Mechanisms Underlying Leptin-induced Cardioprotection

Thesis submitted by

Richard Alastair Dixon

BSc (Hons)

For the degree of

Doctor of Philosophy

In the

Faculty of Medicine,

University College London

The Hatter Cardiovascular Institute

University College London

67 Chenies Mews

London, WC1E 6HX.

ABSTRACT

Background

The tissue damage resulting from myocardial ischaemia/reperfusion (I/R) leads to a large proportion of the deaths in the developed world. Even if patients survive a myocardial infarction, I/R injury is associated with decreased cardiac function and a higher incidence of morbidity, and consequently, a reduced quality of life. Leptin is a cytokine produced by white adipose tissue that has been shown to activate the PI3K-Akt and p44/42 MAPK signalling cascades and protect the murine myocardium. The studies described in this thesis focused on the molecular mechanisms underlying leptin-induced cardioprotection with particular reference to cell signalling.

Methods & Results

Using an isolated rat heart model of I/R injury, it was confirmed that leptin given at reperfusion protects the myocardium. This protection was abolished by the administration of specific blockers of the survival kinases PI3K, JAK and p44/42 MAPK. The time-points at which maximal phosphorylation of Akt, p44/42 MAPK and STAT3 occur in response to leptin given at reperfusion were identified. The key role played by the leptin receptor in leptin-induced cardioprotection was established by employing the Zucker obese rat (*fa/*fa), which has a mutation in the leptin receptor, and its Zucker lean (*fa/*+) counterpart that possesses a functional OB-R. It was found that the Zucker fatty rats were non-responsive to leptin-induced cardioprotection. Furthermore, it was shown that whilst leptin inhibited mitochondrial permeability transition pore (MPTP) opening in

cardiomyocytes from Wistar and Zucker lean rats, MPTP opening in OB-R deficient Zucker fatty rats was unaffected. Finally, leptin was shown to protect against ischaemia-reperfusion injury in an in vivo rat model of I/R injury.

Conclusion

The present study has yielded information concerning the mechanisms underlying the cardioprotective actions of leptin; including the importance of the OB-R. Extending the studies employing *in vivo* models of I/R injury will assist in establishing the potential of leptin as a therapeutic agent

ACKNOWLEDGEMENTS

I would like to express my thanks to my supervisors Dr. Christopher Smith and Professor Derek Yellon for their guidance, support and motivation.

I am also indebted to my colleagues Dr Mihaela Moody, Dr Sean Davidson, Dr Max Lim and Ms Abigail Wynne who have provided invaluable guidance, inspiration and practical support and advice.

I am grateful to Professor Michael Duchen who permitted us to use his Welcome Trust funded confocal microscope.

Finally I would like to thank the British Heart Foundation (BHF) for providing funding for my time at the Hatter Institute.

TABLE OF CONTENTS

ABSTRACT 2

Background 2

Methods & Results 2

Conclusion 3

ACKNOWLEDGEMENTS 4

TABLE OF CONTENTS 5

TABLE OF FIGURES 8

LIST OF TABLES 12

LIST OF ABBREVIATIONS 14

CHAPTER 1 - GENERAL INTRODUCTION 23

1.1 Coronary Artery Disease 23

1.2 Myocardial Ischaemia-Reperfusion (I/R) Injury 25

1.3 Protection Against I/R Injury 41

1.4 Cardiometabolic Disease 56

1.5 The Adipose Tissue and Adipocytokines 58

1.6 Leptin 63

CHAPTER 2 - AIMS & HYPOTHESIS 80

2.1 Aims 80

2.2 Hypothesis 80

CHAPTER 3 - GENERAL METHODS 81

3.1 Animals 81

3.2 Chemicals & Drugs Used 81

3.3 Langendorff Isolated Perfused Rat Heart 82

3.4 Preparation of Isolated Adult Rat Cardiomyocytes 92

3.5 Western Blotting 98

3.6 Rat In Vivo Model of I/R Injury 102

3.7 Method of Randomisation 109

3.8 Statistical Analysis 109

CHAPTER 4 - LEPTIN PROTECTS AGAINST I/R INJURY IN THE WISTAR RAT HEART...110
4.1 Introduction..110
4.2 Aim..110
4.3 Experimental Protocol & Materials 111
4.4 Results 115
4.5 Discussion 124
CHAPTER 5 - LEPTIN-INDUCED CARDIOPROTECTION VIA THE RISK PATHWAY..127
5.1 Introduction..127
5.2 Aim..128
5.3 Experimental Protocol & Materials 129
5.4 Results 132
5.5 Discussion 145
CHAPTER 6 - LEPTIN-INDUCED CARDIOPROTECTION VIA THE JAK-STAT PATHWAY...152
6.1 Introduction..152
6.2 Aim..153
6.3 Experimental Protocol & Materials 153
6.4 Results 159
6.5 Discussion 172
CHAPTER 7 - LEPTIN-INDUCED CARDIOPROTECTION IN ZUCKER LEAN & ZUCKER FATTY RATS................................178
7.1 Introduction..178
7.2 Aim..179
7.3 Hypothesis 179
7.4 Experimental Protocol & Materials 179
7.5 Results 183
7.6 Discussion 197

CHAPTER 8 - LEPTIN-INDUCED INHIBITION OF MPTP OPENING 203

8.1 Introduction ... 203
8.2 Aim ... 205
8.3 Experimental Protocol & Materials ... 205
8.4 Results ... 208
8.5 Discussion ... 209

CHAPTER 9 - LEPTIN-INDUCED MYOCARDIAL PROTECTION IN AN *IN VIVO* RAT MODEL OF I/R INJURY ... 213

9.1 Introduction ... 213
9.2 Aim ... 214
9.3 Experimental Protocol & Materials ... 215
9.4 Results ... 218
9.5 Discussion ... 219

CHAPTER 10 - SUMMARY & CONCLUSIONS ... 221

10.1 Summary of Findings ... 221
10.2 Conclusions ... 221
10.3 Future Directions ... 223

PUBLICATIONS & COMMUNICATIONS ... 226

Abstracts ... 226
Oral Presentations ... 226
Publications ... 226

REFERENCES ... 227

Journals ... 227
Books ... 272
Websites ... 272

APPENDIX ... 273

PUBLICATIONS IN FULL ... 279

TABLE OF FIGURES

Figure 1.1.1 - Schematic diagram depicting the progression of coronary artery disease. 25
Figure 1.2.1 – Schematic representing the changes in the levels of ions, ATP, P_i and NADH during ischaemia and reperfusion 30
Figure 1.2.2 – Schematic of the mitochondrial permeability transition pore (MPTP) 37
Figure 1.3.1 – Schematic of Cardiomyocyte signalling pathways 45
Figure 1.3.2 – Diagram of the Reperfusion injury salvage kinase (RISK) pathway components. 47
Figure 1.3.3 – Diagram of PI3K-Akt pathway. 50
Figure 1.3.4 – Diagram of the Structure of STATs 54
Figure 1.6.1 – Leptin and energy homeostasis diagram. 65
Figure 1.6.2 – Diagram of leptin signalling mechanisms. 71
Figure 3.3.1 – Diagram of the Langendorff apparatus used........ 84
Figure 3.3.2 – Photographs of Langendorff isolated rat heart perfusion apparatus. 85
Figure 3.3.3 – Schematic of standard protocol for isolated rat heart I/R experiment. 87
Figure 3.3.4 – Isolated mounted rat heart photos demonstrating stages of ischaemia reperfusion protocol. 88
Figure 3.3.5 – Example of a heart trace. 89
Figure 3.3.6 - Schematic of heart slice subjected to I/R injury and perfused with Evans blue dye…… 91
Figure 3.3.7 - Figure showing the method of estimating infarct size using computed planimetry…….. 91
Figure 3.4.1 – Micrograph of isolated rat cardiomyocytes 94
Figure 3.4.2 – Photograph of cardiac myocyte isolation rig. 95
Figure 3.4.3 – Photograph of confocal microscope and operating computer 97
Figure 3.6.1 – Rat *in vivo* surgery (1). 106
Figure 3.6.2 – Rat *in vivo* surgery (2) 107

Figure 3.6.3 – Schematic of standard rat *in vivo* protocol. 108
Figure 4.3.1 – Schematic of ischaemic preconditioning protocol. 112
Figure 4.4.1 – Infarct data for the control and preconditioned (IPC) Wistar rat hearts. 119
Figure 4.4.2 – Infarct data for leptin dose response study…………………… 123
Figure 5.3.1 – Protocol for investigating the effects of RISK pathway inhibitors on infarct size. 130
Figure 5.3.2 – Protocol for the collection of samples for Western blot analysis 131
Figure 5.4.1 – Infarct data of hearts treated with leptin in the presence and absence of LY and UO. 136
Figure 5.4.2 – Graph of Akt/serine-473 phosphorylation in the presence and absence of leptin at different time-points during reperfusion.......................... 139
Figure 5.4.3 - Western blots showing phosphorylated Akt/Ser-473 and total Akt levels in tissue taken from Wistar hearts treated with and without leptin (10nM) 140
Figure 5.4.4 – Graph showing the effect of 10nM leptin on Akt/Thr 308 phosphorylation in Wistar rat hearts.. 141
Figure 5.4.5 – Western blots showing phosphorylated Akt/Thr-308 and total Akt levels in tissue taken from Wistar hearts treated with and without leptin (10nM). 142
Figure 5.4.6 – Graph showing the effect of leptin (10nM) on (A) p44 and (B) p42 phosphorylation in Wistar rat hearts.. 143
Figure 5.4.7 – Western blots showing phosphorylated p44/42 MAPK and total MAPK levels in tissue taken from Wistar hearts treated with and without leptin (10nM). 144
Figure 6.3.1 – Schematic of protocol for investigating the effects of AG490, a JAK2 inhibitor, on leptin-induced infarct size reduction.................................. 155
Figure 6.3.2 – Schematic of protocol for the collection of samples for STAT3 determination by Western blot analysis. 157

Figure 6.3.3 – Schematic of protocol for investigating the influence of the JAK2 inhibitor, AG490 (5μM), on STAT3 phosphorylation in the presence and absence of leptin (10nM), as determined by Western blot analysis. 158
Figure 6.4.1 – Graph showing the abrogation of leptin-induced protection by AG490. 163
Figure 6.4.2 – Graph showing leptin-induced STAT3 phosphorylation at different time-points in reperfusion. 166
Figure 6.4.3 – Western blots showing phosphorylated STAT3 and total STAT3 levels in tissue taken from Wistar hearts treated with and without leptin (10nM) 167
Figure 6.4.4 - Graph showing the inhibition of leptin-induced STAT3 phosphorylation by AG490 169
Figure 7.4.1 – Schematic of protocol for investigating the cardioprotective effects of leptin in Langendorff perfused hearts from Zucker lean and *fa/fa* rats. 180
Figure 7.4.2 – Schematic of protocol for the collection of Western blot samples from Zucker lean and *fa/fa* hearts subjected to I/R injury. 182
Figure 7.5.1 – Graph of haemodynamic data for hearts in the Zucker lean and *fa/fa* treatment groups. 186
Figure 7.5.2 – Graph showing the effect of leptin (10nM) on infarct size in hearts from Zucker lean and *fa/fa* rats subjected to I/R injury. 188
Figure 7.5.3 – Graph showing the effect of leptin (10nM) on Akt/Ser-473 phosphorylation in hearts from Zucker lean and *fa/fa* rats. 190
Figure 7.5.4 – Western blots showing phosphorylated Akt/Ser-473 and total Akt levels in tissue taken from Zucker lean and Zucker *fa/fa* hearts treated with and without leptin (10nM). 191
Figure 7.5.5 – Graph showing leptin-induced phosphorylation of Akt/Thr-308 in Zucker lean and *fa/fa* rat hearts. 192
Figure 7.5.6 - Western blots showing phosphorylated Akt/Thr-308 and total Akt levels in tissue taken from Zucker lean and Zucker *fa/fa* hearts treated with and without leptin (10nM). 193

Figure 7.5.7 – Graph showing leptin-induced phosphorylation of p44 MAPK in Zucker lean and *fa/fa* rat hearts. 194
Figure 7.5.8 – Graph showing leptin-induced phosphorylation of p42 MAPK in Zucker lean and *fa/fa* rat hearts of leptin (10nM). 195
Figure 7.5.9 - Western blots showing phosphorylated MAPK p44/42 and total MAPK levels in tissue taken from Zucker lean and Zucker *fa/fa* hearts treated with and without leptin (10nM). 196
Figure 8.3.1 – Schematic of protocol for investigating the effects of leptin (10nM) on MPTP opening 206
Figure 8.3.2 – Photographs showing the depolarisation of isolated rat cardiomyocytes in response to ROS. 207
Figure 8.4.1 – Graph showing MPTP opening in cardiomyocytes isolated from Wistar, Zucker lean and Zucker fatty rat hearts. 209
Figure 8.5.1 – Proposed signalling mechanisms underlying leptin-induced myocardial protection. 212
Figure 9.3.1 - Schematic of protocol for investigating the effects of leptin on myocardial I/R injury in an *in vivo* rat model…………………………………....216
Figure 9.4.1 – Effect of leptin, administered at reperfusion, on infarct size in an *in vivo* rat model of myocardial I/R injury 218

LIST OF TABLES

Table 1.4.1 – The international classification of adult underweight, normal weight, overweight and obese according to BMI. 57
Table 4.4.1 – Characteristics of animals in the treatment groups 115
Table 4.4.2 – Coronary flow rates of the different groups at various time-points during the ischaemia/reperfusion protocol. 116
Table 4.4.3 – Rate pressure product of the different groups at various time-points during the ischaemia/reperfusion protocol. 117
Table 4.4.4 - Characteristics of the animals in the treatment groups 120
Table 4.4.5 – Coronary flow rates (ml/min) of each group at various time-points during the ischaemia/reperfusion protocol. 121
Table 4.4.6 - Rate Pressure Product (x 103mmHg/min) of each group at various time-points during the ischaemia/reperfusion protocol. 122
Table 5.4.1 – Table showing the characteristics of the animals in the different treatment groups. 133
Table 5.4.2 – Coronary flow rate (ml/min) of each group at various time-points during the ischaemia/reperfusion protocol. 134
Table 5.4.3 - Rate Pressure Product (x 103mmHg/min) of each group at various time-points during the ischaemia/reperfusion protocol. 135
Table 5.4.4 - Table showing the characteristics of the animals in the different treatment groups included in the Western blot study. 138
Table 6.4.1 – Characteristics of the animals in the treatment groups. Data are expressed as mean±s.e.m. 160
Table 6.4.2 – Coronary flow rate (ml/min) of each group at various time-points during the ischaemia/reperfusion protocol. 161
Table 6.4.3 - Rate Pressure Product (x 103mmHg/min) of each group at various time-points during the ischaemia/reperfusion protocol. 162
Table 6.4.4 – Table showing the body weights of the animals used in the different treatment groups 165

Table 6.4.5 – Average body weight of the animals in the different treatment groups. 168
Table 7.5.1 – Mean body weights, ventricular volumes and risk zones for the different treatment groups. Data expressed are as mean±s.e.m. 183
Table 7.5.2 – Coronary flow rate (ml/min) of each group at various time-points during the ischaemia/reperfusion protocol. 184
Table 7.5.3 - Rate Pressure Product (x 103mmHg/min) of each group at various time-points during the ischaemia/reperfusion protocol. 185
Table 7.5.4 – Mean body weights for the animals different treatment groups. Data are presented as mean±s.e.m. 189

LIST OF ABBREVIATIONS

The following is a list of abbreviations used in this thesis

%	percentage
AAR	area at risk
ADP	adenosine diphosphate
AdipoR1	adiponectin receptor 1
AdipoR2	adiponectin receptor 2
AgRP	agouti-related peptide
AIF1	apoptosis inducing factor 1
Akt	cellular Akt/ protein kinase B
AMP	5' adenosine monophosphate
AMPK	5' AMP-activated protein kinase
ANOVA	analysis of variance
APAF-1	apoptosis protease-inducing factor-1
APJ	apelin receptor
APS	ammonium persulphate
ATP	adenosine triphosphate

ATPase	ATP synthase
AU	arbitrary units
Bad	Bcl-XL/Bcl-2-associated death promoter
Bax/BAX	Bcl-associated X protein
BAT	brown adipose tissue
BCA	bicinchoninic acid
BMI	body mass index
BSA	bovine serum albumin
Ca^{2+}	calcium ion
CAL	coronary artery ligation
CART	cocaine and amphetamine-regulated transcript
Caspase	cysteine-dependent aspartate specific proteases
CFR	coronary flow rate
CHD	coronary heart disease
CK	creatine kinase
COX-2	cyclooxygenase 2
CRP	C-reactive protein

CsA	cyclosporin-A
CVD	cardiovascular disease
Cyp-D	cyclophilin D
Da	daltons
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
Elk-1	E-26-like protein-1
ELISA	enzyme-linked immunosorbent assay
ENOS	endothelial nitric oxide synthase
EPO	erythropoietin
ERK	extracellular signal-regulated MAPK
ET-1	endothelin-1
ETC	electron transport chain
FA	fatty acid
FADH	flavin adenine dinucleotide
FERM	band 4.1, ezrin, radixin and moesin

G-CSF	granulocyte colony stimulating-factor
GLP-1	glucagon-like peptide 1
GPCR	G-protein coupled receptor
GSK-3β	glycogen synthase kinase-3 beta
H+	hydrogen ion/proton
H_2O_2	hydrogen peroxide
Hg	Mercury
HPA	hypothalamic pituitary axis
IRS	insulin receptor substrate
IL6	interleukin 6
iNOS	inducible nitric oxide synthase
IHD	ischaemic heart disease
IRS	insulin receptor substrate
IPC	ischaemic preconditioning
IPOST	ischaemic postconditioning
I/R	ischaemia/reperfusion
IS	infarct size

JAK	janus kinase
JNK	c-Jun NHP2 terminal kinase
K^+	potassium ion
KATP	ATP-sensitive potassium channel
kDa	kilodalton
KR	Krebs-Ringer
L-NAME	N ω-nitro-L-arginine methyl ester
LAD	left anterior descending
LVH	left ventricular hypertrophy
LY	LY294002
MAPK	mitogen activated protein kinase
Mdm2	murine double minute 2
MetS	metabolic syndrome
Mg^{2+}	magnesium ion
MEK	MAPK/Erk kinase
Mn	manganese
Mn-SOD	manganese superoxide dismutase

MPT	mitochondrial permeability transition
MPTP	mitochondrial permeability transition pore
Na^+	sodium ion
NHE	Na^+/H^+ exchanger/Sodium-hydrogen exchanger
NADH	nicotinamide adenine dinucleotide
nm	nanometre
NF-κB	nuclear factor kappa B
NHE	Na+-H+ exchanger
NPY	neuropeptide Y
NO	nitric oxide
OB-R	leptin receptor
p21	cyclin-dependent kinase inhibitor 1A
p53	tumour protein 53
PBEF	pre-B cell colony-enhancing factor
PCTA	percutaneous transluminal coronary angioplasty
PDK	phosphoinositide dependent kinase
Pi	inorganic phosphate

PIAS	protein inhibitor of activated STAT
PH	pleckstrin-homology
pH	pH
pH(i)	intracellular pH
PHLPP	PH domain leucine-rich repeat protein phosphatase
PI3K	phosphatidyl inositol 3-OH kinase
PKC	protein kinase C
POMC	proopiomelanocortin
PPAR-α	peroxisome proliferator-activated receptor-alpha
PTEN	phosphatase and tensin homolog
p70S6K	70-kDA ribosomal protein S6 kinase
p90RSK	p90 ribosomal S6 kinase
Rac1	Ras-related C3 botulinum toxin substrate 1
Raf	MAPK kinase
RBP4	Retinol Binding Protein 4
Rcf	relative centrifugal force
RhoA	Ras homolog A

RISK	reperfusion injury salvage kinase
ROCK	Rho-associated coiled coil–containing protein kinase
ROS	reactive oxygen species
Rpm	revolutions per minute
RPP	rate-pressure product
RTK	receptor tyrosine kinase
SDS	sodium dodecyl sulphate
SH2	Src homology 2
SHIP2	Src homology 1–containing tyrosine phosphatase
SOCS	suppressor of cytokine signalling
SR	sarcoplasmic reticulum
STAT	signal transducer and activator of transcription
SWOP	second window of protection
TCA	tricarboxylic acid
TEMED	tetramethylethylenediamine
TGFβ-1	transforming growth factor beta-1
TK	tyrosine kinase

TMRM	tetramethyl-rhodamine methyl ester
TNF-α	tumour necrosis factor-alpha
TTC	triphenyl tetrazolium chloride
UO	UO126
WAT	white adipose tissue
WHO	world health organisation

CHAPTER 1 - GENERAL INTRODUCTION

1.1 Coronary Artery Disease

Cardiovascular disease (CVD) is the principal cause of mortality in the United Kingdom, accounting for approximately 1 in 5 deaths in males and 1 in 6 in females. Apart from the human cost, CVD places a large economic burden on the national health system. In 2003 CVD cost the UK healthcare system approximately £15,000 million. Therefore it is clear that a concerted effort is needed to prevent, treat and understand CVD in order to limit its consequences. Basic scientists aim to understand CVD disease at the molecular level and to investigate potential treatments that can then be translated to clinical practice.

CVD ranges in severity from sub-clinical coronary artery disease to acute myocardial infarction (MI), with congestive heart failure and death featuring as the worst manifestations of the disease (Shah & Forrester 1991). According to the leadership of the European Society of Cardiology (ESC), the American College of Cardiology (ACC), American Heart Association (AHA) and the World Heart Federation (WHF), the term myocardial infarction should be used when there is evidence of myocardial necrosis in a clinical setting consistent with myocardial ischaemia. This information was published in an Expert Consensus Document published by the Journal of the American College of Cardiology in 2007 (Thygesen et al. 2007). Myocardial ischaemia is defined as the limitation of blood flow, with increased resistance, to a portion of the heart, which leads to the oxygen demands of the tissue exceeding that provided by the decreased blood flow. Coronary artery disease starts with a gradual build-up of plaque in

the arteries of the heart. This build-up is often exacerbated in sedentary individuals who consume a diet rich in fats and who smoke, however this can also be as a result of genetic factors (Genest, Jr. & Cohn 1995; Poirier et al. 2006; Shah 2006). Blocking of an artery occurs when an unstable atherosclerotic plaque ruptures, resulting in the formation of a thrombotic plug which occludes the vessel and prevents canonical blood flow (figure 1.1.1) (Shah 2002). Cessation of blood flow in one or more coronary arteries presents clinically as an acute myocardial infarction. Rapid reperfusion of ischaemic tissue by application of either thrombolysis or primary percutaneous transluminal coronary angioplasty (PCTA) has been found to be the most effective way of salvaging myocardial tissue at risk (Faxon 2005). Limitation of ischaemic tissue and optimisation of patient prognosis and quality of life is the main goal of these therapies. In the clinical setting reperfusion of the ischaemic myocardium is associated with the following complications: myocardial stunning (Braunwald & Kloner 1982; Bolli & Marban 1999), reperfusion arrhythmias (Manning & Hearse, 1984), endothelial and mircovascular dysfunction (Ito, 2006) and cardiomyocyte death. These pathologies are the consequence of a phenomenon known as reperfusion injury (Piper et al, 1998) (see 1.2).

The goal of scientists working in the field of ischaemic heart disease is to understand the mechanisms by which the disease manifests itself and to identify treatments that will improve patient prognosis. Myocardial infarctions are difficult to predict and therefore it is difficult to apply treatments prior to the

ischaemic insult. Consequently, treatments are usually targeted at either the ischaemic phase or the reperfusion-phase.

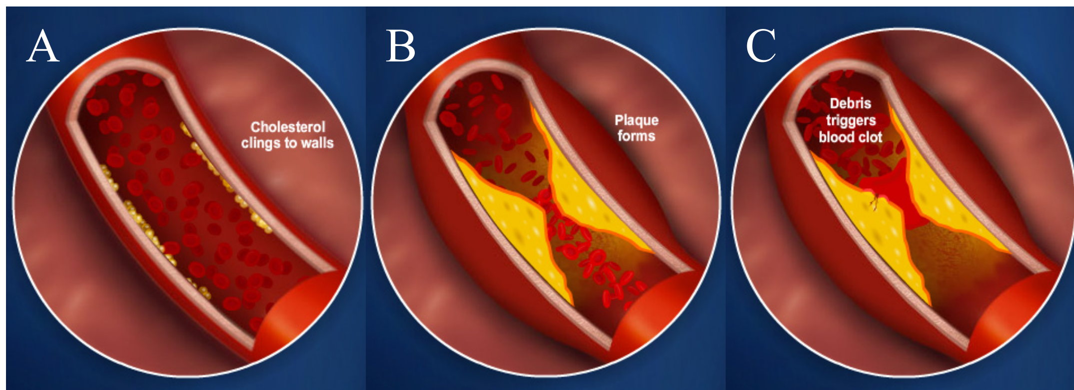


Figure 1.1.1 - Schematic diagram depicting the progression of coronary artery disease. (A) Plaque formation begins with cholesterol adhering to walls of coronary vessels. (B) As plaque becomes larger artery expands and lumen of the vessel decreases. (C) Plaque rupture leads precipitation of a clotting reaction and consequential occlusion of vessel by a thrombotic plug. *Figure adapted from www.nationalgeographic.com.*

1.2 Myocardial Ischaemia-Reperfusion (I/R) Injury

The heart is a complex biomechanical pump, efficient in the conversion of chemical to mechanical energy. Blood flow via the coronary vasculature provides fuel for oxidative metabolism, which in the form of adenine-triphosphate (ATP) provides the majority of the energy required to produce the powerful rhythmic contractions needed to pump the blood around the body. If blood flow is interrupted for a short period, the energy requirements of the heart can be met by glycolytic ATP production until normal oxidative metabolism can be restored (Das & Harris 1990). However, prolonged periods of interrupted blood flow, known as ischaemia, leads to the production of lactate and hydrogen ions (H^+).

The accumulation of these by-products of glycolytic ATP production can irreversibly damage the myocardium (Dennis et al. 1991).

Reperfusion of the ischaemic myocardium is a necessary event in order to salvage cardiac tissue at risk. Although reperfusion provides washout of the damaging lactate and restores pH levels to normal, paradoxically, it is also responsible for damage to ischaemic cells that were previously viable before reperfusion (Piper et al. 1998). Hence, reperfusion has been referred to as a “double edged sword,” not only salvaging myocardial tissue but also exacerbating and accelerating injury caused by ischaemia alone (Braunwald & Kloner 1985). This phenomenon, termed reperfusion injury, was first described by Jennings et al. in 1960. They conducted detailed histological examinations of the damaged reperfused ischaemic canine myocardium and identified cellular swelling, disruption of sarcolemma, contracture of myofibrils and the emergence of intramitochondrial calcium phosphate particles as key features (Jennings et al. 1960).

The existence of reperfusion injury was contested for some time, with sceptics suggesting that reperfusion just exacerbates the damage sustained during ischaemia (Gross & Auchampach 2007). Nonetheless, studies demonstrating that infarct size can be reduced by pharmacological agents delivered at reperfusion have provided evidence for the existence of I/R injury (Bell & Yellon 2003; Mocanu et al. 2000; Andreadou et al. 2008). Studies using animal models of myocardial infarction indicate that lethal reperfusion injury may account for up to 50% of the infarct size. The damage caused by myocardial reperfusion leads

to four different types of cardiac dysfunction. The first of these is myocardial stunning, which is defined as “prolonged post-ischaemic mechanical dysfunction and continues after reperfusion in the absence of irreversible damage” (Braunwald & Kloner 1982). The myocardial dysfunction persists much longer than the time of the original index-ischaemic period. Hence, just 15min of myocardial ischaemia in dogs leads to 24 hours of depressed cardiac function. However, stunning is not usually a major clinical problem unless a significantly large portion of the myocardium is affected (Piper et al. 1998). The second type of cardiac dysfunction associated with myocardial reperfusion are potentially lethal myocardial arrhythmias (Manning & Hearse 1984), however their incidence is low, and should they occur, are easily treated by the clinician (Piper et al. 1998). Mircovascular dysfunction, the third type of cardiac dysfunction, is the result of a combination of endothelial damage, oedema, oxidative stress and microvascular obstruction by microemboli (Ito 2006). No-reflow is the most severe form of microvascular dysfunction in which perfusion of the microvasculature is either reduced or completely prevented (Ito 2006). Lethal reperfusion injury is the last type of cardiac dysfunction (Piper et al. 1998). This is defined as cell death that occurs in the reperfusion-phase and should not be confused with “oncosis,” which is defined as cell death which occurs during the ischaemic phase (Majno & Joris 1995). In the following sections the molecular mechanisms contributing to the four clinical manifestations of reperfusion injury will be examined in detail.

1.2.1 Metabolic and biochemical consequences of myocardial ischaemia

In cardiac ischaemia the myocardium must adopt strategies in order to survive the decrease in blood flow and consequent reduction in oxygen supply (Hochachka et al. 1996). These strategies include a decrease in myocardial contracture coupled with the activation of compensatory metabolic changes, both processes occurring within 10-120 seconds of the onset of severe myocardial ischaemia (Guth et al. 1993). Oxygen deprivation leads to a decrease in the availability of cellular energy. This is reflected by a sharp decline in creatine phosphate levels, which in turn stimulate glycolysis and glycogenolysis (Solaini & Harris 2005). In the canine heart, anaerobic glycolysis is activated within 8 seconds of coronary artery ligation (Kloner & Jennings 2001). Anoxia will allow ATP production to continue via glycolysis (Das & Harris 1990), however, during ischaemia this process leads to the production of lactate and hydrogen ions, i.e. a decrease in intracellular pH, which, in turn, inhibits glycolysis (Solaini & Harris 2005).

Oxygen deprivation also leads to the inhibition of the tricarboxylic acid (TCA) cycle, and results in no energy being available from oxidative phosphorylation, and an accumulation of cytoplasmic nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH) (Solaini & Harris 2005). During ischaemia ATP levels decline slowly with 40-50% being still available after 30min of ischaemia (Ambrosio et al. 1993). In the heart ATP consumption is closely associated with contraction. In cardiomyocytes, for example, the actomyosin contractile apparatus consumes approximately two thirds of the

available ATP (Opie, 1991). The remaining third is used to maintain ionic homeostasis by a variety of ATP-dependent membrane pumps (Opie, 1991). When ATP levels become depleted, as in myocardial ischaemia, these ATP dependent pumps fail and cellular ionic homeostasis is lost. Intracellular levels of Na^+ and Ca^{2+} rise in proportion to the severity of ischaemia (Steenbergen et al. 1990). It is thought that at least part of the cellular burden of Na^+ results from increased activity of the Na^+/H^+ exchanger (NHE) during ischaemia, which is stimulated by acidosis (i.e. increased H^+ levels) (Karmazyn et al. 1999). Recently, however, it has been demonstrated that Na^+ channels are, in part, also responsible for increased intracellular Na^+ levels (Williams et al. 2007). In turn, increased levels of Na^+ trigger the Na^+/Ca^{2+} exchanger, which expels Na^+ from the cell in return for an influx of Ca^{2+} (Anderson et al. 1990).

Increases in both Na^+ and Ca^{2+} are detrimental to the cell. Increased sarcoplasmic Na^+ levels can cause mitochondrial damage and also lead to an increase in osmolarity which can ultimately lead to cellular swelling and even cell rupture (Takeo & Tanonaka 2004). High intracellular Ca^{2+} levels are thought to lead to damage to the contractile apparatus, mitochondrial damage and activation of phospholipases (Bernardi & Rasola 2007).

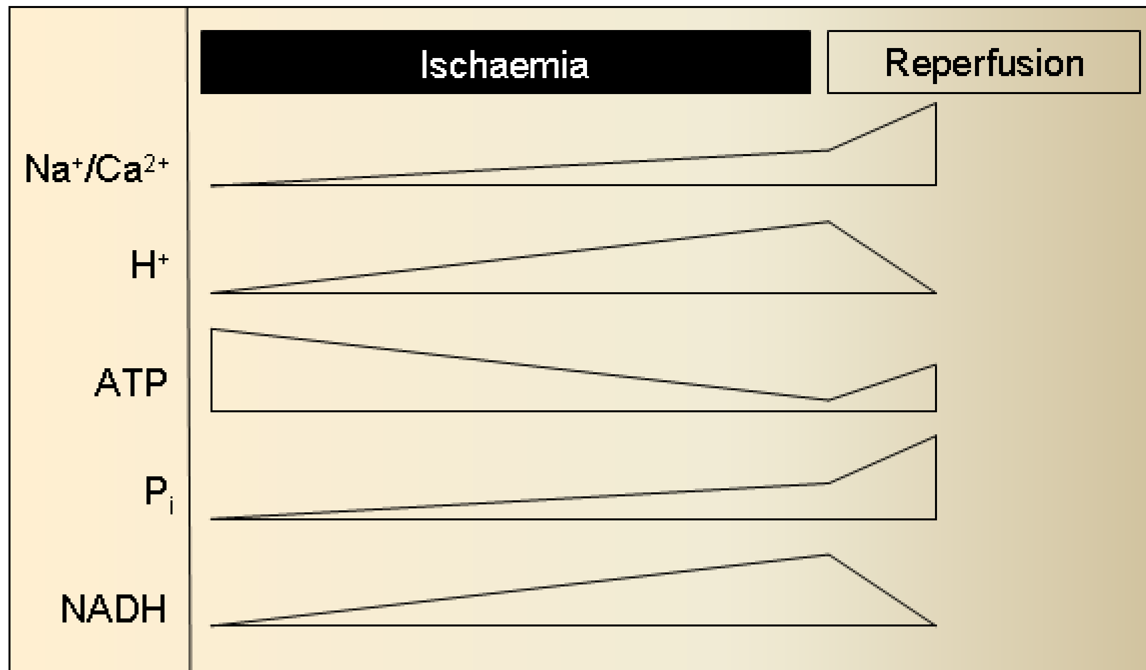


Figure 1.2.1 – Schematic representing the changes in the levels of ions, ATP, P_i and NADH during ischaemia and reperfusion. *Figure adapted from: (www.heartandmetabolism.org)*

1.2.2 Metabolic and biochemical consequences of myocardial reperfusion

Upon reperfusion oxidative phosphorylation and ATP synthesis recommence. Despite reactivation of these processes and the restoration of normal intracellular pH (~7.0), cellular dysfunction is exacerbated (Dennis et al. 1991). The factors contributing to reperfusion injury are discussed below.

1.2.2.1 Calcium overload

Reperfusion of the ischaemic myocardium leads to re-energization of the mitochondria, providing ATP to cation pumps that were inactive during ischaemia (Javadov & Karmazyn 2007). The Ca^{2+}-ATPase of the sarcoplasmic reticulum (SR) is reactivated and Ca^{2+} is sequestered to the SR (Siegmund et al. 1992). If the levels of Ca^{2+}, however, exceed the capacity of the SR, Ca^{2+} is re-released, leading to a cycle of release and uptake (Siegmund et al. 1994). Only if Na^+ homeostasis is achieved, i.e. a Na^+ gradient is re-established, will the Na^+/Ca^{2+} exchanger function again and remove excess calcium from the cell (Siegmund et al. 1994).

The cardiomyocytes that are carrying high intracellular calcium are vulnerable to cell death via hypercontracture and or mitochondrial permeability transition pore (MPTP) opening (see section 1.2.2.4) (Halestrap et al. 1998). Experimental data have demonstrated that inhibitors of the sarcolemmal Ca^{2+} ion channel, the mitochondrial Ca^{2+} uniporter, or the sodium-hydrogen (Na^+/H^+) exchanger (NHE) can block the surge of Ca^{2+} at reperfusion and result in an approximately 50%

reduction in myocardial infarct size (Klein et al. 1989; Carry et al. 1989; Gumina et al. 1999) However, it must be noted that inhibition of the NHE reduces infarct size only when the antagonist is administered during or prior to ischaemia, not at reperfusion (Zeymer et al. 2001). Hence, this approach to infarct size reduction has its limitations in the clinical arena.

1.2.2.2 Oxidative stress

At low concentrations reactive oxygen species (ROS) act as cellular messengers, however, at higher concentrations they are extremely deleterious (Bandyopadhyay et al, 1999). Approximately, 1-2% of normal oxidative phosphorylation leads to ROS production (Gnaiger & Kuznetsov 2002). Paradoxically, the decreased availability of O_2, i.e. decreased partial pressure, leads to increased ROS generation (Solaini & Harris 2005). This effect, also known as the oxygen paradox, is thought to be a result of the action of O_2 within the mitochondrial inner membrane, however, very little is known about the underlying mechanism of this phenomenon (Guzy & Schumacker 2006). Ischaemia leads to increased ROS production (Becker et al. 1999; Vanden Hoek et al. 1997), which is exacerbated on reperfusion (Bolli & Marban 1999). The adverse effects of ROS on cellular function include lipid peroxidation and the oxidation of cardiac proteins (Ferrari et al. 2004). Protective mechanisms, include the enzymes superoxide dismutase (SOD) and manganese superoxide dismutase (Mn-SOD) (in the mitochondria), which act to remove ROS by catalysing the conversion of superoxide to oxygen and hydrogen peroxide (H_2O_2) (Kinnula & Crapo 2004). Furthermore, the H_2O_2 by-product, which is

also deleterious, can be removed by glutathione peroxidase and peroxiredoxin (Nohl & Jordan 1980). Low levels of ROS lead to lipid peroxidation, protein modification and mitochondrial deoxyribonucleic acid (DNA) oxidation (Ferrari et al. 2004). However, high levels of ROS can overwhelm these scavenging systems and lead to cell death by apoptosis or necrosis, thereby contributing significantly to I/R injury (Kang & Izumo 2000). In the ischaemic heart the main sources of ROS include the electron transport chain (ETC), xanthine oxidase and the NADPH oxidase system (Szocs 2004).

1.2.2.3 The pH paradox

The cellular acidosis (pH<7.0) which occurs during ischaemia has been demonstrated to protect against necrotic cell death in practically all cell types examined (Penttila & Trump 1974; Gores et al. 1988; Inserte et al. 2008). By contrast, the rapid return to a normal intracellular pH (pH(i)) that occurs during reperfusion is, in fact, detrimental, contributing to reperfusion injury (Bond et al. 1993). This phenomenon, which is facilitated by the activation of the Na^+/H^+ exchanger and the sodium-bicarbonate symporter, and washout of lactic acid at reperfusion is known as “The pH paradox” (Bond et al. 1994). It is now thought that a mechanism involving the opening of the mitochondrial permeability transition pore (MPTP) may underlie the pH paradox (Lemasters 1999). Return of pH(i) to normal physiological values upon reperfusion has been shown to be associated with MPTP opening, which in turn can lead to cell death (see section 1.2.2.4). In rat hepatocytes, reperfusion with either an acidic buffer (pH=6.2) or with Cyclosporin A (CsA) abrogated the increase in MPTP opening and

preserved cell viability (Qian et al. 1997). Bond el al performed a similar study that demonstrated that ischaemic neonatal rat cardiomyocytes were protected against I/R injury when reperfused with an acidic buffer (Bond et al. 1991). Furthermore, methods that delay the return to a physiological pH level, and thus allow the cell time to recover, have been shown to be protective. For example, treatment of cardiomyocytes with dimethyl amiloride or HOE694, which are inhibitors of the Na^+/H exchanger facilitate cell viability by prolonging the time taken for pH(i) change to occur.

1.2.2.4 Mitochondrial permeability transition

The mitochondrial permeability transition pore (MPTP) is a non-specific pore that occurs between the inner and outer mitochondrial membranes (Bernardi & Petronilli 1996). Under conditions of stress, such as those occurring after reperfusion of the ischaemic myocardium (elevated matrix Ca^{2+}, ROS elevation, elevated P_i and depleted ATP, see sections above) the MPTP opens. Consequently, proteins and metabolites <1.5KDa are allowed to flow down their respective gradients (Halestrap et al. 2004). In the absence of the H^+ gradient, used to drive the F_1F_0-ATPase, mitochondria become depolarised and oxidative phosphorylation is uncoupled (Solaini & Harris. 2005). Furthermore, the ATPase can function in reverse, hydrolysing ATP and leading to a rapid decrease in cellular ATP levels (Jennings et al. 1991). ATP is required to maintain cellular ionic and metabolic homeostasis. Pore closure must occur in cells subjected to I/R to avoid necrotic cell death and so that homeostasis can be re-established (Halestrap 2006). The amount of available ATP is thought to

dictate whether a cell undergoes necrotic, ATP-independent or apoptotic, ATP requiring, cell death (Halestrap et al. 2000)(see section 1.3).

The structure of the pore remains to be fully elucidated. However, it has been comprehensively shown that cyclophilin D (Cyp-D) is, if not the main component, one of the major components of the pore. Thus, it was demonstrated that Cyp-D -/- mice subjected to myocardial I/R develop smaller infarcts than wild-type mice (Baines et al. 2005), indicating that formation of the pore is required for canonical mitochondrial death signalling to occur.

A growing amount of data indicates that inhibition of the MPTP protects myocardial tissue against I/R injury. Cyclosporin A (CsA) and Sanglifehrin A both protect the ischaemic myocardium by binding to Cyp-D, albeit at different locations, and inhibit pore opening (Shanmuganathan et al. 2005; Clarke et al. 2002). Furthermore, it has been demonstrated in isolated cardiomyocytes that cells treated with insulin, which stimulates Akt phosphorylation, are less likely to undergo pore opening and subsequent rigor contraction (Davidson et al. 2006). This indicates that cardioprotectants that inhibit pore opening may do so via a mechanism that activates Akt. The signalling mechanisms operating between Akt and the MPTP are, however, as yet unknown.

Collectively, this body of data suggest that the MPTP may represent an important therapeutic target with regard to cell death, not only in the heart but also in other tissues such as the brain (Deniaud et al. 2006). In a preliminary trial patients who presented with (acute ST-elevation) myocardial infarction were

randomised to receive placebo or Cyclosporin A (CsA) by intravenous bolus (Piot et al. 2008). This study yielded data that mirrored those obtained by experiments utilising CsA in *in vivo* models (Xie & Yu 2007). For example creatine kinase levels were significantly reduced and the mass of the infarcted tissue was also reduced in the group treated with CsA (Piot et al. 2008). The levels of troponin, an important marker of tissue injury, were, however, not significantly reduced. This is an important pilot study that when taken further should provide an important insight into the cardioprotective capacity of CsA in the human myocardium.

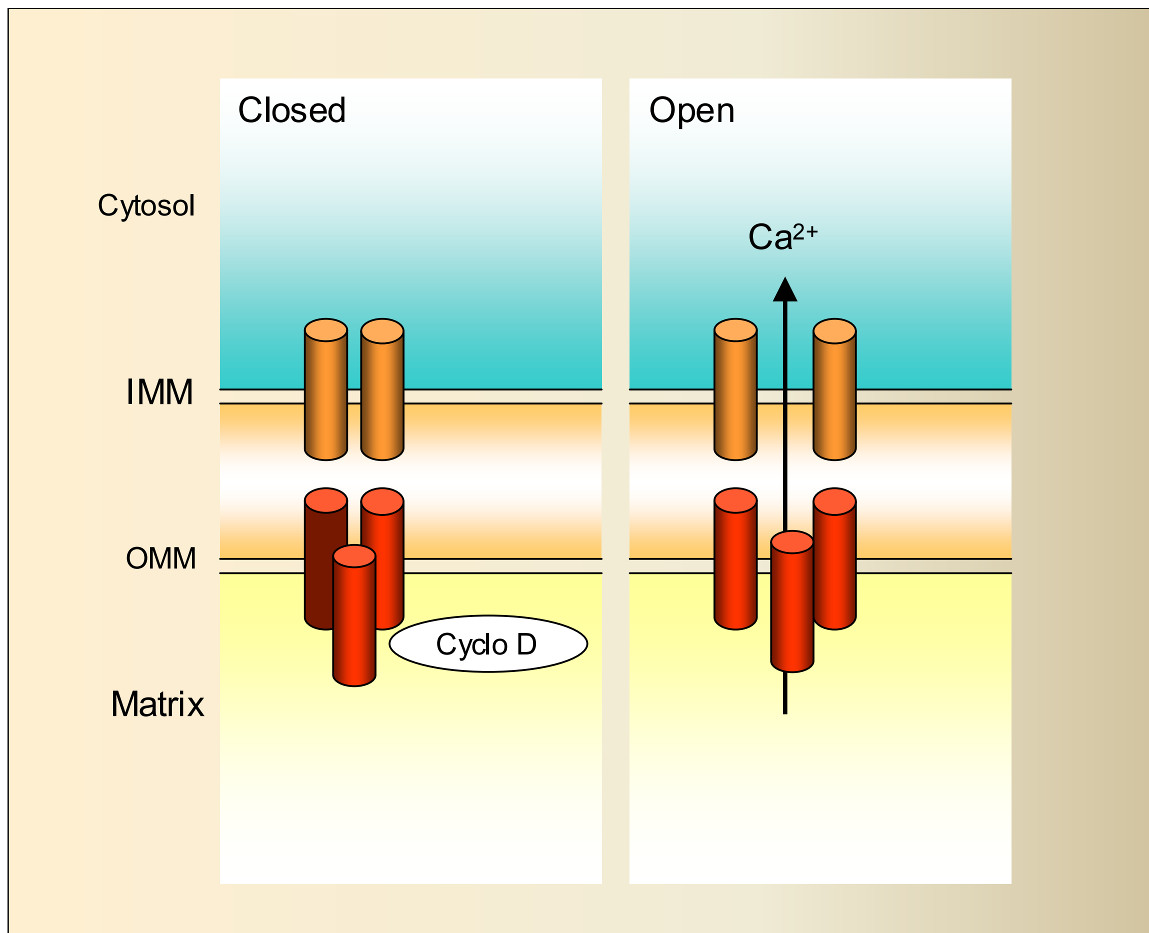


Figure 1.2.2 – Schematic of the mitochondrial permeability transition pore (MPTP). The MPTP is a non-specific pore that forms between the inner mitochondrial membrane and the outer mitochondrial membrane (OMM). Currently the true components that form the pore are unknown. However, cyclophilin D (Cyp D) has been shown to be a regulatory component of the pore. Opening of the pore ultimately leads to cytochrome C release and apoptosis. *Figure adapted from:(Abou-Sleiman et al. 2006)*.

1.2.3 Cell death

Ischaemia-reperfusion injury ultimately leads to cell death. Prior to 1971 cell death was termed necrosis. Subsequently, however, an alternative programmed form of cell death was identified in some tissues, which was ultimately termed apoptosis (Kerr et al. 1972). Thus, it was suggested that cell death took two principle forms, namely necrosis and apoptosis (Gottlieb & Engler 1999).

1.2.3.1 Necrosis

Necrosis is a pathological form of cell death which is characterised by cellular swelling, organelle swelling, membrane permeabilisation and the release of cellular contents, and provokes inflammation (Wei-Xing Zong Tompson 2006). Initially, necrosis was thought to be the predominant form of cell death in ischaemic heart disease (IHD). Subsequently, however, evidence was obtained indicating that apoptosis is responsible for the generation of a larger proportion of infarcted tissue in the context of MI than necrosis (Kajstura et al. 1996). This situation was clarified by studies showing that inhibition of caspases, enzymes integral to the process of apoptosis, leads to significant decreases in infarct size (Mocanu et al. 2000).

1.2.3.2 Apoptosis

In the context of I/R injury apoptosis in the heart was first identified in 1994 (Gottlieb et al. 1994). Unlike necrosis, apoptosis is an energy-dependant, ATP-requiring process that involves a number of biochemical steps (Leist et al, 1997). The process of apoptosis can be triggered by various signals, the origin of which can be intra- or extracellular. Fas ligand and tumour necrosis factor-alpha (TNF-α), for example, induce apoptotic signalling by binding to their respective receptors located on the extracellular membrane (Fas et al. 2006). By contrast, a wide range of intrinsic signals can also promote the apoptotic cascade. These can include heat, radiation, viral infection (Cotran et al, 1998) and high Ca^{2+} concentration (Mattson & Chan 2003). Generally, both the intrinsic and extrinsic pathways stimulate the activation of a group of proteases known as the caspases (Nicholson 1999). Receptor mediated apoptosis, however, typically

activates caspase-8, which in turn stimulates caspase-3 (Baines & Molkentin 2005). By contrast, the intrinsic pathway, which in the case of I/R injury is stimulated by hypoxia, leads to increases in ROS and Ca^{2+} levels and, ultimately, mitochondrial permeability transition (Weiss et al. 2003). Loss of mitochondrial membrane integrity leads to the release of a number of intermembrane proteins including cytochrome C, Smac/DIABLO and apoptosis inducing factor 1 (AIF1) (Zhou et al. 2005). Once released to the cytosol, cytochrome C combines with a protein called apoptotic protease-activating factor-1 (Apaf-1) (Hill et al. 2004) to form a multi-protein structure known as the "apoptosome". The apoptosome stimulates activation of caspase-9, which in turn activates caspase-3 (Adrain et al. 2006). Ultimately the activation of these caspases leads to the cleavage of several caspase substrate proteins including (1) lamins, leading to the degradation of the nuclear lamina (Buendia et al. 1999), (2) topoisomerases, histones, DNase inhibitors and DNA repair enzymes, resulting in the degradation of cellular DNA (Sordet et al. 2004) and (3) structural proteins such as actin and gelsolin, leading to the breakdown of the cytoskeleton (Gourlay & Ayscough 2005). During apoptosis cells go through a series of morphological changes including membrane blebbing, cell shrinkage, nuclear fragmentation and DNA fragmentation (Kerr et al. 1972). Contrasting with necrosis which results in the generation of debris through the lysis of cells, apoptosis creates small, well-defined rounded cell bodies that are easily digested by phagocytosis, thus reducing damage to the surrounding tissues (Zong & Thompson 2006).

1.2.3.3 Other forms of cell death

It is becoming increasingly clear that the processes of apoptosis and necrosis alone are not sufficient to describe the range of cell death mechanisms that have been observed. In addition to apoptosis (type I cell death) and necrosis (type II cell death) a third form of cell death is currently recognised, namely autophagic cell death (type II cell death) (Galluzzi et al. 2008).

1.2.3.3.1 Autophagy

Autophagy is known to fulfil the “self-killing” role when apoptosis is unavailable (Maiuri et al. 2007). Autophagic cell death is characterised by the accumulation of double membrane vesicles, or autophagosomes, within the dying cell. Protein aggregates, expired organelles and cytoplasmic components are sequestered to the autophagosomes where they are destroyed by lysosomal enzymes (Clarke 1990).

1.2.3.3.2 Necroptosis

Necroptosis is another form of cell death that was recently proposed to occur in the ischaemic brain (Degterev et al. 2005). Although characterised by the same morphological conditions as necrosis, necroptosis is described as a regulated form of cell death that occurs in apoptotic-deficient conditions (Hitomi et al. 2008). It is currently understood that necroptosis is activated by external ligands which bind to death receptors. This in turn activates receptor interacting protein 1 (RIP1), a kinase which has been demonstrated to be vital to the mechanism underlying necroptosis (Degterev et al. 2008). Necrostatin, an agent that has been proposed to inhibit necroptosis (Degterev et al. 2005), has been shown to protect against myocardial I/R in both *in vitro* and *in vivo* models in our

laboratory (Smith et al. 2007a). Furthermore, this protection has been demonstrated to involve modulation of MPTP opening (Lim et al. 2007b).

1.3 Protection Against I/R Injury

1.3.1 Ischaemic preconditioning

In 1986 a revolutionary paper reported that the application of short periods of sublethal ischaemia interspersed with reperfusion prior to a period of severe ischaemia led to a considerable decrease in infarct size, as compared with control (Murry et al. 1986). This phenomenon, first demonstrated in a canine model, is now known as ischaemic preconditioning (IPC). IPC has now been demonstrated in various species, including mouse (Sumeray & Yellon 1998b), rat (Liu & Downey 1992), pig (Schott et al. 1990), sheep and human (Yellon et al. 1993). Initially, the end-point for assessing protection afforded by IPC was infarct size (Murry et al. 1986). However, IPC was subsequently shown to decrease the incidence of arrhythmias (Shiki & Hearse 1987) and improve functional recovery (Asimakis et al. 1992).

The preconditioned state achieved in the short-term has been demonstrated to last between 3-4 hours, after which time protection is lost, and has been termed classical preconditioning (Liu et al. 1991). Classical IPC is rendered ineffective if the index ischaemic period continues beyond three hours (Murry et al. 1986). In addition to the protection afforded by IPC in the short term, however, there is also a delayed protective effect that occurs approximately 24 hours following the IPC protocol, which is known as the second window of protection (SWOP)

(Kuzuya et al. 1993; Marber et al. 1993). The SWOP disappears after approximately 72 hours (Zacharowski et al. 1999).

Considering the extent of the damage caused by myocardial infarction (see section 1.1) the potential of IPC as a treatment clinically is attracting considerable interest and has generated in excess of 4000 publications (www.pubmed.com). Many of these studies have focused on possible underlying signalling mechanisms, the aim being to discover pharmacological treatments that activate these same pathways so that convenient therapeutic strategies can be developed. Despite the large amount of research that has been carried out into this phenomenon the mechanisms that underlie IPC are still not fully understood. Some of the proteins involved in transmitting the IPC signal, however, have been identified. These include AKT (also known as protein kinase B, PKB), phosphatidylinositol-3'-OH kinase (PI3K), and protein kinase C (PKC) (see section 1.3.4.1) (Hausenloy et al. 2005; Hausenloy & Yellon 2007a). For key reviews of cardioprotective agents see the following publications (Hausenloy & Yellon 2009; Hausenloy & Yellon 2004; Schultz & Gross 2001).

1.3.2 Postconditioning

IPC is clearly a powerful cardioprotective treatment. Unfortunately, it possesses one major pitfall with respect to the clinical setting, in as much as MI is difficult to predict. It has been discovered, however, that a similar protocol of episodes of sublethal ischaemia and reperfusion but administered subsequent to the index ischaemia also produces a cardioprotective effect, a phenomenon which has

been termed postconditioning (IPost) (Zhao et al. 2003; Vinten-Johansen et al. 2005). Using a canine model of I/R injury Vinten-Johansen and colleagues demonstrated that following a three hour period of ischaemia, the application of three 30 second episodes of reperfusion followed by ischaemia significantly protected the myocardium (Zhao et al. 2003). IPost has been shown to depend upon reperfusion injury salvage kinase (RISK) pathway activation (Tsang et al. 2004). Indeed, Sivaraman et al demonstrated in a human atrial muscle model of I/R that IPost could be abolished by the addition of LY294002 and UO126, inhibitors of phosphatidyl inositol 3-OH kinase (PI3K) and p44/42mitogen-activated protein kinase (MAPK), respectively (Sivaraman et al. 2007). Furthermore, it has been shown that Janus kinases / Signal transducers and activators of transcription (JAK/STAT) signalling is also required for functional recovery in the Langendorff-perfused mouse heart subjected to IPOST (Goodman et al. 2008). JAK/STAT signalling alone, however, was not sufficient to provide protection, PI3K-Akt activation also being required (Goodman et al. 2008). IPost has already been shown to yield benefit in the clinic and recently, in the setting of coronary angioplasty, it was shown to reduce creatine kinase release over 72 hours (Staat et al. 2005).

1.3.3 Pharmacological protection

Various chemically unrelated drugs administered prior to ischaemia and at reperfusion have been shown to mimic the protection afforded by IPC and IPOST, including with respect to intracellular signalling mechanisms (Riess et al. 2004; Gross & Gross 2006). Potential cardioprotective agents, therefore, are

usually selected according to their ability to activate pro-survival pathways and or inhibit pro-apoptotic pathways. Insulin, for example, has been shown to activate the PI3K/Akt pathway, phosphorylate and inactivate the pro-apoptotic factor Bcl- XL /Bcl-2-associated death promoter (BAD), and to stimulate endothelial nitric oxide synthase (eNOS) (Jonassen et al. 2001; Gao et al. 2002). Agents released by the heart itself have also been found to be cardioprotective. Urocortin, for example, is secreted by myocytes in response to ischaemia and has been demonstrated to protect the heart via stimulation of the PI3K/Akt and ERK 1/2 signalling pathways (Brar et al. 2000; Schulman et al. 2002). As outlined above, stimulation of the JAK/STAT pathway has also been shown to be cardioprotective and cardiotrophin, which activates various protective signalling pathways, was also shown to increase JAK/STAT activity (Freed et al. 2003). In the next section these signalling pathways will be examined in more detail.

1.3.4 Cellular signalling mechanisms: their roles in the heart and cardioprotection

Many signalling pathways have been identified in cardiomyocytes. These pathways, activated in response to a number of different stimuli, ultimately lead to changes in growth and function. Numerous signalling pathways in cardiomyocytes are activated in response to stress (see Figure 1.3.1). This thesis will focus on the PI3K-Akt (RISK), MAPK and JAK/STAT pathways.

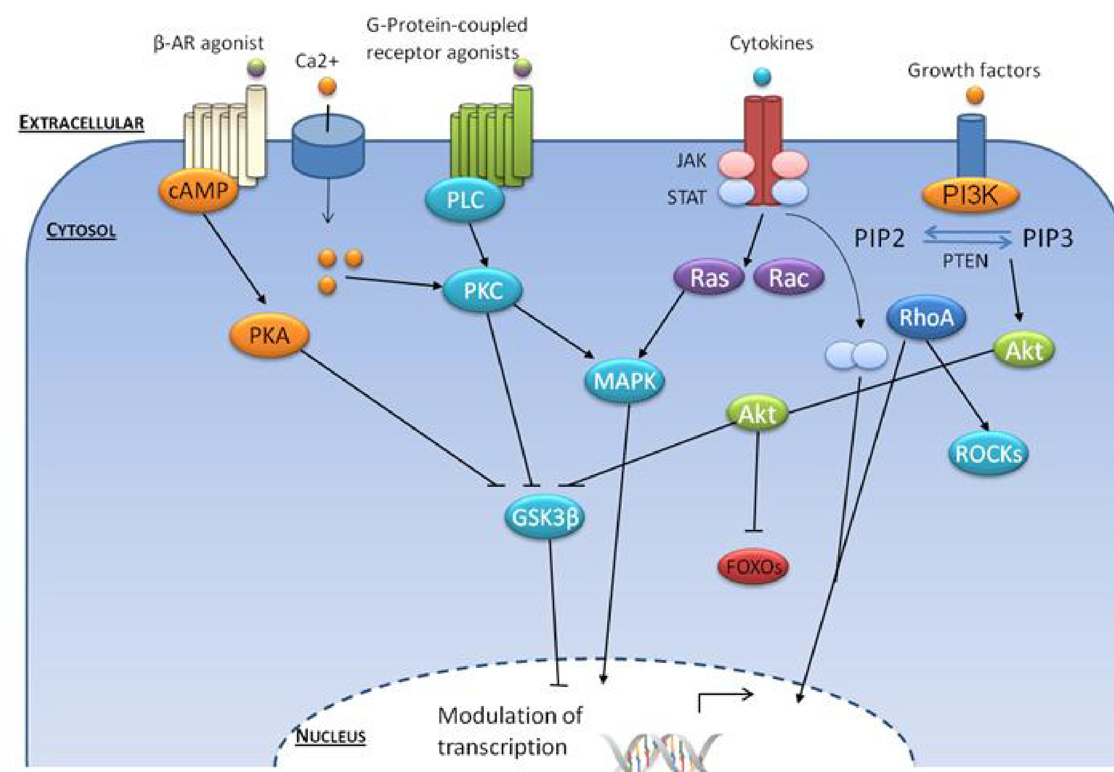


Figure 1.3.1 – Cardiomyocyte signalling pathways. The figure shows a simplified scheme of the signalling pathways that occur in cardiomyocytes. For simplicity, feedback loops and most of the crosstalk between individual pathways and have been omitted. Binding of a ligand to its membrane receptor leads to activation of an associated kinase, which in turn may activate signalling nodes. At this point pathways may converge. In this diagram the examples of signalling nodes are Akt and glycogen synthase β (GSK3β). Ultimately, the activated downstream components modulate cell growth or function. Anchor protein 1 (PKA); cyclic AMP (cAMP); forkhead-box O proteins (FOXO); Janus Kinase (JAK); mitogen-activated protein kinase (MAPK); phosphatidylinositol 3-OH-kinase (PI3K); cAMP-dependent protein kinase (PKA); protein kinase C (PKC); phospholipases C (PLC); phosphatase and tensin homologue (PTEN); Rho-associated (ROCK); signal transducer and activator of transcription (STAT). *Figure adapted from (Mudd & Kass 2008).*

1.3.4.1 Reperfusion injury salvage kinase pathway

The RISK pathway is a term that has been applied to a group of protein kinases that, when activated at reperfusion, are associated with protection against myocardial I/R injury (Schulman et al. 2002; Hausenloy & Yellon 2007b). These pro-survival kinases include protein kinase B (PKB/Akt) and p44/42 MAPK (see sections 1.3.4.1.1 and 1.1.1.1.1 respectively). Originally these kinases were found to be activated by ischaemia/reperfusion injury (Mockridge et al. 2000b; Omura et al. 1999). It has, however, become apparent that additional activation, such as that provided by a pharmacological or mechanical stimulus, confers cardioprotection. *In vitro* and *in vivo* animal studies have demonstrated that the RISK pathway can be stimulated by a myriad of agents including cytokines such as apelin (Simpkin et al. 2007), transforming growth factor beta-1TGFβ-1 (Baxter et al. 2001), erythropoietin (Bullard et al. 2005), leptin (Smith et al. 2006) and visfatin (Lim et al. 2008), and G-protein coupled receptor (GPCR) ligands, including bradykinin (Bell & Yellon 2003), glucagon-like peptide 1 (GLP-1) (Bose et al. 2005b; Bose et al. 2005a) and urocortin (Brar et al. 2002; Brar et al. 2000; Schulman et al. 2002). Mechanical treatments that induce activation of RISK pathway kinases include IPC (Hausenloy et al. 2005), postconditioning (Sivaraman et al. 2007) and low pressure reperfusion (Bopassa et al. 2005).

In order to confer cardioprotection, activation of the RISK pathway must occur within the first few minutes of reperfusion (Jonassen et al. 2001). During these first few minutes intracellular accumulation of ROS and Ca^{2+} leads to MPTP opening (see section 1.1.1), followed by mitochondrial uncoupling and swelling

and consequent cell death (section 1.2). Inhibition of MPTP opening must occur within several minutes of reperfusion, in order for protection to be achieved (Hausenloy et al. 2003). Hence, there exists a critical "window" in which pro-survival kinase activation must occur in order to achieve cardioprotection.

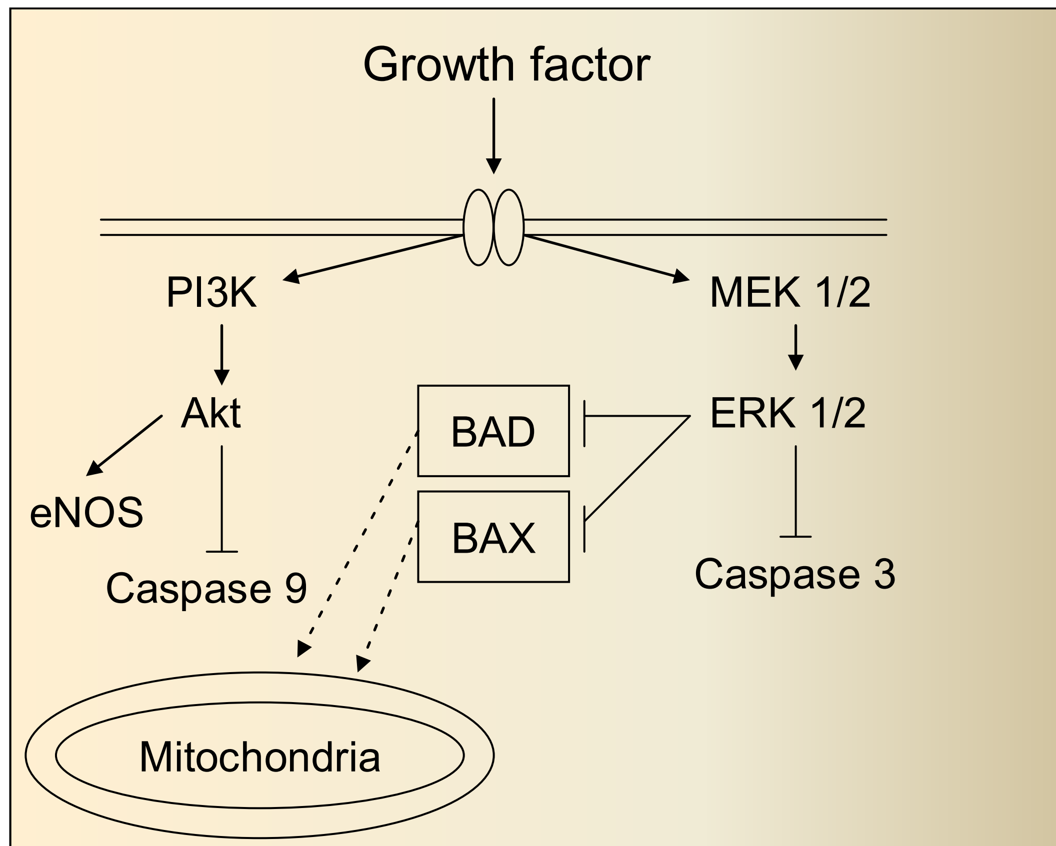


Figure 1.3.2 – Reperfusion injury salvage kinase (RISK) pathway components. Growth factors are known to stimulate PI3K and ERK 1/2, the components of the RISK pathway, which ultimately stimulate cellular survival by inhibiting caspases and pro-apoptotic factors such as BAD and BAX. *Figure adapted from (Hausenloy & Yellon 2004).*

1.3.4.1.1 PI3K-AKT pathway

The PI3K-AKT pathway was one of the first signalling transduction pathways to be associated with cardioprotection and is one of the key components of the RISK pathway (section 1.3.4.1). The PI3K's are a family of conserved lipid

kinases that transduce extracellular signals directly to a broad array of intracellular functions. A key target for PI3K-induced phosphorylation is the serine/threonine kinase Akt, of which there are three isoforms (Datta et al. 1999). The expression of Akt1 and Akt2 occurs in almost all tissues, whilst Akt3 expression occurs primarily in the brain and the testis (Konishi et al. 1995). The isoforms of Akt share 80% homology with respect to their amino acid sequence and have similar domain structures (Garofalo et al. 2003). In the central portion of the protein there is a kinase domain with specificity for serine or threonine residues in target proteins (Bellacosa et al. 1991). The carboxyl terminus comprises proline-rich and hydrophobic domains and the amino terminus contains a pleckstrin-homology (PH) domain, which can facilitate lipid/protein and protein/protein interactions (Kumar & Madison 2005). The Akt isoforms can phosphorylate and activate a large range of downstream proteins, of which, to date, 40 have been identified (Franke 2008). Therefore, it is not surprising to learn that Akt plays many different roles. Studies using transgenic and knock-out Akt mouse models have allowed the elucidation of some of the functions of Akt. These include the modulation of cell growth and proliferation (Skeen et al. 2006), glucose homeostasis, postnatal brain development, heart growth, skin growth, adipogenesis, bone development and lipid metabolism in the mammary gland (Peng et al. 2003; Yang et al. 2004b).

The PI3K-Akt pathway is activated by numerous ligands including cytokines, growth factors, hormones and neurotransmitters (Downward 2004). A significant number of plasma membrane receptors, tyrosine kinase receptors in particular,

have the ability to activate PI3K's (Paez & Sellers 2003). Ligand-mediated receptor activation leads to autophosphorylation of the receptor on tyrosine residues, and subsequent recruitment and activation of PI3K. Active PI3K catalyses the reaction converting membrane lipid phosphatidylinositol 3,4-biphosphate [PI(3,4)P_2] to phosphatidylinositol 3,4,5-triphosphate [PI(3,4,5)P_3]. Akt is then recruited, via its PH domain, to the membrane where it binds to PI(3,4,5)P_3 and is then phosphorylated and activated by phosphoinositide dependent kinase (PDK) 1 and 2 (Stephens et al. 1998). PI3K facilitated Akt activation can be controlled via the tumour suppressor protein phosphatase and tensin homolog (PTEN), which is a phosphatase (Leslie et al. 2008). PTEN suppresses the Akt signal by catalysing the opposite reaction to that catalysed by PI3K, i.e. it de-phosphorylates PI(3,4,5)P_3 to PI(3,4)P_2, hence preventing the formation of the Akt binding site and reducing Akt activation and downstream signalling (Leslie et al. 2008).

Akt acts on a number of downstream targets, some of which function to inhibit apoptosis (Datta et al. 1999). Thus, the cell survival promoting actions of Akt include inhibition of caspase 9 (conducted in human embryonic kidney cells, 293T HEK) (Cardone et al. 1998), inhibition of pro-apoptotic factors, such as Bcl-XL/Bcl-2-associated death promoter (BAD) and Bcl-2-associated X protein (BAX) (conducted in FL5.12 cells, a murine prolymphocytic cell line) (Yamaguchi & Wang 2001), and altering gene transcription factors associated with survival and cell death (conducted in 293T HEK cells)(Matsui & Rosenzweig 2005; Brunet et al. 1999). It should be noted, however, that these studies have been

conducted in a range of cells and the results may not reflect how Akt functions in cardiomyocytes.

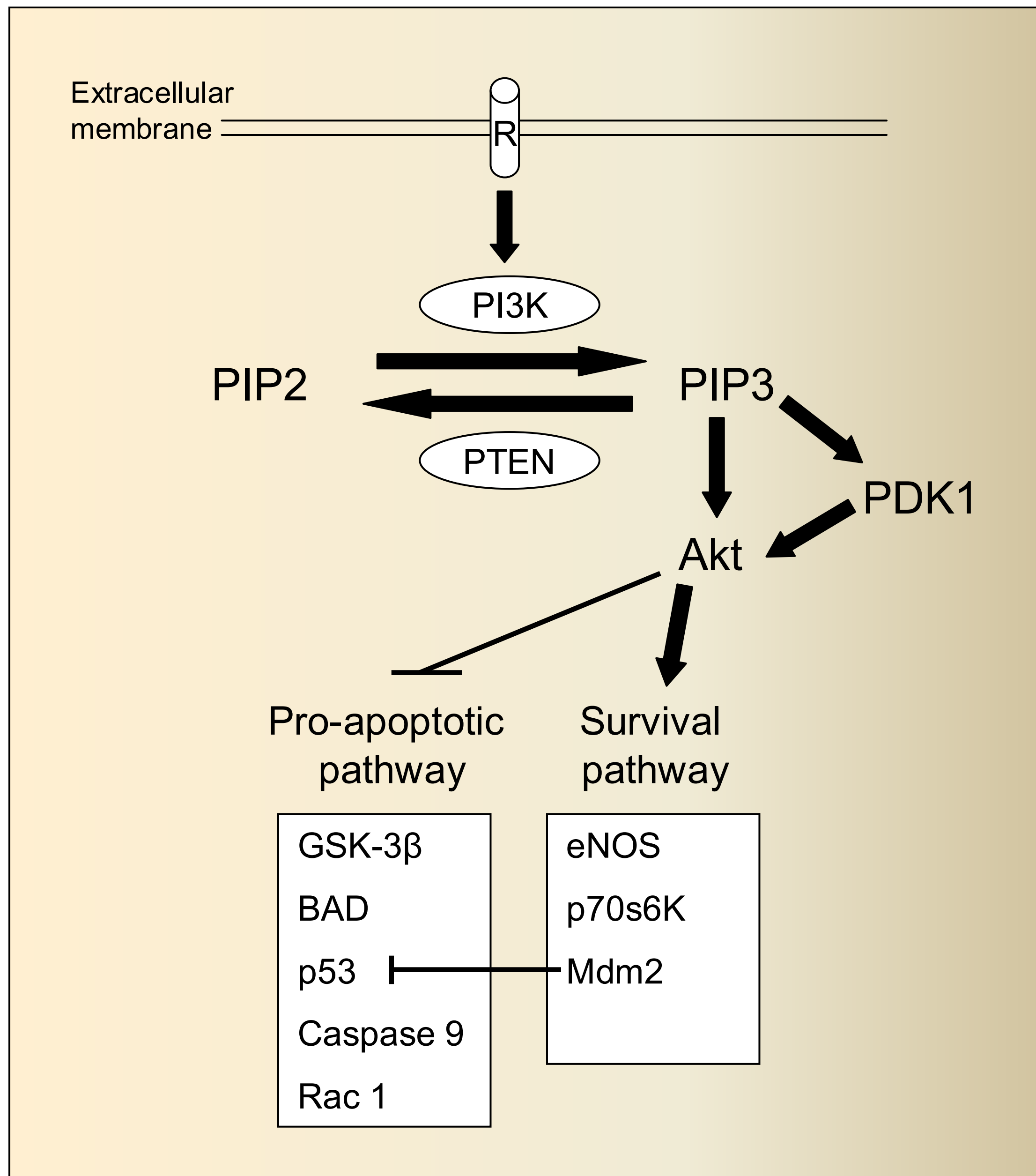


Figure 1.3.3 – PI3K-Akt pathway. Once activated PI3K phosphorylates PIP2 to PIP3. PTEN regulates Akt activity by catalysing the reverse reaction to that catalysed by PI3K, i.e. it de-phosphorylates PIP3 to PIP2. Once formed PIP3 binds Akt where it is phosphorylated and activated by PDK1. Once active, Akt inhibits various pro-apoptotic proteins; such as glycogen synthase kinase-3β (GSK-3 β), tumour protein 53 (p53) and Ras-related C3 botulinum toxin substrate 1 (Rac1) and stimulates anti-apoptotic proteins; such as eNOS and murine double minute 2 (Mdm2). *Adapted from:(Vivanco & Sawyers 2002).*

1.3.4.1.2 ERK 1/2 – p44/42 pathway

The p44/42 extracellular signal-regulated kinases (ERK) are members of the mitogen activated protein kinase (MAPK) family (Schaeffer & Weber 1999). The MAP kinases are serine/threonine kinases that are greatly conserved in eukaryotic cells (Widmann et al. 1999). At least three MAP kinases have been implicated in the MAPK signalling cascade (Schaeffer & Weber 1999). Signalling is initiated by a range of ligands and external stimuli which activate tyrosine kinase (TK) or G-protein receptors (Cross et al. 2000). Stimulation of these receptors leads to activation of MAPK Raf1, which in turn phosphorylates and activates the MEK1/2-Erk1/2 kinase cascade (Kyriakis et al. 1992). With regard to myocardial infarction it is interesting to note that stimulation of the ERK signalling pathway is associated with cardioprotection (Schulman et al. 2002). Furthermore, it has been demonstrated that ERK-mediated cardioprotection can be blocked with the MEK inhibitor, UO126 (Davies et al. 2000). Like Akt, ERK activation has also been shown to be linked to inhibition of caspase 9, a key modulator of apoptosis (Allan et al. 2003), pharmacological inhibition of caspase 9 being associated with infarct size reduction (Mocanu et al. 2000). Apart from its influence on caspase activity, ERK activation also activates P90RSK, which in turn phosphorylates and inhibits the pro-apoptotic factor BAD (Tan et al. 1999). Furthermore, active ERK translocates to the nucleus and stimulates gene transcription via activation of transcription factors such as c-Myc and E-26-like protein-1 (Elk-1) (Davis 1995).

1.3.4.1.3 Downstream mechanisms of the RISK pathway

Additional downstream mechanisms possibly contributing to cytoprotection include regulation of sarcoplasmic reticulum Ca^{2+} uptake and release (Abdallah et al. 2006), activation of NOS (Baxter & Burley 2008), inhibition of glycogen synthase kinase-3β (GSK-3β) (Murphy 2004) and inhibition of the MPTP (Hausenloy et al. 2002). How and if these various processes interact is currently unknown, however, evidence has been obtained indicating that these mechanisms converge on the mitochondrion and inhibit the opening of the MPTP (Halestrap & Pasdois 2009). Although it took some time for the role of the MPTP in cardioprotection to be accepted, the pore is now recognised as an important facilitator of cardiomyocyte death in the setting of I/R injury (Halestrap & Pasdois 2009). However, despite many studies on the pore, the intermediate steps between stimulation of RISK signalling and inhibition of the pore have yet to be revealed (Davidson et al. 2006). Nevertheless, several theories have been proposed. For example, it has been suggested that eNOS may inhibit MPT via the generation of NO (Kim et al. 2004a) or via a PKG-PKC-ε-mKATP channel signalling pathway (Costa & Garlid 2008; Costa et al. 2005). It has also been proposed that GSK-3β may inhibit the pore directly or induce activation of other factors that inhibit the pore (Miura et al. 2009). In addition, inhibition of BAX translocation to the nucleus has been put forward as a mechanism that may prevent MPTP opening (Bagci et al. 2006).

1.3.4.2 JAK-STAT pathway

1.3.4.2.1 JAK-STAT signalling mechanism

The JAK-STAT pathway is currently one of the most comprehensively understood cell signalling mechanisms. The current model of JAK-STAT signalling holds that the interaction of a cytokine ligand with its cytokine transmembrane receptor leads to receptor dimerisation which juxtaposes and activates JAK proteins associated with the intracellular domain of the receptor (Schindler et al. 2007). These JAK binding sites on the cytokine receptor are positioned in close proximity to the extracellular membrane (Behrmann et al. 2004). The association between JAK proteins and cytokine receptors was first documented with respect to interactions between JAK2 and erythropoietin and growth hormone receptors (Witthuhn et al. 1993; Argetsinger et al. 1993). Once activated JAK proteins phosphorylate sites on the cytosolic domain of the receptor, creating binding sites for the Src homology 2 (SH2) domains of STAT proteins. Following their recruitment STAT proteins are phosphorylated at key tyrosine and/or serine residues by JAK proteins and other associated kinases (Schindler et al. 2007). Phosphorylation leads to STAT dimerisation, via SH2 phosphotyrosine interactions, (Figure 1.3.4) dissociation and subsequent translocation to the nucleus, a process which is yet to be fully understood (Murray 2007; Schindler & Darnell, Jr. 1995). It has yet to be established if STAT proteins dimerise whilst bound to the receptor or upon their release (Lim & Cao 2006). In the nucleus STATs bind to specific sequences in the genome and regulate gene expression (Murray 2007). STAT monomers are unable to bind to

DNA, only STAT homodimers or certain heterodimers being capable of participation in such a reaction (Schindler & Darnell, Jr. 1995).

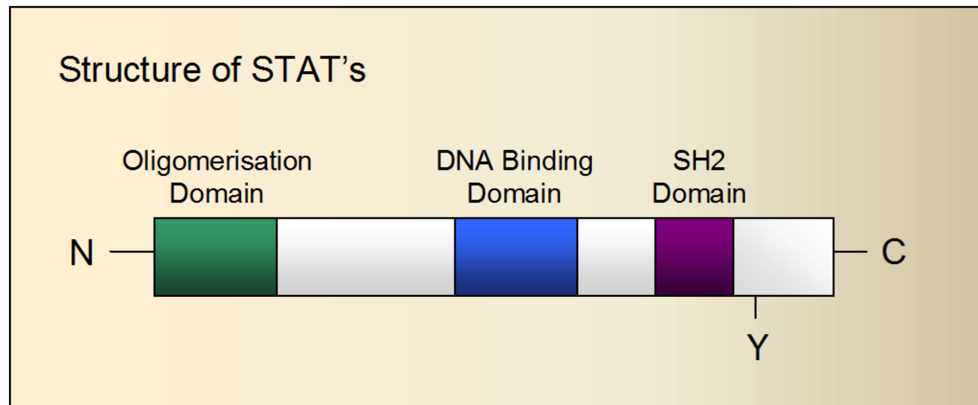


Figure 1.3.4 – Structure of STATs. Following activation by binding of ligands to specific receptors, STATs form dimers by binding to each other via their SH2 domains. Once formed, the dimer migrates to the cell nucleus where it acts as a transcription factor. *Adapted from (Imada & Leonard, 1999)*

1.3.4.2.2 Negative regulation of JAK-STAT signalling

To prevent cytokine signals from reaching excessive levels the JAK-STAT signalling mechanism is negatively regulated by a number of proteins, including protein tyrosine phosphatases such as protein inhibitor of activated STAT (PIAS), Src homology 1–containing tyrosine phosphatase (SHP1) and suppressor of cytokine signalling (SOCS) (Heinrich et al. 2003). Stimulation of these proteins leads to ubiquitin/proteasome mediated degradation, dissociation of receptor associated JAKs and exportation of STATs from the nucleus. Furthermore, phosphorylation of JAK2 at its FERM (band 4.1, ezrin, radixin and moesin) domain, in response to cytokine stimulation, results in its inactivation (Funakoshi-Tago et al. 2006). However, perhaps the most interesting mechanism in relation to the regulation of JAK-STAT signalling involves the inhibition of JAK-STAT by SOCS proteins, although it has yet to be fully elucidated (Croker et al. 2008).

The majority of SOCS proteins are activated in response to cytokine signalling and therefore act in a negative feedback loop, dampening cytokine signal transduction. Once active SOCS proteins, specifically SOCS 1 and 3, act to inhibit JAK-STAT signalling by either binding to and inactivating JAK, or by competing with STAT for receptor docking sites (Cooney 2002)

1.3.4.2.3 JAK-STAT and the heart

The greater part of the literature concerning JAK/STAT signalling in the heart is concerned with the activation of STAT1 and STAT3. Although the role of the JAK/STAT pathway in myocardial injury has yet to be fully elucidated, there are significant data indicating that STAT1 is pro-apoptotic, whilst activation of STAT3 is anti-apoptotic (Stephanou 2004). STAT1 activation is associated with increased expression of the apoptotic target genes cyclin-dependent kinase inhibitor 1A (p21), p53 and FAS ligand, and upregulation of caspases 1, 2, 3 and 7 (Chin et al. 1997; Stephanou et al. 2001; Townsend et al. 2004; Sironi & Ouchi 2004). By contrast, a growing amount of data suggests that activation of STAT3 is cardioprotective. STAT3 activation, for example, confers protection against doxorubicin-induced cardiomyopathy (Kunisada et al. 2000). Furthermore, I/R injury has been shown to be enhanced in STAT3 cardiac-deficient mice compared to control animals, cardiac function also being impaired (Hilfiker-Kleiner et al. 2004).

1.4 Cardiometabolic Disease

1.4.1 21st Century obesity epidemic

Obesity is now a global problem affecting developing countries as well as those in the western world (Hossain et al. 2007). According to the world health organisation (WHO) obesity is defined as having body mass index (BMI) of greater than 30, where BMI is an index of weight and height (equation 1)(table Table 1.4.1)(World Health Organization, 2009). Obesity is usually the result of a sedentary lifestyle combined with a poor diet. These individuals experience a large increase in adiposity, particularly visceral adipose tissue, which places them at an increased risk of developing Type-2 diabetes and cardiovascular disease (Despres et al. 2008; Kriketos et al. 2004). It is currently estimated that there will be 300 million cases of obesity-related type-2 diabetes in the world by the year 2025 (Grant 2005). Since cardiovascular pathologies account for 80% of deaths of patients with type-2 diabetes, this problem is set to increase unless steps are taken to prevent the occurrence of these disorders (Aronson et al. 1997).

BMI	Classification
<18.5	underweight
18.5-24.9	normal weight
25.0-29.9	overweight
>30	obesity

Table 1.4.1 – The international classification of adult underweight, normal weight, overweight and obese according to BMI. *Source: Adapted from (World Health Organisation, 2009).*

BMI = WEIGHT (KILOGRAMS) / HEIGHT (METRES^2)

Equation 1 – Equation for calculation of body mass index

1.4.2 The metabolic syndrome (syndrome X)

The metabolic syndrome (metS), also known as Reaven's syndrome or syndrome X, is characterised by the co-occurrence of abdominal fat, dysfunction of glucose tolerance, dyslipidaemia and hypertension (Alessi & Juhan-Vague 2008). MetS leads to alterations in metabolic homeostasis, which collectively lead to an increased risk of developing type-2 diabetes and cardiovascular disease. In the developed world the incidence of metS is growing rapidly. It is currently estimated that 25% of the population of the USA have metS (Ford et al. 2002). The mechanisms underlying this phenomenon are under close scrutiny and further studies are urgently required if we are to solve this growing problem. A link has now been made between the factors that the adipose tissue secreted,

namely interleukin-1, interleukin-6, TNF-α, leptin and adiponectin, and the MetS (Bullo et al. 2003). These substances are collectively known as the adipocytokines or adipokines (Matarese et al. 2007b; Tilg & Moschen 2006).

1.5 The Adipose Tissue and Adipocytokines

1.5.1 The adipose tissue

Until recently adipose tissue was thought to be an inert storage depot. Now, however, it is known that adipose tissue, especially white adipose tissue (WAT) constitutes an active endocrine organ that responds to a variety of signals, produces a plethora of active peptides and plays an active role in energy homeostasis (Ahima 2006). The largest deposits of WAT are concentrated in the subcutaneous region and around the viscera (Fruhbeck 2008). Gross increases in abdominal WAT, as observed in obese individuals, are associated with biochemical and histological alterations that often lead to inflammation, hypercoagulation, cardiovascular risk and the metabolic syndrome (Matsuzawa et al. 1995; Matsuzawa 2005). Macroscopically, adipocytes are highly vascularised lipid-filled cells held in a scaffold of loose connective tissue in a supporting matrix of collagen (Fruhbeck 2008). Proteins secreted by WAT show a great deal of pleiotropy and have roles in energy homeostasis, the regulation of neuroendocrine function and in autonomic and immune function (Ahima 2006).

1.5.2 The adipocytokines

The term adipocytokine has been used to describe a group of cytokines produced by white adipose tissue and includes peptides such as leptin,

adiponectin, apelin and visfatin (Trayhurn & Wood 2005). Although WAT is the main source of adipocytokines, it must be noted, however, that adipocytokines are also produced by other tissues (Tilg & Moschen 2006). For example, adiponectin is also produced by skeletal cells, cardiomyocytes and endothelial cells (Pineiro et al. 2005; Delaigle et al. 2004; Wolf et al. 2006). Adipocytokines expressed by adipocytes are secreted directly into the circulation where, at least in the case of leptin and adiponectin, they have roles in modulating energy homeostasis (Havel 2002).

1.5.2.1 Adiponectin

Adiponectin, discovered in 1995, has become the subject of intense research due to the discovery that hypoadiponectinaemia is associated with visceral obesity and coronary artery disease (Kumada et al. 2003). Furthermore, low serum levels of adiponectin have been related to insulin resistance (non-alcoholic fatty liver disease), atherosclerosis and type-2 diabetes (Arita et al. 1999b).

Globular adiponectin is abundantly present in the blood plasma of healthy patients, where it exists as either low molecular weight trimers and hexamers (dimer of trimers) or as high molecular weight 12- or 18- mers, accounting for approximately 0.01% of plasma proteins (Arita et al. 1999b). Adiponectin signals through two cell surface transmembrane receptors, adiponectin receptor 1 (AdipoR1) and 2 (AdipoR2) (Yamauchi et al. 2003). The different oligomers of adiponectin trigger slightly different signalling pathways (Tsao et al. 2003). Adiponectin is known to stimulate 5’ AMP-activated protein kinase (AMPK),

cyclooxygenase-2 COX-2, p38 MAPK, and peroxisome proliferator-activated receptor-alpha (PPAR-α) and also stimulates fatty acid oxidation (Yamauchi et al. 2003). Adiponectin has been shown to suppress PTEN signalling, thereby potentiating PI3K-AKT signalling (Chandrasekar et al. 2008).

In 2005 Walsh's group demonstrated that the administration of adiponectin protected against myocardial IR injury in a murine in vivo model via COX-2 and AMPK-dependent mechanisms (Shibata et al. 2005). This has important implications for myocardial infarction given that decreased adiponectin levels are associated with cardiovascular risk factors including obesity, hypertension, raised C-reactive protein (CRP) levels and hyperlipidaemia (Ouchi et al. 2003; Arita et al. 1999a; Kadowaki & Yamauchi 2005).

1.5.2.2 Visfatin

Visfatin acts to accelerate the conversion of triglycerides to glucose, inhibit glucose release and enhance glucose uptake (Adeghate 2008). Visfatin was previously identified as pre-B cell colony-enhancing factor (PBEF), and its expression is increased in obesity (Fukuhara et al. 2005). During hypoxia visfatin expression and secretion is increased in adipocytes through a HIF1-α dependent mechanism (Segawa et al. 2006). Visfatin binds to the insulin receptor at an alternative site to insulin itself and facilitates the activation of the PI3K-Akt and MAPK pathways (Murphy & Bloom 2006). Current literature suggests that visfatin may act as an anti-diabetic drug (Hausenloy 2009). In addition, studies conducted in our laboratory using an *in vivo* mouse model have demonstrated that visfatin is protective against myocardial I/R injury (Lim et al.

2008). Further investigation is therefore required to establish whether visfatin may be used therapeutically in the clinic.

1.5.2.3 Apelin

Apelin, first isolated in 1998 by Tatemoto et al, is the ligand for the apelin receptor (APJ) (Tatemoto et al. 1998). Apelin and its receptor are similar to the other adipocytokines, such as adiponectin and leptin, in that the peptide and its receptor are not only expressed by adipose tissue (O'Carroll et al. 2000). Apelin and APJ, for example, have been found to be expressed in a variety of rodent tissues, including brain, skeletal muscle and the vasculature (Hosoya et al. 2000). Apelin, like leptin, is thought to play a role in energy homeostasis. This hypothesis is borne out by data indicating that apelin secretion is increased by insulin administration in human and mouse adipocytes, and by obesity in the mouse (Boucher et al. 2005). Furthermore, it has been demonstrated that apelin expression is abrogated by fasting (Boucher et al. 2005). Further investigation, however, is required to elucidate the exact role apelin plays in the adipose tissue.

Apelin and the APJ have been found to be expressed in cardiomyocytes (O'Carroll et al. 2000). It has been demonstrated that apelin produces various effects in the heart and vasculature, including regulation of contractility (Szokodi et al. 2002), hypotensive actions (Tatemoto et al. 2001) and ionotropic actions (Berry et al. 2004). Upon completion of gene transcription, the 77 amino acid pre-propeptide of apelin undergoes post-translational modification to yield the following several shorter active polypeptides apelin-36, apelin-17 and apelin 13

(Tatemoto et al. 1998). Apelin-36 and apelin-13 have both been tested for their potential as cardioprotectants in the isolated perfused heart and in an *in vivo* mouse model of myocardial I/R (Simpkin et al. 2007). Both forms were found to be cardioprotective, however, apelin-13, the most physiologically active apelin polypeptide was found to elicit the more potent cardioprotective action (Simpkin et al. 2007).

The levels of apelin and its receptor have been reported to be reduced in patients with chronic heart failure, although in its early stages apelin levels were found to be increased (Foldes et al. 2003). These data combined with apelin's observed regulatory effects on cardiac homeostasis has led to the adipocytokine being investigated as a potential treatment for chronic heart failure (Japp & Newby 2008).

1.5.2.4 Resistin

Resistin is a 12.5 KDa cysteine-based proinflammatory cytokine that is thought to have roles in insulin resistance, adipogenesis and atherosclerosis (Steppan et al. 2001; Kim et al. 2004b). Resistin levels are diminished upon fasting and recover with feeding, suggesting a role in energy homeostasis. Whilst resistin receptors have been found to be located on cardiomyocytes the roles it plays in the heart are not clear (Gao et al. 2007). With regard to myocardial infarction current data are paradoxical. For instance, resistin has been shown to both exacerbate and protect against cardiac reperfusion injury (Rothwell et al. 2006; Gao et al. 2007).

1.6 Leptin

1.6.1 Background

First discovered in 1994 by positional cloning, leptin is the 16 KDa product of the *Obese* (*ob)* gene (Zhang et al. 1994). Leptin was initially proposed as a "cure" for obesity, disappointingly, however, this turned out not to be the case (Bell-Anderson & Bryson 2004). Despite this early disappointment leptin has yielded valuable insights into the mechanisms modulating energy homeostasis and satiety, and is the focus of this thesis.

Leptin is primarily produced by white adipose tissue, nevertheless, it is also secreted by a range of other tissues including the heart (Purdham et al. 2004). Adipocytes secrete leptin in a slow, continuous fashion, however, if stimulated (by insulin and glycolytic substrates, for example) secretion is markedly increased (Cammisotto et al. 2005). Leptin secretion is reduced as a response to starvation or cold with starvation leading to increased sympathetic nervous activity, and, as a consequence leptin production is reduced (Rayner & Trayhurn 2001). Generally, the circulating levels of leptin correlate well with body adipose tissue mass (Schwartz et al, 1996).

1.6.2 Function

Under normal conditions the main function of leptin is to signal low fat mass stores by acting at several sites in the brain (Blevins et al. 2002). However, the predominant site of action is the so-called hypothalamic pituitary axis (HPA) (Pralong & Gaillard 2001). Leptin signalling at the HPA alters the levels of various neuropeptides and neurotransmitters, many of which interact to control

over-feeding behaviour and energy expenditure. The arcuate nucleus of the hypothalamus is the key region involved in these mechanisms, and is a major area of the brain where the expression of neuropeptide Y (NPY) and Agouti-related peptide (AgRP) occurs (Figure 1.6.1). NPY and AgRP increase appetite and decrease energy expenditure, and leptin functions to counteract their actions (Claycombe et al. 2000; Wang et al. 1997; Parker et al. 2002). Leptin also inhibits orexin and galanin production and the synthesis of various cannabinoids, peptides which all stimulate appetite (Wilding 2002). The mechanisms underlying the control of energy homeostasis are complex and have yet to be fully elucidated.

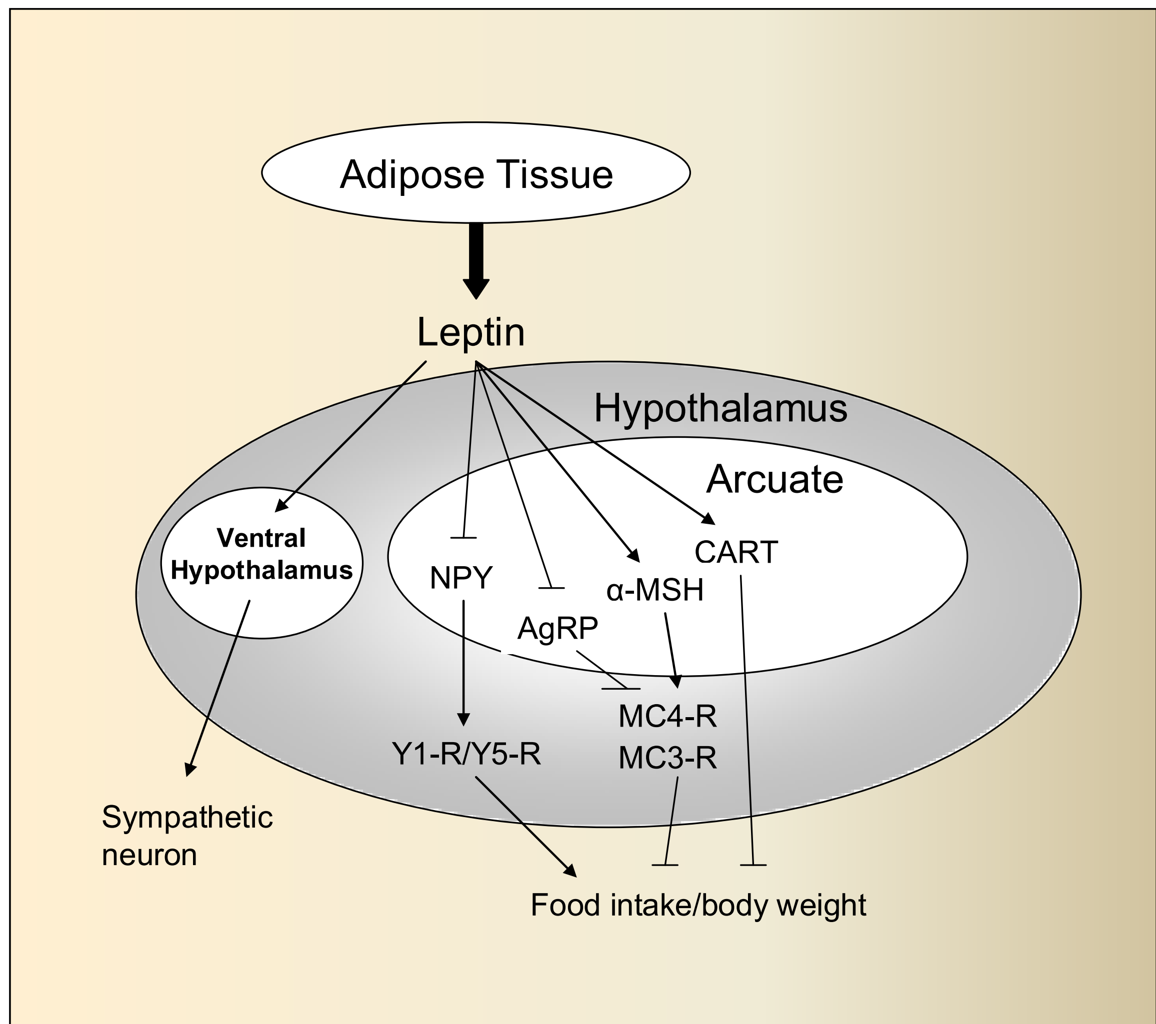


Figure 1.6.1 – Leptin and energy homeostasis. This schematic represents homeostatic leptin signalling in the hypothalamus under normal conditions. The direct action of leptin in the arcuate nucleus leads to the suppression of neuropeptide Y (NPY) and agouti-related peptide (AgRP) and stimulation of α - melanin-stimulating hormone (α-MSH) and cocaine and amphetamine-regulated transcript (CART), both of which ultimately lead to a reduction in food intake and an increase in thermogenesis. NPY normally stimulates feeding by binding to Y1 (Y1-R) and Y5 (Y5-R) receptors. AgRP blocks the anorectic effect of α-MSH by binding to melanocortin subtype-4 receptors (MC4-R). *Source: Adapted from (Ahima 2005).*

Leptin functions within a feedback mechanism. Increases in plasma leptin concentrations lead to the suppression of appetite and increased energy expenditure, whilst decreased leptin has the opposite effects (Bates & Myers, Jr. 2003) Plasma leptin levels are influenced by various factors, for example increased BMI, food intake and glucose uptake all lead to increased leptin in the blood (Grinspoon et al. 1996; Grinspoon et al. 1997) . On the other hand,

increases in age and/or exercise leads to decreased plasma leptin concentrations (Zheng et al. 1996). Plasma leptin concentrations have been reported to correlate with BMI (Schwartz et al, 1996).

The importance of leptin, with regard to energy balance and metabolic status, is clearly demonstrated in the presence of interrupted leptin signalling. Thus, in rodent models of type 2 diabetes abrogation of leptin signalling, through mutation of the long form of the leptin receptor, leads to an obese phenotype characterised by hyperphagia, decreased energy expenditure and increased adiposity (Chua, Jr. et al. 1996; Chen & Wang 2005).

Leptin via a negative feedback mechanism functions in healthy people to regulate body fat stores (Enriori et al. 2006). In obese individuals, however, increased plasma leptin levels resulting from large adipose tissue deposits leads to hypothalamic leptin resistance (see section 1.6.6) (Enriori et al. 2006).

Whilst the hypothalamic ARC has been shown to be an important site of the action of leptin, it should be noted that leptin has also been found to act at other sites in the hypothalamus and other locations in the brain. In the rat brain the long form of the leptin receptor (OB-Rb) has been found to be located within particular nuclei of the hypothalamus other than the arcuate, including the dorsomedial, ventromedial, and ventral premamillary nuclei (Elmquist et al. 1998). Hence, indicating that homeostatic leptin signalling may occur at these particular locations. Indeed, a group of neurons (SF-1 containing neurons) found in the ventromedial nucleus (VMN), for example, have been shown to be

involved in leptin-induced satiety (Dhillon et al. 2006). A recent study demonstrated that mice with a specific deletion of leptin receptors from SF-1 containing neurons in the VMN resulted in increased appetite, increased body weight and development of the metabolic syndrome (Bingham et al. 2008).

In addition, data have been collected that indicate that leptin can suppress appetite by signalling within areas of the caudal brainstem (Grill et al. 2002). Other than the hypothalamus in the rat brain, OB-Rb receptors have been shown to be present in the hindbrain and the hippocampus (Elmquist et al. 1998). Furthermore, mesolimbic areas of the brain known to be involved in reward circuits, namely the substantia nigra and the ventral tegmental area (VTA) have been demonstrated to display OB-Rb receptors. Direct administration of leptin into the VTA has been shown to suppress food intake and reduce body weight (Hommel et al. 2006).

In addition, a recent study by Farooqi et al indicates that leptin may act on mesolimbic areas in the brain to reduce the sensation of food reward (Farooqi et al. 2007). In this study, Farooqi and colleagues used two patients with a congenital leptin deficiency and examined the effect that leptin repletion had on (1) food intake and (2) the activation of mesolimbic areas of the brain using functional magnetic resonance imaging (fMRI) in response to visual stimuli of food. Congenital leptin deficiency in humans is a rare condition that results in hyperphagia, early onset diabetes and metabolic, neuroendocrine and immune dysfunction (Gibson et al. 2004). Results demonstrated that indeed leptin repletion caused a marked reduction in food intake. Interestingly, fMRI studies

revealed that the activation of mesolimbic areas of the brain in response to visual food stimuli could be observed in the fasted and fed states in the individuals with leptin deficiency. However, in the leptin-replaced individuals activation of these areas could only be stimulated in the fasted state. These results indicate the leptin may regulate food intake, at least partially, by altering the perception of food reward.

A secondary major function of leptin has been shown to occur in the process of reproduction (Holness et al. 1999; Forhead & Fowden 2009). It has been well established that leptin and OB-R (leptin receptors) are ubiquitous in the foetus and placental tissues. The role leptin plays appears to go beyond signalling that the body has sufficient energy stores for reproduction to proceed, but also has an effect on foetal growth. For example, it has been found that leptin concentration, prior to birth, correlate well with several measures of foetal growth (Hassink et al. 1997; Varvarigou et al. 1999).

1.6.3 Functional pleiotropy

Apart from its role in energy homeostasis, leptin has been demonstrated to possess a great deal of functional pleiotropy (Fruhbeck et al. 1998). For example, leptin plays roles in angiogenesis (Sierra-Honigmann et al. 1998), bone formation (Ducy et al. 2000; Takeda et al. 2002), blood pressure control (Fruhbeck 1999) and haematopoiesis (Cioffi et al. 1996; Holness et al. 1999).

1.6.4 The leptin receptor

The leptin receptor is a member of the class I cytokine family of cytokine receptors (Tartaglia et al. 1995). This group of receptors include various members of the interleukin receptor family, the granulocyte colony-stimulating factor (GCS-F) receptor and the erythropoietin receptor (Liongue & Ward 2007). They all share a similar three-dimensional folded structure and activate similar signalling pathways (Huising et al. 2006). The leptin receptor (OB-R) occurs in a variety of isoforms; OB-Ra, OB-Rb, OB-Rc, OB-Rd, OB-Re and OB-Rf (Lee et al. 1996). These isoforms all share a highly conserved extracellular receptor binding domain (Baumann et al. 1996). All of the variants, except the secreted OB-Re isoform, have a conserved transmembrane domain (Yang et al. 2004a). By contrast, the intracellular portion of the different receptor isoforms varies (Fruhbeck 2006). The OB-Rb receptor, which has the longest intracellular tail, is thought to be the fully functional and physiologically dominant receptor (Fruhbeck 2006). This hypothesis is supported by the observation that inactivation of the OB-Rb receptor by point mutation leads to an obese phenotype in rodents (Chen et al. 1996). This can be seen in models of type-2 diabetes such as the Zucker (fa/fa) fatty rat and the db/db mouse (Chen & Wang 2005).

1.6.5 Signalling pathways stimulated by leptin

To mediate its many physiological roles, leptin functions by stimulating several different signal transduction pathways. Like other cytokine receptors leptin receptors have a proline rich box-1 motif (Ihle 1995). Janus kinase (JAK) binding is vital to leptin signalling, due to the lack of an intrinsic kinase domain

within the OB-R itself. Binding of JAKs to the box-1 motif allows transphosphorylation between the JAKs and leads to phosphorylation of specific tyrosine residues within the receptor. These phosphorylated tyrosine residues provide docking sites for the mediation of downstream signalling (see figure 1.6.2). Fruhbeck (Fruhbeck 2006) recently reviewed leptin signalling in detail.

1.6.5.1 JAK/STAT

Leptin predominantly transmits its signal through the JAK/STAT pathway (Fruhbeck 2006). As mentioned earlier, the long form of the receptor, which contains an additional motif, known as the box-2 motif, is required for maximal activation of the leptin signalling pathway (Fruhbeck 2006). It has also been shown, however, if the box-1 motif alone or its proximal amino acids are present JAK activation occurs, suggesting that receptor isoforms possessing the box-1 motif alone can also mediate JAK activation (Bahrenberg et al. 2002). Nevertheless, it appears that the presence of both the box-1 and box-2 motif's are crucial for maximal activation of leptin stimulated pathways to occur.

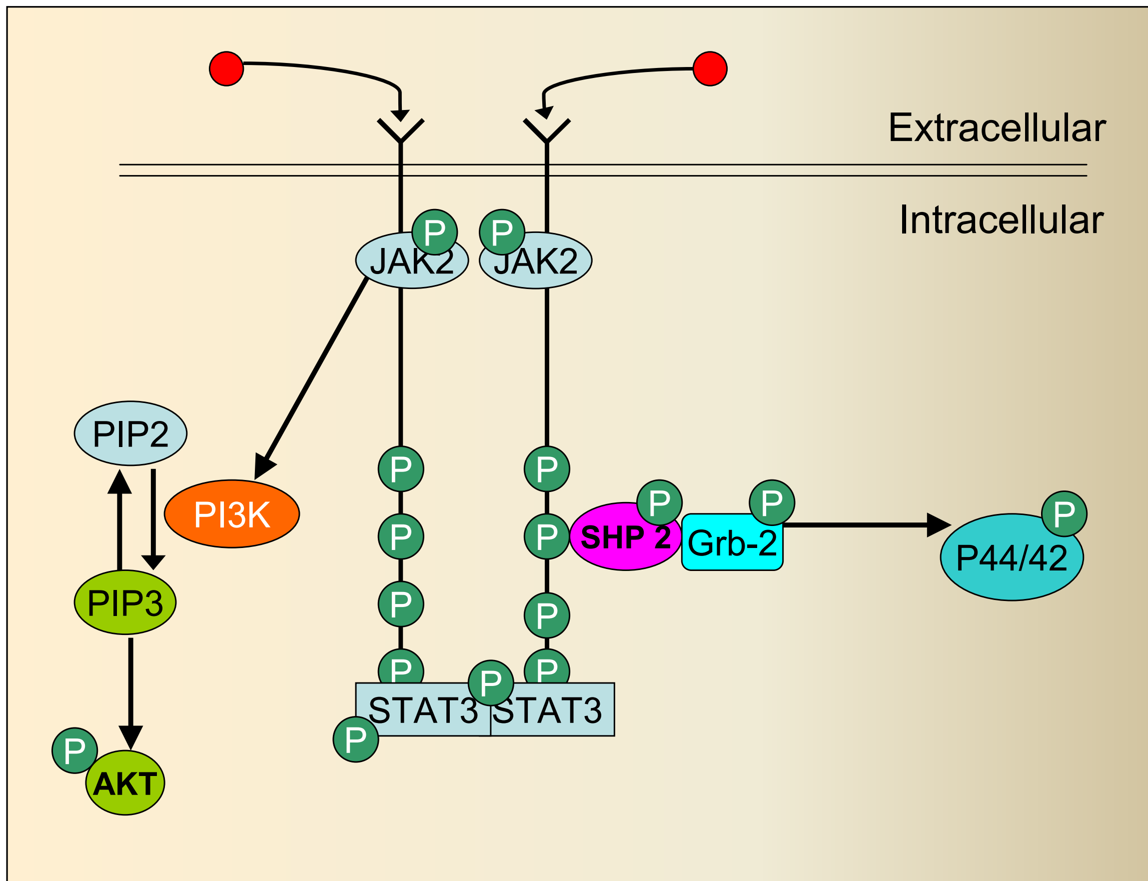


Figure 1.6.2 – Leptin signalling mechanisms. Leptin binding to its receptor leads to the phosphorylation and activation of an associated JAK2 protein. In turn, this causes phosphorylation of various tyrosine residues on the OB-R and subsequent activation of PI3K-Akt, p44/42 MAPK and STAT3. Figure adapted from (Fruhbeck 2006).

1.6.5.2 MAPK

Recent studies have shown that the presence of the Tyr^{985} residue in the OB-Rb is required for leptin-mediated activation of the MAPK pathway to occur (Fruhbeck 2006). Interestingly, it has been reported that OB-Ra binding is also capable of stimulating MAPK pathway activation (Banks et al. 2000). OB-Ra-induced MAPK activation, however, is not as marked as that induced by OB-Rb receptor stimulation (Banks et al. 2000). Bjørbæk et al demonstrated that leptin-dependent ERK phosphorylation, mediated by either OB-Ra or OB-Rb, requires the presence of an intact catalytic domain within the phosphatase SHP-2

(Bjorbaek et al. 2001). These data, therefore, suggest that leptin-induced MAPK activation is transduced via SHP-2. MAPK signalling has been demonstrated to be an important cascade with respect to leptin signaling. All of the upstream and downstream components involved, however, have yet to be identified.

1.6.5.3 PI3K-AKT (PTEN inhibition)

The importance of PI3K with respect to leptin's role in energy homeostasis was indicated by the observation that pharmacological inhibition of PI3K abrogates the anorectic effect of leptin by blocking hyperpolarisation of NPY

/AgRP neurons (Niswender et al. 2001). In the hypothalamus leptin has been shown to stimulate PI3K activity via activation of the insulin receptor subtype-2 (IRS2). Furthermore, PI3K activity was found to be required for leptin-induced activation of the sympathetic nervous system, which in turn has been demonstrated to induce changes in renal sympathetic outflow (Rahmouni et al. 2003). Downstream of PI3K, leptin is known to activate Akt and protein kinase C (PKC) isoforms (Sweeney 2002). Interestingly, recent data indicate that leptin may potentiate the Akt signal by inhibiting PTEN activity (see section 1.3.4.1.1). This observation was made with hepatocytes in which leptin was found to activate casein kinase 2 (CK2), which leads to phosphorylation and as a consequence inactivation of PTEN phosphatase activity (Ning et al. 2006). With PTEN inactivated more PIP_3 can be generated by PI3K, which can then lead to further Akt activation via PDK1/2.

1.6.5.4 AMPK

Leptin is associated with the activation of AMPK and consequential activation of fatty acid oxidation (Unger 2004; Minokoshi et al. 2002). AMPK has been described as a “fuel monitor” and functions to increase nutrient intake through signalling in the hypothalamus (Kahn et al. 2005). A downward shift in the ATP/AMP ratio stimulates AMPK activation, which leads to increased catabolism and decreased anabolism (Kahn et al. 2005). It is thought that leptin and adiponectin activate AMPK by altering the cellular ATP/AMP ratio (Kahn et al. 2005).

1.6.6 Leptin resistance

Leptin resistance is associated with impaired leptin signalling, particularly in the hypothalamus, and leads to increased appetite and decreased energy expenditure (Enriori et al. 2006). Leptin resistance is thought to be an important step in the development of diet-induced obesity and its subsequent problems (Tschop et al. 2007). Two mechanisms have been put forward to explain leptin resistance. First, it has been suggested that it may occur as a result of decreased leptin transport to the brain (Banks et al. 2004) Alternatively, it has been hypothesised that leptin resistance may be caused by an impairment in the OB-Rb signalling cascade (Munzberg & Myers, Jr. 2005; Banks & Farrell 2003). This model of leptin resistance suggests that chronic activation of leptin signalling leads to the upregulation of suppressor of cytokine signalling 3 (SOCS3), which acts to suppress leptin signalling (Munzberg & Myers, Jr. 2005). This theory is supported by an investigation conducted by Dunn and colleagues, that demonstrated that overexpression of SOCS3 leads to reduced OB-Rb

signalling (Dunn et al. 2005). Furthermore, this study also found that mutation of the STAT3 binding site on the OB-Rb receptor abolished leptin resistance. These data, therefore, indicate that chronic STAT3 activation mediated by leptin leads to leptin resistance. SOCS3 is further implicated in this mechanism by a study which showed that reduced levels of SOCS3 in the brain correlate with an increase in leptin sensitivity and resistance to diet induced obesity (Mori et al. 2004). Currently it is unknown whether leptin resistance is a purely neural phenomenon or whether it occurs in other tissues in the body. Recent data, however, suggest that leptin resistance may be tissue specific. Somoza et al found that mice fed on a high fat diet for 8 weeks exhibit leptin resistance in the hypothalamus, but not in the heart (Somoza et al. 2007). Therefore, cardiac tissue may be amenable to leptin-induced cardioprotection even if the subject exhibits hypothalamic leptin resistance.

1.6.7 Leptin and the cardiovascular system

As mentioned above, the leptin receptor has been found to be present on cardiomyocytes (Purdham et al. 2004). Our current understanding regarding the role of leptin signalling in the heart is, however, limited. Leptin has, nevertheless, been implicated in a number of cardiac phenomena, including cardiac hypertrophy and heart failure, and it has been shown to elicit various effects on the vasculature (Purdham et al. 2008; Maruyama et al. 2000; Park et al. 2001).

1.6.7.1 Vascular effects of leptin

In vivo administration of leptin has been demonstrated to cause an elevation in blood pressure and heart rate, which is mediated by the sympathetic nervous system (Shek et al. 1998; Correia et al. 2001). In hyperleptinaemia, however, it is thought that leptin may induce hypertension by alternative mechanisms. Leptin is known, for example, to upregulate the production and secretion of pro-inflammatory cytokines, such as interleukin 6 (IL-6) and TNF-α, which are known to increase blood pressure and promote atherosclerosis (Loffreda et al. 1998). Whilst leptin has been shown to promote hypertension, interestingly, it has also been found to produce vasodilatory effects (Leung & Kwan 2008). In endothelial cells and in anaesthetised rats, for example, leptin has been found to stimulate the production of the vasodilator, NO (Winters et al. 2000; Fruhbeck 1999). In conscious rats, however, leptin failed to elicit a vasodilatory effect (Gardiner et al. 2000). In endothelial cells, leptin has been demonstrated to stimulate eNOS via Akt, in a mechanism independent of PI3K (Vecchione et al. 2002). Whilst leptin stimulates the production of NO, a vasodilator, it has also been demonstrated to stimulate the release of endothelin-1 (ET-1), which is a vasoconstrictor (Quehenberger et al. 2002).

The pro-inflammatory effects of leptin are thought to play a role in atherogenesis (Dubey & Hesong 2006). Indeed, elevations in plasma leptin levels have been found to be associated with coronary artery calcification in type-2 diabetics and in non-diabetics (Reillt et al, 2004) (Qasim et al, 2008). Whether leptin signalling contributes to the calcification process directly, however, has yet to be clarified.

Nonetheless, leptin has been demonstrated to elicit various effects on the vasculature that contribute to the development of atheroma (Beltowski 2006a). In endothelial cells, for example, as well as stimulating the release of ET-1, leptin increases ROS production and promotes cellular proliferation (Quehenberger et al. 2002; Bouloumie et al. 1999). Furthermore, leptin has been shown to promote thrombosis by inducing platelet aggregation (Maruyama et al, 2000).

1.6.7.2 Leptin and myocardial fatty acid utilization

Fatty acids (FA) are the principal “fuel” in the healthy heart, oxidation of which produces the bulk of the ATP used by the contractile apparatus (Noh et al. 2006). Leptin has been shown to increase fatty acid oxidation in the isolated working rat heart via a mechanism independent of changes in the AMPK-acetyl-CoA carboxylase-malonyl-CoA axis (Atkinson et al. 2002). It is thought that hyperleptinaemia in the early stages of obesity may protect against the accumulation of FAs in the heart, known as myocardial steatosis, by increasing FA oxidation (Yang & Barouch 2007). In the later stages of obesity, however, it has been suggested that the development of leptin resistance leads to cardiac FA accumulation, lipotoxicity, contractile apparatus dysfunction and, ultimately, apoptosis of cardiomyocytes (Zhou et al. 2000). Given that it is currently unclear whether leptin resistance occurs in the heart (see section 1.6.6), further investigation is required in order to understand leptin’s role in obesity.

1.6.7.3 Effects of leptin on hypertrophy and remodelling

There appears to be an ever growing amount of evidence indicating that leptin promotes cardiac hypertrophy. Serum leptin levels have been found to correlate with left ventricular hypertrophy (LVH) independent of BMI and blood pressure (Kartal et al. 2008). Furthermore, Purdham et al demonstrated recently that a leptin receptor neutralising antibody inhibited myocardial hypertrophy in rat hearts subjected to coronary artery ligation (CAL) (Purdham et al. 2008). The mechanisms underlying leptin-induced hypertrophy, however, are still unclear. In cardiac myocytes leptin has been shown to induce MAPK and RhoA activation, both of which are thought to play roles in promoting hypertrophy (Rajapurohitam et al. 2003; Tajmir et al. 2004; Zeidan et al. 2006). In addition, leptin-induced hypertrophy was shown to occur following translocation of p38 MAPK from the cytosol to the nucleus via a mechanism that was dependent on the presence of intact caveolae and Ras homolog/Rho-associated coiled coil–containing protein kinase (Rho/ROCK) signalling (Zeidan et al. 2008). Furthermore, in neonatal rat cardiomyocytes leptin-induced hypertrophy was demonstrated to be associated with increased ET-1 and ROS levels (Xu et al. 2004). It is clear that this is an area that needs more research as leptin may prove to be an important drug target in the setting of cardiac hypertrophy.

1.6.8 Tissue protection

Leptin has been demonstrated to have anti-apoptotic effects in various tissues. In the gut, for example, it has been found that leptin protects against I/R induced gastric erosions (Brzozowski et al., 2001). In renal tissue, leptin was shown to

protect against I/R injury by abrogating the deleterious effects of TNF-α (Erkasap et al. 2003).

In salivary gland acinar cells, leptin was found to protect against ethanol-induced cytotoxicity through a mechanism involving the activation of prostaglandin and NO synthase pathways by Src kinase (Slomiany & Slomiany 2008). Protection of the small intestine by leptin was also found to be associated with increased NO synthesis as compared with control (Hacioglu et al. 2005). In hepatic stellate cells, leptin-induced protection against Fas-induced apoptosis was found to be dependent on the presence of the long, OB-RB form of the leptin receptor (Qamar et al. 2006).

Recently, Tang has described how leptin's pro-survival effects and ease of access to the brain make it a suitable candidate as a neuroprotective drug (Tang 2008). Leptin has, indeed, been used as a neuroprotectant in several studies utilising models of neurodegeneration. In a model of Parkinson's disease, for example, leptin was found to protect against 6-hydroxydopamine-induced dopaminergic cell death via ERK1/2 activation (Weng et al. 2007). Furthermore, leptin was shown to protect hippocampal CA1 neurons against I/R injury via stimulation of both Akt and ERK 1/2 (Zhang & Chen 2008). In another study using hippocampal neurons, leptin was shown to protect cells via JAK/STAT3 stimulation and mitochondrial stabilisation (Guo et al. 2008). Protection was lost when cells were treated with either the JAK/STAT inhibitor, AG490, or PI3K/Akt inhibitors (Guo et al. 2008).

1.6.8.1 Cardioprotection

Leptin has been demonstrated to protect cardiac myocyte cultures against hypoxia-induced damage (Erkasap et al. 2006). Furthermore, leptin has been found to protect H9c2 rat cardiomyocytes against H_2O_2-induced apoptosis through a mechanism which involved the suppression of mitochondrial membrane potential changes and inhibition of cytochrome C release (Eguchi et al. 2008). Translocation of the pro-apoptotic factor BAX was also found to be inhibited by leptin administration (Eguchi et al. 2008). In our laboratory, leptin was demonstrated to protect the isolated perfused Langendorff mouse heart against I/R injury, via activation of ERK 1/2 (Smith et al. 2006). Leptin remains, however, to be tested in an *in vivo* model of I/R injury.

Leptin has been the subject of intense study for the past 14 years and in addition to learning about its important role in energy homeostasis we now know that it also stimulates cellular survival in several tissues. Further studies concerning the protective actions of leptin against myocardial I/R injury are a necessity to ascertain the clinical potential of this drug and therefore form the basis of the investigation presented in this thesis.

CHAPTER 2 - AIMS & HYPOTHESIS

2.1 Aims

Examine the signalling mechanisms underlying leptin-induced cardioprotection *in vitro* focusing on the p44/42 MAPK, PI3K-Akt and JAK/STAT pathways.

Explore the importance of the OB-R and adiposity in leptin-induced cardioprotection *in vitro*.

Investigate the cardioprotective effects of leptin in an *in vivo* model of ischaemia reperfusion injury.

2.2 Hypothesis

Leptin, via activation of the RISK pathway and JAK/STAT signalling cascade, protects the myocardium against I/R injury via activation of the leptin receptor (OB-R).

CHAPTER 3 - GENERAL METHODS

3.1 Animals

Prior to undertaking investigations using animals the appropriate education and training, as directed by the Animals (Scientific Procedures) Act 1986, was sought. Modules 1, 2, 3 and 4 (general principles) was successfully passed and subsequently a personal licence was awarded by the Home Office. The licence allowed a number of procedures to be performed including all those performed whilst undertaking this thesis.

Male Wistar, Zucker Lean (*fa/*+) and Zucker fatty (*fa/*fa) rats between the age of thee and four weeks were used in this study. All animals were obtained from Charles River UK Limited (Margate, UK) and received humane care in accordance with The Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 (The Stationary Office, London, UK). Animals were allowed to acclimatise for a minimum of 4-5 days prior to use. They were kept in cages of four and had free access to fresh water and standard pellet chow (RM1 diet), and were subjected to a 12 hour light-dark cycle and maintained at 19-22˚C and 55±10% humidity.

3.2 Chemicals & Drugs Used

Leptin[1] (human) and triphenyltetrazolium chloride (TTC) were provided by Sigma-Aldrich Company Ltd (Gillingham, Dorset, UK). Leptin was reconstituted as advised by Sigma by adding 0.5 ml of 0.2 µm-filtered 15 mM HCl. Once the protein was fully dissolved, 0.3 ml of 0.2 µm-filtered 7.5 mM NaOH was then

[1] It should be noted that human leptin and not rat leptin was used for all experiments, this will be discussed further in section 4.5.2.

added (Sigma-Aldrich, 2009). All constituents of the Krebs-Henseleit buffer were supplied by BDH Laboratory supplies (Poole, Dorset, UK). Protein kinase inhibitors AG490, LY294002 (LY) and UO126 (UO) were obtained from Tocris Bioscience (Avonmouth, UK). All Western blot reagents and tissue culture chemicals were provided by Sigma-Aldrich Company Ltd (Gillingham, Dorset, UK). The BCA protein assay kit was purchased from Pierce Biotechnology (Rockford, IL, USA). Tetramethylrhodamine methyl ester (TMRM) was supplied by Molecular Probes (Eugene, OR, USA).

3.3 Langendorff Isolated Perfused Rat Heart

Rats were anaesthetised with Nembutal (sodium pentobarbital) (55 mg/kg intra-peritoneally, Sagatal-Rhone, Merieux), and were given heparin sodium (300 IU intra-peritoneally, Multiparin CP Pharmaceuticals Ltd.) to prevent the formation of thrombi in the heart and blood vessels. When the rat was sufficiently anaesthetised, as indicated by the loss of the pedal withdrawal reflex, a trans-abdominal incision was made and the thoracic cavity was exposed by a bilateral incision along the lower rib margin. The anterior thoracic cage was then reflected superiorly and the heart gently held between the fingers and excised. The heart was then immediately immersed in ice-cold (4°C) modified Krebs-Henseleit bicarbonate buffer (containing NaCl 118mM, NaHCO3 24mM, KCl 4mM, NaH2PO4 1mM, $CaCl_2$ 1.8mM, MgCl2 1.2mM and glucose 10mM) to arrest the heart and minimise ischaemic injury (Krebs & Henseleit 1932). The heart was then mounted via the aorta onto a 14 gauge cannula (2mm external diameter; approximately 1.5mm internal diameter) connected to the perfusion

apparatus (Figure 3.3.1 & Figure 3.3.2). The time between excision of the heart and perfusion with oxygenated Krebs-Henseleit buffer was kept to a minimum to prevent ischaemic preconditioning of the myocardium. The heart was perfused retrogradely via the aorta at a constant pressure of 100mm Hg with modified Krebs buffer as described above. Retrograde perfusion via the aorta causes the closure of the aortic valve allowing the buffer to flow through the coronary vessels and perfuse the myocardium. Careful oxygenation of the buffer using a 95% O_2 / 5% CO_2 gas mixture resulted in a stable pH of 7.4 ± 0.5, which was carefully monitored using a gas analyser (ABL 700 Radiometer, Denmark).

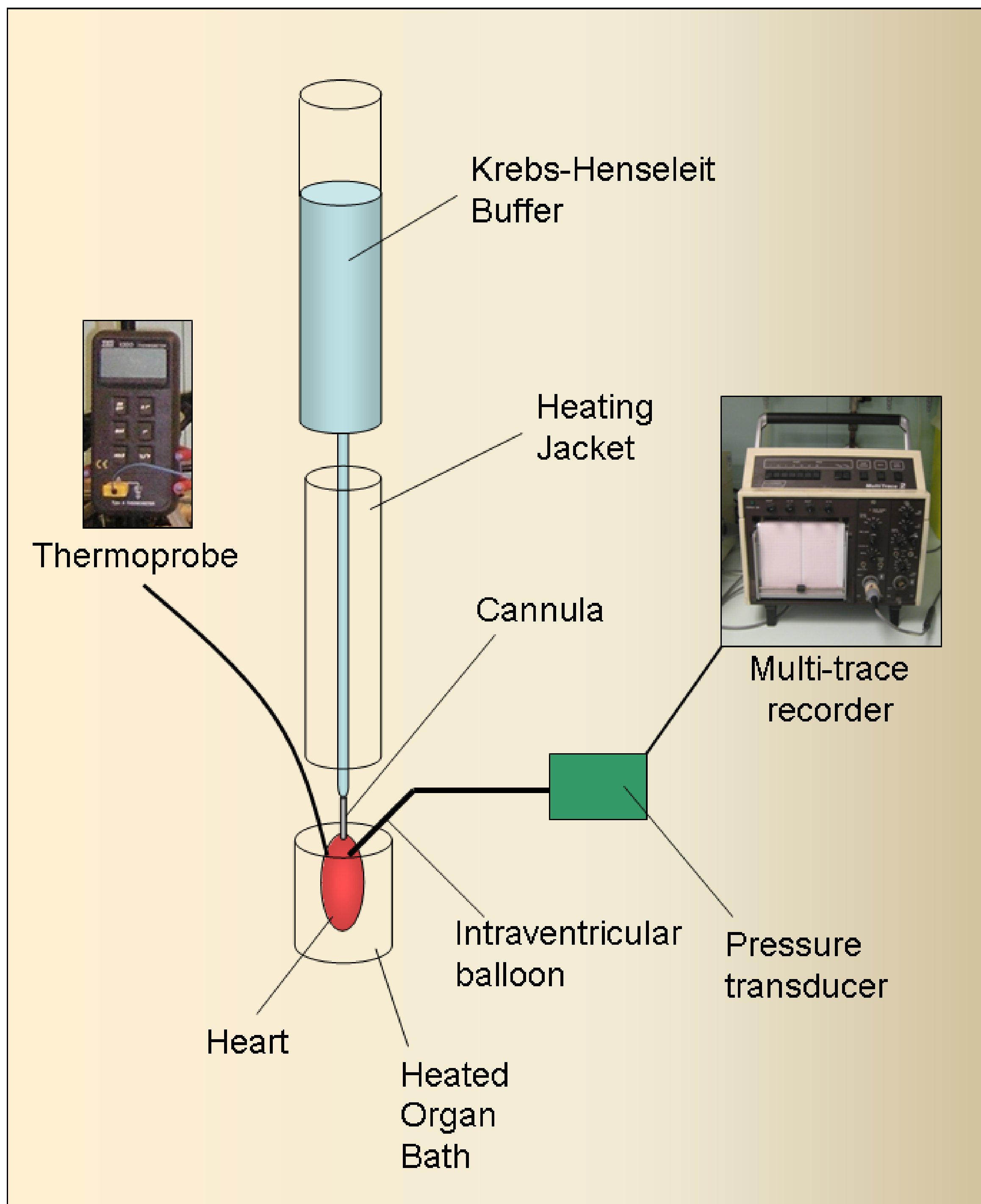


Figure 3.3.1 - Diagram of the Langendorff apparatus used

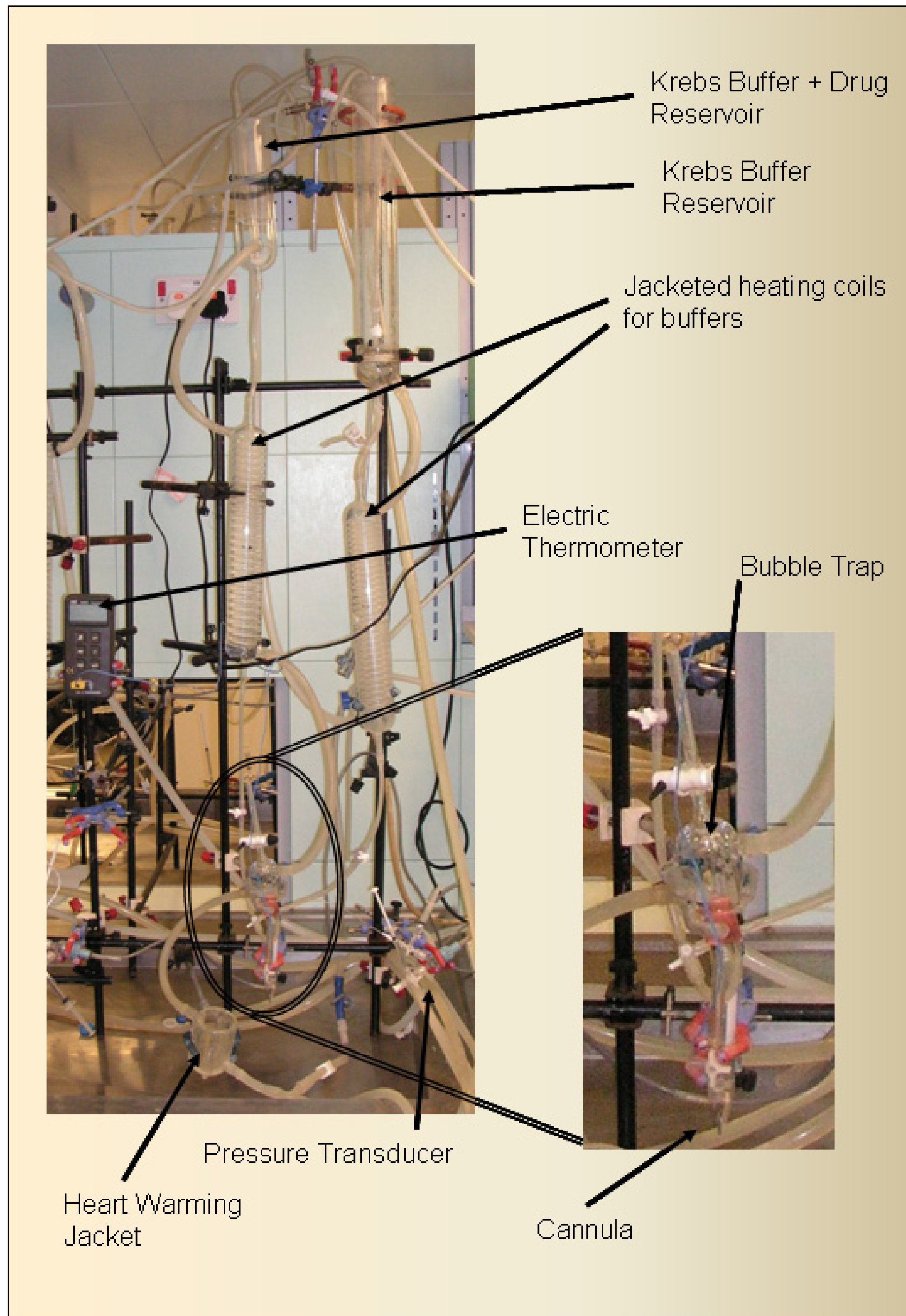


Figure 3.3.2 – Photograph of Langendorff isolated rat heart perfusion apparatus.

In order to induce ischaemia this model requires that one or more of the coronary vessels is obstructed. This is achieved by passing a length of 3.0 Mersilk under the left anterior descending coronary artery (LAD) using a curved round bodied surgical needle. The ends of the suture were placed through a snare constructed from the ends of two Gilson 1ml pipette tips (see Figure 3.3.4 for photographs of the isolated rat heart preparation).

Myocardial temperature was measured using a thermoprobe inserted into a small incision made in the base of the pulmonary trunk. Temperature was maintained at 37 ± 0.5°C using a thermostatically-controlled water-jacket system (C-85A circulator, Techne, USA) that entirely encased the glassware of the perfusion equipment. Coronary flow rate (CFR) was measured by collecting the coronary effluent of a particular heart in a 15ml falcon tube for 30sec and then multiplying the resulting figure by two to obtain the value in ml per min. Heart rate and developed pressure were measured using a latex water-filled balloon, constructed using the tip of a condom (Durex, UK), was inserted into the left ventricle subsequent to the removal of the left atrial appendage. The balloon, connected to a pressure transducer which directed the signal to a chart recorder (Multitrace 2, Lectromed, UK), was inflated such that a left ventricular end diastolic pressure of 5-10mmHg was achieved (see Figure 3.3.5).

After a stabilisation period of 40 min ischaemia was induced by tightening the suture around the LAD and clamping it with the snare. The suture was tightened so that there was at least a 40% reduction in coronary flow and developed pressure. Subsequent to 35 min of regional ischaemia the snare was released

and the myocardium reperfused for a period of 120 min (see Figure 3.3.3). Reperfusion was confirmed by an increase in coronary flow and cardiac function. It is essential that the myocardium is reperfused for 120 min to ensure that removal of dehydrogenase enzymes and cofactors occurs: inadequate wash-out of dehydrogenase enzymes and cofactors as a result of the use of shorter reperfusion times leads to underestimation of the infarct size (Schwarz et al. 2000). Left ventricular developed systolic and diastolic pressures, heart rate, myocardial temperature and coronary flow were noted at regular intervals. The rate pressure product (RPP) was also recorded, which is the product of the diastolic pressure and heart rate, and is used as a measure of ventricular contractility.

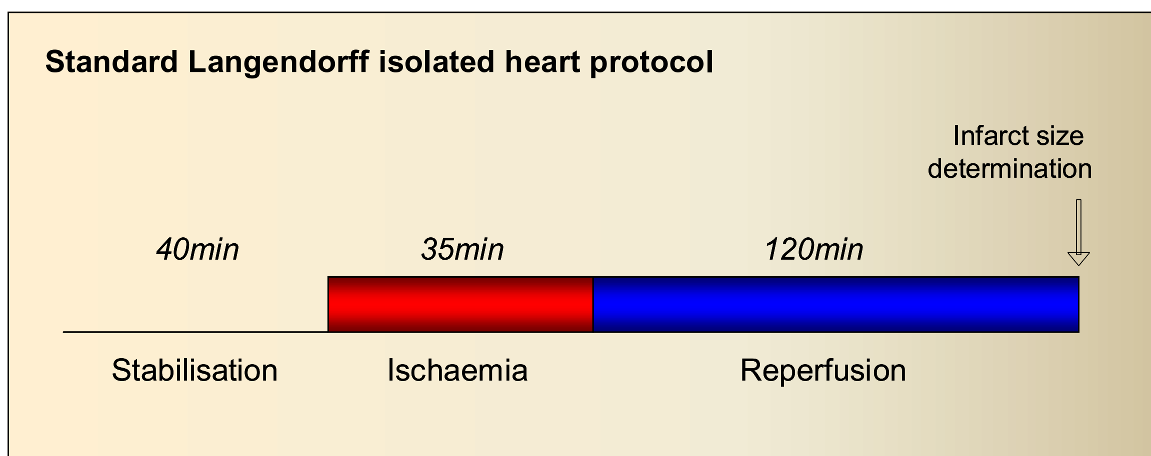


Figure 3.3.3 – Schematic of standard protocol for isolated rat heart I/R experiment. Hearts were stabilised for 40min, subjected to 35min ischaemia and then reperfused for 120min.

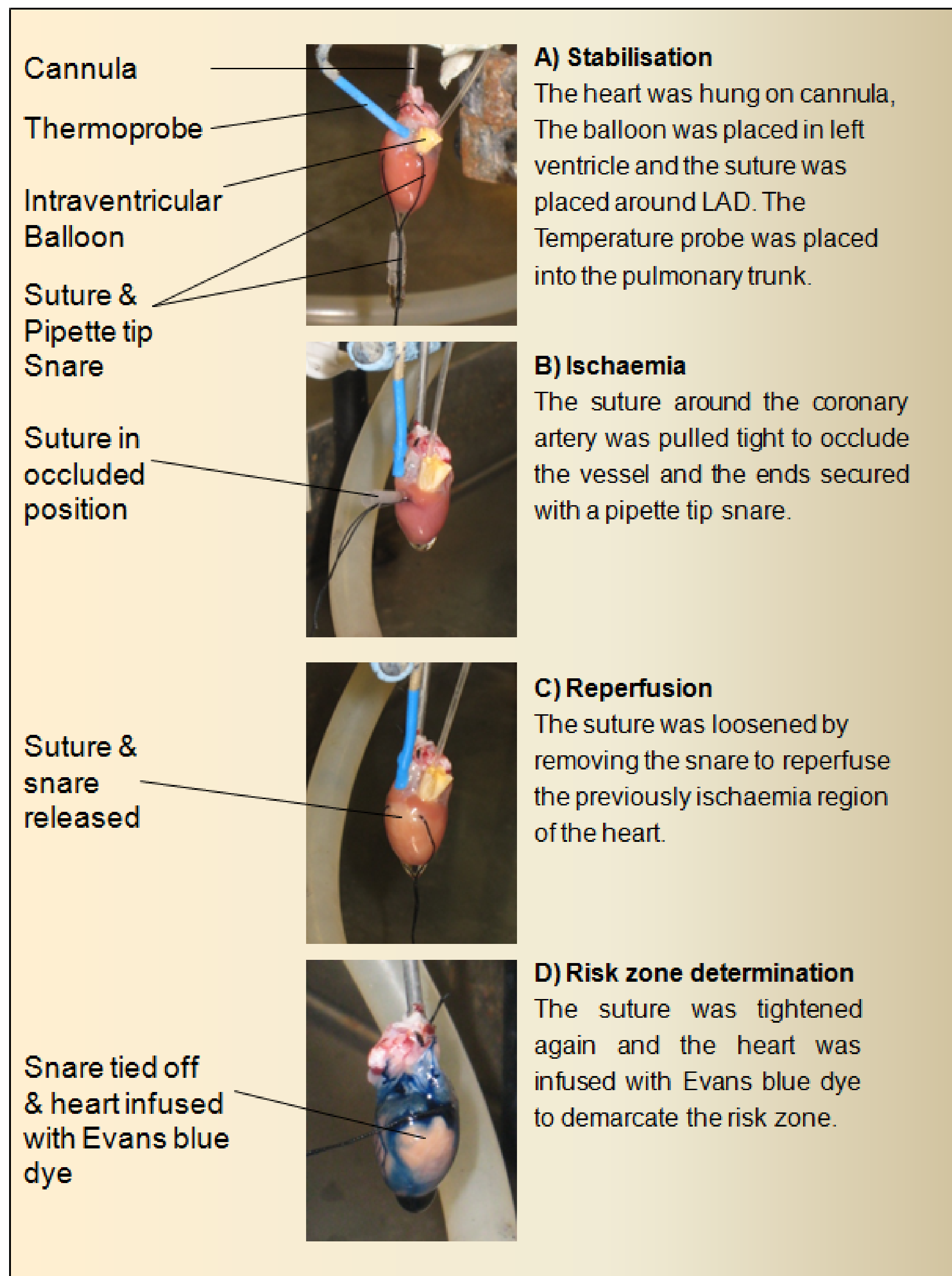


Figure 3.3.4 – Isolated mounted rat heart photos demonstrating stages of ischaemia reperfusion protocol.

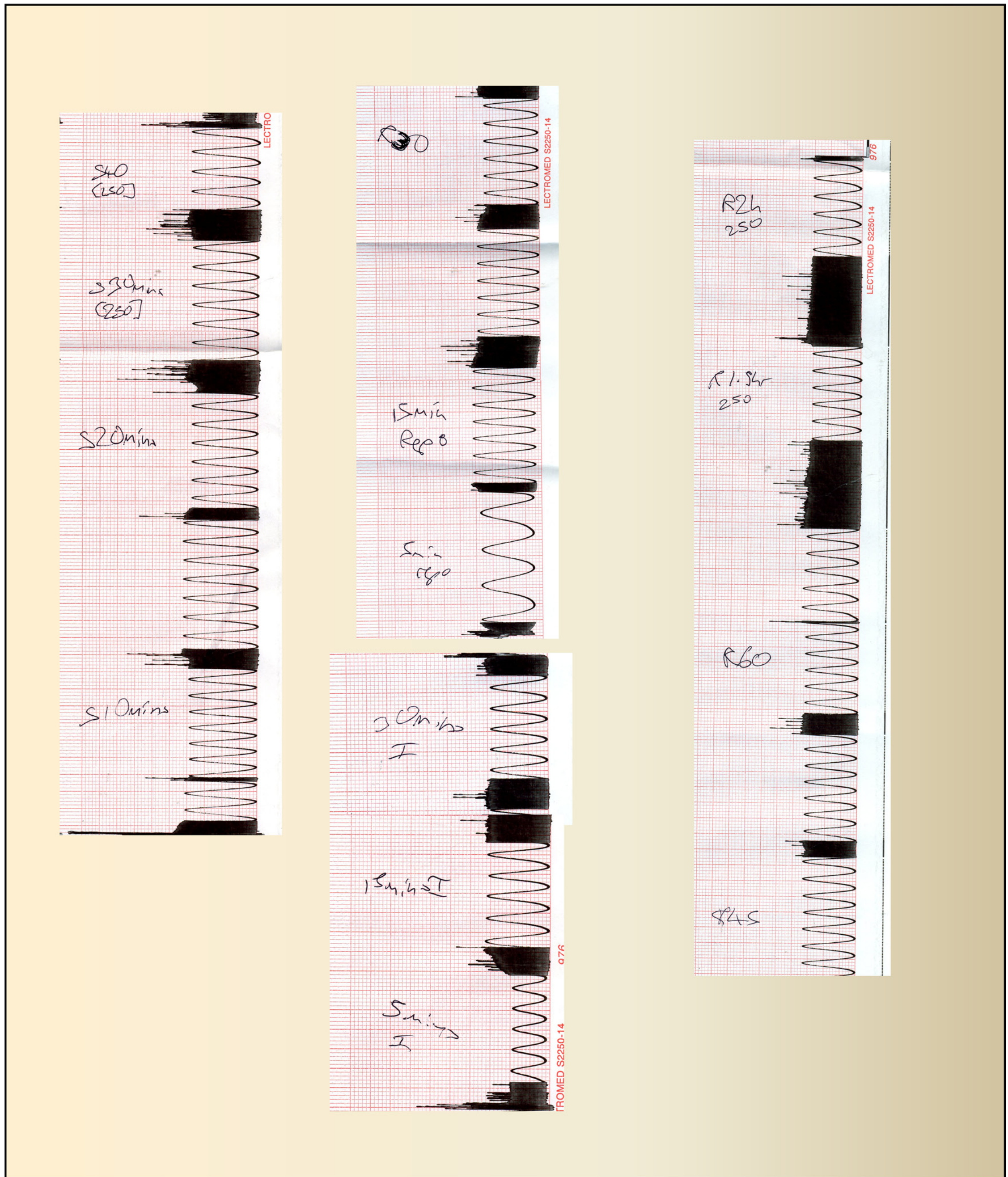


Figure 3.3.5 – Example of a heart trace obtained using latex balloon inserted into the left ventricle during an ischaemia/reperfusion experiment.

3.3.1 Determination of infarct size

Following the reperfusion-phase of the experiment the suture was pulled tight and the heart infused with 2ml saline solution containing 0.25% Evans blue (Sigma Chemicals, Poole, Dorset) to demarcate the non-ischaemic zone of the myocardium (Figure 3.3.). The heart was then weighed and immediately frozen at -20°C. After 1 to 4 hours at -20°C, the hearts were sliced from apex to base into 5 x 2mm-thick transverse sections, and incubated in a triphenyltetrazolium chloride (TTC) solution (1% in phosphate buffer, pH 7.4) at 37°C for 15 minutes. TTC reacts with the dehydrogenases still present in the non-infarcted tissue to produce the red-pigmented azo compound formazan. Infarcted regions of the tissue, by contrast, remain white due to the prior wash-out of the dehydrogenases during reperfusion (Figure 3.3.) (Vivaldi et al. 1985; Hausenloy et al. 2007). Heart slices were then transferred to 10% formalin (BDH Laboratory supplies, Poole, UK) for 24 hours to bleach the infarcted tissue, thus improving the disparity between infarcted and viable tissue. Heart slices were then placed between two sheets of transparent Perspex which were secured using bulldog clips. Slices were then traced onto an acetate sheet using a fine permanent marker by a designated person who was blinded to the protocol each heart had been subjected to. Subsequently, a computerised planimetry package (Summa Sketch III, Summagraphics, Seymour, CT, USA), was used to calculate the percentage of infarcted tissue within the volume of myocardium at risk, and the data expressed as an infarct-risk volume ratio (IS/AAR%) (Figure 3.3.6 & Figure 3.3.7).

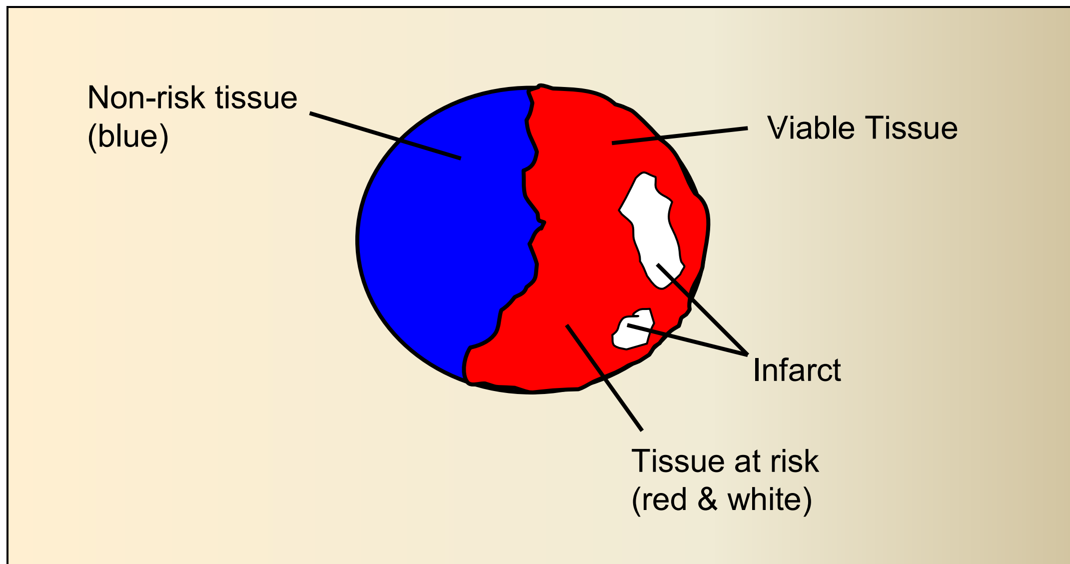


Figure 3.3.6 – Schematic of heart subjected to ischaemia/reperfusion injury infused with Evans blue dye. The blue area represents the tissue that was perfused throughout the experiment and therefore was not at risk. The red and white area represents the portion of the heart that has been subjected to ischaemia and contains viable (red) and infarcted (white) tissue.

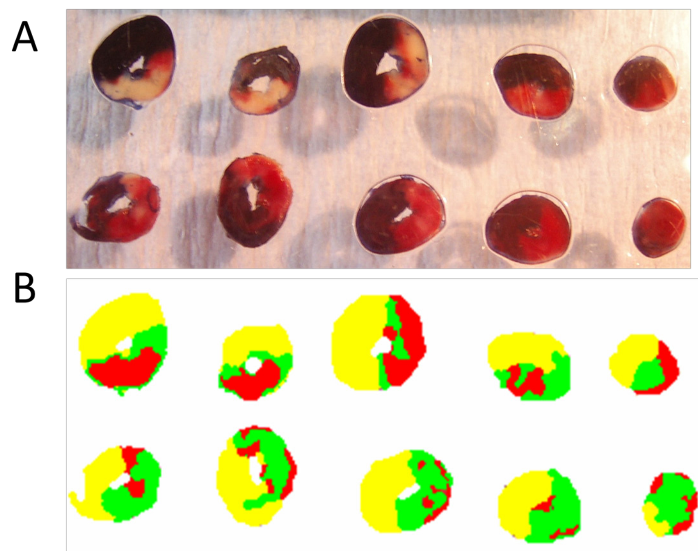


Figure 3.3.7 - Heart slices(A) and computed planimetry(B). Figure (A) shows heart slices which have been stained with TTC and bleached in formalin. Figure B shows the digital analysis for heart slices, in which the yellow area corresponds to non-risk tissue, green to tissue at risk and red to infarct.

3.3.2 Exclusion criteria

Hearts were excluded from the study if either the RPP was less than 17 mmHg min-1 or the coronary flow rate was less than 10 ml/min during stabilisation. Hearts were also excluded if there was no drop in pressure and coronary flow on induction of ischaemia. It should be noted that a reduction in pressure is a reduction in left ventricular developed pressure. In addition hearts were excluded if sustained ventricular fibrillation occurred upon reperfusion, i.e. longer than 5min. Finally, hearts with risk zone volumes of less than 40% or greater than 65% were also excluded.

3.4 Preparation of Isolated Adult Rat Cardiomyocytes

Hearts were mounted on a non-recirculating constant flow (14 ml/min) Langendorff apparatus following excision from Wistar rats (for details of the excision process see section 3.3). The protocol as described previously in (Maddock et al. 2002), involved the perfusion of hearts with low-calcium containing buffers based on Krebs-Ringer's (KR) buffer which consisted of 116mM NaCl, 10 mM Glucose, 5.4 mM KCl, 0.9 mM Na_2HPO_4, 0.4 mM $MgSO_4$, 20 mM taurine and 5 mM pyruvate (Krebs & Henseleit 1932). Hearts were initially perfused for 3-5 mins with "buffer 1", which consisted of the KR buffer described above with the addition of 1 mg/ml BSA and 3.3 μmol/l EGTA. The heart was then perfused for 10min with "buffer 2", i.e. KR buffer with the addition of 1mg/ml collagenase (Type II LS004176, Worthington): the collagenase breaks the peptide bonds in collagen, a key component of the extracellular matrix, and promotes the separation of the myocytes (Tytgat 1994). The ventricles were then harvested by cutting away the atria which remained

attached to the perfusion apparatus. The ventricles were cut into strips, placed in a 50ml Falcon tube and incubated in ~15ml “buffer 2” at 37°C with shaking and bubbling with 100% O_2. After 10min the supernatant was discarded and the remaining undigested tissue was incubated for a further 20min in 15ml fresh “buffer 2” with shaking and O_2. Cells in solution were then transferred to a fresh tube and centrifuged at 600 rpm for 3min. The pellet was then resuspended in 15ml of “buffer 3”, i.e. KR buffer with 44μM calcium, and re-centrifuged. The pellet was then slowly resuspended, drop-wise, in M-199 plating medium (M7653, Sigma Chemicals, Poole, Dorset) to allow the cells to acclimatise to a higher calcium concentration. The plating medium (40ml) was supplemented with the following additives: 1.0g BSA, 0.33g creatine, 0.31g taurine, 0.16g carnitine and 1 % pen-strep. At all stages when cells were being transferred from tube to tube a plastic Pasteur pipette was used to avoid cell membrane rupture. Isolation efficiency and cell viability were monitored at every stage during the protocol by visualising samples under a light microscopy. Preparations with less than 60-70% viable cells were excluded. Viability was assessed by the visualisation of the isolated cardiomyocytes, i.e. viable cardiomyocytes are characterised by their elongated appearance, by contrast non-viable cells appear either contracted or rounded (see Figure 3.4.1) (Egorova et al. 2005). Isolation efficiency was judged to looking at the sample to see how many dead cells were in the preparation. Furthermore, cell counts were performed using a cell count chamber (see http://www.ruf.rice.edu/~bioslabs/methods/microscopy/cellcounting.html for a detailed explanation).

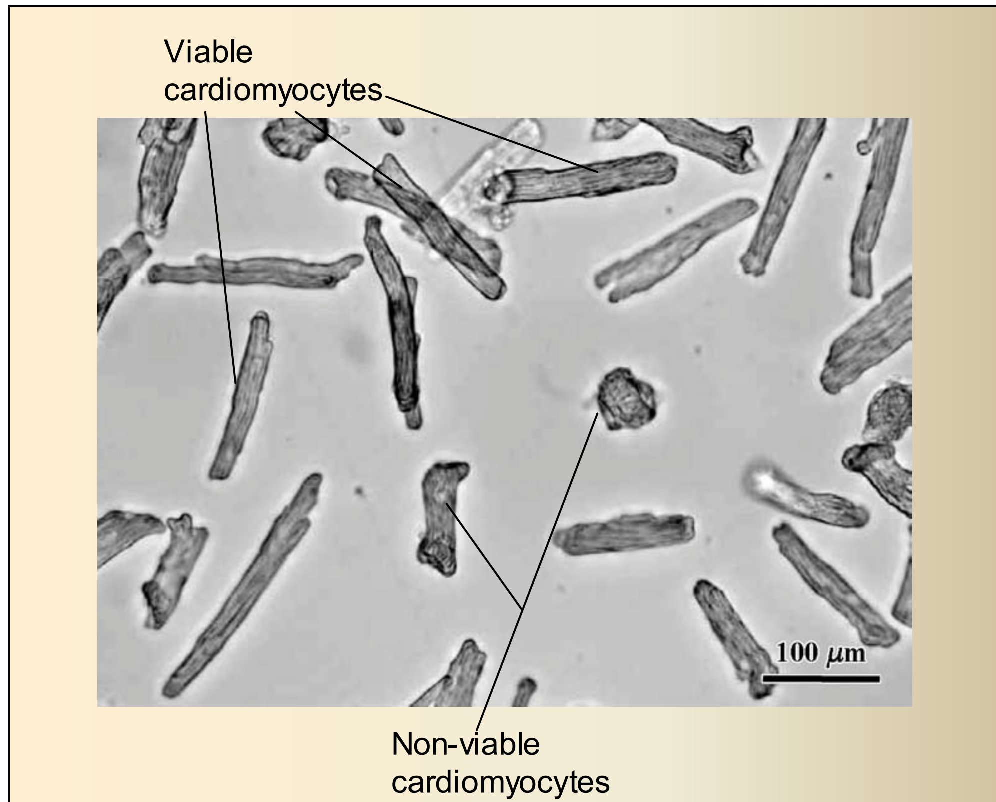


Figure 3.4.1 – Micrograph of isolated rat cardiomyocytes. At the end of the isolation procedure preparations were examined by eye to estimate the percentage yield. Non-viable cardiomyocytes were easily identified by their shortened or rounded appearance when compared to viable cells.

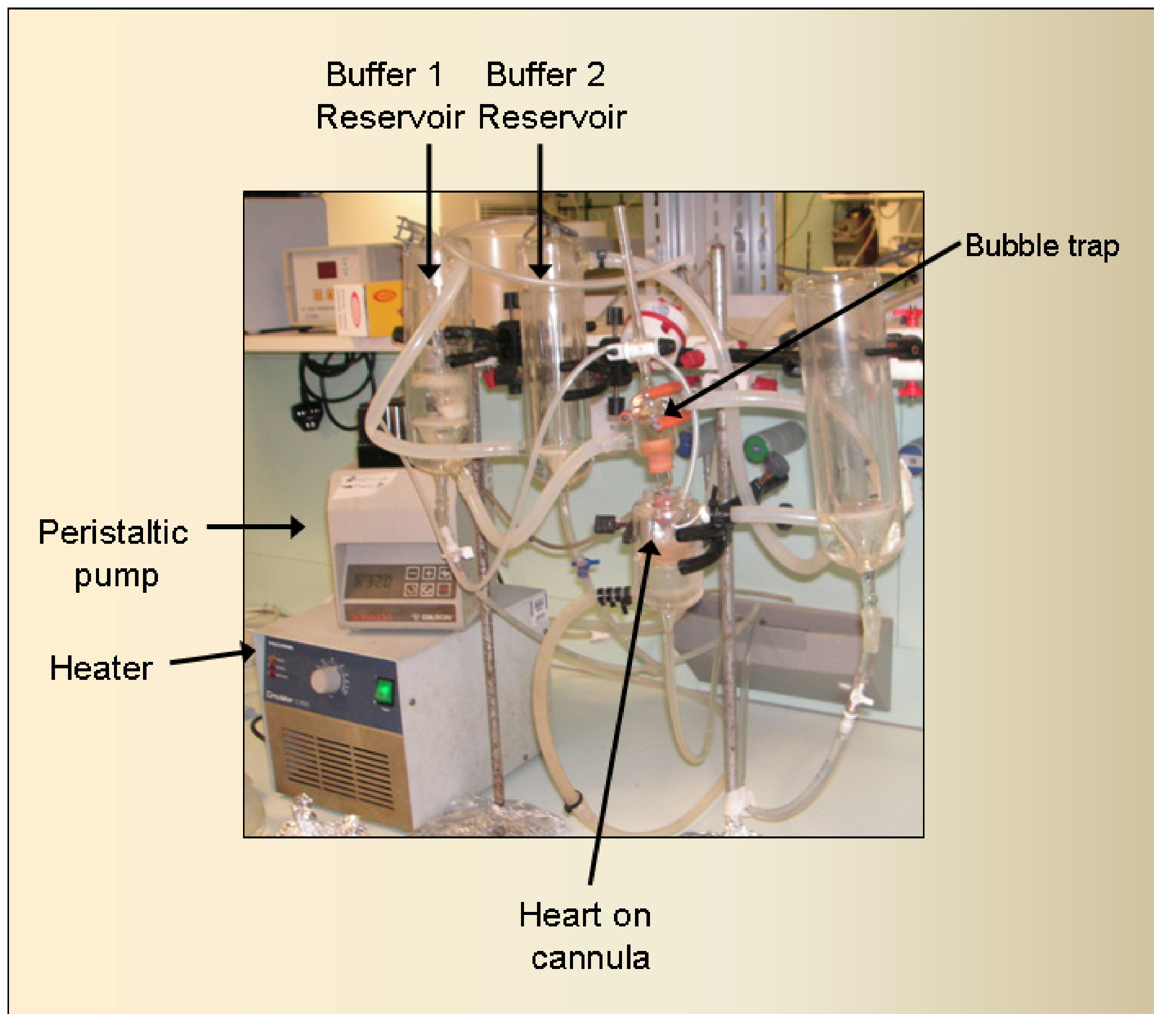


Figure 3.4.2 – Cardiac myocyte isolation rig. The rig has two buffer reservoirs, thus allowing an efficient transition from collagenase-free to collagenase-containing buffers.

3.4.1 Preparation of cardiomyocytes for confocal microscopy

A single 25mm diameter glass coverslip was placed in each well of a sterile six-well plate. The plate was then placed in a UV crosslinker to kill any bacteria present on the coverslips. To provide a structural scaffold for the cells to adhere to, 50µl laminin (1mg/ml diluted in 30 ml of distilled water, Sigma Chemicals, Poole, Dorset) was pipetted onto each coverslip. 200 µl of the cell solution (section 3.4) was then pipetted onto the laminin coated plates which were incubated at 37°C with 5% CO_2 for 45-60 min (incubator CO28IR, New

Brunswick Scientific, USA). The cells were then washed with 1ml M-199 plating medium, which was removed and replaced with 1ml fresh plating medium. Confocal microscopy was performed to investigate how leptin affects the opening of the MPTP (section 1.2.2.4) in isolated cardiomyocytes (Smith et al. 2006). Opening of the MPTP leads to a loss of mitochondrial potential. To visualise this process tetramethyl-rhodamine methyl ester (TMRM), a cationic fluorescent dye that accumulates in the mitochondria due to its charge, was used (Ehrenberg et al. 1988). Exposure of the TMRM dye to a laser leads to the generation of ROS in the mitochondria (Zorov et al. 2000). Increasing levels of ROS in the mitochondria ultimately leads to the induction of MPTP opening, which can be visualised as depolarisation of the mitochondria and dequenching of the TMRM fluorescence (Davidson et al. 2006). Coverslips containing isolated myocytes were incubated with TMRM (3µM) for 15 min, which is sufficient time for mitochondrial TMRM uptake. Cells were then incubated for a further 10min in either (1) KH buffer (control), (2) KH buffer with 10µM leptin or (3) KH buffer with 0.2µM CsA. CsA is an established inhibitor of pore opening and was therefore used as a positive control (Xie & Yu 2007).

3.4.2 Confocal microscopy

Confocal microscopic analysis was performed in the Department of Physiology in collaboration with Professor Michael Duchen, head of the Mitochondrial Biology Group. A Zeiss 510 CLSM microscope, fitted with a henna laser that was filtered to 543nm and TMRM fluorescence measured at 585 nm by using a long pass filter was used for all experiments (Figure 3.4.3). Following the

incubation with TMRM and any drugs used, the cloverslips were placed upon the custom built housing of the microscope and the cells were visualised using a 40x oil immersion objective. Using the light microscope function, a small group of viable cells (usually 2-4) were selected and then the laser was turned on. Exposure of the cells to the laser in the viewing field continued until mitochondrial depolarisation occurred. The time from switching on the laser to mitochondrial depolarisation was recorded and used as a measure of cellular vulnerability. A total of 12-20 cells from at least five hearts per group were analysed.

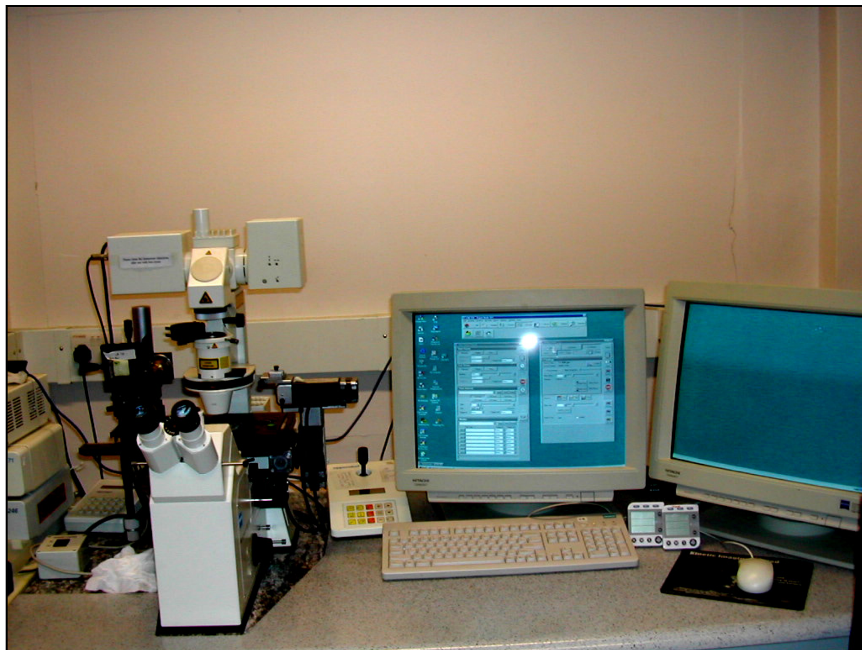


Figure 3.4.3 – Confocal microscope and operating computer

3.5 Western Blotting

3.5.1 Tissue collection

Rat hearts were perfused for 40min to allow them to stabilise. Hearts were then subjected to 35min regional ischaemia and reperfused for 2.5, 5, 10, 15 and 30min, at which time the risk zone was identified using Evans blue dye (see section 3.3). This area was then excised, snap-frozen in liquid nitrogen using Wallenberg clamps to freeze/disrupt the tissue. All samples were stored at -80°C to await analysis.

3.5.2 Protein extraction

Proteins were extracted by homogenising myocardial samples in an ice-cold suspension buffer containing: 100mM NaCl, 10mM Tris (pH 7.6), 1mM ethylenediaminetetraacetic acid (pH 8.0), 2mM sodium pyrophosphate, 2mM sodium fluoride, 2mM β-glycerophosphate and a protease inhibitor cocktail, using a Plytron model T25 homogeniser (IKA Labortechnik T25, Janke & Kunkel GmbH & Co., Germany) set at 24000rpm. Samples were then spun at 11000rpm for 10min in a 5417R centrifuge (Eppendorf, UK) and the supernatants assayed for protein content using a bicinchoninic acid (BCA) assay (Pierce, UK). The remaining supernatant was further diluted 1:1 in sample buffer: Tris 100mM (pH 6.8), DTT 200mM, SDS 2%, bromophenol blue 0.2% and glycerol 20%, and subsequently boiled for 10 minutes at 100°C in a DRI-Block DB-2A heating block (Techne, USA). Boiling the protein mixture in the presence of SDS denatures the protein and allows the detergent to bind to the polypeptide backbone. SDS binds in a uniform manner and therefore the proteins take on a negative charge that positively correlates with its molecular weight.

3.5.3 Protein estimation

The protein concentrations for samples were determined so that the amounts of protein loaded onto polyacrylamide gels for Western blot analysis could be standardised. The bicinchoninic acid (BCA) protein assay works on the principle that peptide bonds associated with cysteine, tryptophan and tyrosine residues are capable of reducing Cu^{2+} in a copper sulphate reagent to Cu^{+} (Wiechelman et al. 1988). Bicinchoninic acid is then able to bind to Cu^{+} to give a purple colouration that is measurable in a linear manner at an absorbance of 562 nm using a spectrophotometer (Jenway 6405 UV/Vis spectrophotometer). The protein contents of samples can then be measured by reference to a standard curve constructed using bovine serum albumin (BSA) (20-2000 µg/ml). In this way protein concentrations for samples, expressed as µg/µl, are obtained.

3.5.4 Polyacrylamide gel electrophoresis

Polyacrylamide gels for protein separation were formed between two glass plates that were cleaned with ethanol to remove dust particles, and were composed of a running gel and a stacking gel. The 12.5% running gel consisted of 12ml ddH_2O, 15ml 30% acrylamide and 9ml running gel base [1.5M Tris, 0.4% SDS in distilled water, pH 8.8]. Gel polymerisation was triggered by adding 40µl 99% tetramethylethylenediamine (TEMED) and 200µl 10% ammonium persulphate (APS). On addition of the polymerisation agent the gel was poured and then left for 30min to set.

To form the wells into which samples were loaded, plastic “combs” were placed above the running gel and a 5% stacking gel poured in to fill the space. The

stacking gel consisted of 4.5ml ddH_2O, 2ml 30% acrylamide, 10µl 8% bromophenol blue and 1.5ml running gel base [0.5M Tris, 0.4% SDS in distilled water, pH 6.8 with HCl acid]. As for the running gel 12µL 99% TEMED and 60µL 10% APS were added to cause the acrylamide to polymerise. The stacking gel was then left for 15min to set, whereupon the combs were removed and the gel placed into the electrophoresis apparatus (Biorad, UK). A running buffer consisting of glycine 14.42 g/l, SDS 1.0 g/l, Tris 3.0 g/l, distilled H_2O 1.0 L was poured into the reservoir of the electrophoresis apparatus so that the sample wells were submerged. Any air bubbles present in the wells were removed using a 21 gauge needle. Running buffer was added to the bottom of the electrophoresis apparatus so that the bottom 3cm of the gels were submerged, this allows the electric current to be applied across the gel and causes the negatively charged SDS-bound proteins to migrate.

In order to establish the molecular weights of the proteins under investigation a molecular weight dual-colour protein marker (Precision Plus Protein Dual Colour Standards, Bio-Rad) was pipetted (15µL) into the first well of each gel run. Quantities of sample equivalent to 30-60µg protein were then loaded into the subsequent wells. Gels were left to run at 200 volts for 2-3hours or until the bromophenol blue marker had migrated sufficiently to ensure that the protein of interest could be visualised.

3.5.5 Protein transfer

After the completion of protein separation by electrophoresis, the stacking gel was trimmed off and the running gel was mounted in a transfer system

containing transfer buffer (200ml methanol, 700ml de-ionised H_20, 100ml 10x transfer buffer [glycine 14.4g/L, Tris 3 g/L, 1 litre ddH_20]). Hybond ECL nitrocellulose membrane (Amersham, UK) was cut to the same size as the gel and placed on the gel. The gel and membrane were then placed between 2 pieces of Whatman 4mm paper taking care to remove any air bubbles. Following this, the proteins were transferred to the membrane by passing a current of 140mA overnight (16-20 hours). Adequate transfer could be determined by staining the membrane with Ponceau Red (Sigma Chemicals, Poole, UK).

3.5.6 Immunoblotting

After the transfer had been completed membranes were washed three times in TBS-Tween (Tris 2.42 g, NaCl 8.0 g, pH 7.6 with HCl, 1 litre deionised H_2O + 1 ml Tween). Membranes were then submerged in TBS-Tween plus 5% milk solution to block non-specific protein binding. After two hours membranes were washed three times for five minutes in TBS-Tween. The membranes were then soaked in a primary antibody specific to the protein of interest at a dilution of 1 in 1000 in a TBS-Tween solution plus 5% BSA for at least one hour. A third set of washes was then performed before placing the membranes in secondary antibody solution (diluted of 1 in 1000 in TBS-Tween plus 5% milk) for at least two hours. A final set of three five minute washes using TBS-Tween was then performed before submerging the membranes in an enhanced chemi-luminescent (ECL) Western blotting detection reagent to visualise the protein bands on exposure of the membrane to Kodak Omat XR photographic film. The

film was developed using a Kodak XOMAT 1000 Film Developer (Serial No. 2225; Kodak House, Hemel Hempstead, HP11JU). All washes and antibody treatments were performed in plastic containers placed on a rocking platform (SSL4 see-saw rocker, Stuart) set at approximately 60rpm. Total and phosphorylated forms of Akt, p44/42 MAPK, AMPK, PTEN and STAT3 were detected using specific primary and secondary antibodies (New England bio-labs, UK).

3.5.7 Quantification of bands

The photographic film was subsequently scanned and the intensities of the protein bands, expressed as arbitrary units (a.u.), determined by computerized densitometry (NIH Image 1.63). The relative changes for the proteins of interest were then established by calculating the ratios of the phosphorylated versus total forms of the protein.

3.6 Rat *In Vivo* Model of I/R Injury

3.6.1 Model

Male rats were anaesthetised by i.p. injection of 50mg/kg pentobarbitone (Sagatal-Rhone, Merieux) and secured to a pre-warmed (37°C) operation table (Peco services Ltd, Cumbria, UK) by the forelimbs. When animals were sufficiently anaesthetised, as indicated by the loss of the pedal withdrawal reflex, a tracheostomy was performed using a 14 gauge catheter, to allow artificial ventilation. Animals were ventilated with room air supplemented with 100% oxygen using a small animal ventilator (Cat no. 50-1718, Harvard apparatus, Kent, UK) set to 70 cycles per min and a tidal volume of 2.0-3.0ml. The carotid

artery and jugular vein were then cannulated, in the case of the former to allow blood pressure to be monitored via a pressure transducer, and the latter to provide a route for drug delivery. When cannulating the carotid artery it was necessary to tie off the vessel using a 3.0 mersilk suture. This served not only to prevent excessive blood loss, but also provided a useful harness by which the vessel could be slightly stretched and secured. A small vessel clip was then applied to the vessel approximately 2cm down from the tie toward the posterior of the animal. The combination of the tie and the clip permitted dissection of the vessel without blood loss. A perpendicular incision was made into the vessel using micro-scissors (Harvard apparatus) and was made large enough so as to allow the introduction of a cannula constructed of a 20cm piece of Portex fine bore tubing, with an inner diameter of 0.58m and outer diameter of 0.96mm (Smiths Medical International Ltd, UK), connected to a 23 gauge (blue) BD Microlance needle. Prior to insertion the cannula was filled with heparinised saline, 0.2ml 5000 I.U./ml heparin (CP Pharmaceuticals Ltd, UK) in 50ml normal saline (0.9% NaCl in 1 litre distilled water), ensuring that air bubbles were not introduced and so avoiding air emboli entering the circulation. Once in place the carotid cannula was connected to the pressure transducer, which was filled with distilled water. The jugular vein was cannulated in a similar fashion, although in this case the cannula was constructed of a 15cm piece of Portex fine bore tubing, with an inner diameter of 0.40mm and an outer diameter of 0.8mm (Smiths Medical International Ltd, UK), connected to a 25-gauge (orange) BD Microneedle. The jugular cannula remained connected to a 1ml syringe throughout the experiment to allow delivery of fluids and drugs. To ensure that

there was adequate illumination during the operative procedures a Photonic PL2000 fibre optic lamp (Photonic, Vienna) was employed.

Electrocardiographic (ECG) recording was achieved by attaching three electrodes to the animal. Thus, two electrodes were attached to the forelimbs whilst a reference electrode was attached to a hind limb to dampen extraneous electrical noise. Arterial BP, heart rate and ECG were recorded using a PowerLab system connected to a computer (ADinstruments, Oxfordshire, UK). Body temperature was monitored via a temperature probe inserted into the anus and maintained at 37 ± 1°C by regulating the temperature of the operation platform.

Following the procedures outlined above a left thoracotomy was performed and the fourth intercostal space was opened carefully to allow visualisation of the heart. Upon visualisation of the left atrial appendage, either the 4th or 5th rib was cut to allow greater access to the thoracic cavity. A 3.0 mersilk suture was tied around the rib, prior to making an incision into the thoracic muscle, in order to tie off the blood vessels in the region and to provide an anchor for holding the operation site open. During the thoracotomy a Change-A-TIP hand held cauterisation device (Bovie Aaron Medical, USA) was used to cut through the pectoralis major and minor muscles and the intercostal muscle to minimise blood loss.

Once sufficient visualisation of the heart had been achieved, the pericardium was gently opened using forceps to allow access to the heart. A 16mm round

bodied needle was used to place a 4.0 prolene suture under the LAD, entering just under the left atrium and exiting under the right pulmonary outflow tract. The vessel was occluded for 30min by tightening a snare constructed using two 1ml Gilson pipette tips (see section 3.3 and Figure 3.3.4). At the conclusion of the occlusion-period the snare was loosened and the area of ischaemic myocardium reperfused for two hours (Figure 3.6.3). The pedal reflex was tested regularly throughout the procedure and additional pentobarbital was given as required. Blood samples were taken at baseline, 15min into reperfusion and just prior to the end of reperfusion to monitor pH, CO_2, O_2 and blood glucose using a gas analyser (ABL 700 Radiometer, Denmark).

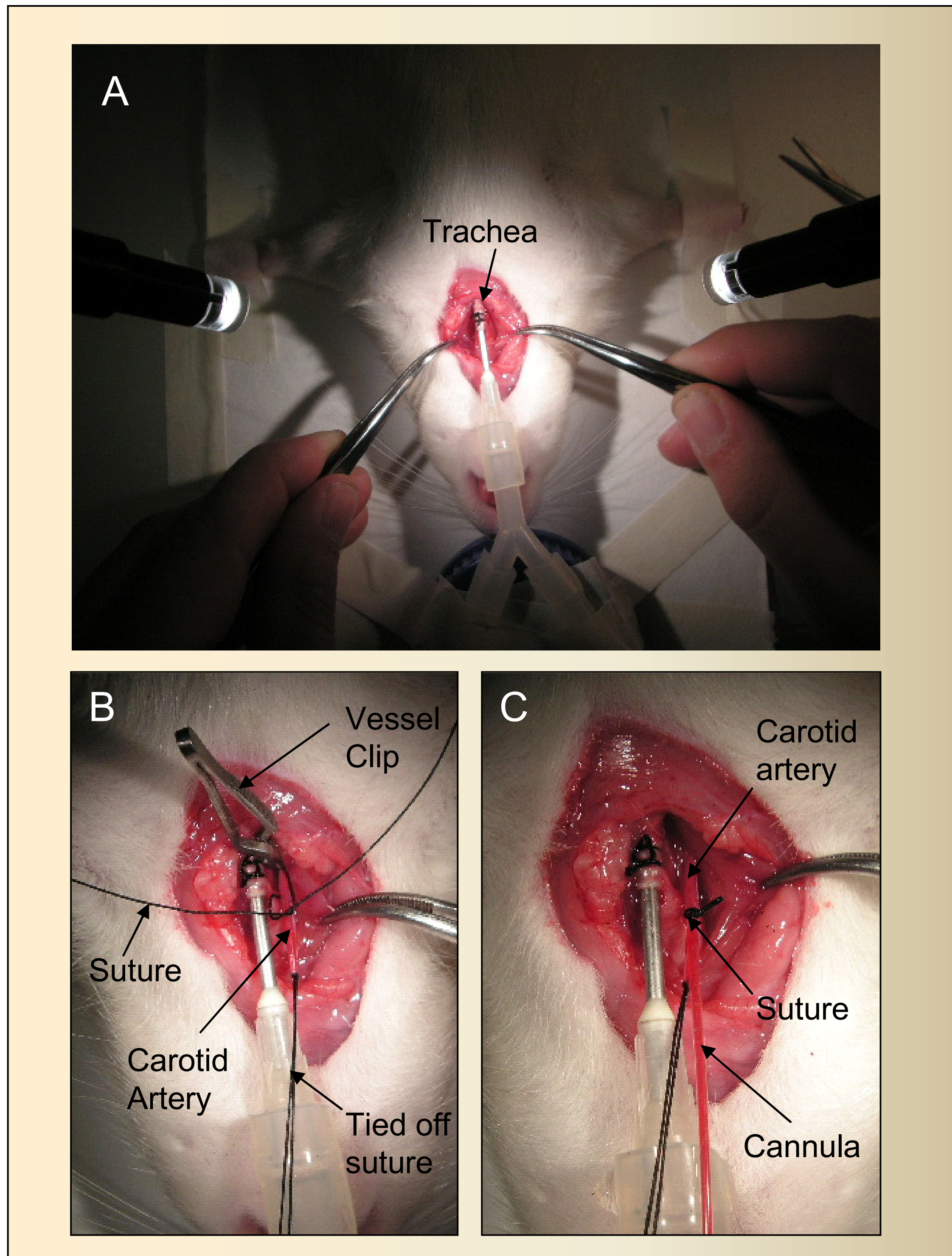


Figure 3.6.1 – Rat *in vivo* surgery (1). (A) A tracheal intubation was performed to allow artificial respiration. (B) The carotid artery was located and separated from its associated nerve. The artery was tied off and a vessel clip placed about 1.5-2cm down from the suture. This allows the vessel to be cut and the cannula positioned without the animal bleeding out. (C) The cannula was positioned and secured in the vessel with a suture. The Jugular vein was cannulated using the same method.

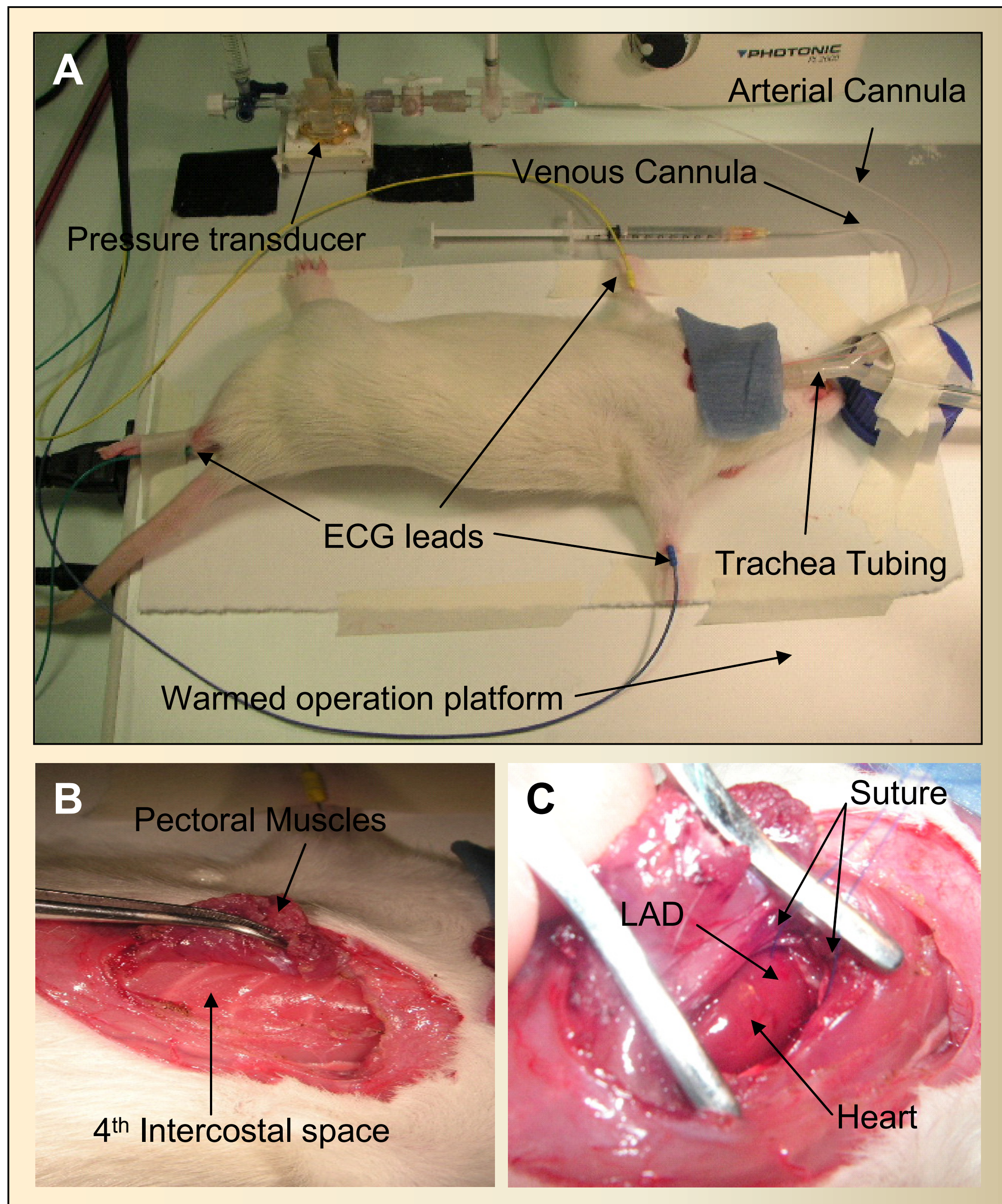


Figure 3.6.2 – Rat *in vivo* surgery (2). (A) Once the carotid artery was cannulated, the cannula was connected to a pressure transducer to record blood pressure and heart rate. (B) Pectoral muscles were cut using a cautery device and reflected to reveal the ribs. (C) The 4th intercostal space was carefully opened and once sufficient visualisation of the heart had been achieved the pericardium was gently opened using forceps. A suture was then placed around the LAD to occlude the vessel.

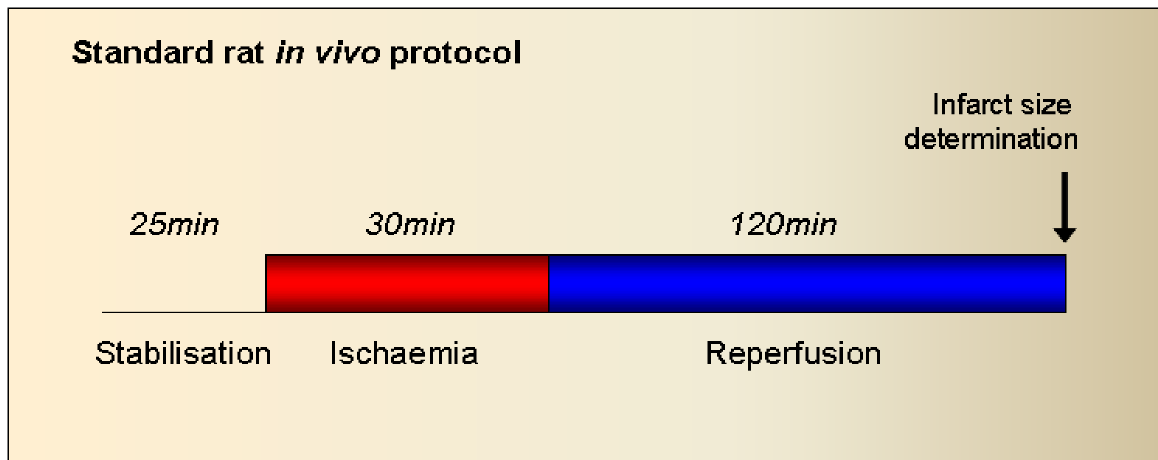


Figure 3.6.3 – Schematic of standard rat *in vivo* protocol. Hearts were stabilized for 25min and then subjected to 30min myocardial ischaemia followed by 120min reperfusion.

3.6.2 Determination of infarct size

At the end of the procedure the heart was excised and placed in iced saline, whereupon it was hung via the aorta on a Langendorff rig in a fashion similar to that described for the isolated perfused heart model (see section 3.3). The heart was then perfused with approximately 15-20ml saline to remove blood cells and debris. Once the saline ran clear from the heart the snare was retightened and the heart was infused with 1.5ml Evans blue dye to delineate the risk zone. Hearts were then frozen and subsequently analysed for infarct using a method identical to that used in the isolated perfused rat heart experiments (see section 3.3.1).

3.6.3 Exclusion criteria

Hearts from animals that did not survive the entire 120min reperfusion period were excluded from the study. In addition, if a drop in blood pressure was not observed upon tightening the suture (for the induction of ischaemia) the animal

was excluded from the study. Hearts were also excluded if they were found to have risk zones smaller than 40% or larger than 65%.

3.7 Method of Randomisation

In each set of experiments the animals were randomised to a particular group. This was carried out by writing the name of each group on a piece of paper, and repeating this for the number needed per group and then placing them in a bag. Animals were assigned to a group by selecting a piece of paper from the bag.

3.8 Statistical Analysis

Data are presented as mean±s.e.m. Generally comparisons between more than two groups were made using factorial, one-way analysis of variance (ANOVA) and the Fisher's protected least significant difference post-hoc test. In some cases (e.g. the MPTP data, see Chapter 8), however, the Kruskal-Wallis analysis of variance method was used followed by the Dunn multiple comparison test Where only two groups were compared the Student's t-test was used. Differences were regarded as statistically significant if a value of $p<0.05$ was obtained.

CHAPTER 4 - LEPTIN PROTECTS AGAINST I/R INJURY IN THE WISTAR RAT HEART

4.1 Introduction

In section 1.3.1 the phenomenon of preconditioning (IPC) was described. IPC is very complex mechanistically and is yet to be fully understood. IPC is, if not the most, one of the most effective methods of protecting myocardial tissue against reperfusion injury. However, due to the unpredictable nature of myocardial infarctions, IPC is not a practical option with respect to the treatment of patients. Current research is aimed at finding therapeutic strategies that can equal the level of protection achieved by IPC. Therefore, in the experimental setting protection afforded by IPC can be used as a good benchmark against which alternative therapies can be compared.

Leptin has been shown to be protective against I/R injury in a variety of settings (Smith et al. 2006; Brzozowski et al. 2001; Zhang & Chen 2008). In particular, in our laboratory it has be shown that leptin is protective against I/R injury in the isolated murine heart (Smith et al. 2006). The concentration of leptin used in this study was chosen on the basis of concentrations previously reported to facilitate protection in alternative tissues and to activate relevant cell signalling pathways (Rajapurohitam et al. 2003; Tajmir et al. 2004).

4.2 Aim

The use of a model that is robust and reproducible is essential to pharmacological experiments. Therefore, the first aim of the studies presented in this chapter was to establish that the isolated perfused rat heart model

performed within acceptable parameters (see section 3.3.2) and responded appropriately to ischaemic preconditioning (IPC). The second aim was to ascertain the optimal concentration of leptin with regard to its myocardial infarct-limiting actions in the Langendorff isolated perfused rat heart when administered at reperfusion.

4.3 Experimental Protocol & Materials

4.3.1 Isolated Langendorff perfused rat heart

All hearts were excised from Wistar animals according to section 3.3. Control hearts were perfused for 40min with no intervention to allow stabilisation and then subjected to 35min regional index ischaemia (Figure 4.3.1). Hearts in the ischaemic preconditioning (IPC) group were stabilised for 10min and then subjected to two cycles of 5min global ischaemia followed by 10min reperfusion (Figure 4.3.1). After the preconditioning protocol had been applied, hearts in this group were subjected to 35min regional ischaemia. Hearts in both groups were allowed to reperfuse for two hours following the index ischaemia. Individual groups were (original group sizes are shown in brackets):

Control (n=8) – no drug, no IPC, 40min stabilisation

IPC (n=8) – no drug, IPC protocol 2 cycles of 5min global ischaemia and 10min reperfusion prior to index ischaemia

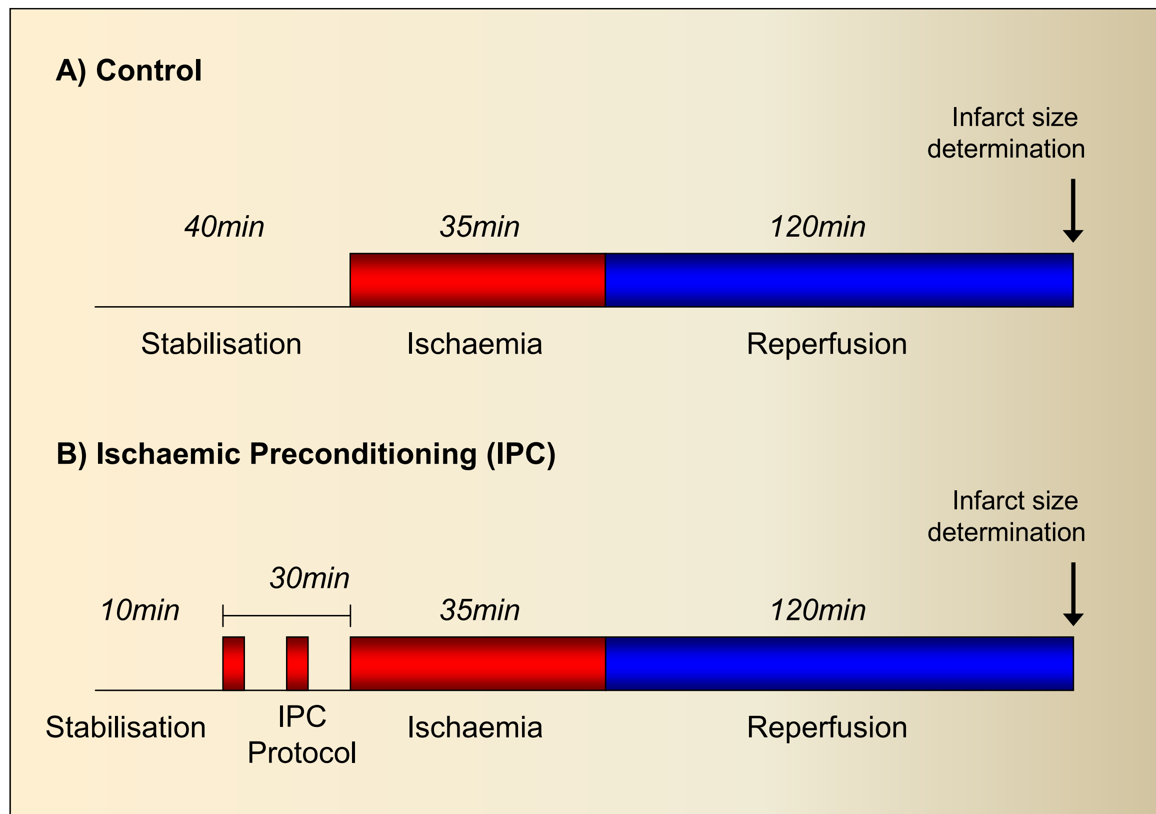


Figure 4.3.1 – Ischaemic preconditioning protocol. (A) Control (B) Ischaemic preconditioning protocol, incorporating two cycles of sublethal ischaemia and reperfusion prior to the index ischaemia.

4.3.2 Leptin dose-response

All hearts were prepared as described in section 3.3. Hearts were perfused for 40min to allow stabilisation and then subjected to 35min ischaemia, except those in the sham group were not made ischaemic (i.e. the suture surrounding the LAD was not tightened), but were perfused for the same period of time as control hearts. After the ischaemic insult, control hearts were reperfused for two hours to provide sufficient washout (see section 3.3). Treated hearts were perfused with 0.1, 1, 10, 30 or 100nM leptin for 30min during the reperfusion-period. Leptin was placed into the rig 5min prior to reperfusion to ensure it

reached the heart immediately upon reperfusion. Individual experimental groups were (original group sizes are shown in brackets):-

Control (n=7) – no drug, 40min stabilisation

Sham (n=7) – no drug, no ischaemia, 40min stabilisation

Leptin (0.1nm) (n=7) – administered 5min prior to, and up until 30min into the reperfusion-phase.

Leptin (1nM) (n=7) - administered 5min prior to, and up until 30min into reperfusion.

Leptin (10nM) (n=7) - administered 5min prior to, and up until 30min into the reperfusion-phase.

Leptin (30nM) (n=7) - administered 5min prior to, and up until 30min into the reperfusion-phase

Leptin (100nM) (n=7) - administered 5min prior to, and up until 30min into the reperfusion-phase.

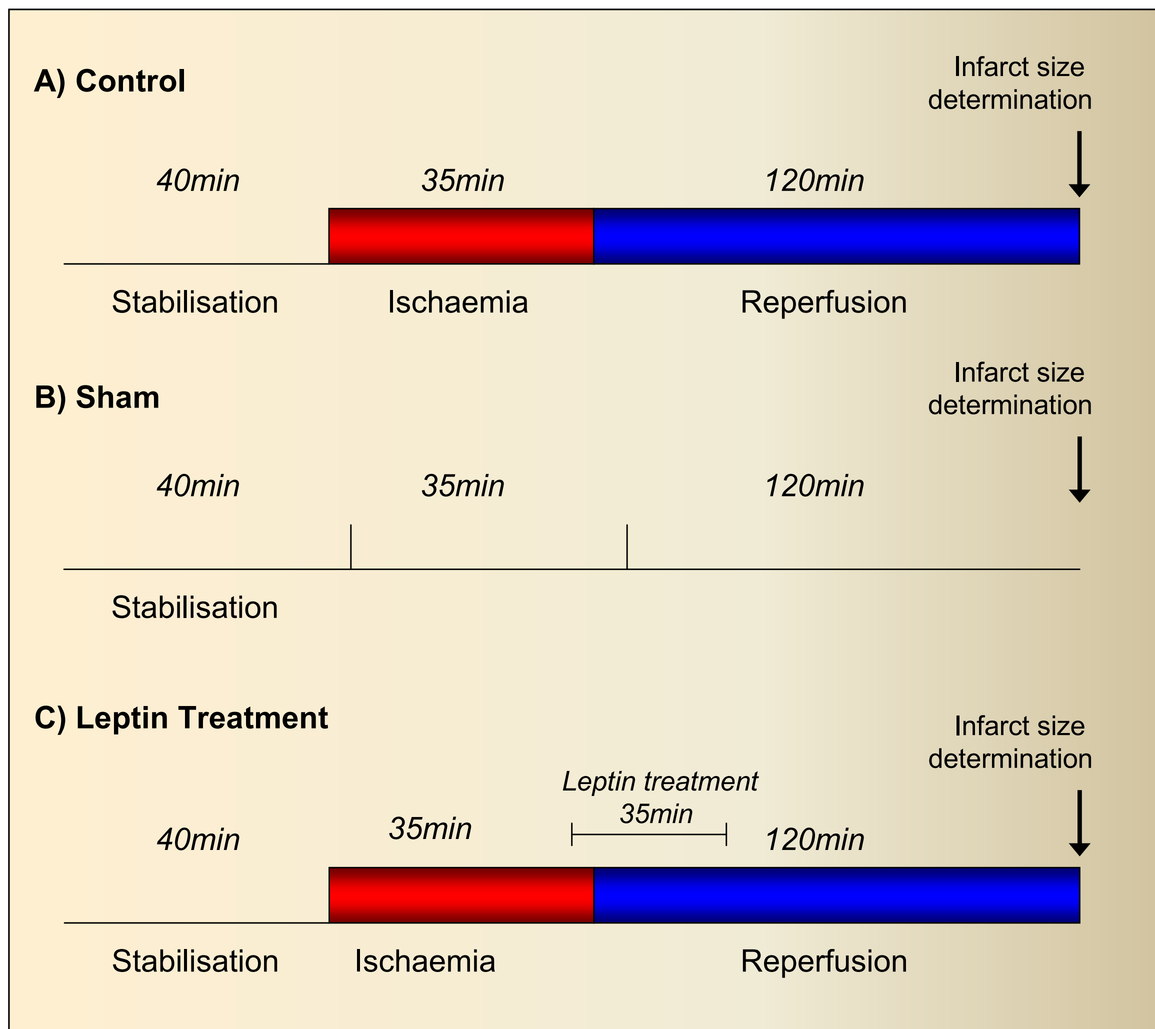


Figure 4.3.1 – Leptin treatment perfusion protocol. (A) Ischaemic control hearts were subjected to the normal protocol (see section 3.4). (B) Sham hearts were perfused for the same period of time as the ischaemic control but were not rendered ischaemic. (C) Leptin-treated hearts were perfused as for the ischaemic control hearts, but leptin was administered 5min before ischaemia and continued until 30min into the reperfusion-phase.

4.4 Results

4.4.1 Preconditioning

4.4.1.1 Animal and Haemodynamic Data

Table 4.4.1 demonstrates that there were no significant differences between the groups with respect to animal weight, ventricular volume or risk zone. Baseline data relating to cardiac function before ischaemia, as assessed by the rate pressure product (RPP) and coronary flow, were similar in both the experimental groups (see table 4.4.2 & 4.4.3). During in ischaemia, however, coronary flow and RPP declined to a similar extent in both groups. Cardiac function and coronary flow increased upon reperfusion, which indicated that re-flow was successful. No significant differences were observed between groups with respect to coronary flow rate and RPP during reperfusion.

Groups	Body Weight (g)	Ventricular Volume (mm3)	Risk Volume (%)
Control (n=10)	378.1±5.9	1791.3±50.3	52.0±2.1
IPC (n=6)	392.5±5.2	1881.6±85.2	51.7±1.9

Table 4.4.1– Characteristics of animals in the treatment groups

Coronary flow rate (ml/min)

	Stabilisation (ml/min)				Ischaemia (ml/min)			Reperfusion (ml/min)						
Time(min) / Groups	**10**	**20**	**30**	**40**	**5**	**15**	**30**	**5**	**15**	**30**	**45**	**60**	**90**	**120**
Control (n=10)	15.0 ±1.5	13.2 ±1.2	13.2 ±1.5	12.9 ±1.4	6.9 ±0.5	7.0 ±0.6	7.1 ±0.8	12.3 ±1.4	11.7 ±1.5	11.3 ±1.4	10.9 ±1.4	10.6 ±1.5	9.0 ±1.6	9.0 ±1.6
IPC (n=6)	17.2 ±0.9	17.0* ±0.9	15.8 ±1.3	15.2 ±1.3	8.7 ±1.2	8.2 ±1.2	7.9 ±1.6	12.0 ±1.7	11.1 ±1.5	11.0 ±1.5	10.4 ±1.5	9.2 ±1.7	8.8 ±1.7	8.7 ±1.6

Table 4.4.2 – Coronary flow rates of the different groups at various time-points during the ischaemia/reperfusion protocol. Values are as mean ±s.e.m. * = $p<0.05$ vs. control *(students t-test)*.

Rate Pressure Product (x 103mmHg/min)

	Stabilisation (x 10^{3}mmHg/min)				Ischaemia (x 10^{3}mmHg/min)			Reperfusion (x 10^{3}mmHg/min)						
Time(min) / Groups	**10**	**20**	**30**	**40**	**5**	**15**	**30**	**5**	**15**	**30**	**45**	**60**	**90**	**120**
Control (n=10)	28250 ±2880	30075 ±2891	30042 ±3102	31658 ±2859	13550 ±1600	19175 ±2839	20808 ±1213	22831 ±2586	23050 ±2816	21363 ±2290	19242 ±2406	18713 ±2907	18483 ±2949	16217 ±3148
IPC (n=6)	32530 ±1800	33780 ±1570	29100 ±4355	25956 ±4663	12366 ±2157	15500 ±1762	15150 ±2471	21075 ±2458	20858 ±1183	22030 ±1746	20554 ±1003	20808 ±1836	17600 ±893	17833 ±1757

Table 4.4.3 – Rate pressure product of the different groups at various time-points during the ischaemia/reperfusion protocol.

4.4.1.2 Exclusions

There were two hearts excluded from the IPC group, one because the infarct size exceeded that set for the exclusion criteria (see section 3.3.2) and one because the risk zone was below 40%. One heart was excluded from the control group due to excessive ventricular fibrillation occurring in ischaemia and which could not be terminated.

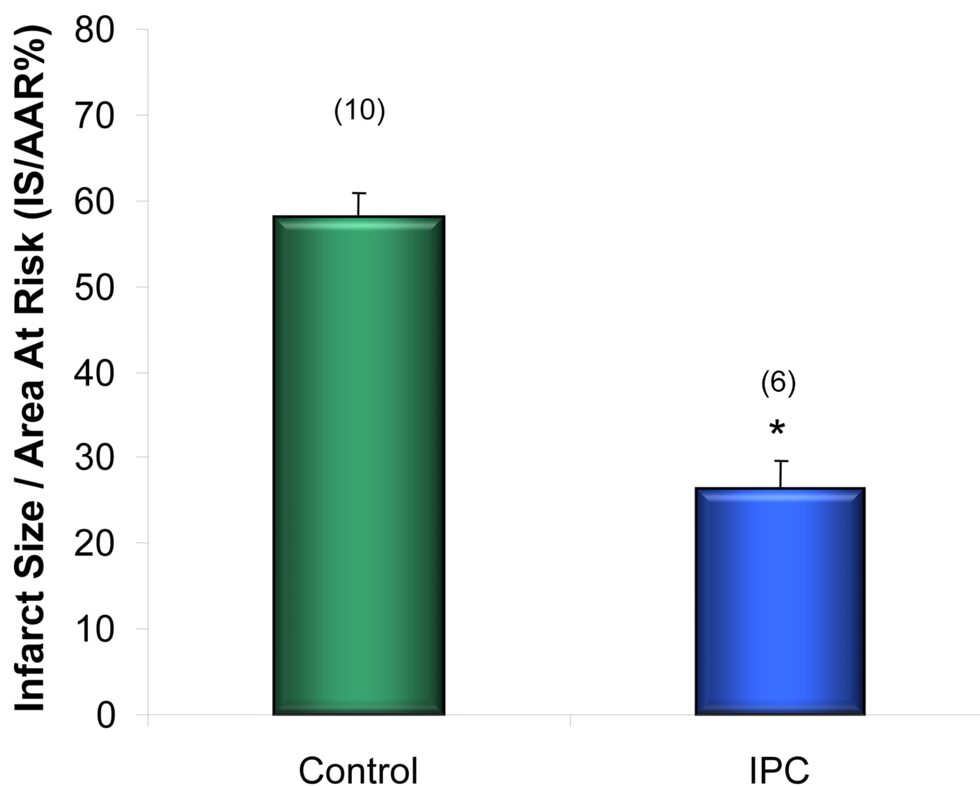


Figure 4.4.1 - Infarct data for the control and preconditioned (IPC) Wistar rat hearts. Hearts were randomly assigned to either the control or preconditioned groups. N is equal to 10 in the control group and 6 in the IPC group. Values are presented as % infarct developed in the risk zone (IS/AAR %) and expressed as mean ±s.e.m. * = p<0.01 vs. control *(students t-test)*.

4.4.1.3 IPC protects the Wistar rat heart against I/R injury

Figure 4.4.1 shows the data obtained for Wistar rat hearts which had been subjected to a control (n=10) or IPC protocol (n=6). The IPC protocol was found to decrease the infarct size by approximately 112% (%I/R 26.4±3.2%, IPC vs. 56.1±2.8%, control; P=<0.01) thus, confirming that the IPC protocol leads to protection and that the model to be used for subsequent experiments is viable.

4.4.2 Leptin dose-response

4.4.2.1 Animal and Haemodynamic Data

Table 4.4.4 demonstrates that there were no significant differences between the groups with respect to animal weights, ventricular volume or myocardial risk zone. . Baseline data relating to cardiac function before ischaemia, as assessed by the rate pressure product (RPP) and coronary flow, were similar in the experimental groups (see table 4.4.5 & 4.4.6). During in ischaemia, however, coronary flow and RPP declined to a similar extent in all the groups. Cardiac function and coronary flow increased upon reperfusion, which indicated that re-flow was successful. No significant differences were observed between groups with respect to coronary flow rate and RPP during reperfusion.

Groups	Body Weight (g)	Ventricular Volume (mm^3)	Risk Zone (%)
Control (n=7)	425±13	2130±84	53.24±3.47
Sham (n=5)	415±22	2074±38	49.15±2.58
Leptin (0.1nM) (n=7)	428±16	2076±84	51.50±3.46
Leptin (1nM) (n=6)	398±18	1928±62	47.26±3.81
Leptin (10nM) (n=9)	396±17	2079±79	50.31±1.88
Leptin (30nM) (n=8)	446±18	1949±69	53.70±43.22
Leptin (100nM) (n=8)	400±30	2023.8±66	51.41±2.87

Table 4.4.4 - Characteristics of the animals in the treatment groups. Data are present as mean±s.e.m.

Coronary flow rate (ml/ml)

	Stabilisation (ml/min)				Ischaemia (ml/min)			Reperfusion (ml/min)						
Groups \ Time(min)	10	20	30	40	5	15	30	5	15	30	45	60	90	120
Control (n=7)	16.4 ±1.0	15.0 ±1.2	14.3 ±1.1	14.1 ±0.9	7.0 ±0.6	7.9 ±0.6	7.1 ±0.8	12.0 ±1.0	11.5 ±1.0	11.3 ±1.2	10.7 ±1.0	10.3 ±1.0	9.0 ±0.8	8.1 ±0.9
Sham (n=5)	15.0 ±1.0	14.9 ±1.2	13.7 ±1.0	13.1 ±0.9	11.1 ±0.9	10.6 ±0.9	11.1 ±1.1	11.8 ±1.0	11.0 ±0.9	10.1 ±0.9	9.7 ±1.1	9.4 ±1.2	8.4 ±0.9	7.6 ±0.8
0.1nM Leptin (n=7)	16.3 ±0.9	14.7 ±0.9	13.7 ±0.7	13.4 ±1.1	8.0 ±0.6	8.0 ±0.5	7.7 ±0.6	11.0 ±1.3	10.9 ±1.5	9.7 ±1.6	8.9 ±1.4	8.1 ±1.1	7.4 ±0.9	7.2 ±1.0
1nM Leptin (n=6)	17.2 ±1.0	16.8 ±1.1	15.0 ±1.1	13.3 ±0.6	8.3 ±0.4	8.3 ±0.6	8.2 ±0.5	11.5 ±0.8	10.0 ±0.7	9.8 ±0.7	9.5 ±0.5	9.3 ±0.4	7.8 ±0.5	6.7 ±0.3
10nM Leptin (n=9)	16.3 ±0.9	14.0 ±1.0	14.0 ±1.0	14.1 ±1.1	7.9 ±0.6	8.0 ±0.5	8.1 ±0.6	11.1 ±0.7	10.0 ±0.9	8.9 ±0.7	8.3 ±0.8	7.6 ±0.7	7.0 ±0.6	5.6 ±0.5
30nM Leptin (n=8)	15.6 ±0.8	14.6 ±0.9	13.4 ±0.9	12.7 ±0.9	7.0 ±0.6	7.4 ±0.5	7.6 ±0.6	11.0 ±0.7	10.0 ±0.8	9.9 ±0.6	8.9 ±0.7	8.3 ±0.7	7.1 ±0.5	5.9 ±0.7
100nM Leptin (n=8)	15.6 ±1.1	15.2 ±1.0	12.8 ±0.8	12.9 ±0.7	7.1 ±0.4	7.4 ±0.4	6.9 ±0.5	10.2 ±0.7	10.3 ±0.9	8.7 ±0.9	7.7 ±1.0	7.0 ±0.9	6.7 ±1.0	6.3 ±0.8

Table 4.4.5 – Coronary flow rates (ml/min) of each group at various time-points during the ischaemia/reperfusion protocol.

Rate Pressure Product (x 103mmHg/min)

Groups \ Time(min)	Stabilisation (x 10^3mmHg/min)				Ischaemia (x 10^3mmHg/min)			Reperfusion (x 10^3mmHg/min)						
	10	**20**	**30**	**40**	**5**	**15**	**30**	**5**	**15**	**30**	**45**	**60**	**90**	**120**
Control (n=7)	35821 ±3324	35554 ±3427	33475 ±3851	35504 ±4523	14900 ±2631	20025 ±2974	19507 ±2453	20425 ±6928	21050 ±3434	22432 ±2552	20236 ±1756	19975 ±2870	17811 ±2070	12900 ±2422
Sham (n=5)	33950 ±1537	32836 ±1257	31814 ±1115	28588 ±1626	25979 ±2492	26214 ±2368	24511 ±2441	22170 ±1996	21725 ±2576	19436 ±1988	20150 ±1975	15821 ±2512	16571 ±1940	14525 ±1401
0.1nM Leptin (n=7)	38107 ±3553	36264 ±4044	36350 ±3046	34211 ±3638	14929 ±1954	19629 ±2292	21107 ±1798	20186 ±2838	21742 ±2160	17664 ±2435	18650 ±2988	19429 ±2831	15593 ±2090	15703 ±2119
1nM Leptin (n=6)	39379 ±4255	41233 ±2640	39017 ±3724	36413 ±3766	15750 ±2003	25425 ±3284	21388 ±2385	24233 ±1913	20070 ±2906	19940 ±2827	19929 ±2333	17542 ±1987	16838 ±1586	18350 ±3427
10nM Leptin (n=9)	36671 ±2310	33921 ±2450	31839 ±2630	30811 ±2140	17500 ±1159	19993 ±1502	19114 ±1282	18492 ±1657	19067 ±2806	18629 ±2943	17557 ±2455	16700 ±2487	15546 ±1775	13533 ±1248
30nM Leptin (n=8)	31900 ±2890	31850 ±2474	30579 ±2305	32975 ±3596	14100 ±2272	19029 ±1486	18107 ±1735	20240 ±1154	17386 ±1560	18650 ±1165	18661 ±1684	15636 ±1534	13986 ±1209	11129 ±1620
100nM Leptin (n=8)	42800 ±2078	36407 ±1545	34293 ±2340	33286 ±2309	13325 ±1545	18829 ±1553	19736 ±1312	21175 ±1980	18914 ±1491	18621 ±1045	18750 ±1328	19057 ±1728	15993 ±1390	14529 ±1402

Table 4.4.6 - Rate Pressure Product (x 103mmHg/min) of each group at various time-points during the ischaemia/reperfusion protocol.

4.4.2.2 Exclusions

There were three exclusions in total, two in the sham group because flow dropped below 10ml/min during reperfusion and one in the 0.1nM leptin group because no reduction of flow was observed upon tightening the suture around the LAD to induce ischaemia.

4.4.2.3 Effect of leptin administered at a range of concentrations on myocardial infarct size

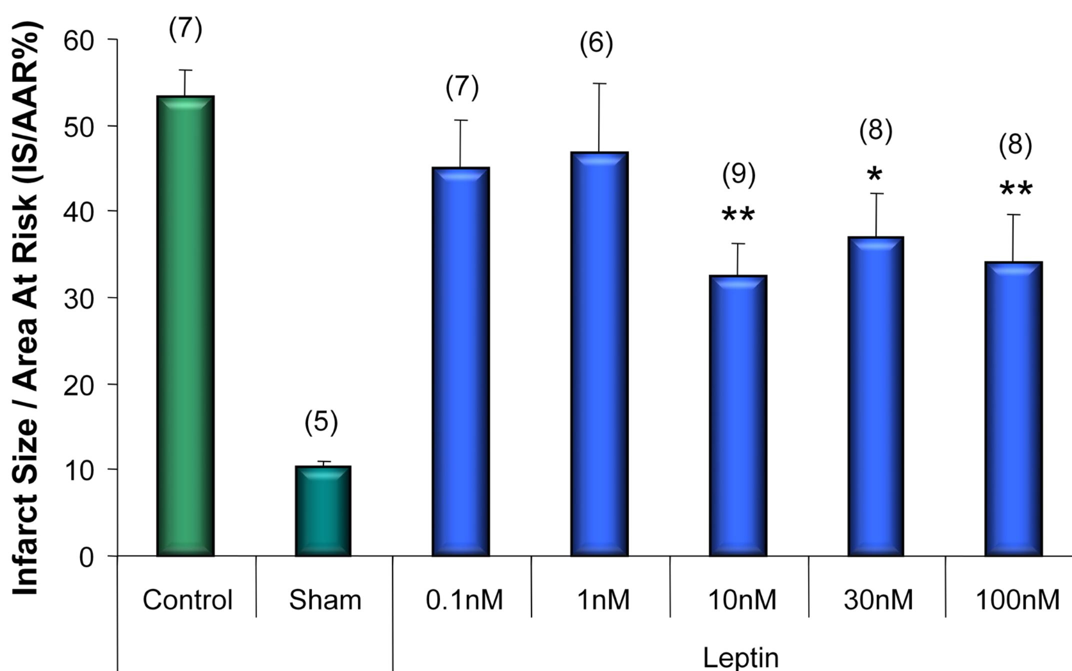


Figure 4.4.2 – The influence of varying concentrations of leptin on infarct size in the Wistar rat heart. A dose-response study was performed using five different concentrations of leptin. The results of the leptin treated hearts were compared with data obtained for control and sham hearts. 10nM, 30nM and 100nM concentrations of leptin were found to induce significant cardioprotection. Data are presented as mean±s.e.m (* = $p<0.05$, ** = $p<0.01$ vs. control) *(one-way ANOVA)*.

Leptin perfused at 10nM (n=9), 30nM (n=8) and 100nM (n=8) was found to produce marked reductions in infarct size in the Wistar rat heart when compared to control (n=7) (53±3.2% control vs. 32.4±3.9%, 36.9±5.0% and 34.1±5.6% for 10nM, 30nM and 100nM leptin, respectively, p=<0.01, p=<0.05 and p=<0.01). By contrast, leptin at 0.1nM (n=7) and 1nM (n=6) failed to induce cardioprotection. In addition, the results obtained with sham hearts indicated that a degree of infarction occurred purely as a result of the excision and normal perfusion of the heart. Thus, in sham operated hearts infarct size reached approximately 10±1.2% as compared to 53±3.2% in control hearts.

4.5 Discussion

4.5.1 Ischaemic preconditioning of the isolated perfused Wistar rat heart

The present study demonstrates that the isolated perfused rat heart model performed within acceptable and well documented parameters (de Leiris et al. 1984). In addition, the infarct data obtained indicated that Wistar rat hearts can be preconditioned with sublethal episodes of ischaemia as demonstrated by Murray et al 1986.

4.5.2 Leptin concentration response studies

Leptin-induced protection against I/R injury has been demonstrated previously in various tissues including the murine heart (Erkasap et al. 2006; Smith et al. 2006). Previous studies, however, have not investigated the relationship between infarct size and leptin concentration. Therefore, prior to embarking on the studies which formed the primary goal of this thesis i.e. an investigation into the mechanisms involved in leptin-induced cardioprotection, experiments were

performed in Wistar rat hearts to identify concentrations of leptin that induced optimal protection against myocardial I/R injury. Protection was observed with concentrations of leptin of 10nM, 30nM and 100nM, with 10 nM leptin yielding optimal extents of infarct size reduction. These data are consistent with the results obtained previously in the murine heart (Smith et al. 2006) and, consequently, for comparative purposes, 10nM leptin was chosen as the standard concentration for all subsequent experiments.

The fact that leptin concentrations lower than 10nM did not elicit responses could be interpreted as indicating that leptin produces an "all or nothing" effect with respect to infarct size limitation. However, as suggested previously for the adipocytokine, apelin, it is equally possible that the effects were missed, that is, if leptin concentrations between 1 and 10nM had been tested, infarct size reductions might have been seen over this range (Simpkin et al. 2007).

It should also be noted that human leptin and not rat leptin was used in the experiments presented in this chapter. This leads to the obvious concern that differences between the structure of rat and human leptin may alter the strength of leptin signalling achieved in the rat heart. However, studies have revealed that leptin shows a high interspecies conservation, for instance human are rat leptin are 84% identical (Ogawa et al. 1995). Furthermore, human leptin has been demonstrated to be active in rodents. In mice and rats, for example, recombinant human leptin has been shown to reduce food intake and body weight and in the latter this effect was demonstrated to occur primarily in the arcuate nucleus (Verploegen et al. 1997; Satoh et al. 1997). Considering that

human leptin has been used to protect the mouse heart and now the rat heart (see dose response above) it was deemed that the recombinant human form of the molecule was active and sufficient to use in subsequent studies.

CHAPTER 5 - LEPTIN-INDUCED CARDIOPROTECTION VIA THE RISK PATHWAY

5.1 Introduction

Protection against myocardial ischaemia reperfusion injury, as stimulated by a range of cytokines, is associated with activation of the RISK pathway, which incorporates the PI3K-Akt and p44/42 MAPK signalling cascades, and inhibition of the MPTP (see section 1.2) (Hausenloy & Yellon 2007b). Leptin has been shown to protect against ischaemia reperfusion injury in a variety of settings including the brain (Zhang et al. 2007), gut (Brzozowski et al. 2001) and kidney (Erkasap et al. 2006). Stimulation of the leptin receptor, particularly the long, OB-Rb isoform, has been directly associated with activation of the JAK-STAT, PI3K-Akt and p44/42 MAPK signalling cascades (Ning et al. 2006; Zabeau et al. 2003; Harvey & Ashford 2003), all of which have been linked to cardioprotection (Hausenloy & Yellon 2006). In cardiomyocytes it has been shown that leptin mediates cellular proliferation in a PI3K and MAPK-dependent manner (Tajmir et al. 2004). Furthermore, leptin has been reported to activate Akt and p44/42 MAPK in an isolated murine model of ischaemia-reperfusion injury (Smith et al. 2006). In this study protein kinase activation was only examined at one time-point, i.e., 10min into the reperfusion-phase. However, the time at which maximal phosphorylation/activation, in response to leptin or many other cardioprotectants, occurs during the reperfusion-phase is uncertain and can therefore result in kinase phosphorylation being missed completely. Different laboratories have often sampled the myocardium for Western blot analysis at different time-points during reperfusion and this may explain some of the

discrepancies observed. In addition, kinase phosphorylation may respond to a stimulus at different times. Furthermore, it cannot be assumed that different drugs activate the survival kinases at the same time-points. For example, in a study in the murine heart, in which apelin was used as the stimulant, the time-point at which maximal phosphorylation of Akt and p44/42 MAPK occurred during the reperfusion-phase was found to vary (Smith et al. 2007b). The strongest phosphorylation signal was found to occur between 2.5 and 5min, perhaps suggesting that sampling at later times e.g. 10-15min could result in a weaker phosphorylation response. Zhao and colleagues found that leptin-induced Akt phosphorylation was weak in hepatocytes, therefore, this may also be the case for cardiomyocytes (Zhao et al. 2000). It is, however, entirely possible that this weak signal may have resulted from sampling at the wrong time-point.

5.2 Aim

The aim of the studies described in this chapter was to investigate leptin-induced Akt and p44/42 MAPK phosphorylation in the setting of cardioprotection in more detail than previously. Western blotting was performed to investigate directly the phosphorylation of the RISK pathway kinases Akt (at both the Ser473 and Thr308 phosphorylation sites) and p44/42 MAPK in response to leptin. These studies were performed using tissue collected from Langendorff perfused Wistar rat hearts, with samples being collected at different time-points during the reperfusion-phase so as to allow a temporal analysis of kinase activation.

5.3 Experimental Protocol & Materials

5.3.1 Protein kinase inhibitors

Hearts from Wistar rats were excised and perfused according to the method described in section 3.3. All hearts were stabilised for 40min and subjected to 35min ischaemia followed by 120min reperfusion. Both inhibitors investigated, i.e. the PI3K inhibitor LY294002 (15μM), and the p44/42 MAPK inhibitor UO126 (10μM), were dissolved in dimethyl sulphoxide (DMSO; final concentration 0.02%). The vehicle, DMSO, was also tested alone to establish if it influenced infarct size itself. Perfusion with all drugs was commenced 5min prior to the reperfusion period to ensure that a sufficient concentration of drug reached the heart immediately upon reperfusion[2]. Individual groups were (original group sizes are shown in brackets):

Control (n=5) – no drug, 40min stabilisation.

DMSO (0.02%) (n=5) – Vehicle administered 5min prior to reperfusion.

LY (15μM) (n=5) – Drug administered 5min prior to reperfusion.

UO (10μM) (n=5) - Drug administered 5min prior to reperfusion.

Leptin (10nM) (n=5) – administered 5min prior to ischaemia up until 30min into reperfusion.

[2] It should be noted that the following experiments were conducted alongside those employing AG490 that are presented in section 6.4.1.3, therefore the same control, DMSO and leptin treated groups appear again. The data were presented in this way to maintain consistency and separate the RISK and JAK/STAT data between different chapters. Furthermore, it should be noted that these experiments were completed within a four week period.

Leptin (10nM) plus LY – LY and leptin co-administered 5min prior to reperfusion

Leptin (10nM) plus UO – UO and leptin co-administered 5min prior to reperfusion

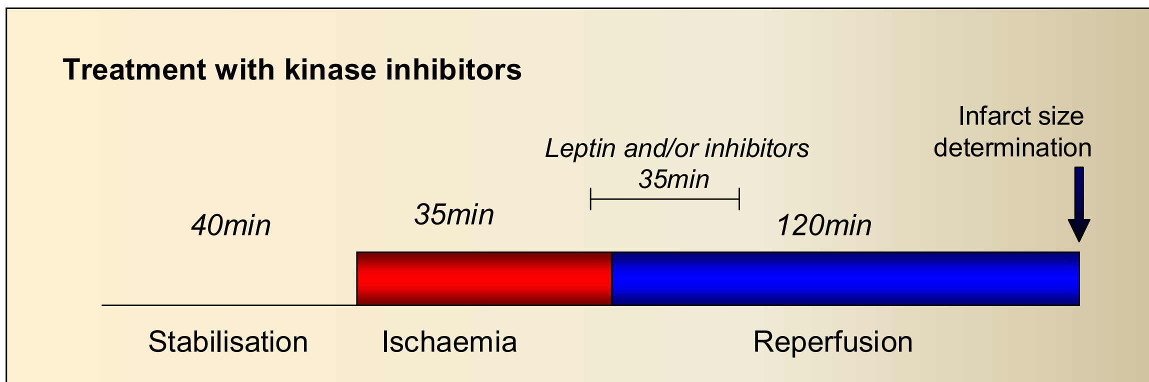


Figure 5.3.1 – Protocol for investigating the effects of RISK pathway inhibitors on infarct size.

5.3.2 Western blots

Hearts from Wistar rats were excised and perfused according to the method described in section 3.3. All hearts were stabilised for 40min and then subjected to 35min ischaemia. Hearts were then reperfused for 2.5, 5, 10, 15 or 30min, at which time the portion of the myocardium at risk was collected for analysis by Western blotting (see section 3.5). Individual groups were as follows (original group sizes are shown in brackets):-

2.5min reperfusion control (n=5)

2.5min reperfusion with leptin (10nM) treated (n=5)

5min reperfusion control (n=6)

5min reperfusion with leptin (10nM) treated (n=6)

10min reperfusion control (n=6)

10min reperfusion with leptin (10nM) treated (n=6)

15min reperfusion control (n=6)

15min reperfusion with leptin (10nM) treated (n=6)

30min reperfusion control (n=5)

30min reperfusion with leptin (10nM) treated (n=6)

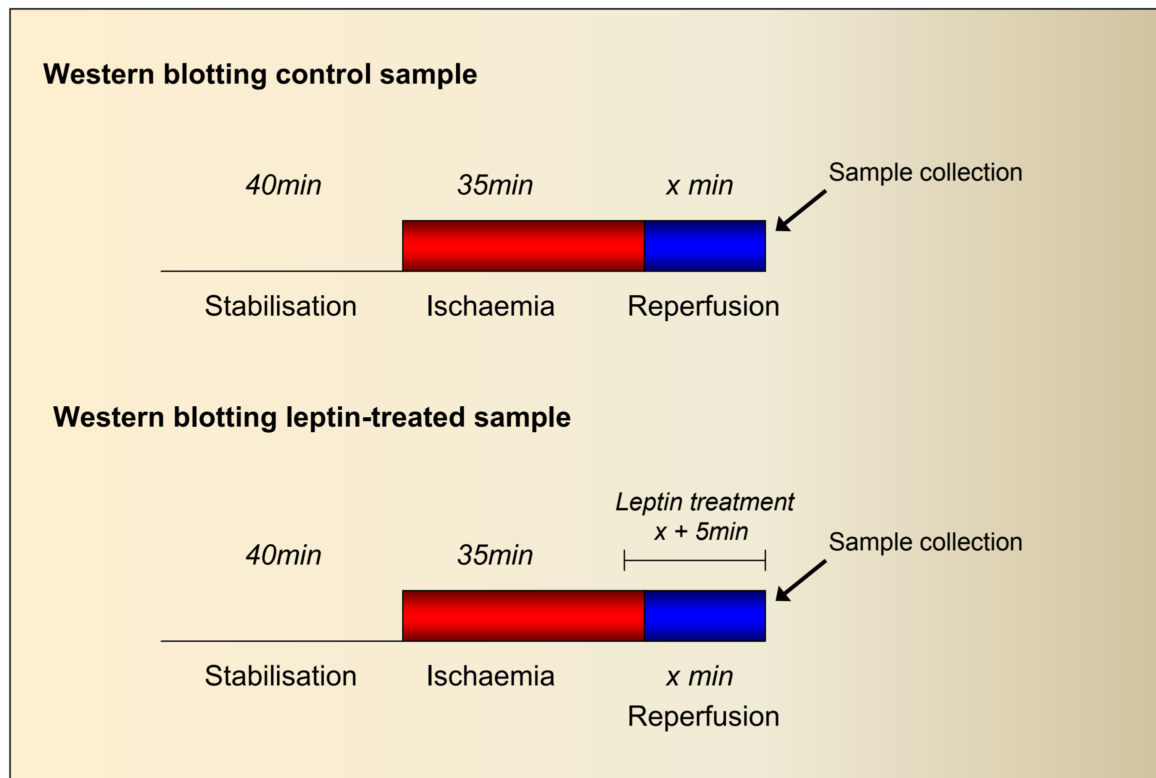


Figure 5.3.2 –Protocol for the collection of samples for Western blot analysis. (A) Control protocol; hearts subjected to 40min stabilisation 35min ischaemia and 2.5, 5, 10, 15 or 30min reperfusion. (B) Leptin-treated protocol hearts subjected to 40min stabilisation, 35min ischaemia and 2.5, 5, 10, 15 or 30min reperfusion. Leptin treatment began 5min before the commencement of reperfusion and continued until the point at which the heart was snap-frozen.

5.4 Results

5.4.1 Protein kinase inhibitors

5.4.1.1 Animal and Haemodynamic Data

Table 5.4.1 demonstrates that there are no significant differences between the groups with respect to animal weight, ventricular volume or risk zone volume. Baseline data relating to cardiac function before ischaemia, as assessed by the rate pressure product (RPP) and coronary flow, were similar in all the experimental groups (see tables 5.4.2 & 5.4.3). During in ischaemia, however, coronary flow and RPP declined to a similar extent in all groups. Cardiac function and coronary flow increased upon reperfusion, which indicated that re-flow was successful. No significant differences were observed between groups with respect to coronary flow rate and RPP during reperfusion.

Groups	Body Weight (g)	Ventricular Volume (mm^3)	Risk Zone (%)
Control (n=5)	417.6±6.1	2130.1±332.1	53.7±4.7
DMSO (n=5)	390.0±28.0	2446.1±445.2	51.1±5.1
LY (n=5)	379.8±22.7	2156.1±472.6	50.2±3.5
UO (n=5)	415.0±17.6	2630.1±454.1	52.6±3.5
Leptin (10nM) (n=10)	385.2±11.5	2211.5±448.1	50.2±5.3
Leptin + LY(n=5)	380.5±26.0	2608.1±344.2	49.89±1.9
Leptin + UO(n=5)	408±9.7	2578.0±362.1	50.0±1.4

Table 5.4.1 – Table showing the characteristics of the animals in the different treatment groups.

Coronary Flow Rate (ml/min)

	Stabilisation (ml/min)				Ischaemia (ml/min)			Reperfusion (ml/min)						
Time(min) / **Groups**	**10**	**20**	**30**	**40**	**5**	**15**	**30**	**5**	**15**	**30**	**45**	**60**	**90**	**120**
Control (n=5)	16.8 ±2.1	16.0 ±2.1	15.5 ±2.1	15.2 ±2.1	7.5 ±1.6	8.3 ±1.6	7.6 ±1.4	13.7 ±0.9	12.6 ±0.8	12.3 ±0.9	11.8 ±0.6	11.2 ±0.7	10.0 ±1.2	9.5 ±1.1
DMSO (n=5)	15.5 ±1.6	14.3 ±1.7	13.0 ±1.4	13.0 ±1.8	7.0 ±1.1	7.5 ±0.9	7.1 ±1.0	12.8 ±1.7	12.7 ±1.3	11.0 ±1.3	10.0 ±0.9	9.3 ±0.9	7.8 ±0.6	6.8 ±0.9
LY (n=5)	13.0 ±1.7	12.6 ±1.3	12.2 ±1.0	12.2 ±1.0	6.4 ±0.7	6.5 ±0.9	6.2 ±0.7	11.2 ±0.9	10.8 ±1.4	9.3 ±0.6	7.8 ±0.7	6.8 ±0.7	6.0 ±0.5	5.4 ±0.5
UO (n=5)	14.6 ±1.0	13.8 ±1.0	12.6 ±1.2	12.2 ±1.3	6.7 ±0.9	7.0 ±0.8	7.0 ±0.8	16.1 ±2.0	15.8 ±2.5	13.4 ±1.7	11.2 ±1.4	9.6 ±1.2	8.0 ±1.5	7.0 ±1.3
Leptin (n=10)	16.5 ±0.9	15.5 ±1.0	14.3 ±1.4	13.8 ±1.5	7.6 ±0.8	7.5 ±0.9	7.4 ±0.9	12.0 ±2.7	12.3 ±2.8	11.0 ±2.7	10.5 ±2.5	10.4 ±2.6	9.8 ±2.8	9.0 ±2.7
Leptin + LY (n=5)	13.8 ±0.3	13.4 ±0.7	12.3 ±0.6	10.8 ±0.8	6.0 ±0.7	5.5 ±0.5	6.0 ±0.4	10.0 ±0.8	9.5 ±0.9	9.3 ±0.9	7.8 ±0.9	6.9 ±1.0	6.6 ±0.8	5.3 ±0.9
Leptin + UO (n=5)	17.0 ±1.3	15.8 ±1.2	14.8 ±1.3	14.1 ±1.5	7.6 ±0.7	8.3 ±1.1	7.6 ±1.0	16.0 ±1.9	15.6 ±2.0	13.6 ±2.0	11.6 ±2.0	9.6 ±1.5	8.2 ±1.2	8.0 ±1.5

Table 5.4.2 – Coronary flow rate (ml/min) of each group at various time-points during the ischaemia/reperfusion protocol.

Rate Pressure Product (x 103mmHg/min)

	Stabilisation (x 10^3mmHg/min)				Ischaemia (x 10^3mmHg/min)			Reperfusion (x 10^3mmHg/min)						
Time(min) / Groups	10	20	30	40	5	15	30	5	15	30	45	60	90	120
Control (n=5)	33717 ±6529	28958 ±4214	30067 ±3147	30820 ±4097	9457 ±2154	15583 ±2384	15567 ±2536	22433 ±3015	19540 ±2163	19883 ±2072	20233 ±2791	17833 ±2451	18733 ±2376	17125 ±1947
DMSO (n=5)	31650 ±5204	31238 ±6530	31719 ±4180	30750 ±4493	19188 ±11661	17395 ±6965	20788 ±3983	22933 ±6769	27800 ±3646	22581 ±4550	20588 ±3580	18375 ±2392	13725 ±4918	15400 ±2459
LY (n=5)	29470 ±5834	33090 ±6300	28950 ±4102	26260 ±4723	14660 ±3818	19425 ±5413	21563 ±3033	14500 ±6154	14875 ±3127	13775 ±2592	15390 ±2335	16420 ±1620	11840 ±2124	10400 ±3107
UO (n=5)	26680 ±3510	26860 ±3487	25430 ±3314	25190 ±2625	6760 ±1356	14065 ±1824	14700 ±1813	22940 ±4079	20160 ±3835	23288 ±2234	18630 ±1954	16350 ±2559	12840 ±1664	10725 ±1289
Leptin (n=10)	38167 ±5868	35250 ±3568	33138 ±3110	29158 ±1304	11333 ±1202	18100 ±2261	15300 ±1060	21013 ±1765	22617 ±1433	22369 ±2118	21725 ±1996	19825 ±2193	19400 ±3439	17500 ±3156
Leptin + LY (n=5)	34438 ±5142	34350 ±5249	31188 ±4634	25883 ±3033	12500 ±1127	16200 ±2700	17600 ±1955	20283 ±1408	19000 ±2003	17850 ±2285	20850 ±2573	19250 ±2341	17325 ±1841	14350 ±2656
Leptin + UO (n=5)	30450 ±3669	29030 ±2828	31720 ±3035	32145 ±3824	14930 ±1885	18160 ±5486	21875 ±2444	29250 ±2605	25472 ±2428	23740 ±3580	20546 ±2595	18520 ±2675	14275 ±3372	11760 ±2951

Table 5.4.3 - Rate Pressure Product (x 103mmHg/min) of each group at various time-points during the ischaemia/reperfusion protocol.

5.4.1.2 Exclusions

One heart was excluded from the leptin group because of excessive ventricular fibrillation during the reperfusion-phase. One heart was excluded from the control group because of poor function, i.e. it did not achieve the required RPP. Two hearts were excluded from the DMSO group, one because of low coronary flow and one because of poor function. Finally, one heart was excluded from the leptin + LY group because of low coronary flow.

5.4.1.3 Effect of protein kinase inhibitors LY294002 and UO126 on leptin-induced cardioprotection

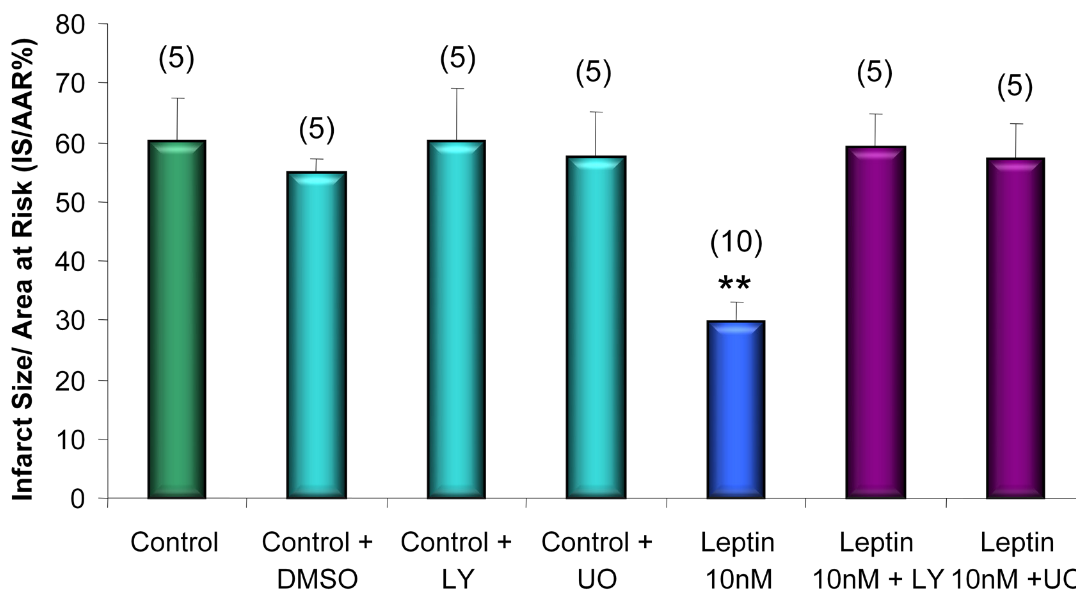


Figure 5.4.1 – Cardioprotection afforded by leptin (10nM) at reperfusion is abrogated by the protein kinase inhibitors LY (15μM) and UO (10μM) given at reperfusion. ** = p<0.01 vs. control *(one-way ANOVA)*.

As seen with the concentration response study, 10nM (n=10) leptin significantly reduced infarct size as compared to control (n=5) (29.86±3.2% vs. 60.05±7.4%, **= $p<0.01$) (Figure 5.4.1). The PI3K inhibitor, LY294002, significantly abolished the infarct-limiting effects of leptin when given in combination with leptin (n=5) (59.06±5.7% vs. 60.05±7.4%, LY plus leptin and control, respectively). By contrast LY294002 (n=5) did not confer protection when administered alone (60.16±9.0% vs. 60.05±7.4%, LY and control, respectively). UO126 (n=5), a p44/42 MAPK inhibitor, did not have any effect on IS when administered alone (57.38±7.7% vs. 60.05±7.4%, UO and control respectively). When administered with leptin, however, UO126 (n=5) abrogated leptin-induced cardioprotection (29.86±3.2%, leptin vs. 57.19±6.08%, leptin + UO). DMSO (n=5), the inhibitor solvent, did not influence infarct size significantly when administered alone (55.04±2.3%, DMSO vs. 60.05±7.4%, control).

5.4.2 Western blotting

5.4.2.1 Body weight of the animals in the different treatment groups

Groups	Body Weight (g)
2.5min Control (n=4)	385±15
2.5min leptin (n=4)	397±12
5min Control (n=6)	425.5±14
5min leptin (n=6)	412.8±26
10min Control (n=6)	419.6±21
10min leptin (n=6)	387.6±24
15min Control (n=5)	390.5±15
15min leptin (n=6)	395.5±8
30min Control (n=6)	405.5±20
30min leptin (n=6)	416.9±17

Table 5.4.4 - Table showing the characteristics of the animals in the different treatment groups included in the Western blot study.

5.4.2.2 Exclusions

One heart was excluded from the 5min control group due to excessive ventricular fibrillation (VF). One heart was excluded from the 10min leptin

treated group due to poor function, i.e. did not achieve the required RPP. Two hearts were excluded from the 15min control group, one due to poor flow and one due to excessive VF. One heart was excluded from the 30min leptin group because of poor function.

5.4.2.3 Leptin stimulates Akt/Serine-473 phosphorylation in hearts subjected to I/R injury

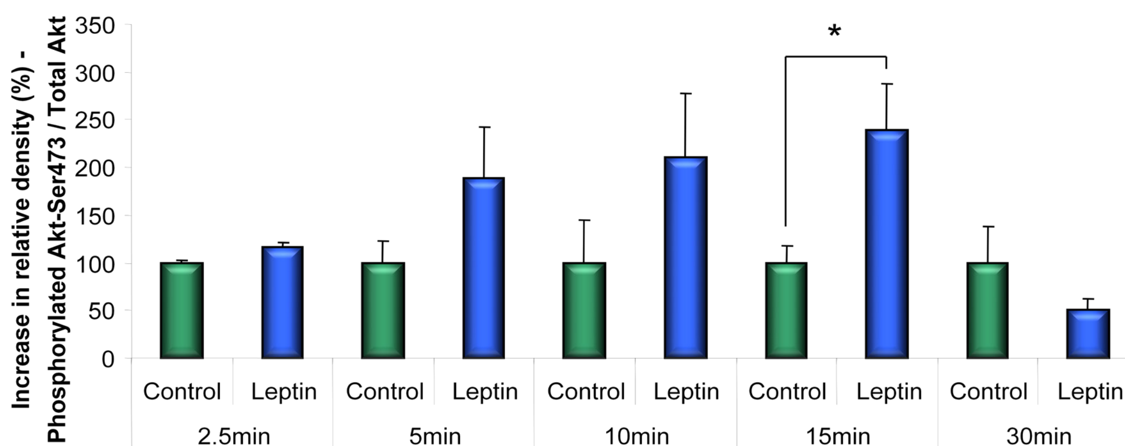
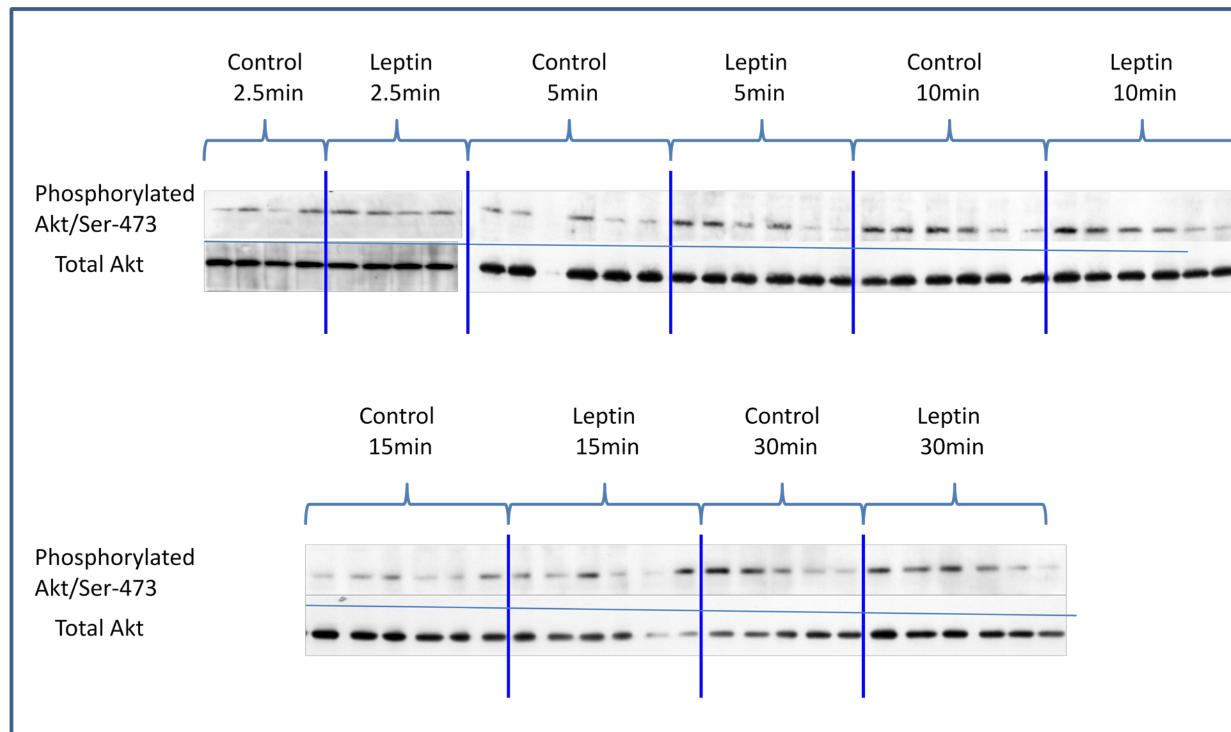


Figure 5.4.2 – Akt/serine-473 phosphorylation in the presence and absence of leptin at different time-points during reperfusion. Total and phosphorylated Akt/serine-473 were determined in extracts derived from rat hearts subjected to I/R under control conditions or in the presence of leptin (10nM). Data are presented as the ratio of phosphorylated to total Akt/serine-473 relative densitometry (RD) values (AU) normalised to control (untreated; 100%) and expressed as mean±s.e.m. (*=P<0.05 vs. control; n=4-6) *(one-way ANOVA)*.

Maximal leptin-induced phosphorylation of Akt/Serine-473 did not occur until 15min into the reperfusion-phase (control n=6, leptin n=6), when a statistically significant (p<0.05), 140% increase was observed (Figure 5.4.2). Non-significant increases in Akt/Serine-473 phosphorylation of 88% and 111% were, however, also seen at 5 min (control n=6, leptin n=6) and 10 min (control n=5, leptin n=5) reperfusion, respectively (Figure 5.4.2).



5.4.3 - Western blots showing phosphorylated Akt/Ser-473 and total Akt levels in tissue taken from Wistar hearts treated with and without leptin (10nM) at various time-points during reperfusion.

5.4.2.4 Leptin stimulates Akt/Threonine-308 phosphorylation in hearts subjected to I/R injury

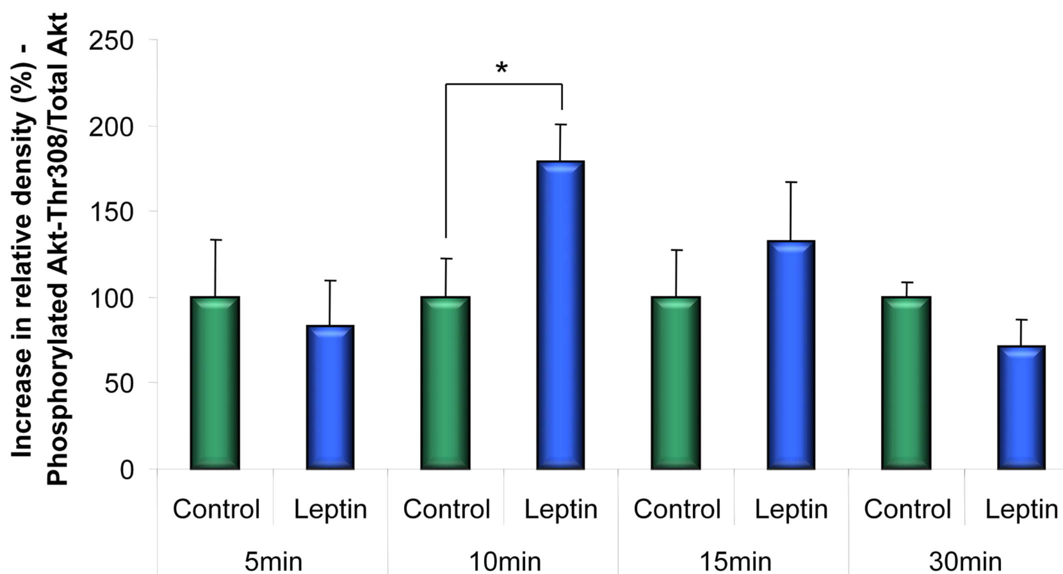
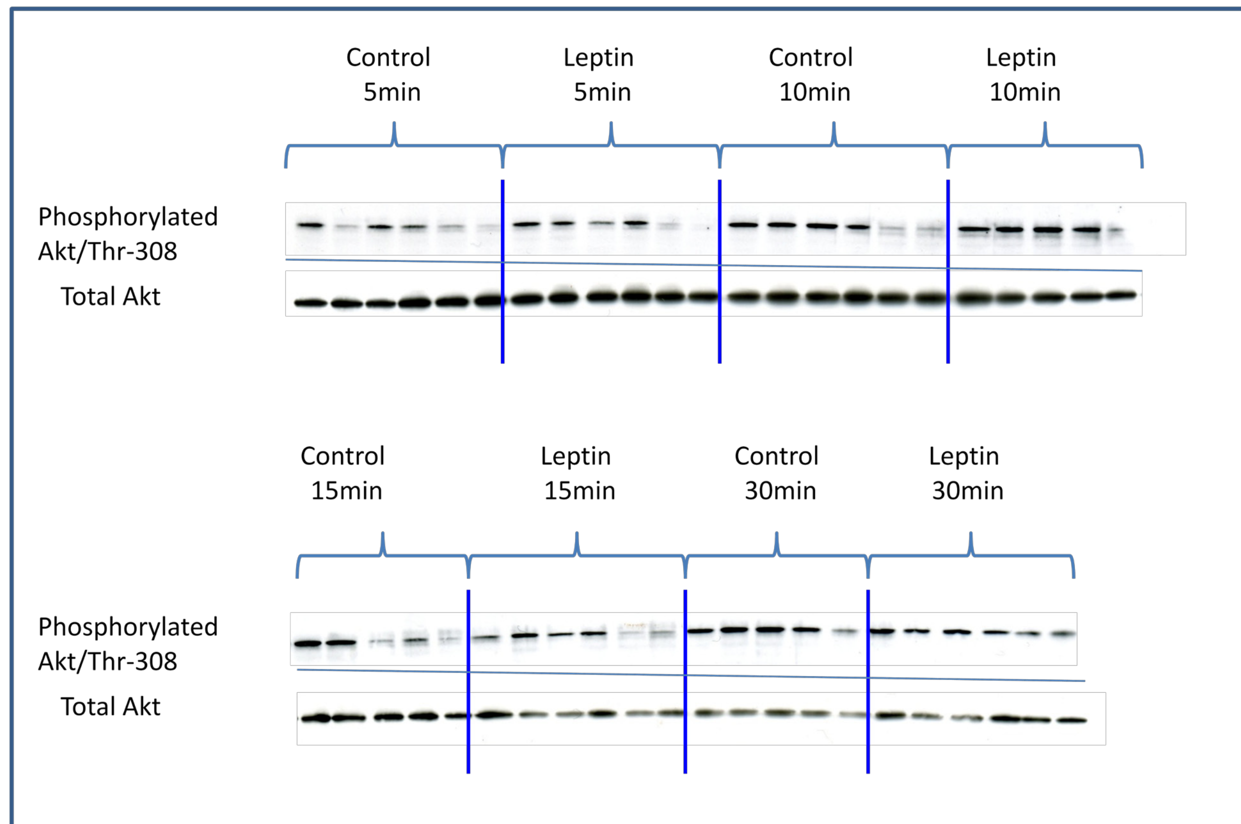


Figure 5.4.4 – Effect of 10nM leptin on the phosphorylation of Akt/threonine-308. Total and phosphorylated Akt/threonine-308 were determined in extracts derived from rat hearts subjected to I/R under control conditions or in the presence of leptin (10nM). Data are presented as the ratio of phosphorylated to total Akt/threonine-308 relative densitometry (RD) values (AU) normalised to control (untreated; 100%) and expressed as mean±s.e.m. (*=P<0.05 vs. control; n=4-6) *(one-way ANOVA)*.

A significant 178% increase (p=<0.05) in the phosphorylation of Akt/Thr308, was observed at 10min (n=4) into the reperfusion-phase. At the 5 (n=6), 15 (n=6) and 30min (n=6) time-points there were no significant changes in the phosphorylation status of the kinase.



5.4.5 - Western blots showing phosphorylated Akt/Thr-308 and total Akt levels in tissue taken from Wistar hearts treated with and without leptin (10nM) at various time-points during reperfusion.

5.4.2.5 Leptin stimulates p44/42 MAPK phosphorylation in hearts subjected to I/R injury

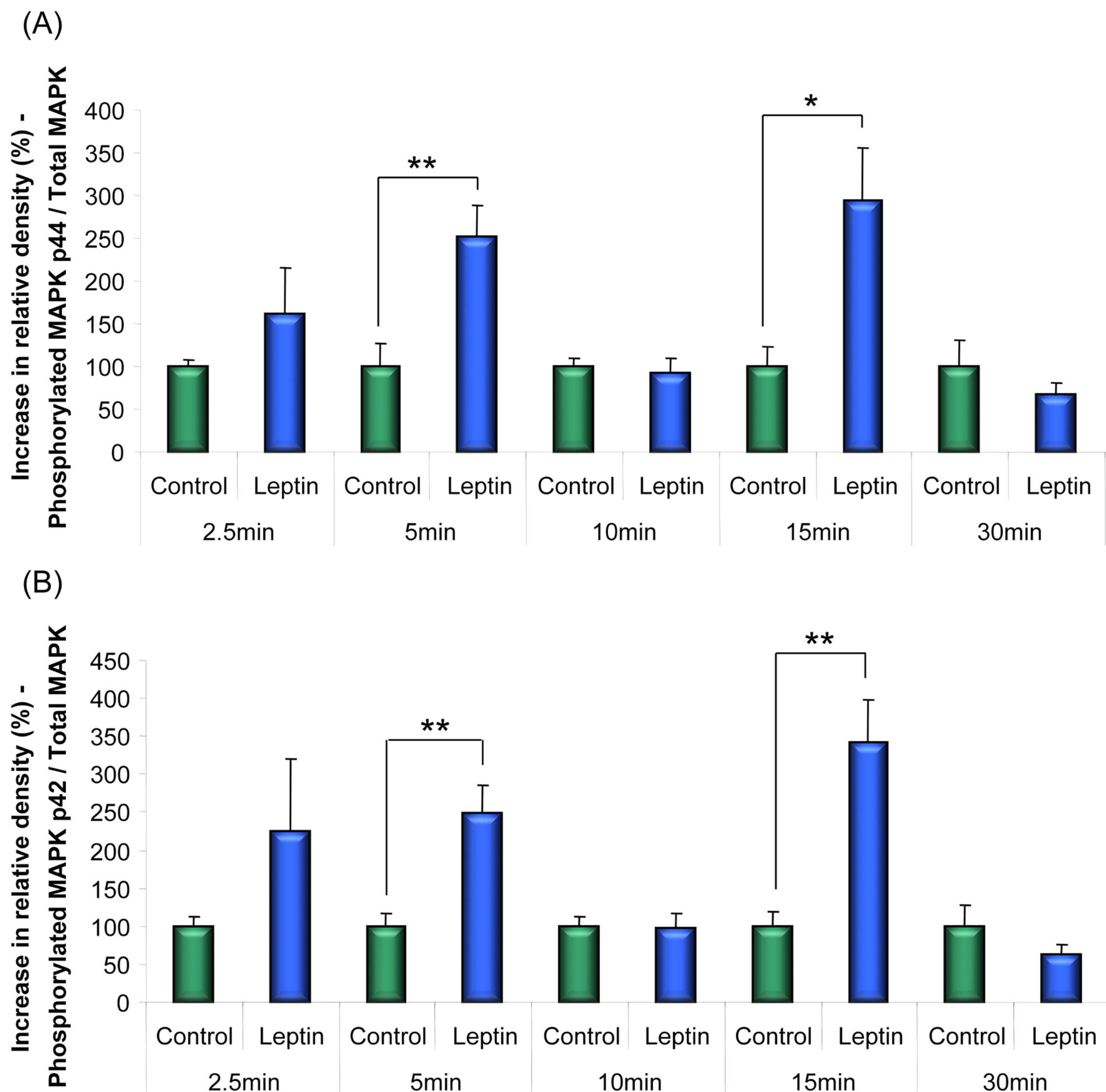
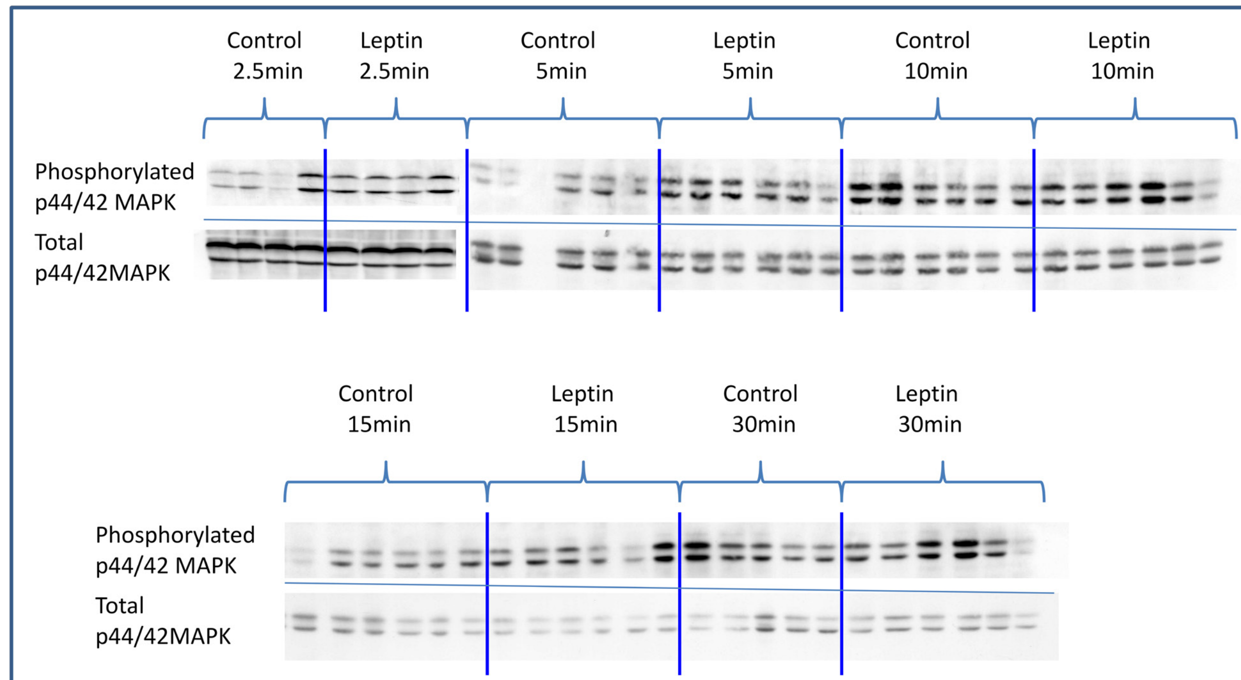


Figure 5.4.6 - Effect of leptin (10nM) on (A) p44 and (B) p42 phosphorylation in Wistar rat hearts subjected to ischaemia–reperfusion. Hearts were stabilised for 40min and then subjected to 35min ischaemia followed by 2.5,5, 10, 15 or 30min reperfusion. Data are presented as the ratio of phosphorylated to total p44 or p42 relative densitometry (RD) values (AU) normalised to control (untreated; 100%) and expressed as mean±s.e.m. (*=P<0.05, **=P<0.01 vs. control; n=4-6) *(one-way ANOVA)*.



5.4.7 - Western blots showing phosphorylated p44/42 MAPK and total MAPK levels in tissue taken from Wistar hearts treated with and without leptin (10nM) at various time-points during reperfusion.

Time course experiments revealed that leptin-induced significant phosphorylation of p44 at 5 (n=6) and 15min (n=6) reperfusion, 152% (p=<0.01) and 294% (p=<0.05) increases, respectively, being observed. Similarly, the phosphorylation of p42 was found to be increased by 248% and 342% at 5 (n=6) and 15min (n=6), respectively (p=<0.01) (Figure 5.4.4). At 2.5min into the reperfusion-phase there appeared to be an increase in p44 and p42 phosphorylation, however, upon statistical analysis these data were found to be non-significant. At 10 (n=6) and 30min (n=6) significant changes in the phosphorylation status of p44 or p42 were not detected.

5.5 Discussion

It has been established in a variety of settings that leptin possesses the signalling capacity to stimulate p44/42 MAPK phosphorylation. Leptin has, for example, been shown to be protective in a cellular model of Parkinson's disease via a p44/42 MAPK-dependent mechanism (Weng et al. 2007). Leptin-induced phosphorylation of Akt occurs via activation of JAK2 and consequent activation of insulin receptor substrate (IRS), which in turn activates the PI3K-Akt cascade (Fruhbeck 2006). This mechanism is evident in microglia, in which leptin-induced interleukin-6 production can be inhibited by transfection with insulin receptor substrate (IRS)-1 small-interference RNA or by the administration of the PI3K inhibitor, LY294002.

5.5.1 Leptin-induced cardioprotection is abolished by the RISK pathway inhibitors LY294002 and UO126

It was hypothesised that leptin-induced infarct size limitation in the Wistar rat heart relies on the activation of Akt and p44/42 MAPK. This hypothesis was tested by the administration of LY294002, a PI3K inhibitor, and UO216, a p44/42 MAPK inhibitor to hearts subjected to I/R injury in the presence and absence of leptin. Indeed, leptin-induced cardioprotection was found to be abolished by the addition of these kinase inhibitors. Having established that activation of these kinases is crucial to the mechanism underlying leptin-induced cardioprotection the decision was made to investigate the temporal nature of kinase phosphorylation/activation in relation to leptin–induced protection.

5.5.2 Leptin-induced phosphorylation of Akt/Serine-473 and Akt/Threonine-308 in response to leptin

It has been shown that the treatment of cardiac myocytes with leptin is associated with increased long-chain fatty acid (LCFA) uptake, together with increased phosphorylation of both Akt/Ser-473 and Akt/Thr-308 (Palanivel et al. 2006). The role of Akt in leptin-induced cardioprotection, however, has not been resolved. Conflicting data concerning the involvement of Akt in leptin-induced cardioprotection has been obtained (Smith et al. 2006). Thus, despite data being obtained indicating that leptin-induced cardioprotection was blocked by LY294002, a PI3K inhibitor, significant Akt phosphorylation was not observed in response to leptin (Smith et al. 2006). Nevertheless, this may have been a reflection of the timing of sample collection, together with the limited number of samples examined.

In the present study, Western blotting revealed that Akt/Ser-473 phosphorylation was significantly increased at 15min into reperfusion. Nevertheless, increases in phosphorylation, although non-significant, were also observed at the 5 and 10min time-points. By contrast the maximal phosphorylation signal for Akt/Thr-308 was observed at 10min into the reperfusion-phase. Phosphorylation of Akt at both the Ser-473 and Thr-308 sites is required for its full activation (Chan et al. 1999). In cardiomyocytes subjected to simulated I/R injury both of these sites are phosphorylated and remain phosphorylated for over an hour into the reperfusion-phase (Mockridge et al. 2000a). However, it is well known that the stimulus provided by I/R alone is not sufficient to confer cardioprotection, and therefore further phosphorylation/activation of Akt by either a pharmacological agent or a mechanical stimulus, such as IPC, is required to produce infarct-limitation. In the present study it was shown that both the Ser-473 and Thr-308 sites are phosphorylated to a significant degree, at 15min reperfusion, in hearts treated with leptin as compared to control hearts. The differences with respect to the times at which maximal phosphorylation of the Ser-473 and Thr-308 sites occurred may reflect the mechanism involved in Akt activation, i.e. it is believed that the Thr-308 site is phosphorylated before the Ser-473 site. Alternatively, maximal phosphorylation of both sites may occur at a time-point between 10 and 15min, and therefore, has been missed in the tissue samples that were analysed. Nevertheless, these data were consistent with those found by (Smith et al. 2006), i.e. leptin-induced cardioprotection could be abolished with a PI3K inhibitor and significant phosphorylation of the Ser-473 site was observed at 15min into the reperfusion-phase as opposed to the earlier time-points assayed.

At 30min reperfusion Akt/serine-473 phosphorylation appeared to be depressed, although this change was not statistically significant. This may be as a result of a negative feedback mechanism employed to prevent excessive kinase phosphorylation/activation. For example, full activation of Akt may, directly or indirectly, activate a phosphatase which dephosphorylates Akt at one or both of its sites, such as PH domain leucine-rich repeat protein phosphatase (PHLPP) (Gao et al. 2005). Alternatively, fully activated Akt may be degraded via ubiquitin mediated proteolysis (Chain et al. 1995). This type of mechanism operates with respect to the negative regulation of Lyn protein-tyrosine kinase by c-Cbl ubiquitin protein ligase in mast cells (Kyo et al. 2003) and works by labelling aberrant or inappropriately active proteins which then undergo disassembly by proteosomal degradation.

5.5.3 Phosphorylation of p44/42 MAPK in response to leptin-treatment

Significant increases in the phosphorylation of both p44 and p42 MAPK were observed at 5 and 15min into the reperfusion-phase or leptin treatment as compared to control, a result that is consistent with previously obtained data (Smith et al. 2006). At 2.5min into the reperfusion-phase there appeared to be a leptin-stimulated increase in p44/42 MAPK phosphorylation, however, these changes were not statistically significant.

Although leptin increased p44/42 MAPK phosphorylation at 5 and 15min reperfusion as compared to control, at 10min no significant change in phosphorylation was observed between control and leptin treated samples.

This data may have occurred due to an error in sampling at this particular time-point. Alternatively, the phosphorylation response profile of control and leptin treated hearts that have undergone a I/R protocol may yield similar results at this specific time, i.e. 10min. In addition, before discounting the data as erroneous it should be noted that several protein kinases show a biphasic phosphorylation response profile. In human embryonic kidney 293 (HEK293), for example, MAPK has been shown to respond in such a manner. The investigators performed a dose-response study of MAPK phosphorylation as stimulated by cadmium and mercury, the results showed two peaks of phosphorylation similar to the result yielded above (Hao et al. 2009).

Phosphorylation of p44 and p42 MAPK appears to be depressed at 30min when treated with leptin as compared to control, a similar result to that obtained for Akt. This may be due to a negative feedback mechanism, similar to that described above (section 5.5.2). A similar phenomenon is observed when treating macrophages with leptin, i.e. MAPK phosphorylation is decreased after a certain concentration threshold of leptin is reached (O'Rourke & Shepherd 2002).

5.5.4 Limitations

5.5.4.1 Western blotting

The technique of Western blotting is associated with limitations, especially with regard to the analysis of RISK kinase activation/phosphorylation. Each blot corresponds to a sample of a heart that has been subjected to I/R injury. Sometimes large variations in the relative density values were obtained, which

may account for some of the non-significant increases in kinase phosphorylation observed. Increasing the number of samples that were analysed may have helped to reduce this variability.

Furthermore, Western blotting samples are taken from the risk zone of the myocardium subjected to I/R injury (see section 3.3). This method does not compensate for the differing ratio of infarct to viable heart tissue that may result when hearts are treated with a cardioprotectant as compared to control. Therefore, phosphorylation increases detected in heart samples that were treated pharmacologically may be the result of sampling a greater amount of viable tissue in a protected heart. Separating viable tissue from infarcted tissue in each heart sample would help to ensure that equal amounts of viable tissue from hearts treated with and without leptin were assayed.

5.5.4.2 Protein kinase inhibitors

Data obtained in which PI3K and p44/42 MAPK were blocked in this study relies on the assumed specificity of the protein kinase inhibitors LY294002 and UO126, respectively. The concentrations used in this study were the same as those used previously in this laboratory (Smith et al. 2006) and have been demonstrated to be within the range known to produce inhibition of these kinases (Vlahos et al. 1994). It should be noted that as well as inhibiting PI3K, LY294002 has been found to block the activation of the following kinases CK2, mTOR, PLK-1, PIM1, PIM3, HIPK2 and GSK3β (Bain et al. 2007). It is therefore possible that one or more of these kinases may be involved in the mechanism of leptin-induced cardioprotection and therefore data collected using LY294002 as

a PI3K antagonist may overestimate the role of PI3K-Akt in this process. Future studies should employ either a more specific PI3K antagonist, if one is available or use another antagonist in simultaneous experiments with LY294002 to help elucidate the role of PI3K phosphorylation in leptin-induced cardioprotection.

CHAPTER 6 - LEPTIN-INDUCED CARDIOPROTECTION VIA THE JAK-STAT PATHWAY

6.1 Introduction

The JAK/STAT pathway, described in section 1.3.4.2, plays a pivotal role in modulating the expression of stress-responsive genes by transducing signals received at the cell membrane to the nucleus (Darnell, Jr. 1997). JAK1 and JAK2 are expressed in cardiomyocytes (Pan et al. 1999), along with all seven isoforms of STAT (Xuan et al. 2001). With regard to cardioprotection, there is a growing body of evidence indicating that the JAK-STAT pathway plays a role in I/R injury. For example, it has been demonstrated in an *in vivo* rat model of I/R injury that I/R injury itself stimulates the phosphorylation of STAT1 and STAT3 (McCormick et al. 2006). It appears, however, that STAT1 and STAT3 play very different roles in the setting of myocardial injury. Various studies have suggested that in the heart STAT3 acts to stimulate survival mechanisms, whilst activation of STAT1 appears to promote apoptotic cell death (Stephanou 2004). Thus, using an *in vivo* model of I/R injury hearts from rats with a cardiac-specific overexpression of constitutively active STAT3 were found to be less susceptible to infarction than control animals. (Osugi et al. 2002; Oshima et al. 2005). It appears that STAT3 may also play an important role in IPost. Goodman et al demonstrated that IPost-enhanced functional recovery, which was associated with increases in STAT3 and Akt phosphorylation, was blocked in hearts from mice with a cardiac-specific STAT3 knockout (Goodman et al, 2008). Functional recovery and the increases in STAT3 and Akt phosphorylation seen in wild-type animals, subjected to IPost were abrogated by the JAK2 inhibitor, AG490

(Goodman et al, 2008). The downstream mechanisms activated by STAT3 are not yet well characterised, although a link between STAT3 phosphorylation and inactivation of Bax and caspase 3 (see section 1.2.3.2) has been demonstrated (Negoro et al. 2000).

Leptin requires activation of JAK for downstream signalling (see section 1.6.5), however, the role of STAT in leptin-induced protection is less well known (Fruhbeck 2006). In a study from this laboratory involving the isolated perfused mouse heart, STAT3 phosphorylation was not found to be increased when hearts were treated with leptin as compared to control (Smith et al. 2006). It was, however, suggested that the maximal signal in response to leptin may have been missed, occurring earlier than the 10min reperfusion time-point at which samples were collected.

6.2 Aim

The aim of the studies described in this chapter was to investigate in more detail the role played by JAK/STAT signalling in leptin-induced cardioprotection, using the isolated perfused Wistar rat heart.

6.3 Experimental Protocol & Materials

6.3.1 JAK2 inhibition

Hearts from Wistar rats were excised and perfused according to the method described in section 3.3. All hearts were stabilised for 40min, subjected to 35min regional ischaemia and then reperfused for two hours. AG490 (5μM, final concentration), the JAK2 inhibitor, was dissolved in dimethyl sulphoxide (DMSO,

final concentration 0.02%). The vehicle, DMSO, was also tested alone to establish if it influenced infarct size by itself. Drug containing buffers were added to the perfusion apparatus 5min before reperfusion was commenced to ensure that the concentration of drug reaching the heart upon reperfusion had achieved the required level. AG490 was administered by itself or with leptin (10nM). Individual treatment groups were as follows (original group sizes are shown in brackets):-

Control (n=6) – no drug

DMSO (0.02%) (n=6) – drug administered for 5min prior to reperfusion.

AG490 (5μM) (n=6) – administered 5min prior to reperfusion and up until 15min into the reperfusion-phase.

Leptin (10nM) (n=6) – administered 5min prior to ischaemia and up until 30min into reperfusion.

Leptin (10nM) with AG490 (10μM) (n=6) – leptin administered with AG490 5min prior to reperfusion. AG490 was stopped 15min into the reperfusion-phase. Leptin was given until 30min into the reperfusion-phase.

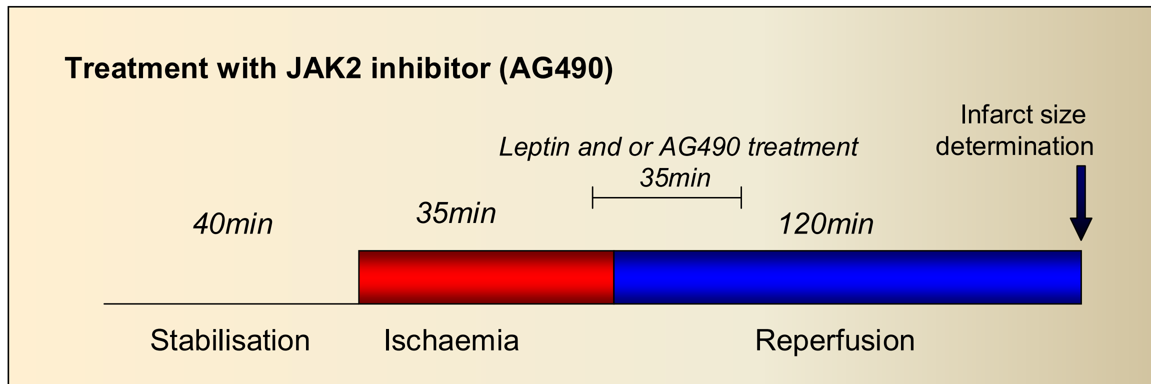


Figure 6.3.1 - Protocol for investigating the effects of AG490, a JAK2 inhibitor, on leptin-induced infarct size reduction.

6.3.2 Western blotting

6.3.2.1 Leptin-induced STAT3 phosphorylation time-course

Hearts from Wistar rats were excised and perfused according to the method described in section 3.3. All hearts were stabilised for 40min and then subjected to 35min regional ischaemia via coronary artery ligation (see section 3.3). Hearts were then reperfused for 2.5, 5, 10, 15 or 30min in the presence and absence of leptin (10nM), at which time the region of the myocardium at risk was collected, snap frozen and stored for subsequent analysis by Western blotting (see section 3.5). Individual treatment groups were as follows (original group sizes are shown in brackets):-

Control (n=5) - **2.5min** reperfusion

Leptin (10nM) (n=5) - **2.5min** reperfusion

Control (n=6) - **5min** reperfusion

Leptin (10nM) (n=6) - **5min** reperfusion

Control (n=6) - **10min** reperfusion

Leptin (10nM) (n=6) - **10min** reperfusion

Control (n=6) - **15min** reperfusion

Leptin (10nM) (n=6) - **15min** reperfusion

Control (n=5) - **30min** reperfusion

Leptin (10nM) (n=6) - **30min** reperfusion

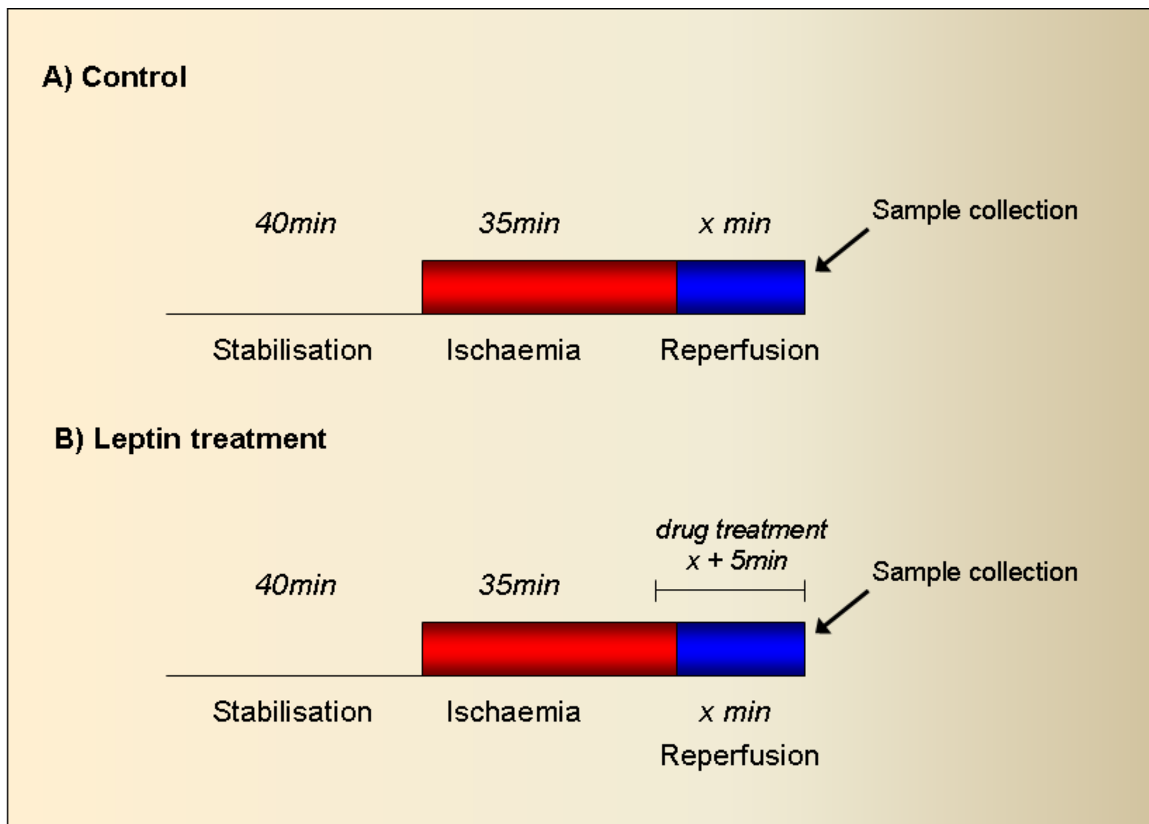


Figure 6.3.2 –Protocol for the collection of samples for STAT3 determination by Western blot analysis. (A) Control protocol in which hearts were subjected to 40min stabilisation, 35min ischaemia and 2.5, 5, 10, 15 or 30min reperfusion. (B) Leptin treatment protocol in which hearts were subjected to 40min stabilisation, 35min ischaemia and 2.5, 5, 10, 15 or 30min reperfusion in the presence of leptin (10μM). Leptin treatment was begun 5min before the commencement of reperfusion and continued until the point at which the heart was snap-frozen.

6.3.2.2 Inhibition of leptin-induced STAT3 phosphorylation by AG490

Hearts from Wistar rats were excised and perfused according to the method described in section 3.3. All hearts were stabilised for 40min and then subjected to 35min ischaemia. Hearts were then reperfused with leptin, AG490 or leptin plus AG490. AG490 (5μM) was dissolved in dimethyl sulphoxide (DMSO; final concentration 0.02%). Hearts were reperfused for a total of 2.5min, at which time the area of the myocardium at risk was collected, snap frozen and stored at

-80°C for subsequent analysis by Western blotting (see section 3.5). Individual groups were as follows (original group sizes are shown in brackets):

Control (n=6)

Leptin (10nM) (n=6)

Leptin (10nM) plus AG490 (5μM) (n=6)

AG490 (5μM) (n=6)

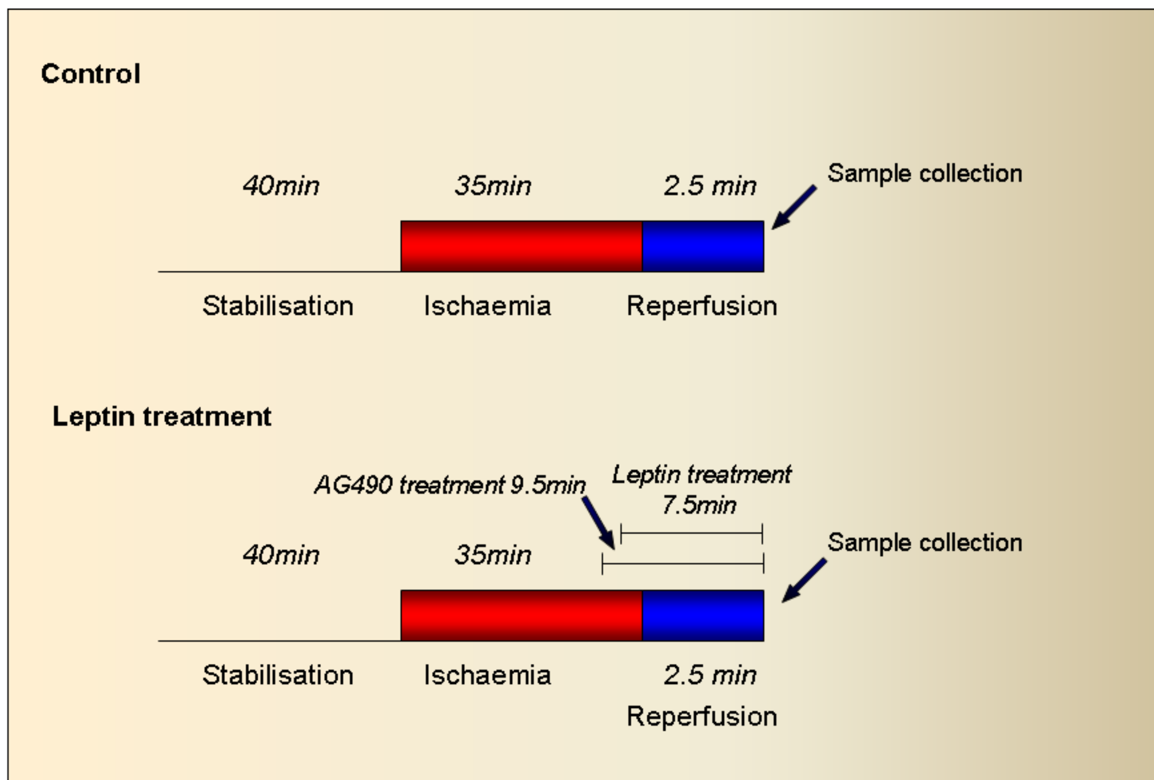


Figure 6.3.3 – Protocol for investigating the influence of the JAK2 inhibitor, AG490 (5μM), on STAT3 phosphorylation in the presence and absence of leptin (10nM), as determined by Western blot analysis.

6.4 Results

6.4.1 Influence of AG490 on leptin-induced cardioprotection

6.4.1.1 Animal data and haemodynamic data

Table 6.4.1 demonstrates that there are no significant differences between the groups with regard to body weight, ventricular volume or risk zone. Baseline data relating to cardiac function before ischaemia, as assessed by the rate pressure product (RPP) and coronary flow, were similar in all the experimental groups (see tables 6.4.1 & 6.4.2). During in ischaemia, however, coronary flow and RPP declined to a similar extent in all groups. Cardiac function and coronary flow increased upon reperfusion, which indicated that re-flow was successful. No significant differences were observed between groups with respect to coronary flow rate and RPP during reperfusion.

Groups	Body Weight (g)	Ventricular Volume (mm^3)	Risk Zone (%)
Control (n=5)	417.6±6.1	2130.1±332.1	53.7±4.7
DMSO (n=5)	390.0±28.0	2446.1±445.2	51.1±5.1
AG490 (n=5)	415.8±23.0	2811.5±323.3	52.8±2.7
Leptin (n=10)	385.2±11.5	2211.5±448.1	50.2±5.3
Leptin + AG490 (n=4)	380.0±26.0	2878.0±362.1	47.7±3.2

Table 6.4.1 – Characteristics of the animals in the treatment groups. Data are expressed as mean±s.e.m.

Coronary flow rate (min/ml)

	Stabilisation (ml/min)				Ischaemia (ml/min)			Reperfusion (ml/min)						
Time(min) / Groups	10	20	30	40	5	15	30	5	15	30	45	60	90	120
Control (n=5)	16.8 ±2.1	16.0 ±2.1	15.5 ±2.1	15.2 ±2.1	7.5 ±1.6	8.3 ±1.6	7.6 ±1.4	13.7 ±0.9	12.6 ±0.8	12.3 ±0.9	11.8 ±0.6	11.2 ±0.7	10.0 ±1.2	9.5 ±1.1
DMSO (n=5)	15.5 ±1.6	14.3 ±1.7	13.0 ±1.4	13.0 ±1.8	7.0 ±1.1	7.5 ±0.9	7.1 ±1.0	12.8 ±1.7	12.7 ±1.3	11.0 ±1.3	10.0 ±0.9	9.3 ±0.9	7.8 ±0.6	6.8 ±0.9
AG490 (n=5)	16.4 ±0.5	12.6 ±0.7	11.6 ±0.7	11.2 ±0.7	6.0 ±0.3	6.4 ±0.8	6.1 ±0.9	13.8 ±0.8	13.2 ±0.8	12.6 ±1.0	9.6 ±0.5	8.8 ±0.5	8.2 ±0.7	7.7 ±0.7
Leptin (n=10)	16.5 ±0.9	15.5 ±1.0	14.3 ±1.4	13.8 ±1.5	7.6 ±0.8	7.5 ±0.9	7.4 ±0.9	12.0 ±2.7	12.3 ±2.8	11.0 ±2.7	10.5 ±2.5	10.4 ±2.6	9.8 ±2.8	9.0 ±2.7
Leptin+AG490 (n=4)	16.2 ±1.2	15.5 ±1.3	15.2 ±1.3	14.5 ±1.1	7.3 ±0.6	7.2 ±0.7	6.9 ±0.7	15.6 ±2.2	17.0 ±2.3	15.3 ±1.8	13.2 ±1.7	11.7 ±1.7	10.5 ±1.7	10.6 ±2.0

Table 6.4.2 – Coronary flow rate (ml/min) of each group at various time-points during the ischaemia/reperfusion protocol.

Rate pressure product (x 103mmHg/min)

	Stabilisation (x 10^3mmHg/min)				Ischaemia (x 10^3mmHg/min)			Reperfusion (x 10^3mmHg/min)						
Time(min) / Groups	10	20	30	40	5	15	30	5	15	30	45	60	90	120
Control (n=5)	33717 ±6529	28958 ±4214	30067 ±3147	30820 ±4097	9457 ±2154	15583 ±2384	15567 ±2536	22433 ±3015	19540 ±2163	19883 ±2072	20233 ±2791	17833 ±2451	18733 ±2376	17125 ±1947
DMSO (n=5)	31650 ±5204	31238 ±6530	31719 ±4180	30750 ±4493	19188 ±11661	17395 ±6965	20788 ±3983	22933 ±6769	27800 ±3646	22581 ±4550	20588 ±3580	18375 ±2392	13725 ±4918	15400 ±2459
AG490 (n=5)	23750 ±2839	24690 ±1501	24630 ±1418	24150 ±2211	8016 ±2791	14990 ±1999	16470 ±1640	21050 ±1750	22180 ±2504	23380 ±2261	19540 ±1678	18380 ±1146	15105 ±1567	14620 ±2614
Leptin (n=5)	38167 ±5868	35250 ±3568	33138 ±3110	29158 ±1304	11333 ±1202	18100 ±2261	15300 ±1060	21013 ±1765	22617 ±1433	22369 ±2118	21725 ±1996	19825 ±2193	19400 ±3439	17500 ±3156
Leptin+AG490 (n=4)	27925 ±3231	29467 ±2479	29267 ±2198	30025 ±2478	12000 ±702	19640 ±2146	18317 ±1761	16960 ±3502	21992 ±1700	22910 ±937	18950 ±1431	16900 ±1125	15783 ±1599	14683 ±2078

Table 6.4.3 - Rate Pressure Product (x 103mmHg/min) of each group at various time-points during the ischaemia/reperfusion protocol.

6.4.1.2 Exclusions

One heart was excluded from the control group because of poor function, i.e. it did not achieve the required RPP. Two hearts were excluded from the DMSO group and AG490, one because of low coronary flow and one because of poor function. Finally, one heart was excluded from the AG490 + leptin group because the risk zone was too small.

6.4.1.3 AG490 abolishes leptin-induced cardioprotection

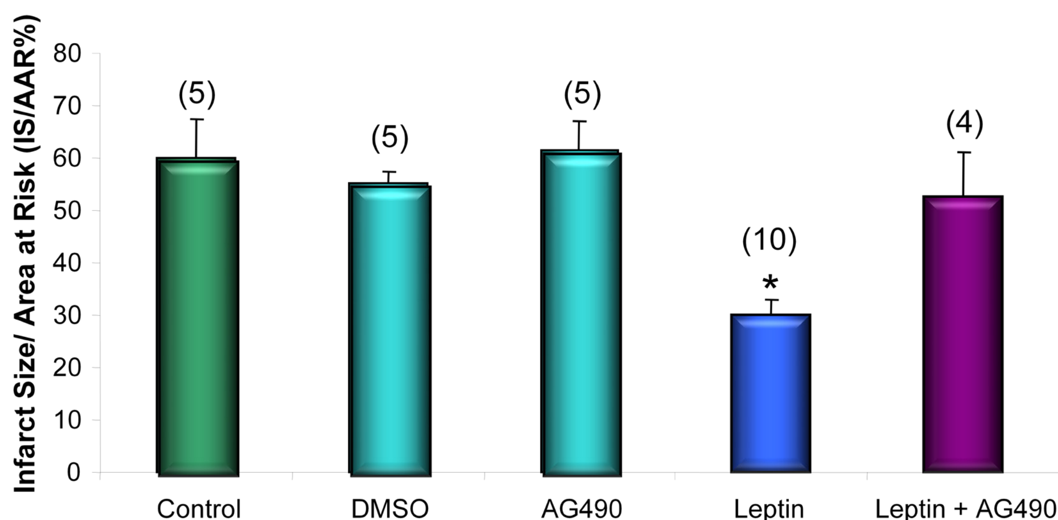


Figure 6.4.1 – Abrogation of leptin-induced protection by AG490. Infarct size is presented as a percentage of the risk zone (%I/R). Isolated rat hearts were reperfused with or without leptin (10nM) in the presence or absence of AG490 (5μM). The data are presented as mean ± s.e.m (*$p<0.05$ vs. control; n = 4-10) *(one-way ANOVA)*.

In hearts treated with leptin (10nM) (n=10) a significant reduction in infarct size, as compared with vehicle-treated control hearts (n=5), was observed (control, 60.05±7.41% vs. leptin-treated, 29.9±3.24%, $p<0.05$, n=4-10) (Figure 6.4.1). The cardioprotective effect of leptin was found to be completely blocked by the

JAK2 inhibitor, AG490 (n=4) (Figure 6.4.1). It should be noted that no difference in infarct size was seen between control hearts and hearts treated with DMSO alone (n=5) (0.02% final concentration); DMSO was used to dissolve AG490 (Figure 6.4.1).

6.4.2 Leptin-induced STAT3 phosphorylation

6.4.2.1 Body weights of animals in the different treatment groups

Groups	Body Weight (g)
2.5min Control (n=4)	385±15
2.5min leptin (n=4)	397±12
5min Control (n=6)	425.5±14
5min leptin (n=6)	412.8±26
10min Control (n=6)	419.6±21
10min leptin (n=6)	387.6±24
15min Control (n=6)	390.5±15
15min leptin (n=6)	395.5±8
30min Control (n=5)	405.5±20
30min leptin (n=6)	416.9±17

Table 6.4.4 – Table showing the body weights of the animals used in the different treatment groups

6.4.2.2 Exclusions

One heart was excluded from the 5min control group due to excessive ventricular fibrillation. One heart was excluded from the 10min leptin treated group due to poor function, i.e. did not achieve the required RPP. Two hearts were excluded from the 15min control group, one due to poor flow and one due to excessive ventricular fibrillation (VF). One heart was excluded from the 30min leptin group because of poor function.

6.4.2.3 Leptin-induced STAT3 phosphorylation

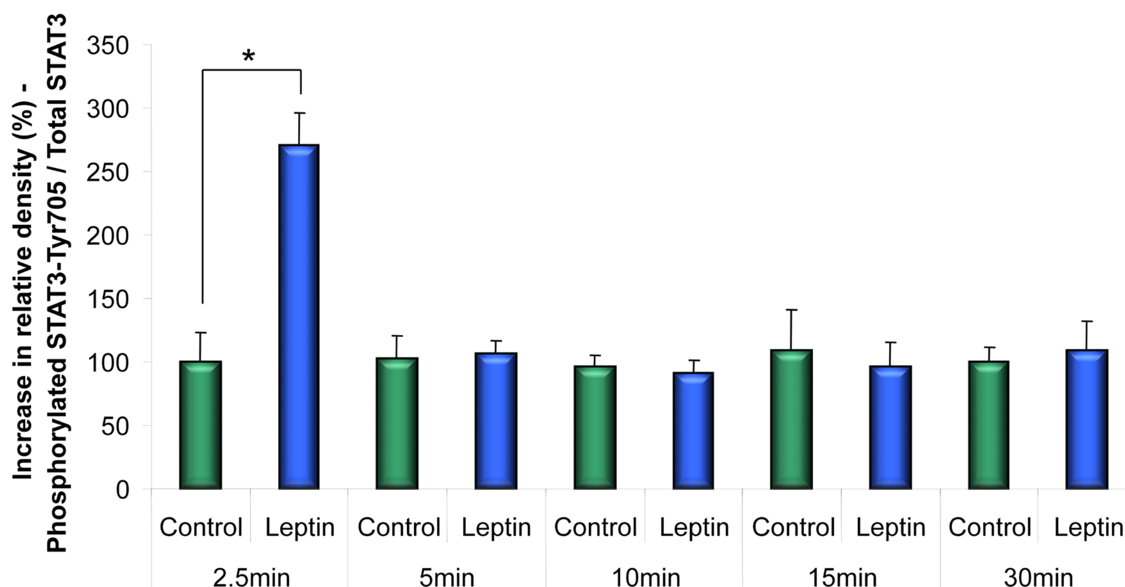
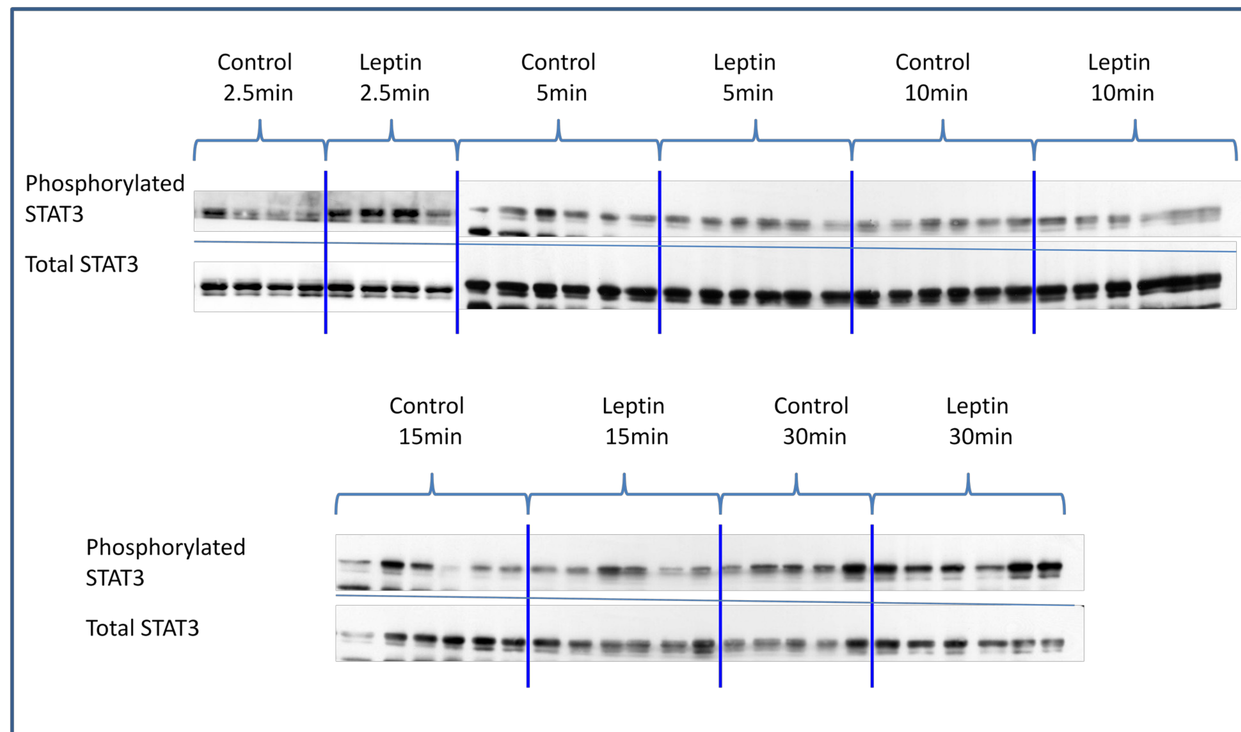


Figure 6.4.2 - Phosphorylation of STAT3/tyrosine-705 in the presence and absence of leptin at different time-points during reperfusion. Total and phosphorylated STAT3 were determined in extracts derived from rat hearts subjected to ischaemia-reperfusion under control conditions or in the presence of leptin (10nM). Data are presented as the mean ± s.e.m of relative densitometry values in arbitrary units (* $p<0.001$ vs. control; n = 5-6) *(one-way ANOVA)*.



6.4.3 - Western blots showing phosphorylated STAT3 and total STAT3 levels in tissue taken from Wistar hearts treated with and without leptin (10nM) at various time-points during reperfusion.

In time course experiments leptin was found to elicit a 171% increase in STAT3/tyrosine-705 phosphorylation at 2.5min reperfusion ($p<0.001$, n=5; see Figure 6.4.2). At the other reperfusion time-points examined (i.e. 5, 10, 15 and 30 min reperfusion), however, significant changes in STAT3 phosphorylation were not detected (n=5-6; see Figure 6.4.2).

6.4.3 Influence of AG490 on the leptin-induced increase in STAT3 phosphorylation

6.4.3.1 Body weights of animals in the different treatment groups

Group	Body weight
Control (n=5)	350±4.7
Leptin (10nM) (n=5)	364±6.8
AG490 (5μM) (n=5)	346±4.7
AG490(5μM) +leptin (10nM) (n=4)	350±5.0

Table 6.4.5 – Average body weight of the animals in the different treatment groups.

Hearts were assigned randomly to one of four treatment groups. Data analysis revealed that there were no statistical differences between the treatment groups with regard to body weight.

6.4.3.2 Exclusions

One heart was excluded from the leptin (10nM) treated group because coronary flow dropped below 10ml/min during stabilisation.

6.4.3.3 Leptin-induced STAT3 phosphorylation is inhibited by AG490

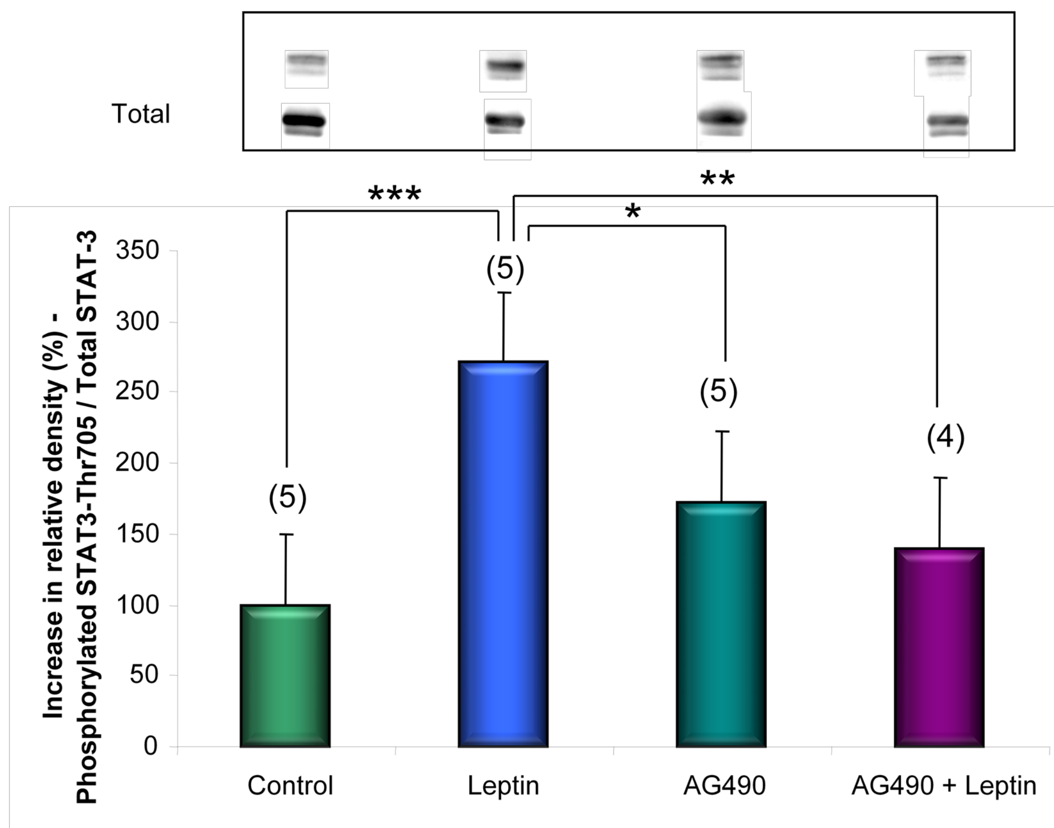


Figure 6.4.4 - Inhibition of leptin-induced STAT3 phosphorylation by AG490. Total and phosphorylated STAT3 were determined in extracts derived from rat hearts subjected to ischaemia-reperfusion with or without leptin (10nM) in the presence or absence of AG490 (5μM). Data are presented as the mean ± s.e.m of relative densitometry values in arbitrary units together with representative Western blots (***$p<0.001$, leptin vs. control, **$p< 0.002$, leptin vs. AG490 + leptin and *$p<0.02$, leptin vs. AG490; n = 4-5) *(one-way ANOVA)*.

As observed with the infarct data, the actions of leptin on STAT3 phosphorylation at 2.5 min reperfusion were blocked by AG490 [leptin (n=5) vs. leptin+AG490 (n=4), $p<0.002$; Figure 6.4.3]. AG490 (n=5) was also found to inhibit STAT3 phosphorylation, albeit to a slightly lesser extent, when administered alone [leptin (n=5) vs. AG490 (n=4), $p<0.02$; Figure 6.4.3].

6.4.4 Influence of AG490 on leptin-induced Akt/serine-473 phosphorylation

6.4.4.1 Body weights of the animals in the different treatment groups

Group	Body weight (g)
Control (n=5)	336.6±6.45
Leptin (10nM) (n=5)	315.0±5.92
AG490 (5μM) (n=6)	311.3±3.01
AG490 (5μM) +leptin (10nM) (n=5)	324.5±7.25

Figure 6.4.5 – Mean body weight of the animals in the treatment groups.

Hearts were assigned randomly to one of four treatment groups. Data analysis revealed that there were no statistical differences between the treatment groups with regard to body weight.

6.4.4.2 Exclusions

One heart from the control treated group was excluded due to poor function.

6.4.4.3 Leptin-induced phosphorylation of Akt/serine-473 is not affected by AG490

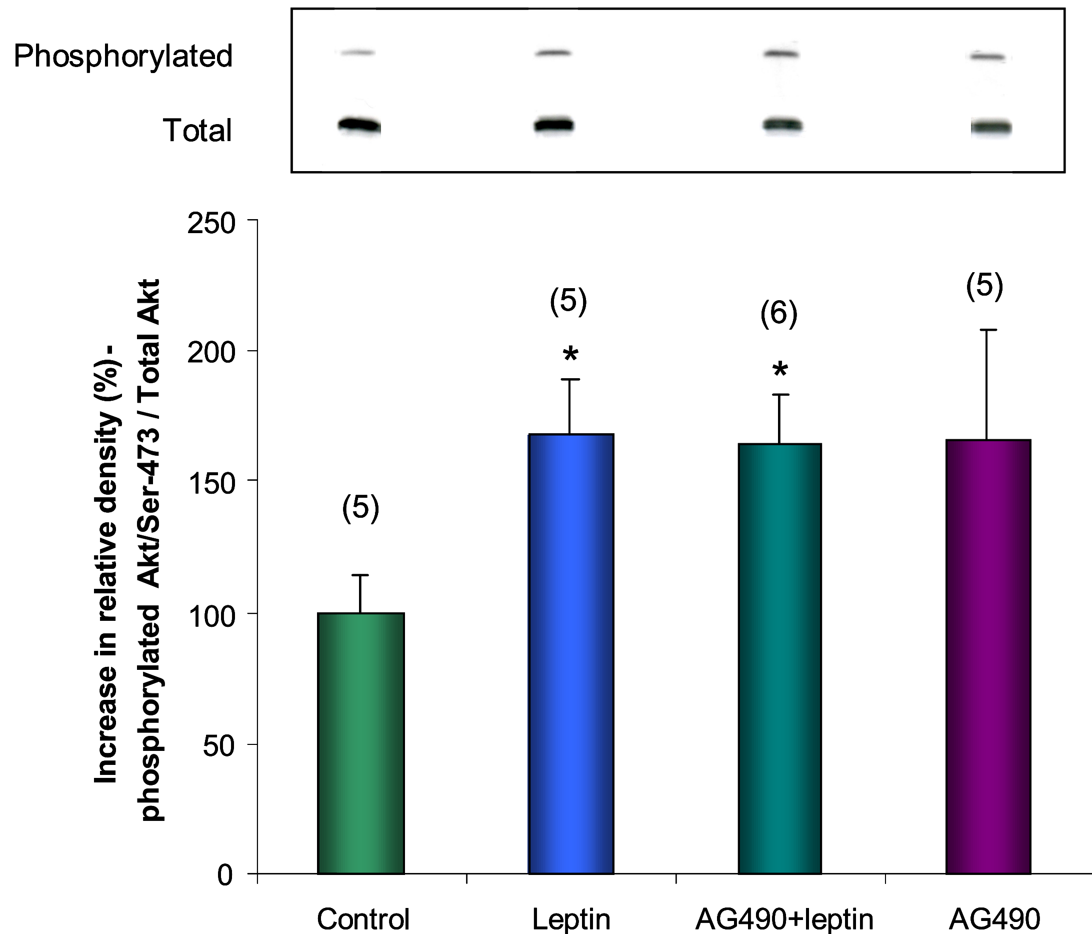


Figure 6.4.5 – Influence of the JAK2 inhibitor, AG490, on leptin-induced phosphorylation of Akt/serine-473. The total and phosphorylated forms of Akt were determined in extracts derived from rat hearts subjected to ischaemia-reperfusion with or without leptin (10nM) in the presence and absence of AG490 (5μM). Data are presented as the mean ± s.e.m of relative densitometry values in arbitrary units together with representative Western blots (*=$p<0.05$ vs. control; n=5-6) *(one-way ANOVA)*.

Leptin (10nM) elicited a 67% increase in Akt phosphorylation [$p<0.05$, leptin (n=5) vs. control (n=5)]. AG490 (5μM) failed to influence the leptin (10nM)-induced increase in Akt/serine-473 phosphorylation. Interestingly, however, the administration of AG490 (5μM) by itself caused an increase in Akt/serine-473 phosphorylation, although this did not achieve statistical significance.

6.5 Discussion

Cardiac JAK/STAT signalling was originally documented in a study in which rat cardiomyocytes were found to respond to treatment with leukaemia inhibitory factor with increased STAT3 activation (Kunisada et al. 1996). A subsequent study demonstrated that both STAT1 and STAT3 were activated in the heart in response to pressure overload hypertrophy (Pan et al. 1997). Evidence indicating that the JAK/STAT pathway is involved in cardioprotection was obtained in 2001. Xuan and colleagues reported that the late phase of IPC was associated with JAK1 and JAK2 activation, resulting in STAT1 and STAT3 recruitment, and subsequent upregulation of nitric oxide synthase (iNOS) (Xuan et al. 2001). The administration of the JAK2 inhibitor, AG490, prior to IPC was found to block these processes. The work of Xuan et al was complimented by that of Hattori and co-workers, who reported that the early phase of IPC was associated with JAK/STAT activation, upregulation of bcl-2 and downregulation of Bax (Hattori et al. 2001). These processes were, once again, abrogated by the administration of AG490.

More recently, Lecour and colleagues demonstrated that mechanical IPC and pharmacological IPC, induced by tumour necrosis factor-α (TNF-α), were associated with early activation of STAT3 during the reperfusion-phase (Lecour et al. 2005). Furthermore, the crucial role played by STAT3 activation in cardioprotection was demonstrated in studies conducted with cardiomyocytes from cardiac-specific STAT3 knockout mice, which failed to respond to IPC (Smith et al. 2004). These data were paralleled by the results of experiments

carried out with Langendorff perfused hearts, in which infarct size limitation conferred by IPC in wild-type mice was found to be lost in hearts from cardiac specific STAT3 knock-out mice (Smith et al. 2004). Very recent data indicates that IPC-induced cardioprotection is inhibited in STAT3 deficient and aged mice (Boengler et al. 2008a). Noteworthy, is the observation that the loss of IPC-induced protection in aged mice was associated with decreased STAT3 phosphorylation (Boengler et al. 2008a). Collectively these data provide evidence that JAK/STAT signalling plays a key role in myocardial preservation, and also indicate a possible mechanism whereby myocardial damage may develop with age (Boengler et al. 2008b).

6.5.1 Leptin-induced cardioprotection is blocked by AG490

In the current study leptin-induced cardioprotection was completely blocked when AG490 was administered at the same time as the adipocytokine, a result that confirms the findings of Xuan et al and provides additional evidence for the involvement of the JAK/STAT pathway in protection (Xuan et al. 2001).

6.5.2 Leptin-induced STAT3 phosphorylation in Wistar rat hearts subjected to I/R injury

STAT3 phosphorylation in response to leptin treatment appeared to occur early in the reperfusion-phase. This was indicated by the observation that a marked increase in STAT 3 phosphorylation occurred 2.5min into reperfusion, following leptin administration. Interestingly, the effect evoked by leptin was lost 5min into the reperfusion-phase, the extent of STAT3 phosphorylation returning to basal levels. This change in STAT3 phosphorylation appears to occur very rapidly, especially when compared to the leptin-induced phosphorylation profiles

obtained for PI3K and MAPK p44/42 (see Chapter 4), in which a gradual increase in phosphorylation levels is observed over 15min and then followed by a decline in phosphorylation. Furthermore, this result appears to be in conflict with leptin-induced STAT3 phosphorylation in other cell types. For example, leptin elicited a peak of STAT3 phosphorylation at 30 min in human hepatic stellate cells (Cao et al. 2004). One must consider that the STAT3 response may occur at different speeds in different tissue of the body. Alternatively, one should consider that STAT3 may play different roles in various tissues. For example, recent data suggest that STAT3 may participate in signalling at or in the mitochondria. Perhaps in cardiomyoctyes leptin elicits an early STAT3 response via JAK2, which results in migration of STAT3 to the mitochondria at which point it may interact with and prevent opening of the MPTP. Subsequently, STAT3 may be degraded in cardiomyocytes, whereas in other tissues it may remain in the cell and therefore can be detected.

Interestingly, it has been speculated that the increase in JAK/STAT activity that occurs following the reperfusion of the ischaemic myocardium is as a result of increased levels of reactive oxygen species (ROS), rather than the upregulation of growth factors or cytokines (Barry et al. 2007). It is possible, therefore, that in the myocardium leptin may not activate STAT3 directly, but via increases in the tissue levels of ROS. The data presented in this study compliment those acquired previously in this laboratory, i.e. it was demonstrated previously that leptin treatment was not associated with increased STAT3 phosphorylation in the Langendorff isolated perfused murine heart at 10min reperfusion (Smith et

al. 2006). Given that maximal STAT3 phosphorylation in the rat heart was observed to occur early in the reperfusion-phase, i.e. at 2.5min, it is not, perhaps, surprising that increases in STAT3 phosphorylation were not seen in the murine heart (Smith et al. 2006).

6.5.3 Leptin-induced STAT3 phosphorylation is blocked by AG490

In this part of the study the aim was to establish if leptin-stimulated STAT3 phosphorylation could be abolished by the administration of the JAK2 inhibitor, AG490. Thus, further evidence for the involvement of the JAK/STAT pathway in leptin-induced myocardial actions was sought. Indeed, as observed by Xuan et al (Xuan et al. 2001) it was found that AG490, apart from abrogating the infarct-reducing effects of leptin, also inhibited STAT3 phosphorylation (Figure 6.4.3). This finding suggests that leptin signalling in the myocardium requires JAK2 phosphorylation, which then leads to STAT3 activation downstream. However, the role of STAT in leptin-induced cardioprotection has yet to be fully elucidated. For example, further studies with the STAT3 specific inhibitor, STATTIC, may lead to a more comprehensive understanding of the role of the JAK/STAT pathway in cardioprotection (Schust et al. 2006).

6.5.4 AG490 does not influence leptin-induced phosphorylation of Akt/serine-473

In chapter 4 data are presented that indicate that maximal leptin-induced phosphorylation of Akt/serine-473 occurred at 15min into the reperfusion-phase. By contrast, maximal leptin-induced STAT3 phosphorylation was found to occur earlier, i.e. at 2.5min into the reperfusion-phase. One interpretation that can be placed on these data is that the JAK/STAT pathway operates upstream of the

RISK signalling cascade. This hypothesis is supported by recent data demonstrating that RISK signalling induced by IPost follows activation of the JAK/STAT pathway, and that JAK/STAT phosphorylation in the absence of RISK activation is insufficient to provide protection (Goodman et al. 2008). It must be noted, however, that this area of research is still very much in its infancy, particularly in the context of cardioprotection, and that interactions between the JAK/STAT and RISK pathways remain to be fully elucidated. Indeed, the data obtained in the present study would appear to be conflicting. Whilst, JAK/STAT phosphorylation occurred earlier than Akt/serine473 phosphorylation (see section 5.4.2.3), indicating that JAK/STAT activation may precede that of Akt activation, the JAK2 inhibitor, AG490 failed to block Akt activation when administered at reperfusion. When considering these data, however, it is important to take account of possible confounding factors such as drug specificity, drug concentration used and the type of JAK2 inhibitor employed.

In conclusion, the data presented in this chapter have clearly demonstrated a role for the JAK/STAT pathway in leptin-induced cardioprotection, where previously the function of STAT3 was unknown. However, many questions currently remain unanswered with regard to the mismatch in timing between STAT3 and Akt phosphorylation. One may speculate that the fact that the peak of STAT3 phosphorylation occurs much earlier than that of Akt indicates that STAT3 is a more important component than Akt in the mechanism that underlies the leptin-induced cardioprotection. Furthermore, this might be, as speculated earlier in the chapter through direct modulation of the MPTP by activated

STAT3. In this suggested mechanism, leptin binds its receptor, which in turn phosphorylates recruited JAK2 proteins, which leads to the recruitment of STAT3 proteins followed by their phosphorylation, dimerisation and migration to the mitochondrion. However, this does by no means suggest the role of Akt is completely redundant in leptin-induced cardioprotection. In a later wave to that of STAT3, Akt may be activated, by PI3K, and converge with STAT3 upon the mitochondrion to prevent MPTP opening.

CHAPTER 7 - LEPTIN-INDUCED CARDIOPROTECTION IN ZUCKER LEAN & ZUCKER FATTY RATS

7.1 Introduction

Leptin has been reported to confer protection against myocardial I/R injury via p44/42 MAPK and Akt signalling (Smith et al. 2006). Studies in a variety of tissues have suggested that these signalling pathways are activated through interaction between leptin and specific leptin receptors, designated OB-R (Fruhbeck 2006). Six isoforms of the OB-R have been described, i.e. OB-Ra, OB-Rb, OB-Rc, OB-Rd, OB-Re and OB-Rf (Tartaglia et al. 1995). The roles played by these receptors with regard to cardioprotection and other cardiac functions have yet to be determined. It has, however, been established that OB-Ra and OB-Rb are expressed in the heart, with the OB-Ra predominating (Purdham et al. 2004). Nevertheless, it is the OB-Rb isoform, which possesses the longer C-terminal domain, that exhibits the greater signalling capacity and could, possibly, play the primary physiological role in the heart (Fruhbeck 2006). It is worth noting that whilst the OB-Ra isoform is capable of signalling via MAPK, it does not possess the ability to stimulate STAT3 (Banks et al. 2000). STAT3 phosphorylation is controlled via a Tyr-(1138) residue, which is found exclusively in the long, OB-Rb, receptor isoform (Banks et al. 2000). The importance of the OB-Rb isoform in leptin signalling is lent credence by the fact that the obese phenotype exhibited by the db/db mouse results from a mutation in the OB-Rb (Chen & Wang 2005). In this study Zucker lean and Zucker fatty (*fa/*fa) rats were employed to investigate how adiposity and OB-R status influence myocardial sensitivity to the protective actions of leptin. The obese

phenotype in the *fa/fa* rat is the result of a glutamine to proline amino acid substitution mutation at the 269 position in the C-terminal domain of all the leptin OB-R isoforms (Phillips et al. 1996). This mutation has no effect on the leptin binding capacity of the receptor (Guan et al. 1997), but is thought to inhibit OB-R dimerisation, a prerequisite for downstream leptin signalling (Kishimoto et al. 1994).

7.2 Aim

The aim in this chapter was to establish the responsiveness of Zucker lean and *fa/fa* hearts to leptin-induced cardioprotection.

7.3 Hypothesis

Given the proposed physiological importance of the OB-Rb isoform and its suggested involvement in obesity, we hypothesised that leptin-induced cardioprotection is dependent on the presence on the myocardium of functional OB-R. This theory was investigated using hearts harvested from Zucker lean and *fa/fa* rats. Zucker lean rats express the functional OB-R (the results of the gene expression in these animals depends on whether animals are homozygous or heterozygous), whereas *fa/fa* rats possess a mutation in the extracellular domain of the OB-R.

7.4 Experimental Protocol & Materials

7.4.1 Langendorff perfused rat hearts

Hearts from Zucker lean and *fa/*fa rats were excised according to the protocol described in section 3.3. Once mounted on the perfusion apparatus, hearts were stabilised for 40min and then subjected to 35min regional ischaemia

induced by coronary artery ligation. Hearts were then reperfused for 120min to ensure sufficient washout of enzymes and cofactors had occurred to enable accurate staining of infarcted and viable tissue (section 3.3). Hearts from both Zucker lean and *fa/fa* rats were reperfused in the presence and absence of leptin. The groups examined were:-

Zucker lean (n=6) - no drug (control)

Zucker lean (n=6) - leptin administered 5min before reperfusion

Zucker fatty (n=6) – no drug (control)

Zucker fatty (n=6) – leptin administered 5min before reperfusion

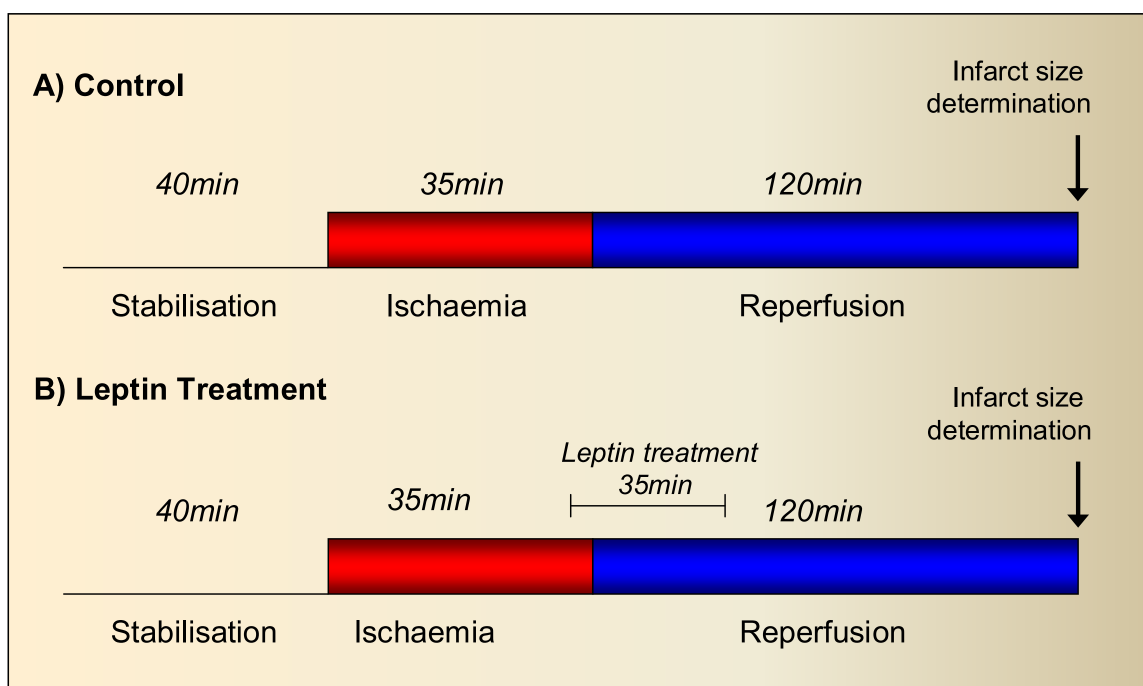


Figure 7.4.1 – Protocol for investigating the cardioprotective effects of leptin in Langendorff perfused hearts from Zucker lean and *fa/fa* rats. (A) Control-perfused with no drug. (B) Leptin-treatment given from 5min prior to and until 30min into the reperfusion-phase.

7.4.2 Western blotting

Hearts from Zucker lean and *fa/fa* rats were extracted according to the protocol described in section 3.3. Hearts were perfused on the Langendorff apparatus for 40min to allow stabilisation and then subjected to 35min regional ischaemia via coronary artery ligation. Following ischaemia hearts were reperfused for a total of 15min in the presence and absence of leptin, after which time the ventricular tissue was harvested, snap-frozen and stored for subsequent Western blot analysis (see section 3.5). Individual groups were as follows:

Zucker lean (n=7) – no drug (Control)

Zucker lean (n=7) – leptin (10nM)

Zucker fatty (n=7) – no drug (Control)

Zucker fatty (n=7) – leptin (10nM)

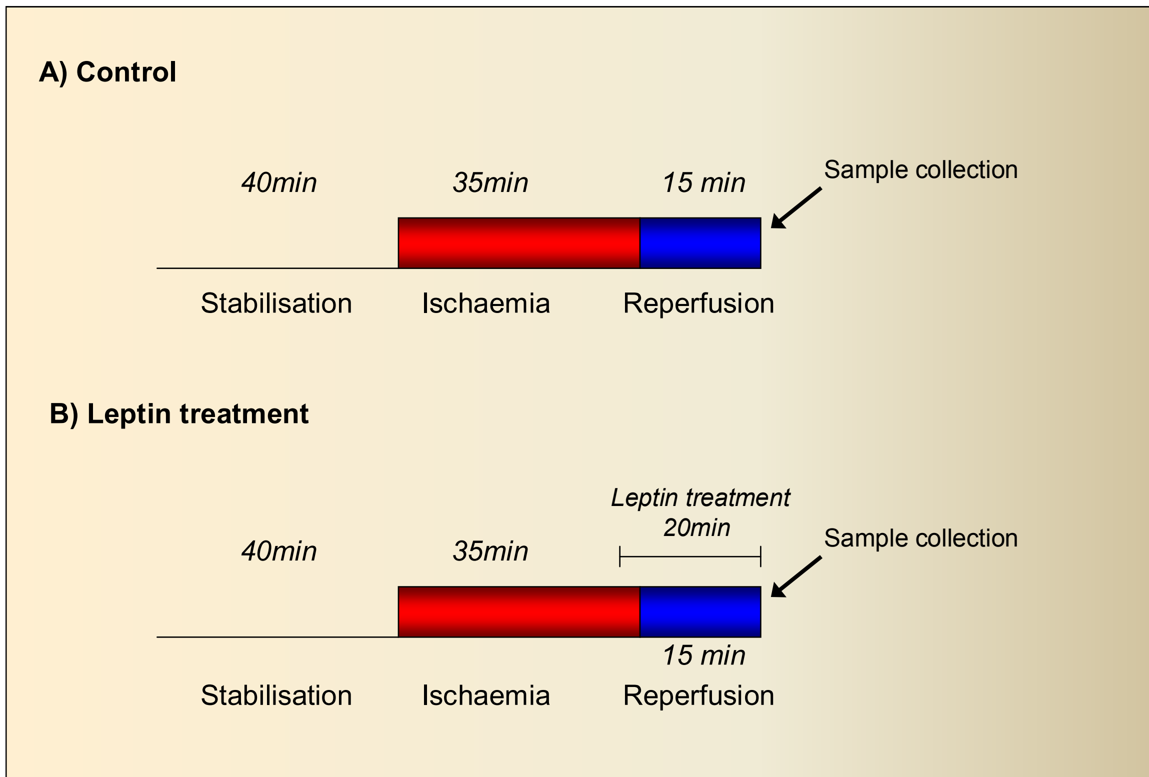


Figure 7.4.2 – Protocol for the collection of Western blot samples from Zucker lean and *fa/fa* hearts subjected to I/R injury. (A) Control hearts were subjected to 40min stabilisation, 35min ischaemia and 15min reperfusion. (B) Leptin-treated hearts subjected to 40min stabilisation, 35min ischaemia and 15min reperfusion with 10nM leptin. Leptin treatment began 5min before the commencement of reperfusion and continued until the point at which the heart was snap-frozen.

7.5 Results

7.5.1 Langendorff perfused rat hearts

7.5.1.1 Body weights and haemodynamic data for the animals/hearts in the different treatment groups

Groups	Body Weight (g)	Ventricular Volume (mm^3)	Risk Volume (%)
Zucker lean (n=5)	329.5±32.8 *	1771.2±256.2	49.5±3.5
Zucker lean + leptin (10nM) (n=6)	361.1±17.3 †	1870.5±342.2	53.9±4.5
fa/fa (n=7)	511.6±14.3 *	1910.4±287.2	51.2±3.2
fa/fa + leptin (10nM) (n=6)	514.9±22.7 †	1852.2±245.2	50.1±4.2

Table 7.5.1 – Mean body weights, ventricular volumes and risk zones for the different treatment groups. Data expressed are as mean±s.e.m.

Animals were weighed prior to the excision of hearts for Langendorff perfusion. Table 7.5.1 demonstrates that there is a significant difference (*/†=p<0.0001) in body weight between the Zucker lean and *fa/fa* groups. The increase in body weight seen in the *fa/fa* rat is directly related to the OB-R mutation which leads to dysfunctional energy homeostasis. Interestingly, despite the increase in body weight in *fa/fa* animals no significant differences in ventricular volume or risk zone volume were apparent between the groups.

7.5.1.1.1 Coronary flow rate (ml/min)

	Stabilisation (ml/min)				Ischaemia (ml/min)			Reperfusion (ml/min)						
Time(min) / **Groups**	**10**	**20**	**30**	**40**	**5**	**15**	**30**	**5**	**15**	**30**	**45**	**60**	**90**	**120**
Zucker lean (n=5)	16.8 ±2.1	16.0 ±2.1	15.5 ±2.1	15.2 ±2.1	7.5 ±1.6	8.3 ±1.6	7.6 ±1.4	13.7 ±0.9	12.6 ±0.8	12.3 ±0.9	11.8 ±0.6	11.2 ±0.7	10.0 ±1.2	9.5 ±1.1
Zucker lean + leptin(10nM) (n=6)	15.5 ±1.6	14.3 ±1.7	13.0 ±1.4	13.0 ±1.8	7.0 ±1.1	7.5 ±0.9	7.1 ±1.0	12.8 ±1.7	12.7 ±1.3	11.0 ±1.3	10.0 ±0.9	9.3 ±0.9	7.8 ±0.6	6.8 ±0.9
fa/fa (n=7)	13.0 ±1.7	12.6 ±1.3	12.2 ±1.0	12.2 ±1.0	6.4 ±0.7	6.5 ±0.9	6.2 ±0.7	11.2 ±0.9	10.8 ±1.4	9.3 ±0.6	7.8 ±0.7	6.8 ±0.7	6.0 ±0.5	5.4 ±0.5
fa/fa + leptin (10nM) (n=6)	14.6 ±1.0	13.8 ±1.0	12.6 ±1.2	12.2 ±1.3	6.7 ±0.9	7.0 ±0.8	7.0 ±0.8	16.1 ±2.0	15.8 ±2.5	13.4 ±1.7	11.2 ±1.4	9.6 ±1.2	8.0 ±1.5	7.0 ±1.3

Table 7.5.2 – Coronary flow rate (ml/min) of each group at various time-points during the ischaemia/reperfusion protocol.

7.5.1.1.2 Rate pressure product (x 103mmHg/min)

	Stabilisation (x 10^3mmHg/min)				**Ischaemia (x 10^3mmHg/min)**			**Reperfusion (x 10^3mmHg/min)**						
Time(min) / Groups	**10**	**20**	**30**	**40**	**5**	**15**	**30**	**5**	**15**	**30**	**45**	**60**	**90**	**120**
Zucker lean (n=5)	35800 ±4582	33850 ±4819	30305 ±3838	28570 ±4215	15988 ±3141	18970 ±3615	16540 ±3285	17280 ±2893	17200 ±3180	15710 ±2750	15475 ±3016	13320 ±3014	10650 ±1894	12325 ±1512
Zucker lean + leptin(10nM) (n=6)	35781 ±2128	34550 ±2223	33313 ±1681	30653 ±2526	18219 ±2138	19371 ±2090	19956 ±1482	22821 ±2213	24900 ±1983	25831 ±1700	24888 ±1733	23075 ±1391	17963 ±1228	16629 ±1184
fa/fa (n=7)	44593 ±2617	34693 ±3437	29207 ±2701	29464 ±3144	13336 ±2128	15714 ±2603	16443 ±2499	17500 ±3643	20921 ±2544	21367 ±1573	19570 ±1742	17983 ±999	16214 ±1587	13139 ±1820
fa/fa + leptin (10nM) (n=6)	38686 ±3836	37843 ±3827	39850 ±4681	38633 ±4017	10543 ±1970	16264 ±2105	16629 ±2540	25050 ±580	20704 ±2254	19907 ±1880	18271 ±1439	18732 ±1332	17451 ±1215	14775 ±1756

Table 7.5.3 - Rate Pressure Product (x 103mmHg/min) of each group at various time-points during the ischaemia/reperfusion protocol.

The haemodynamic data obtained did not reveal any significant differences between the groups during the stabilisation period. By contrast, functional recovery, as indicated by the rate pressure product measurements, occurring during the reperfusion-phase appeared to be enhanced in hearts from Zucker lean animals when treated with leptin (n=6) as compared with hearts from *fa/fa* (n=7) (Figure 7.5.1).

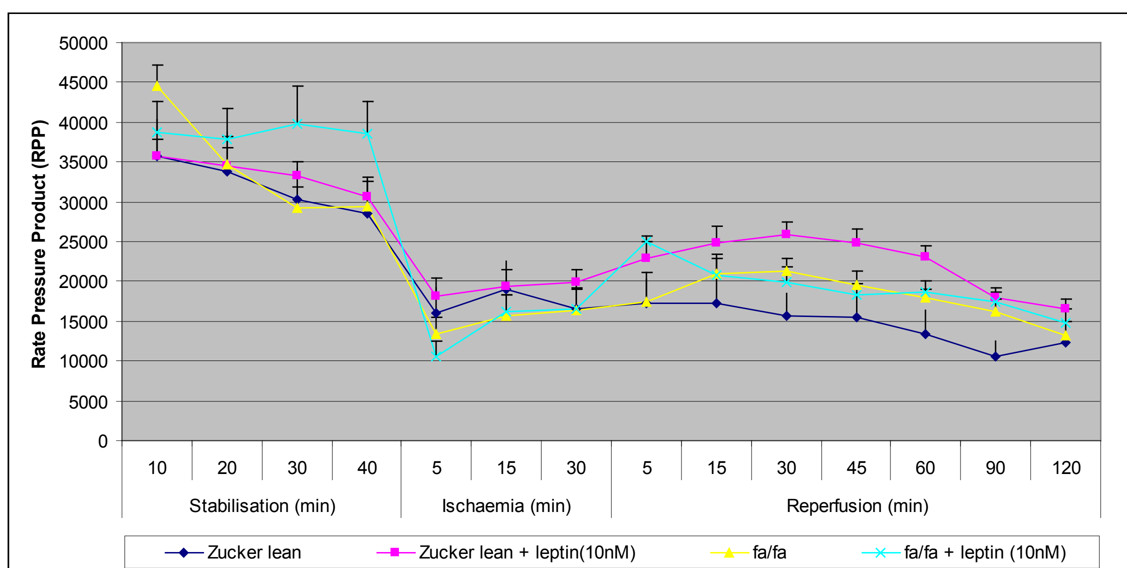


Figure 7.5.1 – Haemodynamic data for hearts in the Zucker lean and *fa/fa* treatment groups.

7.5.1.2 Exclusions

Two hearts was excluded from the Zucker lean group because the risk zones were too small. One heart was excluded from the Zucker lean + leptin group due to low coronary flow. One heart was excluded from the *fa/fa* group because of excessive ventricular fibrillation.

7.5.1.3 Effect of leptin on infarct size in hearts from **Zucker lean** *and* **Zucker fatty** *animals*

Leptin (10nM), administered at reperfusion, substantially reduced infarct size in hearts from Zucker lean rats, which possess at least one functional copy of the OB-Rb gene, whilst hearts from *fa/fa*, which lack a functional OB-Rb, did not exhibit statistically significant decreases in infarct size when treated with leptin (10nM) (Figure 7.5.2). Thus, infarct size in leptin-treated Zucker lean rat hearts was 25.2±3.7% (n=6) as compared with 53.9±11.3% (n=5) in control (untreated) Zucker lean rat hearts ($p<0.05$), whilst infarct sizes for leptin-treated and control *fa/fa* hearts were 40.9±5.5% (n=6) and 50.4±5.0% (n=7), respectively. These data compare with infarct sizes of 32.4±3.9% and 53.2±3.2% ($p<0.01$) for leptin-treated and control hearts from Wistar animals (Figure 7.5.2). Regarding the data obtained with fa/fa hearts, no significant reduction in infarct size was observed.

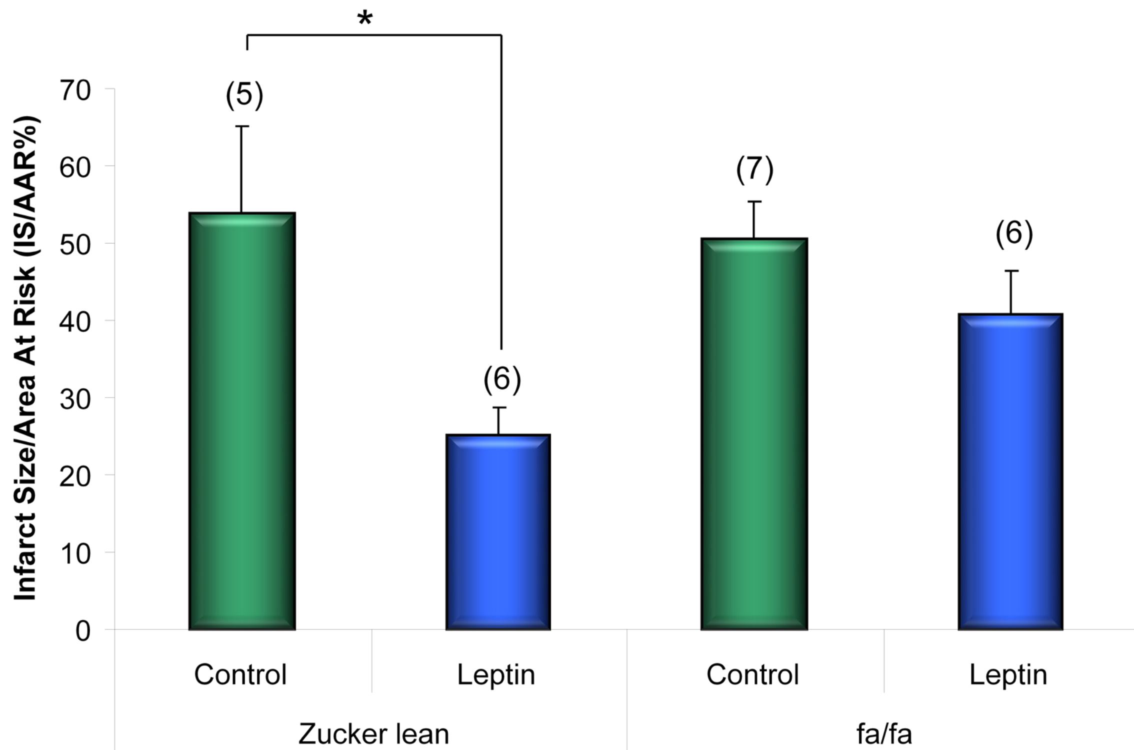


Figure 7.5.2 – Effect of leptin (10nM), perfused during reperfusion (120 min), on infarct size (as a percentage of the area at risk, IS/AAR%) in isolated hearts from Zucker lean and *fa/fa subjected to I/R*. Values are expressed as mean ± s.e.m of 5-7 experiments (** P<0.01) *(one-way ANOVA)*.

7.5.2 Western blotting

7.5.2.1 Body weights for the animals in the different treatment groups

Group	Body Weight (g)
Zucker lean – no drug (n=5)	340.4±8.6
Zucker lean – Leptin (n=5)	344.0±6.0
fa/fa – no drug (n=5)	497.6±6.0
fa/fa – leptin (n=5)	487.4±11.1

Table 7.5.4 – Mean body weights for the animals different treatment groups. Data are presented as mean±s.e.m.

7.5.2.2 Exclusions

There were no exclusions from the treatment groups.

7.5.2.3 The influence of leptin on Akt/Ser-473 phosphorylation in Zucker lean and fa/fa rats

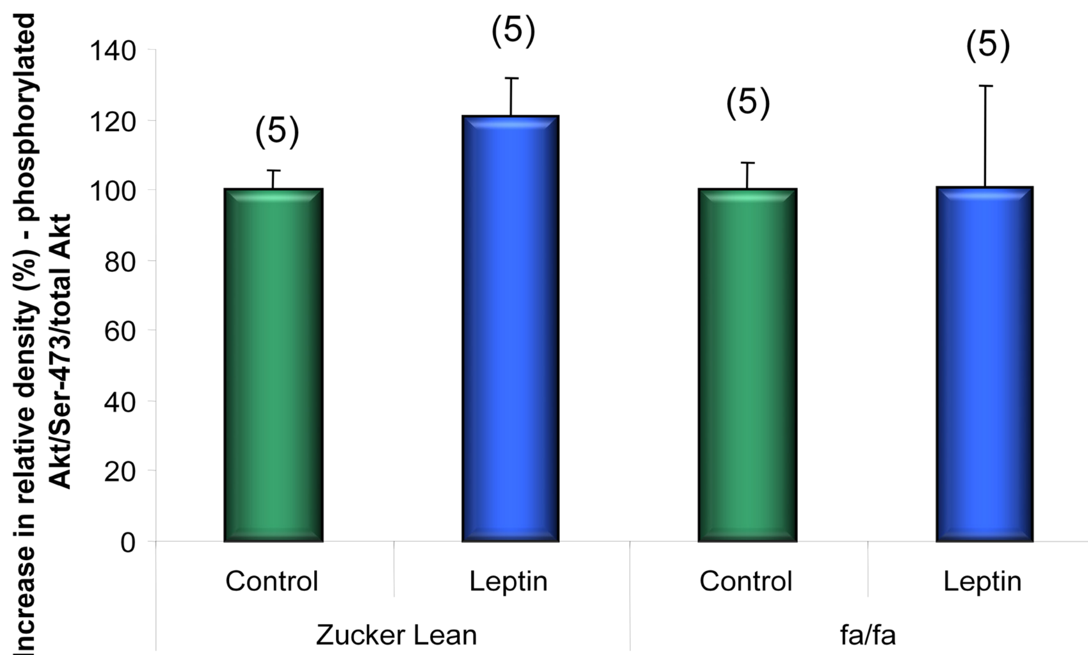


Figure 7.5.3 – Akt/Ser-473 phosphorylation in hearts from Zucker lean and *fa/fa* rats following the administration of leptin. Total and phosphorylated Akt/Ser-473 levels were determined in extracts derived from rat hearts subjected to I/R injury in the presence and absence of leptin (10nM). Data were calculated as the ratio of phosphorylated to total protein relative densitometry values normalised to control (untreated, 100%). Data are presented as the mean ± s.e.m of relative densitometry values in arbitrary units (*=p< 0.05, n = 5) *(one-way ANOVA)*.

In hearts isolated from Zucker lean animals leptin (n=5) elicited a small 20% increase in Akt/Ser-473 phosphorylation, as compared to control (n=5), however this change was insignificant. In the hearts from *fa/fa* animals leptin (n=5) had no effect on Akt/Ser-473 phosphorylation compared to control (n=5) (untreated) hearts.

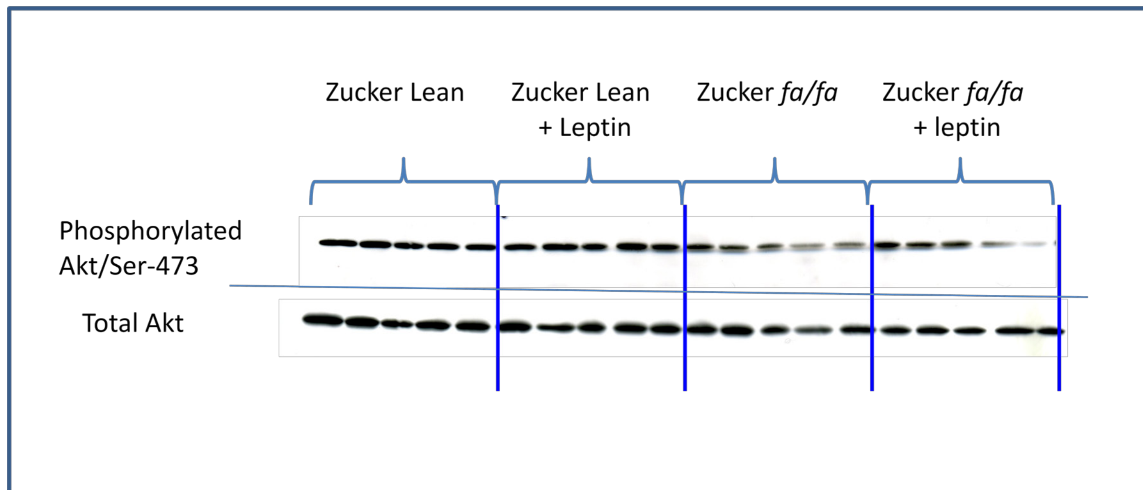


Figure 7.5.4 – Western blots showing phosphorylated Akt/Ser-473 and total Akt levels in tissue taken from Zucker lean and Zucker *fa/fa* hearts treated with and without leptin (10nM).

7.5.2.4 The influence of leptin on Akt/Thr-308 phosphorylation in Zucker lean and fa/fa rat hearts

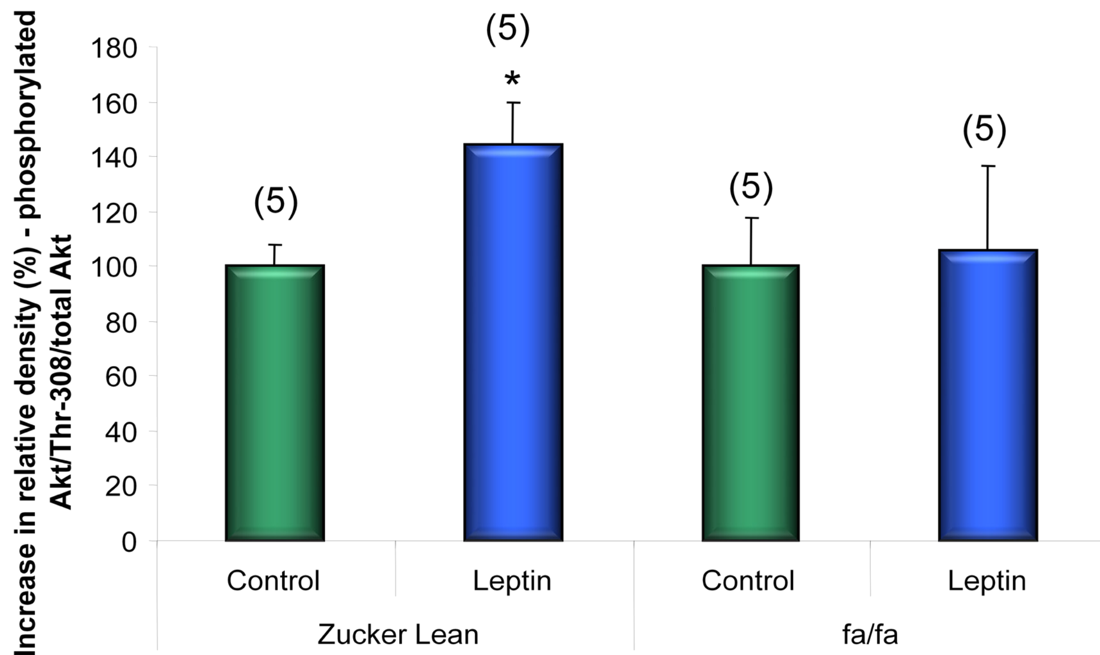


Figure 7.5.5 – Leptin-induced phosphorylation of Akt/Thr-308 in Zucker lean and *fa/fa* rat hearts. Total and phosphorylated Akt/Thr-308 levels were determined in extracts derived from rat hearts subjected to I/R injury in the presence and absence of leptin (10nM). Data were calculated as the ratio of phosphorylated to total protein relative densitometry values normalised to control (untreated, 100%). Data are presented as the mean ± s.e.m (*=$p< 0.05$, n = 5) *(one-way ANOVA)*.

In isolated *fa/fa* hearts treated with leptin (n=5), no change was observed in the phosphorylation status of Akt/Thr-308, as compared to control (n=5). However, in hearts from Zucker lean animals leptin (n=5) elicited a significant 43.9% ($p<0.05$) increase in Akt/Thr-308 phosphorylation when compared to control (n=5).

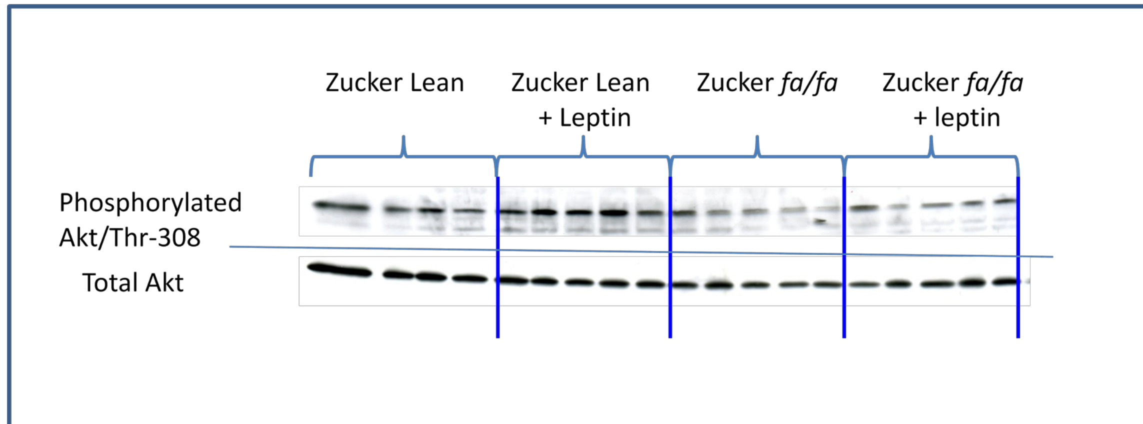


Figure 7.5.6 - Western blots showing phosphorylated Akt/Thr-308 and total Akt levels in tissue taken from Zucker lean and Zucker *fa/fa* hearts treated with and without leptin (10nM).

7.5.2.5 *The influence of leptin on p44/42 MAPK phosphorylation in Zucker lean and fa/fa rat hearts*

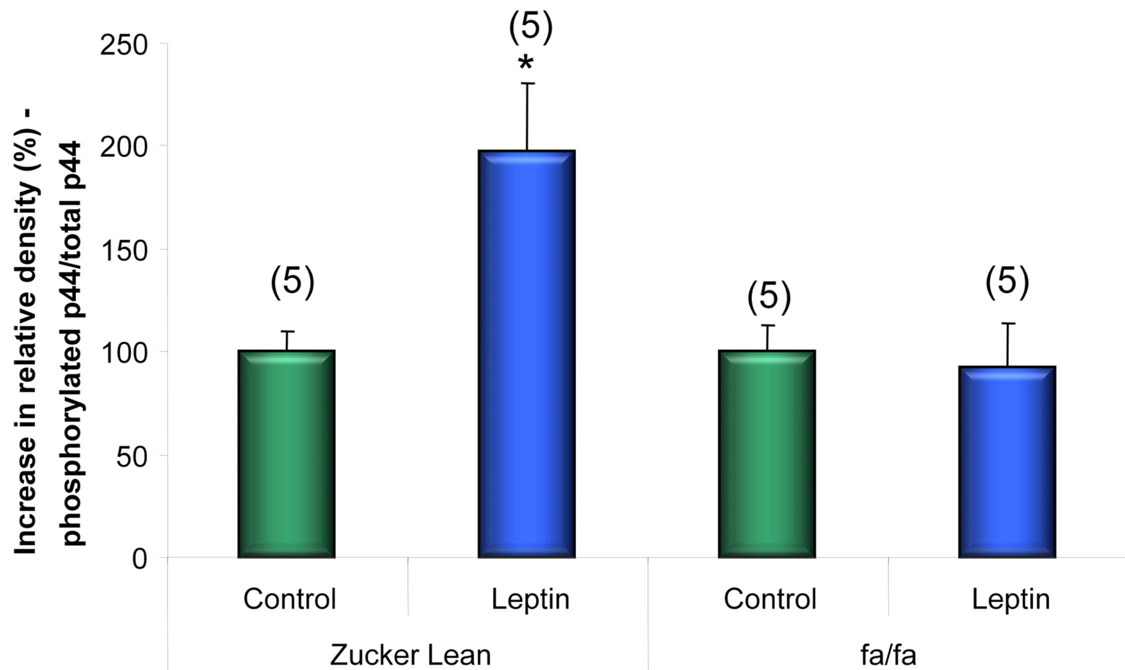


Figure 7.5.7 – Leptin-induced phosphorylation of p44 MAPK in Zucker lean and *fa/fa* rat hearts. Total and phosphorylated p44 MAPK levels were determined in extracts derived from hearts subjected to I/R injury in the presence and absence of leptin (10nM). Data were calculated as the ratio of phosphorylated to total protein relative densitometry values normalised to control (untreated, 100%). Data are presented as the mean ± s.e.m (*=p< 0.05, n = 5) *(one-way ANOVA)*.

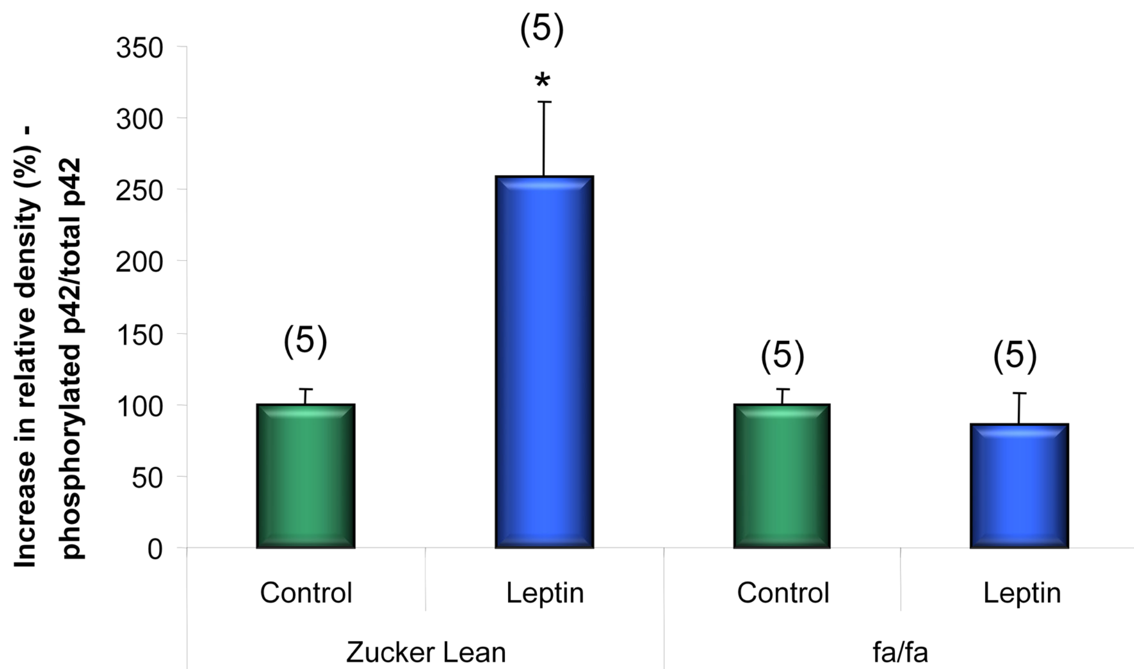


Figure 7.5.8 – Leptin-induced phosphorylation of p42 MAPK in Zucker lean and *fa/fa* rat hearts. Total and phosphorylated p42 MAPK levels were determined in extracts derived from hearts subjected to I/R injury in the presence and absence of leptin (10nM). Data were calculated as the ratio of phosphorylated to total protein relative densitometry values normalised to control (untreated, 100%). Data are presented as the mean ± s.e.m (*=p< 0.05, n = 5) *(one-way ANOVA)*.

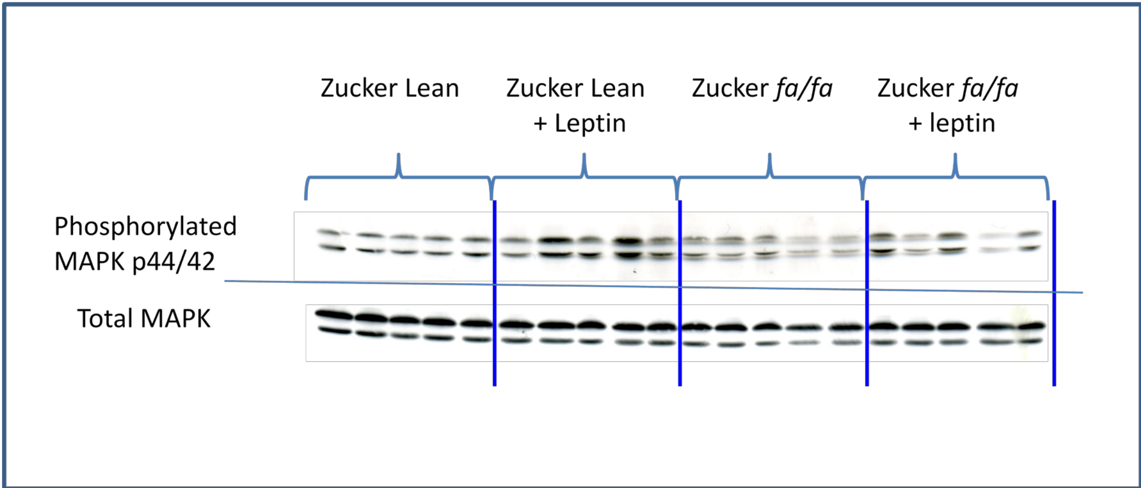


Figure 7.5.9 - Western blots showing phosphorylated MAPK p44/42 and total MAPK levels in tissue taken from Zucker lean and Zucker *fa/fa* hearts treated with and without leptin (10nM).

In hearts isolated from Zucker lean rats leptin (n=5) elicited significant increases in both p44 and p42 MAPK phosphorylation at 15min into the reperfusion-phase (97.1% and 158.1% respectively, $p<0.05$), when compared to untreated control hearts (n=5) (Figure 7.5.5 & Figure 7.5.6). By contrast, in hearts isolated from *fa/fa* leptin (n=5) had no effect on p44 or p42 MAPK phosphorylation when compared to untreated control hearts (n=5) (Figure 7.5.5 & Figure 7.5.6).

7.6 Discussion

As mentioned previously, the Zucker lean rat expresses the normal functional OB-R gene, whilst the *fa/*fa rat expresses a mutated non-functional form of the gene (Chen & Wang 2005). Absence of the functional OB-R results in the animal being unable to maintain normal energy homeostasis (Chen & Wang 2005). Consequently, this has made the Zucker rat an invaluable tool for investigating OB-R signalling with respect to metabolic processes and disease (Chen & Wang 2005). Six isoforms of the leptin receptor have been identified, OB-Ra, OB-Rb, OB-Rc, OB-Rd, OB-Re and OB-Rf (Lee et al. 1996). The available literature concerning leptin signalling indicate that in order to elicit a full cellular response interaction between the adipocytokine and the long form of the leptin receptor (OB-Rb) is required (Fruhbeck 2006). The OB-Ra isoform, which occurs in the heart at levels that exceed those of the OB-Rb, also may play a role in physiological mechanisms, although it has been shown to elicit weaker responses than those mediated by OB-Rb (Banks et al. 2000).

The involvement of the OB-R in energy homeostasis has been well documented (Ahima 2006; Palkovits 2003). By contrast, its role in myocardial function

remains to be fully elucidated. Thus, in the present study the Zucker rat was employed to investigate further the mechanisms underlying leptin-induced cardioprotection. Since leptin has been shown to be cardioprotective against I/R injury (Smith et al., 2006) and given that the OB-R has been demonstrated to be present in cardiac tissue, it was hypothesised that myocardial sensitivity to leptin may be related to adiposity and the presence of functional OB-R in the heart.

7.6.1 Leptin protects against myocardial I/R injury in Zucker lean rats but not in *fa/fa animals*

Leptin significantly protected hearts from Zucker lean animals against I/R injury and to similar extents to those seen with hearts from Wistar animals (see Chapter 4). Both the Zucker lean rat and the Wistar rat express functional OB-Rb receptors. By contrast, hearts from *fa/fa* animals, which do not express the functional OB-R, did not respond significantly to leptin treatment. It should be noted that there appears to be a small trend towards infarct size reduction with leptin treatment in the *fa/fa* heart. This raises the possibility that leptin may be producing an effect in the heart via a mechanism independent of the OB-R. However, assuming that the findings of the present study are valid, one might suggest that these data reflect the general lack of sensitivity to cardioprotective agents in these animals. Nevertheless, evidence opposing this view has been presented, various agents having been found to reduce infarct size in the *fa/fa* heart. Hexarelin, a synthetic enkephalin-derived peptide was found to be protective in hearts from both Zucker lean and *fa/fa* rats (Gennaro-Colonna et al. 2000). Similarly, rosiglitazone, which protects the myocardium in normal

animals (Gonon et al. 2007), was found to reduce infarct size in Zucker lean and *fa/fa* animals via Akt activation (Yue et al. 2005).

7.6.2 Leptin stimulates Akt/Thr-308 phosphorylation in Zucker lean hearts but not in *fa/fa* hearts

Contrasting with the results obtained with hearts from Wistar animals (see Chapter 5), leptin failed to stimulate a significant increase in Akt/Ser-473 phosphorylation in hearts from Zucker lean animals. This observation may relate to the fact that some Zucker lean animals possess only one copy of the functional OB-R gene, i.e. depending on whether they are heterozygous or homozygous for the gene, resulting in decreased expression of functional receptors, and, consequently decreased leptin signalling. The phosphorylation of Akt/Ser-473 in hearts from *fa/fa*, which do not express functional OB-R was also not altered. Contrasting with the results obtained for Akt/Ser-473, a significant increase in the phosphorylation of Akt/Thr-308 was observed in Zucker lean hearts treated with leptin. Again, however, no increases were seen in *fa/fa* hearts. Thus, results obtained with regard to leptin-induced phosphorylation of the Akt sites with Wister and Zucker lean hearts appeared to be conflicting.

Current literature suggests that in the process of Akt activation the Akt/thr308 site is the primary site of phosphorylation, which is followed by phosphorylation of the Akt/ser473 site (Balendran et al. 1999; Kumar & Madison 2005). These phosphorylation reactions are catalysed by the enzymes PDK1 and PDK2, respectively (Balendran et al., 1999). The aforementioned ambiguity observed

in the data presented in this chapter, therefore, may reflect differences between the Wistar and Zucker lean rat hearts with respect to the time-points at which PDK1 and PDK2-catalysed phosphorylation of the Akt/thr308 and Akt/ser473 sites occurred. Hence, the time at which the Akt/thr308 site was phosphorylated in the Wistar rat heart may be earlier than that of the Zucker lean, and therefore missed by our experiments. Similarly, the window of maximal activation of Akt/ser473 may have been later in the Zucker lean than the Wistar, and therefore possibly missed in the tissue samples. Nevertheless, overall the phosphorylation of Akt was increased in the hearts from both types of animal when treated with leptin, indicating that Akt activation may be involved in the mechanism underlying leptin-induced cardioprotection.

7.6.3 Leptin stimulates phosphorylation of p44/42 MAPK in hearts from Zucker lean animals but not *fa/fa* animals

Significant increases in the phosphorylation of p44 and p42 MAPK were observed in hearts from Zucker lean animals treated with leptin (Figure 7.5.5 & Figure 7.5.6). Similar levels of phosphorylation were observed in hearts from Wistar animals. By contrast, no significant changes in the phosphorylation states of either p44 or p42 MAPK were seen with hearts from *fa/fa* animals treated with leptin. These results again reflect the fact that Zucker lean and Wistar animals produce functional OB-Rb and *fa/fa* do not.

7.6.4 Conclusion

Data in this chapter suggests that the presence of the OB-R receptor is a prerequisite for leptin-induced cardioprotection. With reference to the current literature, that indicate that the long OB-Rb isoform is required for full activation

of leptin signalling pathways, one could extrapolate that leptin-induced cardioprotection seen in Zucker lean animals is via the OB-Rb. However, one cannot exclude the possibility that alternative OB-R isoforms may play a role in cardioprotective mechanisms. It is known, for example, that cardiomyocytes express the OB-Ra isoform, which is also reported to be non-functional in the Zucker rat [Phillips et al., 1996]. The OB-Ra have been demonstrated to play various roles in cellular processes including ventricular hypertrophy (Rajapurohitam et al. 2003; Phillips et al. 1996). Future studies utilising specific receptor blockers or antibodies directed at individual leptin receptors, which have only recently become available, will allow the roles of the different OB-R to be elucidated in greater detail. Whatever the precise mechanisms involved, the fact remains that our data are consistent with activation of myocardial leptin receptors being involved in leptin-induced myocardial protection. Of the various leptin receptor-isoforms the OB-Rb is the most likely candidate, given that it is generally accepted that this isoform plays the primary functional role with respect to full leptin-induced cell signalling (Banks et al. 2000).

7.6.5 Limitations

It should be noted that in addition to being the obese the *fa/fa* rat is hyperglycaemic. It has been demonstrated that in order to precondition the diabetic myocardium the IPC stimulus must be increased over that needed to protect the non-diabetic myocardium (Tsang et al. 2005). This data, therefore, indicates that the diabetic myocardium may be compromised with respect to the signalling mechanisms that must be activated to achieve cardioprotection. It

should also be noted, however, that various studies have shown that for a given area at risk the diabetic myocardium is less susceptible to I/R injury than the non-diabetic myocardium (Monteiro et al. 2005). With respect to the data presented in this thesis, the *fa/fa* myocardium may have a lower response to leptin in terms of activating survival signalling pathways and consequently may be more difficult to protect.

CHAPTER 8 - LEPTIN-INDUCED INHIBITION OF MPTP OPENING

8.1 Introduction

In the study described in this chapter, the effects of leptin on MPTP opening were investigated with reference to obesity and OB-R status. Opening of the MPTP during reperfusion of the ischaemic myocardium has been demonstrated to be an important determinant of cell death (Halestrap 2006). Whilst the MPTP remains closed during ischaemia, the conditions that develop intracellularly on reperfusion result in pore opening, ultimately leading to necrosis and apoptosis (Halestrap et al. 1997b). The aforementioned conditions include rectification of low intracellular pH, a decline in ATP levels and increases in intracellular ROS levels (Crompton & Andreeva 1993). Recent studies indicate that inhibition of pore opening may underlie cardioprotection induced by IPC, IPost and various pharmacological agents (Lim et al. 2007a).

The structure of the pore has yet to be fully elucidated. Originally, it was thought to be a multi-component structure incorporating the following proteins adenine nucleotide translocase (ANT), voltage dependent anion channel (VDAC) and cyclophilin D (Halestrap & Brennerb 2003; Vyssokikh & Brdiczka 2003). More recently, however, it has been shown in studies utilising ANT and VDAC knock-out mice that the pore can still be induced to form, perhaps indicating that these components are redundant (Juhaszova et al. 2008; Leung & Halestrap 2008). By contrast, it appears that the cyclophilin D component is vital for MPTP formation and function. This finding is supported by various studies which

demonstrated that MPT can be inhibited by agents that bind to cyclophilin D, such as Cyclosporin A (CsA) and sanglifehrin A (SfA) (Halestrap et al. 1997a; Clarke et al. 2002). In the setting of myocardial I/R injury CsA has been demonstrated to reduce reperfusion induced arrhythmias (Arteaga et al. 1992), normalise ATP/ADP ratios and AMP levels (Griffiths & Halestrap 1993), and limit infarction by up to 50% (Halestrap et al. 1997a). In our laboratory it has been shown that cyclophilin D knockout mice, which are presumed to be dysfunctional with regard to pore formation, are less susceptible to myocardial I/R injury than wild-type animals (Lim et al., 2007). Cardioprotective strategies such as IPC, IPost and diazoxide that protect the myocardium in wild-type animals, interestingly, conferred no protection in cyclophilin D knockout mice (Lim et al., 2007). These data indicate that the pore plays an important role in the underlying mechanisms of both mechanical and pharmacological cardioprotection.

Stimulation of the RISK survival kinases has been linked to inhibition of the pore and cardioprotection. Insulin-induced Akt activation in isolated cardiomyocytes, for example, was associated with a delay in the time to initiation of mitochondrial depolarisation, an indicator of pore opening (Davidson et al. 2006). This delay was abolished by the administration of the Akt inhibitor, LY-294002 (Davidson et al. 2006). However, despite establishing a link between RISK activation and inhibition of the pore, it still remains for the intermediate signalling steps to be elucidated.

Leptin has been reported to delay MPTP opening in cardiomyocytes isolated from Sprague Dawley rats (Smith et al. 2006). In addition, pore opening was found to be abrogated by the administration of LY-294002 or MEK inhibitor 1, inhibitors of Akt and p44/42respectively, providing further evidence for a link between the RISK pathway and the MPTP (Smith et al. 2006). These previous investigations were, however, conducted with lean animals expressing normal OB-R.

8.2 Aim

The aim of the present study, therefore, was to investigate the mechanisms underlying leptin's actions on the pore further. Thus, using cardiomyocytes isolated from *fa/fa*, Zucker lean and Wistar rat hearts, the influence of adiposity and OB-R status on MPTP opening induced by leptin was examined.

8.3 Experimental Protocol & Materials

8.3.1 Cardiomyocyte model of MPTP opening

Adult rat cardiomyocytes were isolated from male *fa/fa*, Zucker lean and Wistar animals and seeded onto 25mm round cover-slips (see section 3.4.1). MPTP opening was examined employing a model of oxidative stress (Davidson et al, 2006). Hence, seeded cardiomyocytes were incubated with the photoactive dye, TMRM, for 15min at room temperature. The cells were then washed with a restoration buffer devoid of TMRM and visualised using confocal microscopy as described in section 3.4.2. TMRM is a lipophilic cation and is therefore taken up by the mitochondria according to their membrane potential (Scaduto, Jr. & Grotyohann 1999). Illumination of the dye with a laser causes the TMRM to

react and produce ROS (Hausenloy et al. 2003). The resulting oxidative stress causes MPTP opening and consequent collapse of the mitochondrial membrane potential, which can be visualised as a wave of depolarisation across the cell (Davidson et al. 2006) (see Figure 8.3.2). The time (seconds) of laser exposure required to trigger the initiation of mitochondrial depolarisation was measured, being related to the quantity of accumulated ROS required to trigger opening of the MPTP (Davidson et al. 2006).

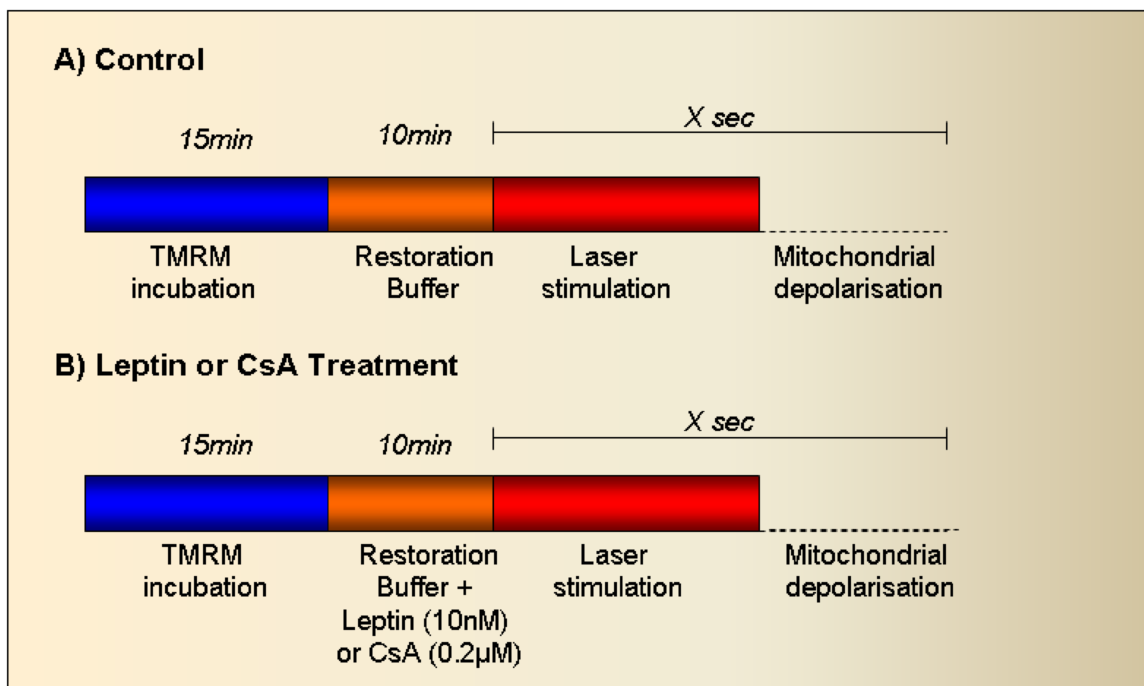


Figure 8.3.1 – Protocol for investigating the effects of leptin (10nM) on MPTP opening. (A) Control cardiomyocytes were incubated in a buffer containing TMRM for 15min and then incubated with a restoration buffer for 10min. (B) Leptin or CsA treated cells, by contrast, were also incubated in a TMRM-containing buffer, but were then incubated in either a restoration buffer containing leptin (10nM) or CsA (0.2µM). The cells were then subjected to laser stimulation until the initiation of mitochondrial depolarisation could be visualized.

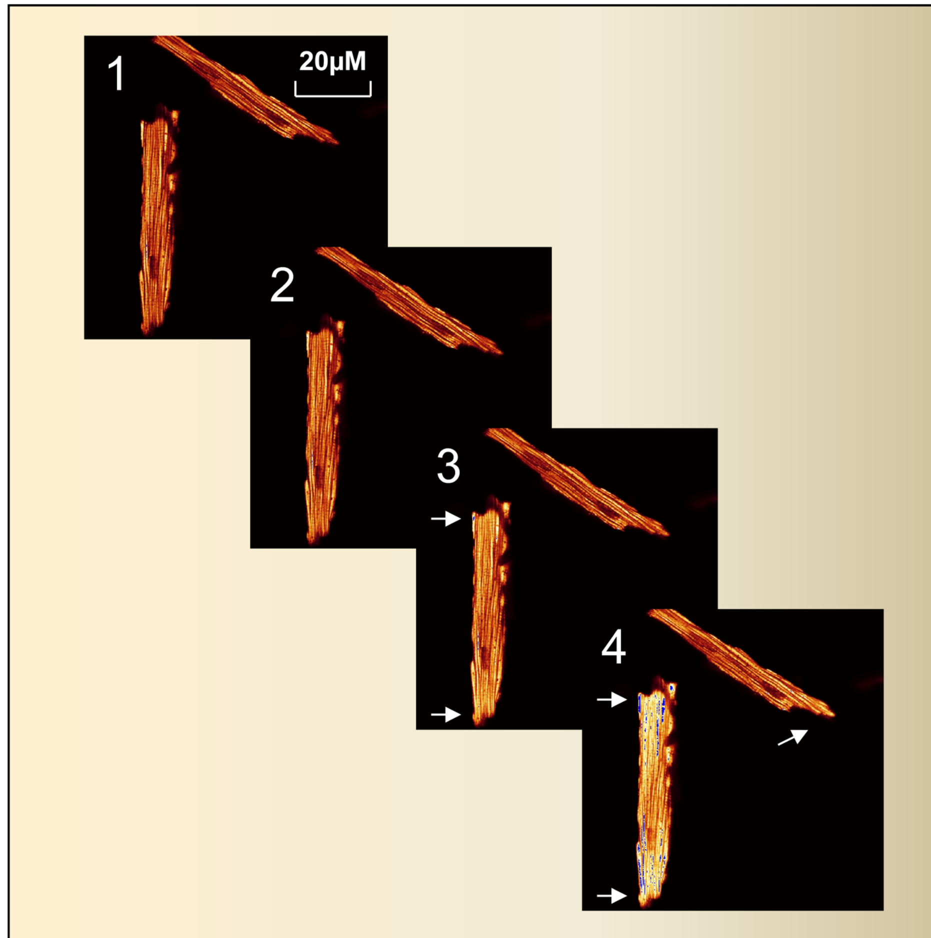


Figure 8.3.2 – Photographs showing the depolarisation of isolated rat cardiomyocytes in response to ROS.

8.3.2 Experimental Protocol for Cardiomyocyte Studies

After TMRM loading cells were randomly assigned to one of the treatment groups below. Cells were incubated for 10min with the drug at room temperature and then subjected to the TMRM-oxidative stress protocol.

Control – incubation in restoration buffer alone.

Leptin – incubation with 10nM leptin.

Cyclosporin A – incubation with 0.2μM CsA, an inhibitor of MPTP opening and used as a positive control.

8.4 Results

8.4.1 Exclusions

A total of seven Wistar rat hearts were used for cardiomyocyte isolation of which 3 were excluded due to poor yields of cells. Six Zucker lean rat hearts were used of which one was excluded owing to poor yields. Similarly, six *fa/fa* hearts were used and one was excluded due to a poor yield.

8.4.2 Effect of leptin on MPTP opening in cardiomyocytes isolated from Wistar, Zucker lean and *fa/fa* hearts

CsA, an established inhibitor of the MPTP, was used as a positive control in all isolated cardiomyocyte experiments. Leptin (10nM) and CsA (0.2μM) delayed the time to MPTP opening in cardiomyocytes isolated from Wistar rat hearts by 43% ($p<0.01$) and 34.5% ($p<0.01$), respectively, as compared with cells treated with vehicle (Fig.6). Similarly, leptin and CsA also delayed the time until MPTP opening in cardiomyocytes isolated from Zucker lean rat hearts i.e. by 30.9% ($p<0.01$) and 28.5% ($p<0.01$). By contrast, whilst cardiomyocytes isolated from fa/fa rat hearts responded to CsA with a delayed time to MPTP opening (+20.3%, $p<0.05$), no changes were observed in leptin-treated cells (Fig.6).

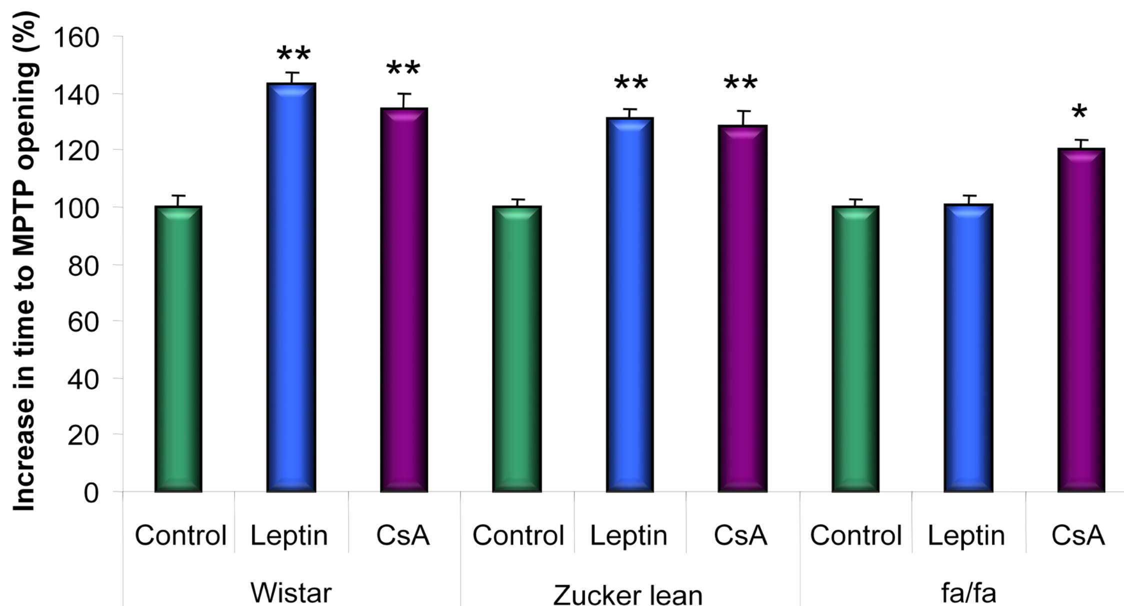


Figure 8.4.1 – MPTP opening in cardiomyocytes isolated from Wistar, Zucker lean and Zucker fatty rat hearts. Data are presented as the percentage increase in time to initiation of MPTP opening, indicated by mitochondrial depolarisation[3]. Values are presented as mean±s.e.m (*=P<0.05, **=P<0.01) and were obtained with a total of 12-20 cells from at least five hearts per group (*Kruskal-Wallis analysis of variance method).*

8.5 Discussion

The MPTP, a non-selective pore that forms in mitochondrial membranes, has been shown to play a key role in the mechanisms leading to cell death in various pathological conditions, including neurodegeneration, brain injury, stroke and myocardial ischaemia reperfusion injury (Youdim et al. 2005; Sullivan et al. 2000; Friberg & Wieloch 2002; Halestrap et al. 2004). Under normal conditions the mitochondrial membrane is only permeable to small ions and metabolites (Halestrap et al. 2002). When the pore forms and opens, however, the free passage of molecules up to 1.5KDa is permitted, leading to mitochondrial

[3] It should be noted that due to large variations in the baseline time to cardiomyocyte depolarisation found on different days, the results were normalised so that the control was equal to 100%. Therefore, any increase in time to depolarisation is reflected by a value over 100%. Raw data are presented in an appendix at the end of this thesis.

uncoupling (Halestrap et al. 2002). Due to increased osmolar load mitochondria swell and, in some cases, cytochrome C is released following mitochondrial membrane rupture (Buki et al. 2000). Once released cytochrome C can bind with the cytosolic protein apaf1 forming an apoptosis-promoting complex known as the apoptosome, which triggers cell death in various tissues (Adrain et al. 2006). Inhibition of the pore, however, both prior to ischaemia and at reperfusion, has been demonstrated to reduce myocardial infarction (Hausenloy et al, 2003).

8.5.1 Leptin delays MPT in Zucker lean and Wistar rat cardiomyocytes but not in *fa/fa* cardiomyocytes

In experiments with cardiomyocytes from Wistar rat hearts leptin was found to delay MPTP opening, as was the case for cells from Zucker lean rat hearts (Chapter 7). These data are, therefore, consistent with the hypothesis that leptin-induced signalling, as indicated by increased Akt, p44/42 phosphorylation (see Chapter 5) and STAT3 (see Chapter 6), converges on the mitochondrion. It should be noted, however, that the cells from Zucker lean rat hearts still responded to leptin despite the apparent lack of Akt/Ser-473 phosphorylation seen in the western blotting studies presented in Chapter 7. This can be reconciled in two ways. Firstly one should note that whilst phosphorylation was not seen at this time-point in reperfusion, i.e. 15 min, the data does not conclusively show that significant phosphorylation of Akt/Ser-473 does not occur at all. For example, phosphorylation may have occurred prior to sampling and therefore may have been missed in our investigation. Secondly, if Akt/Ser-473 is not activated in Zucker lean hearts protection may occur via p44/42 MAPK

and STAT3 pathways. By comparison, it appears that these pathways have not been activated by leptin at all in the cells from *fa/fa* hearts. Therefore, further investigation is needed to clarify the role of Akt in this process. Nonetheless, this data shows that leptin signalling, be it via the RISK or JAK/STAT pathway is linked mitochondria, Figure 8.5.1 depicts this hypothesis. The fact that leptin did not produce a comparable effect on cardiomyocytes from *fa/fa* rats indicates that leptin-induced signalling with respect to MPTP opening, is defective in these animals, thus providing further support for the notion that the OB-R plays a key role in leptin-mediated protection. Cyclosporin A delayed MPT in cardiomyocytes isolated from all three types of animal (Figure 8.4.1). It should be noted, however, that the percentage delay produced by CsA in *fa/fa* cardiomyocytes appeared to be slightly less than that in cardiomyocytes from Wistar and Zucker lean animals. This could indicate that *fa/fa* cardiomyocytes possess a generalised impairment with regard to mitochondrial function. This idea is supported by data indicating that mitochondrial apoptotic signalling is increased in *fa/*fa cardiac muscle (Lu et al. 2007; Peterson et al. 2008). Further investigation, therefore, is needed to establish the mitochondrial functional capacity in cardiomyocytes from *fa/fa* hearts as compared with cells from normal hearts.

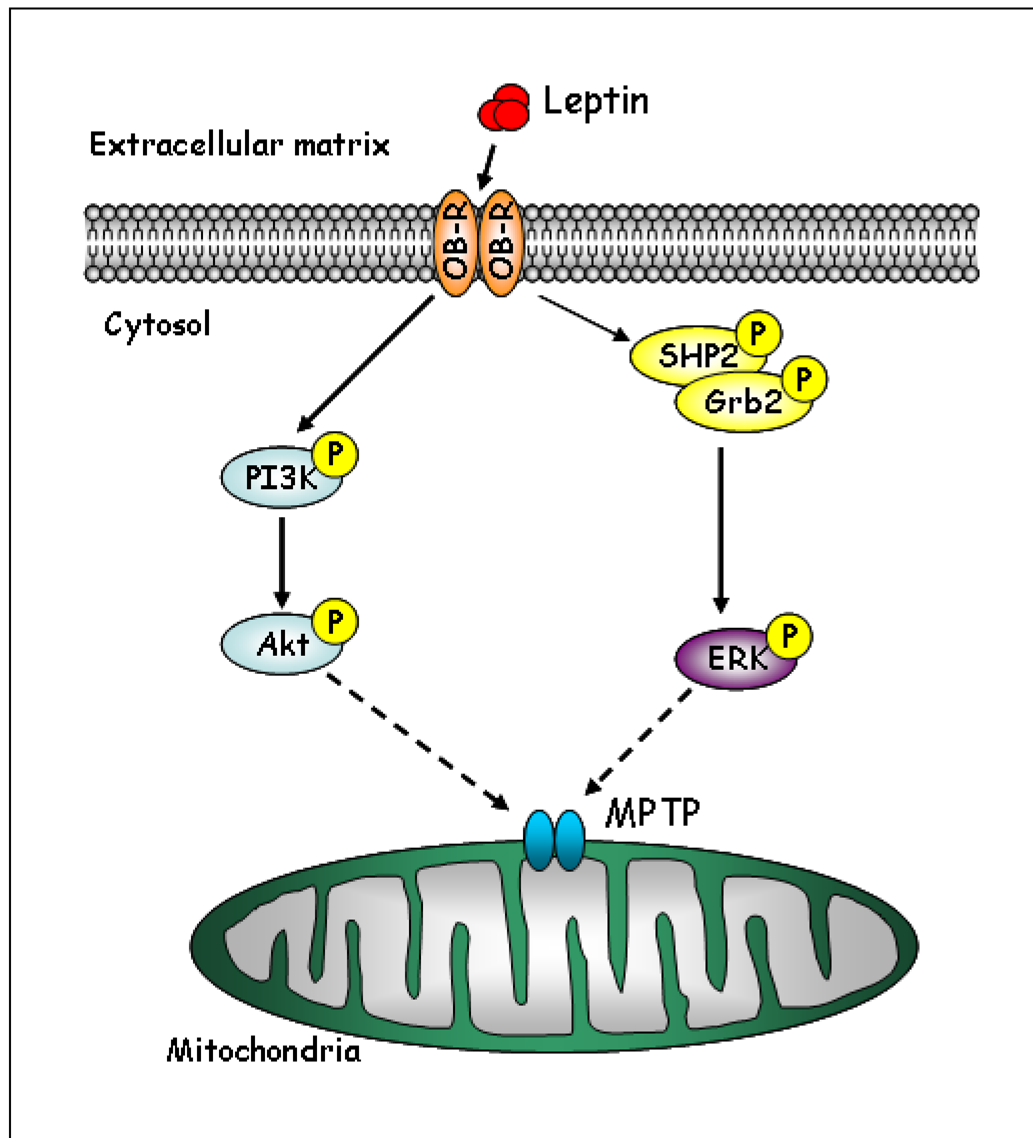


Figure 8.5.1 – Proposed signalling mechanisms underlying leptin-induced myocardial protection. Reperfusion of the myocardium after a period of ischemia results in MPTP opening and subsequent mitochondrial uncoupling, ATP consumption and, ultimately, cell death. It is hypothesized that binding of leptin by leptin receptors (OB-R) present on the cardiomyocyte leads to the phosphorylation of PI3K and subsequently Akt, and/or phosphorylation of Src homology 2 (SHP2) followed by growth factor receptor-bound 2 (Grb2) and p44/42 MAPK extracellular signal-regulated (ERK 1/2). Signalling pathways downstream of these cascades then converge on the mitochondrion to inhibit MPTP opening and, as a consequence, prevent mitochondrial uncoupling and cell death. If OB-R are absent or defective, these mechanisms would not be operative with the consequence that cell death would proceed unimpeded.

CHAPTER 9 - LEPTIN-INDUCED MYOCARDIAL PROTECTION IN AN *IN VIVO* RAT MODEL OF I/R INJURY

9.1 Introduction

In addition to its actions in the central nervous system with respect to the regulation of appetite and energy balance, leptin has been found to produce multiple effects in the peripheral tissues (Lee et al. 2008; Matarese et al. 2007*a*; Park et al. 2001; Harvey & Ashford 2003). The administration of leptin *in vivo*, for example, has revealed that leptin is involved in a wide range of processes including angiogenesis (Anagnostoulis et al. 2008; Liapakis et al. 2008), neurogenesis (Garza et al. 2008) and lung development (Kirwin et al. 2006). Thus, it would appear that leptin functions as a mitogen through the activation of signalling pathways that have been implicated in protection against I/R injury. Leptin has been shown to influence cardiovascular function (see section 1.6.7). It has, for example, been found to stimulate NO production in vascular endothelial cells (Winters et al. 2000) and to increase fatty acid oxidation in the heart (Atkinson et al. 2002). These observations, coupled with reports that leptin acts through signalling pathways implicated in tissue protection led to the original hypothesis that leptin may act as a cardioprotective agent (Smith et al. 2006). Leptin is a pro-inflammatory cytokine that has been linked to atherogenesis (Loffreda et al. 1998), heart failure (Abe et al. 2007) and myocardial hypertrophy (Kartal et al. 2008; Purdham et al. 2008). It is, however, important when considering the multiple actions of leptin to be aware of the fact that factors such as the concentrations of leptin employed, and whether treatment is given acutely or chronically can strongly influence outcomes. In

heart failure, for example, leptin secretion and signalling are increased after coronary artery ligation (CAL), resulting in activation of the JAK/STAT pathway and improved cardiac function (McGaffin et al. 2008). By contrast, Purdham and colleagues demonstrated that the administration of leptin receptor antibodies, which block leptin signalling, following myocardial infarction decreased cardiac hypertrophy and improved function (Purdham et al. 2008). It appears, therefore, that the administration of leptin at specific time-points in the development of heart failure may be protective. Indeed, this is borne out by the observation that the administration of leptin at reperfusion in the isolated perfused mouse heart reduces myocardial I/R injury (Smith et al. 2006).

Whilst the Langendorff isolated perfused heart is a convenient model for investigating the myocardial actions of drugs to the exclusion of humoral factors that may interfere with drug-induced responses, these same “interfering factors” may play important roles in modulating drug-induced actions. Leptin is functionally highly pleiotropic and, therefore, having shown that it reduces I/R injury *in vitro* it is important that the factors influencing leptin-induced cardioprotection are identified so that their involvement in patients may be evaluated. In the present study, therefore, the effects of leptin on myocardial I/R injury in an *in vivo* rat model were investigated so that information that may prove relevant with regard to its clinical application might be obtained.

9.2 Aim

The aim in this chapter was to establish if leptin protects against myocardial I/R injury in an *in vivo* rat model.

9.3 Experimental Protocol & Materials

9.3.1 Materials

Sodium pentobarbital was obtained from Sagatal-Rhone, Merieux. Heparin, used to prevent thrombosis, was obtained from Multiparin CP Pharmaceuticals Ltd. Leptin and triphenyltetrazolium chloride (TTC) were provided by Sigma-Aldrich Company Ltd (Gillingham, Dorset, UK).

9.3.2 *In vivo* rat model of myocardial I/R injury

Male rats were anaesthetised by i.p. injection of 50mg/kg sodium pentobarbital. When animals were sufficiently anaesthetised, as indicated by the loss of the pedal withdrawal reflex, a tracheal intubation was performed to allow artificial ventilation. The carotid artery and jugular vein were then cannulated, in the case of the former to allow blood pressure to be monitored via a pressure transducer, and the latter to provide a route for drug delivery. The jugular cannula remained connected to a 1ml syringe throughout the experiment to allow delivery of fluids (to maintain hydration) and drugs. Electrocardiographic (ECG) recording was achieved by the attachment of electrodes to the two forelimbs and one hindlimb. Left ventricular systolic and diastolic BP, heart rate and ECG were recorded using a PowerLab system connected to a computer (ADinstruments, Oxfordshire, UK). Body temperature was monitored via a temperature probe inserted into the anus and maintained at 37 ± 1°C by regulating the temperature of the operation platform. A left thoracotomy was then performed and the fourth intercostal space was opened carefully to allow visualisation of the heart. The pericardium was then gently opened using

forceps to allow access to the heart. A 16mm round-bodied needle was used to place a 4.0 prolene suture under the LAD, entering just under the left atrium and exiting under the right pulmonary outflow tract. The animal was stabilised for 25min and then subjected to 30min ischemia followed by 120min reperfusion (see Figure 9.3.1)

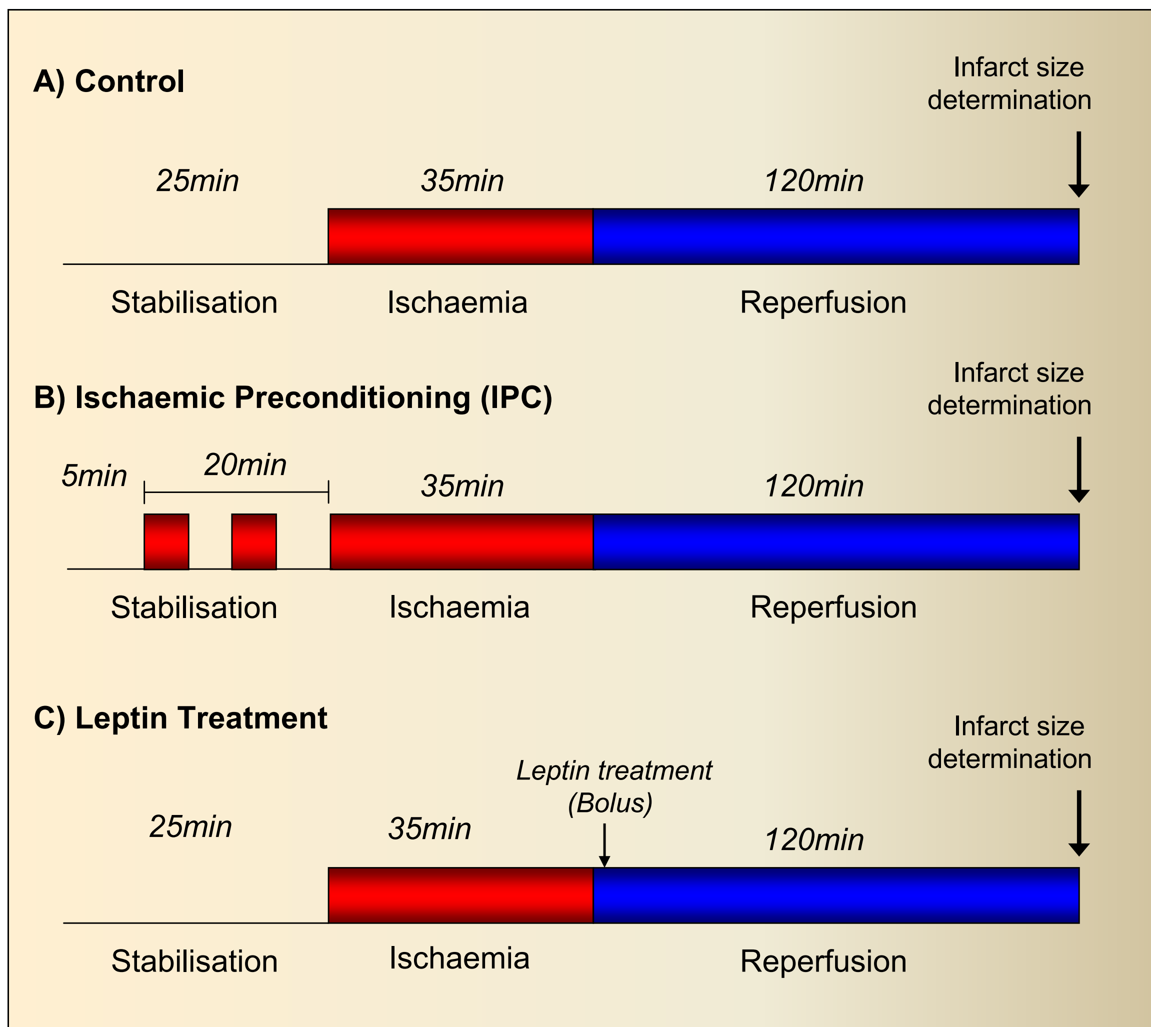


Figure 9.3.1 - Protocol for investigating the effects of leptin on myocardial I/R injury in an *in vivo* rat model. (A) Control rats were stabilised for 25min and then subjected to 35min ischaemia followed by 120min reperfusion. (B) Rats subjected to an IPC protocol were stabilised for 5min followed by two cycles of 5min ischaemia and 5min reperfusion and then subjected to 35min ischaemia and 120min reperfusion. (C) Rats were subjected to a protocol identical to that of control experiments, but were given an intravenous bolus of leptin at 1min prior to reperfusion. In all experiments at the end of reperfusion hearts were harvested and stored at -20°C to await analysis.

9.3.3 Treatment groups

Wistar rats were randomly assigned to one of the following treatment groups (original group sizes are shown in brackets):

Control (n=5) – Rats were subjected to the standard I/R protocol (Figure 9.3.1A)

IPC (n=4) – Rats were subjected to an IPC protocol prior to the index ischaemia and reperfusion.

10µg/kg Leptin treated (n=5) – Rats were subjected to the standard I/R protocol, except that animals received an intravenous bolus of leptin (10µg/kg) 1min prior to reperfusion.

9.3.4 Exclusions

One animal was excluded from the IPC group due to a small risk zone.

9.4 Results

9.4.1 Leptin protects against myocardial I/R injury in an *in vivo* rat model

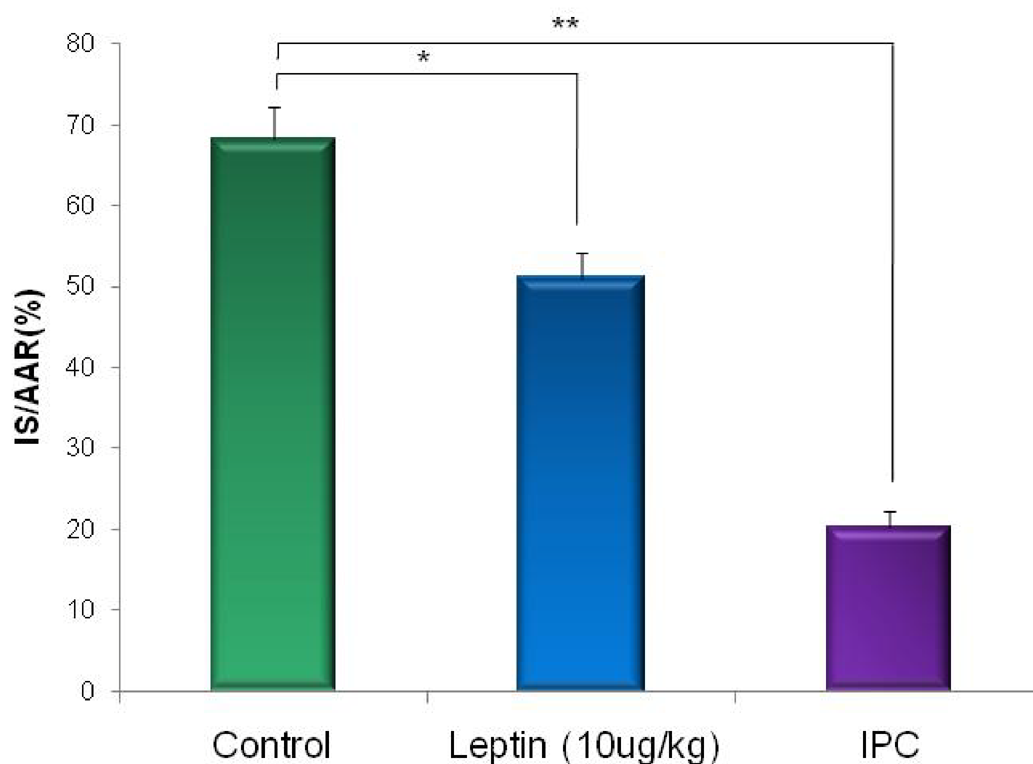


Figure 9.4.1 – Effect of leptin, administered at reperfusion, on infarct size in an *in vivo* rat model of myocardial I/R injury. Values are expressed as mean ± s.e.m (n=3-5 for each group) *(one-way ANOVA)*.

Leptin administered at 10 μg/kg (n=5) produced a significant 25.1% decrease in infarct size when compared to control (n=5) animals (50.9%±3.20 vs. 68.0%±3.97, p=<0.05). By contrast, IPC (n=3) decreases in infarct size that exceeded those seen with 10 μg/kg leptin were observed (IPC, 20.26%±1.83 vs. control, 68%±3.97, p=<0.001).

9.5 Discussion

Recently, Purdham et al reported that antibodies directed against the leptin receptor inhibited hypertrophy in the post-infarcted rat heart (Purdham et al. 2008). These data, perhaps, lead one to conclude that leptin is essentially damaging to the cardiovascular system. There is, however, a growing amount of data indicating that under certain conditions the administration of leptin can be protective (Qamar et al. 2006; Signore et al. 2008). Indeed, this appears to be the case in relation to myocardial I/R injury (Smith et al. 2006; Eguchi et al. 2008) and lipotoxicity (Unger 2005).

9.5.1 Leptin protects the heart against I/R injury in an *in vivo* rat model

Following on from the *in vitro* studies conducted previously in our laboratory (Smith et al. 2006) and those described in earlier chapters of this thesis, the data obtained in the present study with 10 μg/kg leptin clearly demonstrate that leptin is also able to protect the heart against I/R injury *in vivo*. However, the decrease in infarct size by the administration of leptin at this particular concentration was relatively small when compared to the protection action of IPC. Leptin at different concentrations may provide a greater cardioprotective effect. Therefore, detailed dose-response studies, i.e. experiments performed with a wider range of leptin doses, should clarify this situation. Whilst 10 μg/kg leptin produced a significant decrease in infarct size, IPC, used as a positive control, was found to elicit a greater protective effect (Figure 9.4.1), possibly indicating that leptin given at reperfusion is not as effective as IPC with respect to myocardial protection. Alternatively, it could reflect the fact that the most

effective dose of leptin has yet to be identified. The doses of leptin used in the current study were extrapolated from a previous study which focused on the effects of leptin on NO synthesis (Fruhbeck 1999). Thus, it was shown that significant dose-dependent increases in NO synthesis occurred with leptin when it was administered at 10μg/kg, 100μg/kg and 1000μg/kg (Fruhbeck 1999). Obviously, with the findings of the present study, one has to consider the possibility that the doses of leptin that are effective at reducing infarct size may not coincide with those that increase NO production.

9.5.2 Future directions

The present study provides important preliminary data with respect to leptin's potential as a cardioprotectant in the more physiological *in vivo* setting. In the future, however, it is essential that detailed dose-response studies are carried out so that optimal doses of leptin can be identified. Subsequently, studies focusing on the cellular signalling mechanisms activated by leptin in the *in vivo* setting need to be carried out so that the data obtained can be compared with those yielded by *in vitro* studies. Ultimately, the studies conducted with *in vivo* models will establish if leptin might be considered as a potential future cardioprotective treatment in patients.

CHAPTER 10 - SUMMARY & CONCLUSIONS

10.1 Summary of Findings

Leptin administered at reperfusion reduced infarct size in an *in vitro* model of I/R injury in hearts from both Wistar and Zucker lean rats, but not in hearts from *fa/fa* animals.

Leptin delayed the opening of the MPTP in cardiomyocytes from both Wistar and Zucker lean rats, but not from *fa/fa* animals.

The effects induced by leptin were associated with the activation of the JAK/STAT and RISK pathways.

Leptin administered at reperfusion was protective in an *in vivo* rat model of myocardial I/R injury.

10.2 Conclusions

In this investigation the cellular mechanisms underlying the cardioprotective actions of leptin were investigated. In addition, information was obtained which confirms previous findings made in this laboratory, namely that leptin-induced protection involves RISK pathway signalling (Smith et al. 2006). Furthermore, data were obtained which may indicate that leptin administered at reperfusion protects against I/R injury via interaction with the OB-R. Apart from the RISK pathway, the findings of the present investigation appear to indicate that JAK/STAT signalling also plays a significant role, this pathway being known to mediate the metabolic actions of leptin (Harvey & Ashford 2003). Furthermore, the present study would suggest that the cardioprotective actions of leptin rely

upon the presence in the myocardium of a functional OB-Rb. These data may have clinical relevance, especially in relation to patients with leptin resistance and/or hyperleptinaemia who are deficient or defective with respect to the leptin receptor. Finally, leptin was shown to reduce I/R injury in an *in vivo* rat model of myocardial infarction. Further investigations will provide additional information on the potential of leptin as a novel treatment against myocardial I/R injury.

Reperfusion of the ischaemic myocardium is essential if damage to the heart is to be minimised (Piper et al. 2004). The act of reperfusion itself, however, has been demonstrated to be associated with cardiac dysfunction and cell death (Manning & Hearse 1984; Piper et al. 1998; Yellon & Hausenloy 2007). In this respect, the MPTP represents an important target for cardioprotection (Halestrap et al. 2004). It is now known that the conditions that occur following the reperfusion of the ischaemic myocardium, such as high levels of ROS and Ca^{2+}, trigger opening of the MPTP and lead to apoptotic signalling and cell death (Halestrap 2006). Thus it could be hypothesised that leptin signalling in cardiomyocytes may occur via OB-Rb activation and subsequent JAK/STAT, PI3K-Akt and p44/42 MAPK signalling, resulting in inhibition of MPT and decreased infarct size.

The adipocytokines were discovered relatively recently and leptin was the first of this group of hormones to be identified (Zhang et al. 1994). Despite the fact that many of the central and peripheral actions of leptin have been identified much remains to be learnt regarding its cardiovascular actions. Leptin is known to promote various processes which are detrimental to the cardiovascular system

(Purdham et al. 2008; Beltowski 2006a; Beltowski 2006b). Paradoxically, however, leptin has also been shown to produce beneficial effects via stimulation of survival kinase pathways (Smith et al. 2006). It has been reported, for example, that leptin signalling following a chronic ischaemic insult is associated with decreased cardiac dysfunction and remodelling (McGaffin et al. 2008). Furthermore, it appears that the role of leptin in the promotion of coronary heart disease (CHD) may have been overestimated. Recent data suggest, for example, that the correlation between circulating leptin and CHD is moderate and mostly dependent on BMI (Sattar et al. 2009). It appears, therefore, that leptin and possibly other adipocytokines can act as protectants against cardiovascular pathologies. Indeed, high BMI, which is associated with hyperleptinaemia, is associated with improved short-term outcomes with regard to cardiovascular mortality (Gruberg et al. 2002). This phenomenon is known as the "obesity paradox." Leptin, and other adipocytokines could, therefore, form part of an intrinsic protective mechanism in the heart. It has been shown, for instance, that expression of leptin and OB-R are increased in response to I/R injury (Matsui et al. 2007). The notion that hormones secreted by the heart act in an autocrine/paracrine fashion to protect the myocardium remains conjecture and further investigation is needed to clarify the situation with regard to this hypothesis.

10.3 Future Directions

In future studies, the cardioprotective actions of leptin will need to be examined in more detail in *in vivo* models of I/R reperfusion injury so that a judgement can

be made as to its possible value as a therapeutic agent. These studies should include detailed examinations of kinases activated by leptin, other than examined in this study, e.g. PTEN (Ning et al. 2006) and RhoA/ROCK (Zeidan et al. 2008), which are known to be modulated by leptin. Such studies may help to elucidate the mechanisms determining the beneficial as opposed to the detrimental actions of leptin in the heart. Furthermore, the identification of the specific OB-R involved in leptin-induced signalling should prove possible by employing specific leptin receptor antibodies and blockers that have recently become available (www.abbiotec.com). Activation of the different populations of OB-R receptors could account for the high functional pleiotropy of leptin. Hence, with the development of antagonists that target specific OB-R isoforms, it may be eventually prove possible to investigate in detail the physiological consequences of activating different OB-R subtypes.

Employing STAT3 knockout mice will permit more detailed investigations of the role played by the JAK/STAT pathway in leptin-induced cardioprotection. Studies should also be directed at understanding how obesity influences leptin-induced cardioprotection, cardioprotection with respect to hyperleptinaemia and leptin resistance.

In conclusion, it is now recognised that adipose tissue is not merely an energy storage “depot” but, in fact, represents the body’s largest endocrine organ. Further investigation of its principal products, i.e. the adipocytokines, will lead to a greater understanding of the many roles played by these substances, possibly

leading to the formulation of novel therapies for the treatment of cardiovascular disease and the metabolic syndrome.

PUBLICATIONS & COMMUNICATIONS

Abstracts

Dixon, RA, Smith, CCT, and Yellon, DM. (2007). Leptin-induced cardioprotection is dependent on the presence of functional OB-Rb receptors. June 2007, Bologna, Italy meeting of the World Congress of the International Society for Heart Research.

Oral Presentations

Dixon, RA, Smith, CCT, and Yellon, DM. (2008). Leptin-stimulated inhibition of mitochondrial permeability transition is dependent on presence of functional OB-Rb leptin receptor. May 2008, Athens, Greece meeting of the International Society for Heart Research

Publications[4]

Smith, CCT, Mocanu, MM, Bowen, J, Wynne, AM, Simpkin, JC, Dixon, RA, Cooper, MB, Yellon, DM. (2007). Temporal changes in myocardial salvage kinases during reperfusion following ischemia: studies involving the cardioprotective adipocytokine apelin. *Cardiovasc Drugs Ther*. 6:409-414.

Dixon, RA, Davidson, SM, Wynne, AM, Yellon, DM and Smith, CCT. (2009). The cardioprotective actions of leptin are lost in the Zucker obese (*fa/fa*) rat. *J Cardiovasc Pharmacol*. 54:311-317.

[4] These publications are included in full at the end of this thesis.

REFERENCES

Journals

Abdallah Y, Gkatzoflia A, Gligorievski D, Kasseckert S, Euler G, Schluter KD, Schafer M, Piper HM, and Schafer C (2006). Insulin protects cardiomyocytes against reoxygenation-induced hypercontracture by a survival pathway targeting SR Ca2+ storage. *Cardiovasc. Res.* 70**:** 346-353.

Abe Y, Ono K, Kawamura T, Wada H, Kita T, Shimatsu A, and Hasegawa K (2007). Leptin induces elongation of cardiac myocytes and causes eccentric left ventricular dilatation with compensation. *Am. J. Physiol Heart Circ. Physiol* 292**:** H2387-H2396.

Abou-Sleiman PM, Muqit MM, and Wood NW (2006). Expanding insights of mitochondrial dysfunction in Parkinson's disease. *Nat. Rev. Neurosci.* 7**:** 207-219.

Adeghate E (2008). Visfatin: structure, function and relation to diabetes mellitus and other dysfunctions. *Curr. Med. Chem.* 15**:** 1851-1862.

Adrain C, Brumatti G, and Martin SJ (2006). Apoptosomes: protease activation platforms to die from. *Trends Biochem. Sci.* 31**:** 243-247.

Ahima RS (2005). Central actions of adipocyte hormones. *Trends Endocrinol. Metab* 16**:** 307-313.

Ahima RS (2006). Adipose tissue as an endocrine organ. *Obesity. (Silver. Spring)* 14 Suppl 5**:** 242S-249S.

Alessi MC and Juhan-Vague I (2008). Metabolic syndrome, haemostasis and thrombosis. *Thromb. Haemost.* 99**:** 995-1000.

Ambrosio G, Zweier JL, Duilio C, Kuppusamy P, Santoro G, Elia PP, Tritto I, Cirillo P, Condorelli M, Chiariello M, and . (1993). Evidence that mitochondrial respiration is a source of potentially toxic oxygen free radicals in intact rabbit hearts subjected to ischemia and reflow. *J. Biol. Chem.* 268**:** 18532-18541.

Anagnostoulis S, Karayiannakis AJ, Lambropoulou M, Efthimiadou A, Polychronidis A, and Simopoulos C (2008). Human leptin induces angiogenesis in vivo. *Cytokine* 42**:** 353-357.

Anderson SE, Murphy E, Steenbergen C, London RE, and Cala PM (1990). Na-H exchange in myocardium: effects of hypoxia and acidification on Na and Ca. *Am. J. Physiol* 259**:** C940-C948.

Andreadou I, Iliodromitis EK, Koufaki M, Farmakis D, Tsotinis A, and Kremastinos DT (2008). Alternative pharmacological interventions that limit myocardial infarction. *Curr. Med. Chem.* 15**:** 3204-3213.

Argetsinger LS, Campbell GS, Yang X, Witthuhn BA, Silvennoinen O, Ihle JN, and Carter-Su C (1993). Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. *Cell* 74**:** 237-244.

Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, Matsubara K, Muraguchi M, Ohmoto Y, Funahashi T, and Matsuzawa Y (1999b). Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem. Biophys. Res. Commun.* 257**:** 79-83.

Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, Matsubara K, Muraguchi M, Ohmoto Y, Funahashi T, and Matsuzawa Y (1999a). Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem. Biophys. Res. Commun.* 257**:** 79-83.

Aronson D, Rayfield EJ, and Chesebro JH (1997). Mechanisms determining course and outcome of diabetic patients who have had acute myocardial infarction. *Ann. Intern. Med.* 126**:** 296-306.

Arteaga D, Odor A, Lopez RM, Contreras G, Pichardo J, Garcia E, Aranda A, and Chavez E (1992). Impairment by cyclosporin A of reperfusion-induced arrhythmias. *Life Sci.* 51**:** 1127-1134.

Asimakis GK, Inners-McBride K, Medellin G, and Conti VR (1992). Ischemic preconditioning attenuates acidosis and postischemic dysfunction in isolated rat heart. *Am. J. Physiol* 263**:** H887-H894.

Atkinson LL, Fischer MA, and Lopaschuk GD (2002). Leptin activates cardiac fatty acid oxidation independent of changes in the AMP-activated protein kinase-acetyl-CoA carboxylase-malonyl-CoA axis. *J. Biol. Chem.* 277**:** 29424-29430.

Bagci EZ, Vodovotz Y, Billiar TR, Ermentrout GB, and Bahar I (2006). Bistability in apoptosis: roles of bax, bcl-2, and mitochondrial permeability transition pores. *Biophys. J.* 90**:** 1546-1559.

Bahrenberg G, Behrmann I, Barthel A, Hekerman P, Heinrich PC, Joost HG, and Becker W (2002). Identification of the critical sequence elements in the cytoplasmic domain of leptin receptor isoforms required for Janus kinase/signal transducer and activator of transcription activation by receptor heterodimers. *Mol. Endocrinol.* 16**:** 859-872.

Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H, Klevernic I, Arthur JS, Alessi DR, and Cohen P (2007). The selectivity of protein kinase inhibitors: a further update. *Biochem. J.* 408**:** 297-315.

Baines CP, Kaiser RA, Purcell NH, Blair NS, Osinska H, Hambleton MA, Brunskill EW, Sayen MR, Gottlieb RA, Dorn GW, Robbins J, and Molkentin JD (2005). Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature* 434**:** 658-662.

Baines CP and Molkentin JD (2005). STRESS signaling pathways that modulate cardiac myocyte apoptosis. *J. Mol. Cell Cardiol.* 38**:** 47-62.

Balendran A, Casamayor A, Deak M, Paterson A, Gaffney P, Currie R, Downes CP, and Alessi DR (1999). PDK1 acquires PDK2 activity in the presence of a synthetic peptide derived from the carboxyl terminus of PRK2. *Curr. Biol.* 9**:** 393-404.

Banks AS, Davis SM, Bates SH, and Myers MG, Jr. (2000). Activation of downstream signals by the long form of the leptin receptor. *J. Biol. Chem.* 275**:** 14563-14572.

Banks WA, Coon AB, Robinson SM, Moinuddin A, Shultz JM, Nakaoke R, and Morley JE (2004). Triglycerides induce leptin resistance at the blood-brain barrier. *Diabetes* 53**:** 1253-1260.

Banks WA and Farrell CL (2003). Impaired transport of leptin across the blood-brain barrier in obesity is acquired and reversible. *Am. J. Physiol Endocrinol. Metab* 285**:** E10-E15.

Barry SP, Townsend PA, Latchman DS, and Stephanou A (2007). Role of the JAK-STAT pathway in myocardial injury. *Trends Mol. Med.* 13**:** 82-89.

Bates SH and Myers MG, Jr. (2003). The role of leptin receptor signaling in feeding and neuroendocrine function. *Trends Endocrinol. Metab* 14**:** 447-452.

Baumann H, Morella KK, White DW, Dembski M, Bailon PS, Kim H, Lai CF, and Tartaglia LA (1996). The full-length leptin receptor has signaling capabilities of interleukin 6-type cytokine receptors. *Proc. Natl. Acad. Sci. U. S. A* 93**:** 8374-8378.

Baxter GF and Burley DS (2008). Reperfusion and calculated RISKs: pharmacological postconditioning of human myocardium. *Br. J. Pharmacol.* 153**:** 1-3.

Baxter GF, Mocanu MM, Brar BK, Latchman DS, and Yellon DM (2001). Cardioprotective effects of transforming growth factor-beta1 during early reoxygenation or reperfusion are mediated by p42/p44 MAPK. *J. Cardiovasc. Pharmacol.* 38**:** 930-939.

Becker LB, Vanden Hoek TL, Shao ZH, Li CQ, and Schumacker PT (1999). Generation of superoxide in cardiomyocytes during ischemia before reperfusion. *Am. J. Physiol* 277**:** H2240-H2246.

Behrmann I, Smyczek T, Heinrich PC, Schmitz-Van de Leur H, Komyod W, Giese B, Muller-Newen G, Haan S, and Haan C (2004). Janus kinase (Jak) subcellular localization revisited: the exclusive membrane localization of endogenous Janus kinase 1 by cytokine receptor interaction uncovers the Jak.receptor complex to be equivalent to a receptor tyrosine kinase. *J. Biol. Chem.* 279**:** 35486-35493.

Bell RM and Yellon DM (2003). Bradykinin limits infarction when administered as an adjunct to reperfusion in mouse heart: the role of PI3K, Akt and eNOS. *J. Mol. Cell Cardiol.* 35**:** 185-193.

Bell-Anderson KS and Bryson JM (2004). Leptin as a potential treatment for obesity: progress to date. *Treat. Endocrinol.* 3**:** 11-18.

Bellacosa A, Testa JR, Staal SP, and Tsichlis PN (1991). A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science* 254**:** 274-277.

Beltowski J (2006a). Leptin and atherosclerosis. *Atherosclerosis* 189**:** 47-60.

Beltowski J (2006b). Role of leptin in blood pressure regulation and arterial hypertension. *J. Hypertens.* 24**:** 789-801.

Bernardi P and Petronilli V (1996). The permeability transition pore as a mitochondrial calcium release channel: a critical appraisal. *J. Bioenerg. Biomembr.* 28**:** 131-138.

Bernardi P and Rasola A (2007). Calcium and cell death: the mitochondrial connection. *Subcell. Biochem.* 45**:** 481-506.

Berry MF, Pirolli TJ, Jayasankar V, Burdick J, Morine KJ, Gardner TJ, and Woo YJ (2004). Apelin has in vivo inotropic effects on normal and failing hearts. *Circulation* 110**:** II187-II193.

Bingham NC, Anderson KK, Reuter AL, Stallings NR, and Parker KL (2008). Selective loss of leptin receptors in the ventromedial hypothalamic nucleus results in increased adiposity and a metabolic syndrome. *Endocrinology* 149**:** 2138-2148.

Bjorbaek C, Buchholz RM, Davis SM, Bates SH, Pierroz DD, Gu H, Neel BG, Myers MG, Jr., and Flier JS (2001). Divergent roles of SHP-2 in ERK activation by leptin receptors. *J. Biol. Chem.* 276**:** 4747-4755.

Blevins JE, Schwartz MW, and Baskin DG (2002). Peptide signals regulating food intake and energy homeostasis. *Can. J. Physiol Pharmacol.* 80**:** 396-406.

Boengler K, Buechert A, Heinen Y, Roeskes C, Hilfiker-Kleiner D, Heusch G, and Schulz R (2008a). Cardioprotection by ischemic postconditioning is lost in aged and STAT3-deficient mice. *Circ. Res.* 102**:** 131-135.

Boengler K, Hilfiker-Kleiner D, Drexler H, Heusch G, and Schulz R (2008b). The myocardial JAK/STAT pathway: From protection to failure. *Pharmacol. Ther.*

Bolli R and Marban E (1999). Molecular and cellular mechanisms of myocardial stunning. *Physiol Rev.* 79**:** 609-634.

Bond JM, Chacon E, Herman B, and Lemasters JJ (1993). Intracellular pH and Ca2+ homeostasis in the pH paradox of reperfusion injury to neonatal rat cardiac myocytes. *Am. J. Physiol* 265**:** C129-C137.

Bond JM, Harper IS, Chacon E, Reece JM, Herman B, and Lemasters JJ (1994). The pH paradox in the pathophysiology of reperfusion injury to rat neonatal cardiac myocytes. *Ann. N. Y. Acad. Sci.* 723**:** 25-37.

Bond JM, Herman B, and Lemasters JJ (1991). Protection by acidotic pH against anoxia/reoxygenation injury to rat neonatal cardiac myocytes. *Biochem. Biophys. Res. Commun.* 179**:** 798-803.

Bopassa JC, Michel P, Gateau-Roesch O, Ovize M, and Ferrera R (2005). Low-pressure reperfusion alters mitochondrial permeability transition. *Am. J. Physiol Heart Circ. Physiol* 288**:** H2750-H2755.

Bose AK, Mocanu MM, Carr RD, Brand CL, and Yellon DM (2005a). Glucagon-like peptide 1 can directly protect the heart against ischemia/reperfusion injury. *Diabetes* 54**:** 146-151.

Bose AK, Mocanu MM, Carr RD, and Yellon DM (2005b). Glucagon like peptide-1 is protective against myocardial ischemia/reperfusion injury when given either as a preconditioning mimetic or at reperfusion in an isolated rat heart model. *Cardiovasc. Drugs Ther.* 19**:** 9-11.

Boucher J, Masri B, Daviaud D, Gesta S, Guigne C, Mazzucotelli A, Castan-Laurell I, Tack I, Knibiehler B, Carpene C, Audigier Y, Saulnier-Blache JS, and Valet P (2005). Apelin, a newly identified adipokine up-regulated by insulin and obesity. *Endocrinology* 146**:** 1764-1771.

Bouloumie A, Marumo T, Lafontan M, and Busse R (1999). Leptin induces oxidative stress in human endothelial cells. *FASEB J.* 13**:** 1231-1238.

Brar BK, Jonassen AK, Stephanou A, Santilli G, Railson J, Knight RA, Yellon DM, and Latchman DS (2000). Urocortin protects against ischemic and reperfusion injury via a MAPK-dependent pathway. *J. Biol. Chem.* 275**:** 8508-8514.

Brar BK, Stephanou A, Knight R, and Latchman DS (2002). Activation of protein kinase B/Akt by urocortin is essential for its ability to protect cardiac cells against hypoxia/reoxygenation-induced cell death. *J. Mol. Cell Cardiol.* 34**:** 483-492.

Braunwald E and Kloner RA (1982). The stunned myocardium: prolonged, postischemic ventricular dysfunction. *Circulation* 66**:** 1146-1149.

Braunwald E and Kloner RA (1985). Myocardial reperfusion: a double-edged sword? *J. Clin. Invest* 76**:** 1713-1719.

Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, and Greenberg ME (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* 96**:** 857-868.

Brzozowski T, Konturek PC, Pajdo R, Kwiecien S, Ptak A, Sliwowski Z, Drozdowicz D, Pawlik M, Konturek SJ, and Hahn EG (2001). Brain-gut axis in gastroprotection by leptin and cholecystokinin against ischemia-reperfusion induced gastric lesions. *J. Physiol Pharmacol.* 52**:** 583-602.

Buendia B, Santa-Maria A, and Courvalin JC (1999). Caspase-dependent proteolysis of integral and peripheral proteins of nuclear membranes and nuclear pore complex proteins during apoptosis. *J. Cell Sci.* 112 (Pt 11)**:** 1743-1753.

Buki A, Okonkwo DO, Wang KK, and Povlishock JT (2000). Cytochrome c release and caspase activation in traumatic axonal injury. *J. Neurosci.* 20**:** 2825-2834.

Bullard AJ, Govewalla P, and Yellon DM (2005). Erythropoietin protects the myocardium against reperfusion injury in vitro and in vivo. *Basic Res. Cardiol.* 100**:** 397-403.

Bullo M, Garcia-Lorda P, Megias I, and Salas-Salvado J (2003). Systemic inflammation, adipose tissue tumor necrosis factor, and leptin expression. *Obes. Res.* 11**:** 525-531.

Cammisotto PG, Gelinas Y, Deshaies Y, and Bukowiecki LJ (2005). Regulation of leptin secretion from white adipocytes by insulin, glycolytic substrates, and amino acids. *Am. J. Physiol Endocrinol. Metab* 289**:** E166-E171.

Cao Q, Mak KM, Ren C, and Lieber CS (2004). Leptin stimulates tissue inhibitor of metalloproteinase-1 in human hepatic stellate cells: respective roles of the JAK/STAT and JAK-mediated H2O2-dependant MAPK pathways. *J. Biol. Chem.* 279**:** 4292-4304.

Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, and Reed JC (1998). Regulation of cell death protease caspase-9 by phosphorylation. *Science* 282**:** 1318-1321.

Carry MM, Mrak RE, Murphy ML, Peng CF, Straub KD, and Fody EP (1989). Reperfusion injury in ischemic myocardium: protective effects of ruthenium red and of nitroprusside. *Am. J. Cardiovasc. Pathol.* 2**:** 335-344.

Chain DG, Hegde AN, Yamamoto N, Liu-Marsh B, and Schwartz JH (1995). Persistent activation of cAMP-dependent protein kinase by regulated proteolysis suggests a neuron-specific function of the ubiquitin system in Aplysia. *J. Neurosci.* 15**:** 7592-7603.

Chan TO, Rittenhouse SE, and Tsichlis PN (1999). AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu. Rev. Biochem.* 68**:** 965-1014.

Chandrasekar B, Boylston WH, Venkatachalam K, Webster NJ, Prabhu SD, and Valente AJ (2008). Adiponectin blocks interleukin-18-mediated endothelial cell death via APPL1-dependent AMPK activation and IKK/NF-kappa B/PTEN suppression. *J. Biol. Chem.*

Chen D and Wang MW (2005). Development and application of rodent models for type 2 diabetes. *Diabetes Obes. Metab* 7**:** 307-317.

Chen H, Charlat O, Tartaglia LA, Woolf EA, Weng X, Ellis SJ, Lakey ND, Culpepper J, Moore KJ, Breitbart RE, Duyk GM, Tepper RI, and Morgenstern JP (1996). Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. *Cell* 84**:** 491-495.

Chin YE, Kitagawa M, Kuida K, Flavell RA, and Fu XY (1997). Activation of the STAT signaling pathway can cause expression of caspase 1 and apoptosis. *Mol. Cell Biol.* 17**:** 5328-5337.

Chua SC, Jr., Chung WK, Wu-Peng XS, Zhang Y, Liu SM, Tartaglia L, and Leibel RL (1996). Phenotypes of mouse diabetes and rat fatty due to mutations in the OB (leptin) receptor. *Science* 271**:** 994-996.

Cioffi JA, Shafer AW, Zupancic TJ, Smith-Gbur J, Mikhail A, Platika D, and Snodgrass HR (1996). Novel B219/OB receptor isoforms: possible role of leptin in hematopoiesis and reproduction. *Nat. Med.* 2**:** 585-589.

Clarke PG (1990). Developmental cell death: morphological diversity and multiple mechanisms. *Anat. Embryol. (Berl)* 181**:** 195-213.

Clarke SJ, McStay GP, and Halestrap AP (2002). Sanglifehrin A acts as a potent inhibitor of the mitochondrial permeability transition and reperfusion injury of the heart by binding to cyclophilin-D at a different site from cyclosporin A. *J. Biol. Chem.* 277**:** 34793-34799.

Claycombe KJ, Xue BZ, Mynatt RL, Zemel MB, and Moustaid-Moussa N (2000). Regulation of leptin by agouti. *Physiol Genomics* 2**:** 101-105.

Cooney RN (2002). Suppressors of cytokine signaling (SOCS): inhibitors of the JAK/STAT pathway. *Shock* 17**:** 83-90.

Correia ML, Morgan DA, Sivitz WI, Mark AL, and Haynes WG (2001). Leptin acts in the central nervous system to produce dose-dependent changes in arterial pressure. *Hypertension* 37**:** 936-942.

Costa AD and Garlid KD (2008). Intramitochondrial signaling: interactions among mitoKATP, PKCepsilon, ROS, and MPT. *Am. J. Physiol Heart Circ. Physiol* 295**:** H874-H882.

Costa AD, Garlid KD, West IC, Lincoln TM, Downey JM, Cohen MV, and Critz SD (2005). Protein kinase G transmits the cardioprotective signal from cytosol to mitochondria. *Circ. Res.* 97**:** 329-336.

Croker BA, Kiu H, and Nicholson SE (2008). SOCS regulation of the JAK/STAT signalling pathway. *Semin. Cell Dev. Biol.* 19**:** 414-422.

Crompton M and Andreeva L (1993). On the involvement of a mitochondrial pore in reperfusion injury. *Basic Res. Cardiol.* 88**:** 513-523.

Cross TG, Scheel-Toellner D, Henriquez NV, Deacon E, Salmon M, and Lord JM (2000). Serine/threonine protein kinases and apoptosis. *Exp. Cell Res.* 256**:** 34-41.

Darnell JE, Jr. (1997). STATs and gene regulation. *Science* 277**:** 1630-1635.

Das AM and Harris DA (1990). Regulation of the mitochondrial ATP synthase in intact rat cardiomyocytes. *Biochem. J.* 266**:** 355-361.

Datta SR, Brunet A, and Greenberg ME (1999). Cellular survival: a play in three Akts. *Genes Dev.* 13**:** 2905-2927.

Davidson SM, Hausenloy D, Duchen MR, and Yellon DM (2006). Signalling via the reperfusion injury signalling kinase (RISK) pathway links closure of the mitochondrial permeability transition pore to cardioprotection. *Int. J. Biochem. Cell Biol.* 38**:** 414-419.

Davis RJ (1995). Transcriptional regulation by MAP kinases. *Mol. Reprod. Dev.* 42**:** 459-467.

de Leiris J, Harding DP, and Pestre S (1984). The isolated perfused rat heart: a model for studying myocardial hypoxia or ischaemia. *Basic Res. Cardiol.* 79**:** 313-321.

Degterev A, Hitomi J, Germscheid M, Ch'en IL, Korkina O, Teng X, Abbott D, Cuny GD, Yuan C, Wagner G, Hedrick SM, Gerber SA, Lugovskoy A, and Yuan J (2008). Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nat. Chem. Biol.* 4**:** 313-321.

Degterev A, Huang Z, Boyce M, Li Y, Jagtap P, Mizushima N, Cuny GD, Mitchison TJ, Moskowitz MA, and Yuan J (2005). Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. *Nat. Chem. Biol.* 1**:** 112-119.

Delaigle AM, Jonas JC, Bauche IB, Cornu O, and Brichard SM (2004). Induction of adiponectin in skeletal muscle by inflammatory cytokines: in vivo and in vitro studies. *Endocrinology* 145**:** 5589-5597.

Deniaud A, Hoebeke J, Briand JP, Muller S, Jacotot E, and Brenner C (2006). Peptido-targeting of the mitochondrial transition pore complex for therapeutic apoptosis induction. *Curr. Pharm. Des* 12**:** 4501-4511.

Dennis SC, Gevers W, and Opie LH (1991). Protons in ischemia: where do they come from; where do they go to? *J. Mol. Cell Cardiol.* 23**:** 1077-1086.

Despres JP, Cartier A, Cote M, and Arsenault BJ (2008). The concept of cardiometabolic risk: Bridging the fields of diabetology and cardiology. *Ann. Med.* 40**:** 514-523.

Dhillon H, Zigman JM, Ye C, Lee CE, McGovern RA, Tang V, Kenny CD, Christiansen LM, White RD, Edelstein EA, Coppari R, Balthasar N, Cowley MA, Chua S Jr, Elmquist JK, and Lowell BB (2006). Leptin directly activates SF1 neurons in the VMH, and this action by leptin is required for normal body-weight homeostasis. *Neuron* 49**:** 191-203.

Downward J (2004). PI 3-kinase, Akt and cell survival. *Semin. Cell Dev. Biol.* 15**:** 177-182.

Dubey L and Hesong Z (2006). Role of leptin in atherogenesis. *Exp. Clin. Cardiol.* 11**:** 269-275.

Ducy P, Amling M, Takeda S, Priemel M, Schilling AF, Beil FT, Shen J, Vinson C, Rueger JM, and Karsenty G (2000). Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. *Cell* 100**:** 197-207.

Dunn SL, Bjornholm M, Bates SH, Chen Z, Seifert M, and Myers MG, Jr. (2005). Feedback inhibition of leptin receptor/Jak2 signaling via Tyr1138 of the leptin receptor and suppressor of cytokine signaling 3. *Mol. Endocrinol.* 19**:** 925-938.

Egorova MV, Afanas'ev SA, and Popov SV (2005). A simple method for isolation of cardiomyocytes from adult rat heart. *Bull. Exp. Biol. Med.* 140**:** 370-373.

Eguchi M, Liu Y, Shin EJ, and Sweeney G (2008). Leptin protects H9c2 rat cardiomyocytes from H2O2-induced apoptosis. *FEBS J.* 275**:** 3136-3144.

Ehrenberg B, Montana V, Wei MD, Wuskell JP, and Loew LM (1988). Membrane potential can be determined in individual cells from the nernstian distribution of cationic dyes. *Biophys. J.* 53**:** 785-794.

Elmquist JK, Bjorbaek C, Ahima RS, Flier JS, and Saper CB (1998). Distributions of leptin receptor mRNA isoforms in the rat brain. *J. Comp Neurol.* 395**:** 535-547.

Enriori PJ, Evans AE, Sinnayah P, and Cowley MA (2006). Leptin resistance and obesity. *Obesity. (Silver. Spring)* 14 Suppl 5**:** 254S-258S.

Erkasap N, Ikizler M, Shneyvays V, Zinman T, Mamedova LK, Uyar R, and Shainberg A (2006). Leptin protects the cardiac myocyte cultures from hypoxic damage. *Life Sci.* 78**:** 1098-1102.

Erkasap N, Uzuner K, Serteser M, Koken T, and Aydin Y (2003). Gastroprotective effect of leptin on gastric mucosal injury induced by ischemia-reperfusion is related to gastric histamine content in rats. *Peptides* 24**:** 1181-1187.

Farooqi IS, Bullmore E, Keogh J, Gillard J, O'Rahilly S, and Fletcher PC (2007). Leptin regulates striatal regions and human eating behavior. *Science* 317**:** 1355.

Fas SC, Fritzsching B, Suri-Payer E, and Krammer PH (2006). Death receptor signaling and its function in the immune system. *Curr. Dir. Autoimmun.* 9**:** 1-17.

Faxon DP (2005). Coronary interventions and their impact on post myocardial infarction survival. *Clin. Cardiol.* 28**:** I38-I44.

Ferrari R, Guardigli G, Mele D, Percoco GF, Ceconi C, and Curello S (2004). Oxidative stress during myocardial ischaemia and heart failure. *Curr. Pharm. Des* 10**:** 1699-1711.

Foldes G, Horkay F, Szokodi I, Vuolteenaho O, Ilves M, Lindstedt KA, Mayranpaa M, Sarman B, Seres L, Skoumal R, Lako-Futo Z, deChatel R, Ruskoaho H, and Toth M (2003). Circulating and cardiac levels of apelin, the novel ligand of the orphan receptor APJ, in patients with heart failure. *Biochem. Biophys. Res. Commun.* 308**:** 480-485.

Ford ES, Giles WH, and Dietz WH (2002). Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey. *JAMA* 287**:** 356-359.

Forhead AJ and Fowden AL (2009). The hungry fetus? Role of leptin as a nutritional signal before birth. *J. Physiol* 587**:** 1145-1152.

Franke TF (2008). Intracellular signaling by Akt: bound to be specific. *Sci. Signal.* 1**:** e29.

Freed DH, Moon MC, Borowiec AM, Jones SC, Zahradka P, and Dixon IM (2003). Cardiotrophin-1: expression in experimental myocardial infarction and potential role in post-MI wound healing. *Mol. Cell Biochem.* 254**:** 247-256.

Friberg H and Wieloch T (2002). Mitochondrial permeability transition in acute neurodegeneration. *Biochimie* 84**:** 241-250.

Fruhbeck G (1999). Pivotal role of nitric oxide in the control of blood pressure after leptin administration. *Diabetes* 48**:** 903-908.

Fruhbeck G (2006). Intracellular signalling pathways activated by leptin. *Biochem. J.* 393**:** 7-20.

Fruhbeck G (2008). Overview of adipose tissue and its role in obesity and metabolic disorders. *Methods Mol. Biol.* 456**:** 1-22.

Fruhbeck G, Jebb SA, and Prentice AM (1998). Leptin: physiology and pathophysiology. *Clin. Physiol* 18**:** 399-419.

Fukuhara A, Matsuda M, Nishizawa M, Segawa K, Tanaka M, Kishimoto K, Matsuki Y, Murakami M, Ichisaka T, Murakami H, Watanabe E, Takagi T, Akiyoshi M, Ohtsubo T, Kihara S, Yamashita S, Makishima M, Funahashi T, Yamanaka S, Hiramatsu R, Matsuzawa Y, and Shimomura I (2005). Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. *Science* 307**:** 426-430.

Funakoshi-Tago M, Pelletier S, Matsuda T, Parganas E, and Ihle JN (2006). Receptor specific downregulation of cytokine signaling by autophosphorylation in the FERM domain of Jak2. *EMBO J.* 25**:** 4763-4772.

Galluzzi L, Vicencio JM, Kepp O, Tasdemir E, Maiuri MC, and Kroemer G (2008). To die or not to die: that is the autophagic question. *Curr. Mol. Med.* 8**:** 78-91.

Gao F, Gao E, Yue TL, Ohlstein EH, Lopez BL, Christopher TA, and Ma XL (2002). Nitric oxide mediates the antiapoptotic effect of insulin in myocardial ischemia-reperfusion: the roles of PI3-kinase, Akt, and endothelial nitric oxide synthase phosphorylation. *Circulation* 105**:** 1497-1502.

Gao J, Chang CC, Chen Z, Wang H, Xu X, Hamdy C, McMullen JR, Shioi T, Izumo S, and Chua BH (2007). Resistin, an adipocytokine, offers protection against acute myocardial infarction. *J. Mol. Cell Cardiol.* 43**:** 601-609.

Gao T, Furnari F, and Newton AC (2005). PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth. *Mol. Cell* 18**:** 13-24.

Gardiner SM, Kemp PA, March JE, and Bennett T (2000). Regional haemodynamic effects of recombinant murine or human leptin in conscious rats. *Br. J. Pharmacol.* 130**:** 805-810.

Garofalo RS, Orena SJ, Rafidi K, Torchia AJ, Stock JL, Hildebrandt AL, Coskran T, Black SC, Brees DJ, Wicks JR, McNeish JD, and Coleman KG (2003). Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKB beta. *J. Clin. Invest* 112**:** 197-208.

Garza JC, Guo M, Zhang W, and Lu XY (2008). Leptin increases adult hippocampal neurogenesis in vivo and in vitro. *J. Biol. Chem.* 283**:** 18238-18247.

Genest J, Jr. and Cohn JS (1995). Clustering of cardiovascular risk factors: targeting high-risk individuals. *Am. J. Cardiol.* 76**:** 8A-20A.

Gennaro-Colonna V, Rossoni G, Cocchi D, Rigamonti AE, Berti F, and Muller EE (2000). Endocrine, metabolic and cardioprotective effects of hexarelin in obese Zucker rats. *J. Endocrinol.* 166**:** 529-536.

Gibson WT, Farooqi IS, Moreau M, DePaoli AM, Lawrence E, O'Rahilly S, and Trussell RA (2004). Congenital leptin deficiency due to homozygosity for the Delta133G mutation: report of another case and evaluation of response to four years of leptin therapy. *J. Clin. Endocrinol. Metab* 89**:** 4821-4826.

Gnaiger E and Kuznetsov AV (2002). Mitochondrial respiration at low levels of oxygen and cytochrome c. *Biochem. Soc. Trans.* 30**:** 252-258.

Gonon AT, Bulhak A, Labruto F, Sjoquist PO, and Pernow J (2007). Cardioprotection mediated by rosiglitazone, a peroxisome proliferator-activated receptor gamma ligand, in relation to nitric oxide. *Basic Res. Cardiol.* 102**:** 80-89.

Goodman MD, Koch SE, Fuller-Bicer GA, and Butler KL (2008). Regulating RISK: a role for JAK-STAT signaling in postconditioning? *Am. J. Physiol Heart Circ. Physiol* 295**:** H1649-H1656.

Gores GJ, Nieminen AL, Fleishman KE, Dawson TL, Herman B, and Lemasters JJ (1988). Extracellular acidosis delays onset of cell death in ATP-depleted hepatocytes. *Am. J. Physiol* 255**:** C315-C322.

Gottlieb RA, Burleson KO, Kloner RA, Babior BM, and Engler RL (1994). Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *J. Clin. Invest* 94**:** 1621-1628.

Gottlieb RA and Engler RL (1999). Apoptosis in myocardial ischemia-reperfusion. *Ann. N. Y. Acad. Sci.* 874**:** 412-426.

Gourlay CW and Ayscough KR (2005). The actin cytoskeleton: a key regulator of apoptosis and ageing? *Nat. Rev. Mol. Cell Biol.* 6**:** 583-589.

Grant PJ (2005). Diabetes and acute coronary syndromes--a diabetologist's view. *Diab. Vasc. Dis. Res.* 2**:** 103-104.

Griffiths EJ and Halestrap AP (1993). Protection by Cyclosporin A of ischemia/reperfusion-induced damage in isolated rat hearts. *J. Mol. Cell Cardiol.* 25**:** 1461-1469.

Grill HJ, Schwartz MW, Kaplan JM, Foxhall JS, Breininger J, and Baskin DG (2002). Evidence that the caudal brainstem is a target for the inhibitory effect of leptin on food intake. *Endocrinology* 143**:** 239-246.

Grinspoon S, Gulick T, Askari H, Landt M, Lee K, Anderson E, Ma Z, Vignati L, Bowsher R, Herzog D, and Klibanski A (1996). Serum leptin levels in women with anorexia nervosa. *J. Clin. Endocrinol. Metab* 81**:** 3861-3863.

Grinspoon SK, Askari H, Landt ML, Nathan DM, Schoenfeld DA, Hayden DL, Laposata M, Hubbard J, and Klibanski A (1997). Effects of fasting and glucose infusion on basal and overnight leptin concentrations in normal-weight women. *Am. J. Clin. Nutr.* 66**:** 1352-1356.

Gross ER and Gross GJ (2006). Ligand triggers of classical preconditioning and postconditioning. *Cardiovasc. Res.* 70**:** 212-221.

Gross GJ and Auchampach JA (2007). Reperfusion injury: does it exist? *J. Mol. Cell Cardiol.* 42**:** 12-18.

Gruberg L, Weissman NJ, Waksman R, Fuchs S, Deible R, Pinnow EE, Ahmed LM, Kent KM, Pichard AD, Suddath WO, Satler LF, and Lindsay J, Jr. (2002). The impact of obesity on the short-term and long-term outcomes after percutaneous coronary intervention: the obesity paradox? *J. Am. Coll. Cardiol.* 39**:** 578-584.

Guan XM, Yu H, Van der Ploeg LH, and Smith RG (1997). Preparation of functionally active [35S]leptin for mapping its receptors in the brain. *Anal. Biochem.* 247**:** 175-177.

Gumina RJ, Buerger E, Eickmeier C, Moore J, Daemmgen J, and Gross GJ (1999). Inhibition of the Na(+)/H(+) exchanger confers greater cardioprotection against 90 minutes of myocardial ischemia than ischemic preconditioning in dogs. *Circulation* 100**:** 2519-2526.

Guo Z, Jiang H, Xu X, Duan W, and Mattson MP (2008). Leptin-mediated cell survival signaling in hippocampal neurons mediated by JAK STAT3 and mitochondrial stabilization. *J. Biol. Chem.* 283**:** 1754-1763.

Guth BD, Schulz R, and Heusch G (1993). Time course and mechanisms of contractile dysfunction during acute myocardial ischemia. *Circulation* 87**:** IV35-IV42.

Guzy RD and Schumacker PT (2006). Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia. *Exp. Physiol* 91**:** 807-819.

Hacioglu A, Algin C, Pasaoglu O, Pasaoglu E, and Kanbak G (2005). Protective effect of leptin against ischemia-reperfusion injury in the rat small intestine. *BMC. Gastroenterol.* 5**:** 37.

Halestrap AP (2006). Calcium, mitochondria and reperfusion injury: a pore way to die. *Biochem. Soc. Trans.* 34**:** 232-237.

Halestrap AP and Brennerb C (2003). The adenine nucleotide translocase: a central component of the mitochondrial permeability transition pore and key player in cell death. *Curr. Med. Chem.* 10**:** 1507-1525.

Halestrap AP, Clarke SJ, and Javadov SA (2004). Mitochondrial permeability transition pore opening during myocardial reperfusion--a target for cardioprotection. *Cardiovasc. Res.* 61**:** 372-385.

Halestrap AP, Connern CP, Griffiths EJ, and Kerr PM (1997a). Cyclosporin A binding to mitochondrial cyclophilin inhibits the permeability transition pore and protects hearts from ischaemia/reperfusion injury. *Mol. Cell Biochem.* 174**:** 167-172.

Halestrap AP, Doran E, Gillespie JP, and O'Toole A (2000). Mitochondria and cell death. *Biochem. Soc. Trans.* 28**:** 170-177.

Halestrap AP, Kerr PM, Javadov S, and Woodfield KY (1998). Elucidating the molecular mechanism of the permeability transition pore and its role in reperfusion injury of the heart. *Biochim. Biophys. Acta* 1366**:** 79-94.

Halestrap AP, McStay GP, and Clarke SJ (2002). The permeability transition pore complex: another view. *Biochimie* 84**:** 153-166.

Halestrap AP and Pasdois P (2009). The role of the mitochondrial permeability transition pore in heart disease. *Biochim. Biophys. Acta*.

Halestrap AP, Woodfield KY, and Connern CP (1997b). Oxidative stress, thiol reagents, and membrane potential modulate the mitochondrial permeability transition by affecting nucleotide binding to the adenine nucleotide translocase. *J. Biol. Chem.* 272**:** 3346-3354.

Hao C, Hao W, Wei X, Xing L, Jiang J, and Shang L (2009). The role of MAPK in the biphasic dose-response phenomenon induced by cadmium and mercury in HEK293 cells. *Toxicol. In Vitro* 23**:** 660-666.

Harvey J and Ashford ML (2003). Leptin in the CNS: much more than a satiety signal. *Neuropharmacology* 44**:** 845-854.

Hassink SG, de Lancey E, Sheslow DV, Smith-Kirwin SM, O'Connor DM, Considine RV, Opentanova I, Dostal K, Spear ML, Leef K, Ash M, Spitzer AR, and Funanage VL (1997). Placental leptin: an important new growth factor in intrauterine and neonatal development? *Pediatrics* 100**:** E1.

Hattori R, Maulik N, Otani H, Zhu L, Cordis G, Engelman RM, Siddiqui MA, and Das DK (2001). Role of STAT3 in ischemic preconditioning. *J. Mol. Cell Cardiol.* 33**:** 1929-1936.

Hausenloy DJ (2009). Drug discovery possibilities from visfatin cardioprotection? *Curr. Opin. Pharmacol.* 9**:** 202-207.

Hausenloy DJ, Duchen MR, and Yellon DM (2003). Inhibiting mitochondrial permeability transition pore opening at reperfusion protects against ischaemia-reperfusion injury. *Cardiovasc. Res.* 60**:** 617-625.

Hausenloy DJ, Maddock HL, Baxter GF, and Yellon DM (2002). Inhibiting mitochondrial permeability transition pore opening: a new paradigm for myocardial preconditioning? *Cardiovasc. Res.* 55**:** 534-543.

Hausenloy DJ, Tsang A, and Yellon DM (2005). The reperfusion injury salvage kinase pathway: a common target for both ischemic preconditioning and postconditioning. *Trends Cardiovasc. Med.* 15**:** 69-75.

Hausenloy DJ, Wynne AM, and Yellon DM (2007). Ischemic preconditioning targets the reperfusion phase. *Basic Res. Cardiol.* 102**:** 445-452.

Hausenloy DJ and Yellon DM (2004). New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. *Cardiovasc. Res.* 61**:** 448-460.

Hausenloy DJ and Yellon DM (2006). Survival kinases in ischemic preconditioning and postconditioning. *Cardiovasc. Res.* 70**:** 240-253.

Hausenloy DJ and Yellon DM (2007a). Preconditioning and postconditioning: united at reperfusion. *Pharmacol. Ther.* 116**:** 173-191.

Hausenloy DJ and Yellon DM (2007b). Reperfusion injury salvage kinase signalling: taking a RISK for cardioprotection. *Heart Fail. Rev.* 12**:** 217-234.

Hausenloy DJ and Yellon DM (2009). Cardioprotective growth factors. *Cardiovasc. Res.* 83**:** 179-194.

Havel PJ (2002). Control of energy homeostasis and insulin action by adipocyte hormones: leptin, acylation stimulating protein, and adiponectin. *Curr. Opin. Lipidol.* 13**:** 51-59.

Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, and Schaper F (2003). Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem. J.* 374**:** 1-20.

Hilfiker-Kleiner D, Hilfiker A, Fuchs M, Kaminski K, Schaefer A, Schieffer B, Hillmer A, Schmiedl A, Ding Z, Podewski E, Podewski E, Poli V, Schneider MD, Schulz R, Park JK, Wollert KC, and Drexler H (2004). Signal transducer and activator of

transcription 3 is required for myocardial capillary growth, control of interstitial matrix deposition, and heart protection from ischemic injury. *Circ. Res.* 95**:** 187-195.

Hill MM, Adrain C, Duriez PJ, Creagh EM, and Martin SJ (2004). Analysis of the composition, assembly kinetics and activity of native Apaf-1 apoptosomes. *EMBO J.* 23**:** 2134-2145.

Hitomi J, Christofferson DE, Ng A, Yao J, Degterev A, Xavier RJ, and Yuan J (2008). Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. *Cell* 135**:** 1311-1323.

Hochachka PW, Buck LT, Doll CJ, and Land SC (1996). Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. *Proc. Natl. Acad. Sci. U. S. A* 93**:** 9493-9498.

Holness MJ, Munns MJ, and Sugden MC (1999). Current concepts concerning the role of leptin in reproductive function. *Mol. Cell Endocrinol.* 157**:** 11-20.

Hommel JD, Trinko R, Sears RM, Georgescu D, Liu ZW, Gao XB, Thurmon JJ, Marinelli M, and DiLeone RJ (2006). Leptin receptor signaling in midbrain dopamine neurons regulates feeding. *Neuron* 51**:** 801-810.

Hosoya M, Kawamata Y, Fukusumi S, Fujii R, Habata Y, Hinuma S, Kitada C, Honda S, Kurokawa T, Onda H, Nishimura O, and Fujino M (2000). Molecular and functional characteristics of APJ. Tissue distribution of mRNA and interaction with the endogenous ligand apelin. *J. Biol. Chem.* 275**:** 21061-21067.

Hossain P, Kawar B, and El Nahas M (2007). Obesity and diabetes in the developing world--a growing challenge. *N. Engl. J. Med.* 356**:** 213-215.

Huising MO, Kruiswijk CP, and Flik G (2006). Phylogeny and evolution of class-I helical cytokines. *J. Endocrinol.* 189**:** 1-25.

Ihle JN (1995). Cytokine receptor signalling. *Nature* 377**:** 591-594.

Inserte J, Barba I, Hernando V, Abellan A, Ruiz-Meana M, Rodriguez-Sinovas A, and Garcia-Dorado D (2008). Effect of acidic reperfusion on prolongation of intracellular acidosis and myocardial salvage. *Cardiovasc. Res.* 77**:** 782-790.

Ito H (2006). No-reflow phenomenon and prognosis in patients with acute myocardial infarction. *Nat. Clin. Pract. Cardiovasc. Med.* 3**:** 499-506.

Japp AG and Newby DE (2008). The apelin-APJ system in heart failure: pathophysiologic relevance and therapeutic potential. *Biochem. Pharmacol.* 75**:** 1882-1892.

Javadov S and Karmazyn M (2007). Mitochondrial permeability transition pore opening as an endpoint to initiate cell death and as a putative target for cardioprotection. *Cell Physiol Biochem.* 20**:** 1-22.

Jennings RB, Reimer KA, and Steenbergen C (1991). Effect of inhibition of the mitochondrial ATPase on net myocardial ATP in total ischemia. *J. Mol. Cell Cardiol.* 23**:** 1383-1395.

Jennings RB, SOMMERS HM, SMYTH GA, FLACK HA, and LINN H (1960). Myocardial necrosis induced by temporary occlusion of a coronary artery in the dog. *Arch. Pathol.* 70**:** 68-78.

Jonassen AK, Sack MN, Mjos OD, and Yellon DM (2001). Myocardial protection by insulin at reperfusion requires early administration and is mediated via Akt and p70s6 kinase cell-survival signaling. *Circ. Res.* 89**:** 1191-1198.

Juhaszova M, Wang S, Zorov DB, Nuss HB, Gleichmann M, Mattson MP, and Sollott SJ (2008). The identity and regulation of the mitochondrial permeability transition pore: where the known meets the unknown. *Ann. N. Y. Acad. Sci.* 1123**:** 197-212.

Kadowaki T and Yamauchi T (2005). Adiponectin and adiponectin receptors. *Endocr. Rev.* 26**:** 439-451.

Kahn BB, Alquier T, Carling D, and Hardie DG (2005). AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab* 1**:** 15-25.

Kang PM and Izumo S (2000). Apoptosis and heart failure: A critical review of the literature. *Circ. Res.* 86**:** 1107-1113.

Karmazyn M, Gan XT, Humphreys RA, Yoshida H, and Kusumoto K (1999). The myocardial Na(+)-H(+) exchange: structure, regulation, and its role in heart disease. *Circ. Res.* 85**:** 777-786.

Kartal O, Inal V, Baysan O, and Saglam K (2008). [Relationship between serum leptin levels and left ventricular hypertrophy in obese hypertensive patients]. *Anadolu. Kardiyol. Derg.* 8**:** 342-346.

Kerr JF, Wyllie AH, and Currie AR (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* 26**:** 239-257.

Kim JS, Ohshima S, Pediaditakis P, and Lemasters JJ (2004a). Nitric oxide: a signaling molecule against mitochondrial permeability transition- and pH-dependent cell death after reperfusion. *Free Radic. Biol. Med.* 37**:** 1943-1950.

Kim KH, Zhao L, Moon Y, Kang C, and Sul HS (2004b). Dominant inhibitory adipocyte-specific secretory factor (ADSF)/resistin enhances adipogenesis and improves insulin sensitivity. *Proc. Natl. Acad. Sci. U. S. A* 101**:** 6780-6785.

Kinnula VL and Crapo JD (2004). Superoxide dismutases in malignant cells and human tumors. *Free Radic. Biol. Med.* 36**:** 718-744.

Kirwin SM, Bhandari V, Dimatteo D, Barone C, Johnson L, Paul S, Spitzer AR, Chander A, Hassink SG, and Funanage VL (2006). Leptin enhances lung maturity in the fetal rat. *Pediatr. Res.* 60**:** 200-204.

Kishimoto T, Taga T, and Akira S (1994). Cytokine signal transduction. *Cell* 76**:** 253-262.

Klein HH, Pich S, Lindert S, Nebendahl K, Warneke G, and Kreuzer H (1989). Treatment of reperfusion injury with intracoronary calcium channel antagonists and reduced coronary free calcium concentration in regionally ischemic, reperfused porcine hearts. *J. Am. Coll. Cardiol.* 13**:** 1395-1401.

Kloner RA and Jennings RB (2001). Consequences of brief ischemia: stunning, preconditioning, and their clinical implications: part 2. *Circulation* 104**:** 3158-3167.

Konishi H, Kuroda S, Tanaka M, Matsuzaki H, Ono Y, Kameyama K, Haga T, and Kikkawa U (1995). Molecular cloning and characterization of a new member of the RAC protein kinase family: association of the pleckstrin homology domain of three types of RAC protein kinase with protein kinase C subspecies and beta gamma subunits of G proteins. *Biochem. Biophys. Res. Commun.* 216**:** 526-534.

Krebs HA and Henseleit K (1932). Untersuchungen über die Harnstoffbildung im Tierkörper. *Hoppe-Seyler's Zeitschrift fürPhysiol. Chemie.* 210**:** 33-66.

Kriketos AD, Greenfield JR, Peake PW, Furler SM, Denyer GS, Charlesworth JA, and Campbell LV (2004). Inflammation, insulin resistance, and adiposity: a study of first-degree relatives of type 2 diabetic subjects. *Diabetes Care* 27**:** 2033-2040.

Kumada M, Kihara S, Sumitsuji S, Kawamoto T, Matsumoto S, Ouchi N, Arita Y, Okamoto Y, Shimomura I, Hiraoka H, Nakamura T, Funahashi T, and Matsuzawa Y (2003). Association of hypoadiponectinemia with coronary artery disease in men. *Arterioscler. Thromb. Vasc. Biol.* 23**:** 85-89.

Kumar CC and Madison V (2005). AKT crystal structure and AKT-specific inhibitors. *Oncogene* 24**:** 7493-7501.

Kunisada K, Hirota H, Fujio Y, Matsui H, Tani Y, Yamauchi-Takihara K, and Kishimoto T (1996). Activation of JAK-STAT and MAP kinases by leukemia inhibitory factor through gp130 in cardiac myocytes. *Circulation* 94**:** 2626-2632.

Kunisada K, Negoro S, Tone E, Funamoto M, Osugi T, Yamada S, Okabe M, Kishimoto T, and Yamauchi-Takihara K (2000). Signal transducer and activator of transcription 3

in the heart transduces not only a hypertrophic signal but a protective signal against doxorubicin-induced cardiomyopathy. *Proc. Natl. Acad. Sci. U. S. A* 97**:** 315-319.

Kuzuya T, Hoshida S, Yamashita N, Fuji H, Oe H, Hori M, Kamada T, and Tada M (1993). Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. *Circ. Res.* 72**:** 1293-1299.

Kyo S, Sada K, Qu X, Maeno K, Miah SM, Kawauchi-Kamata K, and Yamamura H (2003). Negative regulation of Lyn protein-tyrosine kinase by c-Cbl ubiquitin-protein ligase in Fc epsilon RI-mediated mast cell activation. *Genes Cells* 8**:** 825-836.

Kyriakis JM, App H, Zhang XF, Banerjee P, Brautigan DL, Rapp UR, and Avruch J (1992). Raf-1 activates MAP kinase-kinase. *Nature* 358**:** 417-421.

Lecour S, Suleman N, Deuchar GA, Somers S, Lacerda L, Huisamen B, and Opie LH (2005). Pharmacological preconditioning with tumor necrosis factor-alpha activates signal transducer and activator of transcription-3 at reperfusion without involving classic prosurvival kinases (Akt and extracellular signal-regulated kinase). *Circulation* 112**:** 3911-3918.

Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI, and Friedman JM (1996). Abnormal splicing of the leptin receptor in diabetic mice. *Nature* 379**:** 632-635.

Lee NJ, Wong IP, Baldock PA, and Herzog H (2008). Leptin as an endocrine signal in bone. *Curr. Osteoporos. Rep.* 6**:** 62-66.

Lemasters JJ (1999). The mitochondrial permeability transition and the calcium, oxygen and pH paradoxes: one paradox after another. *Cardiovasc. Res.* 44**:** 470-473.

Leslie NR, Batty IH, Maccario H, Davidson L, and Downes CP (2008). Understanding PTEN regulation: PIP2, polarity and protein stability. *Oncogene* 27**:** 5464-5476.

Leung AW and Halestrap AP (2008). Recent progress in elucidating the molecular mechanism of the mitochondrial permeability transition pore. *Biochim. Biophys. Acta* 1777**:** 946-952.

Leung YM and Kwan CY (2008). Dual vascular effects of leptin via endothelium: hypothesis and perspective. *Chin J. Physiol* 51**:** 1-6.

Liapakis IE, Anagnostoulis S, Karayiannakis AJ, Korkolis DP, Lambropoulou M, Arnaud E, and Simopoulos CE (2008). Recombinant leptin administration improves early angiogenesis in full-thickness skin flaps: an experimental study. *In Vivo* 22**:** 247-252.

Lim CP and Cao X (2006). Structure, function, and regulation of STAT proteins. *Mol. Biosyst.* 2**:** 536-550.

Lim SY, Davidson SM, Hausenloy DJ, and Yellon DM (2007a). Preconditioning and postconditioning: the essential role of the mitochondrial permeability transition pore. *Cardiovasc. Res.* 75**:** 530-535.

Lim SY, Davidson SM, Mocanu MM, Yellon DM, and Smith CC (2007b). The cardioprotective effect of necrostatin requires the cyclophilin-D component of the mitochondrial permeability transition pore. *Cardiovasc. Drugs Ther.* 21**:** 467-469.

Lim SY, Davidson SM, Paramanathan AJ, Smith CC, Yellon DM, and Hausenloy DJ (2008). The novel adipocytokine visfatin exerts direct cardioprotective effects. *J. Cell Mol. Med.*

Liongue C and Ward AC (2007). Evolution of Class I cytokine receptors. *BMC. Evol. Biol.* 7**:** 120.

Liu GS, Thornton J, Van Winkle DM, Stanley AW, Olsson RA, and Downey JM (1991). Protection against infarction afforded by preconditioning is mediated by A1 adenosine receptors in rabbit heart. *Circulation* 84**:** 350-356.

Loffreda S, Yang SQ, Lin HZ, Karp CL, Brengman ML, Wang DJ, Klein AS, Bulkley GB, Bao C, Noble PW, Lane MD, and Diehl AM (1998). Leptin regulates proinflammatory immune responses. *FASEB J.* 12**:** 57-65.

Lu MC, Tzang BS, Kuo WW, Wu FL, Chen YS, Tsai CH, Huang CY, and Lee SD (2007). More activated cardiac mitochondrial-dependent apoptotic pathway in obese Zucker rats. *Obesity. (Silver. Spring)* 15**:** 2634-2642.

Maddock HL, Mocanu MM, and Yellon DM (2002). Adenosine A(3) receptor activation protects the myocardium from reperfusion/reoxygenation injury. *Am. J. Physiol Heart Circ. Physiol* 283**:** H1307-H1313.

Maiuri MC, Zalckvar E, Kimchi A, and Kroemer G (2007). Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nat. Rev. Mol. Cell Biol.* 8**:** 741-752.

Majno G and Joris I (1995). Apoptosis, oncosis, and necrosis. An overview of cell death. *Am. J. Pathol.* 146**:** 3-15.

Manning AS and Hearse DJ (1984). Reperfusion-induced arrhythmias: mechanisms and prevention. *J. Mol. Cell Cardiol.* 16**:** 497-518.

Marber MS, Latchman DS, Walker JM, and Yellon DM (1993). Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. *Circulation* 88**:** 1264-1272.

Maruyama I, Nakata M, and Yamaji K (2000). Effect of leptin in platelet and endothelial cells. Obesity and arterial thrombosis. *Ann. N. Y. Acad. Sci.* 902**:** 315-319.

Matarese G, Leiter EH, and La Cava A (2007a). Leptin in autoimmunity: many questions, some answers. *Tissue Antigens* 70**:** 87-95.

Matarese G, Mantzoros C, and La Cava A (2007b). Leptin and adipocytokines: bridging the gap between immunity and atherosclerosis. *Curr. Pharm. Des* 13**:** 3676-3680.

Matsui H, Motooka M, Koike H, Inoue M, Iwasaki T, Suzuki T, Kurabayashi M, and Yokoyama T (2007). Ischemia/reperfusion in rat heart induces leptin and leptin receptor gene expression. *Life Sci.* 80**:** 672-680.

Matsui T and Rosenzweig A (2005). Convergent signal transduction pathways controlling cardiomyocyte survival and function: the role of PI 3-kinase and Akt. *J. Mol. Cell Cardiol.* 38**:** 63-71.

Matsuzawa Y (2005). White adipose tissue and cardiovascular disease. *Best. Pract. Res. Clin. Endocrinol. Metab* 19**:** 637-647.

Matsuzawa Y, Nakamura T, Shimomura I, and Kotani K (1995). Visceral fat accumulation and cardiovascular disease. *Obes. Res.* 3 Suppl 5**:** 645S-647S.

Mattson MP and Chan SL (2003). Calcium orchestrates apoptosis. *Nat. Cell Biol.* 5**:** 1041-1043.

McCormick J, Barry SP, Sivarajah A, Stefanutti G, Townsend PA, Lawrence KM, Eaton S, Knight RA, Thiemermann C, Latchman DS, and Stephanou A (2006). Free radical scavenging inhibits STAT phosphorylation following in vivo ischemia/reperfusion injury. *FASEB J.* 20**:** 2115-2117.

McGaffin KR, Sun CK, Rager JJ, Romano LC, Zou B, Mathier MA, O'Doherty RM, McTiernan CF, and O'donnell CP (2008). Leptin signalling reduces the severity of cardiac dysfunction and remodelling after chronic ischaemic injury. *Cardiovasc. Res.* 77**:** 54-63.

Minokoshi Y, Kim YB, Peroni OD, Fryer LG, Muller C, Carling D, and Kahn BB (2002). Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. *Nature* 415**:** 339-343.

Miura T, Nishihara M, and Miki T (2009). Drug Development Targeting the Glycogen Synthase Kinase-3beta (GSK-3beta)-Mediated Signal Transduction Pathway:Role of GSK-3beta in Myocardial Protection Against Ischemia/Reperfusion Injury. *J. Pharmacol. Sci.*

Mocanu MM, Baxter GF, and Yellon DM (2000). Caspase inhibition and limitation of myocardial infarct size: protection against lethal reperfusion injury. *Br. J. Pharmacol.* 130**:** 197-200.

Mockridge JW, Marber MS, and Heads RJ (2000a). Activation of Akt during simulated ischemia/reperfusion in cardiac myocytes. *Biochem. Biophys. Res. Commun.* 270**:** 947-952.

Mockridge JW, Marber MS, and Heads RJ (2000b). Activation of Akt during simulated ischemia/reperfusion in cardiac myocytes. *Biochem. Biophys. Res. Commun.* 270**:** 947-952.

Monteiro P, Goncalves L, and Providencia LA (2005). Diabetes and cardiovascular disease: the road to cardioprotection. *Heart* 91**:** 1621-1625.

Mori H, Hanada R, Hanada T, Aki D, Mashima R, Nishinakamura H, Torisu T, Chien KR, Yasukawa H, and Yoshimura A (2004). Socs3 deficiency in the brain elevates leptin sensitivity and confers resistance to diet-induced obesity. *Nat. Med.* 10**:** 739-743.

Mudd JO and Kass DA (2008). Tackling heart failure in the twenty-first century. *Nature* 451**:** 919-928.

Munzberg H and Myers MG, Jr. (2005). Molecular and anatomical determinants of central leptin resistance. *Nat. Neurosci.* 8**:** 566-570.

Murphy E (2004). Inhibit GSK-3beta or there's heartbreak dead ahead. *J. Clin. Invest* 113**:** 1526-1528.

Murphy KG and Bloom SR (2006). Are all fats created equal? *Nat. Med.* 12**:** 32-33.

Murray PJ (2007). The JAK-STAT signaling pathway: input and output integration. *J. Immunol.* 178**:** 2623-2629.

Murry CE, Jennings RB, and Reimer KA (1986). Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 74**:** 1124-1136.

Negoro S, Kunisada K, Tone E, Funamoto M, Oh H, Kishimoto T, and Yamauchi-Takihara K (2000). Activation of JAK/STAT pathway transduces cytoprotective signal in rat acute myocardial infarction. *Cardiovasc. Res.* 47**:** 797-805.

Nicholson DW (1999). Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death. Differ.* 6**:** 1028-1042.

Ning K, Miller LC, Laidlaw HA, Burgess LA, Perera NM, Downes CP, Leslie NR, and Ashford ML (2006). A novel leptin signalling pathway via PTEN inhibition in hypothalamic cell lines and pancreatic beta-cells. *EMBO J.* 25**:** 2377-2387.

Niswender KD, Morton GJ, Stearns WH, Rhodes CJ, Myers MG, Jr., and Schwartz MW (2001). Intracellular signalling. Key enzyme in leptin-induced anorexia. *Nature* 413**:** 794-795.

Noh HL, Yamashita H, and Goldberg IJ (2006). Cardiac metabolism and mechanics are altered by genetic loss of lipoprotein triglyceride lipolysis. *Cardiovasc. Drugs Ther.* 20**:** 441-444.

Nohl H and Jordan W (1980). The metabolic fate of mitochondrial hydrogen peroxide. *Eur. J. Biochem.* 111**:** 203-210.

O'Carroll AM, Selby TL, Palkovits M, and Lolait SJ (2000). Distribution of mRNA encoding B78/apj, the rat homologue of the human APJ receptor, and its endogenous ligand apelin in brain and peripheral tissues. *Biochim. Biophys. Acta* 1492**:** 72-80.

O'Rourke L and Shepherd PR (2002). Biphasic regulation of extracellular-signal-regulated protein kinase by leptin in macrophages: role in regulating STAT3 Ser727 phosphorylation and DNA binding. *Biochem. J.* 364**:** 875-879.

Ogawa Y, Masuzaki H, Isse N, Okazaki T, Mori K, Shigemoto M, Satoh N, Tamura N, Hosoda K, Yoshimasa Y, and . (1995). Molecular cloning of rat obese cDNA and augmented gene expression in genetically obese Zucker fatty (fa/fa) rats. *J. Clin. Invest* 96**:** 1647-1652.

Omura T, Yoshiyama M, Shimada T, Shimizu N, Kim S, Iwao H, Takeuchi K, and Yoshikawa J (1999). Activation of mitogen-activated protein kinases in in vivo ischemia/reperfused myocardium in rats. *J. Mol. Cell Cardiol.* 31**:** 1269-1279.

Oshima Y, Fujio Y, Nakanishi T, Itoh N, Yamamoto Y, Negoro S, Tanaka K, Kishimoto T, Kawase I, and Azuma J (2005). STAT3 mediates cardioprotection against ischemia/reperfusion injury through metallothionein induction in the heart. *Cardiovasc. Res.* 65**:** 428-435.

Osugi T, Oshima Y, Fujio Y, Funamoto M, Yamashita A, Negoro S, Kunisada K, Izumi M, Nakaoka Y, Hirota H, Okabe M, Yamauchi-Takihara K, Kawase I, and Kishimoto T (2002). Cardiac-specific activation of signal transducer and activator of transcription 3 promotes vascular formation in the heart. *J. Biol. Chem.* 277**:** 6676-6681.

Ouchi N, Kihara S, Funahashi T, Nakamura T, Nishida M, Kumada M, Okamoto Y, Ohashi K, Nagaretani H, Kishida K, Nishizawa H, Maeda N, Kobayashi H, Hiraoka H, and Matsuzawa Y (2003). Reciprocal association of C-reactive protein with adiponectin in blood stream and adipose tissue. *Circulation* 107**:** 671-674.

Paez J and Sellers WR (2003). PI3K/PTEN/AKT pathway. A critical mediator of oncogenic signaling. *Cancer Treat. Res.* 115**:** 145-167.

Palanivel R, Eguchi M, Shuralyova I, Coe I, and Sweeney G (2006). Distinct effects of short- and long-term leptin treatment on glucose and fatty acid uptake and metabolism in HL-1 cardiomyocytes. *Metabolism* 55**:** 1067-1075.

Palkovits M (2003). Hypothalamic regulation of food intake. *Ideggyogy. Sz* 56**:** 288-302.

Pan J, Fukuda K, Kodama H, Makino S, Takahashi T, Sano M, Hori S, and Ogawa S (1997). Role of angiotensin II in activation of the JAK/STAT pathway induced by acute pressure overload in the rat heart. *Circ. Res.* 81**:** 611-617.

Pan J, Fukuda K, Saito M, Matsuzaki J, Kodama H, Sano M, Takahashi T, Kato T, and Ogawa S (1999). Mechanical stretch activates the JAK/STAT pathway in rat cardiomyocytes. *Circ. Res.* 84**:** 1127-1136.

Park HY, Kwon HM, Lim HJ, Hong BK, Lee JY, Park BE, Jang Y, Cho SY, and Kim HS (2001). Potential role of leptin in angiogenesis: leptin induces endothelial cell proliferation and expression of matrix metalloproteinases in vivo and in vitro. *Exp. Mol. Med.* 33**:** 95-102.

Parker E, Van Heek M, and Stamford A (2002). Neuropeptide Y receptors as targets for anti-obesity drug development: perspective and current status. *Eur. J. Pharmacol.* 440**:** 173-187.

Peng XD, Xu PZ, Chen ML, Hahn-Windgassen A, Skeen J, Jacobs J, Sundararajan D, Chen WS, Crawford SE, Coleman KG, and Hay N (2003). Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2. *Genes Dev.* 17**:** 1352-1365.

Penttila A and Trump BF (1974). Extracellular acidosis protects Ehrlich ascites tumor cells and rat renal cortex against anoxic injury. *Science* 185**:** 277-278.

Peterson JM, Bryner RW, Sindler A, Frisbee JC, and Alway SE (2008). Mitochondrial apoptotic signaling is elevated in cardiac but not skeletal muscle in the obese Zucker rat and is reduced with aerobic exercise. *J. Appl. Physiol* 105**:** 1934-1943.

Phillips MS, Liu Q, Hammond HA, Dugan V, Hey PJ, Caskey CJ, and Hess JF (1996). Leptin receptor missense mutation in the fatty Zucker rat. *Nat. Genet.* 13**:** 18-19.

Pineiro R, Iglesias MJ, Gallego R, Raghay K, Eiras S, Rubio J, Dieguez C, Gualillo O, Gonzalez-Juanatey JR, and Lago F (2005). Adiponectin is synthesized and secreted by human and murine cardiomyocytes. *FEBS Lett.* 579**:** 5163-5169.

Piot C, Croisille P, Staat P, Thibault H, Rioufol G, Mewton N, Elbelghiti R, Cung TT, Bonnefoy E, Angoulvant D, Macia C, Raczka F, Sportouch C, Gahide G, Finet G, Andre-Fouet X, Revel D, Kirkorian G, Monassier JP, Derumeaux G, and Ovize M (2008). Effect of cyclosporine on reperfusion injury in acute myocardial infarction. *N. Engl. J. Med.* 359**:** 473-481.

Piper HM, Abdallah Y, and Schafer C (2004). The first minutes of reperfusion: a window of opportunity for cardioprotection. *Cardiovasc. Res.* 61**:** 365-371.

Piper HM, Garcia-Dorado D, and Ovize M (1998). A fresh look at reperfusion injury. *Cardiovasc. Res.* 38**:** 291-300.

Poirier P, Giles TD, Bray GA, Hong Y, Stern JS, Pi-Sunyer FX, and Eckel RH (2006). Obesity and cardiovascular disease: pathophysiology, evaluation, and effect of weight loss: an update of the 1997 American Heart Association Scientific Statement on Obesity and Heart Disease from the Obesity Committee of the Council on Nutrition, Physical Activity, and Metabolism. *Circulation* 113**:** 898-918.

Pralong FP and Gaillard RC (2001). Neuroendocrine effects of leptin. *Pituitary.* 4**:** 25-32.

Purdham DM, Rajapurohitam V, Zeidan A, Huang C, Gross GJ, and Karmazyn M (2008). A neutralizing leptin receptor antibody mitigates hypertrophy and hemodynamic dysfunction in the postinfarcted rat heart. *Am. J. Physiol Heart Circ. Physiol* 295**:** H441-H446.

Purdham DM, Zou MX, Rajapurohitam V, and Karmazyn M (2004). Rat heart is a site of leptin production and action. *Am. J. Physiol Heart Circ. Physiol* 287**:** H2877-H2884.

Qamar A, Sheikh SZ, Masud A, Jhandier MN, Inayat IB, Hakim W, and Mehal WZ (2006). In vitro and in vivo protection of stellate cells from apoptosis by leptin. *Dig. Dis. Sci.* 51**:** 1697-1705.

Qian T, Nieminen AL, Herman B, and Lemasters JJ (1997). Mitochondrial permeability transition in pH-dependent reperfusion injury to rat hepatocytes. *Am. J. Physiol* 273**:** C1783-C1792.

Quehenberger P, Exner M, Sunder-Plassmann R, Ruzicka K, Bieglmayer C, Endler G, Muellner C, Speiser W, and Wagner O (2002). Leptin induces endothelin-1 in endothelial cells in vitro. *Circ. Res.* 90**:** 711-718.

Rahmouni K, Haynes WG, Morgan DA, and Mark AL (2003). Intracellular mechanisms involved in leptin regulation of sympathetic outflow. *Hypertension* 41**:** 763-767.

Rajapurohitam V, Gan XT, Kirshenbaum LA, and Karmazyn M (2003). The obesity-associated peptide leptin induces hypertrophy in neonatal rat ventricular myocytes. *Circ. Res.* 93**:** 277-279.

Rayner DV and Trayhurn P (2001). Regulation of leptin production: sympathetic nervous system interactions. *J. Mol. Med.* 79**:** 8-20.

Riess ML, Stowe DF, and Warltier DC (2004). Cardiac pharmacological preconditioning with volatile anesthetics: from bench to bedside? *Am. J. Physiol Heart Circ. Physiol* 286**:** H1603-H1607.

Rothwell SE, Richards AM, and Pemberton CJ (2006). Resistin worsens cardiac ischaemia-reperfusion injury. *Biochem. Biophys. Res. Commun.* 349**:** 400-407.

Satoh N, Ogawa Y, Katsuura G, Hayase M, Tsuji T, Imagawa K, Yoshimasa Y, Nishi S, Hosoda K, and Nakao K (1997). The arcuate nucleus as a primary site of satiety effect of leptin in rats. *Neurosci. Lett.* 224**:** 149-152.

Sattar N, Wannamethee G, Sarwar N, Chernova J, Lawlor DA, Kelly A, Wallace AM, Danesh J, and Whincup PH (2009). Leptin and coronary heart disease: prospective study and systematic review. *J. Am. Coll. Cardiol.* 53**:** 167-175.

Scaduto RC, Jr. and Grotyohann LW (1999). Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. *Biophys. J.* 76**:** 469-477.

Schaeffer HJ and Weber MJ (1999). Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol. Cell Biol.* 19**:** 2435-2444.

Schindler C and Darnell JE, Jr. (1995). Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. *Annu. Rev. Biochem.* 64**:** 621-651.

Schindler C, Levy DE, and Decker T (2007). JAK-STAT signaling: from interferons to cytokines. *J. Biol. Chem.* 282**:** 20059-20063.

Schulman D, Latchman DS, and Yellon DM (2002). Urocortin protects the heart from reperfusion injury via upregulation of p42/p44 MAPK signaling pathway. *Am. J. Physiol Heart Circ. Physiol* 283**:** H1481-H1488.

Schultz JE and Gross GJ (2001). Opioids and cardioprotection. *Pharmacol. Ther.* 89**:** 123-137.

Schust J, Sperl B, Hollis A, Mayer TU, and Berg T (2006). Stattic: a small-molecule inhibitor of STAT3 activation and dimerization. *Chem. Biol.* 13**:** 1235-1242.

Schwarz ER, Somoano Y, Hale SL, and Kloner RA (2000). What is the required reperfusion period for assessment of myocardial infarct size using triphenyltetrazolium chloride staining in the rat? *J. Thromb. Thrombolysis.* 10**:** 181-187.

Segawa K, Fukuhara A, Hosogai N, Morita K, Okuno Y, Tanaka M, Nakagawa Y, Kihara S, Funahashi T, Komuro R, Matsuda M, and Shimomura I (2006). Visfatin in adipocytes is upregulated by hypoxia through HIF1alpha-dependent mechanism. *Biochem. Biophys. Res. Commun.* 349**:** 875-882.

Shah PK (2002). Pathophysiology of coronary thrombosis: role of plaque rupture and plaque erosion. *Prog. Cardiovasc. Dis.* 44**:** 357-368.

Shah PK (2006). Thrombogenic risk factors for atherothrombosis. *Rev. Cardiovasc. Med.* 7**:** 10-16.

Shanmuganathan S, Hausenloy DJ, Duchen MR, and Yellon DM (2005). Mitochondrial permeability transition pore as a target for cardioprotection in the human heart. *Am. J. Physiol Heart Circ. Physiol* 289**:** H237-H242.

Shek EW, Brands MW, and Hall JE (1998). Chronic leptin infusion increases arterial pressure. *Hypertension* 31**:** 409-414.

Shibata R, Sato K, Pimentel DR, Takemura Y, Kihara S, Ohashi K, Funahashi T, Ouchi N, and Walsh K (2005). Adiponectin protects against myocardial ischemia-reperfusion injury through. *Nat. Med.* 11**:** 1096-1103.

Shiki K and Hearse DJ (1987). Preconditioning of ischemic myocardium: reperfusion-induced arrhythmias. *Am. J. Physiol* 253**:** H1470-H1476.

Siegmund B, Ladilov YV, and Piper HM (1994). Importance of sodium for recovery of calcium control in reoxygenated cardiomyocytes. *Am. J. Physiol* 267**:** H506-H513.

Siegmund B, Zude R, and Piper HM (1992). Recovery of anoxic-reoxygenated cardiomyocytes from severe Ca2+ overload. *Am. J. Physiol* 263**:** H1262-H1269.

Sierra-Honigmann MR, Nath AK, Murakami C, Garcia-Cardena G, Papapetropoulos A, Sessa WC, Madge LA, Schechner JS, Schwabb MB, Polverini PJ, and Flores-Riveros JR (1998). Biological action of leptin as an angiogenic factor. *Science* 281**:** 1683-1686.

Signore AP, Zhang F, Weng Z, Gao Y, and Chen J (2008). Leptin neuroprotection in the CNS: mechanisms and therapeutic potentials. *J. Neurochem.* 106**:** 1977-1990.

Simpkin JC, Yellon DM, Davidson SM, Lim SY, Wynne AM, and Smith CC (2007). Apelin-13 and apelin-36 exhibit direct cardioprotective activity against ischemiareperfusion injury. *Basic Res. Cardiol.* 102**:** 518-528.

Sironi JJ and Ouchi T (2004). STAT1-induced apoptosis is mediated by caspases 2, 3, and 7. *J. Biol. Chem.* 279**:** 4066-4074.

Sivaraman V, Mudalagiri NR, Di Salvo C, Kolvekar S, Hayward M, Yap J, Keogh B, Hausenloy DJ, and Yellon DM (2007). Postconditioning protects human atrial muscle through the activation of the RISK pathway. *Basic Res. Cardiol.* 102**:** 453-459.

Skeen JE, Bhaskar PT, Chen CC, Chen WS, Peng XD, Nogueira V, Hahn-Windgassen A, Kiyokawa H, and Hay N (2006). Akt deficiency impairs normal cell proliferation and suppresses oncogenesis in a p53-independent and mTORC1-dependent manner. *Cancer Cell* 10**:** 269-280.

Slomiany BL and Slomiany A (2008). Leptin protection of salivary gland acinar cells against ethanol cytotoxicity involves Src kinase-mediated parallel activation of prostaglandin and constitutive nitric oxide synthase pathways. *Inflammopharmacology.* 16**:** 76-82.

Smith CC, Davidson SM, Lim SY, Simpkin JC, Hothersall JS, and Yellon DM (2007a). Necrostatin: a potentially novel cardioprotective agent? *Cardiovasc. Drugs Ther.* 21**:** 227-233.

Smith CC, Mocanu MM, Bowen J, Wynne AM, Simpkin JC, Dixon RA, Cooper MB, and Yellon DM (2007b). Temporal Changes in Myocardial Salvage Kinases During Reperfusion Following Ischemia: Studies Involving the Cardioprotective Adipocytokine Apelin. *Cardiovasc. Drugs Ther.*

Smith CC, Mocanu MM, Davidson SM, Wynne AM, Simpkin JC, and Yellon DM (2006). Leptin, the obesity-associated hormone, exhibits direct cardioprotective effects. *Br. J. Pharmacol.* 149**:** 5-13.

Smith RM, Suleman N, Lacerda L, Opie LH, Akira S, Chien KR, and Sack MN (2004). Genetic depletion of cardiac myocyte STAT-3 abolishes classical preconditioning. *Cardiovasc. Res.* 63**:** 611-616.

Solaini G and Harris DA (2005). Biochemical dysfunction in heart mitochondria exposed to ischaemia and reperfusion. *Biochem. J.* 390**:** 377-394.

Somoza B, Guzman R, Cano V, Merino B, Ramos P, Diez-Fernandez C, Fernandez-Alfonso MS, and Ruiz-Gayo M (2007). Induction of cardiac uncoupling protein-2 expression and adenosine 5'-monophosphate-activated protein kinase phosphorylation during early states of diet-induced obesity in mice. *Endocrinology* 148**:** 924-931.

Sordet O, Khan QA, and Pommier Y (2004). Apoptotic topoisomerase I-DNA complexes induced by oxygen radicals and mitochondrial dysfunction. *Cell Cycle* 3**:** 1095-1097.

Staat P, Rioufol G, Piot C, Cottin Y, Cung TT, L'Huillier I, Aupetit JF, Bonnefoy E, Finet G, Andre-Fouet X, and Ovize M (2005). Postconditioning the human heart. *Circulation* 112**:** 2143-2148.

Steenbergen C, Murphy E, Watts JA, and London RE (1990). Correlation between cytosolic free calcium, contracture, ATP, and irreversible ischemic injury in perfused rat heart. *Circ. Res.* 66**:** 135-146.

Stephanou A (2004). Role of STAT-1 and STAT-3 in ischaemia/reperfusion injury. *J. Cell Mol. Med.* 8**:** 519-525.

Stephanou A, Scarabelli TM, Brar BK, Nakanishi Y, Matsumura M, Knight RA, and Latchman DS (2001). Induction of apoptosis and Fas receptor/Fas ligand expression by ischemia/reperfusion in cardiac myocytes requires serine 727 of the STAT-1 transcription factor but not tyrosine 701. *J. Biol. Chem.* 276**:** 28340-28347.

Stephens L, Anderson K, Stokoe D, Erdjument-Bromage H, Painter GF, Holmes AB, Gaffney PR, Reese CB, McCormick F, Tempst P, Coadwell J, and Hawkins PT (1998). Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. *Science* 279**:** 710-714.

Steppan CM, Brown EJ, Wright CM, Bhat S, Banerjee RR, Dai CY, Enders GH, Silberg DG, Wen X, Wu GD, and Lazar MA (2001). A family of tissue-specific resistin-like molecules. *Proc. Natl. Acad. Sci. U. S. A* 98**:** 502-506.

Sullivan PG, Thompson M, and Scheff SW (2000). Continuous infusion of cyclosporin A postinjury significantly ameliorates cortical damage following traumatic brain injury. *Exp. Neurol.* 161**:** 631-637.

Sweeney G (2002). Leptin signalling. *Cell Signal.* 14**:** 655-663.

Szocs K (2004). Endothelial dysfunction and reactive oxygen species production in ischemia/reperfusion and nitrate tolerance. *Gen. Physiol Biophys.* 23**:** 265-295.

Szokodi I, Tavi P, Foldes G, Voutilainen-Myllyla S, Ilves M, Tokola H, Pikkarainen S, Piuhola J, Rysa J, Toth M, and Ruskoaho H (2002). Apelin, the novel endogenous ligand of the orphan receptor APJ, regulates cardiac contractility. *Circ. Res.* 91**:** 434-440.

Tajmir P, Ceddia RB, Li RK, Coe IR, and Sweeney G (2004). Leptin increases cardiomyocyte hyperplasia via extracellular signal-regulated kinase- and phosphatidylinositol 3-kinase-dependent signaling pathways. *Endocrinology* 145**:** 1550-1555.

Takeda S, Elefteriou F, Levasseur R, Liu X, Zhao L, Parker KL, Armstrong D, Ducy P, and Karsenty G (2002). Leptin regulates bone formation via the sympathetic nervous system. *Cell* 111**:** 305-317.

Takeo S and Tanonaka K (2004). Na+ overload-induced mitochondrial damage in the ischemic heart. *Can. J. Physiol Pharmacol.* 82**:** 1033-1043.

Tang BL (2008). Leptin as a neuroprotective agent. *Biochem. Biophys. Res. Commun.* 368**:** 181-185.

Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J, Muir C, Sanker S, Moriarty A, Moore KJ, Smutko JS, Mays GG, Wool EA, Monroe CA, and Tepper RI (1995). Identification and expression cloning of a leptin receptor, OB-R. *Cell* 83**:** 1263-1271.

Tatemoto K, Hosoya M, Habata Y, Fujii R, Kakegawa T, Zou MX, Kawamata Y, Fukusumi S, Hinuma S, Kitada C, Kurokawa T, Onda H, and Fujino M (1998). Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. *Biochem. Biophys. Res. Commun.* 251**:** 471-476.

Tatemoto K, Takayama K, Zou MX, Kumaki I, Zhang W, Kumano K, and Fujimiya M (2001). The novel peptide apelin lowers blood pressure via a nitric oxide-dependent mechanism. *Regul. Pept.* 99**:** 87-92.

Thygesen K, Alpert JS, and White HD (2007). Universal definition of myocardial infarction. *Eur. Heart J.* 28**:** 2525-2538.

Tilg H and Moschen AR (2006). Adipocytokines: mediators linking adipose tissue, inflammation and immunity. *Nat. Rev. Immunol.* 6**:** 772-783.

Townsend PA, Scarabelli TM, Davidson SM, Knight RA, Latchman DS, and Stephanou A (2004). STAT-1 interacts with p53 to enhance DNA damage-induced apoptosis. *J. Biol. Chem.* 279**:** 5811-5820.

Trayhurn P and Wood IS (2005). Signalling role of adipose tissue: adipokines and inflammation in obesity. *Biochem. Soc. Trans.* 33**:** 1078-1081.

Tsang A, Hausenloy DJ, Mocanu MM, Carr RD, and Yellon DM (2005). Preconditioning the diabetic heart: the importance of Akt phosphorylation. *Diabetes* 54**:** 2360-2364.

Tsang A, Hausenloy DJ, Mocanu MM, and Yellon DM (2004). Postconditioning: a form of "modified reperfusion" protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. *Circ. Res.* 95**:** 230-232.

Tsao TS, Tomas E, Murrey HE, Hug C, Lee DH, Ruderman NB, Heuser JE, and Lodish HF (2003). Role of disulfide bonds in Acrp30/adiponectin structure and signaling specificity. Different oligomers activate different signal transduction pathways. *J. Biol. Chem.* 278**:** 50810-50817.

Tschop MH, Hui DY, and Horvath TL (2007). Diet-induced leptin resistance: the heart of the matter. *Endocrinology* 148**:** 921-923.

Tytgat J (1994). How to isolate cardiac myocytes. *Cardiovasc. Res.* 28**:** 280-283.

Unger RH (2004). The hyperleptinemia of obesity-regulator of caloric surpluses. *Cell* 117**:** 145-146.

Unger RH (2005). Hyperleptinemia: protecting the heart from lipid overload. *Hypertension* 45**:** 1031-1034.

Vanden Hoek TL, Li C, Shao Z, Schumacker PT, and Becker LB (1997). Significant levels of oxidants are generated by isolated cardiomyocytes during ischemia prior to reperfusion. *J. Mol. Cell Cardiol.* 29**:** 2571-2583.

Varvarigou A, Mantzoros CS, and Beratis NG (1999). Cord blood leptin concentrations in relation to intrauterine growth. *Clin. Endocrinol. (Oxf)* 50**:** 177-183.

Vecchione C, Maffei A, Colella S, Aretini A, Poulet R, Frati G, Gentile MT, Fratta L, Trimarco V, Trimarco B, and Lembo G (2002). Leptin effect on endothelial nitric oxide is mediated through Akt-endothelial nitric oxide synthase phosphorylation pathway. *Diabetes* 51**:** 168-173.

Verploegen SA, Plaetinck G, Devos R, Van der HJ, and Guisez Y (1997). A human leptin mutant induces weight gain in normal mice. *FEBS Lett.* 405**:** 237-240.

Vinten-Johansen J, Yellon DM, and Opie LH (2005). Postconditioning: a simple, clinically applicable procedure to improve revascularization in acute myocardial infarction. *Circulation* 112**:** 2085-2088.

Vivaldi MT, Kloner RA, and Schoen FJ (1985). Triphenyltetrazolium staining of irreversible ischemic injury following coronary artery occlusion in rats. *Am. J. Pathol.* 121**:** 522-530.

Vivanco I and Sawyers CL (2002). The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat. Rev. Cancer* 2**:** 489-501.

Vlahos CJ, Matter WF, Hui KY, and Brown RF (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* 269**:** 5241-5248.

Vyssokikh MY and Brdiczka D (2003). The function of complexes between the outer mitochondrial membrane pore (VDAC) and the adenine nucleotide translocase in regulation of energy metabolism and apoptosis. *Acta Biochim. Pol.* 50**:** 389-404.

Wang Q, Bing C, Al Barazanji K, Mossakowaska DE, Wang XM, McBay DL, Neville WA, Taddayon M, Pickavance L, Dryden S, Thomas ME, McHale MT, Gloyer IS, Wilson S, Buckingham R, Arch JR, Trayhurn P, and Williams G (1997). Interactions between leptin and hypothalamic neuropeptide Y neurons in the control of food intake and energy homeostasis in the rat. *Diabetes* 46**:** 335-341.

Weiss JN, Korge P, Honda HM, and Ping P (2003). Role of the mitochondrial permeability transition in myocardial disease. *Circ. Res.* 93**:** 292-301.

Weng Z, Signore AP, Gao Y, Wang S, Zhang F, Hastings T, Yin XM, and Chen J (2007). Leptin protects against 6-hydroxydopamine-induced dopaminergic cell death via mitogen-activated protein kinase signaling. *J. Biol. Chem.* 282**:** 34479-34491.

Widmann C, Gibson S, Jarpe MB, and Johnson GL (1999). Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol Rev.* 79**:** 143-180.

Wiechelman KJ, Braun RD, and Fitzpatrick JD (1988). Investigation of the bicinchoninic acid protein assay: identification of the groups responsible for color formation. *Anal. Biochem.* 175**:** 231-237.

Wilding JP (2002). Neuropeptides and appetite control. *Diabet. Med.* 19**:** 619-627.

Williams IA, Xiao XH, Ju YK, and Allen DG (2007). The rise of [Na(+)] (i) during ischemia and reperfusion in the rat heart-underlying mechanisms. *Pflugers Arch.* 454**:** 903-912.

Winters B, Mo Z, Brooks-Asplund E, Kim S, Shoukas A, Li D, Nyhan D, and Berkowitz DE (2000). Reduction of obesity, as induced by leptin, reverses endothelial dysfunction in obese (Lep(ob)) mice. *J. Appl. Physiol* 89**:** 2382-2390.

Witthuhn BA, Quelle FW, Silvennoinen O, Yi T, Tang B, Miura O, and Ihle JN (1993). JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. *Cell* 74**:** 227-236.

Wolf AM, Wolf D, Avila MA, Moschen AR, Berasain C, Enrich B, Rumpold H, and Tilg H (2006). Up-regulation of the anti-inflammatory adipokine adiponectin in acute liver failure in mice. *J. Hepatol.* 44**:** 537-543.

Xie JR and Yu LN (2007). Cardioprotective effects of cyclosporine A in an in vivo model of myocardial ischemia and reperfusion. *Acta Anaesthesiol. Scand.* 51**:** 909-913.

Xu FP, Chen MS, Wang YZ, Yi Q, Lin SB, Chen AF, and Luo JD (2004). Leptin induces hypertrophy via endothelin-1-reactive oxygen species pathway in cultured neonatal rat cardiomyocytes. *Circulation* 110**:** 1269-1275.

Xuan YT, Guo Y, Han H, Zhu Y, and Bolli R (2001). An essential role of the JAK-STAT pathway in ischemic preconditioning. *Proc. Natl. Acad. Sci. U. S. A* 98**:** 9050-9055.

Yamaguchi H and Wang HG (2001). The protein kinase PKB/Akt regulates cell survival and apoptosis by inhibiting Bax conformational change. *Oncogene* 20**:** 7779-7786.

Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, Sugiyama T, Miyagishi M, Hara K, Tsunoda M, Murakami K, Ohteki T, Uchida S, Takekawa S, Waki H, Tsuno NH, Shibata Y, Terauchi Y, Froguel P, Tobe K, Koyasu S, Taira K, Kitamura T, Shimizu T, Nagai R, and Kadowaki T (2003). Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* 423**:** 762-769.

Yang G, Ge H, Boucher A, Yu X, and Li C (2004a). Modulation of direct leptin signaling by soluble leptin receptor. *Mol. Endocrinol.* 18**:** 1354-1362.

Yang R and Barouch LA (2007). Leptin signaling and obesity: cardiovascular consequences. *Circ. Res.* 101**:** 545-559.

Yang ZZ, Tschopp O, Baudry A, Dummler B, Hynx D, and Hemmings BA (2004b). Physiological functions of protein kinase B/Akt. *Biochem. Soc. Trans.* 32**:** 350-354.

Yellon DM, Alkhulaifi AM, and Pugsley WB (1993). Preconditioning the human myocardium. *Lancet* 342**:** 276-277.

Yellon DM and Hausenloy DJ (2007). Myocardial reperfusion injury. *N. Engl. J. Med.* 357**:** 1121-1135.

Youdim MB, Bar AO, Yogev-Falach M, Weinreb O, Maruyama W, Naoi M, and Amit T (2005). Rasagiline: neurodegeneration, neuroprotection, and mitochondrial permeability transition. *J. Neurosci. Res.* 79**:** 172-179.

Yue TL, Bao W, Gu JL, Cui J, Tao L, Ma XL, Ohlstein EH, and Jucker BM (2005). Rosiglitazone treatment in Zucker diabetic Fatty rats is associated with ameliorated cardiac insulin resistance and protection from ischemia/reperfusion-induced myocardial injury. *Diabetes* 54**:** 554-562.

Zabeau L, Lavens D, Peelman F, Eyckerman S, Vandekerckhove J, and Tavernier J (2003). The ins and outs of leptin receptor activation. *FEBS Lett.* 546**:** 45-50.

Zacharowski K, Otto M, Hafner G, Chatterjee PK, and Thiemermann C (1999). Endotoxin induces a second window of protection in the rat heart as determined by using p-nitro-blue tetrazolium staining, cardiac troponin T release, and histology. *Arterioscler. Thromb. Vasc. Biol.* 19**:** 2276-2280.

Zeidan A, Javadov S, Chakrabarti S, and Karmazyn M (2008). Leptin-induced cardiomyocyte hypertrophy involves selective caveolae and RhoA/ROCK-dependent p38 MAPK translocation to nuclei. *Cardiovasc. Res.* 77**:** 64-72.

Zeidan A, Javadov S, and Karmazyn M (2006). Essential role of Rho/ROCK-dependent processes and actin dynamics in mediating leptin-induced hypertrophy in rat neonatal ventricular myocytes. *Cardiovasc. Res.* 72**:** 101-111.

Zeymer U, Suryapranata H, Monassier JP, Opolski G, Davies J, Rasmanis G, Linssen G, Tebbe U, Schroder R, Tiemann R, Machnig T, and Neuhaus KL (2001). The Na(+)/H(+) exchange inhibitor eniporide as an adjunct to early reperfusion therapy for acute myocardial infarction. Results of the evaluation of the safety and cardioprotective effects of eniporide in acute myocardial infarction (ESCAMI) trial. *J. Am. Coll. Cardiol.* 38**:** 1644-1650.

Zhang F and Chen J (2008). Leptin protects hippocampal CA1 neurons against ischemic injury. *J. Neurochem.* 107**:** 578-587.

Zhang F, Wang S, Signore AP, and Chen J (2007). Neuroprotective effects of leptin against ischemic injury induced by oxygen-glucose deprivation and transient cerebral ischemia. *Stroke* 38**:** 2329-2336.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, and Friedman JM (1994). Positional cloning of the mouse obese gene and its human homologue. *Nature* 372**:** 425-432.

Zhao AZ, Shinohara MM, Huang D, Shimizu M, Eldar-Finkelman H, Krebs EG, Beavo JA, and Bornfeldt KE (2000). Leptin induces insulin-like signaling that antagonizes cAMP elevation by glucagon in hepatocytes. *J. Biol. Chem.* 275**:** 11348-11354.

Zhao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, Guyton RA, and Vinten-Johansen J (2003). Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. *Am. J. Physiol Heart Circ. Physiol* 285**:** H579-H588.

Zheng D, Wooter MH, Zhou Q, and Dohm GL (1996). The effect of exercise on ob gene expression. *Biochem. Biophys. Res. Commun.* 225**:** 747-750.

Zhou LL, Zhou LY, Luo KQ, and Chang DC (2005). Smac/DIABLO and cytochrome c are released from mitochondria through a similar mechanism during UV-induced apoptosis. *Apoptosis.* 10**:** 289-299.

Zhou YT, Grayburn P, Karim A, Shimabukuro M, Higa M, Baetens D, Orci L, and Unger RH (2000). Lipotoxic heart disease in obese rats: implications for human obesity. *Proc. Natl. Acad. Sci. U. S. A* 97**:** 1784-1789.

Zong WX and Thompson CB (2006). Necrotic death as a cell fate. *Genes Dev.* 20**:** 1-15.

Zorov DB, Filburn CR, Klotz LO, Zweier JL, and Sollott SJ (2000). Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. *J. Exp. Med.* 192**:** 1001-1014.

Books

Cotran; Kumar, Collins (1998). Robbins Pathologic Basis of Disease. Philadelphia: W.B Saunders Company

Opie, L. H. (1991) The Heart: Physiology and Metabolism, Raven Press, NY

Websites

www.pubmed.com

http://www.ruf.rice.edu/~bioslabs/methods/microscopy/cellcounting.html

http://www.heartandmetabolism.org/issues/HM10/hm10_basic_metabolism.asp

APPENDIX

The following section presents the raw data of chapter 8 (please see section 8.4). As aforementioned in the footnote on p.209 in section 8.4, a large amount of variation with respect to the baseline time to depolarisation of cardiomyocytes was observed. Therefore, the average time taken for control myocytes to depolarise was used as 100% and each result was calculated with respect to this value and expressed as a percentage, i.e. if a treated cardiomyocyte took twice the time to depolarise as the control the value assigned to this cardiomyocyte would be 200%. The original data are presented in tables on the following 5 pages.

One	Wistar			Zucker Lean			*fa/fa*		
	Control	Leptin	CsA	Control	leptin	CsA	Control	Leptin A	CSA
	126	180		151	220	173	114	183	157
	140	151		169	326	234	181	145	160
	98	151		171	263	261	200	196	210
	120	150		181	338	311	175	106	163
	33	106		265	348	401	177	104	143
	41	173		118	232	328	204	179	202
	74	165		167	263	322	126	254	261
	167	163		192	289	429	155	75	143
		106		238	277	541	167	160	218
		98		216	269	189	155	177	212
		192		167	303	180	183	147	230
				216	261	189	196	169	140
					344	224	128	110	185
					413		194		238
					326				216
					405				246
Average	99.9	148.6		187.6	304.8	290.9	168.2	154.2	195.3

0.1 – Table showing raw cardiomyocyte depolarisation data

Two	Wistar			Zucker Lean			*fa/fa*		
	Control	Leptin	CSA	Control	leptin	CsA	Control	Leptin	CsA
	175	273	234	139	192	165	84	110	128
	194	265	263	139	226	236	70	112	135
	246	328	287	212	206	332	120	126	116
	200	257	301	212	181	277	124	141	135
	212	295	280	145	224	297	120	135	167
	255	270	289	179	208	181	126	145	80
	252	265	309	190	224	253	70	92	120
	228	356	352	192	281	265	76	155	120
	173	224	346	116	135	110	92	212	110
	175	240	240	84	198	236	92	210	200
	224	326	277	167	226	194	118	114	163
	206	269	299	145	155	135	86.6	122	110
		320	260	128	185	198	112	86.6	
		194		143	175	165	61	118	
		240		163	137	228	63	137	
		313		192	179			102	
		322						114	
Average	211.7	279.8	287.5	159.1	195.8	218.1	94.3	131.3	132.0

0.2 – Table showing raw cardiomyocyte depolarisation data

Three	Wistar			Zucker Lean			*fa/fa*		
	Control	leptin	CSA	Control	leptin	CsA	Control	leptin	CsA
	102	86	153	135	181	183	86.6	82	133
	60	92	153	175	242	232	80.7	100	165
	106	122	206	163	297	228	110	78	202
	80	140	139	169	230	269	126	110	181
	100	100	145	226	185	187	126	118	161
	112	191	100	90	191	157	133	82	147
	112	163	208	198	263	206	124	84	151
	126	108	165	183	263	315	139	120	159
	70	126	190	216	151	185	165	68	153
	106	120	126	147	175	206	147	68	200
	114	150	145	173	216	151	92	80	102
	65		90	151		192	84	80	130
	70		129				116	65	
	80						85	90	
	86						85	102	
	98						84	110	
	112						145	110	
Average	94.1	127.1	149.9	168.8	217.6	209.3	113.4	91.0	157.0

0.3 – Table showing raw cardiomyocyte depolarisation data

Four	Wistar			Zucker Lean			*fa/fa*		
	Control	Leptin	CSA1	Control	leptin	CsA	Control	leptin	CsA
	96.5	213	112	104	181	189	137	151	163
	96.5	196	131	139	208	228	159	129.9	175
	116	271	137	196	224	204	171	177	173
	122	145	150	175	242	193	143	226	212
	104	173	185	177	171	224	179	141	147
	108	238	177	218	202	155	243	204	252
	108	157	189	212	212	196	112	171	130
	80	157	108	169	244	220	153	135	175
	114	189	122	173	135	218	155	143	240
	141	185	114	192	214	179	112	160	133
	234	208	122	181	214	200	155	143	140
	139	256	143	189	214	218	220	160	155
	173	254	157			320	232	118	
		163	131				94	145	
		210	128				94	165	
							194		
Average	125.5	201.0	140.4	177.1	205.1	211.1	159.6	157.9	174.6

0.4 – Table showing raw cardiomyocyte depolarisation data

Five	Zucker Lean			*fa/fa*		
	Control	leptin	CsA	Control	leptin	CsA
	139	252	171	187	228	198
	200	252	226	232	228	238
	204	257	250	232	232	257
	218	281	236	175	235	259
	189	322	271	218	185	198
	206	238	269	124	189	218
	206	271	200	145	222	253
	281	265	257	185	236	218
	297	275	232	224	190	280
	336	316	230	228	220	222
	204	254	277	242	271	236
	206	271	204	261	147	265
	206	242		179	210	170
	244	275		196	234	208
	263	291		220	126	257
		281			171	
					198	
	226.6	271.4	235.3	203.2	207.2	231.8

0.5 - Table showing raw cardiomyocyte depolarisation data

PUBLICATIONS IN FULL

The publications produced whilst working on this thesis are presented in full on the following pages.

Cardiovasc Drugs Ther (2007) 21:409–414
DOI 10.1007/s10557-007-6054-y

Temporal Changes in Myocardial Salvage Kinases During Reperfusion Following Ischemia: Studies Involving the Cardioprotective Adipocytokine Apelin

Christopher C. T. Smith · Michaela M. Mocanu · Jonathan Bowen · Abigail M. Wynne · James C. Simpkin · Richard A. Dixon · Michael B. Cooper · Derek M. Yellon

Published online: 9 October 2007
© Springer Science + Business Media, LLC 2007

Abstract
Introduction Activation of the Reperfusion Injury Salvage Kinase (RISK) pathway, which incorporates phosphatidylinositol-3-OH kinase (PI3K)-Akt/protein kinase B (PKB) and p44/42 mitogen-activated protein kinase (MAPK), underlies protection against ischemia–reperfusion (I/R) injury. The temporal nature of the activation of these RISK pathway components during reperfusion is, however, uncertain. We examined Akt and p44/42 *phosphorylation* in hearts subjected to ischemia and varying periods of reperfusion in the absence or presence of the putative cardioprotectant, apelin-13. Akt *activity* was also measured.
Materials and methods Langendorff perfused C57Bl/6J mouse hearts were subjected to 35 min global ischemia followed by 0, 2.5, 5 or 10 min reperfusion with or without 1 μM apelin-13. Basal and apelin-induced *phosphorylation* of Akt (at both the threonine 308 and serine 473 phosphorylation sites) and p44/42 during the reperfusion phase was determined by Western blotting and Akt *activity* measured using an Enzyme-Linked ImmunoSorbent Assay (ELISA).
Results Basal *phosphorylation* of both Akt and p44/42 increased progressively with time of reperfusion. Apelin enhanced Akt and p44/42 *phosphorylation* at all reperfusion time points. Akt *activity* did not change under basal conditions but was increased by apelin at 5 min (NS) and 10 min ($p<0.05$) reperfusion.
Discussion We conclude that under basal conditions Akt and p44/42 *phosphorylation* increases with time of reperfusion but that this is not accompanied by increased kinase (Akt) *activity.* On application of a cardioprotectant, however, kinase *phosphorylation* and *activity* are enhanced suggesting that it is the combination of these two mechanisms that may underly the tissue preserving actions of such agents.

Key words myocardium · ischemia–reperfusion injury · salvage kinases · temporal changes

C. C. T. Smith · M. M. Mocanu · J. Bowen · A. M. Wynne · J. C. Simpkin · R. A. Dixon · M. B. Cooper · D. M. Yellon (✉)
The Hatter Cardiovascular Institute,
University College London Hospital and Medical School,
67 Chenies Mews,
London WC1E 6HX, UK
e-mail: d.yellon@ucl.ac.uk

Introduction

It is now generally accepted that phosphorylation of the Reperfusion Injury Salvage Kinase (RISK) pathway components Akt/protein kinase B (PKB) and p44/42 mitogen-activated protein kinase (MAPK), is a vital step in the series of events leading to myocardial protection against ischemia–reperfusion (I/R) injury [1]. When the maximal phosphorylation/activation of these kinases occurs during the reperfusion phase is, however, open to conjecture. For example, different laboratories often sample myocardium for Western blot analysis at different time points. The possibility that temporal differences occur between the two kinases with regard to their activation has also not been considered. In addition, it cannot be assumed that different cardioprotective agents necessarily stimulate kinase activation at the same time points.

A variety of chemically diverse substances protect against myocardial injury, as evidenced by reduced infarct size, including endogenous factors such as the adipocytokines adiponectin and leptin [2, 3]. We have reported that another

adipocytokine, namely apelin, also reduces infarct size [4]. Additionally, the administration of apelin within 5 min of reperfusion following a period of lethal ischemia was found to result in significant increases in the phosphorylation states of Akt and p44/42 (increases that were abrogated by LY294002 and UO126, inhibitors of Akt and p44/42, respectively), although we did not establish if these increases were maximal or not [4]. Furthermore, apelin was shown to delay the opening of the mitochondrial permeability transition pore (MPTP) in rat cardiomyocytes via a mechanism that was blocked by LY294002 and MEK inhibitor 1 [4]. Given our previous experience with apelin we, therefore, decided to use this peptide as the cardioprotective agent in the present study to investigate possible temporal changes occurring with respect to kinase phosphorylation and activity during reperfusion. Western blot analysis was used to examine the phosphorylation of Akt, at both its threonine 308 and serine 473 phosphorylation sites, and p44/42 at four time points (0, 2.5, 5 and 10 min) during the reperfusion phase that follows a period of lethal ischemia. Additionally, in the case of one of the kinases, i.e. Akt, a recently introduced Enzyme Linked ImmunoSorbent Assay (ELISA) was employed to investigate if Akt activity reflected the Akt phosphorylation state.

Materials and methods

Animals The current study was carried out in accordance with *The Guide for the care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication no.85–23, revised 1996) and was approved by the UCL Hospitals Ethics Committee.

Murine Langendorff model of ischemia–reperfusion injury Adult male C57Bl/6J mice (ca. 30 g) were given 100 U of heparin by intraperitoneal injection prior to cervical dislocation. Hearts were then excised and perfused retrogradely via the aorta at a constant pressure of 100 mm Hg with oxygenated Krebs-Henseleit buffer, pH 37°C [3, 5]. Myocardial temperature (thermal probe) and heart rate (ventricular balloon) were monitored throughout the perfusion periods. Hearts underwent 30 min stabilisation followed by 35 min global ischemia (achieved by total perfusion arrest) and 0, 2.5, 5 or 10 min reperfusion. Where necessary Krebs buffer containing 1 μM apelin-13 was substituted for normal buffer at reperfusion, these hearts corresponding to the cardioprotectant-treated group. Following the appropriate reperfusion period hearts were snap-frozen and stored at −80°C until analysis.

Western blot analysis Proteins from previously stored cardiac samples (−80°C) were extracted by homogenisation followed by high-speed centrifugation and the resultant supernatants assayed for total protein using a bicinchoninic acid (BCA) assay. Proteins were then separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences). Primary and secondary antibodies (Cell Signaling Technology) and ECL Western blotting reagent (Amersham Biosciences) were then used to detect total and phosphorylated Akt and p44/42 MAPK; in the case of Akt antibodies directed against both the threonine 308 and serine 473 phosphorylation sites were employed [3, 5]. The nitrocellulose membranes were then exposed to photographic film which was scanned and protein band intensity, expressed as arbitrary units (AU), determined by computerised densitometry (NIH Image 1.63).

Akt activity assay Myocardial samples were homogenised as described above (see section on Western blotting) and Akt in the supernatants extracted using a procedure that involves incubation with an Akt antibody (Calbiochem) and adsorption of immunoprecipitated Akt onto Protein A/Protein G-Plus agarose beads (Calbiochem). Akt activity was measured using a recently described K-LISA ™ Akt activity assay kit (Calbiochem), i.e. an ELISA-based activity assay that employs a biotinylated peptide substrate that is phosphorylated on the second serine by Akt1, Akt2, Akt3, SGK (Serum Glucocorticoid Kinase) and MSK1. The extracted tissue samples were incubated together with biotinylated substrate in the presence of ATP in the wells of a streptavidin-coated 96-well plate. The resultant phosphorylated substrate was then detected with a phosphoserine detection antibody followed by an HRP-antibody conjugate and colour development with TMB substrate. The absorbance was then read at 450/570 nm and enzyme activity expressed as optical density per 10 mg protein.

Statistical analysis Data are given as mean±SEM. Comparisons between groups were made using factorial, one-way analysis of variance (ANOVA). Where a significant F-value was obtained the Fisher's protected least significant difference post hoc test was used for between group comparisons. Differences were regarded as statistically significant if a value of $p<0.05$ was obtained.

Results

Akt phosphorylation Western blot analysis revealed that under basal conditions (i.e. in the absence of 1 μM apelin-13) Akt phosphorylation at the serine 473 site increased with time of reperfusion compared to control (0 min reperfusion), increases of 165% ($p<0.001$) and 249% ($p<0.001$) being recorded for 5

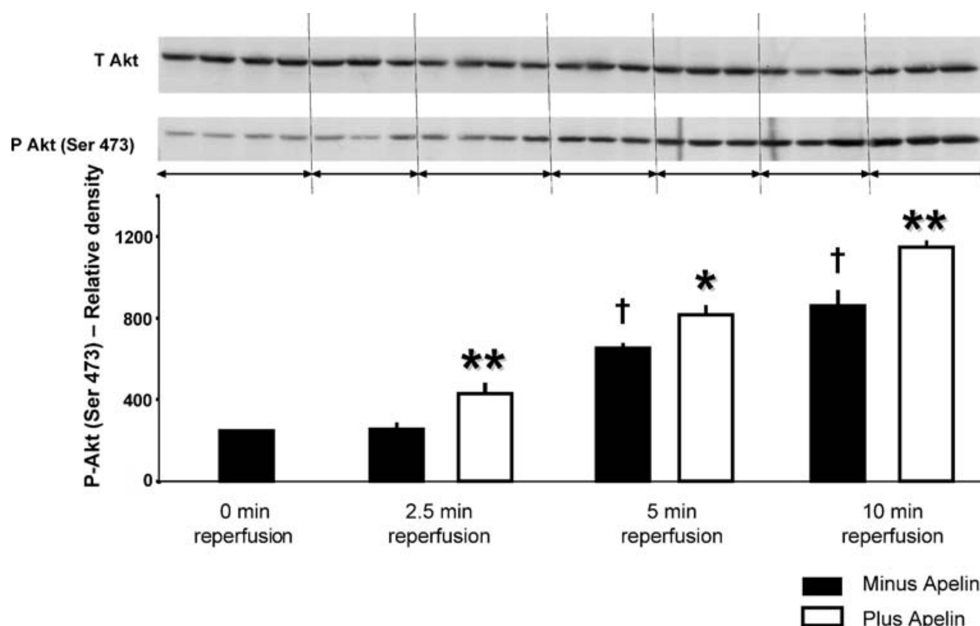


Fig. 1 Akt/serine 473 phosphorylation, determined by Western blot analysis, under basal conditions and in the presence of the cardioprotectant apelin-13 (1 μM). Hearts underwent a protocol that involved 30 min stabilisation followed by 35 min global ischemia (achieved by total perfusion arrest) and 2.5, 5 or 10 min reperfusion with or without apelin. *P* Phosphorylated and *T* total. Data (relative density readings) are presented as mean±SEM (*single asterisk* denotes $p<0.05$, *double asterisk* denotes $p<0.01$, *single dagger* denotes $p<0.001$; $n=3$–4)

and 10 min, respectively (Fig. 1). In the presence of apelin-13, Akt/serine 473 phosphorylation was enhanced relative to the corresponding basal level by 70% ($p<0.01$), 25% ($p<0.05$) and 34% ($p<0.001$) at 2.5, 5, and 10 min reperfusion (Fig. 1).

Akt/threonine 308 phosphorylation increased with time of reperfusion by 271% ($p<0.001$) and 623% ($p<0.0001$) for 5 and 10 min, respectively, compared to control (0 min reperfusion; Fig. 2). On application of apelin-13 Akt/threonine 308 phosphorylation was increased relative to the corresponding basal level by 117% ($p<0.05$) and 42% ($p<0.05$) at 2.5 and 5 min reperfusion (Fig. 2).

Total Akt levels did not differ markedly between the different groups (Figs. 1 and 2).

p44/42 phosphorylation As seen with Akt, p44 and p42 phosphorylation under basal conditions also increased with time of reperfusion (Figs. 3 and 4). Thus, in the case of p44,

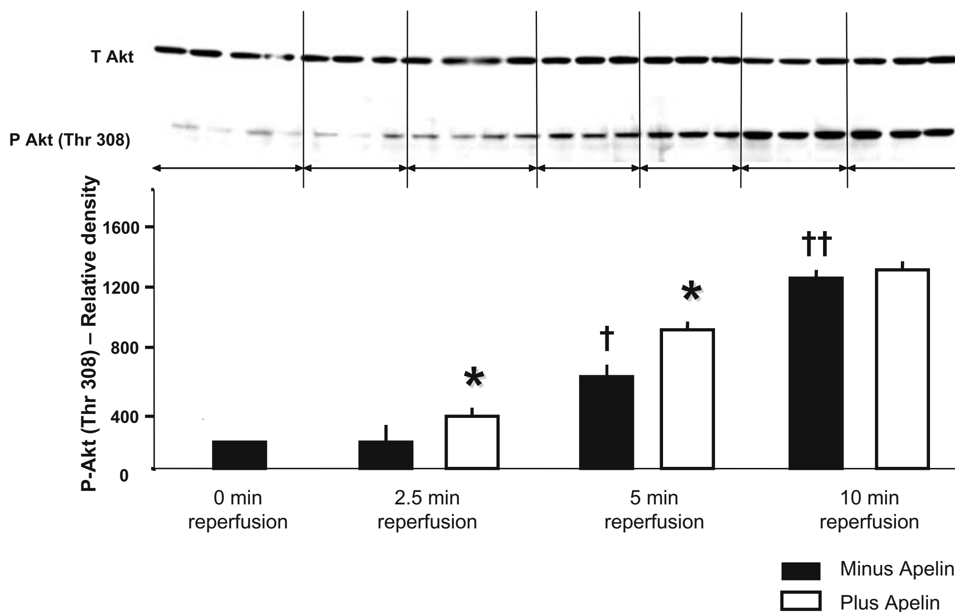


Fig. 2 Akt/threonine 308 phosphorylation, determined by Western blot analysis, under basal conditions and in the presence of apelin-13 (1 μM). Hearts underwent a protocol that involved 30 min stabilisation followed by 35 min global ischemia (achieved by total perfusion arrest) and 2.5, 5 or 10 min reperfusion with or without apelin. *P* Phosphorylated and *T* total. Data (relative density readings) are presented as mean±SEM (*single asterisk* denotes $p<0.05$, *single dagger* denotes $p<0.001$, *double dagger* denotes $p<0.0001$; $n=3$–4)

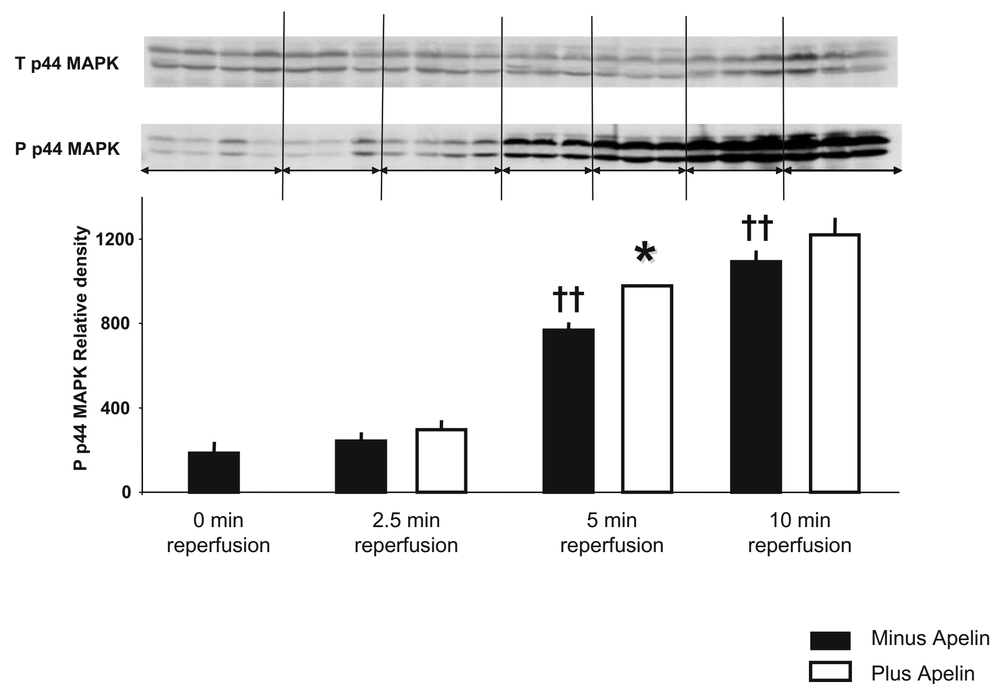


Fig. 3 p44 phosphorylation, determined by Western blotting, in the absence and presence of apelin-13 (1 μM). Hearts underwent 30 min stabilisation followed by 35 min global ischemia and 2.5, 5 and 10 min reperfusion with or without apelin. *P* Phosphorylated and *T* total. Values (relative density) are expressed as mean±SEM (*single asterisk* denotes $p<0.05$, *double dagger* denotes $p<0.0001$; $n=3$–4)

phosphorylation at 2.5, 5 and 10 min was increased by 29% (NS), 310% ($p<0.0001$) and 485% ($p<0.0001$), respectively (Fig. 3). With p42 increases of 28% (NS), 379% ($p<0.0001$) and 692% ($p<0.0001$) were observed (Fig. 4). On addition of apelin-13, p44 and p42 phosphorylation was increased relative to the basal level (Figs. 3 and 4). These changes were associated primarily with the p42 isoform, increases at 2.5 and 5 min reperfusion of 75% ($p<0.05$) and 47% ($p<0.01$), respectively, being observed (Fig. 4). With p44 a less marked but significant increase (+28%, $p<0.05$) was recorded at 5 min reperfusion only (Fig. 3). Again, as for total Akt, total p44 and p42 levels did not exhibit significant differences between the different groups (Figs 3 and 4).

Akt activity Having established that the Akt ELISA was linear on varying the amount of cardiac extract included in the assay (data not shown), Akt activity under basal conditions and in the presence of apelin-13 (1 μM) was investigated. Basal Akt

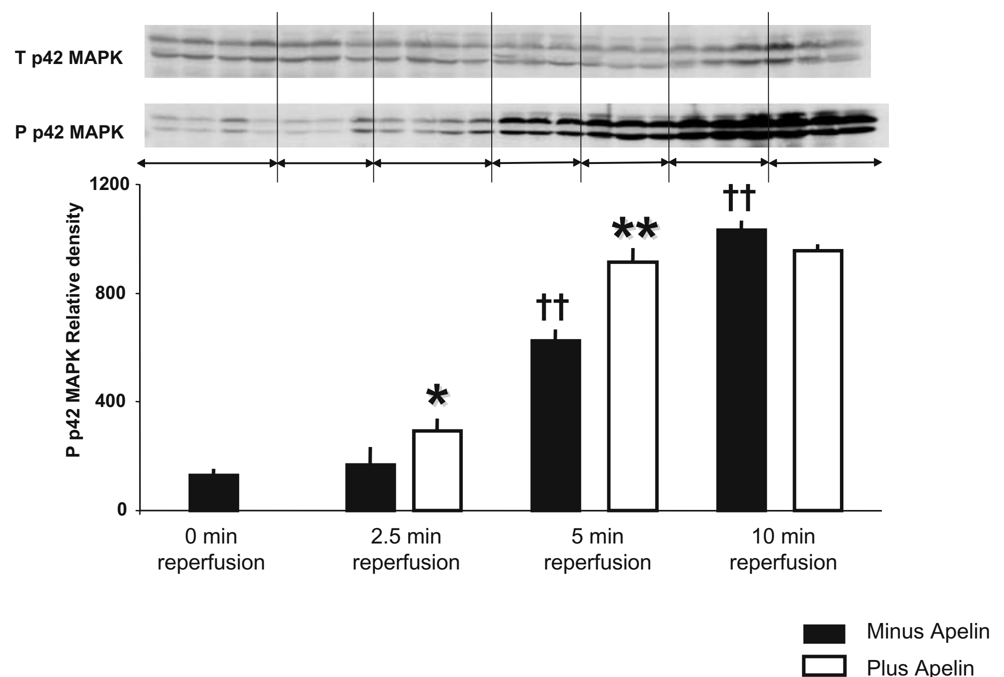


Fig. 4 p42 phosphorylation, determined by Western blot analysis, under basal conditions and in the presence of the cardioprotectant apelin-13 (1 μM). Hearts were perfused for 30 min (stabilisation) followed by 35 min ischemia and 2.5, 5 or 10 min reperfusion with or without apelin. P Phosphorylated and *T* total. Data (relative density readings) are presented as mean±SEM (*single asterisk* denotes $p<0.05$, *double asterisk* denotes $p<0.01$, *double dagger* denotes $p<0.0001$; $n=3$–4)

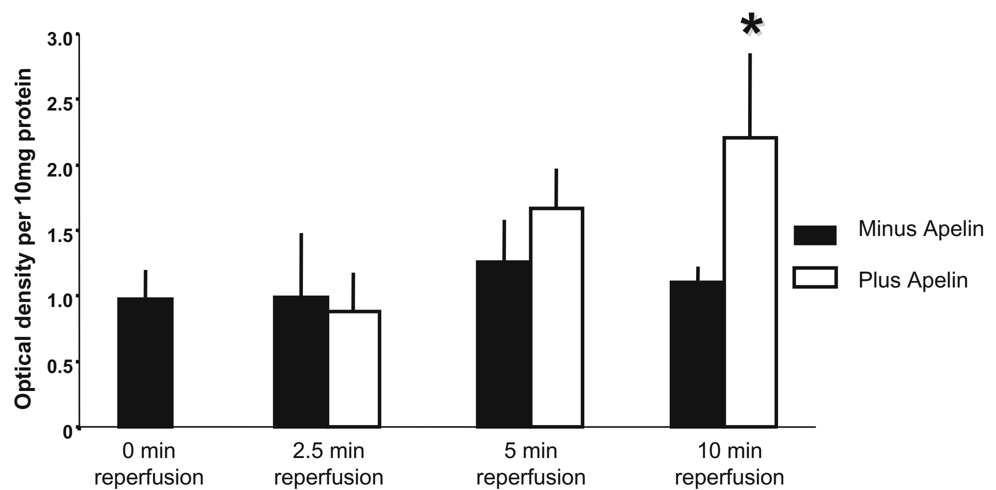


Fig. 5 Akt activity, as determined by ELISA, in the absence and presence of apelin-13 (1 μM). Hearts underwent a procedure which involved 30 min perfusion to achieve stabilisation followed by 35 min global ischemia (total perfusion arrest) and 2.5, 5 or 10 min reperfusion in the absence or presence of apelin. Values (optical density/10 mg protein) are expressed as mean±SEM (*single asterisk* denotes $p<0.05$; $n=3–4$)

activity was not found to vary with time of reperfusion and was not different from that observed under control conditions (0 min reperfusion; Fig. 5). With apelin-13 Akt *activity* was increased over basal levels at 5 min (+ 32%, NS) and 10 min (+100%, $p<0.05$) reperfusion (Fig. 5).

Discussion

In summary, this study has shown that under basal conditions of ischemia–reperfusion (i.e. in the absence of the cardioprotective agent apelin) phosphorylation of the RISK pathway components, Akt and p44/42, increased in a time-dependent fashion in the early minutes of reperfusion. These increases in phosphorylation were, however, not accompanied by increased kinase (i.e. Akt) activity. Contrasting with the results obtained under basal conditions, the application of apelin was found to result in not only increased Akt (serine 473 and threonine 308) and p44/42 phosphorylation (i.e. relative to the relevant controls for each reperfusion time-point) but also increased Akt activity at 5 and 10 min reperfusion, being statistically significant for the later time point.

We have reported previously that apelin-13 administered at reperfusion reduces infarct size compared to control (i.e. in the absence of apelin) and that this is associated with increased Akt and p44/42 phosphorylation at 5 min reperfusion [4]. Interestingly, in the present study kinase phosphorylation was found to increase progressively and, indeed, substantially under basal (i.e. control) conditions with time of reperfusion. Studies in which the cardioprotective effects of atorvastatin were investigated yielded comparable findings for basal Akt and eNOS phosphorylation [6]. One could suggest that such increases reflect an attempt by the myocardium to protect itself from I/R injury. It would appear, however, that in order for significant myocardial preservation to occur a protective agent, such as apelin, needs to be present [4]. Hence, the elevations in kinase phosphorylation that take place under basal conditions are, for whatever reason, not appropriate in the context of RISK pathway-mediated cardioprotection. The data obtained may also indicate that increased kinase phosphorylation is not necessarily accompanied by increased kinase activity. It is conceivable that for kinase activity to be elevated a threshold of phosphorylation must be exceeded, such as that induced by a cardioprotective agent, i.e. kinase phosphorylation under basal conditions may not be sufficient to “kick-start” kinase activity. Previously, Goto et al. [7] have invoked a similar scenario in relation to preconditioning of the myocardium. It was demonstrated that a preconditioning threshold needed to be surmounted in order for G_i protein-receptor-stimulated release of endogenous cardioprotective substances, such as adenosine, bradykinin, noradrenaline and opioids, to occur at levels sufficient to reduce infarction [7]. In the current study support for such a concept was provided by the observations made when Akt phosphorylation (at serine 473 and threonine 308) and Akt activity were compared at the different reperfusion time-points, a significant increase in Akt activity only being observed after 10 min treatment with apelin: of course, one cannot, exclude the possibility that under normal circumstances a delay may exist between kinase phosphorylation and activation of the enzyme. A further point to be considered regarding basal versus stimulus-induced kinase phosphorylation and infarction concerns the observation that although inhibitors of RISK pathway kinases abrogate the reductions in infarct size stimulated by cardioprotective treatments, including apelin [4], these inhibitors do not increase infarct size under basal conditions [1]. As these inhibitors substantially inhibit kinase phosphorylation under basal conditions, as well as that induced by cardioprotective agents, one might have expected a concomitant increase in infarct size relative to control. Nevertheless, this finding is consistent with the proposal that basal kinase phosphorylation is not appropriate in relation to RISK pathway-mediated cardioprotection.

Another theory to be explored in the context of RISK pathway-mediated cardioprotection concerns the particular isoforms of the kinases that are activated under different conditions. Akt, for example, exists as three isoforms designated Akt1, Akt2 and Akt3 [8]. When blotting for phospho-Akt we used antibodies directed against both the threonine 308 and serine 473 sites [9]. These antibodies recognize all three Akt isoforms when phosphorylated at the appropriate sites. Akt1 signaling is generally recognized as playing an important role in myocardial protection [10, 11], including in relation to ischemia–reperfusion injury [12]. It is, however, possible that factors which promote cellular survival may do so via the phosphorylation of Akt2 and Akt3 also, ultimately leading to increased Akt activity. In this regard, it should be noted that whilst the Akt1 isoform predominates in the heart, substantial expression of Akt2 also occurs, whilst Akt3 phosphorylation is upregulated in diseased hearts [13, 14]. Therefore, phosphorylation of the Akt2 and Akt3 isoforms, in addition to Akt1, might occur under specific conditions and influence cardioprotective mechanisms.

An alternative possibility to be considered in attempting to reconcile kinase phosphorylation with kinase activity concerns the particular sites that are phosphorylated on the kinase. As outlined above Akt can be phosphorylated at two sites and it has been suggested that full Akt activation requires two phosphorylation events [9, 15]. Hence, this is the reason why we blotted for both the serine 473 and threonine 308 phosphorylation sites in the present study. Interestingly, we found that the increases, as induced by apelin, observed with respect to threonine 308 phosphorylation appeared generally to exceed those for serine 473 (i.e. at 2.5 and 5 min reperfusion). One could, therefore, hypothesise that phosphorylation of the threonine 308 site might represent a key step in relation to Akt activation in the setting of cardioprotection, although this will need to be investigated rigorously in order to assess the relative contributions made by the two phosphorylation sites to cardioprotection. The fact remains, however, that we observed a temporal difference between peak Akt phosphorylation, including that for the threonine 308 site, and maximal Akt activity.

Turning to p44/42, it is unfortunate that parallel measurements of both the phosphorylation states and activities of this kinase were not made under the different conditions examined. The present study was, however, only preliminary and as commercially available ELISA assays for p44/42 have now come “online” measurements will be carried out in future investigations to establish if similar profiles to those seen with Akt are obtained.

To conclude, we have obtained evidence that the phosphorylation of RISK pathway components and specifically Akt does not necessarily equate with kinase activity. Importantly, kinase phosphorylation under basal conditions, even if substantial, does not appear to be consistent with cardioprotection and the introduction of an added stimulus in the form of pre- or post-conditioning, or a pharmacological agent is necessary in order to tip the balance in favour of myocardial preservation. Future, more detailed studies including measurements of p44/42 kinase activity, as well as Akt activity, should resolve the situation with respect to the relationship between the phosphorylation and activation of RISK pathway components and cardioprotection.

References

1. Hausenloy DJ, Yellon DM. New directions for protecting the heart against ischaemia–reperfusion injury: targeting the reperfusion injury salvage kinase (RISK)-pathway. Cardiovasc Res 2004;61:448–60.
2. Shibata R, Sato K, Pimental DR, Takemura Y, Kihara S, Ohashi K, et al. Adiponectin protects against myocardial ischemia–reperfusion injury through AMPK- and COX-2 dependent mechanisms. Nat Med 2005;10:1384–9.
3. Smith CCT, Mocanu MM, Davidson SM, Wynne AM, Simpkin JC, Yellon DM. Leptin, the obesity-associated hormone, exhibits direct cardioprotective effects. Br J Pharmacol 2006;149:5–13.
4. Simpkin JC, Yellon DM, Davison SM, Lim SY, Wynne AM, Smith CCT. Apelin-13 and apelin-36 exhibit direct cardioprotective activity against ischemia–reperfusion injury. Basic Res Cardiol 2007. DOI 10.1007/s00395-007-0671-2.
5. Efthymiou CA, Mocanu MM, Yellon DM. Atorvastatin and myocardial reperfusion injury: new pleiotropic effect implicating multiple prosurvival signaling. J Cardiovasc Pharmacol 2005; 45:247–52.
6. Bell RM, Yellon DM. Atorvastatin, administered at the onset of reperfusion, and independent of lipid lowering, protects the myocardium by up-regulating a pro-survival pathway. J Am Coll Cardiol 2003;41:508–15.
7. Goto M, Liu Y, Yang X-M, Ardell JL, Cohen MV, Downey JM. Role of bradykinin in protection of ischemic preconditioning in rabbit hearts. Circ Res 1995;77:611–21.
8. Dummler B, Hemmings BA. Physiological roles of PKB/Akt isoforms in development and disease. Biochem Soc Trans 2007; 35:231–35.
9. Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, Hemmings BA. Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J 1996;15:6541–51.
10. Fujio Y, Nguyen T, Wencker D, Kitsis RN, Walsh K. Akt promotes survival of cardiomyocytes in vitro and protects against ischemia–reperfusion injury in mouse heart. Circulation 2000;101:660–67.
11. Taniyama Y, Walsh K. Elevated myocardial Akt signaling ameliorates doxorubicin-induced congestive heart failure and promotes heart growth. J Mol Cell Cardiol 2002;34:1241–7.
12. Shiraishi I, Melendez J, Ahn Y, Skavdahl M, Murphy E, Wech S, et al. Nuclear targeting of Akt enhances kinase activity and survival of cardiomyocytes. Circ Res 2004;94:884–91.
13. Altomare DA, Guo K, Cheng JQ, Sonoda G, Walsh K, Testa JR. Cloning, chromosomal localization and expression analysis of the mouse Akt2 oncogene. Oncogene 1995;11:1055–60.
14. Taniyama Y, Ito M, Sato K, Kuester C, Veit K, Tremp G, et al. Akt3 overexpression in the heart results in progression from adaptive to maladaptive hypertrophy. J Mol Cell Cardiol 2005;38:375–85.
15. Tsurutani J, Fukuoka J, Tsurutani H, Shih JH, Hewitt SM, Travis WD, et al. Evaluation of two phosphorylation sites improves the prognostic significance of Akt activation in non-small-cell lung cancer tumors. J Clin Oncol 2006;24:306–14.

The Cardioprotective Actions of Leptin Are Lost in the Zucker Obese *(fa/fa)* Rat

Richard A. Dixon, BSc, Sean M. Davidson, PhD, Abigail M. Wynne, MSc, Derek M. Yellon, DSc, and Christopher C. T. Smith, PhD

Abstract: Protection against myocardial ischemia–reperfusion injury, including that induced by leptin, involves activation of the reperfusion injury salvage kinase pathway and inhibition of the mitochondrial permeability transition pore. In the current study, we explored the mechanisms underlying leptin-induced cardioprotection further with reference to the leptin receptor (OB-R) and obesity. We examined hearts from Wistar and Zucker lean rats that express functional OB-R and Zucker obese *(fa/fa)* rats with nonfunctional OB-R. In Langendorff experiments, leptin (10 nM) caused significant reductions in infarct size in hearts from Wistar (leptin treated, 32.4% ± 3.9% vs. control, 53.2% ± 3.2%, $P < 0.01$) and Zucker lean animals (leptin treated, 25.2% ± 3.7% vs. control, 53.9% ± 11.3%, $P < 0.01$). By contrast, hearts from *(fa/fa)* did not exhibit significant decreases in infarct size. Leptin increased p44 and p42 phosphorylation in Wistar rat hearts by 103.9% ($P < 0.05$) and 157.3% ($P < 0.001$), respectively, and by 97.0% ($P < 0.05$) and 158.1% ($P < 0.05$) in hearts from Zucker lean rats. Akt/serine-473 phosphorylation was increased in Wistar hearts by 96.7% ($P < 0.05$), whereas Akt/threonine-308 phosphorylation was elevated by 43.9% ($P < 0.05$) in Zucker lean rat hearts. Leptin did not influence Akt or p44/42 phosphorylation in *(fa/fa)* animals. On leptin treatment, mitochondrial permeability transition pore opening was delayed by 43% ($P < 0.01$) and 30.9% ($P < 0.01$), respectively, in cardiomyocytes from Wistar and Zucker lean rat hearts but not in cardiomyocytes from *(fa/fa)*. This study provides the first evidence that myocardial sensitivity to the tissue preserving actions of leptin is influenced by adiposity and OB-R status.

Key Words: ischemia–reperfusion injury, leptin, leptin receptor, obesity

(*J Cardiovasc Pharmacol*™ 2009;53:311–317)

INTRODUCTION

Reperfusion is required for salvage of the ischemic myocardium to occur. Paradoxically, however, reperfusion itself can lead to enhanced damage to the myocardium,[1] and therefore, effective means of reducing this so-called ischemia–reperfusion (I/R) injury are being actively sought. Various treatments have been reported to protect against myocardial I/R injury,[2–4] many of which have been shown to be associated with activation of the reperfusion injury salvage kinase (RISK) pathway, which incorporates phosphatidyl-inositol 3-OH kinase (PI3K) cellular Akt/protein kinase B (Akt) and p44/42 mitogen-activated protein kinase (MAPK) extracellular signal–regulated MAPK (ERK 1/2),[5] opening of mitochondrial ATP-sensitive potassium channels (KATP), and inhibition of the mitochondrial permeability transition pore (MPTP).[6,7]

Obesity, as reflected by excessive deposition of abdominal fat, is reaching epidemic proportions.[8] Although previously thought to be purely an energy storage depot, adipose tissue is now recognized as being an active endocrine organ producing a plethora of cytokines and other bioactive molecules,[9] including the so-called adipocytokines. Leptin, the 16-kDa product of the Obese *(ob)* gene, was the first adipocytokine to be identified and is produced primarily by white adipose tissue.[10] It has important roles in the control of energy homeostasis and satiety[8] and also in bone formation,[11,12] blood pressure control,[13] hematopoiesis, and reproduction[14,15] and has been implicated in myocardial infarction linked to obesity.[16] Previously, we demonstrated that leptin reduces infarct size in the murine heart via activation of PI3K-Akt and p44/42 and inhibits MPTP opening in rat cardiomyocytes.[17] Interestingly, apart from adipose tissue, leptin has been shown to be secreted by the heart in response to I/R injury,[18,19] and we suggested that leptin-induced cardioprotection might occur via an autocrine/paracrine mechanism.[17]

Six isoforms of the leptin receptor (OB-R) have been identified, OB-Ra, OB-Rb, OB-Rc, OB-Rd, OB-Re, and OB-Rf.[20] These isoforms share a highly conserved extracellular leptin-binding domain but differ in the size of the intracellular portion of the receptor,[21] this being reflected by differences in their signaling capacities.[22] OB-Ra and OB-Rb expression occurs to significant extents in isolated cardiomyocytes.[19,23] It is, however, the OB-Rb that is believed to be the most important isoform physiologically, particularly in relation to metabolism.[21] A missense mutation within the OB-R has been linked to the obese phenotype in the *(fa/fa)* Zucker rat.[24] This mutation does not render the receptor incapable of binding

Received for publication November 17, 2008; accepted January 19, 2009.
From The Hatter Cardiovascular Institute, University College London Hospital Medical School, London, United Kingdom.
Supported by grants from the British Heart Foundation.
The authors report no conflict of interest.
Reprints: Prof Derek M. Yellon, DSc, The Hatter Cardiovascular Institute, University College London Hospital and Medical School, 67 Chenies Mews, London WC1E 6HX, United Kingdom (e-mail: d.yellon@ucl.ac.uk).
Copyright © 2009 by Lippincott Williams & Wilkins

leptin but, rather, interferes with the dimerization of receptor components, a prerequisite for cell signaling initiated by the activation of this class of receptors.[25,26] Although the role of the *Ob-R* in hypothalamic function and energy balance has been extensively investigated, the part it plays in cardiac function remains to be fully elucidated.

Given that leptin protects the myocardium against I/R injury[17] and that OB-R expression has been detected in the heart, we hypothesized that leptin's effectiveness as a cardioprotective agent is related to adiposity and the presence on the myocardium of functional OB-R. This theory was investigated using hearts harvested from Wistar, Zucker lean, and Zucker obese *(fa/fa)* rats; Wistar and Zucker lean rats express the normal OB-R gene, the extents of gene expression in the latter depending upon whether animals are heterozygous or homozygous, whereas *fa/fa* possess a mutation in the extracellular domain of all the OB-R isoforms.[24]

MATERIALS AND METHODS

Animals

Male Wistar rats and Zucker obese *(fa/fa)* rats and their lean *(fa/+)* littermates (aged circa 4 months) were used in these studies and were obtained from Charles River UK. Animals were treated in accordance with the Animals (Scientific Procedures) act 1986 published by the UK Home Office and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Heath (Publication No. 85-23, revised 1996).

Langendorff Isolated Perfused Rat Heart Model

Langendorff experiments were carried out as described previously.[27] Rats were anesthetized with sodium pentobarbital by intraperitoneal injection (50 mg/kg). Heparin was administered concomitantly to prevent thrombus formation (300 IU). Hearts were rapidly excised and placed in an ice-cold buffer and then perfused retrogradely via the aorta at a constant pressure of 100 mm Hg with a modified Krebs–Henseleit buffer consisting of: NaCl 118 mM, $NaHCO_3$ 25 mM, KCl 4.8 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 1.7 mM, and glucose 11 mM and aerated with carbogen pH 7.3–7.5 at 37°C. Temperature was monitored via a probe inserted into an incision made in the pulmonary trunk and hearts maintained at 37 ± 0.5°C. Heart rate was monitored via a latex intraventricular balloon inflated, so that an end-diastolic pressure of 5–10 mm Hg was achieved. Hearts underwent a stabilization period of 40 minutes followed by 35 minutes of regional ischemia and 120 minutes of reperfusion. Regional ischemia was induced by total occlusion of the left anterior descending coronary artery and achieved by tightening a 3.0 silk suture that had been placed previously around the vessel, until a substantial reduction in heart rate and coronary flow was observed. At reperfusion, hearts were perfused with normal buffer or a buffer containing leptin with or without 15 μM LY294002 (PI3K inhibitor) or 10 μM UO126 (MAPK inhibitor) for the first 30 minutes of reperfusion. At the end of the reperfusion period, the risk zone was established by retightening the suture and by the introduction of 5% Evans blue dye into the aorta. Hearts were immediately frozen at −20°C and subsequently cut into 2-mm slices. Heart slices were then incubated in a 1% triphenyltetrazolium chloride solution to stain viable tissue. Slices were analyzed using a computerized planimetry package (Summa Sketch II; Summagraphics, Seymour, CT). Infarct size was expressed as a percentage of area at risk (IS/AAR%). The risk zones for both Wistar and Zucker hearts were between 40% and 75% of the area of the left ventricle.

Western Blotting

Rat hearts were perfused for 40 minutes to allow them to stabilize and then subjected to 35 minutes of regional ischemia (as described above). Hearts were then reperfused for 15 minutes at which time the ventricular tissue at risk was excised and snap frozen in liquid nitrogen before being stored at −80°C to await analysis. On analysis, the proteins were extracted by homogenizing samples on ice in a buffer containing: 100 mM NaCl, 10 mM Tris (pH 7.6), 1 mM EDTA (pH 8.0), 2 mM sodium pyrophosphate, 2 mM sodium fluoride, 2 mM β-glycerophosphate, and a protease inhibitor cocktail. Samples were then centrifuged at 12,900 × g for 10 minutes and the supernatants assayed for protein content using a bicinchoninic acid assay. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was then used to separate the proteins, 30–60 μg of total sample protein being loaded per well. Subsequent to separation, the proteins were transferred to Hybond-enhanced chemiluminescence nitrocellulose membranes. Total and phosphorylated Akts (serine-473 and threonine-308) and p44/42 MAPK were detected using appropriate primary and secondary antibodies (Cell Signaling Technology, Hitchin, United Kingdom) and an enhanced chemiluminescence blotting reagent (Amersham Biosciences, Little Chalfont, United Kingdom). The nitrocellulose membranes were then exposed to photographic film, which was scanned, and the intensities of the protein bands, expressed as arbitrary units, determined by computerized densitometry (National Institutes of Heath Image 1.63). The relative amounts of phosphorylated and total proteins were then calculated correcting for differences in protein loading as established by probing for β-actin (Abcam, Cambridge, United Kingdom).

Isolated Cardiomyocytes

Ventricular cardiomyocytes were isolated from Wistar, Zucker lean *(fa/+)*, and *fa/fa* rat hearts as described previously.[28] A procedure involving the perfusion of hearts with a buffer solution containing collagenase (280 U/mg) was used to isolate cardiomyocytes, 1.5–3 million cells being routinely obtained per heart. In a model of oxidative stress, cells were loaded with a dye, tetramethylrhodamine methyl ester (TMRM), for 15 minutes, which was then exchanged for saline. Upon laser stimulation, TMRM generates mitochondrial reactive oxygen species (ROS), mimicking the burst of ROS that occurs as a consequence of reperfusing the ischemic myocardium. Thus, MPTP opening is indicated by the dequenching of TMRM associated with mitochondrial depolarization. The times (seconds) until mitochondrial

© 2009 Lippincott Williams & Wilkins

depolarization were recorded, constituting the end point of the experiment, and the data obtained after drug treatments were normalized versus control (untreated). Leptin (10 nM) was added to the buffer containing TMRM to investigate the influence of these agents on MPTP opening. Cyclosporin (CsA) (200 nM), a recognized inhibitor of the MPTP, was used as a positive control.

Statistical Analysis

Data are presented as mean ± SEM. Generally, comparisons between more than 2 groups were made using factorial 1-way analysis of variance and the Fisher protected least significant difference post hoc test. In some cases (eg, the MPTP data), however, the Kruskal–Wallis analysis of variance method was used followed by the Dunn multiple comparison test. Where only 2 groups were compared, the Student t test was used. Differences were regarded as statistically significant if a value of $P < 0.05$ was obtained.

RESULTS

Effect of Leptin, Administered at a Range of Concentrations, on Myocardial Infarct Size

Before carrying out studies with hearts from Zucker animals, experiments were performed in Wistar rat hearts to identify a leptin concentration that produced optimal protection against I/R injury. Thus, leptin perfused at 10, 30 and, 100 nM and produced marked reductions in infarct size in the Wistar rat heart (Fig. 1). By contrast, leptin at 0.1 and 1 nM failed to induce cardioprotection. As leptin at the higher concentrations produced similar levels of protection, 10 nM leptin was chosen as the standard concentration for all subsequent experiments.

Effect of the Protein Kinase Inhibitors LY294002 and UO126 on Leptin-induced Cardioprotection

As seen previously in the mouse heart,[17] LY294002, the PI3K inhibitor, blocked 10 nM leptin-induced protection in the Wistar rat heart (Fig. 2). Similarly, the p44/42 MAPK inhibitor, UO126, also blocked leptin-induced protection. The inhibitors and their solvent (0.02% dimethyl sulfoxide) did not influence infarct size when administered by themselves (Fig. 2).

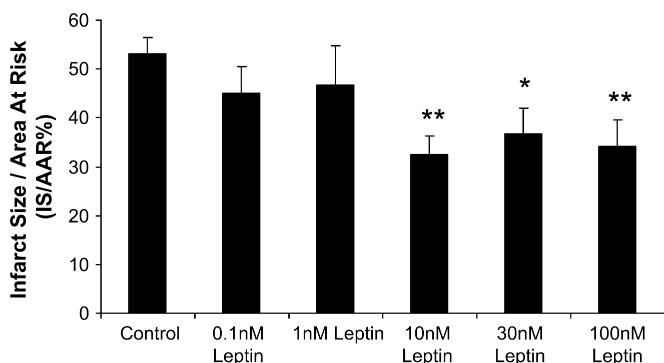


FIGURE 1. The influence of leptin, perfused during reperfusion (120 minutes), at 0.1, 1.0, 10, 30, and 100 nM, on infarct size as a percentage of the area at risk (IS/AAR%) in isolated perfused Wistar rat hearts. Values are expressed as mean ± SEM of 5–9 experiments (*$P < 0.05$, **$P < 0.01$).

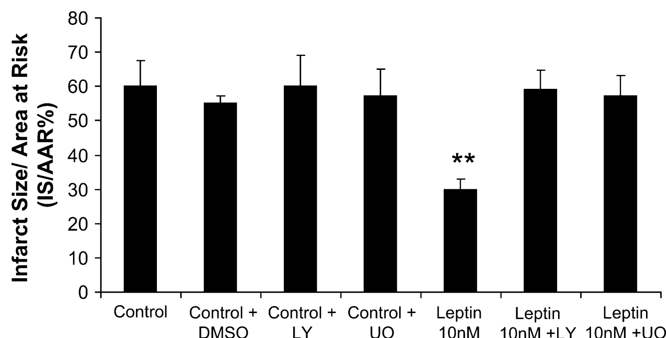


FIGURE 2. The influence of LY294002 and UO126 (inhibitors of PI3K and p44/42 MAPK, respectively) on infarct size (as a percentage of the area at risk, IS/AAR%) reduction induced by 10 nM leptin, perfused during reperfusion (120 minutes) in isolated perfused Wistar rat hearts. Values are expressed as mean ± SEM of 4–9 experiments (**$P < 0.01$).

Effect of Leptin on Infarct Size Limitation in Hearts From Zucker Lean and *fa/fa* Rats

Leptin (10 nM), administered at reperfusion, substantially reduced infarct size in hearts from Zucker lean rats, which express the normal OB-R gene (Fig. 3). By contrast, hearts from *fa/fa*, which lack functional OB-R, did not exhibit statistically significant decreases in infarct size, although a trend toward infarct size reduction was noted (Fig. 3).

Effect of Leptin, Administered at Reperfusion, on the Phosphorylation of Akt and p44/42 MAPK

Western blot analysis revealed that leptin treatment of Wistar rat hearts was associated with statistically significant increases in the phosphorylation of Akt/serine-473, p44, and p42 but not Akt/threonine-308 (Table 1). Contrasting with the observations made with Wistar cardiac samples, significant changes in Akt/serine-473 phosphorylation were not seen in leptin-treated Zucker lean rat hearts (Table 1). A statistically significant increase in Akt/threonine-308 phosphorylation was, however, detected (Table 1). Significant leptin-induced increases in p44 and p42 phosphorylation were also observed in Zucker lean rat hearts (Table 1). In *fa/fa* hearts, phosphorylation of Akt/serine-473, Akt/threonine-308, p44 MAPK, and p42 MAPK was unaltered by leptin treatment (Table 1).

Effect of Leptin on MPTP Opening in Cardiomyocytes Isolated From Wistar, Zucker Lean, and *fa/fa* Rat Hearts

CsA, an established inhibitor of the MPTP, was used as a positive control in all isolated cardiomyocyte experiments. Leptin (10 nM) and CsA (200 nM) delayed the time to MPTP opening in cardiomyocytes isolated from Wistar rat hearts as compared with cells treated with vehicle (Fig. 4). Similarly,

© 2009 Lippincott Williams & Wilkins

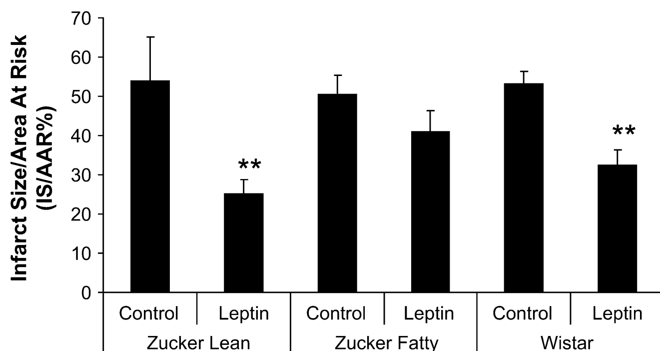


FIGURE 3. Effect of leptin (10 nM), perfused during reperfusion (120 minutes), on infarct size (as a percentage of the area at risk, IS/AAR%) in isolated hearts from Zucker lean *(fa/+)*, Zucker fatty *(fa/fa)*, and Wistar rats. Values are expressed as mean ± SEM of 5–7 experiments (**$P < 0.01$).

both leptin and CsA delayed the time until MPTP opening in cardiomyocytes isolated from Zucker lean rat hearts. By contrast, although cardiomyocytes isolated from *fa/fa* rat hearts responded to CsA with a delayed time to MPTP opening, no changes were observed in leptin-treated cells (Fig. 4).

DISCUSSION

Leptin, an adipocytokine linked to obesity and metabolic disease, protects against I/R injury in various organs including kidney,[29] brain,[30] and gut.[31] We reported that leptin also reduces myocardial injury, its cardioprotective actions being exerted through PI3K-Akt and p44/42 MAPK activation and MPTP inhibition.[17] In the present study, we focused on factors that may determine myocardial sensitivity to the cardioprotective actions of leptin. Consequently, contrasting with the findings obtained with hearts from Wistar and Zucker lean rats that possess functional OB-R, we demonstrated that infarct size, RISK pathway activation, and ROS-induced MPTP opening in hearts from Zucker obese *(fa/fa)* animals, which are compromised with respect to the OB-R, were unaffected by leptin treatment. These data indicate that adiposity and/or OB-R status may influence myocardial responsiveness to leptin.

Dose-response studies performed with isolated hearts from Wistar rats indicated that 10 nM leptin, a concentration equivalent to a pharmacological dose and comparable to serum concentrations occurring in obese humans,[32] yielded optimal extents of infarct size reduction, data consistent with findings made in the murine heart.[17] The fact that leptin concentrations lower than 10 nM did not elicit responses could be interpreted as indicating that leptin produces an "all or nothing" effect with respect to infarct size reduction. However, as suggested previously for the adipocytokine, apelin,[33] it is equally possible that the effects were missed, that is, if leptin concentrations between 1 and 10 nM had been tested, infarct size reductions might have been seen over this range. Nevertheless, the fact remains that 10 nM leptin yielded an optimal response and was consequently chosen for all subsequent experiments involving hearts from Wistar and Zucker lean and *fa/fa* rats.

As described earlier, the Zucker lean rat expresses the normal fully functional OB-R gene, whereas the *fa/fa* rat is compromised with respect to the *Ob-R*, a mutation within the extracellular domain rendering it nonfunctional. The use of Zucker lean and *fa/fa* animals has, therefore, proved valuable in studying metabolic processes and disease and in gauging the importance of the OB-R with respect to leptin-mediated mechanisms. We found that, as observed with hearts from Wistar animals, hearts from Zucker lean rats responded to leptin treatment with significant reductions in infarct size. By contrast, hearts from obese fa/fa rats did not respond to leptin with statistically significant decreases in infarct size, although a small trend toward infarct size reduction was seen. This trend raises the possibility that if leptin concentrations had been increased above 10 nM, protection might have been achieved. Alternatively, the involvement of mechanisms, other than those relating to *Ob-R* activation, in leptin-induced cardioprotection might be indicated. If, however, it is assumed that our findings are valid and that leptin is, indeed, ineffective in reducing infarct size in the *fa/fa* rat heart, one could suggest that this reflects a general lack of sensitivity to cardioprotective agents in these animals, possibly as a consequence of changes in cardiomyocyte plasma membrane lipid composition. Nevertheless, evidence opposing this view has been obtained, various agents having been found to protect the *fa/fa* rat heart. De Gennaro-Colonna et al[34] demonstrated that hexarelin, a synthetic enkephalin-derived peptide, exerted a cardioprotective effect in both lean and obese Zucker rats. Similarly, rosiglitazone, which protects the myocardium in normal animals,[35] was found to reduce infarct size in lean and obese Zucker animals via Akt activation.[36]

TABLE 1. Summary of Western Blot Data

	Wistar		*fa/+*		*fa/fa*	
	Control	Leptin	Control	Leptin	Control	Leptin
Akt/Ser-473 phosphorylation	100 ± 9.6	196.7 ± 33.5*	100 ± 5.6	121.1 ± 10.5	100 ± 7.6	100.6 ± 28.8
Akt/Thr-308 phosphorylation	100 ± 15.2	123.5 ± 25.4	100 ± 8.2	143.9 ± 15.4*	100 ± 17.6	105.7 ± 30.7
p44 phosphorylation	100 ± 14.1	203.9 ± 41.3*	100 ± 10.3	197 ± 33.6*	100 ± 13.0	92.4 ± 21.2
p42 phosphorylation	101.1 ± 12.9	257.3 ± 48.1†	100 ± 10	258.1 ± 52.9*	100 ± 10.8	85.2 ± 22.8

The effect of leptin (10 nM) treatment on Akt/serine-473, Akt/threonine-308, p44, and p42 phosphorylation in hearts from Zucker lean *(fa/+)*, Zucker fatty *(fa/fa)*, and Wistar rats was examined. Data were calculated as the ratio of phosphorylated to total protein, relative densitometry values normalized to control (untreated, 100%). Values are mean ± SEM (*$P < 0.05$, †$P < 0.001$, leptin vs. control; n = 5–10).

 © 2009 Lippincott Williams & Wilkins

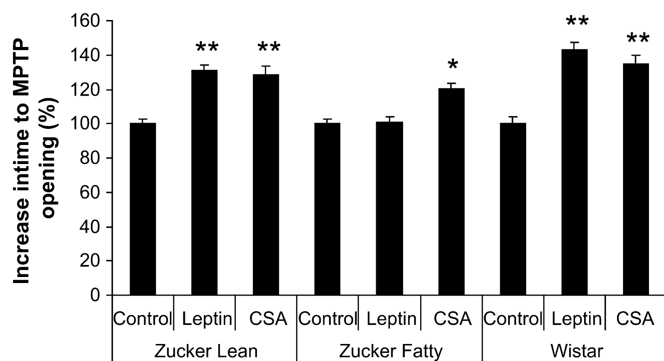


FIGURE 4. The times until the initiation of mitochondrial depolarization, that is, MPTP opening, in the presence and absence of leptin (10 nM) in cardiomyocytes isolated from Zucker lean *(fa/+)*, Zucker fatty *(fa/fa)*, and Wistar rat hearts. Values are presented as mean ± SEM ($*P < 0.05$, $**P < 0.01$) and were obtained with a total of 12–20 cells from at least 5 hearts per group.

In the present study, we considered the possibility that leptin-induced cardioprotection may be influenced by factors such as the metabolic state and myocardial OB-R status. Given its proposed primacy with respect to full cell signaling and the physiological actions of leptin,[21,37] it is tempting to suggest that the OB-Rb plays the principal role in relation to its cardioprotective effects. One, however, cannot exclude the possibility that other *Ob* isoforms may be involved in cardiac function. It is, for example, known that in addition to *Ob-Rb*, cardiomyocytes express *Ob-Ra*, which may also modulate cellular processes[19,23] and are defective in the Zucker rat.[24] *Ob-Ra*, which, interestingly, are expressed at higher levels than *Ob-Rb* in the normal heart,[18] have been linked to ventricular hypertrophy and activation of p38 and p44/42, signaling pathways implicated in cardioprotection.[23] We would suggest, therefore, that the cardioprotective actions of leptin could, to some extent, relate to *Ob-Ra* activation and p44/42 phosphorylation, which, as discussed below, was observed on leptin treatment. Studies involving the use of leptin receptor–blocking peptides or antileptin receptor antibodies may help to resolve the issue as to which OB-R subtype is responsible for modulating the leptin-induced actions observed or, indeed, if the OB-R is involved at all. Purdham et al,[38] for example, reported that treatment of rats with a nonspecific leptin receptor–neutralizing antibody reduces hypertrophy and hemodynamic dysfunction in the postinfarcted heart. Recently, specific antileptin receptor antibodies have come on line, including antibodies against OB-Rb, which should make the task of identifying the receptors involved in mediating the cardiovascular actions of leptin easier.

Using pharmacological kinase inhibitors, data were obtained indicating that the infarct reducing effects of leptin in the Wistar rat heart involved PI3K-Akt and p44/42 MAPK activation, a finding consistent with results obtained previously in the murine myocardium.[17] Meanwhile, Western blot analysis confirmed that Akt, as indicated by Akt/serine-473 phosphorylation, and p44/42 are involved in leptin-induced cardioprotection in the Wistar rat heart, a finding which, again, coincided with our previous observations made in the murine

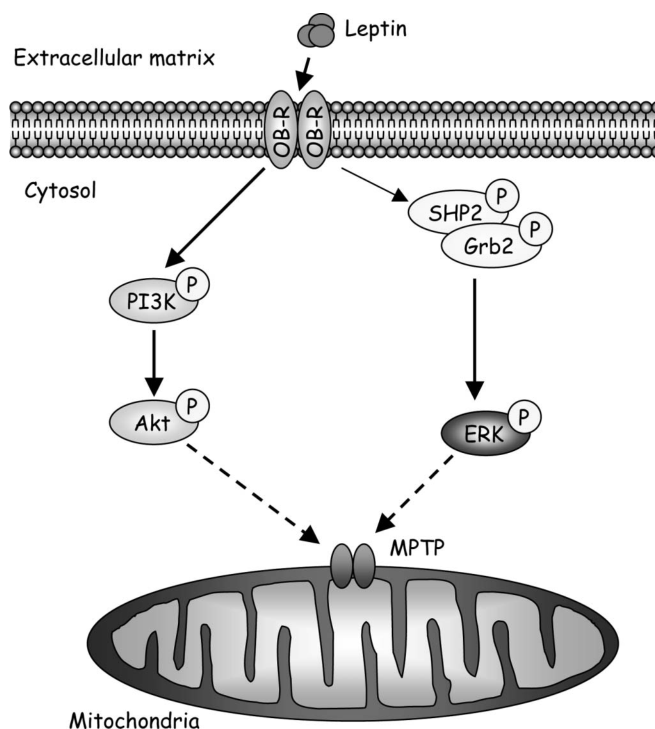


FIGURE 5. Proposed cell signaling mechanisms underlying leptin-induced myocardial protection. Reperfusion of the myocardium after a period of ischemia results in MPTP opening and subsequent mitochondrial uncoupling, ATP consumption, and, ultimately, cell death. It is hypothesized that binding of leptin by leptin receptors (OB-R) present on the cardiomyocyte leads to the phosphorylation of PI3K and subsequently Akt/protein kinase B (Akt) and/or phosphorylation of Src homology phosphatase 2 (SHP2) followed by growth factor receptor–bound protein 2 (Grb2) and p44/42 MAPK extracellular signal–regulated MAPK (ERK 1/2). Signaling pathways downstream of these cascades then converge on the mitochondrion to inhibit MPTP opening and, as a consequence, prevent mitochondrial uncoupling and cell death. If OB-R are absent or defective, these mechanisms would not be operative with the consequence that cell death would proceed unimpeded.

myocardium.[17] Intriguingly, however, although leptin reduced infarct size in Zucker lean rat hearts and increased p44 and p42 phosphorylation to extents comparable to those seen in Wistar hearts, Akt/threonine-308 phosphorylation, rather than Akt/serine-473 phosphorylation, was significantly enhanced: It is possible that these data reflect temporal differences between Wistar and Zucker lean rats with respect to the phosphorylation of Akt at its 2 phosphorylation sites. Regarding the Akt and p44/42 Western blot data obtained with *fa/fa* hearts, these can be interpreted as indicating that leptin-stimulated cardioprotection is dependent upon the presence of OB-R on the myocardium, although a generalized influence of obesity on cardiac function cannot be excluded. Thus, leptin treatment not only failed to reduce infarct size in these animals but also was not associated with RISK pathway activation, as evidenced by our failure to detect increases in Akt (serine-473 and threonine-308) and p44/42 phosphorylation. Of course, the cardioprotective actions of leptin may involve the

© 2009 Lippincott Williams & Wilkins

mobilization of alternative cell signaling pathways, an obvious candidate for future investigation being the Janus kinase-signal transducers and activators of transcription (JAK/STAT) pathway, which mediates many of the metabolic actions of leptin[21,22] and has been implicated in cardioprotection.[39,40]

As described above, the infarct and Western blot data obtained may be consistent with OB-R activation, playing a role in leptin-induced cardioprotection. It has been proposed that signaling via the RISK pathway ultimately converges on the mitochondrion, resulting in MPTP inhibition.[28,41] Evidence for such a mechanism has come from studies focusing on MPTP opening in isolated cardiomyocytes.[6] Indeed, it has been suggested that the MPTP may represent an essential regulatory component with respect to mitochondrial function and a key target in relation to cardioprotection.[6] Studies in cyclophilin-D knockout mice have provided strong evidence for the MPTP playing a key role in cell death.[42] Previously, we reported that leptin delayed MPTP opening in cardiomyocytes from Sprague-Dawley rats and that this was blocked by LY294002 and MAPK/ERK kinase (MEK) inhibitor 1.[17] In experiments with cardiomyocytes from Wistar rat hearts, we again found that leptin delayed MPTP opening, as was the case for cells from Zucker lean rat hearts, consolidating our previous evidence that leptin-induced signaling, as indicated by increased Akt and p44/42 phosphorylation, converges on the mitochondrion[17] (proposed steps involved in leptin-induced signaling in the myocardium are shown in Fig. 5). The fact that leptin did not produce a comparable effect on cardiomyocytes from *fa/fa* rats indicates that leptin-induced signaling, with respect to MPTP opening, is defective in these animals, thus, perhaps, providing further support for the notion that the OB-R plays a role in leptin-mediated protection.

To conclude, we would suggest that the data presented in this article have implications for protection against myocardial injury, especially with respect to individuals with obesity or exhibiting leptin resistance and/or hyperleptinemia.

REFERENCES

1. Gross GJ, Auchampach JA. Reperfusion injury: does it exist? *J Mol Cell Cardiol*. 2007;42:12–18.
2. Lim SY, Davidson SM, Paramanathan AJ, et al. The novel adipocytokine visfatin exerts direct cardioprotective effects. *J Cell Mol Med*. 2008;12: 1395–1403.
3. Mudalagiri NR, Mocanu MM, Di Salvo C, et al. Erythropoietin protects the human myocardium against hypoxia/reoxygenation injury via phosphatidylinositol-3 kinase and ERK1/2 activation. *Br J Pharmacol*. 2008;153:50–56.
4. Sivaraman V, Mudalagiri NR, Di Salvo C, et al. Postconditioning protects human atrial muscle through the activation of the RISK pathway. *Basic Res Cardiol*. 2007;102:453–459.
5. Hausenloy DJ, Tsang A, Yellon, DM. The reperfusion injury salvage kinase pathway: a common target for both ischemic preconditioning and postconditioning. *Trends Cardiovasc Med*. 2005;15:60–75.
6. Davidson SM, Hausenloy DJ, Duchen MR, et al. Signalling via the reperfusion injury signalling kinase (RISK) pathway links closure of the mitochondrial permeability transition pore to cardioprotection. *Int J Biochem Cell Biol*. 2006;38:414–419.
7. Mykyteno J, Reeves JG, Kin H, et al. Persistent beneficial effect of postconditioning against infarct size: role of mitochondrial K ATP channels during reperfusion. *Basic Res Cardiol*. 2008;103:472–484.
8. Katagiri H, Yamada T, Oka Y. Adiposity and cardiovascular disorders: disturbance of the regulatory system consisting of humoral and neuronal signals. *Circ Res*. 2007;101:27–39.
9. Skopkova M, Penesova A, Sell H, et al. Protein array reveals differentially expressed proteins in subcutaneous adipose tissue in obesity. *Obesity*. 2007;10:2396–2406.
10. Zhang Y, Proenca R, Maffei M, et al. Positional cloning of the mouse obese gene and its human homologue. *Nature*. 1994;372:425–432.
11. Ducy P, Amling M, Takeda S, et al. Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. *Cell*. 2000; 100:197–207.
12. Takeda S, Elefteriou F, Levasseur R, et al. Leptin regulates bone formation via the sympathetic nervous system. *Cell*. 2002;111:305–317.
13. Fruhbeck G. Pivotal role of nitric oxide in the control of blood pressure after leptin administration. *Diabetes*. 1999;48:903–908.
14. Cioffi JA, Shafer AW, Zupancic TJ, et al. Novel B219/OB receptor isoforms: possible role of leptin in hematopoiesis and reproduction. *Nat Med*. 1996;2:585–589.
15. Holness MJ, Munns MJ, Sugden MC. Current concepts concerning the role of leptin in reproductive function. *Mol Cell Endocrinol*. 1999;157: 11–20.
16. Heusch G. Obesity—a risk factor or a RISK factor for myocardial infarction? *Br J Pharmacol*. 2006;149:1–3.
17. Smith CCT, Mocanu MM, Davidson SM, et al. Leptin, the obesity-associated hormone, exhibits direct cardioprotective effects. *Br J Pharmacol*. 2006;149:5–13.
18. Matsui H, Motooka M, Koike H, et al. Ischemia/reperfusion in rat heart induces leptin and leptin receptor gene expression. *Life Sci*. 2007;80: 672–680.
19. Purdham DM, Zou MX, Rajapurohitam V, et al. Rat heart is a site of leptin production and action. *Am J Physiol Heart Circ Physiol*. 2004;287: H2887–H2884.
20. Lee GH, Proenca R, Montez JM, et al. Abnormal splicing of the leptin receptor in diabetic mice. *Nature*. 1996;379:632–635.
21. Fruhbeck G. Intracellular signalling pathways activated by leptin. *Biochem J*. 2006;393:7–20.
22. Banks AS, Davis SM, Bates, SH, et al. Activation of downstream signals by the long form of the leptin receptor. *J Biol Chem*. 2000;275:14563–14572.
23. Rajapurohitam V, Gan XT, Kirshenbaum LA, et al. The obesity-associated peptide leptin induces hypertrophy in neonatal rat ventricular myocytes. *Circ Res*. 2003;93:277–279.
24. Takaya K, Ogawa Y, Isse N, et al. Molecular cloning of rat leptin receptor isoform complementary DNAs-identification of a missense mutation in Zucker fatty *(fa/fa)* rats. *Biochem Biophys Res Commun*. 1996;225: 75–83.
25. Kishimoto T, Taga T, Akira S. Cytokine signal transduction. *Cell*. 1994; 76:253–262.
26. Phillips MS, Liu Q, Hammond HA, et al. Leptin receptor missense mutation in the fatty Zucker rat. *Nat Genet*. 1996;13:18–19.
27. Tsang A, Hausenloy DJ, Mocanu MM, et al. Postconditioning: a form of "modified reperfusion" protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. *Circ Res*. 2004;95:230–232.
28. Hausenloy DJ, Wynne A, Duchen M, et al. Transient mitochondrial permeability transition pore opening mediates preconditioning-induced protection. *Circulation*. 2004;109:1714–1717.
29. Erkasap S, Erkasap N, Koken T, et al. Effect of leptin on renal ischemia-reperfusion injury damage in rats. *J Physiol Biochem*. 2004;60:79–84.
30. Zhang F, Wang S, Signore AP, et al. Neuroprotective effects of leptin against ischemic injury induced by oxygen-glucose deprivation and transient cerebral ischemia. *Stroke*. 2007;38:2329–2336.
31. Brzozowski T, Konturek PC, Pajdo R, et al. Brain-gut axis in gastroprotection by leptin and cholecystokinin against ischemia-reperfusion induced gastric lesions. *J. Physiol Pharmacol*. 2001;52:583–602.
32. Considine RV, Sinha MK, Heiman ML, et al. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med*. 1996;334:292–295.
33. Simpkin JC, Yellon DM, Davidson SM, et al. Apelin-13 and apelin-36 exhibit direct cardioprotective activity against ischemia-reperfusion injury. *Basic Res Cardiol*. 2007;102:518–528.
34. De Gennaro-Colonna V, Rossoni G, Cocchi D, et al. Endocrine, metabolic and cardioprotective effects of hexarelin in obese Zucker rats. *J Endocrinol*. 2000;166:529–536.
35. Gonon AT, Bulhak A, Labruto F, et al. Cardioprotection mediated by rosiglitazone, a peroxisome proliferator-activated receptor gamma

 © 2009 Lippincott Williams & Wilkins

ligand, in relation to nitric oxide. *Basic Res Cardiol*. 2007;102: 80–89.
36. Yue T-L, Bao W, Gu J-L, et al. Rosiglitazone treatment in Zucker diabetic fatty rats is associated with ameliorated cardiac insulin resistance and protection from ischemia/reperfusion-induced myocardial injury. *Diabetes*. 2005;54:554–562.
37. Myers MG. Leptin receptor signaling and the regulation of mammalian physiology. *Recent Prog Horm Res*. 2004;59:287–304.
38. Purdham DM, Rajapurohitam V, Zeidan A, et al. A neutralizing leptin receptor antibody mitigates hypertrophy and hemodynamic dysfunction in the post-infarcted rat heart. *Am J Physiol Heart Circ Physiol*. 2008;295:H441–H446.
39. Boengler K, Buechert A, Heinen Y, et al. Cardioprotection by ischemic postconditioning is lost in aged and STAT3-deficient mice. *Circ Res*. 2008;102:131–135.
40. Bolli R, Dawn B, Xuan Y-T. Role of the JAK-STAT pathway in protection against myocardial ischemia-reperfusion injury. *Trends Cardiovasc Med*. 2003;13:72–79.
41. Hausenloy DJ, Yellon DM. Survival kinases in ischemic preconditioning and postconditioning. *Cardiovasc Res*. 2006;70:240–253.
42. Lim SY, Davidson SM, Hausenloy DJ, et al. Preconditioning and postconditioning: the essential role of the mitochondrial permeability transition pore. *Cardiovasc Res*. 2007;75:530–535.

© 2009 Lippincott Williams & Wilkins