabbit heart, using cDNA microarrays, has revealed cardioplegic arrest to downregulate the pro-apoptotic p53 pathway and enhancement of anti-apoptotic heterodimer Bcl-2 family members, with a corresponding upregulation of Bcl-2 and downregulation of the proapoptotic Bak [207]. Furthermore, these same authors show that preservation of mitochondrial ATP synthase mRNA expression accompanies improved indices of functional recovery following reoxygenation/reperfusion when hypothermic cardioplegia is employed. Interestingly, it has also been demonstrated that the high extracellular K\(^+\) concentrations as those used in cardioplegic solutions inhibit cytochrome-c release, with a decreased caspase-3 activation and endonuclease activity, leading to reduced inter-nucleosomal DNA laddering and inhibition of apoptosis [305].
1.10.3 Ischaemic Pre-conditioning

Another method to protect the heart from ischaemic injury is the phenomenon known as *ischaemic pre-conditioning*. With this phenomenon one or more brief (>3 min) periods of ischaemia and reperfusion afford remarkable protection against a subsequent extended ischaemic insult [196]. The benefit is temporary, the acute phase disappearing after about 2 hr (*classic or early preconditioning*). However, the protection reappears about 24 hrs and lasts 24-72 hrs before disappearing altogether (*late preconditioning*). The phenomenon has been shown to exist in all species studied and many have argued that it is one of the most powerful methods of attenuating ischaemic injury. To overcome the practical and ethical problem of inflicting ischaemia in an already diseased heart and to avoid the difficulties that arise from the transient nature and tolerance to this kind of protection, pharmacological preconditioning [48] was successfully introduced with transient exposure to drugs like adenosine, catecholamines, bradykinin, opioids, nitric oxide and angiotensin II. The mechanism of pre-conditioning is via a receptor-mediated, G protein-coupled, signal transduction process involving the activation and translocation of one or more protein kinases which, in turn, mediates the phosphorylation of an end effector which is ultimately responsible for conferring protection. Some investigators [34,49,94] have suggested that one such end effector may be the $K_{ATP}$ channels that are localized in the mitochondrial and sarcolemmal membranes.

So far as I am aware, there is no data in the literature to show that cardioplegia is superior to ischaemic preconditioning alone. It has been shown that hyperkalemic arrest results in inhibition of calcium overload, and the reduction of apoptosis [129], maintenance of
Na/K pump activity [145] together with higher energy and nutrient reserves and an upregulation of transcriptional capacity [207]. This effect of cardioplegia on cell membrane channel function combined with upregulation of the pathways of apoptosis inhibition and in combination with mito-K$_{\text{ATP}}$ channel activation [292] may provide for an enhanced anti-apoptotic myocardial protection when compared to ischaemic preconditioning.

1.10.4 Effect of preconditioning on apoptosis

While several studies have shown that apoptosis contributes to cardiac cell death after ischaemia, emerging evidence indicates that preconditioning may suppress apoptosis in addition to necrosis in intact hearts [312]. The exact mechanism(s) by which preconditioning leads to cardioprotection by inhibition of apoptosis is still unknown, but may be a result of opening of channels in the mitochondrion following activation of intramitochondrial signalling pathways.

The opening of mitochondrial ATP sensitive potassium (mito-K$_{\text{ATP}}$) channels can lead to either the enhancement or attenuation of cardioprotection with the preconditioning mechanism highly dependent on the phasing of channel opening [219]. Thus mito-K$_{\text{ATP}}$ channels may signal protection through: (i) inhibition of cytochrome c release, (ii) the optimization of energy production through depolarisation of the mitochondrial membrane potential with alterations in mitochondrial Ca$_{\text{2+}}$ handling, and/or (iii) the modulation of reactive oxygen species (ROS) production, including oxygen free radicals [20,316] and nitric oxide [128], during ischemia or reperfusion.
Previously, Akao et al [3] have shown that the opening of mito-K$_{\text{ATP}}$ channels inhibits oxidative stress induced apoptosis in rat cardiomyocytes. Using ischaemic and pharmacological preconditioning of human tissue our group has shown that opening of mitochondrial K$_{\text{ATP}}$ channels signals the activation of both PKC and p38MAPK. Both downstream elements have been shown to be inhibited by the antagonists 5-hydroxydecanoate (5-HD: a presumed specific mito-K$_{\text{ATP}}$ channel inhibitor) or chelerythrine and SB203580, respectively [168]. By inducing a sublethal production of ROS, the short ischaemic stimulus associated with preconditioning by ischaemia may confer cardioprotection through opening of mito-K$_{\text{ATP}}$ channels [170] or via activation of protein kinase C isozymes leading to attenuation of ROS [138] and the inhibition of caspase-8 induced apoptosis [251]. In my research design, studies were carried out to determine the effect of ischaemic preconditioning through the inhibition following the activation of antiapoptotic pathways. Using the human right atrial trabeculae model, I attempted to determine whether this intervention lead to cardioprotection through an effect on apoptosis.
1.11 Human models of reperfusion injury and preconditioning

Much work has been done in the context of ischaemia, reperfusion and preconditioning and their relationship to apoptosis. However, many issues still remain unresolved. In contrast to the animals used in experiments that have a normal coronary artery tree, human subjects frequently suffer from coronary artery disease. Furthermore, investigation into the underlying mechanisms of apoptosis- and necrosis-related reperfusion injury and cardioprotection in humans in-vivo is hindered by ethical constraints.

Although isolated human cardiomyocytes have also been successfully used to demonstrate the phenomenon of ischaemic preconditioning [115], they are difficult to obtain, cell to cell interactions are not present and the induction of global ischaemia, reperfusion and preconditioning is technically more difficult to achieve. Isolated cardiomyocytes would however be feasible for the kind of quantitative studies I have performed requiring counting thousands of nuclei as part of one experimental project.

In-vivo models are helpful in studying the patho-physiological short and long-term effects of the pathways under investigation. The percutaneous transluminal coronary angioplasty (PTCA) is an unique in-vivo tool that has been used to study the effects of reperfusion and preconditioning in the human heart [53,57,276]. The major advantage of such a model is the ability to control ischaemia and reperfusion in individual regions of the heart under vision. The clinical response may be apparent promptly and biochemical
markers in the blood can be measured simultaneously. The results should, however, be interpreted with some caution as the confounding effects of collateral coronary circulation cannot be excluded. Furthermore, the technically challenging manoeuvre to retrieve myocardial tissue makes it less attractive to carry out studies on apoptosis with TUNEL and caspase assays.

Cross clamping the aortic arch during open heart surgery induces global ischaemia. The effects of collateral flow are thus eliminated. Pharmacological or ischaemic preconditioning protocols may be applied before this step to look at their effect on clinical outcome. Although some investigators [120] have shown beneficial effects of preconditioning in this situation, others have been unable to do so [136, 226]. Atrial appendage would not be useful here as it can only be taken once at the time of insertion of the venous cannula into the right atrium before the application of cross-clamp. Multiple adequate-sized biopsies of the ventricle would need to be taken before and after interventions to see the effect on cell viability as well as to determine cell injury using the TUNEL and caspase assays.

An in-vitro functional human atrial trabeculae model has been developed in Yellon's laboratory that has enabled to examine the effects of ischaemic and pharmacological preconditioning [288]. Zhang et al [307] characterised the human atrial appendage model at Glenfield Hospital whereas Ghosh et al [82] fully characterised preconditioning in this model system. This model has proved to be a valuable tool to investigate myocardial ischaemia/reperfusion injury, the phenomenon of preconditioning and the underlying
mechanisms in human myocardium [82,168,307]. A superfused in-vitro model, the human atrial appendage model, is less likely to be influenced by haematological and neurohormonal factors than more complex in-vivo models like the Langendorff isolated heart preparation used in rat, mouse, rabbit, pig, hamster, guinea pig and so on. It is ethically impossible to carry out studies with the induction of myocardial ischaemia for longer periods in humans. In-vivo animal heart models have been developed to examine the effects of ischaemia and reperfusion, but it is not always possible to extrapolate the results from these experiments to humans. The right human atrial appendage model is easy to use, economical and readily available means of undertaking studies on ischaemia/reoxygenation in the human myocardium. As this model is central in my thesis, it is discussed in further detail in Chapter 2.

1.12 Hypotheses tested in my thesis

These include:

1. Apoptosis and necrosis occur in the first 24 hours of I/R in the right human atrial appendage model and that I/R is associated with up- and down-regulation of genes.

2. Caspase are activated during I/R of the human atrial myocardium and that apoptosis induction and caspase activation are inhibited with the use of caspase inhibitors.

3. IPC leads to a reduction in apoptosis and necrosis induced by I/R in the right human atrial appendage model and that mitoK\textsubscript{ATP} channels, PKC and p38MAPK play a role in the signal transduction pathway involved.

4. Caspase inhibition and IPC are associated with the up- and down-regulation of genes in the human atrial myocardium.
1.13 Aims of my study

My study has the following specific research aims:

1.13.1 Elucidation of the time-course of apoptosis and necrosis in ischaemia/reoxygenation injury in the human myocardium and to define the effect of myocardial I/R on gene expression.

The significance of the role of apoptosis in I/R injury was determined by elucidating the timing and extent of apoptosis in I/R injury in the human myocardium. Sections of tissue were subjected to simulated graded ischaemia of 0, 30, 90 and 180 minutes followed by 2, 8 and 24 hours of reoxygenation. In this way a model of apoptotic and necrotic cell death during I-R injury was characterised. The I/R period that was found to produce the maximum amount of apoptosis was then used in subsequent experimentation employing caspase inhibition, cardioplegia and preconditioning protocols. The upregulation and downregulation of genes that may have an influence on the injury sustained during I/R were determined by subjecting the atrial muscles to SI and SI/R as compared to aerobically incubated tissue.

1.13.2 Investigation of the role of caspases in apoptosis in ischaemia/reoxygenation of the human myocardium.

This study was designed to determine whether I/R-induced apoptosis and caspase activation could be reduced with caspase inhibitors. To achieve this, experiments were
first carried out to establish the dose-response relationship in the absence and presence of increasing doses of irreversible, cell membrane permeable inhibitors of caspase-3 and caspase-8+9 (z.DEVD.FMK and z.IEDT.FMK+z.LEHD.FMK, respectively). The inhibitors were used throughout the perfusion protocols. Total caspase, caspase-3 and caspase-8+9 were measured in the tissue at the end of 90min ischaemia and 2 hours reoxygenation using caspase fluorometric assay. The most appropriate dose (section 4.5.1, figure 4.2&4.3) of the inhibitors for caspase inhibition was used in a new set of experiments to see the effect on apoptosis, necrosis and caspase activity with similar time-periods.

1.13.3 Evaluation of the effect of ischaemic preconditioning on apoptosis and necrosis sustained during ischaemia/reoxygenation of the human myocardium.

It has been shown in my laboratory that in the human atrial muscle [82] maximal cardioprotection by preconditioning is achieved by a single cycle of 5 minutes of simulated ischaemia followed by 5 minutes of reoxygenation, before the tissue is subjected to further ischaemia. Additional number of cycles of preconditioning stimuli did not confer any additional benefit. I, therefore, used this protocol to evaluate the effect and potency of ischaemic pre-conditioning on myocyte apoptosis and necrosis induced by I/R injury.
1.13.4 Investigation of the role of mitoK\textsubscript{ATP} channels, protein kinase C (PKC) and p38MAP kinase in the reduction of apoptosis by ischaemic preconditioning.

This study was undertaken to investigate whether mitoK\textsubscript{ATP} channels, PKC or p38MAPK, that have been shown to be part of the signal transduction pathway of preconditioning [168] are also mediators of the reduced apoptosis in ischaemic preconditioning in the human myocardium. The inhibitors employed were 5-hydroxydecanoate (5-HD), chelerythrine and SB203580 and the activators used were diazoxide, anisomycin and PMA, respectively.
CHAPTER II

EXPERIMENTAL MATERIALS AND METHODS
2.1 Introduction

In this chapter, I will discuss the different experimental techniques used to carry out my research. First, an outline of the various steps in each of the experimental techniques will be discussed. The chapter ends with an overview of the advantages and limitations of the techniques, in particular, the human right atrial trabeculae model and the commonly used ways of assessing apoptosis.

2.2 Patients

Patients undergoing elective coronary artery bypass grafting and/or aortic valve surgery were included. This allows retrieval of the atrial appendage at the time of application of the purse-string suture in the right atrium before the insertion of the venous cannula (Leicestershire Research Ethics Committee reference no. 7805) The right atrial appendage is generally regarded as ‘surgical waste’ by the end of the operation as the purse-string is tied and is disconnected from the rest of the circulation. Tissue from patients with atrial fibrillation, a poor ejection fraction (EF<30%); those with diabetes receiving oral hypoglycaemic drugs, insulin or potassium channel activators [167] (nicorandil or diazoxide) was excluded.

2.3 The Human Right Atrial Trabeculae Model

2.3.1 Preparation of atrial tissue and solutions

The experiments are based on an in-vitro model of ischaemia/reoxygenation that has been developed in University Clinical Sciences, Cardiac Surgery Division, Glenfield Hospital,
Leicester [307]. The human right atrial appendage from patients undergoing elective coronary artery bypass graft or aortic valve surgery is harvested at the time of cannulation of the right atrium. For this, local ethical approval and patients' informed consent was obtained (Leicestershire Research Ethics Committee reference no. 7805). The appendage is mounted onto an ice cooled ground glass plate with the epicardial surface face down and then sliced freehand with surgical skin graft blades (Shwann-Morton, United Kingdom) to a thickness of between 300 and 500 μm. The specimen and slide are kept moist throughout the procedure. The muscle sections weighing between 30-50 mg are then transferred to conical flasks (25ml Erlenmeyer flasks, Duran, Astell Scientific Sidcup, Kent, UK) containing 10 ml of oxygenated buffered solution. Following this, the flasks are placed in a shaking water bath (figure 2.1) maintained at 37°C. The oxygenation of the incubation medium is maintained by a continuous flow of 95% O₂/5%CO₂ gas mixture to obtain a pO₂ between 25 and 30kPa and a pCO₂ between 6.0 and 6.5 kPa. These sections are equilibrated for 30 minutes in oxygenated Krebs Henseleit Hepes (KHH) buffer containing (in mM) NaCl (118), KCl (4.8), NaHCO₃ (27.2), MgCl₂ (1.2), KH₂PO₄ (1.0), CaCl₂ (1.20), glucose.H₂O (10), HEPES (20) at a pH of 7.4 and a temperature of 37°C. The buffer is supplemented in my experiments with 10% foetal calf serum (FCS: Harlanseralabs #S-0001A) since serum deprivation promotes apoptosis in isolated rat cardiomyocytes [260]. Ischaemia is simulated by bubbling the media with 95% N₂ and 5% CO₂ at pH6.8 in the absence of glucose.
Figure 2.1 Shaking water bath with the atrial slices in Erlenmeyer conical flasks (labelled separately for each experimental protocol) containing 10 mls of the perfusate. Gas ports are seen to enter the flasks through rubber stop corks.
2.4 Experimental Techniques

2.4.1 Collection of human right atrial appendage, preparation of sections and ischaemia-reoxygenation protocols

Once the human right atrial appendage was collected from the patients, the sample was transferred immediately from theatre to the laboratory in oxygenated KHH buffer on ice. Free hand sections of the atrium are cut as described above. The structural and functional properties of the myocardium are preserved in such sections [123]. After equilibration the sections were subjected to the various protocols. At the end of these time-periods, tissue was processed either for cellular viability, preparation of sections in the microtome for labelling or snap frozen in liquid nitrogen for caspase assay.

2.4.2 Measurement of tissue injury by Creatine Kinase (CK) assay.

Damage to the myocardium leads to the release of creatine kinase into blood in humans. Cardiomyocyte injury was measured by myocyte specific creatine kinase (CK) release [234] into the perfusate at the end of 2 hours of reoxygenation. The enzyme activity was measured by a linked-enzyme kinetic assay (IU/gr wet weight) at the end of the experimental protocol employing a commercial assay kit (DG147-K: Sigma Chemicals, Perth, Australia).

The enzymatic reactions involved in the assay are as follows:

Creatine Phosphate + ADP →> Creatine + ATP

ATP + Glucose →> ADP + G-6-P

G-6-P + NADP →> 6-PG + NADPH
During this oxidation an equimolar amount of NADP is reduced to NADPH resulting in an increase in absorbance at 340nm. The rate of change in absorbance is directly proportional to CK activity. Approximately 2% of CK activity measured is lost when the assay mixture is stored for 7 days at 4°C or for 1 day at room temperature.

2.4.3 Measurement of tissue viability by the MTT assay

Tissue viability was assessed by the mitochondrial reduction of 3-[4,5 Dimethylthiazol-2-y1]-2,5 diphenyltetrazolium bromide (MTT) to an insoluble purple formazan dye [284] (M2128-Sigma Chemicals, Perth, Australia) at the end of the reoxygenation period. The tissue was loaded into a 5 ml test-tube (Sarstedt Labware, Leicester, UK) into which 2 ml of phosphate buffer solution (0.05 mol/L) containing MTT (1.25 mg/ml; 3mmol/L at final concentration) was added. After incubation for 30 minutes at 37°C, the tissue was taken and the excess MTT was removed on a chromatography paper. The tissue was then finely diced with iris scissors in 2 ml dimethylsulfoxide (DMSO) and incubated for another 30 minutes. After this, a volume of 0.2 ml of the sample was dispensed into a 96-well flat-bottom microtiter plate (Nunc Brand Products, Rochester, NY, USA). Finally, the absorbance of the blue formazan product was measured on a plate reader (Benchmark, Bio-Rad Laboratories, Hercules, CA, USA) at 550nm and the results were expressed as mM of formazan/g wet weight.
2.4.4 Assessment of apoptosis and necrosis

The muscles were incubated for 10 min on ice with 5 μM propidium iodide (PI) in 0.1 M tri-sodium citrate and 20 mM phosphate buffered saline (PBS) at pH 7.4 to identify the necrotic nuclei. Sections were then fixed twice, first for 30 minutes then with 4% paraformaldehyde in 30% sucrose and 20 mM PBS overnight on ice and at pH 7.4. Following this, serial sections of 10 μm were cut with a Bright cryomicrotome (model OTF) at −25°C in tissue embedding matrix (Tissue Tek® OCT compound). The cryopreserved tissue sections were washed with 20 mM PBS at pH 7.4 for 2 min, then permeabilised in 0.02 mg/ml proteinase-K for 10 min at 37°C, and pre-sensitised for 1 minute in a microwave oven at 800 watts in 0.1% Triton X-100 and 0.1 M sodium citrate at pH 6.0. To assess apoptosis, the terminal deoxynucleotidyl transferase (TdT) was used to incorporate fluorescein (FITC) labelled dUTP oligonucleotides to DNA strand breaks at the 3'-OH termini in a template dependent manner (TUNEL technique) using a commercially available kit (Roche: 1684795, Basel, Switzerland). The FITC fluorescence emission (range 600-630 nm) was measured using argon-ion fluorescence excitation at 488 nm and detected by laser confocal epifluorescence microscopy with a ×10 oil immersion objective. The PI labelled nuclei was excited with helium-neon laser light at 543 nm and fluorescence was detected using an emission range of 680-730 nm in order to abolish fluorescence ‘bleed-through’ from FITC labelled nuclei. Analysis was done using NIH Image software (Scion Corp, Frederick, Maryland, USA) with the Cavalieri-3 macro (G. MacDonald, University of Washington). Fluorescent signals with areas greater than 16 μm² were counted to ensure that only cardiomyocyte nuclei are taken into account and to avoid the inclusion of artefact. Absolute numbers of green fluorescent apoptotic (A)
and necrotic (N) red fluorescent nuclei in any given image field were determined by dividing by the total number of PI labelled nuclei (M) in the next serial, or mirror section. The absolute percentage of apoptotic cells was given by $A/M \times 100\%$ and the percentage of necrotic cells by $N/M \times 100\%$.

### 2.4.5 Quantitation of caspase activity by fluorometric assay.

The muscle sections stored at $-80^\circ\mathrm{C}$ until analyses were thawed in 400μl of cell lysis buffer (in mM: Hepses (100), 10% sucrose, 0.1% Chaps and DTT (10), in the presence of a cocktail of enzyme inhibitors (P2850-Sigma Chemicals, Perth, Australia) at a pH of 7.0 to release the intracellular contents. The sections were diced finely and then homogenised (Ultra-Turrax homogeniser: Janke and Kunkel GmbH, Staufen, Germany) at 13,000 rpm for 1 minute on ice. This was followed by centrifugation (PK121R-ALC International) at 14,000 rpm for 30 minutes. Subsequently, the protein concentration of the soluble supernatant (cell lysate) was measured using a detergent compatible Bio-Rad assay (23225-Pierce, Cheshire, UK). Aliquots of cell lysate were then tested for caspase activity by the addition of a caspase-specific peptide or substrate, DEVD [36], that is conjugated to the chromophore (fluorescent reporter molecule) 7-amino-4-trifluoromethyl coumarin (AFC). The cleavage of the peptide DEVD from DEVD.AFC (final concentration 20μM; Alexis Chemicals, San Diego, CA, USA) releases AFC that when excited by light at 400nm emits fluorescence at 505nm. The level of caspase activity in the cell lysate is detected by fluorescence signal obtained with a fluorometer (Fluostar P401, BMG software, Longmont, CO, USA). The amount of caspase-3 like...
activity was measured by using the effector caspase inhibitor [64] z-VAD.fmk at a final concentration of 10µM in the well of the reader plate and by subtracting the fluorescence obtained by the total fluorescence measured in the absence of the inhibitor. The inherent fluorescence of the aromatic residues in the protein homogenate was subtracted in the final calculations. The results were expressed as units of activity/g wet weight.

2.4.6 cDNA microarrays

(i) RNA preparation:
The right atrial appendage from 3 different patients and subjected to each experimental protocol were pooled. Total RNA was extracted using the RNeasy Total RNA Isolation Kit (741104; Qiagen, Crawley, West Sussex, UK). RNA was quantified spectrophotometrically by reading absorbance at 260 and 280 nm. To assess the quality of the total RNA, 4 µg of each extract was run in a formaldehyde denaturing agarose gel (1.2% agarose, 0.02 M 3-[N-morpholino]propanesulfonic acid (MOPS) pH 7.0, 0.6 M formaldehyde, 5 mM sodium acetate and 1 mM ethylenediaminetetraacetic acid (EDTA)). The gel was first equilibrated by a pre-run for 30 minutes at 7 V/cm in formaldehyde running buffer (0.02 M MOPS pH 7.0, 0.6 M formaldehyde, 5 mM sodium acetate and 1 mM EDTA). Samples (10 µL) comprising 4 µg total RNA, blue dye loading buffer (MBI Fermentas, Hanover, MD, USA) and diethyl pyrocarbonate (DEPC)-treated water were denatured at 65°C before loading in the gel and running at 7 V/cm for 150 minutes. Sizes were assessed using the RNA ladder, High Range Marker (MBI Fermentas, Newington, NH, USA). After electrophoresis, the gel was stained in 0.5 µg/mL ethidium
bromide for 30 minutes, destained for 5 min in DEPC-treated water, and visualized under ultraviolet (UV) light.

(ii) Oligoarray hybridization and scanning:

Affymetrix (Santa Clara, CA, USA) HG-U133A GeneChip® was used which monitors the relative abundance of the mRNA for 22284 full-length human genes. Standardised procedures were followed for the preparation of whole RNA; cDNA copies of the RNA message (probe), fluorescent labelling of the probe, the washing steps and conditions for establishing specificity during hybridization of the probe to the chip-mounted oligonucleotide, the scanning and image analysis of the immobilized fluorescent label; all followed laboratory techniques supplied by the manufacturer. Briefly, labelled cDNA copies of the RNA message for each gene present were fragmented to an average size of 20-200 bases and hybridized to the immobilised oligo-probe arrays at 99°C for 5 minutes, cooling to 45Cs for 5 minutes. Hybridized arrays were scanned in the G2500A Hewlett-Packard (Palo Alto, CA, USA) Gene Array Scanner at a wavelength of 488 nm. The amount of fluorescent light is assumed to be proportional to bound target at each location on the probe arrays.

(iii) Microarray data analysis

Gene induction or repression was considered significant if the change in hybridization intensity was greater than twofold and signal intensity differences between arrays were 50 or greater. Affymetrix Microarray Suite version 4.0 and DNA-chip analyser (dCHIP) version 1.3 was used for data analysis. Similar gene changes obtained with the two softwares are presented in the results section.
2.5 Discussion

2.5.1 Patients

Certain groups of patients were excluded from my studies. This was done to maintain uniformity in the experimental design as the myocardium from these patients has been shown (see below) to behave in a different manner to cardioprotection strategies. This deserves further discussion.

Diabetes Mellitus

Ghosh et al [81] using the human atrial trabeculae model have reported that tissue from patients with diabetes who are on long term oral hypoglycaemics or on insulin are not protected by ischaemic preconditioning, although the injury induced by ischaemia/reoxygenation is not exacerbated in these conditions. This is in agreement with the results reported by Cleveland et al [43] in their functional human atrial model of myocardial stunning who demonstrated that myocardium from diabetic patients on longterm \( K_{\text{ATP}} \) blockers also does not precondition. However, their study revealed that myocardium from insulin-dependent diabetics can be preconditioned, although not to the same extent as non-diabetics. Experimental work in animals in a rat model of streptozocin-induced diabetes supports the notion that diabetes \textit{per-se} results in loss of cardiac protection.

Poor left ventricular function

Ghosh et al [81] have shown that myocardium from hearts exhibiting a LVEF<30% cannot be preconditioned. This is in contrast to a previous study [43] which showed that
isolated ventricular trabeculae obtained from patients undergoing cardiac transplantation can be preconditioned. Similarly conflicting results [44,55] have been shown by different groups in this regard. Because of this, patients with LVEF <30% were excluded from the studies.

**K\textsubscript{ATP} channel openers**

These agents are most commonly used in the clinical setting as drugs for the treatment of angina and hypertension. Nicorandil and diazoxide are the two most commonly used drugs in clinical practice. In many animal studies, nicorandil has been shown to be cardioprotective. There is evidence in the literature purporting to show that the site of pharmacological action in the cardioprotection achieved with nicorandil are the mitochondrial K\textsubscript{ATP} channels [93,195]. Garlid et al [78], in the bovine heart and Liu et al [163], in isolated rabbit ventricular myocytes, have also shown that diazoxide leads to cardioprotection as a result of opening of mitochondrial K\textsubscript{ATP} channels. Furthermore, evidence from our laboratory [83] using the human right atrial trabeculae model has shown that protection of the ischaemic myocardium with 100μM diazoxide, a selective mitochondrial K\textsubscript{ATP} channel opener, was abolished by glibenclamide, a K\textsubscript{ATP} channel blocker. Loubani et al [167] have further shown the inability to precondition the atrial muscle of patients on long-term nicorandil. Because of this, also patients on mitochondrial K\textsubscript{ATP} channel openers were excluded.

46
Chronic Atrial Fibrillation

It has been demonstrated by Aime-Sempe et al [2] in humans that fibrillating atria contain a number of myocyte undergoing myolysis, with 45% of nuclei TUNEL positive. Similar cellular alterations were observed in the atrial appendage of a goat model [14] of pacing-induced atrial fibrillation. These changes were more severe than those seen by Mary-Rabine et al [180] where only approximately 10% dilated and fibrillating atria showed similar cardiomyocyte changes. To avoid the possible confounding effects of this phenomenon, patients suffering with chronic atrial fibrillation were also excluded from these studies.

2.5.2 The Human Right Atrial Trabeculae model

The majority of information obtained in the context of reperfusion injury and preconditioning has been gained from study of various in-vitro and in-vivo animal models. This information so gained has been used to develop pharmacological tools. In-vivo studies in humans are difficult due a variety of technical and ethical constraints. Different models have been exploited in humans to carry out studies on ischaemia-reperfusion and preconditioning. Isolated human myocytes [115], papillary muscle [44] and atrial muscle [243,289] have been used to look into the mechanisms and effects of reperfusion injury in man. Zhang et al [307] characterised the human right trabeculae model in our laboratory at the Clinical Sciences Building, Glenfield Hospital. This model may be used as an intermediary step between animal models and man to test the safety and efficacy of potential beneficial treatments before these are introduced in clinical use.
Advantages

The atrium is easily obtained from patients undergoing open-heart surgery, it is simple to prepare and inexpensive. The atrial tissue is sliced and then nourished and oxygenated in an organ bath using the principles of superfusion, in which both $O_2$ and substrates are provided through diffusion. The section thickness is critical however, since with sections ranging from 0.2 to 0.5mm, it has been shown that vascular integrity is not required to support viability and metabolic function [221].

This preparation has the major advantage of being stable for at least 24 hours [307]. Adherence to strict experimental conditions is vital. The temperature of the media for collection should be 4-10° C, for processing 37° C, the slice thickness no more than 0.5 mm and the $pO_2$ [231] of the media should be 25-30kPa [307].

Studies of the effects of ischaemia in this model [307] have revealed that significant tissue injury is directly proportional to the increasing doses of ischaemia, as measured by MTT reduction and LDH leakage, and that lengthening the ischaemic time to 2 hr lead to loss of more than 75% of the viable tissue and massive LDH leakage.

Comparison with other models

The induction of myocardial ischaemia is not possible in humans. An in-vitro model like the right human trabeculae model is less likely to be influenced by haematological and neurohormonal factors than the more complex in-vivo models. This right human atrial appendage model is easy to use, economical and readily available. Isolated perfused animal hearts such as the Langendorff heart preparation have been extensively used for
short-term studies and in these preparations the confounding effects of collateral circulation cannot be excluded. Isolated and cultured myocytes are more labour intensive, the cell-cell interactions are lacking and the attainment of complete ischaemia is technically more difficult.

Limitations

Atrial and ventricular muscle preparations have different features that may affect the vulnerability to apoptosis and necrosis as a result of ischaemia-reperfusion injury. Potassium channels, which may play a key role in reperfusion injury [219], are shown to be distributed differently in the atrium and the ventricle [9]. Although extrapolation of these results to the ventricular myocardium should be done with considerable caution, it is to be hoped that they may provide a fundamental advance in our knowledge of the effects of ischaemia/reperfusion injury and preconditioning in the human myocardium.

Another limitation of this model may be that ischaemia and reperfusion have been simulated and not by arterial occlusion and release. However, this may be advantageous as the additional vascular effects are eliminated. In addition, the superfusion model allows the introduction of pharmacological tools during the ischaemic period.

By now, many studies [80,82,83,167-169] have been carried out in our laboratory using this model to explore the different signal transduction pathways in the context of pharmacological and ischaemic preconditioning in the ‘normal’ and the pathological human myocardium. Several experimenters have obtained consistent, reproducible and
reliable results. I have, therefore, used this model of human atrial trabeculae to investigate the role of apoptosis and necrosis in reoxygenation-induced injury and preconditioning in the human myocardium.

2.5.3 CK and MTT Assays

The CK-MB and the MTT assays are standard and reliable assays for assessing tissue injury and viability, respectively. Both readouts have been used in the field of biomedical research for several years. Many studies [80,82,83,167-169] have been published from our laboratory in the context of ischaemia, reperfusion and preconditioning in the human myocardium.

It is important to emphasise at this point that I have measured CK-MB levels in the perfusate at the end 2 hours of reoxygenation, even in the 8 and 24 hour reoxygenation protocols in the ischaemia/reoxygenation studies discussed in Chapter 3. The reasons behind this are two-fold. Firstly, Zhang et al [307], in the same model, have shown in their reoxygenation studies that there is no net significant increase in cardiac enzyme leakage over 24 hours to that seen during the first 2 hours of reoxygenation. Second, this approach was taken to ensure that all the sections were treated to similar interventional levels of both ischaemia and reoxygenation. Hence, similar CK-MB release at the end of comparable time-points could be expected to indicate a comparable uniformity of application of the experimental protocols.
2.5.4 Assessment of Apoptosis

Apoptotic cell death plays an important role in different cellular events for example embryogenesis, T-cell selection and so on. These beneficial roles of apoptosis are in contrast to its role in a variety of pathological states including cardiovascular and neurodegenerative disorders. Consequently, the assessment of apoptosis has generated great interest in the field of biomedical research. Various methods are available with inherent limitations with all of them. Here, I briefly describe some of them with main emphasis on the TUNEL technique.

TUNEL staining: advantages and limitations

Apoptotic cells are not easily identified by light microscopy. TUNEL technique [8] is a staining method based on the occurrence of double strand DNA breaks in the nucleus, in which terminal deoxynucleotidyl transferase (TdT) is used to incorporate fluorescein (FITC) labelled dUTP oligonucleotides to DNA strand breaks at the 3’-OH termini in a template dependent manner. It is the method of choice for rapid identification and quantification of apoptotic cell death. TUNEL is considered more sensitive and specific than other in-situ end labelling techniques [85,88,194]. This is due to the assumption that double-strand DNA breaks occur in apoptotic nuclei at a higher frequency than single-strand breaks which are detected by the other in-situ labelling techniques. The beneficial effects of proteinase-K and micro-wave treatments are a result of cleavage of peptide bonds. Negoeescu et al [203] have shown that microwave sensitisation of TUNEL staining increases two-fold over proteinase K treatment. Protein cross-linking as a result of paraformaldehyde fixing (used in my experiments) as well as DNA-DNA crosslinkage
may possibly reduce the accessibility of 3'OH ends to TdT enzyme. Microwave pre-
treatment of sections results in 90% TUNEL staining of morphologically apoptotic cells
in paraformaldehyde-fixed cells [203]. Furthermore, rapid cooling of the samples after
irradiation has also been shown to increase the sensitivity of the TUNEL signal [203].

It is however, crucial to avoid excessive proteolytic treatment [194,203] and high energy
micro-wave (>800 milliwatts) as this may result in high false-positive value of TUNEL
staining or loss of substrate. Control of the concentrations, temperature and exposure
times of fixatives, proteolytic digestion and microwave treatment is of paramount
importance to avoid induced double-strand DNA breaks. Therefore, in my study, TUNEL
labeling was carried out using standardized pretreatments and was considered
representative of apoptosis if the fluorescent signal was bright green and the area was
16μ² (nuclear dimensions) after image normalisation. Prior to TUNEL batch labeling of
atrial tissue sections, positive controls treated with DNase-I were first TUNEL labeled
(figure 2.3) to ensure that the staining protocols were working. Also, in preliminary
studies I showed that maximum TUNEL positivity is attained with 100μM H₂O₂ (figure
2.2), a well known inducer of oxidative stress and apoptosis.

The issue of strongly TUNEL stained normal [79,88,194], repairing [134] and necrotic
[210] cells still remains a matter of ongoing research. It has been argued on the basis of
ultrastructural techniques that TUNEL labelled cells might be apoptotic but they can also
be oncotic [210] or undergoing DNA repair [134].
Necrosis is identified in my experiments by labeling of double stranded DNA with the cell membrane impermeable fluochrome propidium iodide (PI) which acts via intercalation. The staining of the cardiomyocytes with PI first enables the exclusion of nuclear labeling with the TUNEL method. Hence, in the labeling procedures adopted in my experiments, the nuclei can either be stained with PI or TUNEL and not both.
Figure 2.2 Induction of apoptosis (TUNEL staining) after treatment of atrial sections with 100µM hydrogen peroxide.

Figure 2.3 TUNEL staining after digestion of nuclei with DNAse-I.
Comparison with other methods

*Annexin-V* is another method to assess apoptosis based on the translocation of phosphatidylinerine (PS) from the inner to the outer leaflet of the plasma membrane during apoptosis [146]. This is a rapid and simple method of detecting early apoptosis. However, due to increased membrane permeability, cells in a late stage of apoptosis cannot be distinguished from necrotic cells and hence, this method is less specific than TUNEL. Furthermore, Annexin-V is also naturally secreted by cells [116,117,135,187]. Nevertheless, quantification after staining with Annexin-V label is possible but is easier with single cells. This is due to the fact that cells that are in contact with each other, for example atrial tissue or cells in confluent monolayer cultures, are impossible to distinguish by annexin label as a result of the inability to separately identify the sarcolemmal membrane for every cardiomyocyte visualised.

*Electron microscopy* (EM) is considered by many investigators as the ‘gold standard’ for morphological identification of single normal, apoptotic [174] and necrotic [121] cells. The tissue sections employed for EM are thinner (10nm) in comparison to the sections utilised for confocal microscopy (10μm). Several sections cut in a variety of planes are required to visualise >100 cardiomyocyte nuclei. Hence, to carry out quantitation of apoptotic and necrotic nuclei, this technique would be extremely time-consuming and thus impractical to conduct.

The hallmark of apoptosis is the internucleosomal cleavage of spacer DNA resulting in 180-200 base pair fragments [297]. This can be analysed by *agarose gel electrophoresis*,
which measures DNA fragmentation in nuclear extracts showing the characteristic DNA
laddering [50]. However, in the characteristic ladder pattern is seen in our model only
when apoptosis is >20%. Being an averaging method this technique is also unable to
recognise individual apoptotic cells or determine the percentage of apoptotic nuclei
(below ~20% apoptosis). Agarose gel electrophoresis also cannot distinguish between the
various cell types (cardiomyocytes, epithelial cells, fibroblasts, neutrophils). Thus, it is
highly unsuitable for the kind of studies I have carried out for my thesis. Caspase
activation is also a method of detection of apoptotic cell death and is discussed in the
next section.

Despite the apparent limitations of the TUNEL technique, it remains the most widely
used method for detection and quantification of apoptosis as it is commercially
accessible, relatively easy to use, is a method to identify programmed cell death and can
be used to stain hundreds of cardiomyocytes at a time.

2.5.5 Caspase fluorometric assay

Caspases, a key biochemical component of the apoptotic phenomenon in cells, participate
in an enzyme cascade that results in cellular disassembly contained within the cellular
membrane. The substrate for caspases consists of three to four amino acids followed by
an aspartic acid residue, with the cleavage occurring after the aspartate. These proteases
are typically synthesized as inactive precursors, or procaspases (zymogens). Inhibitor
release or cofactor binding activates the caspase by cleavage at internal aspartates
through autocatalysis or by the action of another protease / caspase [274]. The caspase
fluorometric assays used here provide a convenient and sensitive means of analysing caspase activities. Molecular Probes Inc. offer a selection of fluorogenic substrates containing the caspase-3 recognition site Asp-Glu-Val-Asp (DEVD), caspase-8 recognition site Ile-Glu-Thr-Asp (IETD) and the caspase-9 recognition site Leu-Glu-His-Asp (LEHD). Hydrolysis of the amino-fluoro-methylcoumarin (AFC)-derived substrates, which are weakly fluorescent in the UV spectrum, yields the blue product AFC which is highly fluorescent upon proteolytic cleavage. Caspase fluorometric assay kits are used to measure the activity of caspases using a fluorescence microplate reader. Caspase inhibitors were used here to confirm that the observed fluorescence is due to the activity of caspase-3 or caspase-8+9. The inhibitors used were however not completely specific for a given caspase and may interact with other caspases. It is important to avoid misinterpretation of the caspase levels obtained as the results represent ‘activated caspases’ which, depending on the redox status, may not be functionally active.

2.5.6 cDNA microarrays

Identification of the genes and proteins involved in reperfusion injury and ischaemic preconditioning is fundamental to understanding the mechanisms involved in cardioprotection in the human myocardium. Genomic technology is a powerful tool that can be utilised to look at the differential expression of tens of thousands of genes simultaneously and to compare patterns of gene expression with various metabolic processes [56]. This has the ability to provide us with a more comprehensive understanding of the role of potential multiple underlying mechanisms associated with ischaemia and reoxygenation in the human myocardium.
Microarray technology is used in my studies to identify genes that may play pivotal role in reperfusion and ischaemic preconditioning. It must be pointed out at the very outset that this latter investigation is by no means an attempt to fully characterize the molecular basis of reperfusion and ischaemic preconditioning. It is aimed to provide a preliminary sketch of genes expressed in the human myocardium as a result of induced oxidative stress using cDNA microarray. An exhaustive discussion of all the genes up or down regulated was not considered necessary. However, the whole data obtained is very helpful in generating testable hypotheses for future investigations.

Although certain patterns were notably revealed from expression data and may be indicative of functional responses underlying oxidative stress, it is important to appreciate that a change in mRNA expression may not necessarily translate into protein synthesis and is, therefore, not sufficient to establish firm functional associations among proteins. Further studies are undoubtedly indicated to determine whether this leads to translation of respective proteins and to determine their subcellular targets, potential functions and cardiovascular significance, which largely remains unexplored at present in the human myocardium.
CHAPTER III

EFFECT OF THE DEGREE OF ISCHAEMIC INJURY AND REOXYGENATION TIME ON THE TYPE OF MYOCARDIAL CELL DEATH IN MAN: ROLE OF CASPASES AND CHANGES IN GENE EXPRESSION.
3.1 Introduction

Ischaemia/reoxygenation (I/R) of the heart induces apoptosis and necrosis [72,89,131,183]; however, the actual contribution of these two forms of cell death to I/R injury and their time-course have not been established in the human myocardium and remains controversial in experimental animal models. Kajstura et al [131] have reported that apoptosis begins in rat ischaemic myocardium either after a prolonged period of permanent ischaemia or during a much shorter period of ischaemia followed by reperfusion whereas others [72,89] have shown that although apoptosis may be initiated during ischaemia, its detection is increased and may well be accelerated during reperfusion. On the other hand, studies in a dog model of coronary artery ligation have shown that necrosis, quantified histologically, develops rapidly after ischaemia and is directly proportional to the ischaemic time [236], although, there is evidence in the literature suggesting that necrosis may also result secondary to reperfusion injury [183]. Furthermore, it has also been suggested that apoptosis may switch to necrosis below certain critical levels of ATP [7,253].

The mechanisms of I/R injury have been extensively investigated, however its pathophysiology is complex and the involvement of various pathways and their importance also remains unclear. Caspases, a large protein family of cysteine proteases, have been specifically linked to apoptosis, however, Schwab et al [259] have reported that caspases mediate cleavage and inactivation of the plasma membrane Ca\(^{2+}\) pump, which lead to Ca\(^{2+}\) overload and can cause necrosis in neural and non-neural tissues.
Genomic technology is a powerful tool that can be utilised to look at the differential expression of tens of thousands of genes simultaneously and to compare patterns of gene expression [56]. This may enable us to have a more comprehensive understanding of the role of potential multiple underlying mechanisms in I/R injury.

The aims of the present studies were: (i) to investigate the degree and the time-course of apoptosis and necrosis sustained during ischaemia and reoxygenation of the human myocardium; (ii) to examine the role of caspase activation, and (iii) to define the effect of myocardial I/R on gene expression.

### 3.2 Materials and Methods

#### 3.2.1 Patients

Patients undergoing elective coronary artery bypass grafting and/or aortic valve surgery were included. The atrial appendage was retrieved at the time of application of the purse-string suture in the right atrium before the insertion of the venous cannula. Tissue from patients with atrial fibrillation, a poor ejection fraction (EF<30%), diabetics and patients taking potassium channel activators (nicorandil or diazoxide) [167] was excluded due to reasons described in section 2.5.1.
3.2.2 Collection of atrial samples and solutions

The human right atrial appendage [307] from patients undergoing elective coronary artery bypass graft surgery was harvested, prepared and subjected to ischaemia and reoxygenation as described in chapter 2 (section 2.3&2.4).

3.2.3 Experimental Protocols

Study 1: Effect of the intensity of ischaemic injury and the time of reoxygenation

After being equilibrated in oxygenated KHH buffer for 30 mins, the atrial sections were randomly allocated to various protocols (see figure 3.1): 30, 90 and 180 minutes of ischaemia followed by 2, 8 or 24 hours of reoxygenation (n=8/group). Some slices were not subjected to ischaemia and were aerobically incubated for 2, 8 or 24 hours to serve as aerobic controls. Creatine kinase (CK) release into the media was measured during the first 2 hours of reoxygenation and during the first 2 hours of aerobic incubation in the controls. The tissues were taken at the end of the protocols for the assessment of tissue viability, cell necrosis and apoptosis, and caspase activity (see below).

Study 2: The role of caspase activation in cell death

The atrial muscles (n=6/group) were subjected to the following experimental protocols: (i) aerobic perfusion for 240 minutes; (ii) aerobic perfusion for 240 minutes with the caspase inhibitor z.DEVD.FMK (70nM); (iii) 90 minutes simulated ischaemia followed by 120 minutes reoxygenation (SI/R); and (iv) SI/R with 70nM z.DEVD.FMK (70nM). The caspase inhibitor was incubated with the muscles for the entire experimental period.
As before, the CK release was measured in the incubation media during the 120 minutes of reoxygenation or during the last 120 minutes of aerobic incubation in the controls and the tissue was taken at the end of protocols for the assessment of tissue viability and cell necrosis and apoptosis.

**Study 3: The effect of ischaemia/reoxygenation on gene expression**

The right atrial appendage muscles (n=3 specimens from different subjects/group) were subjected to the following experimental protocols. (i): fresh tissue; (ii) aerobic perfusion; (iii) SI alone; and (iv) SI/R. The tissues were taken at the end of the protocols and stored at -80°C until microarray analysis.

3.2.4 Assessment of tissue injury and viability

Tissue injury was measured by myocyte specific creatine kinase (CK) release into the perfusate at the end of 2 hours of reoxygenation in all the protocols as described in section 2.4.2 (chapter 2). Tissue viability was assessed by the MTT assay as described in section 2.4.3 (chapter 2) at the end of the 2, 8 and 24 hours of reoxygenation.

3.2.5 Assessment of apoptosis and necrosis

This was done as described in section 2.4.4 (chapter 2).
3.2.6 Quantitation of caspase activity by flourometric assay

Total caspase and caspase-3 like activities were measured in the tissue homogenate by the assay described in section 2.4.5 (chapter 2). Caspase-3 like activity was detected by adding the irreversible inhibitor z.VAD.fmK at a concentration of 10μM in the well in the reader plate and by subtracting the fluorescence obtained by the total fluorescence measured in the absence of the inhibitor.

3.2.7 cDNA microarrays

This was done as described in section 2.4.6

3.3 Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) was used for comparisons of means (Microsoft® Excel analysis tool pak) with the application of a post-hoc Tukey’s test. A p value of less than 0.05 was considered statistically significant.

3.4 Results

3.4.1. Study 1: Effect of the intensity of ischaemic injury and the time of reoxygenation:

(i) Apoptosis and Necrosis

Figure 3.2 shows a low degree of apoptosis and necrosis in muscles aerobically incubated for 2 hours (3.2±1.3% and 2.8±0.8%, respectively; see photomicrographs in figure 3.7A
and 3.8A) and further increases of the two forms of cell death with the extension of aerobic incubation to 8 and 24 hours. The degree of necrosis increased with extension of ischaemia (photomicrographs in figure 3.8B and 3.8C, however apoptosis was greater than necrosis (32.0±3.2% vs 10.7 ± 1.9; p<0.05) after 90 minutes of ischaemia and two hours of reoxygenation (photomicrographs in figure 3.7C and 3.8B) and the reverse was seen after 180 minutes of ischaemia (12.6±1.9% vs 27.1%±2.8%; p<0.05). A similar pattern of cell death type was observed after 8 hours of reoxygenation (photomicrograph in figure 3.7D) although the extent of apoptosis and necrosis was greater than that after 2 hours of reoxygenation (photomicrographs in figure 3.7B and 3.7C). After 24 hours of reoxygenation, necrosis also increased with the extension of ischaemia (photomicrograph in figure 3.8D) and it was greater than after 2 and 8 hours of reoxygenation, but after this reoxygenation time necrosis was significantly more important than apoptosis.

(ii) MTT Reduction

Figure 3.3 shows that there was a decrease in MTT reduction with increasing periods of ischaemia and that, in contrast with the assessment of apoptosis and necrosis by the TUNEL assay, it was not significantly influenced by increasing the period of reoxygenation.

(iii) CK release

Table 1 demonstrates that the CK release exhibited the lowest values in the aerobic control groups and that in contrast there was an increase in CK release with increasing ischaemia. The mean CK release values during the first two hours of reoxygenation were
similar in the groups with identical ischaemic time suggesting that ischaemic injury was of the same degree in the three reoxygenation protocols

(iv) Caspase activity

Figure 3.4 shows that there was no significant difference in the total caspase activation irrespective of the time of ischaemia or reoxygenation amongst the groups and that, unexpectedly, the highest values corresponded to the fresh tissue.

Figure 3.5 demonstrates that caspase-3-like activation was significantly increased after 2 hours of aerobic incubation when compared to the mean values in the fresh muscles and that although activity increased with the extension of the ischaemic time, values were not greater than the ones seen in the aerobic control group. Importantly, by 8 and 24 hours of reoxygenation the levels of caspase-3-like activation had decreased to levels close to the values observed in the fresh muscles irrespective of the periods of ischaemia.

3.4.2. Study 2: The role of caspase activation in cell death.

(i) Apoptosis and Necrosis

Figure 3.6 shows the percentage of apoptosis and necrosis in atrial tissue after SI/R in the absence and presence of the caspase-3 inhibitor z.DEVD.FMK. z.DEVD.FMK (70nM). Caspase-3 inhibition significantly reduced apoptosis in the muscles aerobically incubated and resulted in almost complete abolition of apoptosis, from 23.3±2.8% to 0.7±0.3% (p<0.05 vs SI/R control), in the muscles subjected to 90 minutes of simulated ischaemia.
and 2 hours reoxygenation. Interestingly, z.DEVD.FMK did not influence the degree of necrosis.

(ii) MTT Reduction

Table 2 shows that there was no significant change in MTT reduction with the addition of z.DEVD.FMK in the muscles aerobically incubated and those subjected to 90 minutes of simulated ischaemia and 2 hours of reoxygenation. Since, as seen above, apoptosis was almost abolished whereas necrosis was unaffected by caspase-3 inhibition, these results suggest that the changes in MTT reduction are not a reflection of apoptosis.

(iii) CK release

Table 2 also shows that the addition of z.DEVD.FMK did not affect the CK release of the aerobically incubated muscle and that the increase in CK release induced by 90 minutes of SI and 2 hours R was unchanged, this suggesting that, as seen with MTT reduction, changes in CK release are not a reflection of apoptosis.

3.4.3. Study 3: The effect of ischaemia/reoxygenation on gene expression

The changes of mRNA expression of at least 2.0 fold on atrial muscles subjected to SI and SI/R as compared to aerobically incubated tissue are presented in table 3. This shows the upregulation and downregulation of genes that may have an influence on the injury sustained during ischaemia and reoxygenation. Genes encoding for pro-inflammatory cytokines such as TNF-α, interleukins 1 and 6, and interferon gamma were down-
regulated by ischaemia and in some cases this downregulation was maintained during reoxygenation. ATP synthase genes were upregulated by ischaemia but not during reoxygenation whereas heat shock proteins (HSP), that have been involved in cardioprotection [160,192,250], were unaffected during ischaemia but were upregulated by reoxygenation. The greater upregulation corresponded to HSP105 that raised to 12.3 fold of the expression seen in aerobic controls. The growth factors IGF-related and VEGF genes were downregulated at the end of ischaemia and during reoxygenation. The only exception was IGF binding protein 6 that was upregulated by 2.5-fold in ischaemia and was unaffected during reoxygenation. As seen in table 2, genes related with apoptosis were either up or downregulated by ischaemia/reoxygenation. Thus, Bcl-2 related gene, that protects against apoptosis [40], was downregulated by more than 6 fold by ischaemia and reoxygenation which may have a pro-apoptotic effect. However, the TNF-related apoptosis-inducing ligand (TRAIL) gene, which has been shown to induce apoptosis in tumour cells [230], and the apoptosis inducer serine/threonine kinase 17a were downregulated by ischaemia whereas the apoptosis repressor nucleolar protein 3 was upregulated by ischaemia which overall may have an anti-apoptotic effect.

3.5 Discussion

The present studies have demonstrated that, depending on the ischaemic insult, apoptosis may be the predominant form of cell death in the human myocardium during the first 8 hours of reoxygenation, so that apoptosis is more important than necrosis when the ischaemic period is <90 minutes but the reverse is true after 180 minutes of ischaemia; however, by 24 hours of reperfusion cell death by apoptosis has subsided and necrosis, that also depends on the degree of ischaemia, becomes the leading cause of cell death.
The implications of these findings for the understanding of the pathophysiology of ischaemia/reoxygenation injury are discussed below.

3.5.1. Effect of the degree of ischaemic injury and the duration of reoxygenation on the type of cell death:

This study is the first to report on the time-course of cell death by apoptosis and necrosis induced by ischaemia and reoxygenation of the human myocardium. The demonstration that apoptosis follows a bell-shaped profile, increasing with the duration of ischaemia up to 90 minutes and then decreasing by 180 minutes of ischaemia, is novel. Previous experimental studies could not show this response because they used limited time-periods of ischaemia [72,186,312]. The finding that apoptosis increases with the duration of reoxygenation from 2 to 8 hours is supported by observations in the dog heart subjected to 60 minutes of ischaemia and reperfused for 6,24,48 and 72 hours [312]. However, whilst my studies have shown a decline in apoptosis when the myocardium was reoxygenated for 24 hours, the latter study [312] reported a progressive increase in apoptosis over the 72 hours of reperfusion. The reason for the differing results of the two studies is unclear but the use of different species (eg, man versus dog) and experimental preparations (eg, in vitro versus in vivo) may be, at least in part, responsible.

The present studies have also shown that, as expected, the degree of cell death by necrosis is directly proportional to the severity of ischaemia. But, in addition, they have demonstrated that necrosis gradually increases with the duration of reoxygenation, which is consistent with the observation that necrosis is a dynamic process that continues over
aperiod of at least 24 hours of reperfusion [309,310]. It should be mentioned, however, that the degree of tissue injury as assessed by the reduction of MTT was not increased with the duration of reoxygenation. This apparent contradiction between the results obtained with the propidium iodide saining and MTT reduction is not clear, but it is possible that while the former reflects the extent of necrosis alone, the latter may represent other forms of cell death. The lack of agreement of these two assays highlights the importance of using more than one index to assess tissue injury.

3.5.2 Role of caspase activation on ischaemia/reoxygenation-induced cell death

The similar mean values of total caspase activity seen after all studied ischaemic and reoxygenation periods may suggest that this pathway may not be an important mechanism of cell death in the human myocardium or, more likely, that only specific caspases may take part in this process, the changes of which may not be of sufficient magnitude to significantly alter the whole pool of caspases. The finding that caspase-3 activity is increased by the degree of ischaemic insult would support the latter thesis. The rapid dissipation of caspase-3 activity with the extension of the reoxygenation period suggests that this enzyme may be activated for a limited time period and that, therefore, the time points investigated in our studies do not provide a complete time-course of changes in enzyme activity. A participation of caspases in apoptosis has also been observed in experimental animal studies [160,283], with the elevation of caspase-3 activity during the early reperfusion period [74,133]. It is of interest to note that in the present studies total caspase activity, but not caspase-3 activity, was elevated in fresh
tissue, a finding that may be explained by the handling and mechanical injury sustained during the sectioning of the muscle.

The almost complete abolition of apoptosis by the specific caspase-3 inhibitor z.DEVD.FMK seen in my studies demonstrates that the induction of apoptosis by ischaemia/reoxygenation in the human myocardium is caspase-3 dependent. This finding in humans is supported by in vitro and in vivo animal experimental studies [90,111,197,299] in which caspase-3 inhibition attenuated apoptosis and reduced reperfusion injury, all suggesting that caspase-3 activation is an obligatory step in the signal transduction pathway of apoptosis induced by ischaemia/reoxygenation. However, necrosis was unaffected by caspase-3 inhibition, which agrees with results reported in adult rat ventricular myocytes [133], although in cancer cells caspase inhibitors also retard necrosis in an in vitro model of chemical hypoxia [253].

Although these studies did not address the mechanism of caspase-3 activation, it has been previously shown that caspases can be activated by oxidative stress [37,62,229,277,282], an important element of ischaemia/reoxygenation injury. Furthermore, the apoptosis elicited by oxidative stress can be reduced by the opening of mitoK_{ATP} channels [3] and the apoptosis and caspase-3 activation induced by ischaemia/reoxygenation can be reduced by overexpression of the heat shock proteins HSP-60 and HSP-10 [160]. It is clear that more investigation is required to elucidate the signalling pathway leading to activation of caspase-3 during ischaemia/reoxygenation.
3.5.3 Effect of ischaemia/reoxygenation on gene expression:

The aim of the microarray analysis here was to provide basic information on the trend of changes in gene expression that may influence ischaemia/reoxygenation injury in the human myocardium; by no means was an attempt to fully characterize the complex participation and interaction of the genes changing expression and their end products. It is also important to clarify that the changes in gene expression cannot be equated to tissue injury and that whereas some of the changes may represent a participation in tissue injury, others may correspond to an intrinsic cardioprotective response of the cardiac muscle. Thus, for example, the down-regulation of pro-inflammatory cytokine genes such as TNF-α, interleukins 1 and 6, and interferon-γ by ischaemia may be interpreted as a protective response of the muscle since these factors depress cardiac contractility and induce apoptosis [1,95,217]. Similarly, the observed up-regulation of ATP synthase genes by ischaemia can be viewed as a response to the reduction in tissue high energy phosphate contents. Interestingly, no significant changes in ATP synthase-associated genes were seen after 2 hours of reoxygenation, a time when the high energy phosphate pool is expected to be partially or completely replenished.

The up-regulation of HSP genes during reoxygenation but not during ischaemia may suggest that this is mainly a response to reoxygenation injury. The 3.8-fold up-regulation of the HSP70 gene was not surprising because the overexpression of this protein improves post-ischaemic myocardial function [54] and reduces infarct size [192]; however, the up-regulation of HSP40, HSP47 and HSP105 genes are novel findings in the human myocardium. HSP40 exists in the cytoplasm and assists HSP70 in carrying out
its chaperone functions [18], whereas HSP47 exists in the endoplasmic reticulum and targets procollagen I and III [249], and HSP105, which exhibited the greatest up-regulation, is found in the cytoplasm. So far, the role of these HSP genes and their encoded products in cardioprotection is unknown and this is an area that would require further investigations.

Apart from increasing cardiac DNA and protein synthesis [61] and being involved in cardiomyocyte proliferation and maturation [75], IGF-1 improves cardiac function after a myocardial infarction in the rat [156] and in healthy human beings [59] and patients with heart failure [60]. It has also been shown that both the administration of IGF-1 [32] and the overexpression of IGF-1 [156] reduces cell death. With this cardioprotective profile, the down-regulation of IGF receptor-1 by ischaemia and during reoxygenation seen in my studies can be interpreted as the loss of an intrinsic protective factor that renders the muscle more susceptible to injury. The down-regulation of the IGF-binding protein 2 and the up-regulation of the IGF-binding protein 6 is more difficult to interpret since the interaction of these factors with IGF-1 leading to its activation or inhibition depends on the local physical and biological environment [32]. This is another area that needs to be further explored. The up-regulation of vascular endothelial growth factor (VEGF) by ischaemia and during reoxygenation in my studies conforms with the literature. Hypoxia and ischaemia, which stimulate angiogenesis, have been found to be strong inducers of VEGF both in vitro and in vivo [100,153,247,262]. It has been reported that a rapid increase in VEGF expression is due to the presence of hypoxia-inducible factor (HIF-1) sensitive elements located in the VEGF promoter, which upregulates the transcription of
VEGF [233]. It has been shown that after 60 minutes of coronary artery ligation, there is a 2.5 fold increase in the expression of VEGF mRNA [153]. Rat ventricular myocardium subjected to hypoxia followed by a 24 hour period of reoxygenation has shown a progressive increase in the intensity of Western blot staining for VEGF with increasing hypoxia [184].

Genes that promote or counteract apoptosis were up- or down-regulated providing a complex picture of which the interactions and outcomes are difficult to predict. Thus, on the one hand, the down-regulation of Bcl-2 related gene may promote apoptosis, a possibility supported by the effect of Bcl-2 in blocking the release of cytochrome c and AIF form mitochondria [143,269] and in regulating $\text{H}_2\text{O}_2$ and menadione-induced oxidative death [110]. Studies in mice have shown that overexpression of Bcl-2 renders the heart more resistant to ischaemia/reperfusion injury and apoptosis [40]. GRIM 19 (bovine homologue sub-unit of mitochondrial NADH: ubiquinone oxidoreductase or complex I), which forms part of the interferon-β and retinoic acid-induced pathway to cell death [71], was up-regulated by ischaemia and this could also favour apoptosis. However, on the other hand, the pro-apoptotic SARP2 (secreted apoptosis related protein 2) [191], the mechanism of action of which remains unknown, was down-regulated during reoxygenation and, similarly, TRAIL receptor 2 (also known as Apo-2L), which has been shown to induce apoptosis in tumor cells [12], was down-regulated by ischaemia. The expression of other apoptosis-related genes, shown in table 3, were also modified by ischaemia/reoxygenation but their role in apoptosis in the present experimental conditions is unclear. A detailed discussion of the potential contribution of
these factors to apoptosis is beyond the scope of the present studies and, clearly, the elucidation of these mechanisms warrants further investigation. It is worth of note that the observed changes in gene expression do not necessarily represent parallel changes of the encoded proteins and that, therefore, they are not sufficient to definitely establish a participation in the pathophysiology of ischaemia/reoxygenation injury. Validation of these results by reverse transcriptase polymerase chain reaction (RT-PCR) is essential before any inference can be made. More studies are certainly required to establish whether the regulation of genes with SI/R in the human atrial trabeculae model results in translation of proteins and also to study the role of these proteins in the signal transduction pathway leading to reperfusion injury in the human myocardium.

3.6 Conclusion

Here I have shown that cell death by apoptosis and necrosis in the human myocardium subjected to ischaemia and reoxygenation depends on the degree of the ischaemic insult and have a different time-course with apoptosis happening early during reoxygenation and necrosis becoming more important later. I have also shown that caspase-3 activation plays a critical role in apoptosis but that it does not affect necrosis, and that a number of genes transcribing for some proinflammatory cytokines, growth factors, metabolic enzymes, heat shock proteins and pro- and anti-apoptotic proteins are significantly down- or up-regulated, changes that may contribute to accelerate or counteract cell death. Once I identified the importance of apoptosis as a form of cell death after ischaemia/reoxygenation injury, I decided to investigate the underlying mechanism. Specifically, in the next chapter I investigated the role of caspases.
Figure 3.1: Experimental protocols for study 1 to investigate the effect of the intensity of ischaemic injury and the time of reoxygenation. Right atrial appendage sections (n=8/group) were equilibrated for 30 min and then subjected to various periods of simulated ischaemia (SI) followed by various periods of reoxygenation (R).

Key: SI  R  

76
Figure 3.2. Percentage of apoptosis (empty columns) and necrosis (solid columns) in right atrial muscles subjected to various periods of simulated ischaemia followed by various periods of reoxygenation. The columns represent the mean of 8 experiments and the bars represent the SEM. *p<0.05 vs aerobic control within the same reoxygenation group. †p<0.05 vs apoptosis with the same time-period.
Figure 3.3. MTT reduction of right atrial muscles subjected to various periods of simulated ischaemia followed by various periods of reoxygenation. The columns represent the mean of 8 experiments and the bars represent the SEM. *p<0.05 vs aerobic control (zero ischaemia) within the same reoxygenation group.
Table 1: CK leakage (IU/gm wet wt) during the first 2 hours of reoxygenation of right atrial muscles (n=8/group) subjected to various periods of simulated ischaemia followed by various periods of reoxygenation. *p<0.05 vs the respective aerobic controls.

<table>
<thead>
<tr>
<th>Reoxygenation group</th>
<th>Aerobic control</th>
<th>Simulated ischaemia (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>2 hours</td>
<td>2.6±0.2</td>
<td>3.5±0.3*</td>
</tr>
<tr>
<td>8 hours</td>
<td>2.8±0.3</td>
<td>4.0±0.5*</td>
</tr>
<tr>
<td>24 hours</td>
<td>2.5±0.4</td>
<td>3.7±0.5*</td>
</tr>
</tbody>
</table>
Figure 3.4. Total caspase activity in right atrial muscles subjected to various periods of simulated ischaemia followed by various periods of reoxygenation. The columns represent the mean of 8 experiments and the bars represent the SEM. *p<0.05 vs fresh muscle
Figure 3.5. Caspase-3-like activity in right atrial muscles subjected to various periods of simulated ischaemia followed by various periods of reoxygenation. The columns represent the mean of 8 experiments and the bars represent the SEM. *p<0.05 vs fresh muscle.
Figure 3.6. Effect of caspase-3 inhibition (C3i) with z.DEVD.FMK (70nM) on apoptosis (empty columns) and necrosis (solid columns) in right atrial muscles subjected to 90 minutes of simulated ischaemia (SI) and 2 hours of reoxygenation (R). The columns represent the mean of 6 experiments and the bars represent the SEM. *p<0.05 vs corresponding group without caspase-3 inhibitor.
<table>
<thead>
<tr>
<th>Group</th>
<th>MTT</th>
<th>P value</th>
<th>CK</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic Control</td>
<td>290.3±32.3</td>
<td></td>
<td>2.5±0.2</td>
<td></td>
</tr>
<tr>
<td>Aerobic Control plus C3i</td>
<td>298.0±30.4</td>
<td>NS</td>
<td>2.7±0.1</td>
<td>NS</td>
</tr>
<tr>
<td>SI/R</td>
<td>212.7±23.1</td>
<td>&lt;0.05</td>
<td>5.4±0.4</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>SI/R plus C3i</td>
<td>220.3±18.4</td>
<td>NS</td>
<td>5.7±0.5</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 2: CK leakage (IU/gm wet wt) during the first 2 hours of reoxygenation and MTT reduction (mM/gm wet wt) of right atrial muscles (n=6/group) in the presence and absence of the caspase-3 inhibitor z.DEVD.FMK (70nM).
NS= not significant versus control without z.DEVD.FMK.
<0.05= significant versus aerobic control
<table>
<thead>
<tr>
<th>No.</th>
<th>Gene</th>
<th>SI</th>
<th>SI/R</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>tumor necrosis factor, alpha-induced protein 3</td>
<td>-8.4</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>tumor necrosis factor, alpha-induced protein 2</td>
<td>-2.2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>tumor necrosis factor, alpha-induced protein 6</td>
<td>-6.1</td>
<td>-3.8</td>
</tr>
<tr>
<td>4</td>
<td>prostaglandin-endoperoxide synthase 2</td>
<td>-8.8</td>
<td>-2.5</td>
</tr>
<tr>
<td>5</td>
<td>interleukin 6 (beta 2 interferon)</td>
<td>-4.4</td>
<td>-2.2</td>
</tr>
<tr>
<td>6</td>
<td>Interferon (gamma-inducible protein)</td>
<td>-3.3</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>interleukin 1-beta (IL1B)</td>
<td>-6.3</td>
<td>-3.7</td>
</tr>
<tr>
<td>8</td>
<td>ATP synthase subunit c</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>ATP synthase delta subunit</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>heat shock protein 40kD</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>heat shock protein 47kD</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>heat shock protein 70kD</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>heat shock protein 105kD</td>
<td>12.3</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>insulin-like growth factor binding protein 2</td>
<td>-4.8</td>
<td>-4.8</td>
</tr>
<tr>
<td>15</td>
<td>insulin-like growth factor 1 receptor</td>
<td>-2.0</td>
<td>-2.8</td>
</tr>
<tr>
<td>16</td>
<td>insulin-like growth factor binding protein 6</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>vascular endothelial growth factor</td>
<td>3.5</td>
<td>4.4</td>
</tr>
<tr>
<td>18</td>
<td>myeloid cell leukemia sequence 1 (BCL2-related)</td>
<td>-6.9</td>
<td>-6.1</td>
</tr>
<tr>
<td>19</td>
<td>cell death-regulatory protein GRIM19</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>secreted apoptosis related protein 2 (SARP2)</td>
<td></td>
<td>-2.7</td>
</tr>
<tr>
<td>21</td>
<td>TRAIL receptor 2</td>
<td></td>
<td>-3.1</td>
</tr>
<tr>
<td>22</td>
<td>nuclear receptor interacting protein 1 (NRIP1)</td>
<td></td>
<td>-2.7</td>
</tr>
<tr>
<td>23</td>
<td>nuclear receptor subfamily 0 (NR0B2)</td>
<td></td>
<td>2.8</td>
</tr>
<tr>
<td>24</td>
<td>nucleolar protein 3 (apoptosis repressor with CARD domain)</td>
<td></td>
<td>2.8</td>
</tr>
<tr>
<td>25</td>
<td>serine/threonine kinase 17a (apoptosis-inducing)</td>
<td></td>
<td>-3.0</td>
</tr>
</tbody>
</table>
Table 3

Effect of simulated ischaemia/reoxygenation of atrial muscles on gene expression. Results are presented as time-fold change of aerobic control. Each group is the pool of 3 specimens obtained from 3 different subjects.
Figure 3.7 Representative TUNEL Images with following treatment protocols.
A. Aerobic control  B. 30 min SI/120 min R  C. 90 min SI/120 min R  D. 90 min SI/8 hr R (Abbreviations: SI – simulated ischaemia, R – reoxygenation).
Figure 3.8 Representative Propidium Iodide images with following treatment protocols:
A. Aerobic control  B. 90 min SI/120 min R  C. 180 min SI/120 min R  D. 180 min SI/120 min R (Abbreviations: SI – simulated ischaemia, R – reoxygenation).
CHAPTER IV

EFFECT OF CASPASE INHIBITION ON APOPTOSIS AND NECROSIS IN ISCHAEMIA AND REOXYGENATION OF THE HUMAN MYOCARDIUM
4.1 Introduction

Reperfusion injury induces a time-dependent oxidative stress in cardiomyocytes [3,22-25,89,289,301], yet the pathophysiological role and significance of reperfusion induced apoptosis for survival of the heart in humans is unknown. By employing experimental studies in animal models [37,251] and in isolated cells [3,188] it has been established that cellular apoptosis induced by oxidant stress is mediated by a family of cysteiny1 aspartate proteases or caspases. Caspase activation is triggered by two main pathways, either externally through the Fas membrane receptor family which leads to the activation of procaspase-8 to caspase-8 (section 1.6.2), or internally via a compromised mitochondrial membrane, leading to release of cytochrome-c and the activation of procaspase-9 to caspase-9 (section 1.6.2). Work in the isolated rat heart [251] has shown that pre-ischaemic infusion of a specific caspase-8 inhibitor lead to inhibition of myocyte apoptosis. However, while it is not known whether caspase-8 and caspase-9 pathways control apoptosis in human heart, activation of downstream effector caspase-3 is required for encapsulation and disposal of the cell contents. In experiments described below, I aimed to establish whether caspase-3 and caspase-8+9 inhibition leads to reduction of SI/R-induced apoptosis in the human atrial myocardium model. However, some authors believe that caspase-independent pathways of cell death could play an important role in cardiomyocytes under certain pathological situations [144]. If that were the case, the extent of reduction in apoptosis or residual apoptosis with caspase inhibitors in these experiments would allow us to determine the contribution of caspase-independent pathways. By measuring total caspase, caspase-3, caspase-8+9, apoptosis and necrosis, the relationship of these measurements with each other as a result of caspase inhibition
can thus be determined. In previous studies (chapter 3), I measured the time course of apoptotic cell death induced by graded SI/R in the human myocardium. Using the same model, I obtained evidence that SI/R induces apoptosis in human atrial myocardium by a process that is mediated by caspase activity. To answer the above-mentioned uncertainties in SI/R in the human myocardium, caspase-3 and caspase-8+9 inhibitors were used and their effect on TUNEL staining and caspase levels was determined.

4.2 Aims of my study

1. To determine the dose response of caspase-3 and caspase-8+9 inhibitors on SI/R-induced total, caspase-3 and caspase-8+9 levels in the human right atrial model.

2. To investigate the effect of pharmacological inhibition of caspase-3 and caspase-8+9 on SI/R-induced apoptosis in the human right atrial model.

4.3 Materials and Methods

4.3.1 Patients

Patients undergoing elective coronary artery bypass grafting were included. Tissue from patients with atrial fibrillation, a poor ejection fraction (EF<30%), those with diabetes receiving oral hypoglycaemic drugs, insulin or potassium channel activators [167] (nicorandil or diazoxide) was excluded for reasons mentioned in chapter 2 (section 2.5.1).
4.3.2 Collection of atrial samples and solutions

The human right atrial appendage [307] from patients undergoing elective coronary artery bypass graft surgery was harvested, prepared and subjected to SI/R as described in chapter 2 (sections 2.3.1 & 2.4.1).

4.3.3 Experimental Protocols

Study 1

The free-hand sections of fresh atrial tissue were allowed to equilibrate in oxygenated KHH buffer for 30 mins and then subjected to 90 min of simulated ischaemia followed by 120 min of reoxygenation (n=4/group) in the absence (controls) and presence of 0.7, 7.0 and 70.0 nM concentrations of specific inhibitors of caspase-3 (z.DEVD.FMK) and caspase-8+9 (z.IETD.FMK+z.LEHD.FMK) (figure 4.1). The inhibitors were used throughout the experimental perfusion.

Study 2

The free-hand sections of fresh atrial tissue were subjected to the following experimental protocols (n=6/group). Group I: Aerobic perfusion without caspase inhibitor, Group II: Aerobic perfusion with 70nM z.DEVD.FMK, Group III: Aerobic perfusion with 70nM z.IETD.FMK + 70nM z.LEHD.FMK, Group IV: SI/R without caspase inhibitor, Group V: SI/R with 70nM z.DEVD.FMK, Group VI: SI/R with 70nM z.IETD.FMK + 70nM z.LEHD.FMK (figure 4.4). The inhibitors were used from the start of equilibration to the end of reoxygenation.
SPECIAL NOTE

The Following Pages Are Scanned As Seen.

Marks on Page Are As Per Original.
4.3.4 Assessment of apoptosis and necrosis

This was done as described in section 2.4.4 (chapter 2).

4.3.5 Quantitation of caspase activity by fluorometric assay

Total caspase, caspase-3 and caspase-8+9 activities were measured in the tissue homogenate by the assay described in chapter 2 (section 2.4.5). Caspase-3 and caspase-8+9 activity was determined by adding z.DEVD.FMK and z.IETD.FMK+z.LEHD.FMK respectively, at a concentration of 10μM to the cell lysate (protein) in the presence of the substrate linked to a fluorescent molecule. The resulting fluorescence yield was corrected by subtracting the fluorescence measured for the protein homogenate alone.

4.4 Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) with the application of a post-hoc Tukey’s test was used for comparisons of means (Microsoft® Excel analysis tool pak) between groups. A p value of less than 0.05 was considered statistically significant.

4.5 Results

4.5.1 Study 1

Figure 4.2 shows that in the tissue there was a dose-dependent reduction in caspase-3 activation with increasing concentration of z.DEVD.FMK. Activation was completely abolished with 70nM of the inhibitor (194000±29000 U/gr wet wt. in control group,
5670±2220 U/gr wet wt. with 70nM z.DEVD.FMK; p<0.05 with respect to the control group). There was no significant difference in the total caspase levels (448000±590100 U/gr wet wt. in the control group, 438000±22000 U/gr wet wt. with 70nM z.DEVD.FMK; p=NS) and caspase-8+9 activity (45700±18700 U/gr wet wt. in the control group, 44600±13900 U/gr wet wt. with 70 nM z.DEVD.FMK; p=NS) with increasing doses of the caspase-3 inhibitor.

Figure 4.3 shows that in the tissue there was a dose-dependent reduction in the measured levels of caspase-8+9 with increasing concentrations of the inhibitors z.IETD.FMK+z.LEHD.FMK which were completely abolished in the presence of 70nM inhibitor (106000±6500 U/gr wet wt in control group, 5250±2410 U/gr wet wt with 70nM z.IETD.FMK+70nM z.LEHD.FMK; p<0.05). However, there was also a significant reduction in total caspase activation (502000±61600 U/gr wet wt in control group, 341000±55400 U/gr wet wt with 70nM z.IETD.FMK+70nM z.LEHD.FMK; p<0.05) and caspase-3 activity (96600±9530 U/gr wet wt in control group, 47800±8800 U/gr wet wt with 70nM z.IETD.FMK+70nM z.LEHD.FMK; p<0.05).

4.5.2 Study 2

i) Apoptosis and Necrosis

Figure 4.5 illustrates the percentage of apoptosis and necrosis in atrial tissue after SI/R in the absence and presence of 70nM z.DEVD.FMK and 70nM z.IETD.FMK+70nM z.LEHD.FMK. It shows a significant reduction in apoptosis from 23.2±2.8% in the
control group to 5.6±1.0 % (p<0.05) in the presence of 70nM z.IETD.FMK+70nM z.LEHD.FMK (photomicrographs in figure 4.6A and 4.6C). Caspase-3 inhibition with 70nM z.DEVD.FMK resulted in almost complete abolition of apoptosis from 23.3±2.8% to 0.7±0.3% (p<0.05 vs SI/R control; photomicrograph in figure 4.6B). A similar pattern of inhibition was seen in the time-matched aerobic group. The amount of necrosis remained unchanged with the addition of 70nM z.DEVD.FMK and 70nM z.IETD.FMK+70nM z.LEHD.FMK (photomicrographs in figure 4.7A, 4.7B and 4.7C).

**ii) Caspase levels**

Figure 4.8 again confirms the findings of study 1, and demonstrates the reduction of caspase-3 activation in the tissue from 99264±21044 U/gr wet wt in SI/R control to 5470±3150 U/gr wet wt (p<0.05 versus SI/R control) in group with 70nM concentration of z.DEVD.FMK without leading to caspase-8+9 inhibition. Furthermore, there was a significant decrease of caspase-3 activity to 52300±11900 U/gr wet wt and caspase-8+9 activity from 43440±5500 U/gr wet wt in SI/R control to 8500±3200 U/gr wet wt (p<0.05 vs SI/R control) in the presence of 70nM concentration of z.IETD.FMK+z.LEHD.FMK. A similar pattern was seen in the aerobic time-matched group.

**4.6 Discussion**

Apoptosis along with necrosis contributes to cardiomyocyte loss in reperfusion injury [74,89,160,170,172,311]. Caspase-activated apoptosis may be initiated by the mitochondria (internal pathway) leading to activation of caspase-9 or by external death receptor binding (for example Fas ligand to the Fas receptor) and activation of caspase-8
Initiator caspases, like caspase-8, either directly or indirectly activate effector caspases such as caspase-3 [46]. These effector caspases then cleave a number of intracellular substrates, such as poly(ADP-ribose) polymerase (PARP) [264] and lamins (proteins lining the inner nuclear membrane) [150] that may trigger apoptosis [265] leading to activation of endonucleases and internucleosomal DNA degradation [204]. Procaspase-9, in the presence of ATP and cytochrome-c, interacts with Apaf-1 to form the apoptosome resulting in the activation of caspase-9 [154] and subsequently the effector caspase-3 [264].

4.6.1 Caspase-8/9 are upstream of caspase-3 in the apoptotic cascade in the human atrium.

As far as I am aware, this is the first account of the dose-response of the human atrial myocardium to caspase-3 and caspase-8/9 inhibitors. In this study, I have shown that total caspases including caspase-3 and caspase-8+9 are activated in our human atrial model of SI/R. There is a dose-dependent inhibition of caspase-3 activation (figure 4.2) which was almost completely abolished with 70nM z.DEVD.FMK. This inhibitor did not affect caspase-8+9 activation. Furthermore, caspase-8+9 inhibition with z.IETD.FMK + z.LEHD.FMK, resulted in almost complete abolition of caspase-8+9 activation at 70nM concentration. In this case however, caspase-3 and total caspase activation were reduced as well. This latter inhibition was, however, incomplete. This finding confirms previous studies in animal models and cell cultures, namely that caspase-8 and 9 are upstream of caspase-3 in the apoptotic cascade [58,65,98,154,179]. In my study, I show that caspase-8 and 9 are activated during reoxygenation. Scarabelli et al [251], however, demonstrated
in rat hearts that caspase-8 is activated only on reperfusion whereas caspase-9 is activated in ischaemia. The lack of change in the total caspase activation on using z.DEVD.FMK rather than z.IETD.FMK+z.LEHD.FMK may be explained by a positive feedback effect of caspase-3 hydrolysis of upstream (initiator) caspases which is unlikely with caspase-8 and -9 inhibition as they are initiator (upstream) caspases.

The concentration of caspase inhibitors (used in my experiments) required to inhibit 50% of the caspase activity (IC$_{50}$) has been reported to be in the picomolar range [64]. However, significant caspase inhibition is not seen in the human right atrial trabeculae model with 0.7nM z.DEVD.FMK or 0.7nM z.IETD.FMK+z.LEHD.FMK. Thus, the IC$_{50}$ of caspase inhibitors determined in solution conditions may not reflect the activity of the inhibitor inside the cardiomyocyte. In the context of cardiomyocytes, the effect of caspase inhibitors relies on the amount of the inhibitor within the cell which seems to require higher concentrations of the inhibitors (my results) than reported in other in-vitro studies [275].

4.6.2 Attenuation of apoptosis by caspase inhibition

In the second set of experiments (figure 4.5 and 4.8), I have shown that there is a significant but incomplete reduction in apoptotic cell death when using z.IEDT.FMK+z.LEHD.CHO. The addition of z.DEVD.FMK during SI/R resulted in complete inhibition of apoptosis. The pattern of caspase activation was similar to that seen in the dose-response studies. This shows that induction of apoptosis in the human atrial appendage model is caspase-dependent and can be inhibited with caspase inhibitors.
Until recently, it was thought that apoptotic signals triggered by death factors lead to irreversible cell death [197]. However, since caspase inhibitors have been shown to attenuate apoptosis in the human right atrial trabeculae model (this study) and in other in vitro [90,299] and in vivo models (rabbits) [111] this may indicate that the 'point of no return' may be downstream of the effector caspases. It has been shown in animal models that caspase inhibition is effective in reducing reperfusion injury in the myocardium following attenuation of apoptosis [299]. Also, Ruetten et al [241] using I/R studies in the isolated rat heart showed that inhibition caspase-3 activation by DEVD.CHO resulted in a substantial improvement of post-ischaemic contractile recovery.

Caspase-3 is thought to be activated by two pathways, one caspase-8 mediated and the other caspase-9 mediated (section 1.6.2). Inhibition of caspase-8 and caspase-9 should, therefore, theoretically result in the achievement of the same reduction in apoptotic cell death as inhibition of caspase-3. However, it is believed that apoptosis inducing factor (AIF) and cytochrome-c (also see section 1.6.2), which reside in the mitochondrial intermembrane space escape via a caspase-dependent and caspase-independent manner. In the cytoplasm, cytochrome-c exerts its effects by regulating the activity of Apaf-1 (section 1.6.2), whereas AIF [268] works in a caspase-independent manner by translocating to the nucleus and binds to DNA thereby initiating its degradation [300]. This may account for the incomplete inhibition of apoptosis seen in the human right atrial trabeculae model with caspase-8+9 inhibition. Furthermore, the role of other caspases thought to be involved in apoptosis, such as caspases-2, -6, -7, and -10 is not known at present following SI/R in the human myocardium.
4.6.3 Effect of caspase inhibitors on necrosis

The use of the irreversible inhibitors z.DEVD.FMK and z.IETD.FMK+z.LEHD.FMK did not have any effect on necrosis in our model of SI/R in the human myocardium. My findings agree with those of Kang et al [133] who observed in adult rat ventricular myocytes (ARVM) that caspase inhibition during reoxygenation leads to inhibition of apoptotic cell death and not necrosis. This is in contrast to Tsujimoto’s group [261] who have shown that caspase inhibitors retard necrotic in their in-vitro system of chemical hypoxia, suggesting that there may be common mediators in apoptotic and necrotic signal transduction. The reasons for this discrepancy may be two-fold: (i) the pathway to cell death leading to necrosis as a result of chemical hypoxia is different from that of SI/R and (ii) the experiments in the two studies were carried out in different cell types (cancer cells versus cardiomyocytes).

4.6.4 Advantages of irreversible inhibitors

My results on the activation of caspase groups 1 (caspase-8 and caspase-9) and 2 (caspase-3) depend on the specificity of peptide caspase inhibitors (discussed in detail in chapter 1). Peptides linked to aldehyde groups (.CHO) act as reversible inhibitors without chemically changing the caspase. They are less permeable to membranes than the .FMK inhibitors. This would be an obvious drawback if inhibition were intended within the atrial tissue I have used. Linking the peptide to fluoro- or chloro-methyl ketones (.CMK, .FMK) groups produces irreversible inhibition. The .CMK/.FMK inhibitors are more membrane permeable than the aldehyde based inhibitors (section 1.9.2).
4.6.5 Limitations of the study

Although the peptide caspase inhibitors in general, are more specific than the cysteine protease inhibitors they can also inhibit other proteases for example cathepsin B [256]. While z.DEVD.FMK is marketed as being largely specific for caspase-3, it may inhibit other groups of caspases as well [77,178]. Although the absolute specificity of this group of compounds may be questioned (section 1.9.2), their use at nanomolar concentrations together with their inhibitory effectiveness in my dose response studies suggests that specific inhibition is being achieved under the conditions employed.

4.7 Conclusion

In this study in the human right atrial trabeculae model [307] I have shown that cardiomyocytes undergo apoptosis via a caspase-dependent pathway and confirmed that caspases-8/9 are upstream of caspase-3 in the apoptotic cascade. Furthermore, apoptosis can be prevented with the use of caspase inhibitors in the human right atrial trabeculae model and that this inhibition proceeds in a dose-dependent manner. What remains uncertain is whether this inhibition leads to an improvement in myocardial contractile function. Further studies are required to delineate the functional role of apoptosis and its inhibition on contractile activity in the human myocardium. After determining that apoptosis as result of SI/R injury in the human atrial myocardium occurs via caspase-dependent pathways, I decided to investigate the effect of protective interventions such as ischaemic preconditioning against apoptotic and necrotic cell death. This study is described in the following chapter.
Figure 4.1. Experimental protocols for study 1 designed to determine the dose-response effect of z.DEVD.fmk (C3i) and z.IETD.fmk+z.LEHD.fmk (C8+9i) on total caspase, caspase-3 and caspase-8+9 activities in ischaemia/reoxygenation. Right atrial appendages (n=4) were equilibrated for 30 min and then subjected to 90 min of simulated ischaemia followed by 120 min of reoxygenation. The inhibitors were used throughout the protocols.

Key: simulated ischaemia ■, reoxygenation □
Figure 4.2 Dose-response effect of caspase-3 inhibitor, z.DEVD.FMK, on total, caspase-3 and caspase-8+9 activities in ischaemia/reoxygenation. Data are expressed as mean±standard error of the mean (SEM) of 4 experiments. *p<0.05 vs corresponding group with no inhibitor. (Abbreviations: C8- caspase-8, C8+9- caspase-8 plus caspase-9, SI- simulated ischaemia, R- reoxygenation, nM- nanomolar)
Figure 4.3. Dose-response effect of caspase-8+9 inhibitors, z.IETD.FMK+z.LEHD.FMK, on total, caspase-3 and caspase-8+9 activities in ischaemia/reoxygenation. Data are expressed as mean±standard error of the mean (SEM) of 4 experiments. *p<0.05 vs corresponding group with no inhibitor. (Abbreviations: C3- caspase-3, C8+9- caspase-8 plus caspase-9, SI- simulated ischaemia, R- reoxygenation, nM- nanomolar).
Figure 4.4. Experimental protocols for study 2 designed to determine the effect of z.DEVD.fmk (C3i) and z.IETD.fmk+z.LEHD.fmk (C8+9i) on apoptosis and necrosis in ischaemia/reoxygenation. Right atrial appendages (n=6) were equilibrated for 30 min and then subjected to 90 min of simulated ischaemia followed by 120 min of reoxygenation. Time-matched aerobic controls were used. The inhibitors were used throughout the protocols.

Key: ■ simulated ischaemia, □ reoxygenation
Figure 4.5 Effect of caspase-3 and caspase-8+9 inhibition on apoptosis and necrosis in ischaemia/reoxygenation. Data are expressed as mean±standard error of the mean (SEM) of 6 experiments. *p<0.05 vs corresponding group with no inhibitor. (Abbreviations: C8- caspase-8, C8+9- caspase-8 plus caspase-9, SI- simulated ischaemia, R- reoxygenation, nM- nanomolar).
Figure 4.6 Representative TUNEL images with the following treatment protocols: A. SI/R  B. 70nM C3i  C. 70nM C8+9i (Abbreviations: SI/R - simulated ischaemia/reoxygenation, nM - nanomolar, C3i - caspase-3 inhibitor, C8+9i - caspase8+9 inhibitor).

Figure 4.7 Representative Propidium Iodide images with the following treatment protocols: A. SI/R  B. 70nM C3i  C. 70nM C8+9i (Abbreviations: SI/R - simulated ischaemia/reoxygenation, nM - nanomolar, C3i - caspase-3 inhibitor, C8+9i - caspase8+9 inhibitor).
Figure 4.8 Effect of caspase-3 and caspase-8+9 inhibition on caspase activities in ischaemia/reoxygenation. Data are expressed as mean±standard error of the mean (SEM) of 6 experiments. *p<0.05 vs corresponding group with no inhibitor. (Abbreviations: C8- caspase-8, C8+9- caspase-8 plus caspase-9, SI- simulated ischaemia, R- reoxygenation, nM- nanomolar).
CHAPTER V

PRECONDITIONING OF THE HUMAN MYOCARDIUM AGAINST APOPTOSIS AND NECROSIS INDUCED BY ISCHAEMIA/REOXYGENATION
5.1 Introduction
Investigations designed to minimise myocardial cell death by apoptosis and necrosis following ischaemia and reperfusion are ongoing and are of great clinical importance. This is particularly true in the context of cardiac surgery where surgeons continue to seek the ideal myocardial protective strategy to minimise ischaemic damage whilst requiring a bloodless field during open heart surgery. Postoperatively, the aim is to restore cardiac contractility to facilitate weaning from cardiopulmonary bypass, especially in high-risk patients. Inability to achieve this may result in low cardiac output syndrome.

Apoptosis and necrosis are two distinct forms of cell death. Both have been shown to be important in reperfusion injury in isolated cardiomyocytes [89]. Preconditioning by a short ischaemic insult or pharmacological means has been demonstrated to result in strong cardioprotection. However, the role of apoptosis and necrosis in the protection afforded by this phenomenon in the heart is undefined. In the present study, the precise contribution of preconditioning by ischaemia to reduce both types of cell death in the human myocardium was investigated.

5.2 Materials and Methods

5.2.1 Patients
Patients were selected as described in section 2.2 (chapter 2).
5.2.2 Preparation of atrial tissue and solutions

The human right atrial appendage [307] from patients undergoing elective coronary artery bypass graft surgery was harvested, prepared and subjected to ischaemia and reoxygenation as described in sections 2.3.1 & 2.4.1 (chapter 2).

5.2.3 Experimental Protocols

The atrial sections (n=6/group) were allowed to equilibrate in oxygenated KHH buffer for 30min and then they were randomly allocated to the following protocols (figure 5.1): (i) aerobic incubation for 210min; (ii) 90min simulated ischaemia (SI); (iii) 90min simulated ischaemia followed by 120min reoxygenation (SI/R); (iv) IPC with 5min SI plus 5min reoxygenation followed by SI/R.

5.2.4 Measurement of tissue injury by the Creatine Kinase (CK) assay

Tissue injury was measured by myocyte specific creatine kinase (CK) release [231] into the perfusate (section 2.4.2). The enzyme was measured by a kinetic assay (IU/gr wet weight) at the end of the experimental protocol employing a commercial assay kit (DG147-K: Sigma Chemicals).

5.2.5 Assessment of apoptosis and necrosis

This was performed as described in section 2.4.4 (chapter 2).
5.3 Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) was used for comparisons of means with the application of a post-hoc Tukey’s test, employing MS Excel (Microsoft Corp) with the statistical Add-In. A p value of less than 0.05 was considered statistically significant.

5.4 Results

i) Creatine Kinase release

As shown in Figure 5.2, SI/R resulted in a significant increase in CK release (4.0±0.3 IU/gr wet wt) when compared to aerobic perfusion (2.3±0.2 IU/gr wet wt; p<0.05). IPC significantly reduced CK leakage to mean values that were similar to those seen in the aerobic control group (2.6±0.3 IU/gr wet wt; p<0.05 versus SI/R).

ii) Apoptosis and necrosis

Figure 5.3 shows that, when compared to aerobic perfusion, SI resulted in small increases in the levels of both apoptosis and necrosis that were not statistically significant. However, SI/R caused significant increases in both apoptosis and necrosis (photomicrographs in figure 5.4A-B and 5.5A-5.5B) but values were greater in the former (29.5±2.9%) than in the latter (12.6±1.6%; p<0.05) and, as expected, IPC resulted in significant reduction of both apoptosis (14.2±3.0%; photomicrograph in figure 5.4C) and necrosis (7.9±0.9%; p<0.05 {photomicrograph in figure 5.5}).
5.4 Discussion

It is well documented that progressive ischaemia can lead to the rapid development of necrosis. Recent studies in animal cardiomyocytes have shown that ischaemia may also lead to cellular apoptosis [26]. Here I show that both apoptosis and necrosis increase in human myocardium following ischaemia and reoxygenation with apoptosis playing a more important role in inducing cellular injury. So far as I am aware, this report is the first to demonstrate that apoptosis is the more important form of ischaemia/reperfusion injury in the human myocardium. Interestingly, these figures equate to values reported for the rat [131,212]. My observations are consistent with the presumption that, in the highly oxygenated human cardiomyocyte, apoptosis primarily occurs as a result of reperfusion induced oxidative stress which it is thought may follow the burst of reactive oxygen species (ROS) released on reperfusion [20,316].

Ischaemic preconditioning, in which a brief period of ischaemia is followed by an equally short period of reperfusion, is well known to confer protection to a myocardium that is subsequently subjected to longer periods of lethal ischaemia [196]. While several studies [74,131,207,215,246] have shown that apoptosis contributes to cardiac cell death after ischaemia, emerging evidence indicates that preconditioning may suppress apoptosis in addition to necrosis in intact hearts [312]. The exact mechanism(s) by which preconditioning leads to cardioprotection by inhibition of apoptosis is still unknown, but IPC can signal the opening of channels in the mitochondrion following triggering of mitochondrial dysfunction (section 6.3, figure 6.3&6.4). Apoptosis or 'programmed cell death' is mediated by the caspase cascade following activation of one or both of two
signal transduction pathways. Signals trigger these pathways, either externally through the Fas membrane receptor family and leading to activation of procaspase-8, or internally via release of cytochrome-c into the cytoplasm and the activation of procaspase-9; the result of a compromised mitochondrial membrane.

The opening of mitochondrial ATP sensitive potassium (mito-K$_{\text{ATP}}$) channels is thought to lead to either the enhancement or attenuation of cardioprotection through the release of ROS with the preconditioning mechanism highly dependent on the phasing of channel opening. Thus mito-K$_{\text{ATP}}$ channels may signal protection through (i) inhibition of cytochrome c release, (ii) the optimization of energy production through depolarisation of the mitochondrial membrane potential ($\Psi_m$) with alterations in mitochondrial Ca$^{2+}$ handling and/or (iii) the modulation of reactive oxygen species (ROS) production, including oxygen free radicals [20,316] and nitric oxide [128] during ischaemia or reperfusion. In the present study there is evidence that ischaemic preconditioning results in the inhibition of apoptosis. These results are in agreement with the previous work of Akao et al [3] who have shown that the opening of mito-K$_{\text{ATP}}$ channels inhibits apoptosis induced by oxidative stress in rat cardiomyocytes. Using ischaemic and pharmacological preconditioning of human tissue it has been shown [168] that opening of mitochondrial K$_{\text{ATP}}$ channels signals the activation of both PKC and p38MAPK. Both downstream elements are shown to be inhibited by the antagonists 5-hydroxydecanoate (5-HD: a presumed specific mito-K$_{\text{ATP}}$ channel inhibitor) or chelerythrine and SB203580, broad spectrum inhibitors of protein kinase C and p38MAPK respectively [168]. The current thinking [21] is that by inducing a sublethal production of ROS, the short ischaemic
stimulus associated with preconditioning by ischaemia may confer cardioprotection through opening of mito-$K_{\text{ATP}}$ channels or alternatively, sublethal generation of ROS could lead to via activation of protein kinase C isozymes the inhibition of caspase-8 induced apoptosis [314].

5.5 Conclusion

In the context of preconditioning in human atrial tissue, the overall results suggest that the cardioprotective effect in the first window of cardioprotection [82] is mediated to a greater extent by a reduction in apoptotic cell death. Data from my previous work (section 3.2.5 and figure 3.2) indicates that necrosis may dominate pathways to cell death following the longer periods (>24hrs) of reoxygenation.

Present techniques of intraoperative myocardial protection are constantly evolving as the current strategies have proved to be suboptimal in high-risk patients. I have confirmed my previous findings that apoptosis is the principal form of cell death in early reperfusion and that ischaemic preconditioning mainly acts via a reduction apoptosis in the human atrial appendage model. Further studies were then designed to delineate the mechanisms underlying this anti-apoptotic effect in the human heart.
### Figure 5.1. Experimental protocols designed to determine the effect of ischaemic preconditioning on apoptosis and necrosis. Right atrial appendage sections (n=6) were equilibrated for 30 min and then subjected to the protocols shown in the figure.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>30' equil</th>
<th>210'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SI</td>
<td>90'</td>
<td></td>
</tr>
<tr>
<td>SI/R</td>
<td>90'</td>
<td>120'</td>
</tr>
<tr>
<td>IPC</td>
<td>5'5'</td>
<td>90'</td>
</tr>
</tbody>
</table>

**Key:**
- Aerobic perfusion
- Simulated ischaemia
- SI - simulated ischaemia
- SI/R - simulated ischaemia/reoxygenation
- IPC - ischaemic preconditioning.
Figure 5.2: CK release in the medium during 2 hours reoxygenation period of right atrial muscles subjected to simulated ischaemia/reoxygenation (SI/R) and ischaemic preconditioning (IPC). The aerobic control serves as time-matched controls. Data are expressed as mean±SEM of six experiments. *p<0.05 vs SI/R group.
Figure 5.3. Percentage of nuclei exhibiting apoptosis and necrosis in right atrial muscles subjected to simulated ischaemia (SI), simulated ischaemia/reoxygenation (SI/R) and ischaemic preconditioning (IPC). The columns represent the mean of 6 experiments and the bars represent the SEM. *p<0.05 vs SI/R group, † P<0.05 vs necrosis in corresponding group.
Figure 5.4: TUNEL staining (A) SI  (B) SI/R  (C) IPC
Figure 5.5: Staining with propidium iodide (A) SI  (B) SI/R  (C) IPC
CHAPTER VI

PRECONDITIONING OF THE HUMAN MYOCARDIUM AGAINST APOPTOSIS AND NECROSIS INDUCED BY ISCHAEMIA/REOXYGENATION: ROLE OF MITOK$\text{ATP}$ CHANNELS, PROTEIN KINASE C AND P38MAPK.
6.1 Introduction

Apoptosis and necrosis are two distinct forms of cell death. Both have been shown to be important in reperfusion injury [89]. Preconditioning by a short ischaemic insult (IPC) or pharmacological means has been demonstrated to result in strong cardioprotection [196]. A variety of endogenous substances are reported to be involved in this protection by IPC. These include adenosine [162], nitric oxide [228], opioids [257], reactive oxygen species (ROS) [15], heat shock proteins (HSP) [177], bradykinin [295] and protein kinases [263]. The involvement of mediators in preconditioning such as PKC and mitoK\textsubscript{ATP} channels is well recognized in man and animals [47,263]. Experimental work in the last two decades has focused on the mechanisms leading to preconditioning related mainly to the limitation of necrosis [162,196,255]. However apoptosis, like necrosis, may independently contribute to irreversible myocardial damage [131]. At present, there is no clear understanding of the mechanistic linkage of apoptotic cell death to the phenomenon of preconditioning in the human myocardium. Although both forms of cell death are thought to be closely connected to altered mitochondrial function [3,281] it is uncertain whether the pathways to apoptosis and necrosis may share parts of the same signal transduction.

The present studies are designed to investigate the role of mitoK\textsubscript{ATP} channels, protein kinase C (PKC) and mitogen activated protein kinase (p38MAPK) on apoptosis and necrosis in the context of IPC in the human myocardium.
6.2 Materials and Methods

6.2.1 Patients

Patients were selected as described in section 2.2 (chapter 2).

6.2.2 Collection of atrial samples and solutions

The human right atrial appendage [307] from patients undergoing elective coronary artery bypass graft surgery was harvested, prepared and subjected to ischaemia and reoxygenation as described in sections 2.3.1&2.4.1 (chapter 2). The following doses of the agents [168] were used in the present studies were: 5-hydroxydecanoate at 1mM, chelerythrine at 10μM, SB203580 at 10μM, diazoxide at 100μM, anisomycin at 1nM and PMA at 1μM. These were purchased from Sigma chemicals (Perth, Australia).

6.2.3 Experimental Protocols

Study 1: The role of mitoKATP channels, PKC and p38MAPK on the anti-apoptotic and anti-necrotic effects of ischaemic preconditioning

Sections of atrial muscle (n=6/group) were equilibrated for 30min and then subjected to the following protocols (figure 6.1): (i) aerobic perfusion for 210min; (ii) SI/R; (iii) IPC. In this study (1A), 5-hydroxydecanoate, chelerythrine and SB203580 were added for the last 10min of the equilibration period and before the induction of ischaemia in the SI/R and IPC groups. In another set of experiments (study 1B), diazoxide, anisomycin and PMA were added for the last 10min of the equilibration period followed by a 5min washout before the induction of ischaemia (figure 6.2).
Study 2: The effect of ischaemic preconditioning and caspase inhibition on gene expression

The right atrial appendages (n=3/group) were subjected to the following experimental protocols: (i) aerobic perfusion; (ii) SI/R; (iii) IPC, and (iv) SI/R with the caspase-3 inhibitor 70nM z.DEVD.FMK.

6.2.4 Measurement of tissue injury by the Creatine Kinase (CK-MB) assay

Tissue injury results in the release of myocyte specific creatine kinase (CK-MB) into the perfusate. For all of the protocols, CK-MB was measured at the end of 2 hours of reoxygenation by a linked-enzyme kinetic assay (Sigma Chemicals) which has been described in chapter 2 (section 2.4.2).

6.2.5 Assessment of apoptosis and necrosis

This was performed as described in section 2.4.4 (chapter 2).

6.2.6 cDNA microarrays

This was done as described in section 2.4.6.
6.3 Results

6.3.1 Study 1: The role of mitoK\textsubscript{ATP} channels, PKC and p38MAPK on the anti-apoptotic and anti-necrotic effects of ischaemic preconditioning.

i) Creatine Kinase leakage

As shown in Figure 6.3, SI/R resulted in a significant increase in CK release, which was not modified by the presence of the inhibitors 5-hydroxydecanoate, chelerythrine and SB203580. In agreement with my previous results (figure 5.2), IPC significantly reduced CK leakage to mean values that were similar to those observed in the aerobic control group. The protection of IPC was abolished by 5-hydroxydecanoate, chelerythrine and SB203580.

Figure 6.4 shows that diazoxide, anisomycin and PMA resulted in a significant reduction of CK release as compared to SI/R, and similar to the values seen with IPC.

ii) Tissue necrosis and apoptosis

Figure 6.5 shows that, SI/R causes a significant increase in both apoptosis and necrosis when compared to the aerobic control group (photomicrographs in figure 6.6A-B and 6.7A-B). Again, the increase in the two forms of cell death was unaffected by the presence of 5-hydroxydecanoate, chelerythrine and SB203580. Interestingly, whilst the anti-necrotic effect of IPC was abolished by the addition of 5-hydroxydecanoate, chelerythrine and SB203580 (photomicrographs in figure 6.7D-F), the anti-apoptotic
effect was abolished only by 5-hydroxydecanoate and chelerythrine (photomicrographs in figure 6.6D-E) but not by SB203580 (photomicrograph in figure 6.6F).

Figure 6.6 shows that the reduction in necrosis by IPC was mimicked by the addition of diazoxide, anisomycin and PMA; however, only diazoxide and PMA, but not anisomycin, resulted in the inhibition of apoptosis. These results were mirror images of the results seen with the 5-hydroxydecanoate, chelerythrine and SB203580.

6.3.2 Study 2: The effect of ischaemic preconditioning and caspase inhibition on gene expression.

Table 1 shows the changes in mRNA expression (e.g., upregulation and downregulation) of at least 2.0 fold on atrial muscles subjected to IPC and caspase-3 inhibition as compared to SI/R that may have an influence on cell death. SI/R caused downregulation of genes encoding for pro-inflammatory cytokines such as TNF and interleukins 1 and 6, and upregulation of heat shock proteins (HSP). The greatest upregulation corresponded to HSP105 with a 13.1-fold increase as compared to aerobic controls. SI/R also resulted in downregulation of the growth factor IGF and upregulation of VEGF, and Bcl-2 related genes, that protect cells from apoptosis [185], were down-regulated by 5.8 fold.

Table 1 also shows that, when compared to SI/R, IPC yielded a 5.4 fold down-regulation of HSP105 gene and a 3.1 fold up-regulation of Bcl-2 related gene. The addition of the caspase-3 inhibitor z.DEVD.fmk to SI/R also resulted in striking changes in gene expression when compared with SI/R alone. Thus, the down-regulation of proinflammatory cytokines were reduced to less than 2-fold as was the upregulation of
heat shock proteins and the changes seen with the expression of growth factors. Importantly, the downregulation of Bcl-2 related genes by SI/R was reversed by C3 inhibition, and the diacylglycerol kinase (delta 130kDa) gene, that encodes for an enzyme that alternates PKC activity [244], was downregulated by 3.5 fold as compared to SI/R alone and IPC.

6.4 Discussion

The present studies have demonstrated that IPC protects the human myocardium against both apoptosis and necrosis and that the anti-necrotic effect is mediated by the opening of the mitoK$_{\text{ATP}}$ channels and activation of PKC and p38MAPK, whereas the anti-apoptotic effect requires opening of the mitoK$_{\text{ATP}}$ channels and PKC activation but is p38MAPK independent. These actions are associated to distinctive changes in gene expression the analysis of which may contribute to a better understanding of the pathophysiology of ischaemia/reoxygenation injury and the mechanisms of cardioprotection. The relevance of these results are discussed below.

6.4.1 MitoK$_{\text{ATP}}$ Channels and IPC:

Animal studies in the rat [94], dog [92] and also in the rabbit [164] and chick embryo cardiomyocytes [159] have previously investigated the role of mitoK$_{\text{ATP}}$ channels in preconditioning against necrosis but not apoptosis. The present studies have demonstrated that, in the human myocardium, mitoK$_{\text{ATP}}$ channels play a key role in reducing apoptosis and necrosis since cardioprotection against the two forms of cell death
was lost by blockade of the channels with 5-HD and elicited by opening of the channels with diazoxide.

Recently my group has shown that mitoK\textsubscript{ATP} channels, PKC and p38MAPK, in that order, are integral part of the signal transduction mechanism of cardioprotection by ischaemic and pharmacological preconditioning of the human myocardium [168]; however, the mechanism by which opening of mitoK\textsubscript{ATP} channels results in cardioprotection remains unclear. We [101] and other investigators [3,4,78] have observed that opening of mitoK\textsubscript{ATP} channels by diazoxide and nicorandil causes mitochondrial membrane potential depolarization and the generation of free radicals, that in turn could activate PKC (see below). An alternative mechanism of mitoK\textsubscript{ATP} channel-induced protection may be the prevention of Ca\textsuperscript{2+} overload seen in rat cardiac mitochondria [112]. Akao et al [4] have demonstrated in neonatal cultured rat cardiomyocytes that opening of mitoK\textsubscript{ATP} channels by diazoxide suppresses caspase activation, the translocation of cytochrome c and the release of poly-ADP-ribosepolymerase (PARP) caused by oxidative stress and that these effects are abolished by 5-HD. However, these actions directly linked to apoptosis may represent the end-effect of the signal transduction pathway initiated by the opening of the mitoK\textsubscript{ATP} channels.

6.4.2 PKC and IPC:

The present results also provide evidence that PKC activation plays a crucial mechanistic role in IPC of the human myocardium by reducing apoptosis and necrosis. There is
agreement in the literature that PKC activation reduces necrosis in in-vivo and in in-vitro animal studies [157,158,292,304], but the results on the role of PKC activation in apoptosis do not agree. Thus, for example, the reported attenuation of apoptosis in rabbit and chick cardiomyocytes by PKC activation [161,170] contrasts with the suggestion that the increased activity of the enzyme induces apoptosis in the salivary epithelium [167]. It is possible that these opposed results may find an explanation in differences in the experimental conditions and type of tissue investigated.

The mechanism leading to PKC activation, the type of PKC isoform involve and the end-effector (s) phosphorylated by PKC and responsible for the cardioprotection are not yet fully elucidated. As discussed earlier, oxygen free radicals are strong candidates linking the opening of mitoK\textsubscript{ATP} channels and the activation of PKC, a thesis supported by the suggestion that the oxygen free radicals released by the mitochondria during brief episodes of hypoxia induce preconditioning in chick cardiomyocytes [281] and by the demonstration that PKC can be activated by selective oxidative modification of the regulatory domain [87]. The PKC isoforms involved is also unclear. My group has demonstrated in the human myocardium that PKC\textepsilon is upstream and PKC\alpha is downstream of the mitoK\textsubscript{ATP} channels [102]. In contrast, other investigators have observed that opening of mitoK\textsubscript{ATP} channels activates the PKC\textepsilon isoform through oxygen free radicals originating in the mitochondria of chick embryonic ventricular myocytes [170].

Regarding the effect of PKC activation, it can be speculated from the literature that the antinecrotic effect of PKC activation may be mediated by regulation of Ca\textsuperscript{2+} channels
[114], by a decrease in intracellular acidification [72] or by another as yet unknown mechanism, while its anti-apoptotic action may be related to the phosphorylation of proteins such as Bcl-2 and Bcl-X [113]. It is clear that there is scarce knowledge in this area and that more research is needed.

6.4.3 p38MAPK and IPC:

My finding that p38MAPK plays a role in the cardioprotection of preconditioning in the human myocardium is consistent with previous studies also from my laboratory showing that p38MAPK is place downstream of mitoK<sub>ATP</sub> channels and PKC [168]. However, in the present studies I have shown for the first time that while the anti-necrotic effect of IPC is p38MAPK-dependent the anti-apoptotic action is p38MAPK-independent, implying that the two forms of cell death may use separate end-effectors. In the literature, the role of p38MAPK in IPC is controversial. My group, here and in previous studies [168], in the human myocardium, and other investigators using experimental animal models such as the rabbit [200], have shown that p38MAPK activation plays a role in preconditioning while no effect of p38MAPK activation on IPC has been seen in studies in the pig [16] and rat [254]. Even inhibition of p38MAPK, as opposed to activation, has been reported to be cardioprotective in the isolated rabbit heart [172] and rat heart [254]. It is possible that species differences and the experimental preparation used may provide an explanation, at least in part, for the disparate results. Tissue specificity and the temporal activation of p38MAPK have also been suggested as potential causes for the variable findings [305].
Although the role of p38MAPK in ischaemic injury and in IPC is not defined, one may be tempted to speculate that any protective action is mediated by the phosphorylation of small heat shock proteins such as HSP27 via the MAPKAP kinase 2 [267], a class of proteins that has been shown to act as chaperonin of Bax and Bcl-X to sequester their translocation during staurosporine-induced apoptosis [176]. It should be emphasized that in many of the reported studies, including the present one, the role of p38MAPK was investigated using blockers such as SB203580, that is highly specific for the α and β isoforms of p38MAPK [96] but insensitive to p38γ and p38δ isoforms [84,293], and activators such as anisomycin that are non-selective agents [221]. Therefore, if p38MAPK isoforms have different physiological roles, it is possible that some of the above controversies can find an explanation on the selectivity of the p38MAPK blockers and activators used. Once again, more research is needed in this area.

It should be mentioned that other biochemical and immunoblotting techniques have been used to assess protein kinase activity in the field of biomedical research. The technique of siRNA can be used to knockout specific mRNA which translate into the protein kinases required to be studied. Radio-active and non-radioactive biochemical assays are available. The radio-active assays are based on the incubation of subcellular fractions with radioisotopes in the presence of specific substrates. The phosphorylated substrate is then isolated on phosphocellulose filters or biotin capture membrane. However, in non-radioactive assays, ELISA or other physical and/or chemical methods are used to detect the phosphorylated peptide. Immunoblotting, which utilizes electrical current to isolate proteins and transfer them to a membrane, can be used by adding primary antibodies
against protein kinases. The presence and location, but not the activity, of the protein kinase can be determined by the luminescence produced by oxidation of luminol by a secondary horseradish peroxidase-tagged antibody. Another method of assessing protein kinase activity is by labelling them with fluorescent probes selective for active enzyme. At present we do not have a single reliable and quantitative method for determining the activity and subcellular localisation of various isozymes of different protein kinases. The development of a combination of isoform-specific immunoblotting technique with a isoform-specific biochemical assay may overcome these limitations and allow us to study the role of individual isozymes of protein kinases involved in regulation of apoptosis and necrosis by ischaemic preconditioning in the human heart.

6.4.4 Changes in gene expression and IPC:

Changes in 22000 genes were followed by human specific cDNA microarrays. The microarray analysis presented here is by no means an attempt to fully characterize the molecular basis of ischaemia/reperfusion and preconditioning. It is aimed to provide a preliminary overview of genes expressed in the human myocardium induced by IPC as compared to SI/R. Since apoptosis and necrosis may have different end-effectors, the effect of caspase-3 inhibition on gene expression was also investigated.

In the present studies, many of the changes in gene expression by SI/R were unaffected by IPC and caspase-3 inhibition. However, the down-regulation of some of the inflammatory cytokines including interleukin-1 (IL-1), interleukin-6 (IL-6) and TNF-α induced protein 6 (TNFAIP6) were attenuated by both IPC and caspase-3 inhibition.
which may be interpreted as a potential protective mechanism against ischaemic/reoxygenation injury by the two interventions. Both the up and down-regulation of the genes encoding for growth factors by SI/R were reversed by IPC and caspase-3 inhibition but the net effect of these changes on cardioprotection are more difficult to interpret.

The observed upregulation of HSP 47, HSP70 and HSP 105 with SI/R in my studies was expected since similar changes have been shown to occur in animal experimental models of myocardial, renal and cerebral ischaemia [139,217,224]. However, the attenuation of their expression by IPC and caspase-3 was unexpected since upregulation of some of the heat shock proteins such as HSP70 have been shown to improve myocardial function [54] and reduce infarct size [303]. The up-regulation of HSP47, which targets procollagen I and III in the endoplasmic reticulum, and of cytoplasmic HSP105, which exhibited the greatest degree of upregulation by SI/R, confirm my previous results (chapter 3, table 3) also in the ischaemic human myocardium [286]. The role of heat shock proteins in the pathophysiology of ischaemia/reoxygenation is not fully defined and therefore their downregulation by IPC and caspase-3 inhibition is of difficult interpretation. This is another area that requires more investigation.

Consistent with my previous studies (chapter 3, table 3) [286], SI/R resulted in significant down-regulation of the Bcl-2 related gene in the present studies. The attenuation of this effect by caspase-3 inhibition and its overexpression by IPC suggest that the anti-apoptotic effect of these interventions may be mediated, at least in part, by ameliorating
the expression of these genes. This thesis is supported by experiments in a transgenic
mice showing that overexpression of Bcl-2 renders the heart more resistant to apoptosis
and I/R injury [40]. Bcl-2 blocks the release of cytochrome-c and AIF from the
mitochondria [143], and this antiapoptotic activity has been thought to be compensatory
to the marked up-regulation of Bcl-2 in in-vivo experimental models of heart failure
[214].

My study is the first to investigate the effect of caspase-3 inhibition on gene expression in
the human myocardium and the significant down-regulation of diacylglycerol kinase
delta (DGKγ) gene when compared to SI/R is a novel finding. DGK is regarded as an
attenuator of PKC activity and may play a role in the metabolism of insulin secretion via
PKC intermediates in the plasmalemmal membrane [244]. Therefore, a downregulation
of DGK would increase PKC activation that in turn, as shown above, can lead to
inhibition of apoptosis. In the literature there are suggestions that some of the DGK
isoforms are involved in healing and LV remodelling after myocardial infarction in the
rat [272]. It is worth noting that the down-regulation in DGKγ only occurred after
inhibition of caspase-3 and not IPC, but the results support the initiation of further studies
to fully elucidate the role of this pathway and the possibility of its clinical exploitation.

It should be clarified that in the present studies I have investigated changes in mRNA
expression and that because this does not translate into protein synthesis one should
exercise caution in the interpretation of the physiological importance of the observed
changes.
6.4.5 Clinical implications and study limitations:

The realisation that the cardioprotective mechanism against apoptotic and necrotic cell death is in part identical but that subsequently diverges into two distinctive pathways may imply that there are two different sets of end-effectors antagonizing each of the two conditions. This opens a window of opportunity from a therapeutical point of view if apoptosis and necrosis can be specifically counteracted and if the unwanted activation of other processes can be avoided. As mentioned earlier in this thesis, the use of human atrial tissue should raise a note of caution before any extrapolation is made to the ventricular myocardium.

6.5 Conclusion

This study has identified a possible mechanistic link between activation of mitoK$_{ATP}$ channels, PKC, p38MAPK and the induction of apoptosis and necrosis in ischaemic preconditioning in the human atrial myocardium. It is thought that the mitochondria play a crucial role in modulating apoptosis and necrosis as is demonstrated by the pharmacological perturbation of mitoK$_{ATP}$ channels. Undoubtedly, further studies are clearly warranted to fully elucidate the various signal transduction pathways involved with apoptosis in reperfusion and ischaemic preconditioning in the human myocardium. The ultimate goal certainly is to be able to translate these findings from laboratory bench to the patient bedside.
Figure 6.1. Experimental protocols for study 1A (n=6/group) designed to investigate the role of mitoKATP channels, PKC and p38MAPK in reducing apoptosis and necrosis in ischaemic preconditioning. The three groups include: i. Aerobic time-matched controls ii. SI/R (no inhibitor, 5-HD, chelerythrine, SB203580) iii. IPC (no inhibitor, 5-HD, chelerythrine, SB203580).

Key: simulated ischaemia ■, reoxygenation □
SI/R- simulated ischaemia/reoxygenation, IPC – ischaemic preconditioning
Figure 6.2. Experimental protocols for study 1B (n=6/group) designed to investigate the role of mitoKATP channels, PKC and p38MAPK in reducing apoptosis and necrosis in ischaemic preconditioning. The six groups include: i. Aerobic time-matched controls ii. SI/R iii. IPC iv. DZX+IPC v. ANS+IPC vi. PMA+IPC.

Key: simulated ischaemia ■ , reoxygenation □

SI/R- simulated ischaemia/reoxygenation, IPC – ischaemic preconditioning, DZX - diazoxide, ANS – anisomycin, PMA.
Figure 6.3. CK release during 2 hours reoxygenation period of right atrial muscles subjected to simulated ischaemia/reoxygenation (SI/R), ischaemic preconditioning (IPC) or the addition of 5-hydroxydecanoate (5-HD), chelerythrine (Chel) and SB203580 (SB) 10 minutes prior to SI. Data are expressed as mean±SEM of six experiments. *p<0.05 vs SI/R group.
Figure 6.4. CK release during 2 hours reoxygenation period of right atrial muscles subjected to simulated ischaemia/reoxygenation (SI/R), ischaemic preconditioning (IPC) or the addition of diazoxide (DZX), PMA and anisomycin (ANIS) administered for 10 minutes and the 5 minutes washout prior to SI. Data are expressed as mean±SEM of six experiments. *p<0.05 vs SI/R group.
Figure 6.5. Effect of blockade of mitoKATP annels, PKC and p38MAPK on the percentage of apoptosis and necrosis in right atrial muscles subjected to simulated ischaemia/reoxygenation (SI/R) and ischaemic preconditioning (IPC). The columns represent the mean of 6 experiments and the bars represent the SEM. *p<0.05 vs corresponding IPC alone group; †p<0.05 vs corresponding SI/R group. 

Abbreviations: 5-HD - 5-hydroxydecanoate, Chel - chelerythrine, SB - SB203580.
Figure 6.6. Effect of activation of mitoKATP channels, PKC and p38MAPK on the percentage of apoptosis and necrosis in right atrial muscles subjected to simulated ischaemia/reoxygenation (SI/R) and ischaemic preconditioning (IPC). The columns represent the mean of 6 experiments and the bars represent the SEM. *p<0.05 vs corresponding SI/R group.

Abbreviations: DZX-diazoxide, ANIS-anisomycin.
Figure 6.7 Representative TUNEL images (A) Aerobic control (B) Simulated ischaemia/reoxygenation (C) Ischaemic preconditioning (IPC) alone (D) 5-hydroxydecanoate+IPC (E) Chelerythrine+IPC (D) SB203580+IPC.
Figure 6.8 Representative Propidium Iodide images (A) Aerobic control (B) Simulated ischaemia/reoxygenation (C) Ischaemic preconditioning (IPC) alone (D) 5-hydroxydecanoate+IPC (E) Chelerythrine+IPC (F) SB203580+IPC.
<table>
<thead>
<tr>
<th>No.</th>
<th>Gene</th>
<th>SI/R (time-fold change of aerobic control)</th>
<th>IPC (time-fold change of SI/R)</th>
<th>SI/R+C3 inhibitor (time-fold change of SI/R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>tumor necrosis factor, alpha-induced protein 6</td>
<td>-3.2</td>
<td>-1.7</td>
<td>-1.1</td>
</tr>
<tr>
<td>2</td>
<td>interleukin 6 (beta 2 interferon)</td>
<td>-2.5</td>
<td>1.1</td>
<td>-1.3</td>
</tr>
<tr>
<td>3</td>
<td>interleukin 1-beta (IL1B)</td>
<td>-4.1</td>
<td>1.0</td>
<td>-1.8</td>
</tr>
<tr>
<td>4</td>
<td>heat shock protein 47kD</td>
<td>2.9</td>
<td>-1.6</td>
<td>1.2</td>
</tr>
<tr>
<td>5</td>
<td>heat shock protein 70kD</td>
<td>5.1</td>
<td>-1.1</td>
<td>1.2</td>
</tr>
<tr>
<td>6</td>
<td>heat shock protein 105kD</td>
<td>13.1</td>
<td>-5.4</td>
<td>-1.8</td>
</tr>
<tr>
<td>7</td>
<td>insulin-like growth factor binding protein 2</td>
<td>-4.8</td>
<td>-1.7</td>
<td>1.0</td>
</tr>
<tr>
<td>8</td>
<td>insulin-like growth factor 1 receptor</td>
<td>-3.2</td>
<td>1.5</td>
<td>-1.2</td>
</tr>
<tr>
<td>9</td>
<td>vascular endothelial growth factor</td>
<td>6.4</td>
<td>1.8</td>
<td>1.9</td>
</tr>
<tr>
<td>10</td>
<td>myeloid cell leukemia sequence 1 (BCL2-related)</td>
<td>-5.8</td>
<td>3.1</td>
<td>-1.1</td>
</tr>
<tr>
<td>11</td>
<td>secreted apoptosis related protein 2 (SARP2)</td>
<td>-2.2</td>
<td>-1.6</td>
<td>1.4</td>
</tr>
<tr>
<td>12</td>
<td>diacylglycerol kinase, delta 130kDa</td>
<td>1.3</td>
<td>1.2</td>
<td>-3.5</td>
</tr>
</tbody>
</table>

Table 1. Gene expression in human atrial muscles as a result of simulated ischaemia-reoxygenation (SI/R) versus aerobic perfusion and ischaemic preconditioning (IPC) and caspase-3 inhibition (SI/R+C3 inhibitor) versus SI/R. Results are presented as time-fold change. Each group is the pool of 3 experiments of atrial muscles obtained from 3 different patients.
CHAPTER VII

CONCLUSIONS
Using the human right atrial trabeculae model, I have determined the time-course of apoptosis and necrosis in ischaemia/reoxygenation injury (section 3.4.1, figure 3.2) and the role of the caspases (sections 3.4.1 & 3.4.2, figures 3.4, 3.5 & 3.6) associated with ischaemia/reoxygenation-induced apoptosis. Both forms of cell death are shown to exist simultaneously. Apoptosis, that is dependent on caspase-3 activation, is the predominant pathway to cardiomyocyte death during the first 8 hours of reoxygenation in contrast to necrosis which is more important at 24 hours reperfusion. In both cases the degree of apoptosis and necrosis depend on the degree of ischaemia (figure 3.2). Here I have established a temporal relationship linking apoptosis and necrosis in the human myocardium for the first 24 hours of reoxygenation. Still, one of the existing problems in studying cardiomyocyte death is that the development of necrosis and apoptosis cannot be accurately followed continuously in time, both in experimental animal preparations and in man. This would require innumerable time-points that would make such studies time consuming and difficult to perform.

Whether apoptosis and necrosis are parallel or consequential processes is still a matter of debate. Since ATP is required for apoptosis to occur, individual ATP levels may determine whether a cell dies by apoptosis or necrosis. It may be that the occurrence of apoptosis is more typical of moderate ischaemia with low levels of ATP whereas necrosis is typical of total ATP depletion after severe ischaemia [252]. The process of apoptosis and necrosis differ in a number of ways and proceed down separate paths. However, there is also suggestion in the literature that there is overlap or cross-over (from apoptosis to necrosis) under certain conditions that share similar mechanisms [312]. The notion that
apoptosis and necrosis are regulated by completely independent molecular mechanisms has been challenged by Shimizu et al [253] who have shown that overexpression of bcl-2 can dramatically inhibit necrosis resulting from chemical hypoxia. On the basis of my studies on the extent of apoptosis and necrosis in I/R at different time-periods in chapter 3, it is not possible to conclude whether these two modes of cell death are a result of separate signal transduction pathways or there is, at least in part, sharing of mechanisms leading to changeover from one mode of cell death to the other. What is clear that both exist simultaneously. In chapter 6 of my thesis, there is evidence that the anti-apoptotic and anti-necrotic effects of ischaemic preconditioning in the human myocardium are mediated by opening of the mitoK\textsubscript{ATP} channels and activation of PKC pointing towards a link between the two pathways to cell death.

It is a well-known fact that cellular apoptosis is known to be mediated by the family of caspases. However, it is uncertain whether reperfusion-induced apoptosis in humans is caspase-dependent or not. It was therefore decided to undertake further studies to ascertain whether caspase-3 and caspase-8/9 activities were required in the human myocardium for the induction of reperfusion-induced apoptosis (as is shown in other cell types and animal models). My studies have shown that the induction of apoptosis in the human myocardium is mainly caspase-3-dependent. They have also demonstrated that caspase-3 inhibition leads to inhibition of apoptotic cell death and not of necrosis. On the basis of my results, caspase inhibitors may seem highly attractive therapeutic tools. However, issues relating to permeability, specificity, potency and delivery need to be addressed and further in vivo research to determine their efficacy in humans is vital.
before these drugs can be introduced clinically for the management of ischaemic heart disease.

It has been demonstrated that ischaemic preconditioning is one of the most powerful methods of attenuating ischaemic injury suppressing both apoptosis and necrosis. In animal experimental models, preconditioning has been shown to limit arrhythmias, post-ischaemic myocardial dysfunction, endothelial cell dysfunction and infarct size [13,47,237]. However, the clinical application of IPC in the human heart has not yet been established. Experimental work in the last two decades has mainly focused on the mechanisms leading to preconditioning related chiefly to the limitation of necrosis [162,196,255]. Using ischaemic and pharmacological preconditioning of non-diabetic human tissue using the human right atrial trabeculae model that opening of mitochondrial $K_{ATP}$ channels and the activation of both PKC and p38MAPK may lead to preconditioning. My laboratory has previously described that mito$K_{ATP}$ channels, PKC and p38MAPK are integral parts of the signal transduction pathways of ischaemic preconditioning. By means of inhibitors and activators of mito$K_{ATP}$ channels, PKC and p38MAPK (section 6.3, figures 6.5 & 6.6) I have demonstrated in this thesis that the anti-apoptotic and anti-necrotic effects of ischaemic preconditioning in the human myocardium are mediated by opening of the mito$K_{ATP}$ channels and activation of PKC; however, whereas the the anti-necrotic action is p38MAPK-dependent, the anti-apoptotic action is p38MAPK-independent. These studies (section 6.3, figures 6.5 & 6.6) point out to the mito$K_{ATP}$ channels and protein kinases as potential targets to combat the apoptosis and necrosis induced by ischaemia/reperfusion of the human myocardium. These findings
have advanced our knowledge on the mechanism of protection by ischaemic preconditioning and it is hoped that they may have a clinical application.

Another interesting pathway that is implicated in I/R and IPC are the protease-activated receptor (PAR)-mediated events. PAR-2, a member of this family of transmembrane domain G protein-coupled receptors, is activated by proteolytic cleavage [208]. It is expressed in the heart and contributes to I/R injury [201]. It has been shown that PAR-2-dependent signalling events are involved early during IPC stimulus in rats and that exposure to the activating peptide of PAR-2 (PAR-2AP) significantly enhances the effect of IPC [202]. More studies need to carried out to explore the effect of caspases on PAR-2-mediated cardioprotection and to determine whether these effects are mediated by mitoK\textsubscript{ATP} channels, PKC and p38MAPK.

Although the microarray analysis performed in this thesis has provided interesting information (chapter 3; table 3, chapter 6; table 1), it is important to appreciate that a change in mRNA expression may not essentially translate into protein synthesis and physiological significance. However, these results give rise to numerous hypotheses that need to be tested in further studies.

**Future directions**

Despite the information obtained with the present studies, the mechanism by which the phenomenon of preconditioning achieves the inhibition of apoptosis and necrosis is yet to be completely understood. Thus, for example, the interaction of the mitoK\textsubscript{ATP} channels
and PKC needs to be clarified. McPherson et al [190] have shown in chick embryonic ventricular myocytes that opening of \( \text{mitoK}_{\text{ATP}} \) channels activates the PKC\( \varepsilon \) isoform through the release of oxygen radicals that are thought to originate in the mitochondria. However, recent studies in my laboratory by another investigator have shown that in the human myocardium the PKC\( \alpha \) isoform is the one downstream of \( \text{mitoK}_{\text{ATP}} \) channels whereas PKC\( \varepsilon \) is placed upstream [102]). Furthermore, it was demonstrated that the superoxide produced by the mitochondria was the radical responsible for the activation of PKC. It is also possible the different isomers of p38MAPK family are involved in the regulation of cell death in the human myocardium. The p38MAPK inhibitor SB203580 used in my studies specifically inhibits the p38\( \alpha \) and p38\( \beta \) isoforms [96], but not the p38\( \gamma \) and p38\( \delta \) isoforms [84,293]. Clearly, further studies are required to clarify the role of different isozymes of the PKC and p38MAPK family in the cardioprotection of preconditioning against cell death.

My laboratory have reported that the myocardium of patients with diabetes and with poor left ventricular function (EF<30%) cannot be protected by ischaemic preconditioning [81]. The investigation of the cause for this lack of cardioprotection and how it can be overcome is of clinical importance because this group of patients has a high risk of cardiovascular complications and poor long term prognosis.
Bibliography


