Effects of DRP-1 Inhibitors in Healthy and Diseased Myocardium

Lauren Wendt

BExSc (Hons)

Menzies Health Institute Queensland
School of Medical Science
Griffith University

Submitted in fulfillment of the requirements of the degree of
Doctor of Philosophy

November 2016
I. Synopsis

Coronary heart disease (CHD) continues to be one of the foremost contributors to morbidity and mortality in developed countries. Leading risk factors that include elevated blood pressure, hypercholesterolaemia, tobacco use, sedentary lifestyles and excess caloric consumption significantly contribute to these alarmingly high mortality rates and prompt the search for targeted interventions that are clinically effective. Myocardial ischaemia, occurring in ischaemic heart disease (IHD) or during cardiac surgery, reduces coronary blood flow to the metabolically active myocardium – the former primarily due to progression of coronary vascular atherosclerosis and associated thromboembolism, the latter due to the need to arrest perfusion of myocardial tissue in different surgical scenarios (e.g. cardiac transplant, by-pass surgery, valve surgery). Insufficient blood flow to the myocardium reduces oxygen and metabolic substrate delivery, and removal of waste products. Since the myocardium is highly oxidative, periods of ischaemia are detrimental, with brief episodes causing cardiac dysfunction and more prolonged insult leading to cell death. Reduced oxygen delivery and subsequent reoxygenation can profoundly impact mitochondrial function, the energy producing ‘powerhouses’ of the cell. Emerging research has confirmed that mitochondria are a network of dynamic organelles undergoing cycles of fragmentation (fission) and elongation (fusion), a quality control process thought to maintain a healthy population of mitochondria matched to prevailing metabolic conditions. Mitochondrially targeted interventions have thus emerged as an exciting therapeutic option across organ systems and diseases, including in the heart and IHD. However, the heart is somewhat unique in its mitochondrial phenotype, with evidence the healthy heart possesses a fragmented mitochondrial population that generates the highest densities and respiratory capacity in mammalian cells. Moreover, these mitochondria do not appear to undergo active cycles of fission/fusion. Thus, the utility of therapy targeting fission/fusion in the heart is unclear.

This doctoral study sought to characterise the impacts of mitochondrial fission inhibitors on myocardial function, signalling and stress responses (in vivo, ex vivo and in vitro). Targeting mitochondrial-remodelling proteins has theoretical potential as a cardioprotective strategy to improve metabolic, respiratory and cardiac function in the diseased heart. The effects of chemical inhibition of the key fission protein DRP-1 were
initially investigated *ex vivo* with myocardial outcomes assessed in both normoxic and post-ischaemic hearts (early and late reperfusion). Mitochondrial remodelling proteins were explored, in addition to examining impacts on the ‘survival kinases’ PI3K/AKT and ERK1/2. The results of these initial studies indicated that pre-treatment with the putative DRP-1 inhibitor dynasore worsened myocardial outcomes from ischaemia, with evidence the agent failed to modify mitochondrial DRP-1 expression. Pre-treatment with 1-5 µM MDIVI-1 did offer some benefit during ischaemia and early reperfusion, though also appeared to dissociate contractile and cell death outcomes. The drug did more selectively target mitochondrial DRP-1, and also inhibited post-ischaemic phospho-activation of ERK1/2. The impacts of ischaemia-reperfusion (I-R) on mitochondrial respiratory activity were largely unaltered by MDIVI-1, though the drug improved respiratory control. These initial studies thus revealed somewhat mixed effects of DRP-1 inhibition in an *ex vivo* perfused heart model.

The mixed effects of DRP-1 inhibition observed in studies in Chapter 3 prompted more detailed *in vitro* investigations (Chapter 4 and Chapter 5). Effects of DRP-1 inhibition in intact hearts were inconsistent with a broadly beneficial or 'cardioprotective' effect, with the agent potentially impairing stress tolerance and respiratory function. Studies were thus performed in a myoblast cell model to assess concentration- and time-dependant effects of H$_2$O$_2$ and DRP-1 inhibition. Interestingly, DRP-1 inhibition did not improve cell survival or respiratory capacity with acute (≤60 min) oxidative stress. However, ATP generation was protected. Similar to observations in intact hearts (Chapter 3), phospho-activation of ERK1/2 signalling during cellular stress was again blocked by DRP-1 inhibition. Conversely, with sustained oxidant stress (to 8 hrs), DRP-1 inhibition improved cell viability or metabolic capacity (MTT assay) but not cell survival (LDH efflux), and worsened proliferation, an effect previously reported in the tumour growth of cancer cells. Nonetheless, MDIVI-1 preserved mitochondrial membrane potential. Expression levels of DRP-1, OPA-1 and MFN-2 were reduced with oxidative stress, changes inconsistently influenced by MDIVI-1. However, effects of prolonged stress on ERK1/2 and AKT phospho-activation were inhibited by MDIVI-1, as were changes in BAX: BCL-2 and PARP cleavage, whereas PARKIN expression and LC3BII/I were augmented. This reveals mixed impacts of MDIVI-1 including suppression of survival kinase signalling, inhibition of apoptosis and paradoxic promotion of autophagy. Additional proteomic interrogation revealed paradoxic shifts in cell death signalling. Sustained DRP-1 inhibition reduced transient
up-regulation of LC3, which may have negatively impacted autophagic signalling. These differential and sometimes opposing effects of MDIVI-1 on kinase, autophagy and apoptosis processes may explain mixed and sometimes negative effects on cardiomyoblast responses to stress. Given still incompletely understood interactions between mitochondrial remodelling proteins, autophagy, apoptosis and survival signalling, it may well be that perturbing the dynamics of fission and fusion is not an effective approach in limiting mitochondrial dysfunction, cell viability and survival.

Finally, to assess the agent in a more clinically relevant disease setting, studies in Chapters 6 and 7 examined effects of MDIVI-1 in the context of hyperglycaemia and type 2 diabetes. Type 2 diabetes mellitus is an increasingly important comorbidity in IHD, with evidence that sustained hyperglycaemia negatively impacts mitochondrial morphology and function. In vitro investigations of cardiomyoblast responses to hypo- and hyper-glycaemia and hyperinsulinaemia (for 24 hr) showed that elevated glucose alone did not impact proliferation or mitochondrial respiratory function, requiring the presence of insulin to influence these parameters. Under conditions of hyperglycaemia + insulin, the DRP-1 inhibitor reduced DRP-1 activation and up-regulated OPA-1, likely promoting fusion under these conditions. In a mouse model of type 2 diabetes mellitus (14 wk high-fat feeding following STZ injection), DRP-1 inhibition partially improved myocardial ischaemic tolerance though not mitochondrial respiratory function. Proteomic interrogation failed to reveal substantial influences on mitochondrial DRP-1, although cytosolic levels were modified. These observations further reveal complex responses to DRP-1 inhibition, and hint at potential non-specific actions distinct from inhibition of mitochondrial DRP-1. However, the drug treatment regime in these latter studies may also have induced negative effects. Injection procedure/vehicle had significant impacts, clouding outcomes and complicating data interpretation, thus highlighting one of the drawbacks of utilisation of MDIVI-1 - its very poor aqueous solubility.

Overall, the findings of this doctoral project do not provide compelling support for DRP-1 inhibition as a broadly useful approach to cardioprotection - improving myocardial resistance to stress and disease. This is not entirely unpredicted, given our evolving understanding of the central role of mitochondria in cell survival and death, and interactions between mitochondrial dynamics, stress signalling, autophagy and multiple cell death processes. The currently detailed findings provide hints of this
complexity, supporting DRP-1/fission dependent survival kinase activation, and modulation of autophagy and apoptosis pathways. Further work is warranted to elucidate roles of DRP-1 and fission in governing myocardial stress responses, and the value of DRP-1 inhibition as a myocardial therapeutic approach.
II. Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

(Signed)_____________________________

Lauren Wendt
# III. Table of Contents

I. Synopsis .......................................................................................................................... 2

II. Statement of Originality ................................................................................................. 6

III. Table of Contents ........................................................................................................... 7

IV. List of Tables and Figures .............................................................................................. 10

V. Acknowledgements .......................................................................................................... 15

Acknowledgment of Assistance Received ........................................................................... 16

List of Abbreviations ............................................................................................................ 17

CHAPTER 1: Background ..................................................................................................... 21

1.0 Introduction ................................................................................................................... 21
1.1 Ischaemic heart disease ............................................................................................... 21
1.2 Mitochondria and myocardial cell death in ischaemia-reperfusion ............................. 24
1.3 Mitochondrial dynamics - a potential therapeutic target? ........................................... 32
1.4 Mitochondrial quality control and dynamics ............................................................... 35
1.5 Mitochondrial fission ................................................................................................... 39
1.6 Mitochondrial dynamics in cardiovascular health and disease ................................. 44
1.7 Summary ....................................................................................................................... 46

Project Aims ....................................................................................................................... 47

CHAPTER 2: Research Methods .......................................................................................... 49

2.0 Animals and ethics ....................................................................................................... 49
2.1 Cell culture studies (in vitro) ...................................................................................... 49
2.2 Cell death analyses ...................................................................................................... 50
2.3 Mitochondrial functional analysis (in vitro) ................................................................. 51
2.4 Mitochondrial respiratory analysis (ex vivo) ............................................................... 53
2.5 Langendorff heart perfusion studies ........................................................................... 55
2.6 Coronary effluent lactate dehydrogenase (LDH) assay ............................................. 56
2.7 Western blot analysis of proteins ................................................................................ 57
2.8 Western immunoblotting ............................................................................................. 57
2.9 Animal studies: type II diabetes mellitus ................................................................. 60
2.10 Statistical analyses ..................................................................................................... 62
CHAPTER 7: Systemic and Cardiac Effects of a Mitochondrial Fission Inhibitor in a Murine Model of Type 2 Diabetes Mellitus ...................................................... 152

7.0 Abstract............................................................................................................... 152
7.1 Introduction ....................................................................................................... 154
7.2 Methods and Materials ..................................................................................... 156
7.3 Results ................................................................................................................. 160
7.4 Discussion .......................................................................................................... 182
Summary ................................................................................................................... 188

Overall Conclusions .................................................................................................... 189

Supplementary Material ............................................................................................ 193

Reference List ............................................................................................................. 203
IV. List of Tables and Figures

Figure 1.1: Coronary atherosclerotic plaque ................................................................. 22

Figure 1.2: Simplified schematic of the mitochondrion .................................................. 26

Figure 1.3: Differentiation of cell death pathways ......................................................... 28

Figure 1.4: Simplified schematic of the extrinsic and intrinsic pathways of apoptosis .... 32

Figure 1.5: Autophagic elimination of mitochondria ...................................................... 34

Figure 1.6: Mitochondrial fission and fusion ................................................................. 37

Figure 1.7: Cellular localisation of mitochondrial fission and fusion proteins ............. 41

Figure 1.8: Post-translational recruitment domains of fission protein DRP-1 ............... 42

Figure 2.1: Mitochondrial oxygen consumption in isolated mitochondria in response to complex I and II substrates and inhibitors ................................................................. 54

Figure 2.2: Langendorff apparatus for isolated hearts ................................................... 56

Table 2.1: Table of Antibodies ....................................................................................... 59

Figure 3.1: Schematic of experimental ischaemia-reperfusion protocol ....................... 66

Table 3.1 Function of ex vivo hearts prior to and post infusion of DRP-1 inhibitor .......... 67

Figure 3.2: Impact of 10 min pre-ischaemic treatment with dynasore hydrate or MDIVI-1 on ischaemic contracture development ................................................................. 68
Figure 3.3: Post-ischaemic recovery of LVDP in hearts administered with MDIVI-1 and
dynasore hydrate pre and post-ischaemia.................................................................70

Figure 3.4: Post-ischaemic outcomes in hearts administered with MDIVI-1 and dynasore
hydrate pre- and post-ischaemia...............................................................................71

Figure 3.5: Late post-ischaemic LDH efflux in MDIVI-1 and dynasore treated
hearts...............................................................................................................................72

Figure 3.6: Early LDH efflux in hearts administered with MDIVI-1 for 20 min pre-
ischaemia........................................................................................................................73

Figure 3.7: DRP-1 expression in post-ischaemic hearts administered with MDIVI-1 (1 µM)
and dynasore hydrate (1 µM) pre- and post-ischaemia................................................74

Figure 3.8: The effects of a DRP-1 inhibitor (MDIVI-1) administered pre-ischaemia on
survival kinase expression and phosphorylation following 10 min reperfusion.........75

Figure 3.9: Mitochondrial oxygen consumption in early and late reperfused hearts
administered with the DRP-1 inhibitor, MDIVI-1, pre-ischaemia.................................78

Figure 3.10: The effects of DRP-1 inhibition on mitochondrial oxygen consumption via
complex I and II in early and late reperfused hearts.....................................................79

Figure 3.11: The effects of DRP-1 inhibition on complex I and II mitochondrial flux
control ratios in early and late reperfused hearts........................................................80

Figure 4.1: Concentration and time dependence of H₂O₂ induced changes in viability of
H9c2 cardiomyoblasts....................................................................................................96

Figure 4.2: Time-course of survival kinase expression/phosphorylation during H₂O₂
challenge in H9c2 cardiomyoblasts................................................................................98

Figure 4.3: Impact of acute 5 min H₂O₂ challenge (± MDIVI-1) on survival kinase
expression/phosphorylation.........................................................................................99

Figure 4.4: Impact of acute 5 min H₂O₂ challenge (± MDIVI-1) on H9c2 cardiomyoblast
DRP-1 and OPA-1 expression....................................................................................101
Figure 4.5: Effects of 60 min H$_2$O$_2$ challenge (± MDIVI-1) on ATP generation in H9c2 cardiomyoblasts. ..........................................................102

Figure 4.6: Effects of acute 5 min H$_2$O$_2$ challenge on routine, leak and ETS respiratory rates in H9c2 cardiomyoblasts. .........................................................103

Figure 4.7: Effects of 60 min H$_2$O$_2$ challenge (± MDIVI-1) on mitochondrial oxygen consumption rate (OCR) and extracellular acidifications rate (ECAR). .........................105

Figure 4.8: Effects of 60 min H$_2$O$_2$ challenge (± MDIVI-1) on the ratio of OCR/ECAR in H9c2 cardiomyoblasts. .................................................................106

Figure 5.1: The Effects of H$_2$O$_2$ and MDIVI-1 on cell viability (MTT assay) and LDH efflux in H9c2 cardiomyoblasts. .................................................................119

Figure 5.2: Real-time analyses of H9c2 viability during a 48 hr H$_2$O$_2$ challenge. ....122

Figure 5.3: Survival kinase activation in H9c2 cardiomyoblasts challenged with H$_2$O$_2$ ± MDIVI-1 over a range of times. .................................................................123

Figure 5.4: Mitochondrial and autophagy protein expression in H9c2 cardiomyoblasts during an 8 hr H$_2$O$_2$ challenge ± MDIVI-1. ........................................125

Figure 5.5: Assessment of pro- and anti- apoptotic protein expression in H9c2 cardiomyoblasts during an 8 hr H$_2$O$_2$ challenge ± MDIVI-1. .............................127

Figure 5.6: Mitochondrial membrane potential in H9c2 cardiomyoblasts during an 8 hr H$_2$O$_2$ challenge ± MDIVI-1. ...............................................................128

Figure 6.1: Cardiomyoblast proliferation under varying glucose and insulin levels. ..................................................................................................................141

Figure 6.2: Mitochondrial respiratory function. ..........................................................143

Figure 6.3: Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in cardiomyoblasts exposed to varying glucose and insulin levels. ..........144
Figure 6.4: Effects of glucose and insulin (± MDIVI-1) on cardiomyoblast expression of fission and fusion proteins..........................................................146

Figure 7.1: Mouse body weight..............................................................161

Figure 7.2: Relative and absolute changes in body weight in mice fed standard or high-fat diets.................................................................163

Figure 7.3: Glucose tolerance.................................................................165

Figure 7.4: Glucose handling in control and high-fat diet fed mice.................................166

Figure 7.5: Non-fasted serum insulin........................................................168

Figure 7.6: Myocardial post-ischaemic outcomes........................................170

Figure 7.7: Post-ischaemic recovery of LVDP in hearts from control and T2D animals.................................................................171

Figure 7.8: Post-ischaemic LDH efflux.....................................................172

Figure 7.9: Baseline and post-ischaemic mitochondrial respiration.........................174

Table 7.1: Relative expression of AKT signalling in healthy and T2D myocardium from untreated and MDIVI-1 treated mice..........................................................176

Table 7.2: Relative expression of survival kinase ERK1/2 in healthy and T2D myocardium from untreated and MDIVI-1 treated mice.........................................................177

Table 7.3: Relative expression of GSK3β in healthy and T2D myocardium from untreated and MDIVI-1 treated mice. ..........................................................178

Table 7.4: Relative expression of myocardial fission and fusion proteins in healthy and T2D myocardium...........................................................................180

Table 7.5: Relative expression of pro- and anti- apoptotic BAX and BCL-2 in healthy and T2D myocardium.................................................................181
Supplementary Figure 1: Mitochondrial functional analysis performed on intact cardiomyoblasts.................................................................193

Supplementary Figure 2: Representative western immunoblot data.........................194

Supplementary Figure 3: Representative western immunoblot data.........................195

Supplementary Figure 4: Representative western immunoblot data.........................196

Supplementary Figure 5: Representative western immunoblot data .......................197

Supplementary Figure 6: Cardiomyoblast proliferation under varying glucose and insulin levels (+ MDIVI-1).............................................................................................................................................198

Supplementary Figure 7: Representative western immunoblot data.........................199

Supplementary Figure 8: A comparative image of visceral adiposity.......................200

Supplementary Table 1: Nutrient composition.........................................................201

Supplementary Table 2: Non-fasted blood lipid profiles and glucose.........................202
V. Acknowledgements

Completion of this doctoral project would not have been possible without the guidance, support, patience and expertise of my supervisors; Professor John Headrick, Associate Professor Jason Peart and Associate Professor Eugene Du Toit. Your willingness to support my transition into the field of cardiovascular research and develop a career as independent researcher would not have been achievable without your mentorship over the past three years of this project. Your continued support and encouragement has provided me with invaluable foundations as a researcher.

I would like to acknowledge Griffith University for awarding me with the GUPRS scholarship throughout my candidature, and the Griffith University Heart Foundation Director (Luke Haseler) for supporting my international travel to allow me to present my research at international conferences. I would also like to acknowledge the School of Medical Science for their continual support and welcoming encouragement throughout the duration of my project.

I would like to acknowledge my fellow colleagues and lab members within the School of Medical Science and Heart Foundation Research Centre who have assisted me throughout various stages of my project, your technical assistance and willingness to help was always appreciated; Dr. Jacob Goodwin, Dr. Jelena Vider, Dr. Elham Alizadeh Pasdar, Dr. Bing Yan, Dr. Weng Endaya, Boris Budiono, Jake Russell and Lana Biviol. My dear friends and colleagues Dr. Louise See Hoe and Dr. Karishma Sachaphibulki, thank you for accompanying me on his journey in parallel to your own. Your technical assistance, entertaining humour, emotional therapy, caffeine support and friendship made this journey possible, amongst all the challenges.

Finally, I would like to thank my dear family for their endless love and constant support throughout this journey. My beloved husband Haroldo, inspiring mother, and my beautiful daughters Jasmyn and Sienna - your continued patience, love and encouragement motivated me daily to make this achievable. I will be forever grateful.
Acknowledgment of Assistance Received

The following contributions were made to studies detailed in this doctoral thesis:

Professor John Headrick for contribution to project design and data analysis for all experimental chapters. Undertaking Langendorff heart perfusions in studies detailed in Chapter 3 and Chapter 7.

Associate Professor Jason Peart for contribution to project design for all experimental chapters and analysis of data for studies detailed in Chapter 3 and Chapter 7.

Associate Professor Eugene Du Toit for contribution to project design and data analysis for studies detailed in Chapters 6 and 7.

Dr. Louise See Hoe for her contribution in performing Langendorff perfusions for studies detailed in Chapter 3 and extensive training in animal handling, and western immunoblotting.

Dr. Karishma Sachaphibulki for assisting in mitochondrial respiratory experiments in Chapter 7 and extensive training in mitochondrial respiratory analysis via the Oroboros oxygraph.

Dr. Jelena Vider for assistance and training in flow cytometry and cardiomyoblasts studies detailed in Chapter 4 and Chapter 5.

Jake Russell for assistance in conducting experiments and data analysis for studies detailed in Chapter 6 and Chapter 7.
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT</td>
<td>Also protein kinase B (PKB)</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis Inducing Factor</td>
</tr>
<tr>
<td>ANT</td>
<td>Adenine nucleotide translocase</td>
</tr>
<tr>
<td>Apaf1</td>
<td>Apoptotic protease activating factor 1</td>
</tr>
<tr>
<td>ATPase</td>
<td>Enzymes that catalyse the conversion of ATP to ADP and phosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>Bad</td>
<td>Bcl-2-associated death promoter</td>
</tr>
<tr>
<td>BAX</td>
<td>Bcl-2-associated X protein</td>
</tr>
<tr>
<td>BCL-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>CI</td>
<td>Complex I</td>
</tr>
<tr>
<td>CII</td>
<td>Complex II</td>
</tr>
<tr>
<td>CaMKI</td>
<td>Calcium/calmodulin-dependent kinase I</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CCCP</td>
<td>Carbonyl cyanide m-chlorophenyl hydrazone</td>
</tr>
<tr>
<td>Cdk1</td>
<td>Cyclin-dependent kinase 1</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CK</td>
<td>Creatine kinase</td>
</tr>
<tr>
<td>COX-IV</td>
<td>Cyclooxygenase-4</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>cypD</td>
<td>Cyclophilin D</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>+dP/dT, -dP/dT</td>
<td>Positive change in pressure over time, negative change in pressure over time</td>
</tr>
<tr>
<td>DRP-1</td>
<td>Dynamin-related protein-1</td>
</tr>
<tr>
<td>ECAR</td>
<td>Extracellular acidification rate</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellular signal-regulated kinases, specifically p44/42 MAPK</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FADH₂</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FIS1</td>
<td>Fusion-1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase-3 beta</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>Class of enzymes that catalyse the conversion of GTP to GDP and phosphate</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>H9c2</td>
<td>Rat cardiomyoblast cell line</td>
</tr>
<tr>
<td>HK</td>
<td>Hexokinase</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>IMM</td>
<td>Inner mitochondrial membrane</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>I-R</td>
<td>Ischaemia-reperfusion</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>KO</td>
<td>Knock out</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LVEDP</td>
<td>Left ventricular end-diastolic pressure</td>
</tr>
<tr>
<td>LVESP</td>
<td>Left ventricular end-systolic pressure</td>
</tr>
<tr>
<td>LVDP</td>
<td>Left ventricular developed pressure</td>
</tr>
<tr>
<td>mmHg</td>
<td>Millimetres of mercury</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MDIVI-1</td>
<td>Mitochondrial division inhibitor-1</td>
</tr>
<tr>
<td>MFN-1/MFN-2</td>
<td>Mitofusin (1 and 2)</td>
</tr>
<tr>
<td>MiD49/51</td>
<td>Mitochondrial dynamics protein 49/51</td>
</tr>
<tr>
<td>MIM</td>
<td>Mitochondrial inner membrane</td>
</tr>
<tr>
<td>mPTP</td>
<td>Mitochondrial permeability transition pore</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>Mx</td>
<td>Interferon-induced GTP-binding protein Mx1</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (oxidised form)</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>Superoxide</td>
</tr>
<tr>
<td>OCR</td>
<td>Oxygen consumption rate</td>
</tr>
<tr>
<td>OMM</td>
<td>Outer mitochondrial membrane</td>
</tr>
<tr>
<td>OPA-1</td>
<td>Optic atrophy-1</td>
</tr>
<tr>
<td>OxPhos</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>Peroxisome proliferator-activated receptor gamma coactivator 1-</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>----------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>alpha</td>
<td></td>
</tr>
<tr>
<td>Phospho</td>
<td>Phosphorylated form</td>
</tr>
<tr>
<td>RIP1/3</td>
<td>Receptor-interacting protein 1/3</td>
</tr>
<tr>
<td>RISK</td>
<td>Reperfusion injury signalling kinase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>T2D</td>
<td>Type II diabetes mellitus</td>
</tr>
<tr>
<td>T1D</td>
<td>Type I diabetes mellitus</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor - alpha</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage dependant anion channel</td>
</tr>
<tr>
<td>Δψm</td>
<td>Mitochondrial membrane potential</td>
</tr>
</tbody>
</table>
CHAPTER 1: Background

1.0 Introduction

Since 1990, the World Health Organisation (WHO) reported that cardiovascular disease (CVD) has remained as one of the leading causes of morbidity and mortality globally than any other non-communicable disease (Mackay and Mensah, 2004). The leading risk factors significantly contributing to the high mortality rates observed in coronary artery disease (CAD) include elevated blood pressure, hypercholesterolaemia, tobacco use, physical inactivity and over-nutrition. Although genetic factors contribute to individual susceptibility to heart disease, 80-90% of those diagnosed with CAD have more than one lifestyle risk factor. Recently, the United Nations predicted that global risk factors attributing to premature CVD death could be reduced from 7.8 million to 5.7 million by 2025 if the aforementioned risk factors are targeted to reduce cardiovascular mortality by ~26% in men and ~23% in women (Who, 2013). Both North America and Western Europe have reported a decline in the prevalence of coronary heart disease, largely due to improved prevention, and advances in diagnosis and treatment in cardiovascular medicine. The Australian Governments National Health Survey reported that cardiovascular disorders, including heart attacks, strokes and vascular diseases account for over one third of all reported deaths in Australia ("Cardiovascular disease: Australian facts," 2011). Pharmacological interventions that could illicit cytoprotective benefits in patients who suffer from myocardial infarction (or cardiac surgery involving ischaemia) would have a major impact on cardiovascular outcomes in the Australian and global population.

1.1 Ischaemic heart disease

Myocardial infarction occurs when coronary blood flow is obstructed or occluded, causing insufficient oxygen and substrate delivery to the myocardial tissue and ultimately resulting in cell death. The most common cause of myocardial infarction is the build up and disruption of atherosclerotic plaque in the coronary arteries (Hausenloy and Yellon, 2013).
Atherosclerotic plaque is composed of cholesterol, fatty deposits (within an inflammatory microenvironment) and fibrous material, which typically evolve over time as a consequence of not only dyslipidaemia but also inflammatory, immune and apoptotic processes (Fig. 1.1). Plaques progress to a stage promoting a high risk of thrombosis which in turn occludes the coronary artery (or breaks away as an embolus to occlude a distal vessel), inducing ischaemia (Andrews et al., 1997). During conditions of ischaemia (defined as an imbalance in oxygen and substrate delivery as a result of impaired blood supply to myocardial cells), the coronary arterioles may dilate in an attempt to counteract the decline in O₂ supply. However, if myocardial cells do not receive sufficient oxygenated blood, both aerobic and anaerobic metabolism are impaired, resulting in complete substrate and intracellular ATP depletion and ultimately the cell death that defines an infarction (commonly termed heart attack).

Figure 1.1: Coronary atherosclerotic plaque. An atherosclerotic plaque is composed of cholesterol, fatty deposits (within an inflammatory microenvironment) and fibrous material, which advance over time due to multifactorial risk factors including; dyslipidaemia, inflammation, immune and apoptotic processes. Plaque formation promotes a high risk of thrombosis, increasing the likelihood of coronary artery occlusion, and the induction of ischaemia (Heart Research Institute, 2016).
Infarction involves death of myocardial cells via necrotic and/or apoptotic death (and recently defined variants of these processes), damaging the myocardium and resulting in myocardial scarring. Restoration of oxygenated blood flow to the heart muscle is termed ‘reperfusion’. This is essential to the restoration of oxidation substrates, normalisation of intra- and extra-cellular pH levels, and the generation of cellular ATP (Headrick, 1996). These critical processes are important for restoration of homeostasis within the cell, however reperfusion also contributes to a paradoxic injury of the myocardium. The sudden return of flow induces a range of injurious mechanisms including uncontrolled calcium entry and activation, induction of a trans-sarcolemma oncotic gradient, generation of reactive oxygen species (ROS), such as superoxide and highly reactive hydroxyl radicals, together with up-regulation of inflammatory cascades, cytokines and catecholamine’s that all contribute to contractile dysfunction and myocardial intracellular abnormalities (Lejay et al., 2014). A wide spectrum of abnormalities arises within the heart during ischaemia-reperfusion, and mitochondria are crucial in governing the progression of injury and the balance of survival vs. death. The pathophysiological events specific to the mitochondria in an ischaemic and reperfused myocardium include: inhibition of the respiratory chain complex (embedded in the inner mitochondrial membrane), increased proton and ionic movement from the inner mitochondrial membrane, dissipation of the membrane potential, mitochondrial calcium imbalance, mitochondrial ROS generation and opening of pores including the mitochondrial permeability transition pore (mPTP), further diminishing respiratory capacity and facilitating the release of apoptosis-inducing proteins (Gottlieb et al., 2011; Hom & Sheu, 2009; Lejay et al., 2014).

1.1.1 Ischaemic preconditioning

Ischaemic preconditioning (IPC) was first identified by Murry et al. (1986), who showed that short periods of ischaemia or coronary artery occlusion followed by reperfusion paradoxically reduced the severity of myocardial injury during subsequent acute myocardial infarction (AMI) in a canine model. Ischaemic preconditioning is widely reported as a powerful cardioprotective therapy, substantially reducing the severity of cellular death while reducing O2 consumption in order to maintain intracellular functioning and ATP expenditure (Headrick, 1996). The effects of ischaemic preconditioning have been
assessed in both experimental and clinical settings, and the response has potential as an intervention to delay and/or prevent myocardial damage and death with ischaemic and other insult (Yellon et al., 1993; Yellon and Downey, 2003; Hoole et al., 2009). However, despite global efforts to develop an efficacious cardioprotective therapy, none have successfully translated to a clinical setting. Further exploration is needed to establish which molecular targets and approaches are effective in the disease context. Whether modulation and maintenance of mitochondrial integrity is a viable therapeutic approach in the prevention of myocardial ischaemia-reperfusion (I-R) injuries remains to be established.

1.2 Mitochondria and myocardial cell death in ischaemia-reperfusion

The primary function of mitochondria is to synthesise ATP through oxidative phosphorylation (OxPhos). Reducing agents NADH and FADH₂ are generated in the tricarboxylic acid cycle (TCA), and are utilised as electron donors in the respiratory chain complex, embedded within the inner mitochondrial membrane (IMM). Under basal conditions, the myocardium preferentially utilises fatty acids to drive oxidative synthesis of ATP, with 10-30% of ATP generated via glycolysis (Marin-Garcia, 2005). These values will vary according to shifts in the levels of circulating carbon substrate level.

Mitochondria are tightly regulated organelles, with an almost impermeable IMM, essential in maintaining the electro-chemical gradient that drives ATP generation - the mitochondrial membrane potential (ΔΨ). The ΔΨ is generated by the electron transport chain (ETC) within the IMM, created via the transfer of protons to the intermembrane space. The movement of these protons back into the matrix is coupled to ADP phosphorylation via ATP synthase, generating ATP (Kroemer and Reed, 2000). Thus, the integrity of the mitochondrial membrane and its selective permeability are essential in maintaining OxPhos, cellular energy, and homeostasis. Either membrane damage or formation/activation of 'pores' disrupts this process.

Although not clearly established, the molecular conformation of the permeability transition pore complex involves several key proteins localised to both the inner and outer mitochondrial membranes. The most abundant proteins responsible for mediating OMM permeabilisation, is the voltage-dependant anion channel (VDAC), which acts by eliminating molecular proteins smaller than ~5 kDa under physiological conditions, while
communicating with adenine nucleotide translocase (ANT), situated within the mitochondrial matrix. Key enzymes, hexokinase (HK) and creatine kinase (CK) are collectively involved in regulating permeabilisation via interaction with the aforementioned (VDAC and ANT) proteins that facilitate permeabilisation. Interactions from either pro and anti-apoptotic regulators, including members of the BCL-2 family, can stimulate changes, thus favouring cell death activation or inhibition (Fig. 1.2). When permeability of both the inner and outer mitochondrial membranes (Fig. 1.2) loses selectivity, the translocation of smaller molecular proteins between the mitochondrial matrix and cytosol is permitted (Riedl et al., 2007). The IMM surrounds the matrix, containing mtDNA (ribosomes, mRNA), respiratory complexes I-V of the ETC, and oxidative enzymes involved in the synthesis of ATP. When cellular homeostasis is compromised as a result of injurious stress or toxicity (e.g. reduced oxygen levels) the permeability of the OMM becomes less selective, allowing the translocation of smaller proteins normally confined within the intermembrane space (Ong et al., 2012). The movement of these proteins from the intermembrane space initiates a signalling cascade that activates cell death pathways (Karbowski et al., 2003).

Once a sufficient population of mitochondria have compromised membrane integrity, cell death is inevitable. The ultimate form of cell death depends upon the extent of de-energisation and mPTP activation, since apoptosis is highly dependent on the availability of ATP. Necrosis is typically unregulated, consequently following the disruption of the plasma membrane and complete ATP depletion (Further detailed in sections 1.2.1, 1.2.2 and 1.2.3).
Figure 1.2: Simplified schematic of the mitochondrion. A) The main structural components that make up the mitochondria include; a outer mitochondrial membrane (OMM) inner membrane space, inner mitochondrial membrane (IMM), the ETC (containing complexes I-V) within the IMM, mitochondrial transition pores (mPTP) (located on the outer and inner mitochondrial membranes) and metabolic exchange shuttles between the cytosolic compartment and the mitochondrial matrix. B) Permeability of the OMM is regulated via the formation of the non-specific mitochondrial permeability transition pore (mPTP) complex via interaction with key proteins including; the voltage gated anion channel protein (VDAC), adenine nucleotide translocase (ANT), hexokinase (HK) and creatine kinase (CK) (Lackner, 2014).
Distinct forms of cell death may arise during ischaemia and/or reperfusion (Fig. 1.3). Cell death ensues after 15-40 min of myocardial ischaemia (though time course can vary according to factors such as coronary collateralisation, myocardial mass, myocardial oxygen consumption ($\text{MVO}_2$), and history of ischaemic events). After 6 hours, few viable cells remain. Regions that are not reperfused develop a pale acellular infarct, sometimes termed ‘mummification’. As leukocytes cannot gain access to necrotic tissue in areas supplied by the occluded coronary artery, myocardial cells autolyze in this type of infarction. Death in reperfused tissue may proceed via several processes, classified according to an increasing array of pathways. However, the most simplified differentiation of cell death is either regulated (e.g. apoptosis) or inactively passive (e.g. oncosis). Parameters that contribute to the latter include loss of ionic balance, depletion of ATP stores, cellular swelling and membrane rupturing/fragmentation, which contribute to an inflammatory response (Liu et al., 2004; Marin-Garcia, 2005). These molecular mechanisms and how they are differentiated in the heart are discussed below. The term necroptosis has also been introduced to describe an alternative mode of programmed cell death, which essentially represents a regulated form of necrosis (Kung et al., 2011).
Figure 1.3: Differentiation of cell death pathways. Apoptosis is ATP dependent and mediated extrinsically via caspase-8 activation, or intrinsically via the mitochondrial release of cytochrome c, which initiates the formation of the apoptosome complex and downstream activation of caspase-9 to initiate apoptosis. Necrotic cell death is not dependent of ATP, therefore favoured when mitochondrial integrity is compromised and oxidative capacity is disabled. Necroptosis is a regulated form of cell death favoured upon caspase-8 inactivation via death receptors, receptor interacting proteins (RIP1 and RIP3).

### 1.2.1 Necrosis

Necrotic cell death has been negatively perceived in the literature as a passive and unregulated form of cell death in which irreversible cell injury results from toxic/chemical stimuli or environmental perturbation. The quintessential morphological characteristic of necrosis is disruption of the plasma membrane, resulting from depletion of cellular ATP stores and cellular swelling, disrupting the homeostatic cell environment (Lockshin and Zakeri, 2004; Whelan et al., 2010). Signals that initiate necrosis include certain cytokines,
which are generally released in response to trauma or toxicity and induce inflammatory activation. A study in a diabetic model, for example, has revealed that both apoptotic and necrotic signalling proteins are up regulated in response to cytokine stimulation, including tumor necrosis factor-alpha (TNF-α), interleukin-1-beta (IL-1β), and interferon-gamma (IFN-γ) (Saldeen, 2000), all of which participate in both apoptotic cell death and necrosis in the heart.

1.2.2 Necroptosis

Necrotic cell death has been further categorised into a form of regulated death termed necroptosis. Necroptosis is a physiologically regulated form of cell death that functions when the regulated apoptotic protein, caspase-8, is inactive. Death receptor activation is initiated via a subset of programmed necroptotic proteins, including protein kinases RIP1 and RIP3 (Sosna et al., 2014). Protein kinases are essential sensors and indicators of cell stress, with adjoining recruitment domains that bind to the death receptors TNFR1, FasR, TRAILR1 and TRAILR2. Although these death receptor molecules function as pro-apoptotic proteins, the ubiquitination of RIP1 determines whether it functions as a pro-survival or pro-death kinase (Declercq et al., 2009). Cyclophilin D (CypD) is a mitochondrial molecular chaperone found to participate in the regulation of the mPTP (Zhou and Yuan, 2014). As such, CypD is a key mediator of ischaemia-reperfusion (I-R) injury and mitochondrial-induced necrosis, governed by a combination of a Ca^{2+} induced overload and an increased ROS production. It has been suggested that there is some mPTP crosstalk with both necrosis and necroptosis pathways, since necroptosis is also characterized by cytosolic Ca^{2+} overload and intracellular acidification (induced by an increase in ROS). Using a CypD-deficient mouse model, Nakagawa and colleagues (2005) induced necrotic cell death via calcium overload, ROS and ischaemia-reperfusion. Mice deficient in CypD showed resistance to I-R injury and Ca^{2+} overload (vs. apoptotic cell death), linking mPTP opening to necrosis.

Since the fate of a cell is highly dependant on the nature of the pathological insult with human disease, the mechanisms of non-apoptotic cell death pathways are not mutually exclusive, with evidence suggesting that there is some overlap in necrosis and necroptosis.
However, regulated cell death pathways (apoptosis) are distinctively conserved and uniform in their pathways, which ultimately leads to the controlled execution of a cell.

### 1.2.3 Apoptosis

Apoptosis (or programmed cell death) in mammalian cells can be mediated via caspase-dependant or caspase-independent signalling. However, it has been well documented that many stimuli promote caspase-mediated apoptosis via extrinsic or intrinsic pathways (Feldstein and Gores, 2005; Garrido *et al.*, 2006; Garrido and Kroemer, 2004). Intrinsically mediated apoptosis is categorised by the release of mitochondrial cytochrome c into the cytosolic compartment whereby it initiates a cascade of signals that enable activation of caspase-3. Extrinsic apoptosis (Fig. 1.4) is categorised by the activation of death receptor ligands that initiate signalling cascades that activate the effector caspase, caspase-8 (Feldstein and Gores, 2005).

Cytochrome c is a water-soluble haem containing protein located within the mitochondria during cellular homeostasis. It functions as an electron carrying protein embedded within the IMM and closely associates with the phospholipid cardiolipin in order to carry out the transfer of electrons (Garrido *et al.*, 2006). The attached haem group of cytochrome c participates as a redox intermediate involved in the transport of electrons between complexes III and IV (Ow *et al.*, 2008). Normally ~85% of cytochrome c is in the inter-cristae space tightly coupled to cardiolipin (~15% in inter-membrane space). Both formation of sufficiently large pores (e.g. mPTP opening) and the detachment of cytochrome c from cardiolipin (for example via cardiolipin oxidation) are required for the release of cytochrome c in order to induce apoptosis. Once in the cytosol, cytochrome c triggers the formation of the apoptosome, a complex involving adapter protein apoptotic protease activity factor (Apaf-1) and caspase-9 (Fig. 1.4).

Caspases are a family of cysteine protease peptidases with catalytic properties selective in cleaving proteins at sites in close proximity to aspartic acid residues (Boatright and Salvesen, 2003). The biochemical action of caspases involves the breaking down of unused or damaged structural components within a cell organelle, which are later ingested phagocytically or digested by other organelles. Depending on when they are activated, caspases function as either initiators or executioners within the cell death pathway (Garrido
and Kroemer, 2004). Initiator caspases include caspases 2, 8, 9, 10, 11 and 12, all which possess different apoptotic substrate recognition sequences. Under normoxic conditions, caspase-9 and -8 exist as inactive pro-caspase monomers within the cytosol, which are activated upon dimerization induced through upstream signalling. Upon a stress or toxic stimulus, the inactive form of caspase-9 becomes activated via dimerization, prompted via the binding to the Apaf-1 to initiate the formation of the apoptosome (complex containing cytochrome c, Apaf-1 and caspase-9). The formation of the apoptosome complex initiates subsequent activation of the effector caspase, caspase-3, governed via a conformational change of the active binding sites. These sites mediate the formation of a functional protease, responsible for cleaving cellular substrates that are either damaged or undergoing stages of early embryonic development. Once an effector caspase is activated, amplified signalling prompts remaining initiator caspases to become activated, inducing rapid cell dismantling (Cohen, 1997; Boatright and Salvesen, 2003; Mcilwain et al., 2013). Early investigations into caspase-9 and the importance of its effects found that knockout reduced apoptotic changes in early embryonic development, causing severe cerebral malformations. A caspase-9 deficiency was found to have a downstream effect on caspase-3 activation, as there was no formation of an apoptosome, an essential process in apoptotic cell death and normal embryonic development (Kuida et al., 1998).

Apoptosis is influenced by apoptosis inducing factor (AIF), a flavoprotein that is confined within the mitochondria during cellular homeostasis. Its role in normoxic conditions is to participate as an intermediate in bio-energetic and redox metabolic reactions (Modjtahedi et al., 2006). Apoptosis inducing factor is important in regulating the respiratory chain complex, crucial for cell survival, cell proliferation and maintaining mitochondrial integrity (Hangen et al., 2010). However, as the result of a pathogenic disease or an ischaemic insult, AIF is released from the mitochondria, translocating first to the cytosol and then binding to the nucleus to induce chromatin condensation and chromatinolysis, a process independent of caspase activation. In addition to its pro-apoptotic effect on isolated nuclei, AIF is also known to promote and release other apoptotic inducing proteins including cytochrome c and pro-caspase-9 (Susin et al., 1999).
Figure 1.4: Simplified schematic of the extrinsic and intrinsic pathways of apoptosis. Apoptosis, a regulatory 'controlled' mode of cell death prompted by various forms of cellular stress or toxicity, is categorised as either an extrinsic or intrinsic mode of cell death. Intrinsically mediated apoptosis is categorised by the mitochondrial release of cytochrome c into the cytosolic compartment where it initiates activation of an initiator caspase, which prompts the downward cascade activation of caspase-3, an effector caspase. Extrinsically mediated apoptosis in mediated via death receptor ligands on the cell surface, which stimulate the activation of the effector caspase-8 (Feldstein and Gores, 2005).

1.3 Mitochondrial dynamics - a potential therapeutic target?

The high mortality rate and costs of CAD demand effective protective therapies. However, cardioprotection remains an elusive clinical goal. Annually, 40 billion American dollars is utilised in preventative and outpatient costs associated with CAD in Northern America. The national impact of CVD induces a massive economic burden; with national health reports estimating $5.9 billion was spent on the disease in 2004-2005 and
representing 34% of all deaths in 2008 (Aihw, 2011). Cardiac functionality in CAD patients can be targeted therapeutically and thus maintained; however damage to the cardiomyocytes alters cardiac physiology and mitochondrial energetics. The mitochondria are essential in supplying the ATP involved in homeostatic maintenance within a healthy heart. These ‘aerobic’ organelles are essential in fuelling the cellular processes of contractile function and force generation, and maintenance of sarcolemma ionic gradients (Bayeva et al., 2013). With mitochondrial impairment, cellular and metabolic processes are compromised, which may initiate the development of disease and mutations of mitochondrial proteins, thus altering cell functionality (both factors that induce cell death). The important role of mitochondria in governing cell survival vs. death renders them an attractive target for therapy. Additional to regulating 'death channel' function discussed above, targeting mitochondrial quality control and dynamics may offer useful therapeutic actions. In healthy tissues, the balance of dysfunctional vs. functional mitochondria is regulated through a range of mechanisms. Mitochondrial morphology is continuously changing via regulatory processes involving fission and fusion signalling to induce elongation and shortening of the mitochondria (further detailed in section 1.4). Both fission and fusion proteins largely contribute to cell proliferation during embryonic development, with fission proteins governing mitochondrial division to stimulate an increase in the mitochondrial population (Chan, 2006).

Mitochondrial fusion is also important in early embryonic development, with both MFN-1 and MFN-2 knockout mice dying in utero as a consequence of placental deficiency (Chen et al., 2003). Conversely, cultured fibroblasts derived from mouse embryos (MFN-1/MFN-2 deficient) remained viable in the absence of regulated fusion mechanisms, with mitochondrial DNA and membrane potential dramatically affected, impacting on ATP production (Chen et al., 2005). Mitochondrial fission and fusion significantly contribute to the quality control of mitochondria in mammalian cells, continuously responding to changes within the cellular environment. When the mitochondrial fission and fusion balance is offset, healthy functioning mitochondria may become compromised, inducing dysfunction in mitochondrial signalling (Youle and Van Der Bliek, 2012).

In addition to the mitochondrial quality control function that fission and fusion proteins provide, autophagy (further detailed in 1.4.1) is another well-established
mechanism that aids in eliminating dysfunctional and mutated mitochondria. Autophagy acts by degrading intracellular components that are utilising energy stores as well as maintaining the mitochondrial population through encapsulation of old, impaired and dysfunctional mitochondria (termed mitophagy). The autophagic removal (Fig. 1.5) of defective mitochondria has been linked to fission and fusion processes, with 20% of daughter mitochondria formed as a direct result of fission recycled through mitophagy if their function is not optimal (Twig et al., 2008).

Figure 1.5: Autophagic elimination of mitochondria. Autophagy contributes significantly to the maintenance of healthy mitochondria by eliminating defective and senescent mitochondria in a process termed mitophagy. Mitophagy works parallel to mitochondrial fission and fusion process as a quality control mechanism (Youle and Van Der Bliek, 2012).

Given the key roles of mitochondrial fission and fusion and mitophagy in maintaining a healthy mitochondrial population, these processes may be useful therapeutic targets. The stimulation of new and healthy produced mitochondria (mitochondrial biogenesis) would be deemed highly beneficial in many age- and lifestyle-related diseases, in which there may be a high incidence of mitochondrial defects.
1.4 Mitochondrial quality control and dynamics

Mitochondrial dynamics refers to a continuous series of fission and fusion events between mitochondria that regulate healthy functioning during a physiological state (Twig et al., 2008; Liesa et al., 2009). Mechanisms that contribute to the elongation (fusion) and shortening (fission) of the mitochondria influence changes in the mitochondrial population, thus serving important functions which influence metabolic regulation, cellular respiration, ionic buffering and apoptosis (Chan, 2012; Ishihara et al., 2012). Emerging evidence suggests that under physiological conditions, the fusion of mitochondria contribute an integral role in development that maintain cellular growth and proliferation, a process in which the demand for ATP greatly drives the oxidative phosphorylation capacity of the cell (Chen and Chan, 2005; Chan, 2012). Furthermore, the fusion protein OPA-1 (further detailed in section 1.4.2) has been independently linked to cristae structure remodelling, deep invaginations within the IMM which contain a diversity of proteins involved in the translocation and synthesis of proteins (Vogel et al., 2006; Kopek et al., 2013). Furthermore, knockout of both OPA-1 and mitofusin proteins (MFN-1/MFN-2) in vivo and ex vivo resulted in severe depletion of mtDNA content, respiratory dysfunction and compromised mitochondrial membrane potential (Chen and Chan, 2005; Chen et al., 2005). Together, this evidence strongly highlights the significance of fusion in maintaining healthy mitochondrial populations with homogeneity in terms of metabolic regulation and mtDNA.

The division of mitochondria are also vital, however its physiological function within a mammalian model remains poorly understood with emerging reports suggesting that it is highly involved in the cell division process during embryonic development and elimination of damaged mitochondria via mitophagy (Ishihara et al., 2009; Kane and Youle, 2010). Together, fission and fusion complement the dynamic nature of mitochondria, which aims to maintain a ‘quality’ functioning population of mitochondria.

1.4.1 Mitophagy

Autophagy (Greek for; ‘self digestion’) is a regulatory process involving catabolic reactions that participate in the degradation of damaged or excess cell organelles and senescent proteins. Under physiological conditions, autophagy is a vital cellular process,
involving the recycling and degradation of macromolecules and organelles (Gustafsson and Gottlieb, 2009)). The autophagosome, a double membrane vesicle, directs cytoplasmic structures or organelles including mitochondria or endoplasmic reticulum to ingesting lysosomes. There are three categories of autophagic process: macroautophagy, microautophagy and chaperone-mediated autophagy. The most commonly cited form of autophagy is macroautophagy, involving formation of the autophagosome, which involves the engulfment of cytoplasmic structures and the IMM, and is regulated by a group of autophagic proteins. Mitophagy contributes to mitochondrial quality control through selective and non-selective degradation of damaged, mutated and senescent mitochondria (Frezza et al., 2006; Mao and Klionsky, 2011).

It is unclear as to whether mitophagy plays a protective or injurious role in cardiomyocytes subject to I-R injury. However, the process is up-regulated in states of energy depletion (Levine and Yuan, 2006). Induction of autophagic activity in cardiomyocytes during I-R has been shown to reduce infarction size by 30\% in vivo and cell death by 41\%, demonstrating that autophagy plays a cytoprotective role in the heart during reperfusion (Cho et al., 2014).

1.4.2 Mitochondrial fusion

The process of mitochondrial fusion is complex and involves a sequential process that the mitochondria undergoes in order to maintain its integrity and unique morphology. Mitochondrial fusion (Fig. 1.6A) involves the tethering of two individual mitochondria in close physical proximity to each other, a process that maintains their individuality without transformation or exchange of properties. The inter-membrane space and mitochondrial membranes (inner and outer) result in one combined structure that encapsulates the two unfused mitochondrial matrices and IMM. The next sequential step involves the fusion of the OMM, followed almost simultaneously by fusion of the IMM between two adjoining mitochondria (Fig. 1.6B) (Seo et al., 2010). The fusion of two mitochondria is highly regulated and sequential, mediated by proteins that include the mitofusins (MFN-1/MFN-2) and OPA-1.
Figure 1.6: Mitochondrial fission and fusion. A) The fusion process requires three steps: docking, fusion of mitochondrial outer membrane (MOM) and fusion of mitochondrial inner membrane (MIM). MFN-1 and MFN-2 are thought to play an important role in docking and MOM fusion. OPA-1 is involved in the formation of cristae junctions and MIM fusion, which occurs in a GTP-dependent manner; and B) The fission protein DRP-1 self-oligomerises and assembles a scission machine around the MOM. The fission protein FIS1 acts as an anchor to the DRP-1 protein, located on the OMM, while DRP-1 is located in the cytosol and translocates to mitochondrial surface to FIS1 (Seo et al., 2010).

Mitofusins: MFN-1 and MFN-2. Mammalian mitofusins are distinct in their roles in fusion of the outer and inner mitochondrial membranes, and function homo- or heterotypically, indicating their interaction is either with the same protein (MFN-1-MFN-1 and MFN2-MFN-2) or between different mitofusins (MFN-1-MFN-2). Investigations of mutations in these mitofusins reveal linkages to the neurodegenerative disease Charcot Marie Tooth Syndrome Type 2A, a group of genetically heterogeneous diseases of the
nervous system involving mutations in the MFN-2 gene (Zuchner et al., 2004; Engelfried et al., 2006). Although this nervous system mutation is not heart specific, it has been reported that mitochondrial cardiomyocytes deficient in MFN-2 are pleomorphic and enlarged, a form of mitochondrial dysfunction. Knockout of MFN-2 produces myocardial hypertrophy and suppressed functional outcomes, yet improved mechanical function upon reperfusion. A delay in the opening of the mPTP as a result of calcium influx and ROS generation is also evident with MFN-2 knockout (Papanicolaou et al., 2012). Cells lacking both MFN-1 and MFN-2 are unable to carry out fusion, resulting in defects including decreases in mitochondrial membrane potential, mitochondrial respiration, and cell proliferation (Papanicolaou et al., 2012). Interestingly, cells lacking either MFN-1 or MFN-2 are still able to maintain fusion, confirming that fusion occurs either at a homotypic or heterotypic level of interaction at the mitochondrial membrane.

**Optic Atrophy-1 (OPA-1).** A ubiquitously expressed protein, optic atrophy 1 (OPA-1) is highly expressed in the heart where it regulates mitochondrial dynamics and integrity in mitochondria-dense myocardium. The OPA-1 protein is one of two GTPases that mediates mitochondrial fusion, and is located on the inner mitochondrial membrane. Loss of function or genetic mutation of both groups of fusions proteins, OPA-1 and MFN (1 and 2), has been found to contribute to the neurological disease Charcot Marie Tooth Syndrome Type 2A. The MFN-1 and 2 and OPA-1 proteins have broader roles in maintaining mitochondrial morphology and integrity, and contributing to other cellular processes (Chen and Chan, 2010). Cells lacking OPA-1 protein, as a result of targeted RNAi, are unable to retain any level of mitochondrial fusion, resulting in mitochondrial fragmentation and associated defects in cellular respiration and cell proliferation, with a decrease in the mitochondrial membrane potential (Piquereau et al., 2012).

However, OPA-1 has also been found to play a maintenance role in the cristae structure of the mitochondria, eliciting a cytoprotective role that inhibits the pro-apoptotic release of cytochrome c into the cytoplasm (Frezza et al., 2006). In cardiac models, Chen and colleagues (2009) found reduced OPA-1 was characteristic in a human heart failure model and a rat cardiomyoblast cell line (H9c2 cells) subjected to simulated ischaemia. Confocal and electron microscopy revealed a significantly higher number of fragmented mitochondria in OPA-1 deficient cells, consistent with low levels of fusion. Furthermore,
an increase in cytochrome c was measured in the cytosol in models with either over- or under-expressed levels of OPA-1 (Chen et al., 2009). Control of the fusion function of OPA-1 may stem from its proteolytic cleavage: OPA-1 is proteolysed into 2 long and 3 short isoforms, which attach differentially to the mitochondrial inner membrane (Ishihara et al., 2006). This OPA-1 processing appears to be governed by reductions in cellular ATP. Long forms of OPA-1 undergo processing within the mitochondrial matrix to generate short forms, potentially via mitochondrial proteases including the ATPase family gene 3-like 2 (Afg3l2) and high temperature requirement protein A2 (HtrA2). Emerging evidence indicates the long forms specifically promote fusion, and that maintenance of a stable inner mitochondrial membrane pool of long OPA-1 prevents mitophagy and maintains mitochondria (Macvicar and Lane, 2014).

1.5 Mitochondrial fission

Mitochondrial fission involves the biological activity of two main molecules that drive the fragmentation of mitochondria. The key fission mediating molecule, dynamin-related-protein-1 (DRP-1) is a cytosolic protein that translocates to the outer mitochondrial membrane and docks with the second group of biological proteins, FIS1 Mff, MiD49 and MiD51. Understanding how these biological proteins influence the dynamic network of mitochondria under varying pathological conditions are an emerging area interest.

1.5.1 Dynamin-related protein-1 (DRP-1) and fission protein-1 (FIS1)

The dynamin-related protein-1 (DRP-1) is a member of a family of GTPases participating in the process of mitochondrial fission. Preliminary explorations into mitochondrial fission regulation were first carried out in yeast by Bleazard et al (1999), and in C.elegans by Labrousse et al (1999). These studies provided a foundation into understanding fission pathways, with subsequent work establishing that mammalian fission is primarily controlled by both DRP-1 and FIS1 proteins (Smirnova et al., 2001; James et al., 2003; Yoon et al., 2003). The DRP-1 protein is a key modulator in mitochondrial fission, expressed in the cytosolic compartment with a small subunit localised to mitochondrial tubules as fission sites (Fig. 1.7). The small protein is another key player in mitochondrial fission, localised to the outer membrane of the mitochondria via a cytosolic
facing c-terminal trans-membrane domain. The DRP-1 molecule acts by constricting the mitochondrial tubules and disassembling the mitochondrial membranes into separate mitochondria.

Until recently, the exact molecular mechanism by which DRP-1 is recruited to the mitochondrial outer membrane was unknown, with FIS1 identified as the only known recruiter of DRP-1 to mediate fission. However, upon inhibition of FIS1, the impact of fission protein DRP-1 remained unchanged, suggesting that additional proteins must participate in recruitment of DRP-1 to the mitochondrial surface (Suzuki et al., 2003; Lee et al., 2004; Stojanovski et al., 2004). Chan and colleagues (2006) proposed three additional proteins involved in the recruitment of DRP-1 during fission - mitochondrial fission factor (Mff), and mitochondrial elongation factor 1 (MIEF1 or MiD51) and 2 (MIEF2 or MiD49). In studies of FIS1-/Mff-null cells, Lonson et al (2013) recently confirmed that Mff and FIS1 have regulatory roles which are important in governing the quantity and dimension of DRP-1 puncta (located on mitochondrial outer membrane). The MiD49/MiD51 recruitment proteins were found to participate in recruitment of DRP-1 in FIS1 and Mff-null cells. Furthermore, DRP-1 recruitment was augmented in cells overexpressing MiD recruitment proteins, with a correlated increase in inhibitory phosphorylation (Ser637) of DRP-1. Treatment with the protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was found to decrease inhibitory phosphorylation and increase recruitment of DRP-1 to mediate mitochondrial fission (Otera et al., 2010; Loson et al., 2013; Richter et al., 2014).
Figure 1.7: Cellular localisation of mitochondrial fission and fusion proteins. The Dynamin-related protein-1 (DRP-1) is localised to the cytosol and translocates to the outer mitochondrial membrane (OMM) upon phosphorylation whereby it mediates fission via recruitment to the docking protein FIS1. The family of fusion proteins optic atrophy-1 (OPA-1), located on the inner mitochondrial membrane (IMM), and mitofusin 1 and 2 (MFN-1/MFN-2) are confined to the OMM. Both fusion proteins are involved in the reorganization of mitochondria, antagonistic of DRP-1.

In order for DRP-1 to drive the changes in the dynamic nature of mitochondria, emerging studies have suggested that DRP-1 is a molecular target of post-translational modifications that enable it to successfully regulate mitochondrial division. Alterations in the phosphorylation of DRP-1 have been linked to processes including; ubiquitination,
SUMOylation and S-nitrosylation, and O-linked-N-acetyl-glucosamine glycosylation (Harder et al., 2004; Chang and Blackstone, 2007; Cribbs and Strack, 2007; Taguchi et al., 2007; Han et al., 2008; Cho et al., 2009; Cho et al., 2013). Each DRP-1 promoter (Fig. 1.8) includes: an N-terminal GTPase domain, a middle assembly domain, B variable domain and C-terminal GTPase effector domain, with modification sites predominantly found in the B variable region (domain). These modifications are mediated in turn by physiological disturbances of cellular homeostasis. Oxidative and pathophysiological stressors can alter the variable domain region of the DRP-1 molecule that distorts the balance of fission/fusion (Chang and Blackstone, 2010; Anderson and Blackstone, 2013; Cho et al., 2013). Evidence indicates that overexpression of the protein SUMO1 increases SUMOylation of DRP-1, a known substrate.

Figure 1.8: Post-translational recruitment domains of fission protein DRP-1. The DRP-1 promoter is composed of 4 regions; an N-terminal GTPase domain, a middle assembly domain, B variable domain and C-terminal GTPase effector domain (Engelfried et al.), with most modification sites predominantly found in the B variable region (domain) (Cho et al., 2013).
SUMOylation induction may be a regulating factor in mitochondrial division through controlling intracellular DRP-1 activity (Harder et al., 2004; Guo et al., 2013). In an ischaemia model, SUMOylation of proteins is of particular interest, with an increase in SUMOylation apparent in experimentally induced ischaemia (Nunez-O'mara and Berra, 2013). The three known human SUMO paralogues (SUMO1-3) are regulated via Ubc9 (E2 complex) and SNEPs (family of isopeptidases), both of which participate in SUMOylation (Anderson and Blackstone, 2013). Guo (2013) revealed that the de-SUMOylating enzyme SENP3 becomes inactive during conditions of ischaemia, an effect mediated via kinase PERK and protease cathepsin B. A decrease or inactivation of SENP3 extends the SUMOylation of mitochondrial fission protein DRP-1 and prolongs translocation to a recruitment protein on the MOM, thus delaying and/or inhibiting fission and the pro-apoptotic release of cytochrome c from the mitochondria. Upon reperfusion, an increase in SENP3 may initiate de-SUMOylation of DRP-1 and its recruitment to a receptor on the MOM. The caspase-mediated release of cytochrome c into the cytosol is also correlated to an increase in SENP3 activation upon reperfusion.

Phosphorylation of DRP-1 through kinase activation has also been suggested as a contributing mediator to DRP-1 regulation. There are currently two known phosphorylation sites on DRP-1: Ser616, phosphorylated by cdk1/cyclin B; and Ser637, phosphorylated by cAMP-dependent protein kinase (PKA) and calcium/calmodulin-dependent kinase I (CaMKI), and dephosphorylated though a calcineurin mediated path. Phosphorylation of DRP-1 (Ser616) is observed in HeLa cells undergoing mitosis, permitting mitochondrial division and translocation of mitochondria into adjoining daughter cells (Taguchi et al., 2007). Phosphorylation of DRP-1 (Ser616) is also implicated in nitric oxide (NO) induced stress, which induces recruitment of DRP-1 to the mitochondrial membrane to facilitate mitochondrial division (Bossy et al., 2010). The administration of a β-adrenergic agonist, or enforced exercise to activate PKA, increases DRP-1 phosphorylation (Ser656 in rat splice variant) in murine hearts (Cribbs and Strack, 2007). The dephosphorylation of DRP-1 (Ser637), via calcineurin regulated pathways, has been shown to increase mitochondrial division through translocation of DRP-1 to the outer mitochondrial membrane to induce fission (Cribbs and Strack, 2007; Cereghetti et al., 2008).
1.6 Mitochondrial dynamics in cardiovascular health and disease

Mitochondrial health is a critical determinant of myocardial functionality, and when the integrity of these dynamic organelles is compromised, cellular respiration is impaired, function modified, and apoptosis may result. Mitochondrial fission and fusion maintains an appropriate number of healthy and dynamic mitochondria, whilst also recycling those mitochondria that may compromise cellular function. The balance of mitochondrial fission/fusion may be disrupted in different disease states, with advanced age, and by stressors that include oxidative stress and myocardial ischaemia (both able to induce cardiac injury via necrosis or apoptosis).

Most sufferers of AMI are >65 years of age and may suffer worsened outcomes as a result of I-R injury. The process of ageing progressively affects the quantity and quality of functional mitochondria, thus significantly impacting metabolic processes and resistance to stress. A majority of the functional proteins involved in mitochondrial biogenesis deteriorate and become inefficient, and are correlated with the age-dependent onset of many pathophysiological conditions. Expression of MFN-2 and DRP-1 genes have been found to be reduced in aged skeletal muscle (Crane et al., 2010). It has also been found that PGC-1α, a key orchestrator of mitochondrial biogenesis and muscle tissue remodelling, is correlated to the regulation of MFN-2 expression, suggesting that mitochondrial morphology may be preserved in senescent cells and other pathological conditions with appropriate pharmacological intervention or exercise. Though advances have been made in understanding these dynamic mitochondrial fission and fusion processes, questions still remain as to how upstream signalling regulates these mechanisms. Characterising mitochondrial fission and fusion activity in age-related pathologies appears an important endeavour.

Heart failure and hypertrophy have also been linked to alterations in mitochondrial dynamics. Mitochondria have a reduced connectivity with other cellular organelles such as the cytoskeleton and sarcoplasmic reticulum, thus attributing to the overall degeneration of cardiomyocytes and alterations in mitochondrial dynamics in a failing heart model (Hom and Sheu, 2009; Gupta et al., 2010). The expression of mitochondrial fusion protein, OPA-1, was substantially reduced in a failing human and murine model with heart failure, with
less significant modifications reported in other key mitochondrial remodelling proteins; DRP-1, FIS1, MFN-1 and MFN-2.

Mitochondrial dysregulation has also been reported in obese, type II diabetes mellitus models (T2D). It has been proposed that alterations in the mitochondrial fusion proteins (OPA-1 and MFN-1 and MFN-2) may be linked to the metabolic changes associated with increased insulin-resistance in obese patients clinically diagnosed with T2D (Bach et al., 2005). Reduced expression of MFN-2 protein has been found in skeletal muscle of T2D subjects classified as clinically obese (Jheng et al., 2012). The MFN-2 protein may also be decreased in clinically obese male and female patients, and in patients with T2D (both lean and obese T2D patients) (Bach et al., 2005). The level of MFN-2 expression in the heart is also reportedly reduced in a diabetic cardiomyopathy rat model, with associated increases in caspase-3 activity (Guan et al., 2012). However, relatively little work has focussed on shifts in cardiac fission and fusion proteins in diabetes.

Alterations in mitochondrial function and dynamics may be crucial to the outcomes of CAD, and experimental studies suggest targeting these processes may be of clinical value in improving outcomes from resultant infarction. The compound, mitochondrial division inhibitor -1 (MDIVI-1), is a quinazolinone derivative that is reportedly selective in inhibiting the fission protein DRP-1 – and found to elicit cardioprotective effects in the ischaemic heart when applied as a preconditioning agent (Cassidy-Stone et al., 2008; Ong et al., 2010). Ong et al. (2010) applied MDIVI-1 in cardiac HL-1 cells subjected to prolonged simulated ischaemia and reperfusion and reported reduced cell death and delayed opening of the mPTP. An equivalent dosage (1.2 mg/kg IV) used in vivo in a murine model also induced similar benefit. These results (Ong et al., 2010) suggest that MDIVI-1 can elicit cardioprotective effects via inhibition of DRP-1. However, little evidence currently exists that characterises these mechanisms in a myocardial ischaemia model. In an attempt to extend the novel findings of Ong et al. (2010), and identify the mechanisms of DRP-1 modulation in settings of a I-R model, Sharp et al. (2014) confirmed that activation of DRP-1 resulted in acute augmentation of left ventricular dysfunction, and exacerbation of ROS production and Ca\(^{2+}\) accumulation following ischaemia. These data further suggest inhibition of DRP-1 dependent mitochondrial division may be of therapeutic benefit in I-R. Mitochondrial imaging additionally revealed that preservation of the mitochondrial network was achieved in isolated cardiomyocytes and Langendorff perfused hearts subjected to
DRP-1 inhibition with MDIVI-1, or treatment with the calcineurin inhibitor FK506 (an indirect inhibitor that acts by preventing dephosphorylation of DRP-1 at Ser637) (Sharp et al., 2014). Whether promotion of fusion itself might be of similar value is unclear. If the balance of fission/fusion is critical, and modified with I-R, benefit might be achieved by targeting different aspects of mitochondrial dynamics.

1.7 Summary

Establishing the precise molecular processes involved in mitochondrial dynamics in cardiac myopathies and lifestyle dependant diseases may aid in the development of mitochondrial targeted therapeutics. Basic and translational research that targets mitochondrial dynamics in the pathophysiology of CVD continues to evolve with the prospect of unravelling the complex nature of these biological processes. This doctoral thesis will assess the effects DRP-1 inhibition in a healthy and diseased myocardial model, and ascertain its effectiveness as a clinically relevant therapeutic that may improve myocardial outcomes and reduce cellular dysfunction.
Project Aims

Project Aim

Inhibition of the key regulator of mitochondrial fission, dynamin related protein-1 (DRP-1) may be a useful approach to cardioprotection in hearts subjected to ischaemia-reperfusion (I-R) injury, oxidative stress or other metabolic insult. This project aims to characterise the effects of DRP-1 inhibition on myocardial and mitochondrial responses to ischaemic, oxidative or metabolic stress, testing both the roles of DRP-1 and fission in governing myocardial phenotype and the potential clinical utility of DRP-1 inhibition to achieve cardioprotection.

STUDY 1: Examine the effects of DRP-1 inhibition (ex vivo) on post-ischaemic myocardial outcomes, mitochondrial function, fission and fusion proteins, and stress kinase signalling.

(i) Examine effects of the putative DRP-1 inhibitors MDIVI-1 and dynasore on myocardial responses to ischaemia-reperfusion

(ii) Test effects of MDIVI-1 and dynasore on expression and phosphorylation of the fission protein DRP-1 in normoxic and post-ischaemic hearts (early and late reperfusion)

(iii) Assess effects of MDIVI-1 on post-ischaemic mitochondrial function

(iv) Assess effects of MDIVI-1 on post-ischaemic survival kinase signalling

STUDIES 2 and 3: Test the effects of DRP-1 inhibition on cardiomyoblast responses to acute and chronic oxidative stress.

(i) Examine effects of MDIVI-1 on cardiomyoblast viability and growth during acute and chronic \( \text{H}_2\text{O}_2 \) exposure.

(ii) Examine effects of MDIVI-1 on mitochondrial function and membrane potential in cardiomyoblasts subjected to \( \text{H}_2\text{O}_2 \) exposure.
(iii) Assess the effects of DRP-1 inhibition on stress kinase signalling and expression of proteins governing apoptosis and autophagy in cardiomyoblasts subjected to H$_2$O$_2$ exposure.

**STUDIES 4 and 5: Test the effects of DRP-1 inhibition in *in vitro and in vivo* models of diabetes.**

(i) Examine the effects of DRP-1 inhibition on cardiomyoblasts viability/growth and respiratory function during hypo- or hyperglycaemia, and hyperinsulinaemia

(ii) Examine the effects of DRP-1 inhibition on fission and fusion protein expression in cardiomyoblasts exposed to hypo- or hyperglycaemia, and hyperinsulinaemia

(iii) Assess cardiac and systemic phenotypes in a murine model of T2D (STZ + high-fat diet feeding), and test for benefit via *in vivo* DRP-1 inhibition.

(iv) Examine effects of *in vivo* DRP-1 treatment on myocardial and mitochondrial responses to ischaemia-reperfusion in hearts from T2D mice.

(v) Assess the effects of DRP-1 inhibition on fission and fusion proteins in hearts from T2D mice.

(vi) Examine the impacts of DRP-1 inhibition on kinase signalling and expression of proteins governing apoptosis, and autophagy in hearts from T2D mice.
CHAPTER 2: Research Methods

2.0 Animals and ethics

All investigations described in this thesis were approved, in accordance with the policy guidelines "The Animal Care and Protection Act 2001", by the Animal Ethics Committee of Griffith University, which is accredited by the Queensland Government. All animal studies are performed in tissue from adult male C57B/6 mice acquired from ARC (Western Australia).

2.1 Cell culture studies (in vitro)

The cardiac cell line used (H9c2) was originally derived from an embryonic rat heart cardiomyoblast line, obtained from the American Type Culture Collection (ATCC; Manassas VA, USA). The cells were maintained and grown in a high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, under 95% O₂ and 5% CO₂ at 37°C. Cell used in all experiments were up to passage 15.

MDIVI-1 treatment (in vitro)

The compound MDIVI-1 was dissolved in dimethyl sulfoxide (DMSO) (<0.5%) and added to DMEM at a final concentration of 50 μM. Experiments were performed in cells incubated with MDIVI-1 or respective vehicle (DMSO <0.5%) for 40 min prior to an acute or sustained oxidative challenge of H₂O₂ at a concentration of 0.4 mM at varying time intervals between 5-60 min (further detailed in Chapter 4) or 1 hr, 2 hr, 4 hr, 6 hr and 8 hr for a sustained chronic challenge (further detailed in Chapter 5). Following oxidative insult (acute or chronic), cells were harvested and centrifuged for 5 min at 600g. This process was repeated and cells were washed with phosphate buffered saline (PBS) before 150 μL of ice-cold lysis buffer (70 mM sucrose, 190 mM mannitol, 20 mM HEPES and 0.2 mM EDTA, 1 mM PMSF, 10 MSF, 1peptin, 3 mM benzamidine, 5 μM pepstatin A and 1 mM NaO) was added. Cell lysates acquired were frozen in -80°C until further analysis.
Acute oxidative challenge via hydrogen peroxide (H$_2$O$_2$)

H9c2 cells were grown in T75 flasks to 70-80% confluence. The cell monolayer was washed in PBS before 1.5 mL of trypsin EDTA (0.25%) was added. Detached cells were resuspended in 5 mL of DMEM before a cell density was determined with a haemocytometer. Cells were then seeded at a density of 2.0x10$^4$ cells in a 100mm plate and left until 70-80% confluence. To assess the effects of oxidative stress, H9c2 cells were challenged to an acute oxidative insult of H$_2$O$_2$ (final concentrations of 0.1, 0.2, 0.4, 0.6, 0.8 or 1 mM) between varying time intervals between 5 - 60 min.

Sustained oxidative challenge via hydrogen peroxide (H$_2$O$_2$)

H9c2 cells were grown in T75 flasks to 70-80% confluence. The cell monolayer was washed in PBS before 1.5 mL of trypsin EDTA (0.25%) was added. Detached cells were resuspended in 5 mL of DMEM before a cell density was determined with a haemocytometer. Cells were then seeded at a density of 2.0x10$^4$ in a 100mm plate and left until 70-80% confluence was reached. To assess the effects of oxidative stress, H9c2 cells were challenged to a sustained oxidative insult of H$_2$O$_2$ (final concentrations 0.4 mM) for 1 hr, 2 hr, 4 hr, 6 hr and 8 hr (further detailed Chapter 5).

2.2 Cell death analyses

Cell viability via MTT assay

The viability of the H9c2 cells was determined using a colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) assay. The MTT tetrazolium dye is reduced to its insoluble formazan by mitochondrial NAD(P)H-dependent oxidoreductases (e.g. succinate dehydrogenase) in viable cells. The violet formazan crystals are solubilised by the addition of a detergent solution and their content assayed colorimetrically. After cells were subjected to control or 'stress' conditions (e.g. H$_2$O$_2$ challenge), MTT solution (200 μL of 5 mg/mL in DMEM) was added to each well followed by incubation in a dark room at 37°C for 2-4 hr. The solvent DMSO was used to dissolve the crystal formations on the plate surface, and absorbance was read at 570nm.
**Lactate dehydrogenase (LDH) cytotoxicity assay**

Cell toxicity was determined quantitatively by assessing the endogenous release of lactate dehydrogenase (LDH) with a colorimetric LDH assay kit (Abcam). As per manufacturers instructions, a 50 μL volume of each sample was transferred into a 96-well plate. Absorbance was measured kinetically in a microplate (Tecan infinite M200 Pro, Mannedorf, Switzerland) reader at 490 nm every 2 min for 1 hr. LDH released into culture medium was quantified to calculate the percentage of LDH release [medium LDH/(medium LDH + cellular LDH) × 100]. After experiments, cardiomyoblasts were lysed with the addition of a lysis buffer before measuring total LDH.

### 2.3 Mitochondrial functional analysis (*in vitro*)

**Assessment of mitochondrial membrane potential (Δψₘ)**

The fluorescent probe, Tetramethylrhodamine methyl ester perchlorate (TMRM), is an indicator of mitochondrial membrane potential. The lipophilic dye is cationic and accumulates in the mitochondria in proportion to the Δψₘ, which is mediated by binding to the inner mitochondrial membrane. Prior to an oxidative insult, cells were pre-treated with MDIVI-1 for 40 min at a final concentration of 50 μM. Cells were then challenged with H₂O₂ for either 1 hr or 4 hr prior to incubation with 100 μM TMRM and protected from light for 20 min at 37°C. Cells were harvested and resuspended in PBS and analysed by the FACS Caliber flow cytometer at an excitation wavelength of 549 nm and an emission wavelength of 575 nm. A cell count of 10,000 was acquired. Data was analysed using FlowJo software, with the mean fluorescent intensity values used to compare the levels of Δψₘ.

**Assessment of ATP production**

An assessment of ATP levels was performed using a luminescent assay (CellTiter-Glo® kit, Promega) according to the manufacturers instructions. In brief, cells were seeded at a density of 1.0 x 10⁴ cells/well in a clear bottom 96 well plate and left to attach overnight and grow to a 75% confluence in 100 μL of DMEM. Cells were pre treated with the pharmacological fission inhibitor MDIVI-1 (at concentration of 50 μM) for 40 min prior to an acute H₂O₂ insult (final concentration of 0.4 mM). An equal volume of the
luminescent reagent was added to each well and placed on an orbital shaker for 2 min to facilitate cell lysis and then left at room temperature for 10 min to stabilise. Luminescence was recorded using a microplate reader (Tecan infinite M200 Pro, Mannedorf, Switzerland). Raw data was analysed using Microsoft Excel, and results normalised to total protein concentration determined via a BCA assay.

Cellular proliferation via xCELLigence system analysis

The xCELLigence system (ACEA Biosciences Inc., San Diego, CA) provides real-time measurement of cell proliferation via changes in electrical impedance in purpose-built culture plates (E-plate) as cell density increases or decreases. The ‘cell index’ employed represents a linear scale for assessing relative changes in cell density. Cells were seeded on an E-plate at a density of 10,000 cells per well, and placed in the RTCA SP instrument located in the cell culture incubator. Cell impedance was measured every 15 minutes for the first 6 hours and then every 30 minutes for up to 48 hours. The initial steep phase of growth categorizes the attachment, followed by a plateau log growth phase. For cell treatment, the e-plate was removed after 18 hours (cell treatments detailed in experimental chapters) and immediately placed back in the RTCA instrument to continue cell impedance readings.

Measurements of cellular oxygen consumption and extracellular acidification via the Seahorse XF-p system

For Seahorse studies H9c2 cells were plated at a density of 1.0 x 10^4 cells per well in 8-well XFp miniplates in DMEM (supplemented with 10% FBS and Penstrep) and left to attach overnight in an incubator (37°C 5% CO₂ and 95% O₂). Cells were supplemented with modified DMEM media containing 25 mM glucose (control), low glucose (5 mM), high glucose (100 mM) or a combination of high glucose (100 mM) and insulin (100 nM) for 24 hours (37°C, 5% CO₂ and 95% O₂). Cells were then washed in PBS and supplemented with XFp modified media (1 mM Pyruvate, 2 mM glutamine, 10 mM glucose, pH 7.4) before incubation in a 37°C non-CO₂ incubator for 45 min prior to assay. A Seahorse XFp Extracellular Flux Analyser (Bioscience, Billerica, MA, USA) was used to measure O₂ consumption rate (OCR) and extracellular acidification rate (ECAR). Measurements were performed over 6 min in measurement cycles of 4 (basal metabolic
rate) or 2 (to assess maximal metabolic rate). After basal OCR was established, oligomycin (1 μM), FCCP (2 μM) and antimycin A (0.5 μM) were injected sequentially to assess OCR and ECAR in each well (Supplementary figure. 1). Measurements were normalised to a cell density of 1.0 x 10^4 cells per well.

2.4 Mitochondrial respiratory analysis (ex vivo)

Cardiac tissue preparation

Mitochondrial respiration was assessed in murine left ventricular myocardium that was shredded using a PBI-Shredder HRR-Set (Oroboros Instruments, Innsbruck, Austria). After undergoing experimentation on a Langendorff apparatus hearts were immediately suspended in cold Mir05 media (in mM): EDTA, 0.5; MgCl_2·6H_2O, 3; K-lactobionate, 60; taurine, 20; KH_2PO_4, 10; HEPES, 20; sucrose, 110; and 1 g BSA (pH to 7.1 at 30°C). Approximately 8-10 mg samples were sectioned from the heart and blot dried on filter paper prior to dissection into 3 or 4 smaller pieces with a scalpel. The heart tissue was then added to the lysis disk of the Shredder-Tube with 800 μL of Mir05 media prior to placement in the shedder apparatus. The lever was set at gear 1 for 10 sec followed by gear 2 for a further 10 sec. The resulting tissue homogenate was immediately transferred to an ice-cold tube. The Mir05 media was used to wash the heart tissue remnants off the Shredder-Tube, and the final volume of the homogenate was adjusted to a concentration between 2 mg/mL - 1 mg/mL (depending on ischaemic or normoxic protocols employed).

Mitochondrial oxygen consumption

Mitochondrial oxygen consumption of shredded ventricular tissue was quantified using an Oxygraph-2k instrument (Oroboros Instruments, Innsbruck, Austria). Chambers ‘A’ and ‘B’ of the apparatus were cleaned prior to use with 70% - 100% ethanol and milliQ water, and were stabilised with Mir05 media. Post stabilisation, heart homogenate was added to each 2 mL chamber (concentration of 1 mg/mL) with the addition of catalase (280 U/ml). Mitochondrial respiratory analysis was undertaken on all heart homogenate samples at 37°C. Analysis of mitochondrial respiration via complex I and complex II function was achieved upon addition of complex specific substrates and inhibitors (Details of the analysis in Fig. 2.1). As a quality control measure, cytochrome c was monitored to validate mitochondrial integrity, and mitochondrial complex inhibitors were used to determine the individual complex function.
Mitochondrial substrates: 5 mM pyruvate, 2 mM malate, 10 mM glutamate (complex I substrates), 10 mM succinate (complex II substrate), 0.01 mM cytochrome c (indicator of mitochondrial integrity), 1 mM ADP (stimulator of OXPHOS capacity). Mitochondrial uncoupler: 0.5 μM FCCP (mitochondrial uncoupler). Inhibitors of mitochondrial function: 0.5 μM rotenone (inhibitor of complex I), 5 mM malonic acid, 2.5 μM antimycin A (inhibitor of complex III).

Figure 2.1: Mitochondrial oxygen consumption in isolated mitochondria in response to complex I and II substrates and inhibitors. Mitochondrial substrates are added sequentially after closure of chambers ‘A’ and ‘B’. 5 mM pyruvate, 2 mM malate, 10 mM glutamate (complex I substrates); 10 mM succinate (complex II substrate); 0.01 mM cytochrome c (indicator of mitochondrial integrity); 1 mM ADP (stimulator of OxPhos capacity). Mitochondrial uncoupler: 0.5 μM FCCP (mitochondrial uncoupler). Inhibitors of mitochondrial function: 0.5 μM rotenone (inhibitor of complex I); 5 mM malonic acid; 2.5 μM antimycin A (inhibitor of complex III).
2.5 Langendorff heart perfusion studies

Hearts from male C57Bl/6 mice (9-12 weeks of age) were perfused on Langendorff apparatus to assess intrinsic cardiac responses to ischaemic insult (Fig. 2.2). Sodium pentobarbital (60 mg/kg i.p.) was used to anaesthetise mice prior to heart dissection. After a thoracotomy hearts were removed into ice-cold buffer and the aorta cannulated for Langendorff perfusion of the coronary circulation using a modified Krebs-Henseleit buffer (119 mM NaCl, 11 mM glucose, 22 mM NaHCO3, 4.7 mM KCl, 1.2 mM MgCl2, 1.2 mM KH2PO4, 1.2 mM EDTA, 0.5 mM and 2.5 mM CaCl2) bubbled with 95% O2/5% CO2 at 37°C (pH 7.4). Contractile function is examined by insertion of a fluid-filled balloon into the left ventricle, which is inflated to an end-diastolic pressure (EDP) of 5 mmHg during heart stabilisation. Coronary blood flow is measured via an ultrasonic flow-probe in the aortic perfusion line, connected to a T206 flow meter (Transonic Systems Inc., Ithaca, NY, USA). A pacing wire, attached to an SD9 stimulator (Grass Instruments, Quincy, MA), is attached to the outer surface of the ventricular cavity for ventricular pacing at approximately 420 beats per minute. A 1-KHz 8 channel MacLab system (ADInstruments Pty Ltd., Castle Hill, Australia) connected to an Apple iMac computer was used to continuously monitor functional data, including: peak systolic and end diastolic pressure, heart rate, flow and + and -dP/dt. Temperature of the perfusate is monitored via a thermal probe connected to a 3-channel Physitemp TH-8 digital thermometer (Physitemp Instruments Inc, Clifton, NJ, USA) A 20 min equilibration period is used to confirm the initial functional stability of the hearts (Peart et al, 2011; Reichelt et al, 2009).

Hearts must have a coronary flow rate of ≤5 mL/min, stable contractility (no detection of cardiac arrhythmias) and a ventricular systolic pressure ≥100 mmHg, otherwise they are excluded from the study. Once stabilised, hearts were switched to ventricular pacing at 420 beats per min for a period of 10 min prior to induction of ischaemia. Drug infusions are administered via the aortic cannula at 1% of coronary flow rate using a peristaltic infusion pump (Pump 22, Harvard Instruments) for relevant periods of time according to experimental protocols. A 25 min period of global normothermic ischaemia was then initiated, followed by 10 or 45 min of aerobic reperfusion (± drug infusion).
Figure 2.2: Langendorff apparatus for isolated hearts. Contractile function is monitored by the insertion of an intraventricular balloon, which upon inflation with fluid generates a left ventricular end-diastolic pressure (LVEDP) of 5 mmHg during stabilization of the heart. Coronary flow is measured via the insertion of an ultrasonic flow-probe, which is connected to a T206 flow meter positioned proximally to the aortic cannula. A pacer wire is inserted on the surface laterally to the ventricle, which stimulates the pacing of the ventricles (Reichelt et al., 2009).

2.6 Coronary effluent lactate dehydrogenase (LDH) assay

Assessment of cell membrane integrity is the most common method of evaluating cell viability or cytotoxicity. The measurement of the LDH is an enzymatic biomarker of cell death in response to ischaemia. For analysis, the rate limiting enzymatic reaction of pyruvate and β-NADH to lactate and β-NAD is monitored spectrophotometrically. The enzymatic reaction produces a decrease in NADH absorbance at 340 nm (at 37°C pH 7.5,
with a 1 cm light path). Change in absorbance is measured over 3 min and enzyme activity expressed in IU/mL/g tissue.

2.7 Western blot analysis of proteins

*Mitochondrial protein extraction*

Protein isolation was performed on whole frozen mouse hearts (~125 mg). Hearts were sectioned into pieces prior to homogenisation in a glass dounce with 1.0 mL of ice-cold isolation buffer containing protease and phosphatase inhibitors: 70 mM sucrose, 190 mM mannitol, 20 mM HEPES and 0.2 mM EDTA, 1 mM PMSF, 10 μM leupeptin, 3 mM benzamidine, 5 μM pepstatin A and 1 mM NaO. Whole homogenate samples were removed and stored with lysis buffer for future analysis. The protein homogenate underwent centrifugation at 600 g (rcf) for 10 min at 4°C. The supernatant was removed and the nuclear pellet obtained was washed in isolation buffer containing protease and phosphatase inhibitors and spun at 600g for 10 min before being resuspended in lysis buffer and stored. The supernatant obtained (containing mitochondria, cytosolic and plasma membrane) was centrifuged at 10,000 g for 30 min. This mitochondria containing pellet was washed in isolation buffer at 600g for 10 min before being re-suspended in lysis buffer and stored at -80°C. The supernatant obtained (cytosol and plasma membrane) was stored at -80°C for future analysis.

2.8 Western immunoblotting

*BCA protein determination*

Fractionated lysates were diluted to an approximate concentration of 1 μg protein per uL (dilutions used; 1:20 for whole homogenate, 1:5 for cytosolic and mitochondrial). Protein concentrations were measured via a BCA assay in a 96-well microplate (Pierce BCA protein assay kit), with absorbance monitored at 540 nm (Tecan infinite M200 Pro, Mannedorf, Switzerland). Aliquots of 20 μg/uL of protein were prepared with appropriate volumes of kinexus buffer containing: 20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 30 mM NaF, 40 mM β-glycerophosphate, 20 mM NaPP, and stored in -80°C.
**Electrophoresis**

Once thawed, samples were prepared in the required volumes with loading dye and denatured at 95°C for 5 min in a heating block. A 28 μL volume of each sample was loaded onto a hand-cast 10% acrylamide gel. Protein separation was achieved by running the gel at a 150 V for 80 min. The transfer of proteins is achieved using a polyvinylidene difluoride fluorescent membrane at a constant amp level of 350 mA for approximately 2 hr and blocked with Odyssey fish serum for an additional 2 hours at room temperature. The transferred proteins were incubated with primary antibody for approximately 15-18 hours overnight in 4°C with gentle rocking. The PVDF membrane was washed in tris-buffered saline (TBS) for 5 min and again washed in TBST for 4 cycles of 5 min before incubation with the corresponding secondary antibody at room temperature in the dark. Membranes were visualised on a Licor Odyssey Infrared Imaging System (Millennium Science, Mulgrave, Australia) and protein densitometry of each sample normalised to an internal standard and a loading control. The loading control used for cytoplasmic and whole cell lysate samples was glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a highly conserved protein across varying species. The loading control used for mitochondrial samples was cytochrome c oxidase (COXIV) located within the inner mitochondrial membrane. Both loading controls were utilised throughout all experimental chapters (list of antibodies detailed in Table 2.1).
<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Animal Source</th>
<th>Supplier</th>
<th>Dilution</th>
<th>Catalogue Number</th>
<th>Corresponding Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFN-1</td>
<td>Mouse</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>ab57602</td>
<td>Anti-Mouse. 680 at 1:30000</td>
</tr>
<tr>
<td>MFN-2</td>
<td>Mouse</td>
<td>abcam</td>
<td>1:1000</td>
<td>ab56889</td>
<td>Anti-Mouse. 680 at 1:30000</td>
</tr>
<tr>
<td>OPA-1</td>
<td>Rabbit</td>
<td>abcam</td>
<td>1:1000</td>
<td>ab42364</td>
<td>Anti-Rabbit. 800 at 1:30000</td>
</tr>
<tr>
<td>DRP-1</td>
<td>Mouse</td>
<td>abcam</td>
<td>1:750</td>
<td>Ab56788</td>
<td>Anti-Mouse. 680 at 1:30000</td>
</tr>
<tr>
<td>Phosphorylated DRP-1 (Ser637)</td>
<td>Rabbit</td>
<td>Cell signalling</td>
<td>1:750</td>
<td>4867</td>
<td>Anti-Rabbit. 800 at 1:30000</td>
</tr>
<tr>
<td>Phosphorylated AKT (Ser473)</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>9271S</td>
<td>Anti-Rabbit. 800 at 1:30000</td>
</tr>
<tr>
<td>Total AKT</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>9272S</td>
<td>Anti-Rabbit. 800 at 1:30000</td>
</tr>
<tr>
<td>Phosphorylated ERK-1/2</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>4094S</td>
<td>Anti-Rabbit. 800 at 1:30000</td>
</tr>
<tr>
<td>Total ERK-1/2</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>9102S</td>
<td>Anti-Rabbit. 800 at 1:30000</td>
</tr>
<tr>
<td>Phosphorylated GSK3β (Ser9)</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>9336S</td>
<td>Anti-Rabbit. 800 at 1:30000</td>
</tr>
<tr>
<td>Total GSK3β</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>9315S</td>
<td>Anti-Rabbit. 800 at 1:30000</td>
</tr>
<tr>
<td>BAX</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>2772S</td>
<td>Anti-Rabbit. 800 at 1:30000</td>
</tr>
<tr>
<td>BCL-2</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>1:500</td>
<td>2876S</td>
<td>Anti-Rabbit. 800 at 1:30000</td>
</tr>
<tr>
<td>PARP</td>
<td>Rabbit</td>
<td>Cell signalling</td>
<td>1:1000</td>
<td>9542</td>
<td>Anti-Rabbit. 800 at 1:30000</td>
</tr>
<tr>
<td>PARKIN</td>
<td>Rabbit</td>
<td>Cell signalling</td>
<td>1:1000</td>
<td>2132</td>
<td>Anti-Rabbit. 800 at 1:30000</td>
</tr>
<tr>
<td>LC3BI/II</td>
<td>Rabbit</td>
<td>Cell signalling</td>
<td>1:1000</td>
<td>2775</td>
<td>Anti-Rabbit. 800 at 1:30000</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Mouse</td>
<td>Santa Cruz</td>
<td>1:5000</td>
<td>sc32233</td>
<td>Anti-Mouse. 680 at 1:30000</td>
</tr>
<tr>
<td>COX IV</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>4844S</td>
<td>Anti-Rabbit. 800 at 1:30000</td>
</tr>
</tbody>
</table>
2.9 Animal studies: type II diabetes mellitus

**STZ administration and high-fat feeding protocol**

A combination of a single dose of streptozotocin (STZ) (75mg/kg in 0.1 M citrate buffer, pH 4.5, Sigma, St. Louis, MO) and a high-fat obesogenic diet (Wensley *et al.*, 2013) was used to induce a T2D murine model for approximately 14 weeks. A purpose made high-fat chow was prepared freshly each week (stored at 4°C) consisting of: 800 g Irradiated Rat & Mouse Powder (*Specialty Feeds*), 300 g condensed milk, 140 g sugar and 125 g blended animal fat (*Supafry*) (nutrient breakdown detailed in Supplementary Table 1). Chow was warmed to soften before being fed to mice daily. Control mice received a placebo injection of citrate buffer (0.1 M, pH 4.5, Sigma, St Louise, MO) at the commencement of the study in combination with standard mouse pellet chow diet.

**Glucose tolerance tests (glucose handling)**

Glucose handling was assessed via a glucose tolerance test (GTT). In order to attain fasting blood glucose concentration, mice were subject to fasting for 11 hours (overnight). A blood sample was obtained via a parallel tail prick, while an Accu-check II glucometer (Roche Diagnostics, Castle Hill, Australia) was used to attain fasting blood glucose. An i.p injection of 20% glucose (2g/kg) was administration and blood glucose was evaluated after 30, 60, 90, 120 and 180 min. Area under the curve (AUC) was calculated in order to compare glucose handling between control and T2D groups. Glucose tolerance tests were performed on mice in week 6 and week 12 of the study. A subgroup of mice received a GTT in week 13, after 4 days of vehicle or MDIVI-1 administration.

**MDIVI-1 administration (in vivo)**

Dimethyl sulfoxide (DMSO) (0.5%) was used to dissolve MDIVI-1. Mice were administered a daily i.p injection of MDIVI-1 (5 mg/kg) or vehicle (DMSO) in week 13 of the study for 7 consecutive days. Administration of MDIVI-1 or vehicle was given after completion of GTT in mice receiving a GTT during the drug administration period. Mice were sacrificed via an i.p injection of sodium pentobarbital (60 mg/kg) one day following
cessation of the treatment regime.

**Blood collection and heart tissue dissection**

Prior to Langendorff perfusion, blood was collected, via a cardiac puncture (from hearts not undergoing perfusion studies) or from the chest cavity during heart excision. Blood lipid profiles were analysed using the Alere Cholestech LDX® system, and blood was stored in heparinized tubes prior to centrifugation at 10,000 g for 5 min to obtain serum. Serum was stored at -80°C until required for analysis. Non-fasted blood glucose was acquired via a blood sample obtained via a tail snip, using a Roche Accu-Check glucometer. Following removal from chest, excised hearts were placed on frozen PBS bricks for Langendorff apparatus. The right ventricle and aorta were removed and the remaining left ventricle was dissected consistently for all hearts. Hearts not subject to Langendorff perfusion had a mid-sagittal cut made, with one half immediately snap-frozen for protein analysis and the other half used for mitochondrial respiration analysis.

**Serum Insulin - Enzyme-Linked Immunosorbent Assay (ELISA)**

Serum insulin was measured using an ultra sensitive mouse ELISA kit purchased from Crystal Chem (IL, USA), according to manufacturer’s instructions:

*Wash buffer:* was prepared by diluting 25 mL of wash buffer concentrate (20x) in 475 mL distilled water. Standard Solutions: Vials of standard were briefly centrifuged and reconstituting in 1 mL of appropriate diluent. Standards were mixed well with inversion and allowed to dissolve for 15 min. A series of 1-in-2 serial dilutions was then performed, generating a minimum of 7 standard concentrations. *Detection Antibody:* Vials were centrifuged prior to use and diluted with the appropriate diluent to the appropriate concentration. *Streptavidin-HRP conjugate:* Vials were briefly centrifuged prior to use and diluted 500-fold using appropriate diluent.

*Analytical Protocol:* Strip-well plates, pre-coated with mouse antibodies for insulin, were loaded with serum samples before sequential addition and incubation with the following reagents; biotin labelled detection antibody, streptavidin-HRP conjugate and 3,3’,5,5’-Tetramethylbenzidine (TMB) substrate (20 min incubation in dark). Plates were washed 4 x with wash buffer between incubations. A stop solution was applied after colour development with optical density determined using a microplate reader (Tecan infinite
M200 Pro, Mannedorf, Switzerland) at 450 nm with a wavelength correction for 570 nm. Insulin concentration was calculated from a standard insulin curve.

2.10 Statistical analyses

Unless otherwise stated differences between two or more groups were tested via one- or two-way ANOVA, with a Newman-Keuls post-hoc test applied when significant effects were detected. Significant differences were accepted for $P<0.05$. All tests were performed with Prism 6 (GraphPad Software Inc., La Jolla, CA).
CHAPTER 3: Impact of DRP-1 Inhibition on Myocardial Ischaemic Tolerance, Mitochondrial Respiration and Stress Signalling

3.0 ABSTRACT

Mitochondrial fission, regulated by proteins including dynamin-related protein-1 (DRP-1), may be a key determinant of myocardial resistance to ischaemia-reperfusion (I-R) and other insults. The current study assessed the impact of I-R with and without DRP-1 inhibition (MDIVI-1 and dynasore hydrate) on myocardial expression of DRP-1, mitochondrial respiratory function, and tissue injury in Langendorff perfused hearts from male C57BI/6 mice. Untreated hearts subjected to 25 min ischaemia/45 min reperfusion exhibited significant cell death and contractile dysfunction (~40 U/L LDH efflux, ~42% recovery of LVDP, ~40 mmHg EDP). Pre-treatment with 1 µM MDIVI-1 significantly reduced contractile and diastolic dysfunction (~55% recovery of LVDP, 30 mmHg EDP) with no change in cell death (37 U/L LDH), whereas a higher 5 µM concentration reduced cell death (LDH efflux) without altering function. These protective effects were not replicated by the inhibitor dynasore hydrate (1 µM). Cardiac mitochondrial O\textsubscript{2} consumption was impaired in early reperfusion, with a ~50% fall in complex I activity that was effectively reversed by MDIVI-1 treatment. These data suggest that MDIVI-1 may elicit cardioprotective benefit in post-ischaemic hearts. Data from western blot analysis of proteins suggest that pre-treatment with MDIVI-1 significantly reduces mitochondrial DRP-1 expression, which may in turn maintain mitochondrial function and limit injury processes in hearts subject to ischaemia-reperfusion.
3.1 INTRODUCTION

Mitochondria are highly dynamic organelles constantly undergoing cycles of fusion and fission, a physiological process that is necessary in maintaining a healthy population of dynamic organelles. Mitochondrial elongation, termed fusion, is facilitated by a family of GTPase dynamin proteins, including mitofusin 1 (MFN-1), mitofusin 2 (MFN-2), and optic atrophy-1 (OPA-1). Mitochondrial fragmentation (fission) is modulated by proteins; dynamin-related protein (DRP-1) and fission 1 homologue protein (FIS1). Stressors including myocardial oxidative stress and ischaemia-reperfusion can compromise this balance, limiting the number of healthy mitochondria and potentially contributing to the initiation and progression of heart disease. Modulating the mitochondrial mediators that alter mitochondrial morphology therefore has the potential to protect the heart from subsequent myocardial injury and dysfunction.

The selective mitochondrial fission inhibitor MDIVI-1 (molecular inhibitor of the fission protein DRP-1) has been found to elicit a cardioprotective effect in the ischaemic heart when applied as a preconditioning agent (Cassidy-Stone et al., 2008; Ong et al., 2010). Sharp et al. (2014) report that activation of DRP-1 results in an acute increase in left ventricular dysfunction, and exacerbates production of ROS and intracellular Ca^{2+} accumulation following ischaemia. Furthermore, mitochondrial imaging revealed that preservation of the mitochondrial network was achieved in isolated cardiomyocytes and Langendorff-perfused hearts subjected to DRP-1 inhibition with MDIVI-1. The calcineurin inhibitor FK506, an indirect inhibitor that acts by preventing the dephosphorylation of DRP-1 (ser637), induced similar effects. There is also evidence DRP-1 inhibition or knockdown can promote cell death during I-R in cardiac (Dong et al., 2016) and other tissues (Zhang et al., 2013; Kumar et al., 2016). Mitochondrial dynamism is not a feature of healthy cardiomyocytes, which do not exhibit mitochondrial networks or dynamic fission/fusion (Song and Dorn, 2015). Rather, small fragmented mitochondria are dense in cardiomyocytes, providing the highest respiratory capacities among mammalian cells (Song and Dorn, 2015). In this study, we therefore investigate whether the mitochondrial fission protein DRP-1 is modified with I-R, and the impact of DRP-1 inhibition on myocardial I-R resistance, survival kinase signalling and mitochondrial respiration.
3.2 MATERIALS AND METHODS

3.2.1 Animals

All investigations were approved in accordance with Animal Ethics Committee of Griffith University, under the guidelines of "The Animal Care and Protection Act 2001, section 757", which is accredited by the Queensland Government, Department of Primary Industries and Fisheries (AEC number MSC/05/13 licensed to Jason N. Peart). Experiments were performed on young (9-12 week) male C57Bl/6 mice.

3.2.2 Chemicals

Mitochondrial division inhibitor-1 (MDIVI-1) and dynasore hydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.2.3 Experimental groups and heart perfusions

Mice were anaesthetised with sodium pentobarbital (60 mg/kg i.p.) and hearts excised via thoracotomy, prior to being Langendorff-perfused as detailed in section 2.5 (Peart et al, 2011; Reichelt et al, 2009). Drug infusions were administered at 1% of coronary flow using a syringe infusion pump (Pump 22, Harvard Instruments) for 10 or 20 min prior to 25 min global normothermic ischaemia and/or throughout reperfusion (either 10 or 45 min aerobic reperfusion; Fig. 3.1). Coronary effluent was collected on ice during reperfusion, in order to assess enzymatic activity of LDH (further detailed in Methods chapter, section 2.6). The following exclusion criteria was applied: i) coronary flow >5 ml/min, ii) unstable (fluctuating) contractile function, iii) left ventricular systolic pressure <100 mmHg, or iv) significant cardiac arrhythmias.
Figure 3.1: Schematic of experimental ischaemia-reperfusion protocol. All experiments were conducted on Langendorff perfused hearts from male C57Bl/6 mice. This study examined the effect of DRP inhibition via two methods; administered pre-ischaemia and throughout reperfusion and administered pre-ischaemia only. **I-R protocol I:** Hearts were infused for 10 min pre-ischaemia and throughout 45 min of reperfusion under the following conditions; control (DMSO), 1 µM dynasore hydrate, 1 µM MDIVI-1 and 5 µM MDIVI-1. **I-R protocol II:** Hearts were infused for 20 min pre-ischaemia and reperfused for 10 mins under the following conditions; control (DMSO), 1 µM MDIVI-1.

### 3.2.4 Western blot analysis of proteins and mitochondrial respiratory analyses

At the end of experimentation hearts were subjected to extraction procedures (as detailed further in Chapter 2) with tissue fractions assessed for expression of total and phosphorylated AKT and ERK1/2 and DRP-1 via immunoblot as detailed in section 2.8 (Peart et al., 2011). For mitochondrial analyses, myocardial tissue was sampled after 10 min of post-ischaemic reperfusion, shredded and assessed for O2 consumption in an Oxygraph-2k instrument (Oroboros Instruments, Innsbruck, Austria). Detailed in section 2.4.

### 3.2.5 Statistical analysis

For post-ischaemic outcomes and cell death analysis, all data are expressed as means ± SEM. Differences between two or more groups were tested via one- or two-way ANOVA, with a Newman-Keuls post-hoc test applied when significant effects were detected. Significant differences were accepted for \( P<0.05 \). All tests were performed with Prism 6 (GraphPad Software Inc., La Jolla, CA).
3.3 RESULTS

3.3.1 DRP-1 inhibition and baseline function

Baseline contractile function and coronary flow were not modified by treatment with either 1 µM or 5 µM MDIVI-1 (P>0.05) (Table 3.1). Similarly, 1 µM dynasore failed to significantly modify cardiovascular function in perfused hearts.

Table 3.1 Function of ex vivo hearts prior to and post infusion of DRP-1 inhibitor

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Timing</th>
<th>EDP (mmHg)</th>
<th>ESP (mmHg)</th>
<th>LVDP (mmHg)</th>
<th>Flow (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTRL</td>
<td>Pre</td>
<td>5 ± 1</td>
<td>134 ± 3</td>
<td>118 ± 16</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>5 ± 1</td>
<td>132 ± 4</td>
<td>116 ± 16</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>DYNASORE</td>
<td>Pre</td>
<td>4 ± 1</td>
<td>135 ± 6</td>
<td>131 ± 6</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>HYDRATE</td>
<td>Post</td>
<td>4 ± 1</td>
<td>133 ± 7</td>
<td>129 ± 7</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
<td>MDIVI-1 [1 µM]</td>
<td>Pre</td>
<td>5 ± 1</td>
<td>122 ± 6</td>
<td>117 ± 6</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>6 ± 1</td>
<td>120 ± 6</td>
<td>114 ± 7</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>MDIVI-1 [5 µM]</td>
<td>Pre</td>
<td>7 ± 1</td>
<td>144 ± 8</td>
<td>124 ± 9</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>6 ± 1</td>
<td>137 ± 8</td>
<td>120 ± 10</td>
<td>2.9 ± 0.2</td>
</tr>
</tbody>
</table>

3.3.2 The effects of DRP-1 inhibition on ischaemic contracture

Ischaemic contracture, defined as an increase of at least 20 mmHg above the resting pre-ischaemic pressure, was evident at ~270 sec in control (untreated) hearts, with a peak diastolic pressure of ~80 mmHg (Fig. 3.2). Treatment with 1 µM MDIVI-1 or dynasore failed to influence contracture, while 5 µM MDIVI-1 (10 min pre-ischaemia) significantly suppressed contracture. Specifically, 5 µM MDIV-1 prolonged times to onset of contracture (~345 sec) and peak contracture (~600 sec), and also reduced the magnitude of peak contracture (to ~60 mmHg).
Figure 3.2: Impact of 10 min pre-ischaemic treatment with dynasore hydrate or MDIVI-1 on ischaemic contracture development. Hearts were untreated (CTRL) or treated with dynasore hydrate (DYN) or MDIVI-1 for 10 min prior to a 25 min ischaemic insult. Effects on rate and extent of ischaemic contracture development were assessed. A) Time to ischaemic contracture; B) time to peak ischaemic contracture; and C) peak ischaemic contracture. Data are means ± SEM (n = 6-8/group). *, P<0.05 vs. CTRL.
3.3.3 Post-ischaemic functional outcomes in hearts treated with a DRP-1 inhibitor pre and post-ischaemia.

Left ventricular developed pressure (LVDP) gradually recovered to ~40% of pre-ischaemic levels during 45 min of reperfusion in untreated hearts (Fig. 3.3). Treatment with 1 µM MDIVI-1 significantly improved the final post-ischaemic recoveries of LVDP (Fig. 3.4A), and systolic and diastolic pressures (Fig. 3.4B). In contrast, 5 µM MDIVI-1 appeared to modestly worsen recovery of LVDP throughout 45 min of reperfusion, while 1 µM dynasore hydrate was without effect (Fig. 3.3). Post-ischaemic diastolic dysfunction was unaltered with 5 µM MDIVI-1 or 1 µM dynasore hydrate, while systolic function appeared to be modestly impaired by treatment with 5 µM MDIVI-1 (dynasore without effect) (Fig. 3.4C).
Figure 3.3: Post-ischaemic recovery of LVDP in hearts treated with MDIVI-1 and dynasore hydrate pre and post-ischaemia. Recovery of LVDP is shown for untreated hearts and hearts treated with the fission inhibitors dynasore hydrate (DYN; 1 μM) or MDIVI-1 (1 and 5 μM) for 10 min pre-ischaemia and throughout 45 min reperfusion. Data are means ± SEM (n = 6-8/group). *, P<0.05; ***, P<0.001 CTRL vs. 5 μM MDIVI-1; φ, P<0.0001 5 μM MDIVI-1 vs. 1 μM MDIVI-1.
Figure 3.4: Post-ischaemic outcomes in hearts treated with MDIVI-1 and dynasore hydrate pre- and post-ischaemia. Inhibitors of DRP-1 were administered for 10 min prior to a 25 min ischaemic insult and throughout 45 min reperfusion. Shown are post-ischaemic recoveries after 45 min reperfusion for: A) left ventricular developed pressure; B) left ventricular systolic pressure; and C) left ventricular end-diastolic pressure. Data are means ± SEM (n = 6-8/group). *, P<0.05; **, P<0.01; ***, P<0.001 vs. CTRL; ϕ, P<0.0001 vs. MDIVI-1 [1 µM].
3.3.4 The effects of DRP-1 inhibition on post-ischaemic LDH release in early and late reperfused hearts

Efflux of LDH was employed as a biomarker of cell death. Post-ischaemic LDH efflux was significantly reduced in hearts treated with 5 µM but not 1 µM MDIVI-1 (Fig 3.5). Conversely, and unexpectedly, LDH efflux was significantly elevated in hearts treated with 1 µM dynasore hydrate. Data in Figure 3.6 depict effects of a longer (20 min) pre-treatment with 1 µM MDIVI-1 on both baseline efflux and early efflux after 10 min of post-ischaemic reperfusion. Efflux of LDH efflux was significantly attenuated in hearts administered with MDIVI-1 at a dose of 1 µM following 10 min reperfusion vs. respective controls. Efflux of LDH was unaltered in normoxic hearts treated with MDIVI-1 (Fig. 3.6)

![Figure 3.5: Late post-ischaemic LDH efflux in MDIVI-1 and dynasore treated hearts.](image)

Lactate dehydrogenase (LDH) efflux was assessed in hearts treated with dynasore hydrate or MDIVI-1 for 10 min prior to 25 min ischaemia, and throughout 45 min reperfusion. Data are means ± SEM (n = 6-8/group). **, P<0.01, ***, P<0.001 vs. CTRL; ϕ, P<0.001 vs. MDIVI-1 [1 µM].
Figure 3.6: Early LDH efflux in hearts treated with MDIVI-1 for 20 min pre-ischaemia. Lactate dehydrogenase (LDH) efflux in hearts treated with 1 µM MDIVI-1 for 20 min prior to 25 min ischaemic insult and 10 min reperfusion. Data are means ± SEM (n = 6-8/group). **, P<0.01 vs. baseline CTRL; ϕ, P<0.05 vs. CTRL (10 min reperfusion).

3.3.5 DRP-1 and survival kinase (ERK1/2, AKT) activation in hearts treated with MDIVI-1 and dynasore hydrate

Expression of DRP-1 was assessed in cytosolic and mitochondrial compartments from post-ischaemic myocardium (25 min ischaemia and 45 min of reperfusion) treated with either dynasore hydrate or MDIVI-1 pre- and post ischaemia. Data suggest DRP-1 inhibition with dynasore hydrate significantly reduces DRP-1 expression within the cytosolic compartment (vs. untreated control) without modifying DRP-1 in the mitochondrial fraction. Conversely, DRP-1 inhibition with MDIVI-1 reduced mitochondrial but not cytosolic DRP-1 expression (Fig. 3.7). Treatment with MDIVI-1 for 20 min pre-ischaemia significantly reduced cytosolic levels of phospho-ERK1/2 and the ratio of phospho: total ERK1/2, whereas mitochondrial expression remained largely unaltered between groups (Fig. 3.8). Post-ischaemic phospho-AKT was unaltered with MDIVI-1 treatment.
Figure 3.7: DRP-1 expression in post-ischaemic hearts treated with MDIVI-1 (1 µM) and dynasore hydrate (1 µM) pre- and post-ischaemia. A) Relative expression of DRP-1 in mitochondrial; and B) cytosolic fractions from post-ischaemic hearts, either untreated (CTRL) or pre-treated with dynasore hydrate or 1 µM MDIVI-1 for 10 min pre-ischaemia and throughout 45 min reperfusion. Data are means ± SEM (n = 6-8/group). *, P<0.05 vs. CTRL; ϕ, P<0.05 vs. DYN.
Figure 3.8 The effects of a DRP-1 inhibitor (MDIVI-1) administered pre-ischaemia on survival kinase expression and phosphorylation following 10 min reperfusion. Mitochondrial and cytosolic fractions from post-ischaemic left ventricular tissue were assessed for total and phosphorylated AKT and ERK1/2 (mitochondrial data normalised to COX IV; cytosolic to GAPDH). Hearts were untreated (CTRL) or treated with 1 µM MDIVI-1 for 10 min prior to 25 min global ischaemia and 10 min reperfusion. Shown is expression of: A) total ERK1/2; B) phosphorylated ERK1/2; C) ratio of phosphorylated ERK1/2: total ERK1/2; D) total AKT; E) phosphorylated AKT; and F) ratio of phosphorylated AKT: total AKT. Data are means ± SEM (n = 6-8/group). *, P<0.05; **, P<0.01 vs. cytosolic CTRL; φ, P<0.05 vs. mitochondrial MDIVI-1; #, P<0.01 vs. mitochondrial CTRL. (Representative blots detailed in supplementary figure 2).
3.3.6 Mitochondrial respiratory function in early and late reperfused hearts treated with the DRP-1 inhibitor, MDIVI-1, pre-ischaemia

Substrate-dependent mitochondrial respiration was assessed in shredded myocardium from hearts subjected to normoxic perfusion, or 25 min ischaemia and either 10 or 45 min reperfusion (untreated or pre-treated with 1 µM of the DRP-1 inhibitor MDIVI-1) (Fig. 3.9). Treatment with MDIVI-1 did not significantly alter O₂ fluxes under either normoxic or post-ischaemic conditions. However, data suggest MDIVI-1 may mediate an inhibitory effect, evidenced by a reduction in O₂ flux after the addition of cytochrome c, a measurement of mitochondrial integrity (Fig. 3.9 C-F). A progressive post-ischaemic reduction in complex I oxidative phosphorylation capacity was evident between 10 and 45 min of reperfusion (Fig. 3.9B). Despite no significant effects of MDIVI-1, some trends were apparent (P>0.14): MDIVI-1 tended to lower complex I and II-linked electron transport system capacity under normoxic conditions (Fig. 3.9D), an effect mimicked by ischaemia-reperfusion (Fig. 3.9E). The MDIVI-1 treated hearts reperfused for 45 min exhibited improved oxidative phosphorylation capacity via complex I (by ~36%) vs. untreated I-R control hearts.

The respiratory O₂ fluxes via complex I and complex II (CI and CII) (Fig. 3.10A and Fig. 3.10B) were also assessed in permeabilised cardiac fibres from normoxic hearts, and 10 min and 45 min reperfused hearts (± MDIVI-1 treatment). The O₂ flux characterising complex I respiration was unaltered by MDIVI-1 in normoxic tissue. Post-ischaemic changes included a trend to reduced CI activity, though this only achieved significance at 45 min reperfusion. However, MDIVI-1 did exaggerate the decline in respiration at 10 min reperfusion, which differed significantly from that in normoxic MDIVI-1 treated tissue. No changes were evident in complex II activity with ischaemia or MDIVI-1 (despite a slight reduction in post-ischaemic tissue).

The flux control ratios, specific to complex I and complex II respiration, were assessed via addition of the uncoupling agent FCCP to stimulate maximal O₂ uptake. The flux control ratios in normoxic hearts were unaltered by MDIVI-1 (Fig 3.11). However, the flux control ratio for complex I activity was significantly reduced at both early and late reperfusion, and this was significantly countered by MDIVI-1 treatment (P<0.05). Conversely, the flux control ratio for complex II appeared to increase proportionally.
(P<0.05) in both early and late reperfused CTRL hearts vs. normoxic CTRL hearts. Treatment with MDIVI-1 did not alter the flux control ratio for complex II in normoxic and early reperfused heart (vs. time matched control), however significantly reduced following 45 min reperfusion.
Figure 3.9: Mitochondrial oxygen consumption in early and late reperfused hearts treated with the DRP-1 inhibitor, MDIVI-1, pre-ischaemia. Hearts were untreated (CTRL) or pre-treated with 1 µM MDIVI-1. Specific functions were assessed as follows: A) Complex I - leak state (addition of 5 mM pyruvate + 2 mM malate + 10 mM Glutamate); B) Complex I - oxidative phosphorylation capacity (addition of 5 mM ADP); C) cytochrome c flux control factor (FCFc), an assessment of mitochondrial integrity (addition of 10 µM cytochrome c); D) Complex I and Complex II oxidative phosphorylation capacity (addition of 10 mM succinate + 5 mM ADP); E) Complex I and Complex II electron transfer system capacity (addition of 0.5 µM FCCP); and F) Complex II electron transfer system capacity (addition of 0.5 µM rotenone + 2.5 µM antimycin A). Data are means ± SEM (n = 4-8/group). No reported values were found to be statistically significant in CTRL vs. MDIVI-1 treated groups.
Figure 3.10: The effects of DRP-1 inhibition on mitochondrial oxygen consumption via complex I and II in early and late reperfused hearts. Data are shown for; A) complex I; and B) complex II supported respiration in left ventricular tissue from hearts perfused under normoxic conditions or reperfused for 10 or 45 min following ischaemia. Hearts were untreated (CTRL) or pre-treated with 1 µM MDIVI-1. Data is representative of mean ± SEM (n = 4-8/group).
Figure 3.11: The effects of DRP-1 inhibition on complex I and II mitochondrial flux control ratios in early and late reperfused hearts. Flux control ratios were determined for; A) complex I; and B) complex II activity in isolated mitochondria from left ventricular tissue from normoxic, early and late reperfused hearts administered with the DRP-1 inhibitor, MDIVI-1, pre-ischaemia. Data are means ± SEM (n = 4-8/group). *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.
3.4 DISCUSSION

The precise roles of DRP-1 in governing cardiac stress-resistance, and the molecular mechanisms by which MDIVI-1 may induce protection against myocardial I-R injury, remain unclear. However, there is much interest in such agents as protectants. In the current study, we assessed whether the putative DRP-1 inhibitor MDIVI-1 can influence myocardial and mitochondrial outcomes from ischaemia in the intact perfused heart. It has previously been reported by Sharp et al. (2014) that inhibition of the fission protein DRP-1 with MDIVI-1 is beneficial when initiated in the post-ischaemic period, preserving mitochondrial morphology via reduced fission and cell death. The current study provides insight into DRP-1 inhibition as a potential determinant of mitochondrial function and I-R injury. This is the first study to assess effects of DRP-1 inhibition with MDIVI-1 on post-ischaemic mitochondrial respiration via complexes I and II, together with functional and cell death outcomes. Data suggest that DRP-1 inhibition has some potential as a preconditioning stimulus to preserve myocardial function via delay/inhibition of contracture development and reduced cell death. However, paradoxical effects of 1 µM vs. 5 µM MDIVI-1 were apparent, with the lower concentration improving post-ischaemic outcomes yet not contracture development, while the higher concentration limited contracture development yet worsened post-ischaemic outcomes. The basis of this concentration-dependence is unclear, though suggests a potentially narrow therapeutic window for such agents.

DRP-1 inhibition as a cardioprotective strategy

The data in this study indicate that MDIVI-1 administered at 1 µM significantly improves I-R tolerance, whereas at a higher 5 µM concentration post-ischaemic recovery is paradoxically worsened. The alternate inhibitor, dynasore hydrate, a molecular inhibitor of GTPase used to target dynamin-1, dynamin-2 and DRP-1 did not appear to influence myocardial I-R tolerance. Increased sensitivity to I-R with the higher 5 µM concentration of MDIVI-1 was unexpected, since Sharp and colleagues (2014) reported significant improvements in post-ischaemic outcomes with 5 and also 25 µM MDIVI-1 (Sharp et al., 2014). Moreover, Ong et al. (2010) report greater effects of 50 vs. 10 µM MDIVI-1 on mitochondrial fission and dysfunction. Nonetheless, there are also reports that DRP-1
inhibition may promote apoptosis (Szabadkai et al., 2004), and post-ischaemic cell death in different tissues (Kumar et al., 2016).

Interestingly, MDIVI-1 administered at 5 μM significantly delayed and reduced ischaemic contracture, an injury process governed by loss of bioenergetic state and shifts in H\(^+\) and Ca\(^{2+}\). Interestingly, these data imply that DRP-1 inhibition is protective during ischaemia. This is consistent with recent evidence, albeit in nervous tissue (Kumar et al., 2016), that a wave of fission arises early in ischaemia, followed by a later wave preceding death in late reperfusion. However, post-ischaemic outcomes after 45 min reperfusion were worsened by 5 μM MDIVI-1. Contrasting studies support benefit via DRP-1 inhibition (Ong et al., 2010; Sharp et al., 2014), this is nonetheless consistent with evidence of exaggerated post-ischemic death in cardiac and neuronal cells. Dong and colleagues (2016) recently established that MDIVI-1 administered at the onset of reoxygenation exaggerated cell death in a cardiomyoblast cell model. Similarly, Zhang et al., (2013) report worsened apoptotic death with DRP-1 inhibition in neuronal cells subjected to simulated I-R. These data suggest that MDIVI-1 treatment may be of benefit when administered pre-ischaemia, allowing the compound time to saturate the intracellular compartments and effectively inhibit translocation of DRP-1 to the OMM during ischaemia and at the onset of reoxygenation when mitochondria are highly fragmented (Kumar et al., 2016).

**DRP-1 inhibition and cell death (LDH Efflux)**

Consistent with previous reports, LDH release was substantially attenuated in hearts treated with MDIVI-1, though effects were only apparent with 5 μM and not 1 μM. This indicates that 5 μM MDIVI-1 reduces myocardial damage/death, although this treatment was paradoxically detrimental to post-ischaemic recovery of contractile function. These data contrast previous observations, with 5 μM and 25 μM MDIVI-1 significantly improving diastolic recovery post-ischaemia (Sharp et al., 2014). While LDH efflux in hearts treated with MDIVI-1 at 5 μM (initiated 10 min pre-ischaemia) was significantly reduced, post ischaemic recovery was worsened. While this data suggest that cell death is reduced in hearts administered with MDIVI-1 at 5 μM, it does not rule out other physiological mechanisms that may contribute to impaired post-ischaemic outcomes. Zhang and colleagues (2013) established that blocking DRP-1 reduced ischaemic damage and had an inhibitory effect on mitophagy, impairing clearance of damaged mitochondria.
These studies suggest that an intracellular increase in MDIVI-1 throughout reperfusion may impair post-ischaemic recovery, while paradoxically reducing LDH release.

Interestingly, dynasore hydrate exaggerated LDH efflux. Dynasore is an alternate DRP-1 inhibitor, implicated as an inhibitor of proteins involved in endocytic pathways. Dynasore inhibits the GTPase activities of dynamin-1, dynamin-2 and DRP-1 (a mitochondrial dynamin). Inhibition of the large dynamin GTPase interferes with vesicle formation and endocytosis (Macia et al., 2006). Whether this effect on LDH efflux reflects exaggerated death is uncertain, with no effects apparent on functional outcomes. Previous reports suggest extracellular LDH is taken into cells via receptor-mediated endocytosis, which may be a factor in elevated LDH efflux in dynasore treated tissue (Smith et al., 1987). The elevation in LDH efflux did not correspond with worsened functional outcomes, suggesting dynasore does not directly influence contractile function. It is important to recognise that dynasore is not a selective dynamin inhibitor, with evidence that it reduces labile cholesterol in the plasma membrane and disrupts lipid raft organisation independently of dynamin (Preta et al., 2015). Given the importance of cholesterol-dependent membrane microdomains to cardiac stress-resistance, cardioprotection and also mitochondrial function (Fridolfsson et al., 2014; Wang et al., 2014), membrane effects of dynasore may be relevant to the mixed outcomes observed. Additionally, while MDIVI-1 appeared to modify mitochondrial DRP-1 expression, a lack of specificity is evidenced with dynasore, which failed to influence mitochondrial DRP-1 expression.

**Impact of DRP-1 inhibition on DRP-1 activation and survival kinase expression**

*Mitochondrial dynamics proteins.* DRP-1 is located primarily in the cytosol and is translocated to the outer mitochondrial membrane to initiate segregation and fission of the OMM (Ong and Hausenloy, 2010). This fission protein has attracted attention as a potential therapeutic target, with emerging data implicating shifts in mitochondrial morphology in different metabolic diseases, including ischaemic heart disease, cardiac myopathy and diabetes. Mitochondrial dynamics and homeostasis is an area of great interest in terms of I-R injury, with studies confirming that ischaemia promotes mitochondrial fragmentation, a process involving translocation of DRP-1 from the cytosol to the OMM. Although exact mechanisms involved remain to be fully elucidated, it is proposed that the changes associated with myocardial reperfusion support a pro-fission mitochondrial network,
impacting on mPTP activity, ROS generation, ATP depletion and respiratory capacity (Chen and Chan, 2005; Gharanei et al., 2013; Hausenloy and Yellon, 2013).

In post-ischaemic myocardium, 1 µM MDIVI-1 significantly decreased levels of mitochondria-associated DRP-1 without altering overall cytosolic levels, consistent with observations of Sharp and colleagues (2014). This apparent inhibition of mitochondrial DRP-1 translocation is thought to confer protection by limiting fission, with mitochondrial elongation shown to decrease mPTP sensitivity and thus pro-death signalling (Sharp et al., 2014; Ong et al., 2015). Interestingly, the alternate multi-protein inhibitor dynasore was ineffective in modifying mitochondrial DRP-1 expression, though cytosolic DRP-1 levels were reduced. This lack of impact on mitochondrial DRP-1 further suggests dynasore may act via other mechanisms to influence myocardial injury and LDH efflux in post-ischaemic hearts.

**Survival kinases (AKT and ERK1/2).** Pro-survival kinases are activated in response to stressors including acute ischaemia, modulating mitochondria-sensitive death pathways and enhancing cell viability (Hausenloy et al., 2011; Hausenloy and Yellon, 2013). Beneficial effects of limiting mitochondrial fission have been linked to suppression of cell death signalling, and potentially improved survival signalling (Sharp et al., 2014; Ong et al., 2015). The PI3K/AKT pathway, and MAPK ERK1/2 are elements of the so-called reperfusion injury salvage kinase (RISK) signalling cascade, and are potential targets for cardioprotection. They may impact a variety of mechanisms to enhance cell survival, including control of downstream mitochondrial modulators (Davidson et al., 2006). The present study assessed impacts of MDIVI-1 on either PI3K/AKT or ERK1/2 signalling in post-ischaemic myocardium. The DRP-1 inhibitor significantly reduced cytosolic levels of phospho-ERK1/2, while mitochondrial levels remained unaltered. Similar findings were reported by Gharanei and colleagues (2013), who showed that co-administration of MDIVI-1 and the toxic cancer therapeutic doxorubicin, reduced myocardial ERK1/2 expression in perfused rat hearts.

Expression, localisation and phospho-activation of AKT were unaltered by MDIVI-1 in post-ischaemic myocardium, thus hinting at a link between mitochondrial dynamics and ERK1/2 stress signalling, rather than via AKT. Consistent with previous reports, ERK1/2 trafficking to mitochondrial membranes governs cardioprotection and is highly dependent on the cytosolic pool of ERK1/2. In addition, microdomains embedded in the plasma
membrane, such as caveolae which contain the protein caveolin-3, have been linked to structures which drive ERK1/2, AKT and GSK3β kinase signalling to the mitochondria, contributing to protection via inhibiting mPTP opening (Segal et al., 1999; Ballard-Croft et al., 2006; Hernández-Reséndiz and Zazueta, 2014).

**Impact of DRP-1 inhibition on post-ischaemic mitochondrial respiratory function**

The constantly beating myocardium is highly oxidative, with high (and highly variable) energy requirements met via a high density of highly fragmented mitochondria (Song and Dorn, 2015). Cardiac energy production is dependent on the oxidation of metabolic substrates coupled to ATP generation within the ETC in the inner mitochondrial membrane (IMM). Synthesis of ATP is driven via the ETC dependent electrochemical (H+) gradient that is harnessed in driving phosphorylation of ADP to ATP. It is clear changes in mitochondrial morphology influence the functional capacity of oxidative phosphorylation, and are implicated in pathological conditions including diabetes and ischaemic heart disease (Chen et al., 2003; Zanna et al., 2008; Schon et al., 2010). Varied myocardial insults lead to mitochondrial damage via oxidative stress, Ca2+ overload, swelling and membrane dysfunction, reducing the mitochondrial transmembrane potential and ATP generation, and ultimately triggering cell death via de-energisation and release of pro-apoptotic factors (Rouslin, 1983). Rouslin (1983) was the first to establish that a large proportion of the impairment to the oxidative phosphorylation (OxPhos) system occurs during ischaemia as opposed to reperfusion, resulting in a highly reduced state and promoting H+ leakage in the absence of O2. Upon reoxygenation, myocardial mitochondrial dysfunction is evident, with reports of significantly reduced complex I activity (Rouslin, 1983; Hardy et al., 1991; Poderoso et al., 1999; Paradies et al., 2004; Chen et al., 2006).

In the present study, I-R and MDIVI-1 modified mitochondrial respiratory capacity, apparently specific to complex I and complex II function. Cardiac OxPhos capacity specific to complex I was reduced in post-ischaemic hearts (an insignificant ~25% reduction at 10 min and >50% reduction at 45 min reperfusion vs. normoxia), which was exaggerated by MDIVI-1. These changes in complex I function are consistent with previous reports (Rouslin, 1983). Interestingly, DRP-1 inhibition did not alter O2 consumption in mitochondria from 10 min reperfused myocardium, while countering dysfunction observed at 45 min reperfusion. This is consistent with reports by Disatnik et al. (2013) who
observed improvements in state 3 respiration (equivalent to complex I OxPhos activity) with a different DRP-1 inhibitor (P110). The post-ischaemic reductions in complex I activity may be attributed to enhanced superoxide (O$_{2}$.') production, a consequence of I-R. Both complex I and III are major sites for O$_{2}$. production, in both the mitochondrial matrix and intermembrane space and also implicated in the pathophysiology of chronic disease conditions including atherosclerosis, cancers and diabetes (Becker et al., 1999). Augmentation of complex I activity by MDIVI-1 at 45 min reperfusion is likely associated with a reduction in mitochondrial fission, which has been linked to a reduction in ROS generation (Jheng et al., 2012; Sharp et al., 2014; Wang et al., 2014).

Shifts in complex II supported respiration with I-R are well defined, with complex II and III showing greater resistance to I-R than complex I activity (Rouslin, 1983; Lesnefsky et al., 1997; Chen et al., 2007). While I-R failed to significantly impair myocardial complex II OxPhos capacity, MDIVI-1 produced a ~25% fall in complex II OxPhos under normoxic conditions (similar to the modest fall early post-ischaemia). Interestingly, MDIVI-1 tended to modestly depress complex II activity across all groups, suggesting that fission inhibition with MDIVI-1 may manipulate mitochondrial bioenergetics independently of ischaemia. Whether or not this is cardioprotective remains unclear.

**Summary**

Pre- and post-ischaemic treatment with 1 µM MDIVI-1 significantly reduced contractile and diastolic dysfunction, whereas a higher 5 µM concentration reduced cell death (LDH efflux) independently of contractile benefit. Complex I OxPhos capacity was severely impaired in early-reperfused hearts, and MDIVI-1 appeared to counter overall dysfunction in mitochondrial O$_{2}$ consumption. Application of a selective DRP-1 inhibitor modestly depressed complex II OxPhos across all groups. Whether or not this effect itself might be cardioprotective remains unclear. The mechanistic action in which mitochondrially-targeted therapies prevent mitochondrial dysfunction remains equivocal and requires further investigation in order to establish whether DRP-1 is a key determinant. While current data indicate that I-R favours a pro-fission environment, thus impairing mitochondrial bioenergetics and enhancing the likelihood of cell death, the specific impacts...
of DRP-1 inhibition on the balance of fission and fusion and in turn cellular injury mechanisms warrants further investigation. These outcomes prompted *in vitro* analyses undertaken in Chapter 4, assessing effects of DRP-1 inhibition in a cell model of acute oxidative.
4.0 ABSTRACT

The clinical manifestations of diseases/co-morbid conditions associated with ischaemic heart disease (e.g. hypertension, obesity, hyperlipidaemia, hyperglycaemia, hyperinsulinaemia) are linked with mitochondrial dysfunction and abnormal ROS production, which may be associated with mitochondrial fragmentation. Nonetheless, precisely how oxidant stress translates to mitochondrial fragmentation and influences respiratory function remains incompletely defined. The current study established that acute exposure to \( \text{H}_2\text{O}_2 \) (0.1 to 1 mM) concentration- and time-dependently impairs H9c2 myoblast viability, triggers transient though substantial (3-fold) phospho-activation of ERK1/2, and inhibits cellular ATP generation by 40-50%. In contrast, AKT expression and phosphorylation were unaltered. Co-treatment with 50 µM MDIVI-1 moderately reduced H9c2 viability, negated \( \text{H}_2\text{O}_2 \)-dependent ERK1/2 phosphorylation, and partially limited the decline in ATP generation. Total and phospho-DRP-1 and total OPA-1 levels were unchanged by exposure to either \( \text{H}_2\text{O}_2 \) or MDIVI-1. However, \( \text{H}_2\text{O}_2 \) reduced mitochondrial 'routine', 'leak' and ETS related O2 fluxes together with peak respiration. Curiously, these inhibitory effects were mimicked by MDIVI-1 treatment. Moreover, while \( \text{H}_2\text{O}_2 \) alone failed to influence extracellular acidification rates, MDIVI-1 sensitised H9c2 cells to \( \text{H}_2\text{O}_2 \), resulting in >50% reductions in acidification rate. Thus, inhibition of DRP-1 to attenuate fission appears to worsen cell viability, based on a measure of metabolic activity, and impair stress kinase signalling during oxidative stress, whilst also impacting mitochondrial respiratory capacity. Such effects are inconsistent with a beneficial 'cardioprotective' effect of this compound and molecular strategy.
4.1 INTRODUCTION

The morphology of mitochondria is governed by dynamic fission and fusion, opposing homeostatic quality control mechanisms that govern mitochondrial structure and function within the cell. Mitochondrial fission and fusion occur in a continuous cycle, eliminating damaged and senescent mitochondria, regulating efficient energy production via oxidative phosphorylation, and cell cycle processes (Chen and Chan, 2005). Imbalances in mitochondrial fission and fusion have been implicated in disease states including obesity (Bach et al., 2005), diabetes mellitus (Westermeier et al., 2015), stroke (Cassidy-Stone et al., 2008) and ischaemia-reperfusion (Ong and Gustafsson, 2012), all of which are associated with oxidative stress and pro-apoptotic signalling.

Mitochondrial fission is regulated via the dynamin related GTPase molecule DRP-1, a cytosolic protein that translocates to the outer mitochondrial membrane in response to differing (stressful) stimuli, where it triggers segregation and constriction of the membrane via interaction with FIS1. Highly fragmented networks of mitochondria are associated with activation of apoptosis via release of cytochrome c and other pro-apoptotic factors, resulting in subsequent activation of caspase cascades. High levels of fission are also associated with impaired or defective mitochondrial function (Chen and Chan, 2005), facilitating ROS formation and associated toxicity. The pharmacological inhibition of DRP-1 has been widely reported as a novel approach to cytoprotection, manipulating the machinery involved in control of mitochondrial dynamics (Lackner and Nunnari, 2010; Andreux et al., 2013). The MDIVI-1 compound prevents DRP-1 translocation to the outer mitochondrial membrane, thus promoting an interconnected mitochondrial phenotype via a shift in the balance of fission/fusion signalling. A pro-fusion environment is considered beneficial, facilitating efficient oxidative energy production (Youle and Van Der Bliek, 2012). That said, it has also emerged that a healthy myocardium may not exhibit active fission/fusion, and possess a high density of fragmented mitochondria that provide the highest respiratory capacity in mammalian cells (Chan, 2005). Thus, how perturbation of this unique system will impact stress responses in cardiac cells is not entirely clear. Studies in Chapter 3 evidenced some benefit via MDIVI-1 during myocardial I-R, associated with reduced mitochondrial DRP-1. To further test the importance of mitochondrial fission, and potential benefits of DRP-1 inhibition, studies in this chapter assess concentration- and
time-dependent effects of H$_2$O$_2$ dependent oxidative insult on stress kinases, mitochondrial respiratory parameters and ATP production in H9c2 cardiomyoblasts, and effects of MDIVI-1 on these responses.
4.2 METHODS AND MATERIALS

4.2.1 Cell culture

The H9c2 cell line was obtained from the American Type Culture Collection (ATCC, U.S.A.) as detailed in the Research Methods (Chapter 2). Cells were maintained and grown in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and kept under 95% O₂ and 5% CO₂ at 37°C. Cells used in experiments were passaged up to 15 times.

4.2.2 Reagents

Quercetin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), hydrogen peroxide (H₂O₂), MDIVI-1, β-mercaptoethanol, DMSO, MOPS, HEPES, Tween 20, EGTA, EDTA, tris, bis-tris, tricine, BSA, MgCl₂, KCl, NaCl, ethidium bromide, and glycine, sodium fluoride, β-glycerophosphate, sodium tetra-pyrophosphate, leupeptin, potassium phosphate, pepstatin A, benzamine, phenylmethanesulfonyl fluoride, sodium orthovanadate, catalase, sucrose, sodium pyruvate, malate, SDS, glutamate, succinate, cytochrome c, ADP, carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone (FCCP), oligomycin, rotenone, malonic acid and antimycin A were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Lactobionic acid was purchased from Acros Organics (Thermo-Fischer Scientific, NJ, USA). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), PBS and other tissue culture reagents were obtained from Gibco (Life Technologies, Carlsbad, CA, USA).
4.2.3 Oxidative challenge with \( H_2O_2 \)

H9c2 cells were grown in 100 mm plates to 70-80% confluence. The cell monolayer was washed in phosphate buffered saline (PBS) before 1.5 mL of trypsin EDTA (0.25%) was added. Detached cells were resuspended in 5 mL of DMEM before cell density was determined with a haemocytometer. Cells were then seeded at a density of 2.0x10^4 cells per well in 24-well plates and left to incubate overnight. To assess the effects of oxidative stress, H9c2 cells were challenged with acute \( H_2O_2 \) for 1 hr (final concentrations of 0.1, 0.2, 0.4, 0.6, 0.8 or 1 mM).

The MDIVI-1 compound was dissolved in DMSO and added to DMEM at a final concentration of 50 μM (final concentration of DMSO <0.5%). Experiments were performed in cells incubated with MDIVI-1 or respective vehicle (DMSO <0.5%) for 40 min prior to \( H_2O_2 \) challenge (0.4 mM, for 5 to 60 min).

4.2.4 Cell viability (MTT assay)

Viability of H9c2 cells was determined using a colorimetric MTT assay. Cell viability was assessed in cells acutely challenged with \( H_2O_2 \) (± MDIVI-1) at varying times from 5 to 60 min. A MTT solution (200 μL of 5 mg/mL in DMEM) was added to each well followed by incubation in a dark room at 37°C for 2-4 hrs. DMSO was then used to dissolve the crystal formations on the plate surface and absorbance was read at 570 nm on a microplate reader (Tecan infinite M200 Pro, Mannedorf, Switzerland).

4.2.5 Measurement of cellular mitochondrial respiration

*Oxygraph analysis in suspended H9c2 cells.* Oxygen consumption was quantified using an Oxygraph-2k instrument (Oroboros Instruments, Innsbruck, Austria). Chambers ‘A’ and ‘B’ of the apparatus were cleaned prior to use with 70% ethanol and milliQ water, and underwent stabilisation with MiR05 media. Post stabilisation, 2 mL of serum-free DMEM was used to re-suspend cells (1 x 10^6 cells/mL) in the chambers. Assessment of respiration in a suspension of whole cardiomyoblast cells enables evaluation of total cellular \( O_2 \) consumption, and \( O_2 \) consumption in both coupled and uncoupled mitochondria. Addition of 2 μg/ml oligomycin (ATPase inhibitor) was used to stimulate ‘Leak’ respiration and 0.5 μM FCCP (mitochondrial uncoupler) was used to stimulate electron transport.
transfer system (ETS) capacity. Respiration values were normalised to residual oxygen consumption, the respiration due to oxidative side reactions. Resulting O₂ fluxes are corrected to the instrument background readings, which were acquired during stabilisation of the instrument. DatLab software (Oroboros Instruments, Innsbruck, Austria) was used for quantitative analysis of mitochondrial O₂ consumption.

**Seahorse XF-p analysis of oxygen consumption and extracellular acidification in H9c2 cells.** H9c2 cells were prepared as detailed in Methods chapter - section 2.3. Cells were treated with the DRP-1 inhibitor MDIVI-1 (50 µM) or vehicle (DMSO) for ~40 min, prior to an acute 1 hr H₂O₂ challenge (0.4 mM). The DMEM was then removed and cells washed in PBS and supplemented with XFp modified media (1 mM pyruvate, 2 mM glutamine, 10 mM glucose, pH 7.4) and incubated at 37°C in a non-CO₂ incubator for 45 min prior to assay. A Seahorse XFp Extracellular Flux Analyser (Bioscience, Billerica, MA, USA) was used to measure oxygen consumption rate (OCR). Measurements were performed over 6 min intervals in measurement cycles of 2 or 4. After basal OCR was established, oligomycin (1 µM), FCCP (2 µM) and antimycin A (0.5 µM) were injected sequentially to assess specific OCR values (Supplementary figure 1). Measurements were normalised to cell density.

**4.2.6 Assessment of cellular ATP levels**

An assessment of ATP levels was performed using a luminescent assay (CellTiter-Glo® kit, Promega) as detailed in section 2.2. Cells were pre-treated with the pharmacological fission inhibitor MDIVI-1 (50 µM) for 40 min prior to acute H₂O₂ insult (0.4 mM). Luminescence was recorded using a microplate reader (Tecan infinite M200 Pro, Mannedorf, Switzerland). Raw data was analysed using Microsoft Excel, and results normalised to total protein concentration determined via a BCA assay.
4.2.7 Western immunoblotting

Western immunoblot analysis was performed as previously described in section 2.7. Cells were harvested by centrifugation and washed twice with PBS prior to being resuspended in 150 µL cell lysis buffer containing protease and phosphatase inhibitors; 0.1% Triton-X; Mops, 20 mM; EGTA, 2 mM; EDTA, 5 mM; sodium fluoride, 30 mM; β-glycerophosphate, 40 mM; sodium tetra-pyrophosphate, 20 mM; leupeptin, 10 µM; pepstatin A, 5 µM; benzamidine, 3 mM; phenylmethanesulfonyl fluoride, 1 mM; and sodium orthovanadate, 1 mM. Cell lysates were frozen at -80°C until further analysis. A sample of 20 µg (28 µL) was loaded onto a hand-cast 10% acrylamide gel. Protein separation was achieved by running gels at 150 V for 80 min. The transfer of proteins was achieved using a polyvinylidene difluoride fluorescent membrane at a constant amperage level of 350 mA for ~2 hrs, and blocked with Odyssey fish serum for an additional 2 hr at room temperature. The membranes were incubated in their primary antibody (detailed in chapter 2 - table 2.1) for 15-18 hr overnight at 4°C with gentle rocking. The PVDF membrane was washed in TBS for 5 min and again washed in TBST for 4 cycles of 5 min before incubation with the corresponding secondary fluorescent antibody at room temperature in the dark. Membranes are visualised on a Licor Odyssey Infrared Imaging System (Millennium Science, Mulgrave, Australia) and densitometric analysis of each protein normalised to an internal standard and loading control.
4.3 RESULTS

4.3.1 Cell viability with H$_2$O$_2$ challenge: effects of MDIVI-1

Sensitivity of H9c2 cardiomyoblasts to an acute H$_2$O$_2$ challenge was assessed in triplicate using a routine MTT assay (Fig. 4.1A). Effects of 1 hr incubation with H$_2$O$_2$ on H9c2 viability (as % of untreated control) were concentration-dependent, viability progressively declining with increases in H$_2$O$_2$ concentration ranging from 0-1mM. Having established the concentration-dependence of H$_2$O$_2$ mediated loss of cell viability in a set of time course experiments, the effects of the DRP-1 fission protein inhibitor MDIVI-1 (50 µM) were assessed in cells treated with 0.4 mM H$_2$O$_2$ for periods of: 5, 15, 30 or 60 min (Fig. 4.1B). The results suggest that pre-treatment with the DRP-1 inhibitor MDIVI-1 prior to an acute H$_2$O$_2$ exposure did not improve cell viability. Indeed, viability appeared to be moderately reduced in response to the DRP-1 fission protein inhibitor.
Figure 4.1: Concentration and time dependence of H$_2$O$_2$ induced changes in viability of H9c2 cardiomyoblasts. A) Data are shown for concentration-dependent changes in viability; and B) time dependent H$_2$O$_2$ induced toxicity at 5, 15, 30 and 60 min of pre-treatment with MDIVI-1 at a concentration of 50 µM for 40 min. Data is representative of mean ± SEM ($n = 3$ experiment repeated 3 times). *, $P<0.05$; **, $P<0.01$; *** $P<0.001$ vs. untreated CTRL; *, $P<0.05$; vs. time matched H$_2$O$_2$ treated.
4.3.2 Survival kinase expression during H₂O₂ challenge: effects of MDIVI-1

H9c2 cardiomyoblasts were acutely treated with H₂O₂ for varying times (5, 15 and 30 min) at a concentration of 0.4 mM (established in initial concentration-response analysis; Fig. 4.1A). Cell lysate proteins were separated by SDS-PAGE and immunoblotted with antibodies for total and phosphorylated ERK1/2 and AKT expression. Phospho-ERK1/2 achieved peak expression at 5 min following a H₂O₂ challenge (Fig. 4.2C), while phospho-AKT was marginally reduced (Fig. 4.2D). The ratio of phosphorylated to total ERK1/2 expression was significantly heightened at 5 min following an acute H₂O₂ challenge. Having identified the acute changes in kinase expression with H₂O₂ alone, H9c2 cardiomyoblasts were administered with the DRP-1 inhibitor MDIVI-1 (50 µM) prior to an acute challenge of H₂O₂. Pre-treatment with MDIVI-1 significantly negated early modifications in ERK1/2 phosphorylation (Fig. 4.3C). While, treatment with MDIVI-1 failed to govern any changes in the phosphorylation of AKT expression (Fig. 4.3D).
Figure 4.2: Time-course of survival kinase expression/phosphorylation during H$_2$O$_2$ challenge in H9c2 cardiomyoblasts. Effects of acute H$_2$O$_2$ (0.4 mM) challenge on survival kinase protein expression were assessed in H9c2 cardiomyoblasts (5-30 min of H$_2$O$_2$ treatment). Cell lysates were assessed for total and phosphorylated survival kinase (AKT and ERK1/2) expression. A) Total-ERK1/2; B) total-AKT; C) phosphorylated-ERK1/2; D) phosphorylated-AKT phosphorylated-ERK1/2; E) ratio of phosphorylated: total ERK1/2; and F) ratio of phosphorylated: total AKT. Data are means ± SEM (n = 3). **, $P<0.01$; ***, $P<0.001$. 
Figure 4.3: Impact of acute 5 min H$_2$O$_2$ challenge (± MDIVI-1) on survival kinase expression/phosphorylation. Cardiomyoblasts were pre-treated with 50 µM MDIVI-1 for 40 min prior to a 5 min challenge with H$_2$O$_2$. Cell lysate was assessed for survival kinase (AKT and ERK1/2) expression. A) Total-ERK1/2; B) total-AKT; C) phosphorylated-ERK1/2; D) phosphorylated-AKT; E) ratio of phosphorylated: total ERK1/2; and F) ratio of phosphorylated: total AKT. Data are means ± SEM (n = 3 experiments repeated 3 times). *, P<0.05; **, P<0.01. (Representative immunoblots detailed in supplementary figure 3)
4.3.3 Fission and fusion protein expression during H$_2$O$_2$ challenge: Effects of MDIVI-1

To assess whether mitochondrial fission plays a role in cardiomyoblast responses to oxidant stress, H9c2 cells were subjected to an acute H$_2$O$_2$ challenge ± DRP-1 inhibition with MDIVI-1, and expression of total- and phospho-DRP-1 and the fusion protein OPA-1 assessed (Fig. 4.4). Results indicate that neither total (Fig. 4.4A) nor phospho-DRP-1 (Fig. 4.4B) is significantly altered in response to acute H$_2$O$_2$ (vs. control), either in the absence or presence of MDIVI-1. Similarly, OPA-1 expression was unaltered by acute H$_2$O$_2$ ± MDIVI-1 (Fig. 4.4C). This suggests that an acute oxidative stress does not influence mitochondrial proteins implicated in mitochondrial fission and fusion.
Figure 4.4: Impact of acute 5 min H2O2 challenge (± MDIVI-1) on H9c2 cardiomyoblast DRP-1 and OPA-1 expression. H9c2 cardiomyoblasts were pre-treated with vehicle or 50 µM MDIVI-1 for 40 min prior to acute 5 min challenge with 0.4 mM H2O2. Cell lysates were assessed for expression of: A) Total DRP-1; B) phosphorylated-DRP-1; and C) OPA-1. Data are means ± SEM (n = 3 experiments, each repeated 3 times).
4.3.4 ATP production during H$_2$O$_2$ challenge: effects of MDIVI-1

Generation of ATP occurs predominantly via oxidative phosphorylation. However, ATP is also generated via glycolysis and β-oxidation of fatty acids. The relative production of ATP was assessed using a luminescence assay in H9c2 cardiomyoblasts treated with 50 μM MDIVI-1 for 40 min prior to acute H$_2$O$_2$ exposure for 60 min (ATP levels normalised to control values). Data suggest that ATP production (Fig 4.5) is attenuated in H9c2 cells exposed to either MDIVI-1 or H$_2$O$_2$ alone or in combination (vs. respective controls). There was a significant difference ($P<0.05$) in ATP production in cardiomyoblasts challenged with H$_2$O$_2$ in the presence vs. absence of MDIVI-1. Data suggests MDIVI-1 may improve ATP generation during an oxidative challenge, despite exerting inhibitory effects in non-stressed myoblasts.

![Graph showing ATP production](image)

**Figure 4.5**: Effects of 60 min H$_2$O$_2$ challenge (± MDIVI-1) on ATP generation in H9c2 cardiomyoblasts. Generation of cellular ATP was assessed using a luminescence assay. Cells were exposed to vehicle (CTRL) or 50 μM MDIVI-1 for 40 min prior to a 60 min H$_2$O$_2$ challenge (0.4 mM). Data are means ± SEM ($n = 4$ experiments, each repeated 3 times).

*, $P<0.05$; ***, $P<0.001$. 
4.3.5 Mitochondrial dysfunction during H$_2$O$_2$ challenge: effects of MDIVI-1

**Oroboros O2k-Oxygraph.** In order to assess cellular respiration, rates of cellular O$_2$ consumption were determined using an O2k-Oxygraph (Oroboros Instruments, Innsbruck, Austria). Experiments were performed in H9c2 cardiomyoblasts exposed either to vehicle or MDIVI-1 for 40 min prior to an acute 5 min H$_2$O$_2$ challenge. Results suggest that H$_2$O$_2$ alone significantly reduces routine (physiological coupling state) respiration by more than 50% (vs. control), with a marginal improvement observed in cells pre-treated with MDIVI-1 (Fig. 4.6). A significant reduction in 'leak' (non-phosphorylating resting state) and electron transfer system (ETS) respiration was detected in all H$_2$O$_2$ treated cardiomyoblasts, and also in cells pre-treated with MDIVI-1 prior to H$_2$O$_2$ (vs. control).

![Graph showing effects of acute H$_2$O$_2$ challenge on routine, leak and ETS respiratory rates in H9c2 cardiomyoblasts.](image)

**Figure 4.6: Effects of acute 5 min H$_2$O$_2$ challenge on routine, leak and ETS respiratory rates in H9c2 cardiomyoblasts.** Oxygen consumption values were assessed using the Oxygraph-2k (O2k) in chambers containing 10$^6$ cells/ml. H9c2 cells were pre-treated with vehicle (CTRL) or 50 μM MDIVI-1 40 min prior to 5 min challenge with 0.4 mM H$_2$O$_2$. Data are means ± SEM (n = 3 experiments repeated 3 times). *, P<0.05; ***, P<0.001; and ****, P<0.0001 vs. CTRL.
Seahorse XFp extracellular flux analyser. The Seahorse XFp extracellular analyser was employed to assess the metabolic effects of H₂O₂ ± MDIVI-1. An advantage of the Seahorse XFp system is that fewer cells are required (vs. the Oroboros oxygraph) and therefore multiple replicates can be performed per plate. Prior to undertaking cellular respiration experiments on H9c2 cardiomyoblasts in the XFp miniplate, cell density optimisation was carried out to confirm the optimum number of cells to seed per well. This is important since over-confluent wells can induce cellular stress and generation of ROS. Cells were plated at a density of 5x10³, 7.5x10³, 1x10⁴ and 1.5x10⁴. A density of 1x10⁴ per well was used for all Seahorse experiments with cells growing to 75% confluence overnight post seeding.

Another advantage of the seahorse XFp extracellular flux system is the simultaneous measurement of both mitochondrial O₂ consumption rate (OCR) and extracellular acidification rate (ECAR), the latter an indicator of glycolytic metabolism. To test whether varying concentrations of H₂O₂ shift metabolic activity from oxidative metabolism to glycolysis, the ratio of OCR/ECAR can be calculated. Values of OCR/ECAR between 5-8 are considered physiological, indicating that aerobic metabolism is the major metabolic producer of ATP (Buck et al., 2016). Values of OCR/ECAR <5 indicate metabolic activity has shifted towards glycolysis, producing higher yields of protons (H⁺) that contribute to acidification.

Experiments were undertaken in cardiomyoblasts exposed to vehicle or the fission inhibitor MDIVI-1 prior to H₂O₂ challenge (Fig. 4.7). The compound MDIVI-1 and H₂O₂ alone moderately reduced basal respiration in cardiomyoblasts, while MDIVI-1 in combination with H₂O₂ significantly attenuated basal respiration by ~46% (vs. control). Maximal respiration was reduced by ~38% with H₂O₂ alone and by ~42% with MDIVI-1 alone (vs. control). MDIVI-1 in combination with H₂O₂ significantly reduced maximal respiration by ~64% (vs. control). MDIVI-1 in combination with H₂O₂ significantly reduced non-mitochondrial respiration (vs. control), a parameter that was relatively insensitive to other treatment conditions. Interestingly, MDIVI-1 substantially increased mitochondrial ATP production by ~75% (vs. control), with a ~64% reduction detected in cells treated with MDIVI-1 + H₂O₂ (vs. control). MDIVI-1 alone increased the OCR/ECAR ratio from a value of ~5.0 (in control cells) to 7.0 (Fig 4.8). The ATPase inhibitor oligomycin reduced the OCR/ECAR ratio in MDIVI-1 treated cells by ~60% vs. ~34% when administered in combination with H₂O₂.
Figure 4.7: Effects of 60 min H$_2$O$_2$ challenge (± MDIVI-1) on mitochondrial oxygen consumption rate (OCR) and extracellular acidifications rate (ECAR). A) The OCR was measured in H9c2 cells pre-treated with vehicle (CTRL) or 50 µM MDIVI-1 40 min prior to 60 min challenge with 0.4 mM H$_2$O$_2$ via the addition of; oligomycin, FCCP, antimycin A and rotenone; and B) Extracellular acidification rate upon addition of; oligomycin, FCCP, antimycin A. Data are means ± SEM (n = 3). *, P<0.05; **, P<0.01; ***, P<0.001 vs. CTRL.
Figure 4.8: Effects of 60 min H$_2$O$_2$ challenge (± MDIVI-1) on the ratio of OCR/ECAR in H9c2 cardiomyoblasts. The OCR/ECAR was measured under basal conditions and after addition of: 2.5 μM oligomycin (ATP synthase inhibitor); 4 μM FCCP (uncoupler); 0.5 μM rotenone (complex I inhibitor); and 2.5 μM antimycin A (complex III inhibitor). H9c2 cells were plated at 1x10$^4$ cells/well in an XFp cell culture miniplate 24 hrs. Cells were then incubated for 40 min with vehicle (CTRL) or 50 μM MDIVI-1 prior to 60 min challenge with 0.4 μM H$_2$O$_2$. Data are means ± SEM (n = 3 experiments, each repeated 3 times). **, $P<0.001$ (MDIVI-1 vs. CTRL); ϕ, $P<0.0001$ (MDIVI-1 + H$_2$O$_2$ vs. CTRL).
4.4 DISCUSSION

Mitochondria are critical to cell survival during stress and disease. Understanding mitochondrial dynamics and its influence on respiratory function and bioenergetics during stress is critical in unravelling the mechanisms of injury and survival in healthy vs. diseased myocardium. Proteins that regulate mitochondrial dynamics have been implicated as key players in mitochondrial dysfunction and in governing severity of injury with ischaemia-reperfusion or related oxidative stress. This study sought to characterise the effects of DRP-1 inhibition (with MDIVI-1) on time-dependent effects of \( \text{H}_2\text{O}_2 \) on cardiomyoblast viability, stress-kinase signalling and mitochondrial respiratory parameters.

**Effect of DRP-1 inhibition on survival kinase (ERK1/2) expression in cardiomyoblasts acutely challenged with \( \text{H}_2\text{O}_2 \)**

Initial studies revealed that an acute \( \text{H}_2\text{O}_2 \) challenge rapidly and transiently activated ERK1/2 kinase signalling, a response negated by MDIVI-1. This acute change in ERK1/2 signalling is consistent with Bogoyevitch *et al.* (2000) who first characterised an early activation of ERK1/2 signalling in cardiomyoblasts acutely challenged with \( \text{H}_2\text{O}_2 \). Reactive oxygen species, including \( \text{H}_2\text{O}_2 \), are predominately derived from the activity of NADPH oxidase enzymes within the mitochondria and plasma membrane, a subclass of ROS that increases following ischaemic episodes, often associated with the onset of molecular processes compromising myocardial tissue and functionality (Bogoyevitch *et al.*, 2000; Park *et al.*, 2013). Recent findings by Prieto *et al.* (2016) established that ERK1/2 signalling influences mitochondrial homeostasis via activation of DRP-1 in mouse embryonic fibroblasts. Activation of ERK1/2 was found to promote DRP-1 phosphorylation and its recruitment to the OMM, triggering an early state of mitochondrial reprogramming. While the current data demonstrates a transient increase in ERK1/2 signalling in response to acute oxidative stress, this may in fact be detrimental rather than cardioprotective, thus promoting DRP-1 activation. On the contrary, treatment with MDIVI-1 repressed ERK1/2 signalling, potentially inhibiting ERK1/2 activation and downstream phosphorylation DRP-1. These findings indicate a feedback control loop between DRP-1 and ERK1/2, whereby inhibition of DRP-1 impairs ERK1/2 signalling, while ERK1/2 promotes DRP-1 activation. Whether this pharmacokinetic manipulation of
mitochondrial dynamics is cardioprotective remains elusive, since interruption of homeostatic mitochondrial control mechanisms may result in an accumulation of dysfunctional mitochondria. In others studies, the effects of MDIVI-1 on doxorubicin induced cardiac dysfunction was found to significantly attenuate the phosphorylation of ERK1/2 in an ischaemic heart (Gharanei et al., 2013). Taken together, the current data suggest ERK1/2 activation may influence mitochondrial dynamics in cardiomyoblasts challenged with acute oxidative stress. However, it remains unclear whether ‘survival’ kinase activation is detrimental towards mitochondrial dynamics.

**Mitochondrial dynamics protein during acute oxidative stress**

Studies in endothelial and neuronal cells have demonstrated that mitochondrial dynamics are modified in the presence of ROS (Giedt et al., 2012). However, the effects on mitochondrial dynamics in cardiomyoblasts subject to an acute oxidative challenge remains unexplored. Current data show that H$_2$O$_2$ alone failed to significantly modify total or phosphorylated DRP-1 expression, while MDIVI-1 moderately ($P>0.05$) reduced DRP-1 expression in cells pre-treated with MDIVI-1. Data from western blot analysis of proteins suggest that mitochondrial remodelling may be time dependant in response to injurious stimuli, requiring chronic/sustained oxidative stress in order to modify mitochondrial dynamics. Treatment with H$_2$O$_2$ has been found to induce mitochondrial fragmentation in C$_2$C$_{12}$ mouse myocytes at 5-6 hours post stimulus, and at 4, 6 and 24 hr of reperfusion following global brain ischaemia (Fan et al., 2010; Kumar et al., 2016). While the current study examined the effects of acute oxidant stress, the later studies suggest that mitochondrial dynamics are unable to be modified under conditions of acute oxidative stress but rather throughout chronic/sustained oxidant stress, which are more clinically relevant.

**Mitochondrial bioenergetics in response to DRP-1 inhibition**

In order to establish whether inhibition of DRP-1 has an impact on the bioenergetic machinery embedded within the mitochondrial membrane, mitochondrial respiration was assessed in cardiomyoblasts acutely challenged with H$_2$O$_2$ ± MDIVI-1. Treatment with H$_2$O$_2$ significantly impacted the mitochondrial respiratory capacity (~50%), however was not improved by MDIVI-1 in cardiomyoblasts challenged with H$_2$O$_2$/oxidative stress. Consistent with previous reports (in non cardiac models), MDIVI-1 has been found to have
inhibitory effect on the OCR, demonstrating a role for DRP-1 as a modifier of bioenergetics (Hong et al., 2013; Buck et al., 2016). In an attempt to understand the complex nature of this compound, Qian et al. (2014) established that MDI VI-1 acts synergistically with the chemotherapeutic drug cisplatin in a carcinoma cell line. Treatment with MDI VI-1 repressed mitochondrial respiration, increased acidification and impaired DNA replication, indicating a modification in energy production pathways, thus favouring glycolytic metabolism. Further supporting this concept, Buck et al. (2016) proposed that mitochondria potentially remodel in response to metabolic stress. Altering cristae structure and producing highly fragmented mitochondria in T cells, while reducing ETC efficiency and ATP production. A reduction in electron transfer causes NADH to accumulate that may trigger metabolically stressed cells to favour ATP production via glycolytic substrates (as denoted by reduction in the ratio of OCAR/ECAR). The current data demonstrates an overall reduction in the OCR in cells challenged with H$_2$O$_2$ or MDI VI-1 alone. A reduction in the OCR suggests ATP producing pathways may shift from OxPhos, favouring glycolysis due to mitochondrial dysfunction and ETC perturbations. Thus, it remains questionable as to whether the therapeutic manipulation of mitochondrial fission protein, DRP-1, prevents or facilitates ETC dysfunction and reduced respiratory capacity.

While this study does not directly assess the level of glycolytic enzymes and substrates activity, the current bioenergetic data indicate that metabolic modifications are likely, and may compensate for a reduction in ETC function due to ROS and other elements of oxidant stress.

The effect of DRP-1 inhibition on ATP production in cardiomyoblasts challenged with H$_2$O$_2$

Emerging research suggests that modifications in mitochondrial dynamics are diverse depending on cell stressor and cell type. Conditions of nutrient deprivation and hypoxia in HL-1 cells (in vitro) and cardiomyocytes (in vivo) support a highly fragmented mitochondrial network (Ong et al, 2010; Kim et al, 2010), while Gomes et al. (2011) established that phosphorylation of DRP-1 (ser637) favoured a pro-fusion network in fasted MEF cells. Interestingly, the current data indicate that H$_2$O$_2$ alone reduces cellular ATP production, with MDI VI-1 inducing a similar trend. The production of ATP in H$_2$O$_2$ challenged cells was significantly improved with MDI VI-1 pre-treatment. Although unexpected, an oxidative stressor in combination with a DRP-1 inhibitor may be
advantageous for mitochondrial-dynamics, thus preventing a substantial decline in ATP production. On the contrary, unperturbed and healthy mitochondria that are administered with a DRP-1 inhibitor may induce unfavourable outcomes, which prompt cellular dysfunction.

**Summary**

Understanding the complex bioenergetic properties of cardiomyoblasts during acute oxidative injury is valuable in developing awareness of the pathophysiological impact that ischaemic heart disease has on the heart. Cellular metabolism is highly dependent on adequate substrate availability and ATP production under varying workloads and conditions of stress. With the use of bioenergetic profiling, this study highlights phenotypic changes during acute oxidative stress and the impacts of the DRP-1 inhibitor MDIVI-1. Interestingly, transient modifications in stress kinase signalling (ERK1/2) associated with oxidative stress were reversed with MDIVI-1 treatment, while ATP generation was preserved with MDIVI-1 treatment. The current data suggest that DRP-1 inhibition modifies both stress kinase signalling and energy production, however the exact mechanism remains undefined.

As an acute stress model in a relatively quiescent cultured cell model, these findings may not necessarily reflect changes associated with more chronic stresses, as occurs in cardiovascular disease. Thus, studies detailed in Chapter 5 assess the effects of chronic oxidative stress.
CHAPTER 5: The Impact of DRP-1 Inhibition in Cardiomyoblasts Subjected to Chronic Oxidative Stress

5.0 ABSTRACT

A potential therapeutic target, that may influence mitochondrial remodeling and quality control, is dynamin-related protein-1 (DRP-1). This protein may be a key modulator of myocardial stress resistance and adaptation in pathological conditions of sustained oxidative or other stresses. To assess the impacts of chronic oxidative stress, this study examines protein expression and cell viability in H9c2 cardiomyoblasts challenged with hydrogen peroxide (H$_2$O$_2$) up to 8 hr (0.4 mM) in the presence or absence of the DRP-1 inhibitor MDIVI-1. Together with stress kinases (AKT, ERK1/2), a western blot analysis of mitochondrial remodeling proteins (DRP-1, OPA-1, MFN-1, MFN-2) and autophagic proteins (LC3, PARKIN) were undertaken. Cell viability (MTT, LDH and xCELLigence assays) and mitochondrial membrane potential were assessed to test whether DRP-1 inhibition (50 µM MDIVI-1) protects against functional and proteomic impacts of oxidative stress. Contrasting effects of brief H$_2$O$_2$ exposure in prior chapters, sustained exposure to H$_2$O$_2$ led to depression of total AKT and ERK1/2 levels coupled with increased phospho-activation. This latter response was modulated by MDIVI-1 in a biphasic manner, initially augmenting phospho-activation (from 2 hrs), and thereafter-depressing phospho-activation (≥4 hrs). Expression of DRP-1 was also repressed with prolonged H$_2$O$_2$ exposure, a response transiently modified by MDIVI-1 treatment. Exposure to H$_2$O$_2$ also reduced expression of the fusion proteins OPA-1 and MFN-2 (not MFN-1), changes that were in part augmented by MDIVI-1 treatment. Expression of the mitophagy protein PARKIN increased modestly with H$_2$O$_2$ challenge, an effect augmented by MDIVI-1. Activation of LC3 (LC3B-II/LC3B-I ratio) transiently increased at 1 hr, a response countered by MDIVI-1, and then subsequently declined. Pro- and anti- apoptotic markers BAX, BCL-2 and PARP (cleaved and uncleaved) were also examined. Oxidant stress increased the BAX:
BCL-2 ratio, an effect initially augmented (yet subsequently inhibited) by MDIVI-1. Uncleaved PARP (116 kDa) was significantly reduced throughout H$_2$O$_2$ exposure whereas cleaved PARP (89 kDa) levels were substantially increased. This increase in PARP cleavage was initially inhibited by MDIVI-1, though failed to modify PARP cleavage from 4 hrs of H$_2$O$_2$ exposure. The mitochondrial membrane potential ($\Delta\psi$) was reduced by ~25% after 1-4 hrs of H$_2$O$_2$ challenge, with MDIVI-1 reversing this inhibitory effect. The basis of these paradoxic observations remains unclear and raises questions regarding the utility of MDIVI-1 as a protectant in conditions of chronic oxidative stress.
5.1 INTRODUCTION

Oxidative stress is a key underlying mechanism driving the pathophysiology of many cardiovascular and metabolic diseases, including chronic heart failure, cardiomyopathy, ischaemic heart disease, atherosclerosis, hypertension, and diabetes. Oxidative stress is induced by excess production of reactive oxygen species (ROS), contributing to the development and/or progression of cardiovascular diseases (Ong and Hausenloy, 2010; Ong et al., 2010; Sharp et al., 2014). The accumulation of ROS is primarily governed via the electron transport chain (ETC), xanthine oxidase, NADPH oxidase, lipooxygenase, and nitric oxide synthase (Nordberg and Arnér, 2001; Rudzka et al., 2015), with associated cellular dysfunction prompting progression of apoptotic cell death. Sustained oxidative stress can induce mitochondrial dysfunction and release of cell-death mediators, activating pro-apoptotic pathways. Emerging studies suggest that an increased generation of ROS can induce an imbalance of mitochondrial remodeling proteins that normally maintain optimal mitochondrial functionality via the opposing processes of fission and fusion, together with mitophagy. Mitochondrial fission/fusion governs mitochondrial morphology, restoration of damaged mitochondria, cellular division and the efficiency of oxidative phosphorylation (Chen and Chan, 2005). Previous reports suggest oxidative injury induces apoptosis via increased mitochondrial fragmentation, reduced mitochondrial membrane potential and release of cytochrome c and other death factors via mitochondrial outer membrane permeabilisation (MOMP) and the mitochondrial permeability transition pore (mPTP) (Wu et al., 2011). The key mitochondrial fission protein dynamin-related protein-1 (DRP-1) translocates to the mitochondrially bound docking protein FIS1, positioned on the outer mitochondria membrane surface (Westermann, 2012), promoting the fission process in response to differing forms of stress. Ischaemic heart disease, which entails impaired myocardial perfusion and enhanced oxidative stress, induces a network of highly fragmented and dysfunctional mitochondria, impairing bioenergetic state and in turn activating multiple cell-death signalling.

This study examines the impacts of DRP-1 inhibition on proteins governing fission, fusion, mitophagy and apoptotic death, together with mitochondrial function, in cardiomyoblasts exposed to prolonged periods (up to 48 hrs) of oxidant stress (H\textsubscript{2}O\textsubscript{2} challenge).
5.2 METHODS AND MATERIALS

5.2.1 Cell culture

The H9c2 cell line used, initially derived from an embryonic rat heart cardiomyoblast line, was obtained from the American Type Culture Collection (ATCC, U.S.A.). Cells were maintained and grown in high glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and kept under 95% O₂ and 5% CO₂ at 37°C. Cell used in experiments were passaged up 15 times.

5.2.2 Reagents

Hydrogen peroxide (H₂O₂) and MDIVI-1 were purchased from Sigma Chemical Co. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and other tissue culture reagents were obtained from Life Technologies Inc. Tetramethylrhodamine methyl ester perchlorate (TMRM) was purchased from Molecular Probes (Thermo-Fischer Scientific, NJ, USA)

5.2.3 Oxidative challenge with H₂O₂

H9c2 cells were grown in 100 mm plates to 70-80% confluence. MDIVI-1 was dissolved in DMSO (<0.5 %) and added to DMEM at a final concentration of 50 μM for 40 min prior to oxidative insult with a single bolus dose of H₂O₂ administered at a concentration of 0.4 mM and incubated for 1, 2, 4, 6 or 8 hrs. Cells were then harvested via centrifugation, washed with PBS and suspended in 150 μL of ice-cold lysis buffer (0.1% Triton-X; Mops, 20 mM; EGTA, 2 mM; EDTA, 5 mM; sodium fluoride, 30 mM; sodium tetra-pyrophosphate, 20 mM; leupeptin, 10 μM; pepstatin A, 5 μM; benzamidine, 3 mM; phenylmethylsulfonyl fluoride, 1 mM; and sodium orthovanadate, 1 mM). Cell lysates were frozen at -80°C until further analysis.

5.2.4 MTT cell viability assay

Viability of H9c2 cells was determined using a colorimetric MTT assay. Viability
was assessed in cells challenged with H$_2$O$_2$ (± MDIVI-1) for 1-8 hrs. An MTT solution (200 µL of 5 mg/mL in DMEM) was added to each well before incubation in a dark room at 37°C for 2-4 hrs. Addition of DMSO dissolved crystal formations on the plate surface and absorbance was then read at 570 nm on a microplate reader (Tecan infinite M200 Pro, Mannedorf, Switzerland).

5.2.5 Lactate dehydrogenase efflux

Cell toxicity was determined quantitatively by assessing the release of intracellular lactate dehydrogenase (LDH) using a colorimetric LDH assay kit (Abcam). A 50 µL volume of each sample was transferred into each well of a 96-well plate, as per manufacturers instructions. Absorbance kinetics (at 490 nm) were monitored every 2 min over 1 hr in a microplate reader (Tecan infinite M200 Pro, Mannedorf, Switzerland). The release of LDH into culture medium was calculated as mU/mL.

5.2.6 Western immunoblotting

Cell signalling and protein expression was assessed over 8 hrs of oxidative stress. Since analyses in Chapter 4 did not encompass autophagy, apoptosis and the expression of other fission/fusion proteins, we additionally assess these proteins after 1 hr in the current study.

Sample preparation. Post treatment, cells were harvested via centrifugation, washed with PBS and suspended in 150 µL of ice cold lysis buffer (0.1% Triton-X; Mops, 20 mM; EGTA, 2 mM; EDTA, 5 mM; sodium fluoride, 30 mM; sodium tetra-pyrophosphate, 20 mM; leupeptin, 10 µM; pepstatin A, 5 µM; benzamidine, 3 mM; phenylmethanesulfonyl fluoride, 1 mM; and sodium orthovanadate, 1 mM). Cell lysates were frozen at -80°C until further analysis.

Protein quantification. Sample protein concentrations were measured via a BCA assay in a 96-well microplate (Pierce BCA protein assay kit), with absorbance changes monitored at 540 nm (Tecan infinite M200 Pro, Mannedorf, Switzerland).
**Electrophoresis.** Once thawed, samples are prepared in required volumes with loading dye, and denatured at 95°C for 5 min in a heating block. A sample of 28 µL was loaded onto a hand-cast 10% acrylamide gel. Protein separation was achieved by running the gel at 150 V for 80 min. The transfer of proteins was achieved using a polyvinylidene difluoride fluorescent membrane at a constant amperage of 350 mA for ~2 hours, and blocked with Odyssey fish serum for an additional 2 hours at room temperature. The transferred proteins were incubated in primary antibody (further detailed in Chapter 2) for 15-18 hours overnight at 4°C with gentle rocking. The PVDF membrane was washed in TBS for 5 min and again washed in TBST for 4 cycles of 5 min before incubation with the corresponding secondary antibody at room temperature in the dark. Membranes were visualized on a Licor Odyssey Infrared Imaging System (Millennium Science, Mulgrave, Australia) with protein densitometry of each sample normalised to an internal standard and loading control.

5.2.7 Cellular proliferation via xCELLigence system analysis

The xCELLigence system (ACEA Biosciences Inc., San Diego, CA) provides real-time measurement of cell proliferation via changes in electrical impedance in purpose-built culture plates (E-plate) as cell density increases or decreases (further detailed in Chapter 2 – section 2.3). Cells were seeded on an E-plate at a density of 10,000 cells per well, and placed in the RTCA SP instrument located in the cell culture incubator. Cell impedance was measured every 15 minutes for the first 6 hours and then every 30 minutes for up to 48 hours. The initial steep phase of growth categorizes the attachment. After 18 hours cells were pre-treated with MDIVI-1 for 40 min, which was dissolved in DMSO (0.5%) and added to DMEM at a final concentration of 50 µM. H2O2 was added at a final concentration of 0.4 mM and the cell impedance was continually monitored. As a control measure, baseline impedance was recorded in plates containing DMEM only and used to correct for background impedance of the wells.
5.2.8 Assessment of mitochondrial membrane potential (Δψₘ)

Prior to oxidative insult for either 1 or 4 hrs, cells were pre-treated with 50 µM MDIVI-1 for 40 min. Following experiments cells were incubated with 0.1 mM TMRM (further detailed in Methods chapter, section 2.3) and protected from light for 20 min at 37°C. Cells were harvested and re-suspended in PBS and analysed by the FACS Caliber flow cytometer at an excitation wavelength of 549 nm and emission wavelength of 575 nm. A count of 10,000 cells was obtained. Data was analysed using FlowJo software, with mean fluorescent intensity values used to compare levels of Δψₘ.
5.3 RESULTS

5.3.1 Effects of long-term \( \text{H}_2\text{O}_2 \) challenge ± MDIVI-1 on \( \text{H}9\text{c}2 \) viability/cytotoxicity.

The time-dependent effects of \( \text{H}_2\text{O}_2 \) on cell viability and cytotoxicity were evaluated in \( \text{H}9\text{c}2 \) cardiomyoblasts. A MTT assay was used to assess cell viability over long-term exposure to \( \text{H}_2\text{O}_2 \) ± 40 min pre-treatment with 50 \( \mu \text{M} \) MDIVI-1 (Fig. 5.1A). Extending previous assessment of cell viability in myoblasts subject to acute \( \text{H}_2\text{O}_2 \), these data indicate that \( \text{H}_2\text{O}_2 \) progressively reduces cell viability over 8 hr, with a significant ~50% reduction apparent after 4 hr. Pre-treatment with the fission inhibitor MDIVI-1 modestly improved cell viability (vs. \( \text{H}_2\text{O}_2 \) alone) over the initial 6 hr, but failed to improve final viability at 8 hr exposure to \( \text{H}_2\text{O}_2 \). Thus, MDIVI-1 appears to delay rather than prevent cell loss. To test whether these shifts in viability correspond with alterations in cell death, release of the enzyme LDH was assessed. A biphasic increase in LDH release was evident, with a plateau (50-55% increase) sustained between 2-4 hr of \( \text{H}_2\text{O}_2 \) challenge (vs. untreated control) (Fig. 5.1B). Pre-treatment with MDIVI-1 failed to significantly modify \( \text{H}_2\text{O}_2 \) dependent LDH release. Thus, effects of MDIVI-1 on viability determined via the MTT assay may reflect shifts in metabolic capacity rather than actual cell death.
Figure 5.1: The affects of H$_2$O$_2$ and MDIVI-1 on cell viability (MTT assay) and LDH efflux in H9c2 cardiomyoblasts. H9c2 cardiomyoblasts were pre-treated with vehicle or 50 µM MDIVI-1 for 40 min prior to H$_2$O$_2$ exposure (0.4 mM) for 8 hrs. A) Progressive changes in cell viability; and B) cytotoxicity (LDH efflux) were assessed. Data are means ± SEM (n = 3). *, P<0.05; **, P<0.01; ***, P<0.001 vs. time matched H$_2$O$_2$ treated only.
5.3.2 Proliferation of cardiomyoblasts during a prolonged 48 hr exposure to \( \text{H}_2\text{O}_2 \pm \text{MDIVI-1} \)

To assess the effects of \( \text{H}_2\text{O}_2 \) toxicity and MDIVI-1 on cardiomyoblast proliferation, cells were seeded at a density of \( 7.5 \times 10^3 \) cells/well and growth monitored continuously over 48 hr using an xCELLigence\textsuperscript{TM} RTCA DP instrument (ACEA, Biosciences) as detailed in the Methods chapters, section 2.3. The H9c2 cardiomyoblasts were seeded in a 96X microplate (E-plate) and left to attach for up to 18 hr prior to treatment with \( \text{H}_2\text{O}_2 \) (0.05, 0.1 or 0.4 mM). Cellular impedance was measured in real time every 30 min for the first 6 hr and then every hour thereafter for a total of 48 hrs \( \text{H}_2\text{O}_2 \) exposure. Data demonstrate that cardiomyoblast proliferation is highly sensitive to \( \text{H}_2\text{O}_2 \), with the cell index abruptly declining by \( \sim 50\% \) in the first 4 hr of \( \text{H}_2\text{O}_2 \) exposure (Fig. 5.2). However, pre-treatment with MDIVI-1 did not alter cardiomyoblast sensitivity to \( \text{H}_2\text{O}_2 \), and itself induced a significant marked reduction in viability or proliferation.
Figure 5.2: Real-time analyses of H9c2 viability during a 48 hr H_2O_2 challenge. Proliferation of H9c2 cardiomyoblasts was assessed in real time using the xCELLigence™ RTCA DP instrument. H9c2 cardiomyoblasts were subjected to a single bolus dose of H_2O_2 at varying concentrations ± pre-treatment with vehicle (0.5% DMSO) or 50 μM MDIVI-1. A) Control (vehicle) and MDIVI-1; B) 0.05 mM H_2O_2 + MDIVI-1; C) 0.1 mM H_2O_2 + MDIVI-1; and D) 0.4 mM H_2O_2 + MDIVI-1. Data are means ± SEM (n = 4). ϕ, P > .0001 vs. time matched H_2O_2 + MDIVI-1.
5.3.3 Survival kinase expression in cardiomyoblasts subject to oxidative stress.

Having established the acute effects (<60 min) of H$_2$O$_2$ administration on survival kinase expression (Chapter 4), the aim of this study was to characterise expression and activation of survival kinases AKT and ERK1/2 in cardiomyoblasts subjected to prolonged H$_2$O$_2$ exposure (2-8 hr), and evaluate the impacts of MDIVI-1 on these responses. In contrast to acute effects of brief H$_2$O$_2$ exposure (Chapter 4), total AKT expression was reduced by H$_2$O$_2$ from 2 to 8 hr. Interestingly, AKT expression was also modestly reduced by MDIVI-1 in non-stressed cardiomyocytes at baseline, with mixed effects during H$_2$O$_2$ exposure. Also contrasting insensitivity of AKT signalling to brief H$_2$O$_2$ challenge (Chapter 4), data in Fig 5.3B reveal profound phospho-activation of AKT from 2-8 hr of oxidant challenge. Treatment with MDIVI-1 exerted a biphasic effect on this activation pattern: AKT phospho-activation was augmented by MDIVI-1 at 2 hr, and reduced by MDIVI-1 from 4-8 hr. As with AKT, baseline ERK1/2 expression was reduced by MDIVI-1, and H$_2$O$_2$ exposure reduced ERK1/2 levels. This effect was initially exaggerated by MDIVI-1 treatment, though later changes were partially inhibited by the agent (Fig. 5.3C). Phospho-activation of ERK1/2 was increased with H$_2$O$_2$, plateauing from 2-4 hr exposure before declining at 6-8 hr. Treatment with MDIVI-1 also exerted a biphasic effect on ERK phosphorylation, initially exaggerating and subsequently suppressing ERK1/2 phospho-activation (Fig. 5.3D).
Figure 5.3: Survival kinase activation in H9c2 cardiomyoblasts challenged with H₂O₂ ± MDIVI-1 over a range of times. Cardiomyoblasts were pre-treated with vehicle or 50 μM MDIVI-1 for 40 min prior to sustained H₂O₂ challenge (0.4 mM). Cell lysates were assessed for total and phosphorylated ERK1/2 and AKT expression. A) Total AKT; B) ratio of phosphorylated: total AKT; C) total ERK1/2; and D) ratio of phosphorylated: total ERK1/2. Data are means ± SEM (n = 3). *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001 vs. time matched H₂O₂. (Representative immunoblots presented in supplementary figure 4)
5.3.4 Proteomic remodeling in cardiomyoblasts subjected to sustained H₂O₂ exposure (± MDIVI-1).

Having established (Chapter 4) that cardiomyocyte expression of fission protein DRP-1 is not altered with acute H₂O₂ insult (± MDIVI-1), the current study characterises mitochondrial fission, fusion and autophagy proteomics in cells subjected to sustained H₂O₂ stress for up to 8 hr. Exposure to H₂O₂ reduced DRP-1 expression by ~50% after 1 hr, with expression repressed for the remainder of the exposure period Baseline expression of DRP-1 was moderately augmented by MDIVI-1 treatment (Fig. 5.4A), which moderately altered early changes during H₂O₂ exposure but not final expression levels. Expression of MFN-2, the mitochondrial outer membrane fusion protein, was progressively attenuated by H₂O₂ over 8 hr (Fig. 5.4B), a change exaggerated by treatment with MDIVI-1. Expression of MFN-1 (Fig. 5.4C) was relatively insensitive to prolonged exposure to H₂O₂, with MDIVI-1 inducing small changes in expression between 2-6 hr but not at the final time point. Interestingly, the expression of fusion protein OPA-1 increased after 1 hr of H₂O₂ exposure before progressively falling below baseline levels (Fig. 5.4D). Treatment with MDIVI-1 reduced OPA-1 expression at 1-2 hrs of H₂O₂ exposure.

The mitophagy proteins PARKIN (Fig. 5.4E) and microtubule-associated protein 1A/1B-light chain 3 (LC3) (Fig. 5.4F) were assessed in parallel with mitochondrial quality control proteins. Peak expression of PARKIN was evident between 2-4 hr of H₂O₂ exposure, while MDIVI-1 reduced PARKIN activation at 1, 2 and 6 hrs. The ratio of LC3B-II/LC3B-I tended to decline over the H₂O₂ exposure period, with MDIVI-1 substantially augmenting the ratio at 8 hr (Fig. 5.4F).
Figure 5.4: Mitochondrial and autophagy protein expression in H9c2 cardiomyoblasts during an 8 hr H\textsubscript{2}O\textsubscript{2} challenge ± MDIVI-1. Cardiomyoblasts were pre-treated with vehicle or 50 μM MDIVI-1 for 40 min prior to exposure to 0.4 mM H\textsubscript{2}O\textsubscript{2} for up to 8 hr. Cell lysates were assessed for mitochondrial fission, fusion and autophagy protein expression. A) Total DRP-1; B) MFN-2; C) MFN-1; D) OPA-1; E) PARKIN; and F) ratio of LC3B-II/LC3B-I. Data are means ± SEM (n = 3). *, P>0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001 vs. time matched H\textsubscript{2}O\textsubscript{2}. (Representative immunoblots presented in supplementary figure 4).
5.3.5 Survival and death proteins in cardiomyoblasts subjected to sustained \( \text{H}_2\text{O}_2 \) stress (± MDIVI-1).

Relative expression levels of pro-apoptotic BAX and anti-apoptotic BCL-2 were quantified and expressed as a ratio to evaluate the apoptotic state of cells exposed to \( \text{H}_2\text{O}_2 \). A higher BAX to BCL-2 ratio is indicative of a pro-apoptotic state, while a low BAX to BCL-2 ratio is indicative of a pro-survival phenotype. At 1 hr and 2 hr, MDIVI-1 treatment in combination with \( \text{H}_2\text{O}_2 \) was significantly heightened vs. \( \text{H}_2\text{O}_2 \) treatment alone. Conversely, MDIVI-1 treatment in combination with \( \text{H}_2\text{O}_2 \) significantly reduced the BAX to BCL-2 ratio vs. \( \text{H}_2\text{O}_2 \) alone at 6 hr (Fig. 5.5A).

Expression of total and cleaved poly-ADP-ribose polymerase (PARP) was also assessed. Under basal conditions, the nuclear protein PARP is primarily responsible for routine repair of DNA breaks in response to diverse stresses. However, PARP yields several cleavage fragments (differing in molecular weight) that participate in pro-death signalling. Therefore, PARP (Fig. 5.5B/5.5C) was assayed in both its pro-survival uncleaved (116 kDa) and pro-death cleaved (89 kDa) forms to assess potential involvement in \( \text{H}_2\text{O}_2 \) induced toxicity. Uncleaved PARP (116 kDa) was reduced by 30-40% during \( \text{H}_2\text{O}_2 \) challenge, an effect unaltered by MDIVI-1. In comparison, cleaved PARP (Fig. 5.5C) (identifiable at 89 kDa) expression was low under basal conditions and increased significantly at all \( \text{H}_2\text{O}_2 \) exposure times. Treatment with MDIVI-1 negated the initial increase in cleaved PARP expression over the initial 2 hrs of \( \text{H}_2\text{O}_2 \) exposure but failed to alter cleaved PARP from 4-8 hrs.
Figure 5.5: Assessment of pro- and anti-apoptotic protein expression in H9c2 cardiomyoblasts during an 8 hr H₂O₂ challenge ± MDIVI-1. Cardiomyoblasts were pre-treated with vehicle or 50 μM MDIVI-1 40 min prior to sustained exposure to 0.4mM H₂O₂. Cell lysate was assessed for pro- and anti-apoptotic protein expression. A) Ratio of BAX/BCL-2; B) uncleaved PARP (116 kDa); and C) cleaved PARP (89kDa). Data are means ± SEM (n = 3). *, P>0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001 vs. time matched H₂O₂. (Representative immunoblots presented in supplementary figure 4).
5.3.6 Mitochondrial membrane potential in cardiomyoblasts subject to prolonged H$_2$O$_2$ exposure (± MDIVI-1).

Effects of H$_2$O$_2$ and MDIVI-1 on the mitochondrial membrane potential ($\Delta \psi_m$) were assessed in H9c2 cells subject to 1 or 4 hr of H$_2$O$_2$ challenge. Exposure of H9c2 cells to H$_2$O$_2$ significantly reduced TMRM fluorescence, reflecting a fall in potential $\Delta \psi_m$. This effect was countered by pre-treatment with MDIVI-1 (Fig. 5.6A and Fig. 5.6B).

Figure 5.6: Mitochondrial membrane potential in H9c2 cardiomyoblasts during an 8 hr H$_2$O$_2$ challenge ± MDIVI-1. Cardiomyoblasts were pre-treated with vehicle (CTRL) or 50 μM MDIVI-1 40 min prior to exposure to 0.4 mM H$_2$O$_2$ for up to 4 hr. Analysis was performed via flow cytometry, with fluorescence emitted at 588 nm detected via the FL2-H channel. A) Median FL2-H fluorescence in individual samples; and B) relative change in fluorescence. Data are means ± SEM ($n = 2$). **, $P<0.01$; vs. untreated CTRL; #, $P<0.01$ vs. time matched H$_2$O$_2$. 
5.4 DISCUSSION

While sustained oxidative stress is involved in the pathogenesis of myocardial ischaemia-reperfusion injury, the extent to which dysfunctional mitochondrial are ‘remodelled’ and involved in activating cell death pathways in heart disease remains to be more fully detailed (Cho et al., 2009; Ong and Gustafsson, 2012). The present study aimed to characterise proteome shifts in mitochondrial remodelling, cell death signalling and survival proteins during prolonged oxidant challenge in cardiomyoblasts, and test the effects of the mitochondrial fission inhibitor MDIVI-1.

**DRP-1 inhibition and its impact on cell viability**

The present findings demonstrate that H9c2 cardiomyoblast viability is significantly reduced (by ~50%) following a 4 hr H$_2$O$_2$ challenge, while MDIVI-1 salvaged cardiomyoblast cell viability in a time dependant manner (up to 8 hours of sustained oxidative stress). Together with MTT, the release of LDH was used as an indicator of cytotoxicity in cardiomyocytes challenged with hydrogen peroxide. Treatment with H$_2$O$_2$ alone, substantially increased LDH release by ~50% after 1 hr, remaining elevated after 4 hours of sustained oxidative stress. This data is consistent with others, confirming that sustained H$_2$O$_2$ exposure produces substantial cytotoxicity (Miguel et al., 2009; Zhang et al., 2013; Park et al., 2014). Pre-treatment with MDIVI-1 failed to reduce the cytotoxicity of H$_2$O$_2$, with similar LDH efflux of LDH across the period of oxidative stress. Contrary to effects on LDH, treatment with MDIVI-1 did enhance cell viability based on the MTT cell viability assay. This suggests no major survival effect of MDIVI-1 in this setting, though the agent appears to significantly delay the progression of cellular dysfunction (based on MTT data). Previous studies have established that the MTT assay is highly sensitive, based on the enzymatic reaction of the active compound MTT in mitochondria (Fotakis and Timbrell, 2006), compared to the release of LDH, which is mediated after the rupturing and/or damage to the cell membrane. Since this study implemented a mitochondrially-targeted therapy, the MTT cell viability assay may be sensitive to the effects of MDIVI-1 (rather than cell membrane rupture).
Real time analysis of cell proliferation via the xCELLigence system was used as an additional method to evaluate cytotoxic affects of a single bolus dose of H$_2$O$_2$. The xCELLigence system complements conventional cytotoxic assays and provides a physiological functional experimental system. Over a period of 48 hr, cardiomyoblast proliferation (determined via cell index) declined as the concentration of H$_2$O$_2$ was increased (from 0.05 to 0.4 mM). This is consistent with the data of Guan et al. (2012) who established that H$_2$O$_2$ significantly impacts the cell index at concentrations greater than 0.1 mM, with reductions evident between 2-6 hr of H$_2$O$_2$ treatment. Interestingly, MDIVI-1 alone substantially impeded the cell index (an arbitrary measure of cell attachment), a finding consistent with others who have shown MDIVI-1 to produce an anti-proliferative and cytotoxic affect in proliferative cancer cells (Qian et al., 2012; Qian et al., 2014; Qian et al., 2015; Suzuki-Karasaki et al., 2015). Findings of these previous studies revealed that treatment with MDIVI-1 interferes with both DNA replication and cell cycle progression via anti-proliferative and pro-apoptotic mechanisms, while reducing O$_2$ consumption and mitochondrial respiratory capacity. While this is the first study to assess the effects of MDIVI-1 in H9c2 cells, a reduction in proliferation may reflect metabolic modifications rather than an index of ‘cell death’, impulse the highly metabolic characteristics of these cardiomyoblast cells.

**Stress kinase signalling during sustained oxidative stress**

*ERK1/2*. The activation of stress kinase signalling under conditions of oxidative stress has been well documented, with acute H$_2$O$_2$ treatment serving as a protective preconditioning stimulus (Han et al., 2001; Sharma and Singh, 2001). Prior data in Chapter 4 confirms a transient ERK1/2 activation in the initial minutes of oxidative stress, consistent with previous reports (Angeloni et al. 2011). These transient acute changes in ERK1/2 may reflect a key defence mechanism against injurious stimuli (i.e. ischaemia-reperfusion injury) that stimulate oxidative injury, mitochondrial dysfunction and opening of the mPTP (Aikawa et al., 1997; Uchiyama et al., 2004). However, the current data suggest a secondary and slower activation of ERK1/2 beyond 2 hr of H$_2$O$_2$ treatment. There is evidence that ERK1/2 activates fission in myocytes (Yu et al., 2011), neuronal cells (Gan et al., 2014) and embryonic fibroblasts (Prieto et al., 2016); ERK1/2 also acts downstream of DRP-1 in control of cell proliferation (Lim et al., 2015); and DRP-1 inhibitors impair myocardial ERK1/2 signalling and augment cardiac and skeletal AKT signalling (Jheng et
These reports are consistent in part with effects of MDIVI-1 in the current study. Blockade of early ERK1/2 activation may contribute to suppression of fission with MDIVI-1 (Yu et al., 2011), and/or stem from associated reduction of mitochondrial dysfunction and ROS generation (Jheng et al., 2012). However, it should be acknowledged that ERK1/2 can both promote and inhibit cell survival, and exaggeration of late ERK1/2 phosphorylation in stressed cells may worsen death outcomes (Koinzer et al., 2015).

AKT. The role of AKT in cardiac and mitochondrial protection has received considerable attention, with more recent evidence suggesting activation of the PI3K/AKT pathway is protective by supporting a pro-mitochondrial fusion network (Ong and Hausenloy, 2010). An increase in phospho-AKT by MDIVI-1 has been documented by Gharanei et al. (2013), who explored the efficacy of MDIVI-1 as a cardioprotectant in doxorubicin induced toxicity in both Langendorff perfused hearts and HL-1 cells. A transient increase in AKT signalling, induced by MDIVI-1, may potentially contribute a pre-conditioning and pro-survival effect, improving mitochondrial stress resistance in the acute stages of oxidative injury. However, while the initial activation of AKT was augmented by MDIVI-1 in the current study, later phospho-activation was impaired. A pattern of augmentation of early survival kinase activation followed by inhibition of late kinase activation emerges with MDIVI-1. Exaggerated AKT phosphorylation is predicted to be protective, potentially promoting fusion vs. fission (Ong and Hausenloy, 2010) However, insensitivity of AKT to MDIVI-1 in post-ischaemic hearts and un-stressed myoblasts is inconsistent with this proposal. Differential disruption of survival kinase signalling with MDIVI-1 may additionally contribute to mixed impacts of DRP-1 inhibition on stress responses.

The impact of sustained oxidative stress on DRP-1 activation and pro-apoptotic mediators

Mitochondrial fragmentation (fission) is implicated in apoptosis, however the time course of pro- and anti- apoptotic events during prolonged oxidative stress are not well defined. This current study demonstrated that expression of DRP-1 is substantially reduced with prolonged oxidant stress, a response modified in a complex manner by MDIVI-1 (which counters and exaggerates this change at different times). Emerging evidence
suggests that inhibition of DRP-1 translocation to the OMM prevents pro-apoptotic signalling and promotes activation of caspases, though the time-dependence of these changes remains to be clarified (Zhang et al., 2013; Park et al., 2015). Interestingly, a peak in DRP-1 expression at 2 hr following H₂O₂ treatment was accompanied by an increase in the BAX/BCL-2 ratio, suggesting pro-apoptotic signalling may be associated with DRP-1 activation. Activation of the pro-apoptotic BCL-2 family member, BAX, was substantially increased following 1 hr of H₂O₂ treatment, consistent with sustained oxidative stress promoting apoptotic signalling. Previous studies have reported that BAX activation is prompted by a loss in the mitochondrial membrane potential, a precursor to the subsequent release of cytochrome c, formation of the apoptosome and downstream activation of caspases and PARP cleavage (Oettinghaus et al., 2016). The current data suggest that the cleavage of PARP correlates with the peak activation of BAX at 1-2 hrs of oxidative stress.

Treatment with MDIVI-1 significantly suppressed PARP cleavage in the early stages of oxidant stress. Cleavage between Asp214 and Gly215 is related to caspase-3 activation, initiating cellular disassembly with execution of apoptosis. Based on these observations, it is plausible that MDIVI-1 acts independently to mitochondrially induced BAX activation, inhibiting PARP cleavage by caspase-3. These findings suggest that MDIVI-1 may limit pro-apoptotic signalling via PARP, thus delaying irreversible cellular dysfunction (mediated via apoptosis) under conditions of sustained oxidative stress.

**DRP-1 inhibition and control of mitophagy**

**PARKIN.** PARKIN, a ubiquitin-protein ligase, is implicated in mitochondrial quality control via autophagy, mediating clearance of degraded mitochondria and associated proteins which assist in maintaining mitochondrial morphology. Reduced activation of PARKIN has been associated with abnormal enlargement of mitochondria and decreased DRP-1 activation (Poole et al., 2008). Kim and colleagues (2016) examined the neuroprotective affects of DRP-1 inhibition (via MDIVI-1) in mice, reporting reductions in PARKIN mediated degradation, DRP-1 activation, the BAX/BCL-2 ratio and seizure activation. In contrast to such findings, the present study reports a monophasic increase in PARKIN expression following H₂O₂ treatment, alone or in combination with MDIVI-1. Consistent with Won et al. (2015), our current findings indicate that H₂O₂ treatment for a period of >2 hr induces both mitophagy and pro-apoptotic signalling in association with mitochondrial dysfunction. Interestingly, increased PARKIN expression with sustained
oxidant stress may be indicative of up-regulation and recruitment to the outer mitochondrial membrane to initiates mitophagy and the removal of damaged mitochondria. In contrast, previous studies suggest PARKIN contributes an anti-apoptotic function with various injurious stimuli (Kubli et al., 2013). Whether up-regulated PARKIN may be an adaptation to limit cell death in the current model is unclear.

**LC3B.** Under conditions of oxidative stress, the cellular process of autophagy (removing/digesting senescent and damaged organelles) is implicated in mitochondrial dysfunction (Gottlieb and Carreira, 2010; Dutta et al., 2013). The autophagy marker, LC3B, was substantially increased with H2O2 exposure, consistent the study of Yin and colleagues (2015), who identified LC3B activation in cardiomyoblasts subjected to prolonged 24 hr of oxidative stress. The transient increase in LC3B activation with oxidant stress preceded peak PARP cleavage, and was positively correlated with increased pro-apoptotic signalling and a reduction in mitochondrial membrane potential (Δψm). Consistent with prior work from Solesio and colleagues (2013), the present study shows that activation of autophagic pathways precede cell death signalling. Prior studies also indicate that blockade of autophagic cell death is beneficial, prolonging cellular function under conditions of ischaemia (Adhami et al., 2006; Wang et al., 2011; Wen et al., 2014). However, treatment with MDIVI-1 negated the transient activation of LC3B, which negatively correlated with activation of pro-apoptotic changes (BAX induction and PARP cleavage). This may suggest that inhibition of LC3 may in fact be detrimental, promoting apoptotic signalling and recruitment of BAX to the OMM to induce cell death.
Summary

In summary, the current data suggest oxidative stress triggers mitochondrial dysfunction, and activates stress kinase and autophagic signaling, changes that are modified in a complex manner by the DRP-1 inhibitor MDIVI-1. Nonetheless, the experimental oxidative insult used has limitations. Cardiomyocytes are conventionally subjected to a bolus of hydrogen peroxide on initiation of stress and it is likely the level will decline from this point in a time-dependent manner due to breakdown/metabolism. This may cloud final cell death outcomes, and in any experiment of this nature it is unclear what the precise final \( \text{H}_2\text{O}_2 \) concentration is. Treatment with MDIVI-1 reduces transient activation of LC3, potentially impacting apoptotic signaling. Although the mechanism by which MDIVI-1 negates LC3 activation remains unclear, analysis of protein translocation would be of value in clarifying the complex nature of these proteomic shifts. The use of MDIVI-1 as a therapeutic agent in preventing and/or delaying the onset of mitochondrial dysfunction in the pathogenesis of cardiovascular disease remains contentious, requiring further investigation.

Studies detailed within Chapter 4 and 5 identified mixed effects with the use of DRP-1 inhibitor under conditions of acute and chronic oxidant stress. Mitochondrial dynamics under perturbed conditions are complex in nature, as evidenced by the mixed outcomes identified with the use of a DRP-1 inhibitor. Mitochondrial dysfunction has been highlighted in models of disease. Type 2 diabetes mellitus is a comorbidity of CVD, with recent work highlighting that sustained conditions of hyperglycaemia increase myocardial stress that may promote mitochondrial dysfunction. Therefore, studies detailed in Chapter 6 examine the effects of DRP-1 inhibition in cardiomyoblasts maintained in modified glucose and insulin conditions.
CHAPTER 6: H9c2 Cardiomyoblast
Responses to Glucose and Insulin: Effects
of DRP-1 Inhibition with MDIVI-1

6.0 ABSTRACT

Influences of glucose and insulin levels on mitochondrial dynamics and respiratory function are relevant to the pathogenesis of diabetic cardiomyopathy, though incompletely defined. In this study cell viability mitochondrial respiratory function, fission and fusion proteins were studied in H9c2 cardiomyoblasts maintained in low to high glucose and insulin for 24-48 hrs. Effects of the mitochondrial fission inhibitor MDIVI-1 were also assessed. Hyperglycaemia (100 mM glucose) in combination with hyperinsulinaemia (100 nM) stimulated cardiomyoblast cell proliferation at 24-36 hrs incubation ($P<0.05-0.01$). Analysis of mitochondrial function revealed that low glucose (5 mM), and hyperglycaemia + hyperinsulinaemia (yet not hyperglycaemia alone) increased basal and maximal respiratory rates, spare respiratory capacity, and ATP generation. Oxygen consumption and extracellular acidification data also support a glycolytic shift in cells maintained in modified glucose conditions as attributed to the ratio (OCR/ECAR). No significant changes in fission or fusion proteins were observed with shifts in glucose or insulin, though hyperglycaemia alone appeared to lower the MFN-1/DRP-1 ratio (suggesting a pro-fission effect). While effects of MDIVI-1 were investigated, marked inhibition of H9c2 proliferation with both MDIVI-1 and vehicle (DMSO) raises questions as to the selectivity of MDIVI-1 in this model. Treatment with MDIVI-1 appeared to suppress DRP-1 expression and enhance OPA-1 expression in myoblasts exposed to hyperglycaemia + hyperinsulinaemia, and increased the MFN-1:DRP-1 ratio in hyperglycaemic myoblasts, suggesting suppression of fission vs. fusion under these conditions. These results demonstrate that elevated glucose alone does not impact cardiomyoblast proliferation or mitochondrial respiratory function, whereas elevation in both glucose + insulin enhances
both parameters. Treatment with a DRP-1 inhibitor may paradoxically promote a fusion vs. fission environment via shifts in DRP-1, MFN-1 and OPA-1 expression in these cells, though potential effects of DMSO vehicle complicate interpretation of outcomes. Finally, increases in respiratory function observed in low glucose conditions may be relevant to improved mitochondrial respiratory capacity under conditions of caloric reduction.
6.1 INTRODUCTION

Conditions of sustained high glucose ± insulin (chronic diabetes) are associated with increased myocardial oxidative stress and cardiac dysfunction, promoting the development of diabetic cardiomyopathy and also ischaemic heart disease (IHD). Recent research links increased generation of myocardial free radicals to mitochondrial dysfunction (Maimaitijiang et al., 2016). However, influences of hyperglycaemia and/or hyperinsulinaemia on mitochondrial morphology and dynamics are poorly defined. The work of Sebastián et al. (2012) highlights the essential role of fusion protein MFN-2 in insulin signalling in both liver and skeletal muscle, with ROS and endoplasmic reticulum (ER) largely contributing to insulin-resistance in MFN-2 deficient mice. This important finding links metabolic disturbances to impairment of mitochondrial dynamics. Dysfunctional mitochondria have been linked to reduced proliferation, survival kinase signalling and oxidative phosphorylation capacity, all of which will influence metabolic homeostasis and mitochondrial regulation. An increase in mitochondrial division, governed by DRP-1 translocation to the outer mitochondrial membrane (OMM), has been linked to impaired mitochondrial respiration and correlates with increased mitochondrial ROS production (Smith et al., 2013). Since the impact of DRP-1 inhibition on mitochondrial and cellular function under hyperglycaemic or hyper-insulinaemic conditions remains to be explored, this study valuates effects of glucose and insulin (individually and together) on cell proliferation, mitochondrial function and expression of proteins governing mitochondrial fission and fusion.
6.2 METHODS AND MATERIALS

6.2.1 Cell culture and experimental protocol

The H9c2 cell line was obtained from the American Type Culture Collection (ATCC, U.S.A.) as further detailed in Chapter 2. Cells were maintained and grown in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and kept under 95% O₂ and 5% CO₂ at 37°C. Cells used in experiments were up to passage 15. Cells were maintained for 24 hr under conditions of: low (5 mM), moderate (25 mM) and high (100 mM) glucose; hyperinsulinaemia (100 nM); or combined hyperglycaemia (100 mM) and hyperinsulinaemia (100 nM). Note that the recommended ('normal') levels of glucose in media for H9c2 cells is 25 mM. Cells were exposed to 50 µM MDIVI-1 or 0.5% DMSO vehicle.

6.2.2 Reagents

Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS) and tissue culture reagents were obtained from Gibco (Life Technologies, Carlsbad, CA, USA). All other chemicals were acquired from Sigma Chemical Co. (St Louis, MO, USA).

6.2.3 Mitochondrial functional analysis

H9c2 cells were plated in DMEM (supplemented with 10% FBS and penstrep) at a density of 10,000 cells per well in 8-well XFp mini-plates, and left to attach overnight in an incubator (37°C 5% CO₂ and 95% O₂). Cells were then exposed to modified DMEM media containing differing glucose and insulin levels for 24 hr (37°C, 5% CO₂ and 95% O₂). The cells were washed in PBS and supplemented with XFp modified media (1 mM Pyruvate, 2 mM glutamine, 10 mM glucose, pH 7.4) and incubated in a 37°C non-CO₂ incubator for 45 min prior to assay. A Seahorse XFp Extracellular Flux Analyser (Bioscience, Billerica, MA, USA) was used to evaluate O₂ consumption rate (OCR). Measurements were performed in 6 min cycles to establish basal and maximal metabolic rates. After basal OCR was measured, oligomycin (1 µM), FCCP (2 µM) and antimycin A (0.5 µM) were added
sequentially to wells for interrogation of specific functions. Measurements of OCR were normalised to cell density seeded per well (supplementary figure 1).

6.2.4 Western immunoblotting

**Extraction procedure.** Post treatment, cells were harvested by centrifugation and washed twice with PBS prior to re-suspension in 150 µL cell lysis buffer containing protease and phosphatase inhibitors; 0.1% Triton-X; Mops, 20 mM; EGTA, 2 mM; EDTA, 5 mM; sodium fluoride, 30 mM; β-glycerophosphate, 40 mM; sodium tetra-pyrophosphate, 20 mM; leupeptin, 10 µM; pepstatin A, 5 µM; benzamidine, 3 mM; phenylmethanesulfonyl fluoride, 1 mM; and sodium orthovanadate, 1 mM.

**Protein quantification.** Protein concentrations of lysate samples were determined via a BCA assay performed in a 96-well microplate (Pierce BCA protein assay kit), with absorbance changes monitored at 540 nm (Tecan infinite M200 Pro, Mannedorf, Switzerland). Aliquots of 20 µg protein were then prepared with the appropriate volume of Kinexus buffer containing: 20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 30 mM NaF, 40 mM β-glycerophosphate, 20 mM NaPP, and stored in -80°C.

**Electrophoresis.** Once thawed, samples are prepared in required volumes with loading dye and denatured at 95°C for 5 min in a heating block. A protein sample of 20 µg (28 µL volume) was loaded onto a hand-cast 10% acrylamide gel. Protein separation was achieved by running gels at 150 V for 80 min. The transfer of proteins was achieved using a polyvinylidene difluoride fluorescent membrane (PVDF) at a constant amperage level of 350 mA for ~2 hr, and blocked with Odyssey fish serum for an additional 2 hr at room temperature. The transferred proteins were incubated in primary antibody (further detailed in Chapter 2) for 15-18 hrs at 4°C with gentle rocking. The PVDF membrane was washed in TBS for 5 min and again washed in TBST for 4 cycles of 5 min before incubation with corresponding secondary (fluorescent) antibody at room temperature in the dark. Membranes are visualized on a Licor Odyssey Infrared Imaging System (Millennium Science, Mulgrave, Australia), and protein densitometry normalised to an internal standard and loading control.
6.2.5 Cellular proliferation via xCELLigence system analysis

The xCELLigence system (ACEA Biosciences Inc., San Diego, CA) provides real-time measurement of cell proliferation via changes in electrical impedance in purpose-built culture plates (E-plate). The ‘cell index’ is a dimensionless, relative measure reflecting changes in electrical impedance due to cell viability, number, morphology and degree of adhesion: the more cells attached to the surface the greater the impedance and cell index (further detailed in Chapter 2 section 2.3). The H9c2 cells were harvested with trypsin/EDTA, washed with DMEM, and resuspended in the modified DMEM (Control glucose (25 mM), low glucose (5 mM), high glucose (100 mM), high glucose (100 mM) + hyperinsulinaemia (100 nM), supplemented with 10% FBS. Cells were seeded on an E-plate at a density of 10,000 cells per well, and placed immediately in the RTCA instrument to measure cell impedance over 48 hours. In a second experiment, cells were seeded and maintained in modified DMEM (as described) and placed in the RTCA instrument to monitor cellular impedance. After 18 hours (to allow for cell attachment) cells were incubated with 50 µM MDIVI-1 or vehicle (0.5% DMSO) and placed back into the RTCA instrument in which cellular impedance was measured every 30 minutes over 48 hours. Baseline impedance was recorded in plates containing DMEM only. Changes in impedance (thus the cell index) were corrected for background impedance of each well.
6.3 RESULTS

6.3.1 Effects of glucose and insulin (± MDIVI-1) on cardiomyoblast proliferation

Growth of cardiomyoblasts was assessed using the xCELLigence™ RTCA DP system. At 24 hr post seeding, a divergence in cell index was evident in cardiomyoblasts maintained in hyperglycaemia + hyperinsulinaemia conditions, which was maintained until 36 hr (vs. control) (Fig. 6.1). An insignificant trend to increased proliferation was also observed with both low (5 mM) and high (100 mM) glucose (vs. 25 mM). Treatment with MDIVI-1 significantly reduced cell proliferation though a similar effect was apparent with vehicle (supplementary figure 2).

Figure 6.1: Cardiomyoblast proliferation under varying glucose and insulin levels. An assessment on H9c2 cardiomyoblast proliferation was undertaken over 48 hr exposure to: Control glucose (25 mM), low glucose (5 mM), high glucose (100 mM), high glucose (100 mM) + hyperinsulinaemia (100 nM), *, P<0.05; **, P<0.01; high glucose (100 mM) + insulin (100 nM) vs. CTRL. Data are means ± SEM (n = 4).
6.3.2 Effects of glucose and insulin on the OCR/ECAR ratio

The Seahorse XFp system can simultaneously assess extracellular acidification rate (ECAR) and OCR (Fig. 6.2A and Fig. 6.2B). The ECAR is a measure of glycolytic H⁺ generation and accumulation under varying conditions. Thus, to gain insight into potential shifts in glycolytic vs. oxidative metabolism the ratio of OCR and ECAR was assessed (Fig. 6.2C). Under basal conditions, the OCR/ECAR values ranged between 4.5 and 5.24, indicative of predominantly oxidative ATP generations. Inhibition of mitochondrial ATP synthase with oligomycin significantly reduces the OCR/ECAR ratio (by ~50%), reflecting impaired oxidative vs. glycolytic metabolism. An OCR/ECAR value <5 indicates increased dependence on glycolytic metabolism for ATP production.

Low glucose alone elevated ECAR and significantly reduced the OCR/ECAR ratio (vs. control), while the OCR/ECAR ratio in high glucose conditions was modestly reduced (by ~15%). Addition of oligomycin substantially reduced the ratio of OCR/ECAR under all modified conditions, although to be expected since ATPase activity is inhibited with the compound oligomycin. A large reduction in the OCR/ECAR ratio suggests a glycolytic shift stimulated under conditions that are inhibitory to OxPhos capacity, which were exaggerated in modified glucose conditions vs. CTRL.

6.3.3 Effects of glucose and insulin on mitochondrial respiratory function

The O₂ consumption rate (OCR) was assessed in H9c2 cardiomyoblasts, maintained under varying levels of glucose and insulin. A mitochondrial ‘stress test’ was undertaken using the Seahorse XFp system, with sequential addition of oligomycin (ATP synthase inhibitor), FCCP (mitochondrial uncoupler) and a combination of antimycin A and rotenone (complex I and III inhibitors) to assess specific mitochondrial functions. A pattern of respiratory stimulation with both low glucose and high glucose + insulin was observed, whereas high glucose alone did not significantly modify respiration. Both basal and maximal OCR values were elevated by low glucose and high glucose + insulin (vs. 25 mM glucose). Estimated ATP production was also elevated, as was the spare respiratory capacity (SRC) (though the latter effect was not significant for high glucose + insulin). Non-mitochondrial OCR was unaltered by varying glucose and insulin levels (Fig. 6.3).
Figure 6.2: Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in cardiomyoblasts exposed to varying glucose and insulin levels. Cardiomyoblasts were exposed to 25 mM glucose (control), 5 mM glucose (hypoglycaemia), 100 mM glucose (hyperglycaemia) and 100 mM glucose (hyperglycaemia) + 100 nM insulin (hyperinsulinaemia). Mitochondrial function was assessed via addition of: oligomycin, FCCP and antimycin A in combination with rotenone, which were added sequentially over 60 min. A) Oxygen consumption rate; B) ECAR; and C) ratio of OCR: ECAR. Data are means ± SEM (n = 2 per group).
Figure 6.3: Mitochondrial respiratory function. Oxygen consumption was assessed in H9c2 cardiomyoblasts subjected to varying levels of glucose and insulin. Control (CTRL, 25 mM glucose); high glucose (HG, 100 mM); low glucose (LG, 5 mM); high glucose (HG, 100 mM) + insulin (INS, 100 nM). Details of respiratory parameters are provided in the Chapter 2 (Supplementary figure 1). Data are means ± SEM (n = 2 per group). *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001; vs. CTRL (individual measurable parameters).
6.3.4 Mitochondrial fission and fusion proteins in cardiomyoblasts maintained under varying glucose and insulin levels (± MDIVI-1).

Alterations in glucose and insulin failed to significantly modify fission or fusion protein expression in cardiomyoblasts (Fig. 6.4). The ratio of MFN-1/DRP-1, an indicator of propensity to fusion over fission, was also unaltered, though appeared suppressed with high glucose alone. Treatment with MDIVI-1 reduced basal expression of MFN-1 in control cells, and in cells exposed to high glucose + insulin MDIVI-1 reduced DRP-1 expression (Fig. 6.4B) while elevating OPA-1 expression (Fig. 6.4D). The MFN-1: DRP-1 ratio (Fig. 4.4C) in cells exposed to hyperglycaemia was also increased by MDIVI-1. Thus, there is a tendency for MDIVI-1 treatment to promote fusion over fission under conditions of elevated glucose.
Figure 6.4: Effects of glucose and insulin (± MDIVI-1) on cardiomyoblast expression of fission and fusion proteins. Cardiomyoblasts were maintained with control glucose (CTRL, 25 mM), low glucose (LG, 5 mM), high glucose (HG, 100 mM), insulin (100 nM) and combined insulin (100 nM) + high glucose (100 mM). Cells were exposed to 50 µM MDIVI-1 or 0.5% DMSO vehicle for 40 min, before analysis of: A) MFN-1; B) DRP-1; C) ratio of MFN-1: DRP-1; and D) OPA-1. Data are means ± SEM (n = 2-3 per group). **, P<0.01; ***, P<0.001 (vs. CTRL under paired treatment conditions).
6.4 DISCUSSION

Conditions of sustained hyperglycaemia induce cellular stress and damage, involving increased production of ROS together with promotion of protein glycosylation and accumulation of advanced glycation end-products (AGEs). These changes ultimately compromise myocardial and mitochondrial function. The current study assessed impacts of glucose and insulin on cardiomyoblast proliferation, mitochondrial respiratory function and expression of fission and fusion proteins. The data reveal stimulatory effects of elevated glucose on myoblast proliferation, and demonstrate that hyperglycaemia alone is insufficient to impact mitochondrial function, requiring the presence of insulin. Interestingly, hypoglycaemic conditions also promote mitochondrial respiratory function, an effect potentially relevant to beneficial mitochondrial impacts of fasting/calorie restriction. Unfortunately, effects of the fission inhibitor MDIVI-1 are clouded by potential toxicity of the vehicle (0.5% DMSO), rendering interpretation of outcomes problematic. Nonetheless, data suggest MDIVI-1 may induce a pro-fusion vs. fission state in cells exposed to altered glucose and insulin.

Effects of glucose and insulin and MDIVI-1 on cardiomyoblast proliferation

The cell proliferation assay employed here, involving use of the xCELLigence system (ACEA Biosciences), offers the advantage of real-time measurements of cell viability or growth with minimal experimental disturbances. Cell proliferation was enhanced in cardiomyoblasts maintained in hyperglycaemia and hyperglycaemia + hyperinsulinaemia, with significant differences in growth evident between 24-36 hr. However, this enhanced growth was not maintained, with proliferation reducing across all groups 36 hr after seeding. Of course, adult cardiomyocytes do not proliferate. Nonetheless, these data for the cardiomyoblast line support increased cellular proliferation with elevated glucose that may be relevant to cardiac remodelling and hypertrophy in diabetes.

Treatment with MDIVI-1 appeared to markedly suppress the cell index across all groups, supporting the importance of DRP-1 and fission in cell division/proliferation (Qian et al., 2012; Qian et al., 2014; Qian et al., 2015; Suzuki-Karasaki et al., 2015). However, a similar inhibitory effect was evident with the 0.5% DMSO vehicle. Cytotoxic effects of high concentrations of DMSO are well established with a multitude of effects reported in
murine and aquatic organisms (Hanslick et al., 2009; Galvao et al., 2014; Xiaoqin et al., 2016). The 0.5% level applied in the current study was not predicted to induce toxicity. For example, Galvao et al. (2014) recently reported concentration-dependent toxicity in vitro in a retinal neuronal cell line at concentrations >1%. The Environmental Protection Agency in the United States specifies maximal allowable concentrations for DMSO between 0.01-0.05%. Whether the toxicity of DMSO may differ under different experimental conditions (in vivo vs. in vitro) remains to be determined. Thus, the impact of DRP-1 inhibition per se is unfortunately unclear given apparent toxicity with DMSO alone. While confounding interpretation of the effects of MDIVI-1 in this study, valuable information is nonetheless acquired regarding effects of glucose and insulin on respiratory function (and cell proliferation).

Effects of glucose and insulin on mitochondrial function

Evidence indicates that models of both T2D and T1D exhibit mitochondrial dysfunction potentially induced by mitochondrial ROS and oxidative stress (Kelley et al., 2002; Hojlund et al., 2008; Makino et al., 2010; Huang et al., 2014). Previous reports suggest hyperglycaemic conditions facilitate ROS over production via the electron transport donors NADH and FAHD₂, stimulating electron flux within the ETC and leading to limitation of electron transport via complex III and accumulation of electrons within coenzyme Q to drive reduction of O₂ to superoxide (Nishikawa et al., 2000; Lambert and Brand, 2004; Rolo and Palmeira, 2006). Mitochondrial analyses reveal several interesting findings, including the insulin-dependence of stimulatory effects of hyperglycaemia, together with paradoxic respiratory stimulation via hypoglycaemia. These data contrast evidence of mitochondrial dysfunction in cardiomyocytes maintained for 48 hrs in high (33 mM) glucose (Dassanayaka et al., 2015), an inhibition that appears independent of protein O-GlcNAcylation. In other cell types chronic hyperglycaemia progressively suppresses mitochondrial OCR (Sen et al., 2015), in association with increased ROS production. The 48 hr treatment period of hyperglycaemia here may be insufficient to depress mitochondrial respiratory capacity.

Combined hyperglycaemia and hyperinsulinaemia produced a significant stimulatory affect on mitochondrial respiration, whereas hyperglycaemia alone was largely ineffectual. Unfortunately effects of insulin alone were not measured, a limitation attributed to being unable to undertake multiple conditions per plate, thus it cannot be definitively
concluded that this reflects the effects of combined hyperglycaemia and hyperinsulinaemia rather than independent effects of elevated insulin alone. Insulin may stimulate respiration in other tissues via increased transcription and expression of mitochondrial enzymes (Boirie et al., 2001; Stump et al., 2003). However, although pronounced in skeletal muscle, other evidence suggests this may not arise in cardiac myocardium (Boirie et al., 2001). Nonetheless, Parra et al. (2014) demonstrate that 3 hr of insulin treatment (in vivo and in vitro) increases mitochondrial respiratory function, membrane potential and intracellular ATP level in cardiomyocytes, and may promote mitochondrial fusion via increased OPA-1 expression. Irrespective of whether insulin acts independently of glucose, the current findings demonstrate that 24 hrs of hyperglycaemia does not appear to influence mitochondrial respiratory function (or may indeed increase ATP generation). This may be of relevance to differing outcomes with both types I or II diabetes, in which circulating insulin levels (and insulin signalling) may be distinct. Thus, it may be that insulin signalling is critical in determining the impacts of hyperglycaemia on mitochondrial function and dynamics.

Interestingly, hypoglycaemia was overtly stimulatory with a significant ~2 fold elevation in maximal respiration, spare respiratory capacity and ATP generation. Increased maximal respiratory function and spare respiratory capacity has been previously reported in skeletal muscle maintained in low glucose for 7 days (Elkalaf et al., 2013). There is evidence that non-cardiac cells can increase their respiratory rate in response to reduced glucose levels (Lo et al., 2011; Fink et al., 2012; Elkalaf et al., 2013) though this is not yet reported for cardiac myocytes. The basis of this effect remains unclear, and again there are no studies to date in cardiac myocytes. Studies in skeletal myoblasts suggest there is no change in mitochondrial density with low glucose, but that respiratory enzymes may be induced and chain activities increased (Elkalaf et al., 2013). There is also evidence from skeletal muscle cells that low glucose induces an 'oxidative' phenotype in which isocitrate dehydrogenase (rather than glucose-6-phosphate dehydrogenase) becomes the major source of mitochondrial NADPH (Mailloux and Harper, 2010), a compensatory mechanism for reduced glucose availability. Investigations in non-cardiac cells also report protective benefits of low glucose conditions, which reduced superoxide production and enhanced anti-oxidant defences (Lo et al., 2011). Reduced glucose may also be cardioprotective, enhancing mitochondrial respiration and potentially contributing to the cardiac protection apparent with fasting or caloric restriction.
Effects of glucose and insulin on cardiomyoblast expression of fission and fusion proteins

Mitochondrial dynamics and its modulation in chronic disease states is an important and growing area of research, with evidence implicating altered mitochondrial fission in disease pathogenesis. Both in vitro and in vivo models are fundamentally important in dissecting and understanding mitochondrial physiology, pathogenesis of chronic diseases, and specifically the cardiac impacts of disease. Mitochondrial dynamics in cardiac tissue appears to be sensitive to hyperglycaemia, with cell imaging revealing a highly fragmented mitochondrial network in hyperglycaemic cells (Yu et al., 2006; Yu et al., 2008; Yu et al., 2011). Increased mitochondrial ROS production, with I-R or diabetes, influences mitochondrial remodelling and may favour both fission and pro-death signalling (Yu et al., 2011). The fission protein DRP-1 has been widely reported as promoting apoptosis under conditions of hyperglycaemia in vitro and in vivo (Wasiak et al., 2007; Tanaka and Youle, 2008; Thomas and Jacobson, 2012; Zhang et al., 2013; Clerc et al., 2014; Park et al., 2015; Oettinghaus et al., 2016). Nonetheless, it is also known that normal healthy myocardium exhibit small and so-called ‘fragmented’ mitochondria (Song and Dorn, 2015), thus the relevance of mitochondrial fragmentation is unclear.

The present study characterised the expression of mitochondrial fission and fusion proteins (DRP-1, MFN-1, OPA-1) and the ratio of MFN-1: DRP-1 in cardiomyoblasts subjected to varying levels of conditions of glucose and insulin. Conditions of hypoglycaemia and hyperinsulinaemia failed to significantly modify expression of MFN-1, OPA-1 and DRP-1, while hyperglycaemia generally favoured fission, as indicated by a reduced MFN-1: DRP-1 ratio. This contrasts Peng et al. (2012), who report increased OPA-1 expression with insulin. Although there were no apparent modifications in the mitochondrial dynamics following the 24 hr treatment regime, a “chronic” stimulus may be warranted in order to manipulate mitochondrial remodelling under modified glucose and insulin conditions.

Treatment with MDIVI-1 appeared to promote a pro-fusion vs. fission environment in myoblasts exposed to hyperglycaemia, with differential changes in DRP-1 and OPA-1 expression and the MFN-1: DRP-1 ratio. Targeting DRP-1 therapeutically to prevent translocation to the OMM may shift the balance of mitochondrial dynamics towards fusion, which may be beneficial in limiting mitochondrial dysfunction with chronic stress. Canfield et al. (2016) established that MDIVI-1 elicits protective benefit in cardiomyocytes derived...
from human-induced pluripotent stem cells maintained in high glucose conditions. Treatment with MDIVI-1 prevented ROS induced phosphoactivation of DRP-1, thus restoring the biological balance of mitochondrial fission and fusion and reducing cell death. Taken together, the current data suggest MDIVI-1 may induce a shift towards fusion in conditions of hyperglycaemia.

**Summary**

Targeting mitochondrial fission in myoblasts exposed to abnormal glucose and insulin levels may be useful in limiting cellular dysfunction. The present study shows hyperglycaemia and hyperinsulinaemia produce a stimulatory effect on cardiomyoblast proliferation and mitochondrial respiratory rates and ATP generation. Intriguingly, hyperglycaemia alone was insufficient to markedly alter respiration, only exerting effects in the presence of inulin. This may be relevant to differences in cardiac outcomes with types 1 vs. 2 diabetes, where insulin levels and responses vary. However, there were no apparent shifts in mitochondrial fission and fusion proteins with alterations in glucose and insulin, though the DRP-1 inhibitor tended to promote fusion over fission in this setting. Consistent with prior studies in Chapters 3-5, these data support mixed effects of DRP-1 inhibition in a model of metabolic stress. To further examine impacts of diabetic disorders on mitochondrial dynamics, and the potential benefits of targeting these changes, investigations in vivo models are necessary. Thus, studies detailed in Chapter 7 assess the effects of DRP-1 inhibition in an STZ/high-fat diet model of type 2 diabetes mellitus in mice.
CHAPTER 7: Systemic and Cardiac Effects of a Mitochondrial Fission Inhibitor in a Murine Model of Type 2 Diabetes Mellitus

7.0 ABSTRACT

Obesity, insulin-resistance and type II diabetes mellitus (T2D) are at epidemic levels in developed countries, and are both key risk factors for development of ischaemic heart disease and may negatively impact disease outcomes. Roles of mitochondrial fission and fusion in these outcomes are ill defined. This study thus sought to characterise effects of a mitochondrial fission inhibitor (MDIVI-1) on systemic and the myocardial stress phenotypes in a murine model of T2D. To simulate a T2D state, C57Bl/6 mice received a single injection of 75 mg/kg streptozotocin (STZ) and were placed on a high-fat diet (60%) for 12 weeks. Non-diabetic mice received vehicle and were maintained on standard rodent chow. All groups were then subjected to 7 daily injections of the DRP-1 inhibitor MDIVI-1 or vehicle (DMSO). Hearts were excised or Langendorff perfused to assess ischaemic tolerance. The T2D model was associated with ~66% weight gain, hyperglycaemia and impaired glucose tolerance. At the level of the heart, functional recovery from I-R was worsened, and post-ischaemic LDH efflux exaggerated ~22% in T2D vs. non-diabetic hearts. Baseline respiratory function was unaltered in T2D hearts, while T2D specifically sensitised complex II to inhibitory effects of ischaemia. These changes were associated with T2D-dependent shifts in AKT, ERK1/2 and GSK3β expression, increased BAX:BCL-2, and decreased DRP-1 and MFN-1 expression. In vivo treatment with MDIVI-1 partially restored functional ischaemic tolerance in T2D hearts without modifying LDH efflux or post-ischaemic reductions in complex I or II activity. Interestingly, MDIVI-1 reduced cytosolic but not mitochondrial DRP-1 expression, and augmented stress kinase signalling. The data suggest limited cardiac benefits with in vivo MDIVI-1 in a model of T2D.
However, the MDIVI-1/vehicle treatment regime may have inadvertently suppressed the T2D phenotype, complicating data interpretation.
7.1 INTRODUCTION

Globally, physical inactivity and obesity are key risk factors in the development of insulin-resistance and type II diabetes mellitus (T2D), both of which are linked with poor prognosis (Cai and Kang, 2003; Hojlund et al., 2008; Turnbull, 2013; Fuentes-Antras et al., 2015). Development of T2D significantly increases risk of developing coronary artery disease (CAD), often accompanying additional risk factors that include hypercholesterolemia and hypertension, worsening both CAD incidence and clinical outcomes. In the absence of clinical intervention and/or accompanying lifestyle changes myocardial damage is unavoidable, including development of a diabetic cardiomyopathy, increased risk of cardiac ischaemia, and worsened myocardial and mitochondrial damage (Kersten et al., 2000; Rolo and Palmeira, 2006; Hojlund et al., 2008; Cleland, 2012; Sayon et al., 2013). Myocardial dysfunction and injury to the T2D myocardium is thought to induce profound changes in mitochondrial function, including increased superoxide production, inflammation and severe respiratory dysfunction due to ATP depletion, thus depleting the population of healthy mitochondria to impact metabolic capacity and also ROS generation (Rouslin, 1983; Lambert and Brand, 2004; Feve and Bastard, 2009; Miki et al., 2012). The quality of mitochondria is important in meeting the metabolic demand, which in turn dictates metabolic function, protein function, biogenesis, degradation, cell cycle regulation and motility. The phenotype or ‘health’ of mitochondrial populations is tightly regulated by fundamental processes of fission and fusion.

Mitochondrial fission is regulated by dynamin-related protein-1 (DRP-1), and has been implicated as a key determinant of cardiac resistance to ischaemia-reperfusion (I-R). Recent studies have established that both genetic and dietary models of obesity in rodents are associated a pro-fission mitochondrial network (Jheng et al., 2012). These findings suggest that manipulation of the fission protein DRP-1 might improve glucose tolerance and alter insulin signalling to balance mitochondrial dynamics in obesity/T2D. However, reduced mitochondrial fusion protein activity may also induce severe cellular stress, with prior studies reporting major reduction in both growth and energy generation in fibroblasts deficient in fusion proteins MFN-1 and MFN-2 (Chen et al., 2005). It is unclear whether mitochondrial fission is important in the systemic and cardiac changes with T2D. Certainly T2D may disrupt survival and injury kinase signalling (Gross et al., 2007) to exacerbate
mitochondrial dysfunction, whilst modifying expression/function of pro- and anti-apoptotic proteins (Maedler et al., 2004; Lee and Pervaiz, 2007). Here, changes in mitochondrial function, cardiac ischaemic tolerance, stress kinase and cell death signalling were assessed in hearts from control and T2D mice untreated or subjected to 7 day treatment with the fission inhibitor MDIVI-1.
7.2 MATERIALS AND METHODS

7.2.1 Animals

Investigations were approved in accordance with Animal Ethics Committee of Griffith University, under the guidelines of "The Animal Care and Protection Act 2001, section 757", AEC number MSC/05/13. Experiments were initiated in 6 week C57Bl/6 male mice obtained from the Animal Resources Centre (Perth, Australia) and housed with 2 or 4 animals per cage with a 12 hr light/dark cycle. Mice were randomly assigned to one of four groups: control (vehicle) (n = 6); control (MDIVI-1) (n = 8); T2D (vehicle) (n = 8); or T2D (MDIVI-1) (n = 8).

7.2.2 Chemicals

All chemicals utilised in this study were purchased from Sigma Chemical Co (St Louis, MO), unless otherwise indicated.

7.2.3 STZ administration and obesogenic high-fat diet

For induction of a T2D-like state mice received a single intraperitoneal (i.p) injection of STZ (75 mg/kg) and were placed on an obesogenic high-fat chow diet for 14 weeks. A high-fat chow diet was prepared freshly, containing: 800 g irradiated Rat & Mouse Powder (Specialty Feeds), 300 g condensed milk, 140 g sugar and 125 g blended animal fat (Supafry). Non-diabetic (control) mice received a vehicle injection (Na-citrate solution) and were fed standard mouse pellet chow (supplementary table 1).

7.2.4 Glucose tolerance tests

Glucose tolerance tests (GTT) were undertaken at weeks 6 and 12 of the study. Mice were fasted overnight for 11 hours, before blood was taken via a tail prick and glucose measured using a Roche Accu-check II glucometer (Roche Diagnostics, Castle Hill, Australia) to attain baseline fasting glucose. A 20% glucose (2 g/kg) solution was administered via an i.p injection and blood glucose measured after 30, 60, 90, 120 and 180 min. Glucose clearance in control and T2D groups was assessed from area under the curve
(AUC) calculations to contrast glucose handling. A GTT was also performed on a sub-
group of mice after 4 days vehicle or MDIVI-1 administration, testing potential effects of
MDIVI-1 treatment on glucose handling.

7.2.5 MDIVI-1 administration

Dimethyl sulfoxide (DMSO) (0.5%) was used to suspend MDIVI-1, and 5 mg/kg
MDIVI-1 (or DMSO vehicle) was administered via i.p injection for 7 consecutive days,
commencing in week 13. Mice undertaking a GTT during this treatment period, were given
the 5th dose of MDIVI-1/vehicle on completion of the GTT. Animals were sacrificed after
an i.p injection of sodium pentobarbital (60 mg/kg) one day after the final injection of
MDIVI-1/vehicle.

7.2.6 Serum Insulin - Enzyme-Linked Immunosorbent Assay (ELISA)

Serum insulin was measured using an ultra-sensitive mouse ELISA kit purchased
from Crystal Chem (IL, USA), according to manufacturers instructions. Details of working
solutions and materials are detailed in Chapter 2 (section 2.9).

7.2.7 Langendorff heart perfusions

Mice were anaesthetised with sodium pentobarbital (60 mg/kg i.p.) and hearts
excised via thoracotomy, prior to Langendorff-perfusion with Krebs–Henseleit buffer (119
mM NaCl, 11 mM glucose, 22 mM NaHCO$_3$, 4.7 mM KCl, 1.2 mM MgCl$_2$, 1.2 mM
KH$_2$PO$_4$, 1.2mM EDTA, 0.5 mM and 2.5 mM CaCl$_2$) gassed with 95% O$_2$/5% CO$_2$ at 37°C
(pH 7.4). Buffer was delivered via the aorta at a pressure of 80 mm Hg. The left ventricle
was fitted with a balloon inflated to yield a diastolic pressure of 5 mmHg during a 20 min
stabilisation period. Hearts were switched to ventricular pacing at 420 bpm and after 10
min were subjected to 25 min global normothermic ischaemia followed by 45 min
reperfusion. Coronary effluent was collected on ice during reperfusion for assessment of
LDH efflux. The following exclusion criteria were applied to hearts after stabilization: i)
coronary flow >5 ml/min, ii) unstable (fluctuating) contractile function, iii) left ventricular
systolic pressure <100 mmHg, or iv) significant cardiac arrhythmias.
7.2.8 Whole blood collection and heart tissue dissection

Blood was collected via cardiac puncture in hearts not undergoing Langendorff perfusion, or from the chest cavity during cardiac excision for Langendorff perfusion. Blood was analysed immediately using an Alere Cholestech LDX® analysis to attain blood lipid levels. Non-fasted blood glucose was analysed in blood collected via tail snips, using a Roche Accu-Check II glucometer (Roche Diagnostics, Castle Hill, Australia). Hearts were placed on bricks of frozen PBS following removal from chest or Langendorff apparatus, and the left ventricle dissected with one half immediately snap-frozen in liquid N₂.

7.2.9 Western blot analysis of proteins and mitochondrial respiratory analyses

A western blot analysis of proteins in baseline myocardial tissue was assessed in left ventricular cardiac tissue. Post sacrifice, cardiac tissue was immediately extracted from the thoracic cavity and immediately frozen in liquid N₂ (further detailed in Chapter 2). Whole, cytosolic, and mitochondrial fractions were assessed for expression of total and phosphorylated stress kinase (AKT, ERK1/2, GSK3β), mitochondrial fission/fusion proteins (DRP-1, MFN-1, MFN-2, OPA-1) and apoptosis related proteins (BCL-2, BAX) via immunoblot (representative blots – supplementary figure 3).

Mitochondrial respiratory analysis

Baseline mitochondrial functional analyses. Left ventricular myocardial tissue was sampled directly from the thoracic cavity immediately post sacrifice. Hearts were washed in ice-cold PBS and resuspended in ice-cold mitochondrial MiR05 buffer prior to tissue homogenisation with the PBS Shredder and assessed for O₂ consumption.

Post-ischaemic mitochondrial functional analyses. Following 45 min reperfusion, hearts were immediately placed in ice-cold MirR05 buffer prior to tissue homogenisation with the PBS tissue shredder and assessment of O₂ consumption in an Oxygraph-2k instrument (Oroboros Instruments, Innsbruck, Austria) as detailed in the Chapter 2.
7.2.10 Statistical analysis

Data are expressed as means ± SEM. Differences between two or more groups were tested via one-way or two-way ANOVA, with a Newman-Keuls post-hoc test applied when significant effects were detected. Significant differences were accepted for $P<0.05$. All tests were performed with Prism 6 (GraphPad Software Inc., La Jolla, CA).
7.3 RESULTS

7.3.1 T2D mouse model

The body weights of C57Bl/6 mice were measured weekly over the 14 wk duration of the study (Fig. 7.1A) There were no significant body weight differences between control and high-fat fed animals over the initial 6 wks of the protocol. However, divergence in body weights was evident from week 7 on. Between weeks 11-14, high-fat diet fed animals were substantially heavier (~35 g body wt) than respective controls (~32g), prior to MDIVI-1 or vehicle administration. To view effects of modified feeding on relative weight change, weight was expressed relative (%) to starting weight (Fig. 7.1B). A similar trend in weight gain (g) was evident between control and high-fat diet animals as a % of starting weights over the initial 6 wks. Weight gain was significantly higher ($P<0.05$ - $P<0.0001$) in high-fat diet vs. control animals between weeks 7-14. On completion of the feeding regime (week 14), prior to drug/vehicle administration, high-fat diet fed animals had a mean weight gain of ~66% vs. ~48% in controls (Fig. 7.2A).
Figure 7.1: Mouse body weight. A) Weekly body weights (g); and B) relative changes in body weights (%) over 14 weeks of standard or high-fat feeding. Data are means ± SEM (n = 28-32 per group). *, P<0.05; ***, P<0.001; ****, P<0.0001 vs. standard diet (at respective week).
During the 7-day MDIVI-1/DMSO injection regime, mice had access to their respective standard chow and high-fat diets. Interestingly, at cessation of treatment (7-days post MDIVI-1/vehicle) body weight differences between control and T2D groups were eliminated. This change in body weight, post treatment regime, suggests that MDIVI-1 may have contributed to weight loss in all groups, though a similar effect was evident with DMSO vehicle. Relative to starting weights at week 1, final weight gain at the end of the treatment regime was ~55% in T2D animals vs. ~40% in control animals ($P>0.05$). At week 15, T2D animals administered with vehicle recorded a significant weight gain (%) in comparison to non-diabetic mice receiving either MDIVI-1 or vehicle (Fig. 7.2B and Fig. 7.2C).
Figure 7.2: Relative and absolute changes in body weight in mice fed standard or high-fat diets. A) Initial and final body weights (g) of mice fed a standard chow or obesogenic diet (*n = 29-31 per group). ****, *P*<0.0001 vs. week 1 body weight; #, *P*<0.0001 vs. standard-diet (week 14); B) Body weights (g) pre- and post-MDIVI-1 or vehicle treatment (*n = 11-16 per group*). *, *P*<0.05 vs. CTRL (MDIVI-1); #, *P*<0.01 vs. CTRL (vehicle); and C) Relative changes in body weight at week 14 (pre-treatment) and 15 (post-treatment) (*n = 11-16 per group*). **, *P*<0.01; ****, *P*<0.0001; vs. CTRL (vehicle); #, *P*<0.01 vs. CTRL (MDIVI-1). Data are means ± SEM.
Glucose handling (glucose tolerance tests): To assess glucose clearance/handling, glucose tolerance tests (GTT) were performed on animals at week 6 (Fig 7.3A), week 12 (Fig 7.3B) and week 13 + 4 day treatment (MDIVI-1/vehicle) (Fig. 7.3C). A modest divergence in glucose clearance (Fig. 7.3A) was evident at week 6, with significantly higher blood glucose measured in animals fed high-fat vs. standard chow diets. Glucose clearance was significantly impeded in high-fat vs. control diet-fed animals at wk 12. There was no significant shifts in the GTT AUC at week 6 (Fig. 7.4A) in animals fed a high-fat diet (vs. control diet), while a significant increase in the AUC was evident at week 12 (Fig. 7.4A). To establish if MDIVI-1 treatment had any effects on glucose clearance, a GTT was performed in animals at 13 week + 4 days of MDIVI-1/vehicle treatment. A modest reduction in the AUC in T2D (MDIVI-1) vs. T2D (vehicle) was evident (P>0.05) (Fig. 7.4B).

Blood glucose concentrations were assessed under fasted and non-fasted (post-sacrifice) conditions in control and high-fat T2D animals. Fasted blood glucose was significantly elevated in T2D vs. control animals, however no significant shifts were evident in blood glucose concentrations under non-fasted conditions (Fig. 7.4C).
Figure 7.3: Glucose tolerance. Blood glucose clearance was assessed over 180 min following i.p administration of 20% glucose solution (2 mg/g) in mice fed a standard or high-fat diet for A) 6 or B) 12 week; and C) 13 weeks + 4 days (MDIVI-1/vehicle treatment). Data are means ± SEM. (n = 20-32 per group for). *, P<0.05; ***, P<0.001; ****, P<0.01 vs. time-matched standard-chow diet.
Figure 7.4: Glucose handling in control and high-fat diet mice. A) Combined GTT area under the curve (AUC) in animals at 6 (n=14-16) and 12 weeks (n=14-16). ***, $P<0.001$ vs. respective control at week 12; #, $P<0.01$ vs. obesogenic diet (week 6); B) 13 weeks + 4 days of MDIVI-1 or vehicle administration (n = 6). *, $P<0.05$ vs. CTRL (vehicle); and C) Fasted blood glucose obtained at 13 weeks + 4 days treatment (via tail prick) and non-fasted glucose obtained post sacrifice (via tail snip) (n = 8-11) **, $P<0.01$, vs. CTRL vehicle (non fasted). Data are means ± SEM.
7.3.2 Non-fasted blood lipid profiles

Non-fasted blood lipid profiles were assessed immediately post sacrifice in all animals (supplementary table 2). Blood was collected via cardiac puncture and the following analytes assayed: low-density lipoprotein, non-low-density lipoprotein, high-density lipoprotein, LDL/HDL ratio, total cholesterol and triglycerides. In T2D animals, a reduction was evident in LDL, non-LDL and the ratio of LDL/HDL vs. respective control animals. Levels of HDL were moderately elevated in T2D vs. controls (vehicle or MDIVI-1), and triglycerides insignificantly elevated in MDIVI-1 treated T2D vs. control mice. Total cholesterol was unaltered across groups. A decline in LDL was recorded in T2D vs. control mice (vehicle or MDIVI-1), resulting in reduced LDL/HDL ratios in T2D mice. Overall, the lipid data provided a snapshot of lipid profiles values across all 4 experimental groups. However, the apparatus used to undertake the lipid profile analysis failed to accurately measure all blood lipids (which fell outside the detection range) from each animal post sacrifice. As a result of this, n values across 4 groups were severely compromised, reducing statistical power.
7.3.3 Non-fasted serum insulin

Insulin concentrations were determined using an ultra-sensitive mouse ELISA kit (Crystal Chem, Downers Grove, IL) in blood sampled from non-fasted mice (Fig. 7.5). Serum insulin concentrations were not significantly altered in untreated control vs. T2D animals, and were significantly higher in MDIVI-1 treated T2D vs. control mice.

![Figure 7.5: Non-fasted serum insulin](image)

Figure 7.5: Non-fasted serum insulin. Non-fasted serum insulin concentrations were assessed in non-diabetic control (CTRL) and T2D animals receiving vehicle or MDIVI-1 for 1 wk. Data are means ± SEM (n = 6-8). *, P<0.05 vs. CTRL (vehicle).
7.3.4 Functional responses to ischaemia-reperfusion in hearts of non-diabetic and T2D mice

Functional recoveries of hearts subjected to a 25 min ischaemic insult were assessed in control and T2D groups receiving either MDIVI-1 or vehicle. Final recoveries of left ventricular end-systolic pressure (LVESP) were comparable across groups, while left ventricular end-diastolic pressure (LVEDP) was significantly worsened in hearts from MDIVI-1 treated control mice (vs. vehicle). Post-ischaemic LVEDP was elevated in T2D vs. control hearts (Fig 7.6A), with MDIVI-1 not modifying recovery in the T2D group (P>0.05). The recovery of LVDP in T2D hearts (vehicle and MDIVI-1) following 20 min reperfusion (early reperfusion) was significantly impaired (vs. vehicle), while MDIVI-1 impeded the early recovery in the control group, however this was not significant (Fig. 7.6C and Fig 7.7).
Figure 7.6: Myocardial post-ischaemic outcomes. Post-ischaemic recoveries for A) left ventricular end systolic pressure; B) left ventricular end diastolic pressure; and C) early (20 min) and final (45 min) recoveries for left ventricular developed pressure (LVDP) *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$ vs. respective group (early recovery); #, $P<0.05$; vs. CTRL (vehicle) early recovery. Data are means ± SEM ($n = 4-6$ per group).
Figure 7.7: Post-ischaemic recovery of LVDP in hearts from control and T2D animals. Post-ischaemic recovery of LVDP is shown for control and T2D Langendorff perfused hearts from mice untreated (vehicle) or treated for 1 wk with MDIVI-1 (5 mg/kg). Hearts were subject to 25 min ischaemia followed by 45 min reperfusion. *, P<0.05 CTRL (vehicle) vs. T2D (vehicle); #, P<0.01 vs. T2D (MDIVI-1). Data are means ± SEM (n = 4-6/group).
7.3.5 Cellular disruption/ lactate dehydrogenase leakage

The post-ischaemic efflux of LDH was assessed enzymatically, demonstrating a significant increase in LDH release in hearts from T2D (vehicle or MDIVI-1) vs. control mice. Treatment with MDIVI-1 did not alter LDH efflux in either CTRL or T2D animals vs. respective vehicle groups (Fig. 7.8).

Figure 7.8: Post-ischaemic LDH efflux. Total LDH efflux into the coronary effluent throughout 45 min reperfusion was assessed as a marker of myocardial death/disruption. Data are means ± SEM. (n = 4-6 per group). *, P<0.05.
7.3.6 Mitochondrial respiratory function

**Baseline respiratory function (non-perfused myocardium).** In order to assess mitochondrial function, isolated mitochondria was extracted from left ventricular tissue removed from the chest of control and T2D animals (MDIVI-1 or vehicle treated). The O$_2$ flux via mitochondrial complex II was substantially higher than via complex I (Fig. 7.9A and Fig. 7.9B). Baseline mitochondrial respiration in normoxic myocardium, assessed from complex I and II activities, was not altered by either T2D or MDIVI-1 treatment.

**Post-ischaemic respiratory function.** Post-ischaemic mitochondrial activity was assessed at 45 min reperfusion. Complex I activity was highly sensitive to ischaemia, substantially reduced to ~10% of baseline activity in control tissue and ~5% in T2D hearts (Fig 7.9C). Complex II was relatively insensitive to ischaemia-reperfusion in control hearts but declined significantly in T2D tissue (relative to baseline activity) (Fig 7.9D). This T2D sensitisation of complex II activity to ischaemic insult was not modified by MDIVI-1 treatment.
Figure 7.9: Baseline and post-ischaemic mitochondrial respiration. A) Baseline and post- I-R mitochondrial complex I activity; B) Baseline and post- I-R mitochondrial complex II activity; C) relative (%) post- I-R changes in complex I; and D) complex II activities. Data are means ± SEM (n = 4-6 per group). *, P<0.05; **, P<0.01; ****, P <0.0001; vs. respective baseline.
7.3.7 Myocardial protein expression in (non-perfused) high-fat diet fed/STZ mouse model administered with a DRP-1 inhibitor

*AKT.* Myocardial expression of AKT was assessed in whole cell lysates, cytosolic and mitochondrial fractions (Table 7.1). Total expression of AKT in whole cell lysate was comparable across all groups, whereas cytosolic and mitochondrial expression levels declined in T2D hearts. Treatment with MDIVI-1 significantly increased the phosphorylation of AKT in whole cell lysate in control hearts. While the phosphorylation of AKT in whole cell T2D tissue substantially increased. The ratio of phospho: total AKT in whole cell and cytosolic T2D tissue increased, however statistical significance was not obtained (albeit insignificant) (Table 7.1).

*ERK1/2.* The expression of total-ERK1/2 was not significantly modified by T2D or MDIVI-1 treatment (Table 7.2). However, T2D reduced cytosolic levels of phospho-ERK1/2, an effect mimicked by MDIVI-1 treatment in controls. The ratio of phospho: total ERK1/2 tended to be reduced in T2D tissue (across fractions), though this did not achieve statistical significance (Table 7.2).

*GSK3β.* There were no statistically significant changes in GSK3β expression patterns (Table 7.3). However, total GSK3β expression moderately reduced in cytosolic and mitochondrial fractions in T2D tissue (albeit significant). The ratio of phospho: total GSK3β declined with T2D in both whole cell and cytosolic fractions. Treatment with MDIVI-1 failed to influence GSK3β expression.
Table 7.1: Relative expression of AKT signalling in a healthy and T2D myocardium from untreated and MDIVI-1 treated mice.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Total AKT</th>
<th>Phospho-AKT</th>
<th>Phospho/Total Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole</td>
<td>Cytosol</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Standard-chow fed animals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTRL (Vehicle) (n=4)</td>
<td>100 ± 16</td>
<td>100 ± 5</td>
<td>100 ± 22</td>
</tr>
<tr>
<td>CTRL (MDIVI-1) (n=6)</td>
<td>105 ± 14</td>
<td>53 ± 8 †</td>
<td>81 ± 19</td>
</tr>
<tr>
<td>High-fat diet fed animals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2D (Vehicle) (n=6)</td>
<td>101 ± 9</td>
<td>60 ± 9 *</td>
<td>57 ± 4 *</td>
</tr>
<tr>
<td>T2D (MDIVI-1) (n=6)</td>
<td>113 ± 10</td>
<td>45 ± 4</td>
<td>56. ± 8</td>
</tr>
</tbody>
</table>

Expression of total AKT, phospho-AKT and the ratio of phospho: total AKT was quantified in whole cell lysate, cytosolic and mitochondrial cellular compartments. Data is representative of mean ± SEM (n = 4-6). *, P<0.05 vs. respective CTRL; †, P<0.05 vs. respective vehicle.
Table 7.2: Relative expression of survival kinase ERK1/2 in a healthy and T2D myocardium from untreated and MDIVI-1 treated mice.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Total ERK1/2</th>
<th></th>
<th></th>
<th>Phospho-ERK1/2</th>
<th></th>
<th></th>
<th>Phospho/Total Ratios</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole</td>
<td>Cytos</td>
<td>Mitochondria</td>
<td>Whole</td>
<td>Cytos</td>
<td>Mitochondria</td>
<td>Whole</td>
<td>Cytos</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Standard-chow fed animals</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTRL (Vehicle) (n=4)</td>
<td>100 ± 17</td>
<td>100 ± 19</td>
<td>100 ± 17</td>
<td>100 ± 18</td>
<td>100 ± 26</td>
<td>100 ± 28</td>
<td>100 ± 12</td>
<td>100 ± 29</td>
<td>100 ± 35</td>
</tr>
<tr>
<td>CTRL (MDIVI-1) (n=6)</td>
<td>100 ± 13</td>
<td>71 ± 9</td>
<td>91 ± 19</td>
<td>98 ± 10</td>
<td>35 ± 11 †</td>
<td>100 ± 20</td>
<td>111 ± 15</td>
<td>70 ± 15</td>
<td>103 ± 29</td>
</tr>
<tr>
<td>High-fat diet fed animals</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2D (Vehicle) (n=6)</td>
<td>109 ± 13</td>
<td>81 ± 6</td>
<td>83 ± 8</td>
<td>100 ± 10</td>
<td>41 ± 10 *</td>
<td>86 ± 23</td>
<td>97 ± 11</td>
<td>69 ± 10</td>
<td>81 ± 26</td>
</tr>
<tr>
<td>T2D (MDIVI-1) (n=6)</td>
<td>112 ± 15</td>
<td>81 ± 5</td>
<td>98 ± 15</td>
<td>104 ± 15</td>
<td>36 ± 11</td>
<td>60 ± 14</td>
<td>89 ± 7</td>
<td>70 ± 15</td>
<td>78 ± 21</td>
</tr>
</tbody>
</table>

Expression of total ERK1/2, phospho-ERK1/2 and the ratio of phospho: total ERK1/2 was quantified in whole cell lysate, cytosolic and mitochondrial cellular compartments. Data is representative of mean ± SEM (n = 4-6). *, P<0.05 vs. respective CTRL; †, P<0.05 vs. respective vehicle.
Table 7.3: Relative expression of GSK3\( \beta \) in healthy and T2D myocardium from untreated and MDIV1-1 treated mice.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Total GSK3( \beta )</th>
<th>Phospho-GSK3( \beta )</th>
<th>Phospho/Total Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole</td>
<td>Cytosol</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>High-fat diet fed animals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2D (Vehicle) ((n=6))</td>
<td>138 ± 16</td>
<td>83 ± 6</td>
<td>65 ± 11</td>
</tr>
<tr>
<td>T2D (MDIV1-1) ((n=6))</td>
<td>149 ± 21</td>
<td>78 ± 8</td>
<td>58 ± 11</td>
</tr>
<tr>
<td>Standard-chow fed animals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTRL (Vehicle) ((n=4))</td>
<td>100 ± 11</td>
<td>100 ± 25</td>
<td>100 ± 28</td>
</tr>
<tr>
<td>CTRL (MDIV1-1) ((n=6))</td>
<td>136 ± 16</td>
<td>79 ± 15</td>
<td>85 ± 19</td>
</tr>
</tbody>
</table>

Expression of total GSK3\( \beta \), phospho-GSK3\( \beta \) and the ratio of phospho: total GSK3\( \beta \) in whole cell lysate, cytosolic and mitochondrial compartments. Data is representative of mean ± SEM \((n = 4-6)\).
Mitochondrial fission and fusion proteins. Expression of MFN-1, MFN-2, DRP-1 and OPA-1 was assessed in cytosolic and mitochondrial compartments (Table 7.4). The cytosolic expression of the fission protein DRP-1 was significantly reduced across all T2D (MDIVI-1 and vehicle) groups and with MDIVI-1 treatment in control animals. Unexpectedly, expression of mitochondrial DRP-1 was unaltered (indeed insignificantly elevated) by MDIVI-1 in control and T2D groups. Cytosolic levels of the outer mitochondrial fusion protein MFN-1 were unaltered across groups, while significant reductions in mitochondrial expression (~25%) were detected in the T2D groups, and with MDIVI-1 treatment in controls. The DRP-1 inhibitor, MDIVI-1, reduced the mitochondrial expression of MFN-2 by ~32% (vs. control vehicle) in T2D animals and increased it by ~16% in the control group. In T2D animals, the expression of mitochondrial OPA-1 reduced by ~17% in vehicle and ~23% in MDIVI-1 treatment groups. MDIVI-1 augmented OPA-1 expression in control groups, while significantly reducing expression in T2D. Since mitochondrial dynamics are highly dependent on an appropriate balance between mitochondrial fission and fusion, our study quantified the ratio of MFN-1 to DRP-1 as a means of quantifying a state of fission. The cytosolic MFN-1 to DRP-1 ratio almost doubled (~100%) in control (MDIVI-1) and T2D groups, while the ratio of mitochondrial MFN-2 to DRP-1 remained unchanged (Table 7.4).

Apoptosis regulators. Mitochondrial expression of pro- and anti-apoptotic BAX and BCL-2 were assessed across all groups, and the BAX: BCL-2 determined as an indicator of apoptotic sensitivity. There was an increase in the BAX: BCL-2 ratio (Table 7.5) in both MDIVI-1 groups (control and T2D) and T2D (vehicle), suggestive of a pro-apoptotic state, however these changes were deemed insignificant.
Table 7.4 Relative expression of myocardial fission and fusion proteins in healthy and T2D myocardium.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>GROUP</th>
<th>Total DRP-1</th>
<th>MFN-1</th>
<th>MFN-2</th>
<th>OPA-1</th>
<th>MFN-1/DRP-1 Ratios</th>
<th>MFN-2/DRP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cytosol</td>
<td>Mitochondria</td>
<td>Cytosol</td>
<td>Mitochondria</td>
<td>Mitochondria</td>
<td>Cytosol</td>
</tr>
<tr>
<td>Standard-chow fed animals</td>
<td>CTRL (Vehicle) (n=4)</td>
<td>100 ± 23</td>
<td>100 ± 15</td>
<td>100 ± 18</td>
<td>100 ± 12</td>
<td>100 ± 19</td>
<td>100 ± 9</td>
</tr>
<tr>
<td></td>
<td>CTRL (MDIVI-1) (n=6)</td>
<td>43 ± 10 †</td>
<td>124 ± 33</td>
<td>96 ± 15</td>
<td>71 ± 10</td>
<td>116 ± 15</td>
<td>109 ± 11</td>
</tr>
<tr>
<td>High-fat diet fed animals</td>
<td>T2D (Vehicle) (n=6)</td>
<td>46 ± 7 *</td>
<td>85 ± 15</td>
<td>93 ± 12</td>
<td>72 ± 7 *</td>
<td>88 ± 9</td>
<td>83 ± 10</td>
</tr>
<tr>
<td></td>
<td>T2D (MDIVI-1) (n=6)</td>
<td>53 ± 9</td>
<td>134 ± 32</td>
<td>87 ± 11</td>
<td>69 ± 7</td>
<td>68 ± 10 *</td>
<td>77 ± 6 *</td>
</tr>
</tbody>
</table>

Expression of cytosolic and mitochondrial DRP-1 and MFN-1, mitochondrial MFN-2 and OPA-1 and the ratios of MFN-1 and MFN-2 to DRP-1 respectively. Data are means ± SEM (n = 4-6 per group). *, P<0.05 vs. respective CTRL; †, P<0.05 vs. respective vehicle.
Table 7.5: Relative expression of pro- and anti- apoptotic BAX and BCL-2 in healthy and T2D myocardium.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>BCL-2</th>
<th>BAX</th>
<th>BAX/BCL-2 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mitochondria</td>
<td>Mitochondria</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Standard-chow fed animals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTRL (Vehicle)</td>
<td>100 ± 22</td>
<td>100 ± 6</td>
<td>100 ± 59</td>
</tr>
<tr>
<td>(n=4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTRL (MDIVI-I)</td>
<td>111 ± 32</td>
<td>111 ± 20</td>
<td>197 ± 69</td>
</tr>
<tr>
<td>(n=6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-fat diet fed animals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2D (Vehicle)</td>
<td>101 ± 26</td>
<td>92 ± 7</td>
<td>184 ± 46</td>
</tr>
<tr>
<td>(n=6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2D (MDIVI-I)</td>
<td>75 ± 21</td>
<td>78 ± 10</td>
<td>204 ± 59</td>
</tr>
<tr>
<td>(n=6)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Expression of mitochondrial pro- apoptotic protein BAX and anti- apoptotic protein BCL-2, and the ratio of BAX: BCL-2. Data is representative of mean ± SEM (n = 4-6).
7.4 DISCUSSION

The growing prevalence of T2D has drawn much attention from clinicians and scientists alike, and is a major risk factor for development and progression of cardiovascular disease. The central aim of this study was to characterise a murine model of T2D, and to test whether in vivo treatment with the mitochondrial fission inhibitor MDIVI-1 may beneficially influence systemic and cardiac phenotype in this pathological model (including effects on ischaemic tolerance, mitochondrial respiratory function and signalling proteins). In brief, data support emergence of an early T2D phenotype following an intermediate dose of STZ and 14 weeks of high-fat feeding, evidenced in substantial weight gain, hyperglycaemia and impaired glucose handling. While baseline mitochondrial function was unaltered, myocardial and mitochondrial responses to ischaemic insult were exaggerated in T2D, in association with shifts in stress kinase, apoptotic, fission and fusion proteins. Nonetheless, emergence of the systemic and cardiac disease phenotype appears largely insensitive to in vivo treatment with MDIVI-1, which again exerted quite mixed effects.

Murine model of T2D

A combination of a single dose of STZ and high-fat feeding produced significant features of T2D, including obesity, hyperglycaemia and impaired glucose disposal. Accumulating evidence suggests that combination of STZ and high-fat feeding (or high-fat feeding alone) is a suitable model for exploring the underlying pathophysiology of the metabolic disease T2D (Reed et al., 2000; Du Toit et al., 2005; Srinivasan et al., 2005). By week 7, a significant divergence in weight gain was evident in animals consuming a high-fat diet (g or % of starting weight) vs. controls, with a substantial ~66% weight gain by week 14. Consistent with previous findings, significant shifts in body weight between standard vs. high-fat diet fed mice were accompanied by impaired glucose handling (week 6 and week 12), and increased visceral adiposity upon dissection and the cessation of the feeding regime (Supplementary figure 4) (Schreyer, 1998; Winzell and Ahren, 2004; Gilbert et al., 2011). Interestingly, although our study reported significantly impaired glucose disposal in high-fat fed animals, non-fasted serum insulin was similar between
groups, with the exception of the T2D (MDIVI-1) group, which had moderately elevated non-fasted serum insulin. Although there was compelling evidence of obesity and impaired glucose handling, non-fasting insulin is not an ideal measure and may obscure potential differences. Longer periods of high-fat feeding may also be required to induce hyperinsulinaemia to mimic a frank diabetic state associated with pancreatic production of insulin. Morris et al. (2016) recently reported a 7-fold increase in plasma insulin levels in C57Bl/6 mice administered a modified high-fat diet for 30 weeks, representing a more pronounced model of T2D. Furthermore, as a result of daily i.p injections in the final week of the study, a significant fall in weight occurred in all mice receiving MDIVI-1 or vehicle. The effect suggests an untoward, potentially stressful impact of the injection regime. This weight reduction was more pronounced in T2D animals (both vehicle and MDIVI-1), and unfortunately may reduce or reverse metabolic progression of the T2D phenotype, attenuating cellular or molecular modifications. This observation suggests potential issues with the drug treatment regime, and complicates data interpretation.

The T2D model did not exhibit any alterations in baseline respiratory function in cardiac tissue, although was associated with specific post-ischaemic depression of complex II activity, and exaggeration of the inhibitory effects of I-R on complex I activity. From a proteomic perspective, the T2D state was associated with reductions in cytosolic and mitochondrial AKT expression, although modest (insignificant) elevations in total cell and cytosolic phosphorylation were also evident. The latter may reflect an early adaptation to altered AKT expression. While total ERK1/2 expression was not substantially modified, cytosolic and mitochondrial levels of phosphorylated ERK1/2 were lower in T2D hearts, while relative phosphorylation of GSK3β was reduced in whole cell lysates and the cytosolic fraction. This mix of changes in stress kinases certainly supports emergence of signal dysfunction in early T2D that may be relevant to altered ischaemic tolerance and shifts in mitochondrial tolerance to stress. This was associated with a pro-apoptotic phenotype, with T2D elevations in the BAX: BCL-2 ratio. Previous reports have suggested that PI3K/AKT signalling is strongly influential in determining the fate of cell survival, with AKT reductions associated with dysfunction in insulin secretory cells (pancreatic β-cells), characteristically observed in the pathophysiology of T2D (Tuttle et al., 2001; Srinivasan et al., 2005). A reduction in pro-survival AKT signalling suggests that apoptotic signalling is easily triggered. Thus, without AKT activation intrinsically mediated
apoptosis and phosphorylation of pro-apoptotic mediators including; Bad, Bid and Bik members trigger the apoptotic cascade involving the release of cytochrome c and subsequent activation of caspase-3 (Sun et al., 1999). Conditions of hyperglycaemia severely induce mitochondrial dysfunction in pancreatic β-cells, thus consequently inducing pro-apoptotic signalling (Men et al., 2009). Consistent with current data, T2D animals showed reduced activation of total and phospho mitochondrial AKT expression and heightened DRP-1 expression. Thus, activation of mitochondrial fission protein DRP-1 may abolish pro-survival effects of mitochondrial AKT signalling in the diabetic heart. Interestingly, there is evidence that link mitochondrial fusion protein OPA-1 to reductions observed in in diabetic models, thus contributing to metabolic dysfunction, impaired glucose-ATP production and mitochondrial respiratory dysfunction (Zhang et al., 2011; Parra et al., 2014). While myocardial OPA-1 expression reduced in T2D animals ($P>0.05$), this may account for a disturbance in the mitochondrial dynamics inducing β-cell dysfunction.

**Effect of MDIVI-1 on ischaemic tolerance in T2D myocardium**

Evidence supports severe left ventricular dysfunction and atrial enlargement in clinical populations presenting with T2D, often accompanied by an increased prevalence of atherosclerotic plaque formation and ventricular hypertrophy (Cleland, 2012; Zhang et al., 2016; Zoppini et al., 2016). The metabolic alterations present in diabetic myocardium significantly contribute to worsened outcomes following I-R, promoting mitochondrial dysfunction and associated cell death. In the current study, the T2D model exhibited moderately impaired tolerance to myocardial ischaemia, which was evident when assessing recovery of LVDP (% baseline) early in reperfusion. In support of this, diastolic dysfunction and increased LDH leakage (post-ischaemia) were evident in the T2D model. Although data indicate I-R tolerance is impaired in high-fat vs. standard-chow diet fed mice, these impairments were not as pronounced as previously reported by Gross et al. (2007) and Aoyagi et al. (2015). This may again reflect suppression of the T2D phenotype with weight loss during the week of drug or vehicle treatment.

Previous studies implicate mitochondrial dysfunction in models of obesity and T2D, and the DRP-1 inhibitor MDIVI-1 has been reported to reduce insulin-resistance in obesity and T2D (Jheng et al., 2012). The current study is the first to assess impacts of *in vivo*
MDIVI-1 treatment on myocardial tolerance to ischaemia in T2D. Pharmacological inhibition of DRP-1 failed to improve post-ischaemic myocardial outcomes vs. for vehicle-treated T2D hearts. The dose of 5 mg/kg employed here failed to substantially improve myocardial outcomes, while previous studies have reported therapeutic benefits in MDIVI-1 \((in\ vivo)\) at doses ranging between 0.24 mg/kg - 50 mg/kg (Givvimani et al., 2012; Grohm et al., 2012; Marsboom et al., 2012; Rappold et al., 2014; Huang et al., 2015; Sharp et al., 2015). While outcomes in prior studies may vary, it remains to be established why the efficacy of MDIVI-1 may have been impeded in a T2D model. During the treatment period, a number of contributing factors including animal weight loss, vehicle toxicity and MDIVI-1 solubility, may have resulted in its lack of efficacy as a potential therapeutic. Toxicity of DMSO has been reported in \(in\ vivo\) studies when utilised as a solvent for pharmacological agents (Hanslick et al., 2009; Patil, 2013; Galvao et al., 2014). Although we were unable to confirm a role for DMSO toxicity, it cannot be ruled out as a causative factor in reducing the efficacy of MDIVI-1 as a novel treatment strategy in improving post-ischaemic outcomes. Clearly the treatment regime requires additional refinement.

**Effects of T2D and MDIVI-1 on mitochondrial respiratory function**

Results presented in Chapter 3 established the impact of 25 min of global ischaemia on mitochondrial respiratory function, repressing complex I activity 25 to 50% in early and late reperfusion The current study assesses myocardial mitochondrial respiratory function in a T2D model both at baseline and at 45 min of post-ischaemic reperfusion. The impact of T2D on mitochondrial function in human atrial tissue was documented by Anderson et al. (2009), who reported significant reductions in mitochondrial substrates which drive respiratory capacity, substantially impairing mitochondrial function. No change in mitochondrial \(O_2\) consumption (complex I and II) was observed at baseline in T2D vs. control hearts, while (consistent with studies in Chapter 3), complex I activity was highly sensitive to ischaemia, with an \(~85\%\) reduction in activity after 45 min reperfusion in control hearts and a \(~95\%\) reduction in T2D hearts. This reduction in complex I function is likely provoked by increased oxidative stress upon reperfusion and re-introduction of \(O_2\). Mitochondrial dysfunction as a result of excess ROS production results in a fall in the mitochondrial membrane potential and activation of pro-apoptotic signalling, a cascade of events which leads to ETC dysfunction and ATP depletion (Chouchani et al., 2014). The current data indicate that myocardial complex I respiratory function in T2D animals is
sensitised to ischaemia. As evidenced by other studies, mitochondrial ROS toxicity contributes to severe complex I inhibition and reduction in GSH levels in the hearts of T2D patients presenting with slow myocardial infarctions (Hernandez-Mijares et al., 2013). Post ischaemia, the T2D model was also associated with sensitisation of complex II (±MDIVI-1), exhibiting a post-ischaemic fall in complex II activity. The exact mechanisms involved in reducing oxygen consumption, via complex II, have been inconclusive, however a recent study by Sverdlov et al. (2016) reported that mice administered a high-fat high-sucrose diet for 4 months had a ~62% reduction in complex II activity and an increase in H$_2$O$_2$ production, thus contributing to the onset of metabolic heart disease. Consistent with current data, a reduction in complex II activity may be attributed to a reduction in succinate oxidase activity, which has been previously reported to decrease by up to ~50% in T2D patients (Mogensen et al., 2007), thus reducing the overall OxPhos capacity. It may be plausible that oxidative stress may reduce substrate utilisation and ADP phosphorylation, thus compromising overall respiratory capacity.

Interestingly, MDIVI-1 treatment did not alter complex I or II activities under baseline or post-ischaemic conditions. Absence of changes in baseline respiratory function, and lack of impact of MDIVI-1, suggest a lack of shift in fission/fusion balance and mitochondrial function. However, pronounced complex I inhibition across control groups, and T2D-dependent sensitization of complex II to effects of I-R confirm mitochondrial dysfunction with I-R and T2D. The lack of effect to MDIVI-1 suggests either that these I-R and T2D perturbations do not involve altered fission/fusion balance and/or that effects of DRP-1 inhibition may be mixed (as per Chapter 3). Restoration of mitochondrial dynamics via therapeutic inhibition of DRP-1 has been reported to reverse mitochondrial dysfunction associated with chronic and inherited diseases (including reductions in mtDNA content and impaired respiratory capacity) (Chen and Chan, 2005; Chen et al., 2005; Lackner and Nunnari, 2010). This is the first study to assess the effects of MDIVI-1, in vivo, on mitochondrial respiratory function and ischaemic tolerance in a T2D model. The current data suggest a lack of efficacy of MDIVI-1 in this setting. Nonetheless, issues with the model and drug treatment regime cloud interpretation.

**Effects of T2D and MDIVI-1 on cell signalling**

*Stress kinase signalling.* Modifications in stress kinase signalling proteins in
diabetic myocardium have been well documented (Gross et al., 2007; Donner et al., 2013; Westermeier et al., 2015). However, interactions between kinase signalling, mitochondrial remodelling and cell death signalling remain unclear. Prior studies report reductions in PI3K and AKT signalling, which act downstream of insulin receptors (Boudina et al., 2009). Data here support a modest reduction in activation state of cytosolic AKT in T2D hearts (untreated or MDIVI-1), while MDIVI-1 significantly reduced AKT expression in control hearts. Similarly, both total and phospho-activation of cytosolic ERK1/2 was blunted in T2D hearts (control and MDIVI-1), while MDIVI-1 negated ERK1/2 activation in control hearts. Consistent with data presented in Chapter 3, MDIVI-1 (ex vivo) moderately reduced cytosolic AKT and ERK1/2 activation, providing evidence that DRP-1 inhibition reduces activation of survival kinases, which are also involved in insulin signalling, cell growth and protein synthesis (O’neill et al., 2007; Boudina et al., 2009). Boudina et al. (2009) demonstrated that impaired insulin signalling, mediated via reductions in reperfusion injury salvage kinase pathway (RISK) signalling proteins, AKT and GSK3β, contributed to mitochondrial dysfunction in insulin receptor deficient mice (CIRKO). Activation of total-GSK3β has a pro-death effect, with both insulin resistant and obese animals being more sensitive to alterations in GSK3β and ERK1/2 signalling. Phospho-inhibition of GSK3β has a cytoprotective function, limiting the opening of the mPTP associated with I-R injury and cell death (Ghaboura et al., 2011). Interestingly, whole cell expression of GSK3β was increased by 30-40% in T2D hearts, while in vivo MDIVI-1 treatment appeared to increase relative phosphorylation of mitochondrial GSK3β in both control and T2D tissue. These data indicate that MDIVI-1 may promote a cytoprotected state and preserve mitochondrial integrity via phospho-inhibition of GSK3β, thus delaying the opening of the mPTP and subsequent release of cytochrome c. Nonetheless, the mitochondrial BAX: BCL-2 ratio was increased in T2D and with MDIVI-1, indicative of a pro-apoptotic state that is not improved with MDIVI-1. These modest shifts in stress kinase and cell death proteins are consistent with emergence of stress intolerance and enhanced cell death in a model of T2D, and reveal potential benefits via MDIVI-1 treatment.

_Fission and fusion proteins._ Recent investigations of mitochondrial phenotype in rodent models of genetic or dietary obesity reveal the presence of smaller and ‘fragmented’ mitochondria, suggesting a pro-fission state (Jheng et al., 2012). Metabolic disorders
induced via high-fat diets and sustained hyperlipidaemia and hyperglycaemia also impair mitochondrial function and dynamics (Bonnard et al., 2008). The current data reveal significant reductions (~50%) in cytosolic DRP-1 and mitochondrial MFN-1 levels in T2D hearts, while mitochondrial MFN-2 and OPA-1 and cytosolic MFN-1 are unaltered ($P>0.05$). Consistent with Jheng et al (2012), these data suggest activation of mitochondrial fission signalling in T2D myocardium, whereas fusion proteins remain unchanged. Pharmacological inhibition of DRP-1 significantly reduced cytosolic DRP-1 expression in control hearts, consistent with other findings (Gharanei et al., 2013; Sharp et al., 2014; Sharp et al., 2015), though failed to alter mitochondrial DRP-1. This contrasts prior findings in Chapter 6, and suggests MDIVI-1 may selectively inhibit cytosolic DRP-1 GTPase activity rather than mitochondrially bound DRP-1. Treatment with MDIVI-1 did shift the cytosolic MFN-1: DRP-1 ratio, supporting a pro-fusion state. However, MDIVI-1 treatment did not modify the mitochondrial MFN-2: DRP-1 ratio, suggesting mitochondrial dynamics are insensitive to MDIVI-1 in this T2D model.

**Summary**

Data from the current study support emergence of an early T2D phenotype in mice receiving a single dose of STZ coupled with 14 weeks of high-fat feeding. The T2D model displayed substantial weight gain and impaired glucose handling, and myocardial tolerance to I-R was moderately impaired. While the T2D state did not appear to influence baseline respiratory function, the model was associated with select sensitisation of complex II to the inhibitory effects of I-R (and may also exaggerate effects of I-R on complex I). The T2D model was also associated with shifts in AKT, ERK1/2 and GSK3β signalling, the ratio of BAX: BCL-2, and cytosolic and mitochondrial levels of DRP-1 and MFN-1. Nonetheless, *in vivo* treatment with the DRP-1 inhibitor MDIVI-1 failed to substantially improve either ischaemic tolerance or mitochondrial respiratory function. That said, the *in vivo* treatment regime was problematic, and changes with vehicle may have modified the final T2D phenotype and clouded somewhat the interpretation of the effects of MDIVI-1. Further work is needed to better delineate the effects of DRP-1 in this model. Nonetheless, there is little compelling evidence that DRP-1 inhibition would be a useful approach to limiting evolution of cardiac abnormalities in T2D.
Overall Conclusions

The circulating function of the myocardium is fundamental to survival. While the average human myocardium beats regularly to eject blood into the circulation more than 30 million times per year, prolonged exposure to many modifiable and non-modifiable CVD risk factors ultimately compromises its physiological function, contributing to the leading global cause of mortality and morbidity. Ischaemic heart disease results when the coronary circulation becomes obstructed, resulting in myocardial injury, inflammation and reduced delivery of oxygen. The mitochondria are the primary producers of oxidative energy, governing cell functionality and also death vs. survival. Depending on the pathogenic insult and/or stress, the mitochondria undergo morphological cycles of fusion-mediated elongation and branching vs. fission-mediated 'fragmentation' between initial biogenesis and ultimate disassembly. This cycle, coupled with ongoing molecular repair (eg. mitochondrial unfolded protein response), may be critical to maintenance of healthy mitochondrial populations. These roles, and evidence of dysregulation with aging and disease, have focussed attention on fission/fusion as therapeutic targets (Palaniyandi et al., 2010; Biala et al., 2015; Dorn, 2015). While many conventional therapies improve mitochondrial integrity and function, pleiotropically or distal to upstream molecular (eg. altered oxidant stress, substrate metabolism) and functional changes (eg. coronary/peripheral vasodilatation), specific mitochondrial targeting offers considerable potential across organ systems and diseases (Picard et al., 2016).

It is important to recognise that healthy cardiomyocytes possess small fragmented mitochondria and have the highest mitochondrial densities and respiratory capacities among mammalian cells (Song and Dorn, 2015). This steady-state, weighted to fission, may nonetheless be unbalanced with diseases (or drugs), promoting dysfunction and death. However, despite fission having being ‘associated’ with cell death (Frank et al., 2001; Taguchi et al., 2007), causal involvement is less clear. Enhanced fission via fusion protein knockout does not initiate death (Chen et al., 2005; Song and Dorn, 2015), with evidence suggesting that DRP-1 dependent fission may suppress apoptosis (Szabadkai et al., 2004), worsen post-ishaemic necroptosis in myocytes (Dong et al., 2016) and promote apoptosis in neuronal cells (Zhang et al., 2013). While these latter studies highlight the complexity of
mitochondrial function, fission, autophagy and differing paths of cell death, it remains undefined as to whether manipulation of these dynamic organelles warrant potential in a diseased setting? The primary goal of this doctoral project was thus to characterise the effects of DRP-1 inhibition in healthy and stressed or diseased myocardium, examining the broader utility of DRP-1 inhibition in clinical cardioprotection.

The two dynamin inhibitors employed in this study (MDIVI-1 and dynasore), reportedly inhibit DRP-1 activation, fission and also autophagy (Macia et al., 2006; Hong et al., 2013; Sharp et al., 2015). However, initial analyses in Chapter 3 revealed distinct effect of the 2 agents. These initial studies revealed specific inhibition of mitochondrial DRP-1 with MDIVI-1 vs. modulation of cytosolic rather than mitochondrial DRP-1 with dynasore in post-ischaemic myocardium. The agents induced mixed effects on I-R outcomes and cell signalling, appearing to dissociate functional and death outcomes and exerting both protective and injurious effects. Impacts of MDIVI-1 may be more pronounced during ischaemia/early reperfusion, consistent with emerging evidence that DRP-1 inhibition may be detrimental during reperfusion rather than during ischaemia. In vitro analyses undertaken in Chapter 4 and 5 support complex and sometimes paradoxic effects of DRP-1 inhibition on autophagy (increased), apoptosis (increased) and kinase signalling (biphasic increase) under acute and chronic conditions. The high degree of integration in mitochondrial dynamics, autophagy, apoptosis and oncosis raises questions regarding the broader utility of MDIVI-1/DRP-1 inhibitors as potential cardioprotective agents.

Investigations undertaken in Chapter 7, murine model of type 2 diabetes mellitus (T2D), established evidence of cardiac dysfunction, ischaemic intolerance, mitochondrial respiratory impairment and down regulation of pro-survival signalling, suggestive of a pro-apoptotic environment. These findings support emergence of cardiac pathophysiology in the early stages of development of T2D. In vivo administration of MDIVI-1 for 7 days was ineffective in modifying myocardial stress phenotype in T2D hearts, and indeed appeared to worsen ischaemic tolerance. While suggesting chronic manipulation of mitochondrial remodeling proteins in a diabetic heart may not be a useful therapeutic approach, untoward impacts of the treatment regime (including effects of vehicle) clouded data interpretation.
This doctoral work revealed a complex array of somewhat mixed outcomes of DRP-1 inhibition in different experimental models, raising important questions that warrant future investigation. However, some limitations are worth highlighting. The dose of MDIVI-1 employed in this study (in vivo and ex vivo) was lower than in previous cardiac focused studies (Ong et al., 2010; Gharanei et al., 2013; Hong et al., 2013). Sharp et al. (2014) reported improved post-ischaemic outcomes at a dose of 50 μM, however drug solubility is highly problematic and a lower and more selective concentration was studied in ex vivo hearts (1 and 5 μM) in studies detailed in Chapter 3. This limitation should not be ignored, with Rappold et al. (2014) reporting the very poor aqueous solubility of MDIVI-1, requiring use of a suspension rather than solution in vivo. Data from in vivo studies suggest that DRP-1 inhibition did not promote cardioprotection, though potentially clouded by apparent DMSO toxicity. Therefore, alternative administration (i.e. utilisation of subcutaneous implants) of DRP-1 inhibitors may be deemed worthwhile in assessing the efficacy of DRP-1 inhibition in a diseased model.

The current data indicate DRP-1 inhibition with MDIVI-1 but not dynasore does inhibit mitochondrial expression of DRP-1. However, direct assessment of mitochondrial morphology via electron microscopy would further support our understanding of how mitochondrial machinery interacts and co-localizes with potential downstream effectors. Furthermore, quantifying the rate of mitochondrial fragmentation vs. fusion would clarify the sequence of events that modify mitochondrial morphology in settings of disease, I-R and oxidative injury. That said, the present mix of findings is not an endorsement of DRP-1 inhibition as a protective therapy. This is not entirely unexpected as we begin to appreciate the unique features of mitochondrial fission in myocardium, and the integration of this process in cell death and survival responses. It may be naïve to assume we can perturb such a key regulatory process to achieve only beneficial outcomes. The divergent effects of MDIVI-1 in varying models may be ineffective at targeting these tightly regulated organelles, thus favoring pro-death signalling and dysfunction. These findings support important roles for DRP-1/fission in governing myocardial survival kinase signaling during ischaemic and oxidative insult, together with influencing processes of autophagy and apoptosis. Further unraveling the links between mitochondrial dynamics, cell signaling, damage management and cell death may not only advance our fundamental understanding of myocardial stress responses and mechanisms governing death and survival, but provide a
more rationalised basis for implementing still elusive clinical cardioprotection.
Supplementary figure 1: Mitochondrial functional analysis performed on intact cardiomyoblasts. Cardiomyoblasts were subjected to a Mitochondrial Stress Test (Seahorse, which permitted a functional analysis of; basal respiration, ATP production, proton leak, maximal respiration, spare respiratory capacity and non-mitochondrial respiration. The compounds applied in the mitochondrial stress test target the complexes embed within the electron transport chain of the mitochondria. Rotenone, antimycin A and oligomycin inhibit complexes I, III and V, respectively. The compound, FCCP, is an uncoupling agent, thus driving oxygen consumption from ATP production.
Supplementary Figure 2. Representative western immunoblot data. Representative western blots for protein expression in total and phosphorylated (Phospho) forms of ERK1/2 and AKT and COXIV (loading control) from heart cytosolic and mitochondrial cellular fractions detailed in Chapter 3 (Fig. 3.8). Alternating lanes (left-right) containing: C (control) and M (MDVI-1) for mitochondrial and cytosolic fractions. Significant changes were detected in phosphorylated ERK1/2 (cytosolic loading control GAPDH; mitochondrial loading control COXIV).
Supplementary Figure 3. Representative western immunoblot data. Representative western blots for protein expression in total and phosphorylated (Phospho) forms of ERK1/2, AKT and GAPDH (loading control) from Chapter 4 (Fig. 4.3). **Lane 1** - C (control); **Lane 2** - M (MDIVI-1 treated); **Lane 3** - H₂O₂ (H₂O₂ treated) and; **Lane 4** - H₂O₂ + M (H₂O₂ and MDIV-1 treated) in whole cell lysate from H9c2 cardiomyoblasts. Significant changes were detected in total and phosphorylated ERK1/2 (loading control GAPDH).
Supplementary Figure 4. Representative western immunoblot data. Representative western blots for protein expression in total and phosphorylated (Phospho) forms of ERK1/2 and AKT from Chapter 5 (Fig. 5.3). Left to right: Lane 1 - C-H (CTRL + H₂O₂); Lane 2 - C-M (CTRL + MDIVI-1); Lane 3 - 1 hr-H (1 hr H₂O₂); Lane 4 - 1 hr M (1 hr H₂O₂ + MDIVI-1); Lane 5 - 2 hr-H (2 hr H₂O₂); Lane 6 - 2 hr M (2 hr H₂O₂ + MDIVI-1); Lane 7 - 4 hr-H (4 hr H₂O₂); Lane 8 - 4 hr M (4 hr H₂O₂ + MDIVI-1); Lane 9 - 6 hr-H (6 hr H₂O₂); Lane 10 - 6 hr M (6 hr H₂O₂ + MDIVI-1); Lane 11 - 8 hr-H (8 hr H₂O₂); Lane 12 - 8 hr M (8 hr H₂O₂ + MDIVI-1). Whole cell lysate from H9c2 cardiomyoblasts.
Supplementary Figure 5. Representative western immunoblot data. Representative western blots for protein expression in MFN-1, MFN-2, OPA-1, DRP-1, BAX, BCL-2, Parkin and PARP1/2 from Chapter 5. Left to right: Lane 1 - C-H (CTRL + H$_2$O$_2$); Lane 2 - C-M (CTRL + MDIVI-1); Lane 3 - 1 hr-H (1 hr H$_2$O$_2$); Lane 4 - 1 hr M (1 hr H$_2$O$_2$ + MDIVI-1); Lane 5 - 2 hr-H (2 hr H$_2$O$_2$); Lane 6 - 2 hr M (2 hr H$_2$O$_2$ + MDIVI-1); Lane 7 - 4 hr-H (4 hr H$_2$O$_2$); Lane 8 - 4 hr M (4 hr H$_2$O$_2$ + MDIVI-1); Lane 9 - 6 hr-H (6 hr H$_2$O$_2$); Lane 10 - 6 hr M (6 hr H$_2$O$_2$ + MDIVI-1); Lane 11 - 8 hr-H (8 hr H$_2$O$_2$); Lane 12 - 8 hr M (8 hr H$_2$O$_2$ + MDIVI-1). Whole cell lysate from H9c2 cardiomyoblasts.
Supplementary Figure 6: Cardiomyoblast proliferation under varying glucose and insulin levels (+MDIVI-1). An assessment on H9c2 cardiomyoblast proliferation was undertaken over 48 hr exposure in combination with MDIVI-1 or respective vehicle: Control glucose (25 mM), low glucose (5 mM), high glucose (100 mM), high glucose (100 mM) + hyperinsulinaemia (100 nM), low glucose (5 mM), high glucose (100 mM) and high glucose (100 mM) + insulin (100 nM).
Supplementary Figure 7: Representative western immunoblot data.

**Representative images of western immunoblots** from data presented in Chapter 7. **Lane 1**-CTRL (vehicle); **Lane 2**-CTRL (MDIVI-1); **Lane 3**-T2D (vehicle) and **Lane 4**-T2D (MDIVI-1). Significant changes were detected in mitochondrial fractions for MFN-1, OPA-1 and total AKT (loading control COX IV). Significant changes were detected in cytosolic fractions for total DRP-1, total AKT, phosphorylated-ERK1/2 (loading control GAPDH).
Supplementary Figure 8: A comparative image of visceral adiposity. Visceral adiposity in mice administered a standard chow diet vs. obesogenic diet 15 wk post feeding regime (post-sacrifice).
Supplementary Table 1: Nutrient composition. Nutrient composition of standard-chow diet and obesogenic diet administered to male C57Bl6 male mice during the feeding regime (Wensley et al., 2013)

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Standard Chow Diet</th>
<th>Obesogenic Diet</th>
<th>Fold-change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kJ)</td>
<td>1844</td>
<td>2047</td>
<td>1.11</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>22.85</td>
<td>13.78</td>
<td>0.6</td>
</tr>
<tr>
<td>Fat-total (g)</td>
<td>7.26</td>
<td>17.29</td>
<td>2.38</td>
</tr>
<tr>
<td>Saturated (g)</td>
<td>1.06</td>
<td>8.11</td>
<td>7.63</td>
</tr>
<tr>
<td>Monounsaturated (g)</td>
<td>3.42</td>
<td>6.99</td>
<td>2.04</td>
</tr>
<tr>
<td>Polyunsaturated (g)</td>
<td>2.5</td>
<td>1.24</td>
<td>0.5</td>
</tr>
<tr>
<td>Trans (g)</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>Nil</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>69.93</td>
<td>69.04</td>
<td>0.99</td>
</tr>
<tr>
<td>Sugar (g)</td>
<td>36.34</td>
<td>54.62</td>
<td>1.5</td>
</tr>
<tr>
<td>Sodium (g)</td>
<td>1632.96</td>
<td>1336.31</td>
<td>0.82</td>
</tr>
</tbody>
</table>
Supplementary Table 2: Non-fasted blood lipid profiles and glucose. Whole blood lipid profiles were assessed via the Alere Cholestech LDX machine in mice immediately post sacrifice in blood collected from the chest cavity or cardiac puncture.

<table>
<thead>
<tr>
<th></th>
<th>HDL (mmol/L)</th>
<th>LDL (mmol/L)</th>
<th>Non-LDL (mmol/L)</th>
<th>Triglycerides (mmol/L)</th>
<th>Total cholesterol (mmol/L)</th>
<th>LDL/HDL (mmol/L)</th>
<th>Non-fasted glucose (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (Vehicle)</td>
<td>n =6</td>
<td>1.11 ± 0.110</td>
<td>1.31 ± 0.09</td>
<td>1.73 ± 0.14</td>
<td>1.2 ± 0.08</td>
<td>2.90 ± 0.07</td>
<td>1.20 ± 0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (MDIVI-1)</td>
<td>n =6</td>
<td>1.12 ± 0.06</td>
<td>0.99 ± 0.05</td>
<td>1.47 ± 0.03</td>
<td>0.98 ± 0.06</td>
<td>2.77 ± 0.07</td>
<td>0.76 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2D (Vehicle)</td>
<td>n =6</td>
<td>1.54 ± 0.06*</td>
<td>0.92 ± 0.09</td>
<td>1.04 ± 0.03</td>
<td>1.00 ± 0.17</td>
<td>2.96 ± 0.07</td>
<td>0.80 ± 0.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2D (MDIVI-1)</td>
<td>n =6</td>
<td>1.58 ± 0.14*</td>
<td>0.30 ± 0.11†*</td>
<td>1.28 ± 0.13</td>
<td>1.43 ± 0.12</td>
<td>3.02 ± 0.07</td>
<td>0.15 ± 0.03*</td>
</tr>
</tbody>
</table>

Low-density lipoprotein (LDL), non-low-density lipoprotein (non-LDL), high-density lipoprotein (HDL), LDL/HDL ratio, total cholesterol and triglycerides. Data are means ± SEM (n = 6 per group). *, P<0.05 vs. respective control; †, P<0.05 vs. respective vehicle; **, P<0.01 vs. cardiac puncture (respective group).
REFERENCE LIST


