Targeting the Increase in Oxidative Stress in Diabetic Cardiomyopathy Using Coenzyme Q₁₀

KARINA HUYNH
Bachelor of Biomedical Sciences (Honours)

Heart Failure Pharmacology Laboratory,
BakerIDI Heart and Diabetes Institute

&

Department of Medicine, Central and Eastern Clinical School
Monash University, Alfred Hospital, Melbourne, Australia

Submitted in total fulfilment of the requirements of the degree of Doctor of Philosophy
Notice 1
Under the Copyright Act 1968, this thesis must be used only under the normal conditions of scholarly fair dealing. In particular no results or conclusions should be extracted from it, nor should it be copied or closely paraphrased in whole or in part without the written consent of the author. Proper written acknowledgement should be made for any assistance obtained from this thesis.
Table of Contents

Abstract vii
General Declaration ix
Acknowledgements xi
Publications Manuscripts xiii
Abstracts xiv
Abbreviations xvii

Chapter 1 – General Introduction

1.1 Diabetic Cardiomyopathy

1.1.1 Introduction and overview of type 1 and type 2 diabetes 2
1.1.2 Microvascular and macrovascular complications of diabetes 4
1.1.3 Features of the diabetic heart 6
  1.1.3.1 Diastolic dysfunction 6
  1.1.3.2 Systolic dysfunction 9
  1.1.3.3 Cardiomyocyte hypertrophy 9
  1.1.3.4 Myocardial fibrosis 10
  1.1.3.5 Apoptosis 11
  1.1.3.6 Microvascular abnormalities 11

1.2 Pathophysiologic triggers of diabetic cardiomyopathy

1.2.1 Hyperglycemia 12
1.2.2 Insulin resistance 12
1.2.3 Metabolic disturbances in the diabetic heart 13
1.2.4 Altered Ca^{2+} handling in the diabetic heart 15
  1.2.4.1 Impaired Ca^{2+} handling and cardiac dysfunction in diabetes 15
  1.2.4.2 SERCA2a modulates contraction/relaxation of cardiomyocytes 17
1.2.5 Oxidative stress in the development of diabetic cardiomyopathy 18
  1.2.5.1 Sources of ROS in the heart 18
1.2.5.2 Role of ROS in heart disease
   1.2.5.2.1 ROS as a driver of cardiac hypertrophy
   1.2.5.2.2 ROS as a driver of cardiac fibrosis
1.2.5.3 Role of ROS in diabetic cardiac complications
1.2.5.4 Mechanisms of hyperglycemia-induced oxidative stress
1.2.5.5 Oxidative stress, hyperglycemia and cell death
1.2.5.6 Cardioprotective effect of antioxidant administration
   1.2.5.6.1 Superoxide dismutase
   1.2.5.6.2 Catalase
   1.2.5.6.3 Glutathione peroxidise
   1.2.5.6.4 Thioredoxin
   1.2.5.6.5 Vitamin C & Vitamin E
   1.2.5.6.6 Metallothionein
   1.2.5.6.7 Coenzyme Q10

1.2.6 RAAS and the use of ACE-Is in treating diabetes
   1.2.6.1 Overview of the RAAS
   1.2.6.2 Mechanisms of RAAS blockade
      1.2.6.2.1 Mechanism of action and pharmacology of ACE-Is
   1.2.6.3 The role of ACE-I in the prevention of diabetic cardiomyopathy
      1.2.6.3.1 ACE-Is and cardiovascular disease
      1.2.6.3.2 ACE-Is for the management of diabetes

1.3 Role of PI3K(p110α) in preventing diabetic cardiomyopathy
   1.3.1 Physiological vs pathological hypertrophy
   1.3.2 IGF1-PI3K(p110α)-Akt signaling in heart disease
      1.3.2.1 Cardioprotective IGF-1 signaling in cardiovascular disease
      1.3.2.2 Cardioprotective PI3K(p110α) signaling in cardiovascular disease
   1.3.3 IGF1-PI3K(p110α)-Akt signaling in diabetic cardiomyopathy

1.4 Experimental models of diabetes
   1.4.1 Common type 1 diabetic rodent models
      1.4.1.1 Streptozotocin (STZ) model
      1.4.1.2 OVE26 mouse model
1.4.1.3 Biobreeding Wistar rat model
1.4.1.4 Akita (Ins2\textsuperscript{Akita}) mouse model

**1.4.2 Common type 2 diabetic rodent models**

1.4.2.1 \textit{ob/ob} mouse model
1.4.2.2 \textit{db/db} mouse model
1.4.2.3 Zucker Fatty Rat and Zucker Diabetic Fatty Rat
1.4.2.4 Diet-induced diabetic models
1.4.2.5 Knock-out mouse models of insulin resistance

**1.4.3 Summary of the rodent models of diabetic cardiomyopathy**

1.5 **Summary**

1.6 **Aims**

---

**Chapter 2 – Coenzyme Q\textsubscript{10} Attenuates Diastolic Dysfunction, Cardiomyocyte Hypertrophy and Cardiac Fibrosis in the \textit{db/db} Mouse Model of Type 2 Diabetes**

**Declaration and general information**

**Abstract**

**Introduction**

**Methods**

- Animal model
- Analysis of LV function \textit{in vivo}
- Tissue collection and histology
- Gene expression and protein analysis
- Detection of lipid peroxidation and superoxide generation
- Statistical analysis

**Results**

- Hyperglycemia is evident in \textit{db/db} mice
- Coenzyme Q\textsubscript{10} preserves diastolic function
- Cardiac fibrosis is reduced in coenzyme Q\textsubscript{10}-treated diabetic mice
- Coenzyme Q\textsubscript{10} limits diabetes-induced cardiomyocyte hypertrophy
- Coenzyme Q\textsubscript{10} prevents diabetes-induced cardiomyocyte death
- Coenzyme Q\textsubscript{10} attenuates oxidative stress in \textit{db/db} mice
Chapter 3 – Targeting the Upregulation of Reactive Oxygen Species Subsequent to Hyperglycemia Prevents Type 1 Diabetic Cardiomyopathy in Mice

Declaration and general information 87
Abstract 90
Introduction 90
Methods 91
Animal model 91
Analysis of cardiac function in vivo 91
Tissue collection and histology 91
Analysis of gene expression 92
Western analysis 92
Detection of superoxide generation and lipid peroxidation 92
Statistical analysis 92
Results 92
Systemic characteristics 92
Coenzyme Q_{10} attenuates type 1 diabetes-induced oxidative stress 93
Coenzyme Q_{10} attenuates LV inflammation and apoptosis in type 1 diabetes 94
Diastolic function is improved with coenzyme Q_{10} administration 94
Cardiac remodeling is limited by coenzyme Q_{10} administration 94
Discussion 95
Conclusion 97
References 98

Chapter 4 – Coenzyme Q_{10} protects the Heart against Diastolic Dysfunction and Structural Remodeling in mice with Diminished PI3K(p110α) Signaling
4.1 Introduction

4.2 Methods

4.2.1 Animal model

4.2.2 Glucose measurements

4.2.3 Analysis of cardiac function in vivo

4.2.3.1 M-mode and baseline echocardiography

4.2.3.2 Cardiac catheterization

4.2.4 Tissue collection

4.2.5 Histology

4.2.5.1 H&E staining

4.2.5.2 Picrosirius red staining

4.2.6 Detection of superoxide generation and lipid peroxidation

4.2.6.1 Superoxide generation

4.2.6.2 MDA assay

4.2.7 Analysis of gene expression

4.2.8 Statistical analysis

4.3 Results

4.3.1 Systemic data

4.3.2 Coenzyme Q\textsubscript{10} reduces oxidative stress in dnPI3K diabetic mice

4.3.3 Improved diastolic function with coenzyme Q\textsubscript{10} administration in Ntg and dnPI3K mice

4.3.4 Coenzyme Q\textsubscript{10} administration attenuated diabetes-induced cardiomyocyte hypertrophy in both Ntg and dnPI3K mice

4.3.5 Diabetes-induced cardiac fibrosis is reduced with coenzyme Q\textsubscript{10} administration

4.4 Discussion

4.5 Limitations and Future Directions

Chapter 5 – General Discussion

5.1 Coenzyme Q\textsubscript{10} attenuated diabetes-induced oxidative stress

5.2 Coenzyme Q\textsubscript{10} attenuated diabetes-induced diastolic dysfunction and cardiac fibrosis

5.3 Coenzyme Q\textsubscript{10} reduced cardiomyocyte hypertrophy in diabetic mice
5.4 Beneficial effect of coenzyme Q₁₀ in mice with reduced PI3K(p110α) signaling 134
5.5 Comparing the efficacy of coenzyme Q₁₀ with an ACE-I in preventing diabetic cardiomyopathy 136
5.6 Future directions 137
5.7 Conclusion 138
References 139

Appendices (Other publications during PhD candidature)

Appendix 1 - Cardiac-specific IGF-1R transgenic expression protects against cardiac fibrosis and diastolic dysfunction in a mouse model of diabetic cardiomyopathy 174

Appendix 2 - Enhanced PI3K(p110α) activity prevents diabetes-induced cardiomyopathy and superoxide generation in a mouse model of diabetes 184

Appendix 3 - Ubiquinone (coenzyme Q₁₀) prevents renal mitochondrial dysfunction in an experimental model of type 2 diabetes 198
Abstract

Diabetes is one of the fastest growing chronic diseases of the 21st century. Diabetic patients are at increased risk of developing heart failure. Diabetic cardiomyopathy, characterized by early diastolic dysfunction and adverse morphological changes, develops independently of macrovascular and microvascular complications. The development of these changes are often attributed to increased oxidative stress, resulting from elevated reactive oxygen species (ROS) production and diminished antioxidant defences. Supplementation with the potent endogenous antioxidant coenzyme Q10 has been shown to be beneficial in improving cardiac function and reducing left ventricular (LV) hypertrophy outside of diabetes; its benefit in protecting the diabetic heart remains unknown.

The first aim of the project was to determine the efficacy of coenzyme Q10 supplementation in protecting the heart from type 2 diabetes-induced damage, using the spontaneous db/db mouse model (obese and hypertensive). Mice were treated with coenzyme Q10, or with the angiotensin-converting enzyme inhibitor (ACE-I) ramipril daily for 10 weeks. Untreated db/db mice exhibited significant diastolic dysfunction in vivo, as well as cardiomyocyte hypertrophy, cardiac fibrosis and apoptosis. These adverse cardiac changes were accompanied by a significant upregulation in systemic lipid peroxidation and cardiac superoxide (•O2-) generation, with concomitant modest hypertension. Supplementation with coenzyme Q10 administration significantly attenuated diastolic dysfunction and was associated with reduced markers of hypertrophy, fibrosis and apoptosis in diabetic mice. Interestingly, coenzyme Q10 treatment in diabetic animals also tended to modestly improve glycemic control and lower blood pressure in diabetic animals. The efficacy of coenzyme Q10 and ramipril were comparable in this model.

The second aim of the project was to determine the efficacy of coenzyme Q10 in protecting the non-obese, non-hypertensive type 1 diabetic heart from diabetes-induced damage. Insulin-deficient streptozotocin (STZ) mice were treated with either coenzyme Q10 or ramipril. As evident in db/db mice, untreated STZ type 1 diabetic mice exhibited diastolic dysfunction, cardiomyocyte hypertrophy, elevated cardiac collagen deposition and enhanced cardiomyocyte apoptosis. Markers of oxidative stress, including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit expression (Nox2, p22phox, p47phox), 3-nitrotyrosine (3-NT) protein levels, NADPH-driven •O2- generation and systemic lipid
peroxidation were all upregulated in untreated STZ mice. These mice also exhibited increased expression of pro-inflammatory markers tumor necrosis factor $\alpha$ (TNF$\alpha$) and interleukin-1$\beta$ (IL-1$\beta$). Coenzyme Q$_{10}$ attenuated all the adverse functional and structural changes in the diabetic heart, as well as reducing all markers of oxidative stress and inflammation.

We have evidence that increased phosphoinositide 3-kinase(p110$\alpha$) [PI3K(p110$\alpha$)] signaling is protective (and diminished PI3K(p110$\alpha$) signaling, detrimental) to the diabetic heart, and that this may be linked to PI3K(p110$\alpha$) negative regulation of ROS generation. Hence, the third aim of the project was to determine whether coenzyme Q$_{10}$ protects the diabetic dnPI3K heart from damage. Diabetic dnPI3K untreated mice exhibited reduced diastolic function and evidence of adverse cardiac remodeling, as previously observed. Coenzyme Q$_{10}$ treatment of diabetic dnPI3K mice significantly attenuated diastolic function and the adverse changes in cardiac morphology. Interestingly, NADPH-driven $\cdot$O$_2^-$-generation was exacerbated in dnPI3K diabetic mice compared to non-transgenic (Ntg) diabetic mice. Coenzyme Q$_{10}$ supplementation also reduced LV $\cdot$O$_2^-$ generation and diminished systemic lipid peroxidation in treated dnPI3K diabetic animals as part of its cardioprotection.

In summary, this study highlighted the characteristic features of the type 1 and type 2 diabetic heart, which includes early diastolic dysfunction, and morphological changes including cardiomyocyte hypertrophy, cardiac fibrosis and enhanced apoptosis. This study showed for the first time that coenzyme Q$_{10}$ was able to attenuate diastolic dysfunction and structural remodeling in the diabetic heart, likely via reduced LV $\cdot$O$_2^-$ generation and lipid peroxidation. Coenzyme Q$_{10}$ was also effective in protecting the hearts of diabetic dnPI3K transgenic mice (in spite of exacerbated ROS levels). As coenzyme Q$_{10}$ was at least as effective as ramipril in protecting the hearts of diabetic mice against damage, coenzyme Q$_{10}$ may be a possible alternative or adjunct therapy to ACE-Is for the treatment of diabetic cardiomyopathy in the clinical setting.
General Declaration

Declaration for thesis based or partially based on conjointly published or unpublished work

In accordance with Monash University Doctorate Regulation 17 Doctor of Philosophy and Research Master’s regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes two original papers published in peer reviewed journals. The core theme of the thesis is the use of antioxidants for treating diabetic cardiomyopathy. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Heart Failure Pharmacology laboratory under the supervision of A/Prof Rebecca Ritchie and A/Prof Julie McMullen.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of chapters two and three my contribution to the work involved the following:

**Chapter Two:** 80% of experiments were carried out by myself, and I was responsible for all data and statistical analysis. I wrote the manuscript with intellectual input and assistance from co-authors.

**Chapter three:** 80% of experiments were carried out by myself, and I was responsible for all data and statistical analysis. I wrote the manuscript with intellectual input and assistance from co-authors.
<table>
<thead>
<tr>
<th>Thesis chapter</th>
<th>Publication title</th>
<th>Publication status*</th>
<th>Nature and extent of candidate’s contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Coenzyme Q\textsubscript{10} Attenuates Diastolic Dysfunction, Cardiomyocyte Hypertrophy and Cardiac Fibrosis in the \textit{db/db} Mouse Model of Type 2 Diabetes</td>
<td>Published</td>
<td>Performed 80% of experiments, wrote manuscript with assistance from co-authors</td>
</tr>
<tr>
<td>3</td>
<td>Targeting the Upregulation of Reactive Oxygen Species Subsequent to Hyperglycemia Prevents Type 1 Diabetic Cardiomyopathy in Mice</td>
<td>Accepted for publication</td>
<td>Performed 80% of experiments, wrote manuscript with assistance from co-authors</td>
</tr>
</tbody>
</table>

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: ................................................................................................................................................

Date: ........................................................................
Acknowledgements

Firstly, I’d like to express my gratitude towards my supervisors A/Prof Rebecca Ritchie and A/Prof Julie McMullen for their guidance, encouragement and unwavering support throughout these 5 years, from Honours until the completion of this PhD thesis. Rebecca – thank you for taking me under your wing these 5 years, I could not have done it without you. Julie – thank you for your wisdom and understanding. You both continue to inspire me with your work ethic and commitment to scientific research – I hope I have done you proud.

I would also like to extend my deepest thanks to Prof Xiao-Jun Du and Dr Helen Kiriazis from the Experimental Cardiology lab for your assistance with mouse echocardiography and catheterization, as well as their helpful advice and discussions in the preparation of manuscripts for publication. Thanks also to Nicole Jennings for your assistance with echocardiography.

My sincere appreciation also goes out to Prof Karin Jandeleit-Dahm and A/Prof Josephine Forbes from the Diabetic Complications lab for taking your time to participate in discussions pertaining to my project. A special thanks to Kylie Gilbert for your technical assistance with the db/db animals.

A big thank you to both past and present members of the Heart Failure Pharmacology and Cardiac Hypertrophy laboratory, in particular Dr Helena Qin and Dr Nga Cao. You offered me words of encouragement and understanding during the most turbulent times, and I am endlessly grateful for your support.

In these 5 years at Baker IDI, I have made many lifelong friends which have made this experience all the more memorable. To Ouda, Amy, Tracey and Nick – thanks for the laughs and the good times. You have been such a big part of this experience, and I am so thankful to have met you all in the process.

To my best friends in the world, Fiona, Melanie, Kerry, Lan, Phillida and Je – thank you for your love, friendship and encouragement throughout all these years. Whilst I doubt any of you have any clue as to what my thesis is actually about, or what it is I do in the lab (up until recently, Fiona thought that the Baker was a pub I frequented most days of the week), your laughter and support has kept me sane through trying times.
To my fiance Han, I am so grateful to have your support, encouragement and understanding. Thank you for always being there to cheer up me at the end of a long, stressful day at the lab, and always reminding me to put things into perspective.

Finally, I’d like to thank my parents and my sister for your unconditional love and guidance. Your belief in me has pushed me to keep trying and to never give up. I dedicate all my achievements to you.
Publications

Manuscripts


Signaling Pathways. Pharmacology and Therapeutics (invited 8/6/12, submission deadline 30/4/13). IF 8.562.

Invited Conference Presentations

**Huynh K**, Kiriazis H, Du XJ, Love JE, Jandeleit-Dahm KA, Forbes JM, McMullen JR, Ritchie RH (2012). Diabetes-Induced Apoptosis and Superoxide Generation is Reduced With Coenzyme Q_{10} Administration in a Mouse Model of Type 1 Diabetes. 6th Australian Health and Medical Research Congress 2012, Adelaide (28/11/12). Invited talk.

Abstracts


3) **Huynh K**, Kiriazis H, Du XJ, Love JE, Jandeleit-Dahm KA, Forbes JM, McMullen JR, Ritchie RH (August 2010). Coenzyme Q_{10} Inhibits Cardiomyocyte Hypertrophy, Myocardial Fibrosis and Diastolic Dysfunction in a Mouse Model of Type 2 Diabetes. Joint scientific meeting of Cardiac Society of Australia and New Zealand/International Society (Australasian section), Adelaide. Shortlisted for ISHR student oral prize (one of four finalists).

4) **Huynh K**, Bowden MA, Kiriazis H, Du XJ, Love JE, Jandeleit-Dahm KA, Forbes JM, McMullen JR, Ritchie RH (November 2010). Coenzyme Q_{10} Rescues Diastolic
Dysfunction, Cardiomyocyte Hypertrophy and Cardiac Fibrosis in Female *db/db* Type 2 Diabetic Mice. American Heart Association Scientific Sessions Chicago, Illinois, USA. Poster presentation.


9) **Huynh K**, Kiriazis H, Du XJ, Love JE, Jandeleit-Dahm KA, McMullen JR, Ritchie RH. (2012) Coenzyme Q₁₀ Protects Against Diabetes-Induced Diastolic Dysfunction and Adverse Cardiac Remodeling in a Mouse Model of Type 1 Diabetes. AMREP post-graduate symposium 2012, Melbourne (shortlisted for student prize).

and Adverse Cardiac Remodeling in a Mouse Model of Type 1 Diabetes. Alfred Research Week 2012. Winner of Baker IDI Heart and Diabetes Institute Prize for Diabetes Research.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-NT</td>
<td>3-nitrotyrosine</td>
</tr>
<tr>
<td>ACE-I</td>
<td>angiotensin converting enzyme inhibitor</td>
</tr>
<tr>
<td>AGE</td>
<td>advanced glycation end-product</td>
</tr>
<tr>
<td>AMI</td>
<td>acute myocardial infarction</td>
</tr>
<tr>
<td>Ang II</td>
<td>angiotensin II</td>
</tr>
<tr>
<td>ANP</td>
<td>atrial natriuretic peptide</td>
</tr>
<tr>
<td>BB</td>
<td>biobreeding</td>
</tr>
<tr>
<td>BNP</td>
<td>B-type natriuretic peptide</td>
</tr>
<tr>
<td>BW</td>
<td>body weight</td>
</tr>
<tr>
<td>CaPI3K</td>
<td>constitutively active phosphoinositide 3-kinase (p110α)</td>
</tr>
<tr>
<td>CIRKO</td>
<td>cardiomyocyte-restricted knock-out of the insulin receptor</td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>copper/zinc superoxide dismutase</td>
</tr>
<tr>
<td>CTGF</td>
<td>connective tissue growth factor</td>
</tr>
<tr>
<td>DCM</td>
<td>dilated cardiomyopathy</td>
</tr>
<tr>
<td>dnPI3K</td>
<td>dominant negative phosphoinositide-3 kinase</td>
</tr>
<tr>
<td>E/A</td>
<td>early/atrial wave ratio</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ecSOD</td>
<td>extracellular superoxide dismutase</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ET-1</td>
<td>endothelin-1</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>FADH₂</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FFAs</td>
<td>free fatty acids</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>GHB</td>
<td>glycated hemoglobin</td>
</tr>
<tr>
<td>GLUT4</td>
<td>glucose transporter 4</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
</tr>
<tr>
<td>GPx1</td>
<td>glutathione peroxidase 1</td>
</tr>
<tr>
<td>HbA₁c</td>
<td>glycated hemoglobin</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HW</td>
<td>heart weight</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor -1</td>
</tr>
<tr>
<td>IL-1β</td>
<td>interleukin-1β</td>
</tr>
<tr>
<td>I-R</td>
<td>ischemia-reperfusion</td>
</tr>
<tr>
<td>IVRT</td>
<td>isovolumetric ventricular relaxation time</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>LV</td>
<td>left ventricular</td>
</tr>
<tr>
<td>LVEDP</td>
<td>left ventricular end diastolic pressure</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MDA</td>
<td>malondialdehyde</td>
</tr>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>MMPs</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MnSOD</td>
<td>manganese superoxide dismutase</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetylcysteine</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>Ntg</td>
<td>non-transgenic</td>
</tr>
<tr>
<td>•O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>superoxide</td>
</tr>
<tr>
<td>•OH</td>
<td>hydroxyl radical</td>
</tr>
<tr>
<td>ONOO&lt;sup&gt;-&lt;/sup&gt;</td>
<td>peroxynitrite</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PLB</td>
<td>phospholamban</td>
</tr>
<tr>
<td>PPAR-α</td>
<td>peroxisome proliferator-activated receptor alpha</td>
</tr>
<tr>
<td>RAAS</td>
<td>renin-angiotensin-aldosterone system</td>
</tr>
<tr>
<td>RAGE</td>
<td>receptor for advanced glycation end products</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>SERCA2a</td>
<td>sarcoplasmic reticulum calcium-ATPase isoform 2a</td>
</tr>
<tr>
<td>SHR</td>
<td>spontaneously hypertensive rat</td>
</tr>
</tbody>
</table>
SOD  superoxide dismutase
SR   sarcoplasmic reticulum
STZ  streptozotocin
T1DM type 1 diabetes mellitus
T2DM type 2 diabetes mellitus
TGF-β transforming growth factor beta
TIMPs tissue inhibitor of matrix metalloproteinase
TL   tibia length
Tfam transcription factor A
TNFα tumour necrosis factor alpha
Trx  thioredoxin
UCP3 mitochondrial uncoupling protein 3
ZDF Zucker diabetic fatty rat
CHAPTER ONE

General Introduction
1.1 Diabetic cardiomyopathy

1.1.1 Introduction and overview of type 1 and type 2 diabetes

Diabetes mellitus is a major threat to human health in the 21st century due to its alarming rise in incidence over the past two decades, which has attracted considerable attention. This rise in incidence has been largely attributed to environmental and lifestyle changes, where an increased occurrence of obesity is accompanied by an increasing number of people diagnosed with diabetes mellitus [1]. An estimated 285 million adults globally were burdened by this chronic disease in 2010; this number has been projected to increase to 439 million by 2030 [2]. Diabetes is regarded as the 5th leading cause of death worldwide, following infectious diseases, cardiovascular disease, cancer and trauma [3]. The rise in incidence and prevalence of diabetes also imposes a significant economic burden globally, particularly in developed countries, with an estimated 12% of the worldwide health care expenditure spent on the treatment and prevention of diabetes [4].

Diabetes is a chronic, progressive metabolic disorder characterized by insulin deficiency and/or resistance, resulting in elevated plasma glucose levels. There are two predominant types of diabetes; type 1 diabetes mellitus (T1DM), formerly known as insulin-dependent or juvenile diabetes and type 2 diabetes mellitus (T2DM), otherwise known as non-insulin-dependent or adult-onset diabetes. T1DM, which accounts for approximately 5-10% of all cases of diabetes [5], has a steadily increasing incidence worldwide [6]. Autoimmune mechanisms (at least in genetically-predisposed individuals) and/or environmental risk factors are regarded as key triggers of T1DM [1, 7] (Figure 1.1). T2DM, characterized by insulin resistance, accounts for the remaining ~90% of all cases of diabetes, and occurs predominantly, but not exclusively, in the older population [1]. The global rate of mortality attributable to T2DM has been estimated at 2.9 million, or 5.2% of all deaths [3, 8]. In contrast to developed countries (where T2DM is most evident in individuals over 60 years of age), people aged 40-60 years old make up the majority of T2DM cases in developing countries [2], thus likely to impact on productivity in those of working age. The rising incidence of T2DM is strongly associated with environmental factors including obesity and sedentary lifestyle [1] that accompany an increasingly ‘westernised-diet’, higher in fat, sugar and energy density [9] (Figure 1.2). Genetic factors such as family history of diabetes and ethnic background are also important for the development of the disease [8]. Insulin resistance is the primary metabolic abnormality amongst T2DM patients, resulting in both
Figure 1.1 Current understanding of the pathophysiology of type 1 diabetes.
T1DM results from the destruction of insulin-producing pancreatic β-islet cells, predominantly via T cell-mediated autoimmune attack against pancreatic islet autoantigens [7]. β-cells are destroyed via apoptosis, by the recruitment and release of cytotoxic molecules such as cytokines by CD4 and CD8 T cells, and subsequent activation of the caspase pathway. Complete β-cell destruction results in insulin deficiency, and thus diminished glucose uptake in fat and muscle cells. ROS, reactive oxygen species; GLUT4, glucose transporter 4; •O₂⁻, superoxide. See text for references.
hyperglycemia and hyperinsulinemia. Pancreatic β-islet cell dysfunction has also been implicated in the progression of T2DM [10, 11]. Glucotoxicity and lipotoxicity may further impair and reduce the rate of insulin secretion from dysfunctional β-islet cells [8].

1.1.2 Microvascular and macrovascular complications of diabetes

As has been widely reviewed, both T1DM and T2DM are associated with increased risk of macrovascular and microvascular complications [12]. These vascular injuries often coexist and can result in hypertension, altered vascular permeability and ischemia [13, 14]. Common microvasculature defects evident in diabetes include retinopathy, nephropathy and peripheral neuropathy, each of which can impart debilitating consequences. Diabetic retinopathy can lead to blindness [15], diabetic nephropathy can result in end-stage renal failure [13] and diabetic neuropathy can progress to peripheral nerve dysfunction in distal regions such as the hands and feet [16]. Diabetic retinopathy also serves as a marker of generalised hyperglycemic damage in the microvasculature [15]. In addition to these widespread defects in the diabetic systemic microvasculature, macrovascular complications such as coronary heart disease, stroke and peripheral vascular disease are thought to be the primary causes of morbidity and mortality in diabetic patients [12]. Increased plaque formation, atherosclerosis progression and vasodilator/vasoconstrictor dysregulation are evident in larger arteries [17], as well as impairments at the level of platelet function (hyperaggregability, reduced fibrinolysis, etc) and alterations in blood flow. The combination of all of these vascular defects contributes to the high incidence of cardiovascular disease, cerebrovascular disease (including stroke) and peripheral arterial disease in diabetes [18].

Independent of the microvascular and macrovascular complications of the disease, both clinical and experimental studies have highlighted the existence of a specific diabetic cardiomyopathy, in which alterations at the level of the cardiomyocyte are evident [19]. Rubler et al. first described diabetic cardiomyopathy as a distinct entity 40 years ago, in a small cohort of diabetic patients with adverse myocardial structural changes at post-mortem, in the absence of coronary arterial disease, hypertension or valvular complications [20]. Considerable attention was dedicated to identifying and characterizing this distinct cardiomyopathy in the following four decades, thus profoundly increasing the awareness of diabetes as a risk factor for heart failure. The Framingham Heart study had established early
Figure 1.2 Current understanding of the pathophysiology of type 2 diabetes

An increase in the consumption of ‘westernized foods’ (high fat, high sugar, and energy-dense processed foods), coupled with increasingly sedentary lifestyles are thought to have contributed to the diabetes epidemic of the 21st century. Increased glycemic load and genetic susceptibility contributes to insulin resistance, and pancreatic β-islet cell dysfunction and death, culminating in impaired glucose uptake into the cell [10]. See text for additional references.
epidemiological links between diabetes and increased risk of heart failure [21]. When compared to age-matched control subjects, the relative risk of developing heart failure was 2-fold greater in diabetic males, and 5-fold greater in diabetic females, independent of age, obesity, dyslipidemia and coronary heart disease. More recent retrospective analysis has suggested that diabetes not only escalates the risk of heart failure, but also increases its incidence > 2.5-fold [22, 23]. Prevalence of heart failure continues to increase with age; for every elderly diabetic subject free of heart failure, approximately 12 will develop heart failure over the ensuing year, with 6 mortalities [24]. As a result, diabetic patients account for 15% to 35% of patients in heart failure clinical trials [25], and diabetes is an independent predictor of poor outcome in heart failure, as demonstrated in the Studies of Left Ventricular Dysfunction (SOLVD) trials and others [26-28]. This strong association between diabetes and heart failure is further supported by evidence from experimental models, where diabetes induces cardiac structural, metabolic and functional change [29-31]. These disturbances that characterize diabetic cardiomyopathy likely contributed to the high incidence of cardiac mortality in diabetic patients [32-34]. Therefore, the mechanisms underlying the development of diabetic cardiomyopathy warrant urgent and detailed investigation, in order to improve disease management strategies and reduce morbidity and mortality rates in affected patients.

1.1.3 Features of the diabetic heart

1.1.3.1 Diastolic dysfunction

LV diastolic dysfunction is one of the first signs of diabetic cardiomyopathy, often developing before systolic dysfunction [35, 36]. The prevalence of diastolic heart failure, also known as heart failure with preserved ejection fraction, is continually rising. It is estimated to account for one third to one half of all heart failure occurrences [25, 37-39], with similar prognosis as systolic heart failure [40, 41]. As recently reviewed by Wood et al., systolic and diastolic dysfunction can result in differences in gross cardiac morphology, associated for example with a dilated ventricle versus an absence of chamber dilation, respectively [42]. There remains a debate regarding the existence of isolated diastolic dysfunction as an indicator of diabetic cardiomyopathy, as patients in the early stages of diabetes do not routinely receive evaluation of diastolic function. Furthermore, the cardiac complications of diabetes are usually investigated only after overt symptoms are evident.
Diastolic dysfunction, characterized by impaired and prolonged isovolumetric ventricular relaxation time (IVRT) [43], can be reliably detected using imaging techniques such as Doppler echocardiography, tissue Doppler and magnetic resonance imaging (MRI) [32, 44, 45]. Doppler echocardiography measures peak blood flow velocity across the mitral valve, to permit regional assessment of myocardial filling, using the ratio of the peak initial (E, early) and second (A, or atrial) blood flow velocity across the mitral valve (Figure 1.3). A reduced E/A ratio coupled with prolonged IVRT, indicative of diastolic dysfunction, is commonly observed in diabetic patients [36, 46, 47]. Doppler echocardiography is commonly used as a non-invasive technique for the assessment of diastolic function [44] in both T1DM [36] and T2DM settings [52]. This technique has been used to demonstrate that impairments in LV relaxation are evident in at least half of asymptomatic diabetic subjects without overt cardiovascular disease or microvascular complications [46, 48]. In contrast, pulse-wave tissue Doppler imaging measures the movement of a particular segment of cardiac muscle tissue relative to the sensor. Diastolic early (E’, to distinguish it from conventional transmitral E flow) and late (A’, as distinct from conventional transmitral A flow) myocardial tissue velocities are derived by integrating these distances over time. LV filling pressure can also be estimated from tissue Doppler imaging, as the ratio of conventional early (E) transmitral flow velocity to diastolic early (E’) tissue velocity (E/E’). This technique has been used to demonstrate that LV filling (on E/E’) is impaired in patients with T2DM [34, 45, 49], independent of LV mass, systolic function or microvascular complications. Cardiac MRI has now emerged as another non-invasive technique for the measurement of cardiac function by providing a 3-dimensional representation of the structure of the heart. This technique yields the same indices of diastolic function as Doppler echocardiography (E/A, deceleration time, LV filling pressures), but with increased sensitivity and reproducibility. In asymptomatic patients with T2DM, cardiac MRI detected impairments in diastolic function, including reductions in reduced LV end-diastolic volume, E and E/A [50].

Diastolic dysfunction has been similarly well characterized in various rodent experimental models of T1DM and T2DM (described in detail in section 1.4). Streptozotocin (STZ)-induced T1DM rats and mice exhibited impairments in diastolic function, as shown on Doppler echocardiography (E/A, deceleration time, and IVRT) and cardiac catheterization (elevated LV end-diastolic pressure, LVEDP) in vivo [30, 51-53]. Similar evidence has been obtained using Doppler echocardiography and cardiac catheterization in mouse models of
Figure 1.3 Features of the diabetic heart

The diabetic heart is characterized by diastolic dysfunction (as shown by a reduction in E/A ratio), cardiomyocyte hypertrophy, myocardial fibrosis (collagen stains red, indicated by arrow) and increased cardiomyocyte apoptosis (apoptotic cardiomyocytes stain blue, indicated by arrow).
T2DM, including the db/db mouse [54-56]. Together, these various imaging techniques provide a powerful means for the early detection of diabetes-induced diastolic dysfunction in both clinical and experimental settings. Diabetic cardiomyopathy, as a distinct entity from associated microvascular diseases, is evident even in individual cardiomyocytes [19, 57, 58]. Isolated LV diastolic dysfunction likely represents its initial stage of progression, although the mechanisms underlying this remain to be fully resolved. Likely contributors include insulin resistance [37], increased cardiac collagen deposition [55, 59], alterations in both glucose metabolism [49], generation of ROS [60] and Ca\(^{2+}\) homeostasis [19, 58], as discussed in detail below.

### 1.1.3.2 Systolic dysfunction

The majority of evidence regarding diabetic cardiomyopathy considers diastolic dysfunction to largely occur in isolation, or prior to systolic dysfunction, as outlined above. A smaller number of studies have however suggested that abnormal systolic function may also be evident, in both clinical [34, 61] and experimental settings [45, 54, 62]. Subclinical systolic dysfunction has been demonstrated in patients with T2DM, who exhibited regional impairments in LV systolic function, on both longitudinal and radial systolic velocities despite normal ejection fraction, peak systolic velocity and functional reserve [34]. The development of systolic dysfunction in experimental diabetic settings may be model-dependent; systolic function is preserved in STZ-induced T1DM mice when using multiple low doses of STZ on the FVB/N strain [51, 52], yet 1-2 higher doses of STZ in C57Bl/6 mice can decrease stroke volume, cardiac output, LV ejection fraction and fractional shortening [55].

### 1.1.3.3 Cardiomyocyte hypertrophy

The functional alterations evident in the diabetic heart are closely associated with molecular and histopathological evidence of both cardiomyocyte hypertrophy and fibrosis (Figure 1.3). LV hypertrophy is a common structural hallmark in patients with diabetes, and is of clinical significance as it is a strong predictor of myocardial infarction (MI), stroke and death from heart failure [63]. The development of LV hypertrophy initially occurs as an adaptive response to elevated hemodynamic stress [64], reduced numbers of functional contractile cardiomyocytes [65] and neurohormonal activation [67]. Although LV
hypertrophy is frequently associated with increased afterload in diabetic patients with hypertension [66], it can also occur independent of pressure-overload [32, 51, 67]. Echocardiographic evidence revealed increased LV posterior wall thickness and mass, in addition to a greater ratio of wall thickness to chamber radius in diabetic patients, even in the absence of coronary artery disease or hypertension [67]. The risk of elevated LV mass in patients with diabetes was increased in the order of 1.5-fold, independent of body mass [71]. Similar evidence of diabetes-induced hypertrophy has been obtained from animal studies, with increases in heart-to-body weight ratio (HW/BW) and cardiomyocyte size and/or upregulated hypertrophic gene expression (e.g. β-myosin heavy chain, atrial natriuretic peptide ANP, B-type natriuretic peptide BNP), often in the absence of hypertension [51, 52, 56, 68-70].

1.1.3.4 Myocardial fibrosis

Another structural hallmark of the diabetic myocardium is the development of interstitial and/or perivascular fibrosis. Extracellular matrix (ECM) is composed of collagen, elastin, laminin and fibronectin, which normally provide a scaffold for cardiomyocytes. Collagen is an integral component of the ECM, as it facilitates connections between cells and muscle bundles, maintaining myocardial structure, shape and chamber thickness [71, 72]. Myocardial fibrosis is a result of abnormally elevated ECM deposition, in particular collagen, which increases myocardial stiffness. This impairs LV relaxation, with subsequent compromise in the efficiency of LV contraction. In the diabetic setting, a decrease in the activity of ECM-degrading enzymes, matrix metalloproteinases (MMP), coupled with an increase in activity of their tissue inhibitors (TIMP) has been proposed as a mechanism underlying ECM accumulation [73, 74]. Furthermore, diabetes has been shown to increase the proportion of collagen that is in the insoluble form [72]. Elevated myocardial content and gene expression of ECM proteins (particularly collagen) is often observed in experimental models of both T1DM [51, 52, 56, 70, 73, 75] and T2DM [55, 68, 76], and is closely associated with impairments in LV diastolic filling. As reviewed by Asbun & Villarreal [77], collagen type I and III were increased in the epicardial and perivascular regions of the human diabetic heart, whereas the endocardium accumulated collagen type IV. Diabetes-induced cardiac fibrosis was accompanied (and likely triggered) by the upregulation of transforming growth factor (TGF-β), its receptor TGF-β receptor II, and its downstream mediator, connective tissue growth factor (CTGF) [55, 73, 78, 79]. Perhaps not surprisingly, alterations
in glucose metabolism are an important contributing mechanism to diabetes-induced cardiac remodeling. Hyperglycemia per se was sufficient to directly increase cardiac fibroblast and vascular smooth muscle cell proliferation [80, 81], in addition to elevating pro-growth signaling in cultured cardiomyocytes [82].

1.1.3.5 Apoptosis

Apoptosis is the most well-known form of programmed cell death; tightly-controlled regulation of apoptosis is essential for maintaining tissue homeostasis under normal physiology [83, 84]. Dysregulation of apoptosis has been implicated in various pathologies, including diabetes. Apoptotic pancreatic β-islet cell death is a likely causal factor in both T1DM and T2DM [83, 85]. Overwhelming evidence from both clinical and experimental settings now indicates that cardiomyocyte apoptosis also plays an important role in the development of diabetic cardiomyopathy [52, 84, 86-88]. Diabetes-induced cardiomyocyte apoptosis often occurs concomitantly with other structural anomalies including increased interstitial fibrosis [52] and myofiber disarray [75, 84], and is likely to be a direct result of hyperglycemia [86].

1.1.3.6 Microvascular abnormalities

Although the debilitating characteristics of diabetic cardiomyopathy can develop independent of the macrovascular complications of the disease, structural and functional changes at the level of the coronary vasculature are a common comorbidity in diabetic patients, which can further aggravate diabetic cardiomyopathy. Sustained hyperglycemia is associated with endothelial dysfunction [89, 90]. As a result, the risk of enhanced microvascular permeability, impaired microvascular blood flow and subsequent tissue ischemia is increased [91, 92]. This process is exacerbated by changes in the expression and bioavailability of vasoactive factors released from the endothelium, including upregulation of endothelin-1 (ET-1) and downregulation of nitric oxide (NO) [89, 93]. Endothelial-derived NO is a potent endogenous vasodilator and negative regulator of abnormal cardiomyocyte growth [64, 94-96]. Numerous studies have reported the diabetes-induced impairment in endothelium-dependent vasodilatation [90, 97-100], reduced capillary density and impaired coronary microvascular perfusion [75, 101], which have implications for the susceptibility of the diabetic heart to ischemic damage [89]. Therapeutic approaches targeting impairments at
the level of the coronary microvasculature may thus offer favorable benefits in the setting of diabetic cardiomyopathy.

1.2 Pathophysiologic triggers of diabetic cardiomyopathy

1.2.1 Hyperglycemia

Hyperglycemia represents one of the central drivers of the metabolic, functional and structural alterations present in the diabetic heart. Aberrations in glucose control itself is sufficient to trigger an array of maladaptive processes including hyperinsulinemia and insulin resistance [102], glucose transporter 4 (GLUT 4) depletion [103], changes in free fatty acid (FFA) oxidation [104, 105], accumulation of advanced glycation end products (AGEs) [106, 107], altered Ca\(^{2+}\) handing [108], increased generation of ROS [107, 109] and activation of the renin-angiotensin-aldosterone system (RAAS) pathway [110, 111]. All of these mechanisms have been implicated in the development and progression of diabetic cardiomyopathy [112]. Several clinical studies have demonstrated the clear correlation between glycemic control and the risk of heart failure development and associated events, on both fasting plasma glucose and glycated hemoglobin (HbA\(_{1c}\)) levels [113-115]. Further, uncontrolled hyperglycemia in type 2 diabetic patients without overt cardiomyopathy was associated with diastolic LV dysfunction, independent of obesity, dyslipidemia and systemic arterial hypertension [116]. Together, these studies emphasize the importance of effective glycemic control in the prevention of cardiovascular events in diabetic patients.

1.2.2 Insulin Resistance

Insulin resistance, and the usually-concomitant hyperinsulinemia, are significant risk factors for the development and progression of cardiovascular disease. Evidence exists to indicate a causal relationship between hyperinsulinemia, hypertension and coronary artery disease [117-119]. Untreated essential hypertension is linked to higher fasting and postprandial insulin levels and impaired insulin sensitivity in both hypertensive patients and in animal models with a genetic predisposition to hypertension [117-119]. Activation of the sympathetic nervous system is one mechanism considered to underlie the development of high blood pressure under insulin-resistant settings [120]; renal sodium retention and increased proliferation of vascular smooth muscle cells may also contribute. Hyperinsulinemia is positively correlated with the risk of developing coronary artery disease,
as demonstrated by a number of studies [121-123]. The incidence of coronary artery disease associated with high triglycerides and low high-density lipoprotein levels is only significantly increased when accompanied by insulin resistance, even in the absence of diabetes [124]. Furthermore, impaired insulin sensitivity can be detected even in well-managed T2DM patients free of coexistent comorbidities [125].

Cardiac abnormalities, including LV hypertrophy, fibrosis and cardiomyocyte dysfunction are often already apparent in the pre-diabetic, insulin-resistant stage, as observed in animal models in vivo [78, 126]. Although much attention has been devoted to elucidating the mechanisms underlying peripheral insulin resistance, whether these mechanisms are also responsible for cardiac insulin resistance is unclear. In the diabetic heart, diminished activities of GLUT4 results in reduced glucose utilization and impaired insulin signaling. This subsequently increases energy demand from FFA oxidation, raising myocardial oxygen demand and reducing cardiac efficiency, accompanied by dyslipidemia and lipotoxicity [127, 128]. Other potential drivers of cardiac insulin resistance include mitochondrial dysfunction, inflammation, cytokine upregulation, endoplasmic reticulum stress and stress kinase signaling [129]. As metabolic derangements and insulin resistance precede the development of cardiac dysfunction and remodeling, these likely predispose the diabetic heart to damage.

1.2.3 Metabolic disturbances in the diabetic heart

Disturbances in energy metabolism of the heart have been implicated as important contributors to diabetic cardiac complications [49, 130]. For the purpose of this thesis, I have provided a short summary on the changes in metabolism evident in diabetes. For an extensive review of altered substrate metabolism in diabetes, please refer to [131, 132]. Hypertriglyceridemia is a common feature of T2DM, characterized by decreased clearance of triglyceride-rich lipoprotein, due to a reduction in the levels of lipoprotein lipase or alterations in circulating lipoproteins [133]. Various human and animal studies have been conducted to correlate the level of triglycerides with the degree of myocardial damage. The Framingham Study identified plasma triglyceride levels as a strong risk factor for development of diabetes-induced cardiac disease [134]. More recently, elevated triglyceride levels were found to correlate with the severity of atherosclerosis and coronary heart disease in diabetic patients [135, 136]. In animal models of diabetes, non-specific plasma lipid-
lowering approaches (hydralazine and L-carnitine) resulted in a concomitant prevention of both hyperlipidemia and cardiac dysfunction [137, 138].

FFA levels are also elevated in T2DM, which may shed light on the mechanistic relationship between increased fat, insulin resistance, impaired glucose tolerance and central obesity. FFAs are the primary energy substrate used by the heart, and are supplied to cardiac cells via both endogenous sources (lipolysis of triglycerides in the heart) or exogenous sources (from the blood, as FFA bound to albumin, or via lipoproteins) [133]. Under insulin-deficient states, there is an increase in adipose tissue lipolysis and thus increased FFA released into the plasma. FFA levels are elevated in pre-diabetic patients with impaired glucose tolerance [139], and both acute and chronic increases in FFA levels are sufficient to induce insulin resistance [132]. Energy metabolism in the diabetic heart is abnormal and inefficient; lower glucose, pyruvate and lactate concentrations give way to an increased amount of fatty acid extraction and storage [140]. The accumulation of triglycerides and non-esterified fatty acids are thought to be involved in potentiating diabetes and its associated complications. High circulating and cellular FFAs can directly elevate peripheral insulin resistance, stimulate apoptosis and trigger a harmful build-up of toxic intermediates which result in lipotoxicity. These deleterious effects can contribute to impaired cardiac function and adverse remodeling in the diabetic myocardium [141-143].

The diabetic heart is also characterized by reduced utilization of glucose for energy production. FFAs can inhibit glucose oxidation by activating proliferation activated receptor-α (PPAR-α) which increased the expression of pyruvate dehydrogenase kinase-4, mediating enhanced mitochondrial fatty acid uptake and reduced glucose oxidation [144]. Cardiac glucose utilization is also reduced through depleted GLUT4 expression and activity, which further contributes to aberrant myocardial morphology [145]. The inability to switch to glucose oxidation renders the heart susceptible to damage and dysfunction under settings of reduced oxygen delivery, such as in myocardial ischemia [146]. Reduced glucose uptake and metabolism post-ischemia may compromise the capacity of the diabetic heart to recover, and several in vivo studies have demonstrated greater LV dysfunction and severe structural remodeling in diabetic animals following short-term occlusion of the coronary artery [29, 147, 148].
1.2.4 Altered Ca\textsuperscript{2+} handling in the diabetic heart

1.2.4.1 Impaired Ca\textsuperscript{2+} handling and cardiac dysfunction in diabetes

The disturbance of Ca\textsuperscript{2+} regulation in the diabetic heart was first established more than two decades ago, where depressed myofibrillar ATPase activity and sarcoplasmic reticulum (SR) function were identified as a contributing factor to the development of diabetic cardiomyopathy [149-151]. A multitude of studies in experimental diabetic animals have since linked the contractile defects observed in the diabetic myocardium with an altered ability of the heart to regulate Ca\textsuperscript{2+}. Abnormal Ca\textsuperscript{2+} metabolism has been identified in both experimental diabetic models [152-154] and diabetic patients [155-157]. It involves a defect in one or more mechanisms which regulate intracellular Ca\textsuperscript{2+} concentration, including the sarcolemmal L-type Ca\textsuperscript{2+} channel, the SR Ca\textsuperscript{2+} release channel, the SR Ca\textsuperscript{2+}-ATPase (SERCA2a), the SERCA2a regulator phospholamban (PLB), and the sarcolemmal Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger [158]. Early studies using isolated myocytes from diabetic animals have reported contradictory results on the relative Ca\textsuperscript{2+} content of the diabetic heart [159-161]. More recent studies using diabetic experimental models have supported the role of reduced total Ca\textsuperscript{2+} levels in the diabetic heart. In the \textit{db/db} spontaneous model of type 2 diabetes, systolic dysfunction was associated with a transient reduction in intracellular Ca\textsuperscript{2+} in cardiomyocytes, accompanied by diminished SR Ca\textsuperscript{2+} load and decreased expression of functional ryanodine receptor Ca\textsuperscript{2+} channels (RyRs) [162]. Similarly, the number of functional RyR was reduced by 37\% in STZ-induced type 1 diabetes, in addition to dysynchronous release of Ca\textsuperscript{2+} by stimulated ventricular myocytes, reduced SR Ca\textsuperscript{2+} load and delayed rate of caffeine-induced SR Ca\textsuperscript{2+} release [163].

As noted earlier, the development of diastolic dysfunction often precedes the development of systolic dysfunction in the diabetic heart; this is apparent in both experimental diabetic models and in diabetic patients [36, 164]. Although overt systolic dysfunction is often absent during the early stages of diabetic cardiomyopathy, it is believed that intrinsic changes in contractile function of individual myocytes occur early in the disease. The regulation of Ca\textsuperscript{2+} signaling is a critical determinant of contractile performance. Choi \textit{et al.} examined the link between defective intracellular Ca\textsuperscript{2+} signaling and cardiomyopathy in 12-week old STZ-diabetic rats and in isolated myocytes [158]. In the heart \textit{in vivo}, both fractional shortening and chamber dimensions were unaltered in the diabetic rats. Ejection rate was however depressed compared to control animals, suggestive of early systolic dysfunction. Assessment of isolated single myocytes revealed reduced rise and fall of
intracellular free Ca\(^{2+}\) concentrations, in parallel with a reduced rate of contraction and relaxation [158]. Ivanics and colleagues have shown that 4 weeks of diabetes is insufficient to induce any changes in Ca\(^{2+}\) handling or hemodynamic function [165]. However, by 6 weeks post-STZ administration, the hearts of diabetic rats showed reduced inotropy and lusitropy, elevated end-diastolic Ca\(^{2+}\) levels and impaired transport capacity of SERCA2a activity [108]. Diastolic dysfunction in diabetes has also been attributed to the impairment of myocardial Ca\(^{2+}\) handling. Eight weeks following diabetes induction using STZ, diabetic rats presented with subclinical diastolic dysfunction (evident on mitral flow patterns) and preserved systolic function [166]. In isolated ventricular myocytes, action potential duration was significantly prolonged. These myocytes also exhibited decreased Ca\(^{2+}\) transient amplitude, prolonged Ca\(^{2+}\) transient decay, a 50% reduction in SR Ca\(^{2+}\) load and a fall in SERCA2a protein levels.

The processes underlying the dysregulation of basal Ca\(^{2+}\) levels inside the cardiomyocyte and Ca\(^{2+}\) oscillations during the contraction-relaxation cycle of the heart are largely attributable to changes in Ca\(^{2+}\) transport mechanisms. Diabetes affects both the enzymatic transport and the passive buffering of Ca\(^{2+}\) in and out of the cell [167]. The SR Ca\(^{2+}\) pump SERCA2a, which plays a central role in the regulation of Ca\(^{2+}\), has been shown to be defective in type 1 diabetes [168-170]. Consequently, the uptake of Ca\(^{2+}\) into the SR is also depressed. These studies also demonstrated that insulin supplementation can normalize SERCA2a activity in insulin-deficient animals [169, 171]. More recent reports have highlighted the relationship between defective SERCA2a activity and myocardial dysfunction in insulin-resistant diabetes. In a study by Belke et al., SERCA2a activity was depressed in \(db/db\) animals, where increased Ca\(^{2+}\) leakage from the SR was accompanied by reduced rates of contraction, relaxation and pressure development, as well as diminished diastolic and systolic levels of Ca\(^{2+}\) in the diabetic heart [172]. Sucrose-fed insulin-resistant rats also exhibited depressed SR activity as evidenced by a decline in SERCA2a Ca\(^{2+}\) uptake, which was associated with a significant decrease in cAMP-dependant protein kinase phosphorylation of PLB [173]. These changes occurred even in the absence of overt type 2 diabetes, suggesting that abnormalities in contractile function and SERCA2a performance are present in the insulin-resistant, pre-diabetic stage. Impaired SERCA2a function has implications for both diastolic and systolic performance. Reduced SR Ca\(^{2+}\) uptake may eventuate in a prolonged rate of relaxation in the diabetic heart, whilst slower Ca\(^{2+}\) release reduces the Ca\(^{2+}\) availability for force generation, thus resulting in weaker contraction [174].
1.2.4.2 SERCA2a modulates contraction/relaxation of cardiomyocytes

As previously described, restoration of cytoplasmic Ca\(^{2+}\) concentrations following contraction is predominantly mediated through activation of SERCA2a and to a lesser extent, the sarcolemmal Na\(^+\)-Ca\(^{2+}\) exchanger. In this way, SERCA2a facilitates cardiac relaxation, allowing sufficient Ca\(^{2+}\) available for the following wave of contraction [175]. Adequate SERCA2a activity is pivotal in preserving a balanced contraction-relaxation cycle [176]. SERCA2a and PLB form a complex whereby an increased SERCA2/PLB ratio results in increased reuptake of Ca\(^{2+}\). Many investigators have suggested that a decreased SERCA2a/PLB activity ratio is the principle factor responsible for slowed cardiac relaxation in the diabetic heart [176-178]. Indeed, approaches to elevate SERCA2a expression [179] and/or ablate PLB activity [180] have resulted in improved contractility, by increasing the number of functional Ca\(^{2+}\) SR pumps, and thus increasing the capacity to sequester Ca\(^{2+}\) back to the SR during relaxation. Transgenic overexpression of SERCA2a in diabetic rats accelerated SR Ca\(^{2+}\) uptake, and partially restored diabetes-induced contractile dysfunction [177]. Moreover, overexpression of SERCA2a alone was sufficient to attenuate ROS-induced damage in mouse myocardium, highlighting the protective effects of SERCA2a against ROS-induced contractile dysfunction [179]. Despite these observations, other studies have revealed no change in SERCA2a expression levels within diabetic hearts [181, 182], suggesting that dysfunctional cardiac relaxation in diabetic cardiomyopathy cannot be entirely attributable to a decrease in SERCA2a/PLB activity ratio.

SERCA2a may also be susceptible to diabetes-induced posttranslational modifications as a result of ROS. The role of antioxidants in preventing SERCA2a activity loss and diastolic dysfunction is exemplified in a study by Shao et al., who demonstrated that reactive carbonyl species present in diabetic animals can react with exposed arginine, lysine and histidine residues on SERCA to form carbonyl adducts [183]. Increased formation of carbonyl adducts on SERCA proteins was associated with diastolic impairment in the diabetic animals. Treating the diabetic mice with pyridoxamine, a scavenger of reactive carbonyl species, prevented SERCA2a dysfunction and enhanced diastolic function [183]. Adverse SERCA2a post-translational modifications may be a critical causal factor of SERCA2a dysfunction, and on a larger scale, may be a primary contributor to myocardial dysfunction in diabetes.
1.2.5 Oxidative stress in the development of diabetic cardiomyopathy

Despite the importance of diabetic cardiomyopathy as a clinical entity, the pathological cellular and molecular mechanisms driving the adverse changes in diabetic myocardial structure and function have not been fully resolved. The development and progression of diabetic complications is frequently attributed to increased oxidative stress. Molecular oxygen in its ground state is relatively inert and therefore is unable to accept electrons from other biological molecules [184]. However, the enzyme-driven addition of electrons to the oxygen molecules greatly increases their reactivity, giving rise to a family of ROS. The oxidizing capability of ROS is used by aerobic cells to modulate the function and activity of cell signaling molecules; this process, however, must be tightly regulated by antioxidants to prevent oxidative damage [184-186]. Oxidative stress thus results from an imbalance between the generation of ROS and the ability of the biological system to detoxify reactive intermediates [187]. ROS include free radicals (which have at least one unpaired electron) such as the parent ROS superoxide (•O₂-) and hydroxyl radical (•OH), and non-radicals which are able to generate free radicals [i.e., hydrogen peroxide (H₂O₂)] [188]. One cellular source of •O₂- generation is as a byproduct of mitochondrial respiration. Under physiological conditions, endogenous superoxide dismutase (SOD) enzymes degrade •O₂- to form the more stable species, H₂O₂, and O₂. H₂O₂ is further broken down to water by the antioxidants glutathione peroxidase (Gpx) and catalase. In pathological states, H₂O₂ can also generate the highly reactive •OH. Peroxynitrite (ONOO⁻) is also produced in excess under pathological settings, as a result of the reaction between •O₂- and nitric oxide (NO) [188]. ROS may induce numerous pathophysiological effects on the cell, depending on their concentration and location. Excessive production of ROS is thought to play a significant role in the development of various pathologies, due to their ability to directly oxidize DNA, proteins and lipid membranes [187, 189]. In addition to this ability to inflict direct molecular damage, ROS may also activate stress-sensitive pathways of cellular damage [190]. Many studies have linked diabetes to increased generation of ROS, and/or a reduction in antioxidant defenses [185, 186, 191]. The predominant sources of ROS in the heart are likely to be derived from nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, mitochondrial sources and uncoupled NO synthases (NOS), as discussed below (Figure 1.4).

1.2.3.1 Sources of ROS in the heart

The membrane-bound enzyme complex NADPH oxidase is a major source of ROS in
**Figure 1.4 Major sources of ROS in the heart**

The NADPH oxidase enzyme complex, the mitochondrial respiratory chain and eNOS-mediated •O$_2$- release comprise the three major sources of •O$_2$- in the heart. Once formed, •O$_2$- can be converted to other ROS, including •OH and ONOO$^-$. •O$_2$-, superoxide; •OH, hydroxyl radical; NO, nitric oxide; ONOO$^-$, peroxynitrite; eNOS, endothelial nitric oxide synthase; SOD, superoxide dismutase, BH$_4$, tetrahydrobiopterin. See text for references.
cardiomyocytes, vascular smooth muscle cells, endothelial cells and adventitial fibroblasts [184, 192]. Activity of NADPH oxidase can be triggered by a range of stimuli, including angiotensin II (Ang II), ET-1 and TNFα [193-197]. NADPH oxidase is the only source of ROS whose explicit role is to specifically generate ROS (rather than as a byproduct of other processes). Each NADPH oxidase complex is comprised of a membrane-bound flavocytochrome, encompassing p22\textsuperscript{phox} and a Nox subunit (usually either Nox1, Nox2 or Nox4), in addition to four regulatory subunits: p47\textsuperscript{phox}, p67\textsuperscript{phox}, p40\textsuperscript{phox} and Rac1 [192] (Figure 1.4). The primary Nox subunit isoforms in cardiac cells are Nox2 (also known as gp91\textsuperscript{phox}) and Nox4 [68], although Nox1 expression is inducible in certain pathologies [68]. Upon activation, phosphorylation of p47\textsuperscript{phox} ensues, and integrates with p67\textsuperscript{phox}, p40\textsuperscript{phox} and Rac1 to form an active NADPH oxidase enzyme complex [198, 199], resulting in release of •O\textsubscript{2}⁻.

NADPH oxidase activity and expression are significantly upregulated in pressure-overload LV hypertrophy [192, 200, 201] and in advanced heart failure [192, 202, 203]. Furthermore, increased expression of the Nox1 and Nox2 subunits of NADPH oxidase has been observed in T1DM, accompanied by myocardial hypertrophy and fibrosis [198, 204, 205]. The NADPH oxidase inhibitor apocynin, which acts by impeding the assembly of p47\textsuperscript{phox} and p67\textsuperscript{phox} subunits into the fully functioning NADPH oxidase enzyme complex, prevented the activation of NAPDH oxidase and apoptosis in Ang II-treated cardiomyocytes [202], highlighting the role of this enzyme complex in mediating Ang II-induced cell death. The Nox5 subunit, expressed in humans, is absent from the rodent genome, and thus, relatively little is known about its vascular physiology and pathophysiology [206]. Interestingly, Nox5 is considered to not be influenced by the known NADPH oxidase regulatory subunits, but is instead regulated by cytosolic Ca\textsuperscript{2+} concentrations [207]. Guzik et al.’s study reported a strong association between Nox5 activity and coronary artery disease in patients [208], however Nox5’s role in diabetic cardiomyopathy remains poorly understood.

The mitochondrial respiratory chain is another major myocardial source of ROS in the heart. Mitochondria are the sites of oxidative phosphorylation, where ATP is generated via the electron transport chain, using the electron carriers NADH and FADH\textsubscript{2}. •O\textsubscript{2}⁻ is a by-product of this energy-generating pathway [209]. The small amount of ROS produced during mitochondrial respiration is usually broken down by endogenous antioxidants to form water in non-disease states. However, impaired antioxidant capacity in pathological settings, such as in the failing myocardium, may aggravate ROS accumulation and lead to increased
oxidative stress [210]. A study conducted by Ide et al. showed that blocking the transport of electrons at complex I of the mitochondrial respiratory chain resulted in enhanced levels of •O$_2$- [211]. Importantly, they also discovered that mitochondria in the failing heart produced more •O$_2$- than in non-failing hearts in the presence of an NADH carrier. Another study demonstrated that mitochondrial ROS production was prevented using an inhibitor of an electron transfer complex, an uncoupler of oxidative phosphorylation or manganese superoxide dismutase (MnSOD) [212]. Normalizing mitochondrial •O$_2$- levels with each of these agents effectively blunted glucose-induced formation of AGEs, nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) activation and sorbitol accumulation, all proposed mechanisms of oxidative stress-induced damage.

Endothelial nitric oxide synthase (eNOS)-dependant •O$_2$- generation represents another source of ROS in the diabetic setting. eNOS, a homodimer enzyme made up of a reductase domain and an oxygenase domain, is the primary driver of NO production in the vasculature. Under physiological conditions, it functions to transfer electrons from NADPH (via the flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) cofactors) to heme in the oxygenase domain. The substrate L-arginine, in the presence of cofactor tetrahydrobiopterin (BH$_4$) is subsequently oxidized to L-citrulline and NO [213]. The tight regulation of electron transfer in eNOS is crucial in preventing the uncoupling of O$_2$ reduction from NO generation, as each of the redox donors present in NOS enzymes (FAD, FMN, heme and BH$_4$) are able to transfer electrons to O$_2$. In its uncoupled state, electrons being transferred from the reductase to the oxygenase domain of eNOS are donated to molecular oxygen instead of L-arginine, producing •O$_2$-. eNOS uncoupling as a potent source of ROS generation has been observed in various disease settings, including diabetes and hypertension [214-216]. STZ-diabetic rats have been shown to exhibit significant endothelial dysfunction, manifest as an impaired relaxation response to acetylcholine, in association with increased •O$_2$- production and a reduction in BH$_4$ levels [217]. BH$_4$-treated diabetic patients showed improved endothelium-dependant vasodilation in response to acetylcholine, indicating that prevention of eNOS uncoupling by increasing bioavailability of the essential cofactor BH$_4$ is critical in maintaining endothelial function [218]. Although other enzyme sources of ROS may be present in the heart, including lipoxygenase, cyclooxygenase and xanthine oxidase, NADPH oxidase, dysfunctional mitochondria and uncoupled eNOS are considered the major contributors to cardiac ROS [219].
1.2.5.2 Role of ROS in heart disease

1.2.5.2.1 ROS as a driver of cardiac hypertrophy

Increased ROS can result in hypertrophy via several mechanisms, including elevated release of vasoactive peptides such as Ang II and ET-1, activation of redox-sensitive protein kinases such as c-jun N-terminal (JNK) and p38 protein kinases, and mechanical stretch [220-223]. Several studies examining the efficacy of antioxidant treatment in reducing cardiac hypertrophy have demonstrated a link between the hypertrophic process and an increased generation of ROS in cardiomyocytes. TNF-α and Ang II-induced cardiomyocyte hypertrophy was blunted on addition of antioxidants (butylated hydroxyanisole, vitamin E), suggesting that TNF-α and Ang-II-mediated cardiomyocyte hypertrophy was mediated by increased generation of ROS [224]. Furthermore, antioxidants such as probucol, tempol and N-acetylcysteine (NAC) have proved effective in reducing cardiomyocyte hypertrophy by preventing Ang II-induced de novo protein synthesis and ANP expression, through inhibition of myocardial •O₂− production [194]. Increased NADPH oxidase-derived ROS production and enzyme activity were also paralleled by the progression of cardiac hypertrophy in a pressure-overload model of LVH in vivo [192]. Treatment of aldosterone-induced cardiac hypertrophy with the NADPH oxidase inhibitor apocynin significantly decreased p22^phox subunit activity and expression, accompanied by normalization of LV weight [225]. Similarly, Ang II-induced cardiac hypertrophy was blunted in a Nox2−/− mouse model, on assessment of hypertrophic markers including heart weight/body weight ratio, LV myocyte area and expression of ANP and β-myosin heavy chain [226]. Collectively, these studies suggest that increased ROS production, in particular NADPH oxidase-dependant ROS generation, plays an important role in the pathophysiology and progression of cardiac hypertrophy.

1.2.5.2.2 ROS as a driver of cardiac fibrosis

Enhanced interstitial collagen deposition is an important feature of cardiac remodeling. Various studies have suggested that oxidative stress is an important regulator of pro-fibrotic processes [226-231]. In addition to cardiac hypertrophy, NADPH oxidase-dependant ROS production is also thought to contribute to the development of interstitial fibrosis. Ang II-infused wild-type mice exhibited increased myocardial collagen content; this increase was completely blocked in Ang-II infused Nox 2−/− mice [226]. Ang II-infused Nox
2−/− mice also showed lower levels of procollagen I and III expression, as well as suppressed MMP2 activation compared with their wild-type counterparts [228]. Aldosterone-infused rat hearts exhibited significantly higher myocardial procollagen I expression and enhanced interstitial and perivascular fibrosis [225]. The NADPH oxidase inhibitor apocynin significantly reduced procollagen I expression and prevented perivascular collagen deposition. Furthermore, Nox 2−/− mice were protected from augmented interstitial fibrosis and increased procollagen I and III expression induced by aortic banding, compared to wild-type mice [232]. Several mechanisms are thought to be implicated in the NADPH oxidase-dependant pro-fibrotic effect, including increased activation of MMPs, expression of pro-fibrotic genes and activation of NF-κB [188]. Reduction of ROS generation and restoration of redox balance may be important in preventing or treating myocardial fibrosis in the failing heart.

1.2.5.3 Role of ROS in diabetic cardiac complications

It is widely accepted that the pathophysiology of diabetic cardiomyopathy is complex and multifactorial. Myocardial remodeling, comprising LV hypertrophy, interstitial fibrosis and myocyte cell death feature prominently in the diabetic heart, resulting in diastolic (and often also, systolic) dysfunction [35, 56, 233, 234]. Several studies have highlighted hyperglycemia-induced alterations in redox state as a key stimulator of these cardiac impairments. As the diabetic heart possesses a lower content of antioxidants in comparison to other organs, it is particularly susceptible to oxidative damage [235]. Aragno et al. reported increased staining for collagen I and IV and increased TGF-β1 protein and gene expression in T1DM, indicative of elevated myocardial fibrosis [236]. This was inhibited by treatment with the steroid hormone dehydroepiandrosterone, which has been shown to possess antioxidant properties [236, 237]. Cardiac-specific GLUT-4 knockout (KO) mice, a model of cardiac insulin resistance, also highlighted the association between NADPH oxidase upregulation and the development of structural impairments in the diabetic heart. GLUT-4 KO mice exhibited increased markers of cardiac hypertrophy (increased heart weight/body weight and β-myosin heavy chain expression), accompanied by increased expression of Nox1 and Nox2 [68]. In the clinic, Gonzalez-Vilchez and colleagues reported a significant correlation between serum levels of the antioxidant enzyme glutathione peroxidise (suggestive of a state of oxidative stress) and LV dysfunction in diabetic patients [238].
Increased ROS formation also plays an integral role in cardiac contractile dysfunction, possibly as a result of impaired function of SERCA2a. SERCA2a is a crucial modulator of cardiomyocyte contractile function, and its dysfunction is a contributing factor to impaired diastolic relaxation in diabetes [181]. An increase in the ratio of SERCA2a to PLB results in increased reuptake of Ca\(^{2+}\) by the SR. Many investigators have attributed the slowing of the cardiac relaxation in diabetes to reduced SERCA2a expression and/or activity [166, 239]. Overexpression of SERCA2a in diabetic rats resulted in increased SR Ca\(^{2+}\) uptake and partial restoration of diabetes-induced contractile dysfunction [177]. Moreover, overexpression of SERCA2a alone is sufficient to attenuate \*OH-induced damage in the murine myocardium, highlighting the protective capacity of SERCA2a against ROS-induced contractile dysfunction [179]. Despite these observations, other studies have revealed no change in SERCA2a expression levels within diabetic hearts. This suggests that dysfunctional cardiac relaxation in diabetic cardiomyopathy can not be entirely attributable to decreased SERCA2a expression; perhaps SERCA2a activity levels are more important [240]. SERCA2a may also be susceptible to diabetes-induced post-translational modifications as a result of elevated ROS generation [241].

### 1.2.5.4 Mechanisms of hyperglycemia-induced oxidative stress

Elevated glucose levels have been linked to several biological pathways involved in the oxidative stress-induced complications of diabetes. Hyperglycemia is thought to directly increase mitochondrial generation of ROS [212]. In a study by Nishikawa et al., hyperglycemia-induced ROS generation was blocked by incubating aortic endothelial cells with an inhibitor of the mitochondrial electron transport complex II. In addition to this, overexpression of MnSOD, a mitochondrial antioxidant enzyme, significantly abated the hyperglycemia-induced elevation in ROS generation [212]. Mitochondrial respiration requires nicotinamide adenine dinucleotide (NADH) as a substrate, in contrast to NADPH oxidase, which uses NADPH as its substrate. Increased glucose oxidation in the cytoplasm increases the levels of reducing equivalents, including NADH, which in turn increases the mitochondrial proton gradient, contributing to elevated production of mitochondrial •O\(_2\)- [242, 243]. Another proposed mechanism of diabetes-induced oxidative stress is the metabolism of glucose at high intracellular concentrations through the polyol pathway [244]. Glucose is reduced by the enzyme aldose reductase to form sorbitol, which is further oxidized
to form fructose. Aldose reductase requires the co-factor NADPH, and thus increased glucose metabolism through the polyol pathway leads to a concomitant decrease in intracellular stores of NADPH [186]. A third, but essential, mechanism of glucose-induced oxidative stress is protein glycation and the formation of AGEs. AGEs are formed by covalent binding between ketone or aldehyde groups of sugars, in most cases to the free amino groups of proteins [243]. As glycated proteins, AGEs can generate ROS via activation of its receptor, RAGE. Activation of RAGE induces ROS generation through a series of complex biochemical pathways, which involve activation of redox-sensitive transcription factors (including NFκB) and pro-inflammatory mediators [242, 243].

In addition to these direct mechanisms of hyperglycemia-induced oxidative stress, the production of ROS can also be stimulated indirectly by several glucose-sensitive signaling pathways. Numerous studies have shown that hyperglycemia can activate NADPH oxidase in a protein kinase C (PKC)-dependent manner, which is a direct contributor to cardiovascular and renal dysfunction [19, 245-247]. PKC inhibitors have been demonstrated to be beneficial in reducing both cardiac and renal complications associated with diabetes [248-250]. Diabetes is also characterized by increased formation of Ang II, a well-established stimulator of NADPH oxidase activity [226, 251-253]. Ang II-mediated oxidative damage has been implicated as a contributor to diabetic cardiomyopathy, and thus its inhibition has shown to be effective in treating diabetic complications (refer to section 1.2.5.2.2).

### 1.2.5.5 Oxidative stress, hyperglycemia and cell death

Defective governing of programmed cell death is implicated in various pathologies, including diabetes. Increased myocyte apoptosis is involved in the process of transition from the compensated to decompensated hypertrophic state in the diabetic heart [84]. Studies have shown that both hyperglycemia and oxidative stress can induce apoptosis, impairing myocardial structure and function independently and synergistically [84, 87]. In a study by Fiordaliso et al., the highest blood glucose level in STZ diabetic rats occurred in parallel with the greatest degree of myocyte apoptosis, in addition to an increase in RAAS activation [87]. Mitochondria also play a significant role in inducing apoptosis under pro-oxidant conditions, such as in diabetes. The release of mitochondrial cytochrome c leads to the activation of caspase-3, downstream of caspase-8 [86]. Activation of this caspase pathway is critical for initiating apoptosis [86, 254]. Previous studies have demonstrated that glucose-treated
endothelial cells were associated with elevated ROS levels, coupled with increased caspase-3 activation and apoptosis [255]. Further, Cai et al. demonstrated that high glucose increased caspase-3 activation, ROS generation and apoptosis in cardiac myoblasts, which was inhibited using a caspase-3 inhibitor [86]. Collectively, these studies demonstrate that both hyperglycemia and increased ROS can directly induce apoptotic cell death in the heart.

1.2.5.6 Cardioprotective effect of antioxidant administration

The balance between the generation of ROS and their removal via antioxidant degradation is critical for the maintenance of cardiovascular health. Under settings of oxidative stress, ROS that are not eliminated from cellular compartments by endogenous antioxidant defences can cause irreparable damage to cellular macromolecules including nucleic acids, lipids and proteins [256]. Aerobic organisms maintain a sophisticated antioxidant defence system to counteract the physiological generation of ROS, which work both enzymatically and non-enzymatically to eliminate free radical intermediates or repair oxidation of cellular macromolecules [185]. Hyperglycemia is thought to directly impair the endogenous antioxidant defence system [186]. Exogenous supplementation of antioxidant nutrients and pharmacological agents that mimic the actions of endogenous antioxidant enzymes have been shown to confer protection against diabetes-induced oxidative damage, which translated to both improved structure and function of the heart in settings of diabetic cardiomyopathy [68, 186, 224, 257-259]. Endogenous enzymatic and nonenzymatic antioxidants include SOD, glutathione peroxidase, catalase, thioredoxin, vitamin E and coenzyme Q$_{10}$ [185, 260] (Table 1.1).

| Table 1.1 – Mammalian antioxidants |
|-------------------------------|-----------------------------------|
| **Antioxidant**               | **Function**                      |
| Superoxide dismutase          | Converts $\cdot$O$_2^-$ to O$_2$ and H$_2$O$_2$ |
| Catalase                       | Converts H$_2$O$_2$ to O$_2$ and H$_2$O |
| Glutathione peroxidase         | Converts hydroperoxides to H$_2$O and alcohols |
| Thioredoxin                    | Reduction of protein cysteine-thiol disulfide bonds |
| Vitamin E                      | Protects membranes from oxidation |
| Ubiquinol (Coenzyme Q$_{10}$)  | Protects membranes from oxidation |
1.2.5.6.1 Superoxide dismutase

SOD enzymes are metalloproteins responsible for catalysing the dismutation of $\cdot$O$_2^-$ radical to molecular oxygen and hydrogen peroxide, thereby reducing intracellular $\cdot$O$_2^-$ levels. There are 3 major isoforms of SOD, distinguished by their localization in different cellular compartments. Copper-Zinc SOD (CuZnSOD) is located in the cytoplasm and in the intermembrane space of the mitochondria [261], MnSOD is found in the mitochondrial matrix [210], and extracellular SOD (ecSOD) resides in the extracellular matrix of tissues [262]. As $\cdot$O$_2^-$ is the most abundant ROS in the cell, being released under physiological states from NADPH oxidase, the mitochondrial respiratory chain and eNOS uncoupling, the antioxidant properties of SOD are critical for maintaining oxidative balance. Homozygous mutant mice lacking MnSOD died within the first 10 days of life with massive hypertrophy and endocardial fibrosis, consistent with dilated cardiomyopathy, as well as marked hepatic steatosis and fatty deposits in skeletal muscles [263]. Mitochondrial function was also severely depressed in these mutant mice, emphasising the importance of MnSOD in protecting mitochondrial enzymes against $\cdot$O$_2^-$ released as a by-product of oxidative phosphorylation. Mice deficient in CuZnSOD showed normal growth and development through to early adulthood, however had an overall reduced lifespan [264]. Autopsies revealed extensive oxidative damage in the cytoplasm, nucleus and mitochondria, coupled with elevated cell proliferation, linked to the increased incidence of hepatocarcinogenesis observed later in life [264]. In Ntg animals, ROS-induced destruction of $\beta$-islet cells as a result of alloxan administration resulted in a steady increase in blood glucose levels; this increase was significantly attenuated in animals with transgenic CuZnSOD expression [265].

Numerous pre-clinical studies have also shown that administration of SOD is protective against various pathologies, including cardiac ischemia-reperfusion (I-R) injury and chronic heart failure [262, 266]. Craven and colleagues demonstrated that CuZnSOD transfection prevented the increase in collagen accumulation induced by high glucose [267]. However, in vivo studies involving exogenous SOD administration do not always show benefit and is fraught with limitations, predominantly due to their large molecular size (restricting cell permeability) and short half-life [262]. To overcome this, various SOD mimetics with lower molecular mass have been developed, including tempol. Tempol (4-hydroxy-2,2,6,6 tetramethylpiperidine-N-oxyl) is a hydrophilic, low molecular weight nitroxide which functions as both a SOD mimetic and a $\cdot$O$_2^-$ spintrap [68]. Tempol was able to blunt pressure-overload-induced increases in heart weight/tibia length (HW/TL) and lipid
peroxidation, as well as improving systolic function in high fructose-fed mice [268]. Tempol also prevented myocardial structural damage in an insulin-resistant GLUT4-deficient mouse model of T2DM [68]. Hypertrophic markers including HW/BW and the gene expression of β-myosin heavy chain and BNP were all decreased following tempol administration [68]. Upregulation of NADPH oxidase (both Nox2 and Nox1) and the pro-fibrotic gene pro-collagen III were also suppressed in GLUT4-deficient mice following treatment with tempol [68].

1.2.5.6.2 Catalase

Like SOD, catalase is capable of scavenging ROS and breaking redox cycles which are involved in the generation of ROS [185]. Catalase functions as a catalyst of the decomposition of H₂O₂ to O₂ and H₂O. An example of the importance of catalase for normal glucose regulation was exemplified by a study involving a group of catalase-deficient Hungarian patients who were more susceptible to developing diabetes compared to the general population; increased frequency of diabetes was coupled with an increase in SOD levels, indicative of elevated oxidative stress [269]. In the experimental setting, overexpression of catalase improved cardiac morphology, mitochondrial structure and myofibrillar substructure, as well as cardiomyocyte contractility in a mouse model of T1DM (OVE26) [258]. This improvement was associated with a significant reduction in ROS levels. Furthermore, cardiac-specific overexpression of catalase improved cardiomyocyte contraction, and blunted the increase in ROS generation and apoptosis in STZ diabetic mice [270]. Catalase transgenic mice were also protected from the diabetes induced decrease in the phosphorylation of the cell survival kinase, Akt.

1.2.5.6.3 Glutathione Peroxidase

Accumulation of H₂O₂ and its decomposition product •OH is harmful for all aerobic organisms, facilitating the need for rapid and efficient removal via various antioxidant systems. Glutathione peroxidase, like catalase, is an antioxidant enzyme capable of reducing H₂O₂ and lipid peroxides to water and lipid alcohols, using glutathione as a hydrogen donor [271]. Glutathione peroxidase-1 (GPx1) is the most abundantly expressed isoform, and is involved in most of the detoxification of H₂O₂ in the cytoplasm [271]. Transfection of cell lines have been frequently used to determine the role of this particular antioxidant in the
prevention of oxidative stress. Overexpression of GPx1 \textit{in vitro} has been shown to protect both pancreatic β-islet cells and human endothelial cells against damage and death induced by H$_2$O$_2$ [272, 273]. In \textit{in vivo} studies, GPx1 protein levels were reduced in diabetic rats, and could be subsequently restored with insulin and/or antioxidant therapy [274]. Overexpression of GPx1 specifically in the pancreatic β-islet cells of \textit{db/db} mice delayed the progression of hyperglycemia, and by 20 weeks of age, their blood glucose levels were not different to non-diabetic \textit{db/+} mice. This reduction in blood glucose levels was also accompanied by an increase in pancreatic β-islet cell volume [272]. Overexpression of GPx1 has also been shown to be cardioprotective in the diabetic setting. GPx1 transgenic diabetic mice exhibited improved LV diastolic function, which was accompanied by the attenuation of myocyte hypertrophy, cardiac fibrosis and apoptosis, compared with Ntg diabetic controls [275]. Furthermore, mice lacking both Gpx1 and apolipoprotein E displayed accelerated atherosclerosis, via upregulation of pro-inflammatory and pro-fibrotic pathways [276].

\subsection*{1.2.5.6.4 Thioredoxin}

One of the methods employed by the antioxidant defence system to lessen oxidative stress is by reversing oxidative damage from cellular macromolecules. GPx4 can detoxify lipid peroxides, whilst thioredoxins (Trx) can detoxify peroxides through peroxiredoxins [277]. The Trx system, comprising thioredoxin, thioredoxin reductase and NADPH, is essential for the prevention of protein oxidation and damage, which under physiological conditions functions to maintain proteins in its reduced state [277]. Trx reduces oxidized cysteine residues present on proteins, forming a disulfide bond, which is subsequently reduced by thioredoxin reductase and NADPH. Trx exists in two forms in the mammalian cell: Trx1 and Trx2, which reside in the cytoplasm and in the mitochondria, respectively [278]. Trx activity can be induced under conditions of stress, including viral infection, ischemic insult, UV light, as well as in disease settings including heart disease and diabetes [277, 279]. Cardiac overexpression of a redox-inactive, dominant-negative Trx mutant significantly increased oxidative stress under both basal and pathological conditions [280]. In addition, pathological cardiac hypertrophy was observed in these transgenic mice under basal settings, which further worsened following pressure-overload. Conversely, overexpression of Trx in mice reduced the degree of hypertrophy and oxidative stress in response to pressure-overload [280]. Trx has also been explored in the context of diabetes. Targeted overexpression of Trx in mouse pancreatic β-cells significantly reduced the incidence of
diabetes in response to STZ compared with Ntg littermates, suggesting a protective effect of increased Trx activity against STZ-induced destruction of pancreatic β-islet cells [279]. Murine cardiomyocytes incubated in high glucose medium exhibited significantly increased •O₂⁻ and ONOO⁻ production, along with enhanced Trx nitration and reduced Trx activity. These cells sustained even greater injury after being subjected to I-R compared to cardiomyocytes in normal glucose, suggesting that restoration of Trx activity in the diabetic heart may impart protection against cardiac injury [281].

1.2.5.6.5 Vitamin C & Vitamin E

The efficacy of therapy with non-enzymatic antioxidant micronutrients such as Vitamin C, E and zinc in preventing the development of cardiovascular complications in diabetic patients has been extensively researched, and results have been largely inconclusive [282, 283]. Vitamin C, or ascorbic acid, functions as a water-soluble antioxidant by acting as a reducing agent to reverse oxidation. After loss of one of its two electrons, vitamin C becomes a free radical known as ascorbyl radical, which is less reactive and fairly stable [284]. Ascorbyl radical can react with a wide variety of harmful oxidants including •O₂⁻ and •OH to form the more stable dehydroascorbic acid. In most mammalian species (excluding humans), vitamin C is synthesized from the hepatic metabolism of glucose. Despite the absence of this pathway in humans, glucose and insulin is involved in the regulation of vitamin C cellular uptake [285]. The potential of vitamin C supplementation to treat pathophysiologicals related to hyperglycemia has thus been recognised, and has been the subject of much research [286-288]. Diabetic patients supplemented with 1000mg of vitamin C daily exhibited a reduction in fasting blood sugar, triglycerides, total cholesterol, low and high density lipoproteins, HbA1C and serum insulin [289]. Vitamin C, administered in conjunction with metformin therapy also improved fasting and post-meal glucose levels, and HbA1C compared with metformin and placebo treatment [288]. In addition to its benefits in glycemic control, vitamin C has also been shown to improve diabetes-associated cardiovascular complications. Both STZ-induced cardiac contractile dysfunction and endothelial-dependant vasodilator defects in diabetic patients were attenuated with vitamin C administration [287, 290]. Whilst various small studies, like those mentioned above, have observed a beneficial effect of vitamin C supplementation on diabetes and its associated complications, large well-controlled, prospective studies have generally failed to find a similar benefit [291, 292]. The clinical efficacy of vitamin C administration thus still remains
unconfirmed, necessitating additional studies that focus specifically on the pharmacokinetics of orally-administered vitamin C.

Like vitamin C, vitamin E’s potential in preventing heart disease and diabetes by reducing oxidative stress has been examined. Vitamin E is a lipid-soluble antioxidant, which functions as a lipid peroxyl radical scavenger \textit{in vivo} and a potential regulator of various cell signaling pathways [282]. Vitamin E is localized to the cell membrane, where it functions to protect the cell against lipid peroxidation. Low plasma vitamin E concentrations have been associated with an increased risk of developing both T1DM and T2DM [293, 294]. Conversely, studies have shown that high serum levels of vitamin E are associated with reduced risk of cardiovascular disease [295, 296], implicating the important role of vitamin E in both the development of diabetes and heart disease. The Cambridge Heart Antioxidant Study (CHAOS) showed that high dose vitamin E supplementation markedly reduced the rate of non-fatal MI in patients with coronary atherosclerosis in a randomized, prospective clinical trial [296]. Vitamin E supplementation also significantly reduced apoptosis, lipid peroxidation and protein oxidation induced by STZ diabetes in rats, in addition to preserving normal cardiac function [297]. Despite these promising results, other studies reporting conflicting results have questioned the true efficacy of vitamin E therapy in treating diabetes and heart disease [259, 298-300]. The risk of developing type 2 diabetes was not altered by vitamin E supplementation in a double-blind, placebo-controlled primary prevention trial involving more than 38000 participants over a 10 year follow-up period [301]. Consistent with this study, patients with a high-risk for a cardiovascular event enrolled in the Heart Outcomes Prevention Evaluation (HOPE) study did not benefit from 4.5 years of vitamin E supplementation [282]. It has even been suggested that vitamin E additionally possesses some pro-oxidant abilities, and that when taken in high dosages can result in an increase in all-cause mortality [302]. Furthermore, vitamin E is thought to be a relatively weak antioxidant, and unlike more potent antioxidants such as tempol, requires electron donation following a single interaction with a ROS [300, 303]. Given the evidence for a causal role of ROS in the cardiac and other complications of diabetes, clinical studies examining the therapeutic promise of more potent antioxidant approaches than vitamins C and E are warranted.

1.2.5.6.6 \textit{Metallothionein}

\textit{Metallothionein} (MT) are cysteine-rich metal-binding proteins which also have antioxidant properties. The antioxidant actions of MT in the hearts of intact animals were
demonstrated in recent years [304-306]. Overexpression of the human MT-IIA gene specifically in cardiomyocytes protected them from I/R-induced oxidative damage [305]. Several laboratories have shown that MT is associated with a reduction in diabetes-induced cardiac damage [307-309]. Overexpression of MT specifically in the hearts of diabetic rodents prevented disturbances in Ca$^{2+}$ handling [310], protected the heart from adverse structural remodeling [309] and reduced apoptotic cell death [311]. The mechanisms underlying MT’s ability to protect against diabetic cardiomyocyte remain largely unknown, however it has be largely attributed to its antioxidant action, its ability to restore cardiomyocyte contractility and its bound zinc [312].

1.2.5.6.7 Coenzyme Q$_{10}$

A number of small studies have also exploited the antioxidant properties of the mitochondrial electron carrier coenzyme Q$_{10}$ to rescue cardiovascular disease [313-315]. Coenzyme Q$_{10}$ is localized in the hydrophobic region of the phospholipid bilayer, where it functions primarily as an electron transfer intermediate in the mitochondrial respiratory chain [316]. Coenzyme Q$_{10}$ is a critical component of the electron transfer chain, receiving electrons from various oxidoreductases including complex I, complex II and electron transfer flavoprotein-ubiquinone oxidoreductase, which subsequently reduce ubiquinone to ubiquinol by the addition of two electrons and two protons [317]. In addition to its role as an electron transfer intermediate, coenzyme Q$_{10}$ also acts as an antioxidant in its reduced form, stimulates the uncoupling of proteins, and regulates membrane permeability [317] (Figure 1.5). Its antioxidant actions rely on its ability to inhibit lipid peroxidation, thereby preventing the reaction of •OH and •O$_2^-$ with neighbouring lipid and protein molecules [317]. Many studies have drawn parallels between the antioxidant properties of vitamin E and coenzyme Q$_{10}$, as they are both lipid-soluble (thus localized to the cell membrane) and both function to minimize lipid peroxidation. Indeed, various studies have utilized coenzyme Q$_{10}$ and vitamin E conjointly to study their interaction and to compare their relative efficacy in preventing oxidative stress [318-320]. The chain-breaking antioxidant properties of coenzyme Q$_{10}$ allows it to interfere with both the initiation and propagation steps involved in lipid peroxidation. In contrast, vitamin E is only capable of preventing the propagation step. Coenzyme Q$_{10}$’s localization to the hydrophobic region of the membrane phospholipid bilayer, and its direct access to the ROS-generating mitochondrial respiratory chain, renders it
Figure 1.5 Known cellular functions of coenzyme Q₁₀

Coenzyme Q₁₀ 1) protects the cell membrane against lipid peroxidation due to its lipid solubility, 2) improves activity of antioxidant enzymes and regulates growth factor activity, 3) improves cellular bioenergetics by increasing ATP production and enhancing mitochondrial function, 4) inhibits apoptosis and protects the cell against DNA damage and 5) protects the cell against oxidative damage by scavenging ROS. Inset represents the inner membrane of the mitochondria. e⁻, electron. See text for references.
particularly favorable for both preventing and scavenging lipid peroxyl radicals [317]. Coenzyme Q\textsubscript{10} can further reduce lipid peroxidation by recycling vitamin E directly from the α-tocopherolxy radical, a process dependent on the availability of water-soluble antioxidants such as ascorbate [321, 322].

The benefits of exogenous coenzyme Q\textsubscript{10} supplementation has emerged from both \textit{in vitro} and \textit{in vivo} settings of coenzyme Q\textsubscript{10} deficiency. In cultured fibroblasts harbouring the Pdss2 mutant responsible for coenzyme Q\textsubscript{10} deficiency (as the Pdss2 gene encodes for an enzyme which synthesizes the prenyl side-chain of coenzyme Q\textsubscript{10}), ROS production and lipid oxidation was enhanced, accompanied by an increase in cell death [323]. Coenzyme Q\textsubscript{10} treatment restored bioenergetic status by normalizing ATP/ADP ratios, as well as reducing •O\textsubscript{2}- production and cell death [323, 324]. Similar observations were obtained in clinical cases of coenzyme Q\textsubscript{10} deficiency. Two brothers with autosomal-recessive myopathic coenzyme Q\textsubscript{10} deficiency presented with severe exercise intolerance, seizures and marked lactic acidosis [325]. Muscle biopsies revealed increased accumulation of lipid droplets and enhanced activation of pro-apoptotic Fas proteins and caspase 3, accompanied by increased apoptosis on TUNEL staining. Following 8 months of coenzyme Q\textsubscript{10} administration, excessive lipid storage was resolved, and the number of apoptotic cells was reduced.

In both basic and clinical research areas, coenzyme Q\textsubscript{10} is most known for its use in the treatment of cardiovascular disease. The past 40 years has seen a steady increase in the number of publications focused on coenzyme Q\textsubscript{10} and its efficacy in reducing cardiomyopathy, hypertension and ischemic damage in both the clinical and experimental setting. More than 34 controlled clinical studies featured in a systematic review by Langsjoen \textit{et al.} accumulatively suggested that coenzyme Q\textsubscript{10} supplementation has the potential to diminish the risk of heart disease through its maintenance of cellular and mitochondrial function in cardiomyocytes [326]. Importantly, the authors noted that apart from transient nausea, no side effects directly resulted from coenzyme Q\textsubscript{10} administration, and improvements were sustained throughout the treatment period. Soja and Moretensen, in their meta-analysis of placebo-controlled cardiovascular disease trials in the years 1986-1995, reported that approximately 73% of patients treated with coenzyme Q\textsubscript{10} exhibited improved cardiac output, 76% displayed increased stroke volume and ejection fraction exhibited improvement in 92% of patients [327].
In addition to its ability to improve cardiac function, coenzyme Q\textsubscript{10} supplementation also significantly reduced posterior and ventricle wall thickness in a group of patients with hypertrophic cardiomyopathy [328]. The effect of coenzyme Q\textsubscript{10} on the levels of serum α-lipoprotein has also been examined in 35 patients diagnosed with acute coronary artery disease. Increased α-lipoprotein levels were strongly associated with both the occurrence of cardiac death and MI [329]. Patients in the treatment group received a 120 mg dose of coenzyme Q\textsubscript{10} twice daily for 28 days, and results showed that compared to placebo, coenzyme Q\textsubscript{10}-treated patients exhibited significantly lower levels of fasting blood glucose, malondialdehyde (MDA), lipid peroxides and α-lipoproteins [329]. Improvement in long-term survival was also observed in coenzyme Q\textsubscript{10}-treated patients with congestive heart failure [330, 331]. Individual patient response to the antioxidant therapy was varied however; some patients saw improvements as early as 30 days of treatment, whilst others did not show improvements until after 60-90 days of treatment. It is interesting to note that in this study, congestive heart failure patients receiving coenzyme Q\textsubscript{10} were found to relapse when treatment ceased [330, 331]. Patients with ischemic heart disease have also benefited from the addition of coenzyme Q\textsubscript{10} to their standard treatment regime [332]. In comparison to the placebo group, coenzyme Q\textsubscript{10}-treated patients exhibited significant improvements in cardiac output, cardiothoracic ratio, frequency of angina attacks and exercise duration. Side effects amongst treated patients were negligible. The efficacy of coenzyme Q\textsubscript{10} has also been explored in the context of cardiac surgery; previous clinical studies have demonstrated that coenzyme Q\textsubscript{10} may afford protection against myocardial injury if administered pre-operatively [333]. By increasing myocardial and cardiac mitochondrial coenzyme Q\textsubscript{10} levels, exogenously administered coenzyme Q\textsubscript{10} enhances mitochondrial efficiency and improves tolerance to hypoxia-reoxygenation stress.

Several researchers have theorized that improvements in cardiac function observed in coenzyme Q\textsubscript{10}-treated patients with cardiac disease may be the result of improved cellular bioenergetics. Moreover, studies which reported a relapse of adverse cardiac symptoms upon cessation of coenzyme Q\textsubscript{10} treatment suggests that coenzyme Q\textsubscript{10} deficiency may be a major contributor to the onset of cardiomyopathy [330]. Indeed, due to the high energy requirements of cardiomyocytes, coenzyme Q\textsubscript{10} is known to be highly concentrated in the heart, and severity of heart failure has been strongly correlated with the severity of coenzyme Q\textsubscript{10} deficiency [334]. Congestive heart failure has also been strongly linked to low blood and tissue levels of coenzyme Q\textsubscript{10} [335]. Improvements in cellular bioenergetics may also explain
coenzyme Q₁₀’s ability to lower blood pressure in hypertensive patients. A meta-analysis performed on 12 clinical trials found that systolic pressure was reduced by approximately 17 mmHg, whilst mean diastolic blood pressure was reduced by 10 mmHg [313] in patients treated with coenzyme Q₁₀. The increase in production of •O₂⁻ in diabetic endothelium likely increases its reaction with NO to form ONOO⁻, reducing the ability of the endothelium to induce NO-mediated relaxation and thus resulting in vasoconstriction and increased blood pressure. Coenzyme Q₁₀ has the potential to counteract vasoconstriction in the diabetic endothelium via its •O₂⁻-scavenging ability.

Under experimental settings, coenzyme Q₁₀ has also been shown to be beneficial for improving various cardiac pathologies, including hypertension and I-R injury. The stroke-prone, spontaneously hypertensive rat (SHR) is the most commonly used animal model of essential hypertension, which at a later age also develop cardiovascular disease characteristics such as cardiac hypertrophy [289]. Coenzyme Q₁₀ treatment in SHR attenuated the elevation of blood pressure, the degradation of membrane phospholipid and the increase in the activity of renal phospholipase A₂ [336]. The study suggested that those beneficial effects may be the result of coenzyme Q₁₀’s membrane-stabilizing properties. Various reports have also indicated a protective role of coenzyme Q₁₀ against I-R injury. Nakamura et al. [337] examined I-R myocardial injury in dogs subjected to left anterior descending coronary artery occlusion. The degree of I-R injury was assessed by determining myocardial ATP levels. Animals pre-treated with coenzyme Q₁₀ prior to the occlusion procedure exhibited significantly higher levels of ATP content in ischemic myocardium compared to control animals [337]. The authors proposed that coenzyme Q₁₀ may be capable of preventing mitochondrial damage and disturbance in ATP production associated with ischemia. In addition to canine hearts, the effect of coenzyme Q₁₀ on I-R injury has also been examined in swine hearts. Pigs were fed with a regular diet supplemented with either coenzyme Q₁₀ or a placebo for 30 days [314]. At the end of the treatment period, hearts were isolated and subjected to 75 minutes of ischemia, followed by 120 minutes of reperfusion. Coenzyme Q₁₀-fed pigs exhibited smaller infarcts, accompanied by improved postischemic LV contractile function and reduced MDA concentration (marker of lipid peroxidation). These results demonstrate that supplementation of coenzyme Q₁₀ effectively reduced I-R injury, possibly by reducing the production of ROS.

As elevated oxidative stress is a major contributor to the pathogenesis of diabetes mellitus, various studies have also examined the efficacy of coenzyme Q₁₀ in preventing an
increase in diabetes-induced systemic redox stress. Lim and colleagues measured plasma coenzyme Q$_{10}$ concentration and composition as an indicator of oxidative burden in individuals with varying severity of glucose intolerance [338]. Diabetic individuals appeared to be in a state of coenzyme Q$_{10}$ deficiency, evidenced by overall reduced plasma total coenzyme Q$_{10}$/total cholesterol levels. In addition, profound changes were also observed in the composition of coenzyme Q$_{10}$, where an incremental increase in the ubiquinone/ubiquinol ratio correlated with an increasing degree of glucose intolerance (from patients with normal glucose tolerance to impaired glucose tolerance to established type 2 diabetes) [338]. In patients with normal glucose tolerance, approximately 90% of total coenzyme Q$_{10}$ existed as the reduced form ubiquinol, which acts as a lipophilic antioxidant. This is in direct contrast to diabetic patients, where only a quarter of total coenzyme Q$_{10}$ existed as the ubiquinol form. The increase in the conversion of ubiquinol to ubiquinone amongst diabetic patients is thought to be indicative of elevated oxidative burden. In another study, Rauscher et al. reported diminished activity of SOD in STZ diabetic rat kidneys following treatment with coenzyme Q$_{10}$, reflecting a lower level of •O$_{2}$- generation [316]. A possible mechanism of this •O$_{2}$- reduction may be via coenzyme Q$_{10}$’s ability to enhance the efficiency of electron transfer from the cytoplasm to the mitochondrial respiratory chain, thus diminishing NADH levels and lessening the reductive power that mediates •O$_{2}$- production [339]. Furthermore, coenzyme Q$_{10}$ is the only lipophilic antioxidant to be biosynthesized and thus is capable of endogenously scavenging free radicals [317]. Exogenous coenzyme Q$_{10}$ can protect cells from oxidative stress by converting to its reduced antioxidant form using cellular reductases.

Coenzyme Q$_{10}$’s ability to lower blood pressure has also been explored in the context of diabetes. Coenzyme Q$_{10}$ supplementation reduced systolic blood pressure by approximately 6mmHg, and diastolic blood pressure by approximately 3mmHg in 74 diabetic patients [340]. This reduction was more modest compared to previous studies utilizing coenzyme Q$_{10}$ in hypertensive patients, however this may be the result of the diabetic patients having lower baseline blood pressure measurements. Coenzyme Q$_{10}$’s ability to enhance endothelial dysfunction has been suggested as a possible mechanism of its protection against rising blood pressure. Patients with dislipidemia and type 2 diabetes exhibited improved abnormal endothelium-dependent vasodilator tone in their brachial artery when treated with coenzyme Q$_{10}$ orally [341]. This improvement may reflect increased endothelial release and/or activity of NO due to the coenzyme Q$_{10}$-induced reduction of oxidative stress.
Despite the existence of numerous studies demonstrating the benefits of coenzyme Q\textsubscript{10} treatment in treating various cardiomyopathies, the impact of coenzyme Q\textsubscript{10} on structural and functional defects caused by diabetic cardiomyopathy still remains to be elucidated. Shen \textit{et al.} reported that overexpression of mitochondrial SOD in T1DM mice improved both cardiac function and structure via protection of cardiac mitochondria [210]. As coenzyme Q\textsubscript{10} is located in the mitochondria, its main site of action, it may possess a direct antioxidant effect against elevated levels of mitochondrial \textbullet\text{O}_2^- in the disease setting.

1.2.6 The renin-angiotensin-aldosterone system (RAAS) and the use of angiotensin-converting enzyme inhibitors (ACE-I) in treating diabetes

1.2.6.1 Overview of the RAAS

The RAAS plays a pivotal role in the preservation of hemodynamic stability, through its ability to regulate blood pressure and maintain water and electrolyte balance. This hormonal cascade is triggered by the synthesis and release of renin from the kidney in response to a fall in blood volume and renal perfusion [342]. Renin is produced and secreted in a tightly-regulated manner by the juxtaglomerular apparatus to elicit both local (kidney) and systemic actions. Angiotensinogen is cleaved by renin to form angiotensin I (Ang I). Ang I is cleaved and converted to the biologically-active peptide Ang II by the zinc metallopeptidase angiotensin-converting enzyme (ACE). ACE is predominantly synthesised in the lungs, however it has also been detected in endothelial cells and in the heart, kidneys and brain [343]. Apart from being a potent vasoconstrictor, Ang II also interacts with the sympathetic nervous system to increase vascular tone, and triggers aldosterone secretion and sodium retention, which subsequently leads to an increase in arterial blood pressure [342]. Ang II also inhibits the release of renin to form a negative feedback loop. The major physiologic actions of Ang II, which include cell proliferation and hypertrophy [344, 345], are predominantly mediated through stimulation of angiotensin type 1 (AT\textsubscript{1}) [and possibly also type 2 (AT\textsubscript{2})] receptors [346].

The RAAS is critical for the maintenance of electrolyte balance and prevention of hemodynamic collapse, and its rapid activation is critical in response to threats which may jeopardize blood pressure control, including loss of blood volume, excessive water and sodium excretion and insufficient intake of sodium [343, 347]. Overactivation of the RAAS however, can result in excessive vasoconstriction [343]. Dysregulation of this complex
system may have severe pathological implications, including hypertension, congestive heart failure, atherosclerosis, and exaggerated diabetic complications [342, 348, 349]. Due to the importance of the RAAS in the regulation of blood pressure, ACE-I were first developed as a therapeutic agent for the treatment of hypertension [350]. ACE-I have since found use for the treatment of additional clinical cardiac indications, including congestive heart failure, left ventricular dysfunction and atherosclerotic vascular disease, both in the presence and absence of diabetic complications [351-354]. In addition to ACE-I, AT₁ receptor antagonists and aldosterone inhibitors have also been used in the clinic to treat hypertension and heart disease [355-357]. For the purpose of this thesis however, I will be focusing on reviewing the use of ACE-I for the treatment of cardiovascular disease in the diabetic and non-diabetic setting.

1.2.6.2 Mechanisms of RAAS blockade

1.2.6.2.1 Mechanism of action and pharmacology of ACE-I

As noted previously, ACE is involved in the cleavage of Ang I to form the biologically active peptide Ang II. ACE (also known as kininase II), catalyses the breakdown of bradykinin, a physiologically active peptide involved in the promotion of vasodilation by triggering the synthesis of prostacyclin, NO and endothelium-derived hyperpolarizing factor in vascular endothelium [358]. Bradykinin also causes natriuresis in the kidney, which along with vasodilation, contributes to a fall in blood pressure. ACE therefore has a critical role in maintaining the balance between the prevention of vasodilation by bradykinin, and the promotion of vasoconstriction by increased Ang II signaling. Short-term ACE inhibition can thus result in a dose-dependent reduction in Ang II levels, a fall in both systolic and diastolic pressure (with little or no change in heart rate and cardiac output) [359], and inhibition of the breakdown of bradykinin [347, 360].

The pharmacokinetic profiles of different ACE-I are distinguished by their biochemical structure and bioavailability, dose, plasma half-life, route of elimination and their distribution and affinity for tissue-bound ACE [347, 361]. ACE-I can be divided into 3 subgroups, based on the molecular structure of their active moieties (Table 1.2).

Whilst ACE-I have proved to be the gold standard in the treatment of various renal and cardiovascular complications, they can also give rise to an array of adverse side effects. Although most side effects of ACE-I therapy are non-life threatening, they often cause
sufficient disturbance to the patient to justify discontinuation of the drug [362]. The most common side effect is cough, occurring in 15-30% of patients, which is the result of an increase in bradykinin levels [363, 364]. Angioedema, another secondary consequence of bradykinin accumulation, can occur in around 0.1% - 0.2% of all patients undergoing ACE-I treatment [362, 365, 366]. Angioedema is characterized by swelling of the lips, tongue, nose, throat and other localized areas on the face; whilst uncommon, it is potentially life-threatening. ACE-I use also needs to be carefully monitored in the elderly, or individuals with heart failure who are susceptible to hypotension; dosages are usually reduced in these patients [347]. In patients with impaired kidney function or reduced renal blood flow, hyperkalemia is another potential side effect of ACE-I usage. Diabetic patients with uncontrolled glucose and renal tubular acidosis are at particular risk for hyperkalemia [367], which can be further exacerbated with concomitant use of ACE-I and angiotensin receptor blockers, aldosterone blockers, potassium supplements, or sodium-restricted diets [367]. A further contraindication to ACE-I therapy is pregnancy, as the drug has been shown to cause fetal defects, including pulmonary hypoplasia, growth retardation, and possible death if administered in the second or third trimester of pregnancy [368, 369].

**Table 1.2 Biochemical classification of ACE Inhibitors** from [347]

<table>
<thead>
<tr>
<th>Sulphydryl-containing</th>
<th>Dicarboxyl-containing</th>
<th>Phosphorus-containing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Captopril</td>
<td>Lisinopril</td>
<td>Fosinopril</td>
</tr>
<tr>
<td>Fentiapril</td>
<td>Benazepril</td>
<td></td>
</tr>
<tr>
<td>Pivalopril</td>
<td>Qinapril</td>
<td></td>
</tr>
<tr>
<td>Zofenopril</td>
<td>Moexipril</td>
<td></td>
</tr>
<tr>
<td>Alacepril</td>
<td>Ramipril</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spirapril</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Perindopril</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pentopril</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cilazapril</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trandolapril</td>
<td></td>
</tr>
</tbody>
</table>

1.2.6.3 The role of RAAS blockade via ACE-I in the prevention of diabetic cardiomyopathy

Despite being originally developed to treat hypertension, ACE-I have since been utilized for the treatment of congestive heart failure, coronary artery disease and renal failure.
In addition, several studies have also suggested that ACE-I are able to reduce the incidence of type 2 diabetes in patients with hypertension and heart failure [370-372].

1.2.6.3.1 ACE-I and cardiovascular disease

Hypertension is a significant cardiovascular risk factor, and when treated successfully, confers a 50% reduction in the incidence of heart failure, a 35-40% decrease in stroke, and a 20-25% decrease in MI [373]. The RAAS plays a critical role in blood pressure regulation, in part by maintaining the balance between Ang II and bradykinin, which are involved in vasoconstriction and vasodilation, respectively. Overactivation of the RAAS has been suggested as an additional cardiovascular risk factor in patients with essential hypertension [342, 374]. As a result, ACE-I are recommended as the first drug of choice to treat hypertension in patients with MI, congestive heart failure, stroke, diabetes and renal failure [375].

In addition to hypertension, the RAAS has also been implicated in the development of MI. Activation of the RAAS after an acute MI (AMI) event is thought to be a necessary adaptive response to preserve normal blood pressure and perfusion levels; prolonged activation however is detrimental to cardiac function [376]. Lack of oxygen delivery to the myocardium following AMI is exacerbated by Ang II-induced vasoconstriction, resulting in irreversible myocardial injury. Numerous major clinical trials have assessed the benefits of using ACE-I following AMI. In the Survival and Ventricular Enlargement (SAVE) and Trandolapril Cardiac Evaluation (TRACE) studies, patients with MI and LV systolic dysfunction treated with captopril and trandolapril experienced a ~30% reduction in mortality compared with patients receiving the placebo [351, 353]. A study by the ACE Inhibitor Myocardial Infarction Collaborative Group reported that ACE inhibition was associated with a 7% reduction in mortality within 30 days post-AMI, which corresponded to 5 lives saved for every 1000 patients treated [377]. The RAAS is also an important therapeutic target for the treatment of systolic heart failure. Dysregulation of major neurohormonal systems, including the sympathetic nervous system and RAAS, are involved in the development and progression of heart failure [378]. Several large, randomized, placebo-controlled trials have shown convincing evidence that ACE inhibition is associated with increased survival rates in patients with congestive heart failure due to systolic dysfunction. The Cooperative North Scandinavian Enalapril Survival Study (CONSENSUS) trial was the first to identify a link between ACE-I and reduced mortality in patients with systematic severe systolic heart
failure; enalapril therapy was linked to a 40% decrease in mortality after 6 months [379]. These observations were further supported by the SOLVD trial, which additionally demonstrated a fall in the incidence of heart failure and rate of related hospitalisation in enalapril-treated patients with LV dysfunction [354, 380].

Ang II is a well-known mediator of cardiac hypertrophy [381, 382], cardiac fibrosis [383] and cardiomyocyte apoptosis [384]. Pathological LV cardiac hypertrophy is an important risk factor for the development of systolic heart failure, contributing to declining systolic function, abnormal diastolic filling in addition to disturbances in heart rhythm [385]; its regression is thus associated with a more favorable prognosis [386]. Both experimental and clinical studies have demonstrated benefits in treating cardiac hypertrophy with ACE-I [221, 387, 388]. In a rodent model of long term MI, treatment with captopril was associated with diminished LV volumes and LV filling pressure, consistent with prevention of adverse structural LV remodeling [389]. Attenuation of ventricular enlargement with long-term ACE-I therapy was also associated with prolonged survival. Inhibitors of ACE have been shown to promote regression of pathological LV hypertrophy to a greater degree than other antihypertensive therapies [390]. A meta-analysis of 39 clinical studies found that LV mass was reduced 13% with ACE-Is, compared to 9% with calcium channel blockers, 7% with diuretics and 6% with beta-blockers [390]. ACE-I’s ability to reverse pathological LV hypertrophy, secondary to its antihypertensive properties, may be in part due to removal of Ang II’s trophic effect on vascular smooth muscle [391].

\[1.2.6.3.2\] ACE-I for the management of diabetes

ACE inhibitors are widely used in patients with cardiovascular disease who are at high risk of developing T2DM, due to the potential added benefits of improving insulin sensitivity and limiting insulin resistance [392, 393]. Several independent trials have suggested that agents blocking RAAS activation, including ACE-Is, are able to prevent or delay the onset of diabetes. The Captopril Prevention Project (CAPPP) reported that captopril treatment was associated with a 30% reduction in the rate of new-onset diabetes in comparison to patients receiving conventional diuretic and/or β-blocker-based therapy, when correcting for age, glucose, body mass index, blood hemoglobin and systolic blood pressure [394]. The HOPE trial reaffirmed the role of ACE inhibition in preventing the onset of diabetes through observations that ramipril treatment reduced the incidence of diabetes in
high-risk individuals [395]. In the placebo group, 5.4% of patients had developed T2DM within the 4.5 year duration of the study, compared to only 3.6% of patients in the ramipril group. Similar observations were obtained in the 2nd Australian National Blood Pressure study (ANBP-2), where ACE-I treatment was associated with a 31% risk reduction in the incidence of new-onset diabetes in elderly patients, compared with diuretic treatment [396]. A subanalysis of the SOLVD trial also demonstrated enalapril’s ability to reduce the incidence of diabetes. Approximately 6% of patients undergoing enalapril therapy for LV dysfunction developed type 2 diabetes within the 2.9-year duration of the study, compared to 22% of patients receiving a placebo [371].

The ability of RAAS blockade to improve peripheral insulin sensitivity and glucose metabolism provided a starting point in identifying the mechanisms underlying ACE-I’s protection against the development of diabetes. The fall in the incidence of new-onset diabetes with ACE-I treatment was usually accompanied by a concomitant improvement in glycemic control. The HOPE study reported a significant reduction in HbA1c in ramipril-treated diabetic patients [393]. In the UK Prospective Diabetes Study (UKPDS), patients randomized to receive captopril treatment had lower levels of HbA1c compared to patients on β-blocker or diuretic therapy [352]. This was consistent with findings in the Study to Evaluate Carotid Ultrasound changes in patients treated with vitamin E (SECURE) trial, where fasting glucose levels were higher in placebo-treated control patients (15.8mg/dL) compared with ramipril-treated patients (9.6mg/dL) [397].

Hypertensive patients have been shown to have an increased propensity to developing T2DM [398, 399]; indeed, a large proportion of hypertensive subjects experience a degree of glucose intolerance, where an amplified plasma insulin response consistently took place following a glucose load [400]. Several mechanisms have been proposed to explain this phenomenon. Alterations in the composition of skeletal muscle (enhanced number of slow-twitch insulin-sensitive muscle fibers) and changes in skeletal muscle vasculature (i.e., increased vasoconstriction), which diminishes delivery of glucose and insulin to skeletal muscle, may contribute to impaired glucose tolerance [401]. In addition, abnormalities in signaling kinases downstream of the insulin receptor (i.e., changes in PI3K and Akt signaling induced by Ang II) may also result in reduced glucose sensitivity [402]. Interruption of the RAAS through use of ACE-Is has indeed shown to improve insulin signaling and reduce insulin resistance in hypertensive, non-diabetic and type 2 diabetic patients, via at least two separate mechanisms [403]. ACE inhibition can afford beneficial hemodynamic effects on the
microcirculation by reducing Ang II-mediated vasoconstriction and vascular resistance, and thus increase blood flow through skeletal muscle and pancreatic β-islet cells. This not only can increase delivery of insulin from the pancreas to the circulation, but can also improve both glucose and insulin tissue uptake [404]. Furthermore, leptin and adiponectin, hormones involved in enhancing insulin sensitivity, have been shown to increase upon RAAS blockade with ACE-I. However, not all of ACE-I’s insulin-sensitizing effects involve the microcirculation. ACE inhibition may prevent some of the inhibitory effects of Ang II on PI3K/Akt signaling downstream of insulin and insulin-like growth factor [405].

As described previously, experimental and clinical studies have suggested a role for ACE inhibition in preventing cardiovascular disease. Importantly, the use of ACE-I also offered protection against both diabetes-related microvascular and macrovascular disorders. The microalbuminuria, cardiovascular and renal outcomes (MICRO) HOPE substudy demonstrated ramipril’s ability to lower the risk of various cardiovascular outcomes in high-risk individuals with diabetes [393]. Ramipril diminished the risk of MI by 22%, stroke by 33%, cardiovascular death by 37% and total mortality by 24%. Consistent with this data, the CAPPP study found that diabetic patients treated with captopril had a significantly diminished risk of having a primary endpoint (defined as fatal or non-fatal MI, fatal or non-fatal stroke, or sudden death), as well as a lower overall risk for a cardiac event [350, 394]. The outcome of microvascular conditions were also improved, with ACE-I treatment lowering the risk of nephrophathy and renal failure.

### 1.3 The role for PI3K(p110α) in preventing diabetes-induced cardiac dysfunction

An extensive number of studies have examined both the beneficial and detrimental role of protein kinases in the regulation of cardiac growth, and their impact on cardiac function. PI3Ks are a family of lipid kinases which are activated by phosphorylating phosphoinositides on the 3’OH (D3) position of the inositol ring to generate lipid second messengers [406]. PI3Ks regulate a diverse range of intracellular signaling pathways, including those which mediate cell growth and survival, protein trafficking and cell adhesion [407, 408]. In particular, PI3Ks have been reported by various studies to be pivotal in the regulation of cardiac growth and hypertrophy which is physiological in nature.
1.3.1 Physiological vs pathological hypertrophy

Cardiac hypertrophy can be categorized into two distinct phenotypes, each possessing divergent structural and molecular properties (Figure 1.6). Adaptive, or physiological cardiac hypertrophy takes place as a feature of normal or regular postnatal growth, as well as in response to physiological stimuli such as exercise [409, 410]. Physiological hypertrophy is beneficial in nature; it is characterized by regular cardiac structure and a normal pattern of gene expression [409]. Hypertrophy in the hearts of athletes develops as an adaptive response to increased metabolic requirements. By reducing resting and submaximal heart rates and increasing filling time and venous return, demands for more oxygen are satisfied whilst normal function is maintained [411]. Exercise-induced cardiac hypertrophy is accompanied by normal systolic function, and not associated with an increase in interstitial fibrosis [412]. Signaling in adaptive hypertrophic hearts is governed primarily by the insulin-like growth factor 1 (IGF-1)-PI3K pathway and is associated with a relatively normal expression of fetal genes such as ANP, BNP and β-myosin heavy chain [407, 413]. Physiological hypertrophy does not eventuate in decompensated heart function and heart failure (Figure 1.6).

Conversely, maladaptive, or pathological cardiac hypertrophy occurs in response to chronic pressure or volume overload in disease settings such as hypertension, MI and valvular disorders [414]. Pathological hypertrophy initially takes place as a compensatory response to increased load, in an attempt to meet oxygen requirements and normalize wall stress. Prolonged pathological stimulation of hypertrophy eventuates in decompensation of cardiac function, where the heart undergoes adverse remodeling and inevitably progresses onto heart failure [415] (Figure 1.6). A major difference between the structural morphology of adaptive and maladaptive hypertrophy is the increased fibrosis in maladaptive hypertrophic hearts. This contributes to impaired elasticity and stiffening of the heart, and may reduce LV compliance and prolong relaxation [416]. Pathological cardiac hypertrophy is also distinct at the molecular level. Re-expression of fetal genes, such as BNP, ANP, ET-1 and β-myosin heavy chain in the heart is often observed in settings of pathological cardiac growth [414, 417]. Furthermore, in contrast to physiological hypertrophy, the development of pathological hypertrophy is mediated by G-protein coupled receptor (GPCR) signaling, following activation by hormones such as Ang II and ET-1 [418] (Figure 1.6).

The distinction between physiological and pathological hypertrophy at the molecular level has been examined in various experimental models. McMullen et al. subjected mice to
Figure 1.6 Physiological vs pathological hypertrophy (adapted from [63])

Pathological hypertrophy is mediated by GPCRs and characterized by adverse structural remodeling (i.e., cardiac fibrosis, apoptosis) and depressed cardiac function, whilst physiological hypertrophy is governed by IGF-1R-PI3K(p110α) signaling and characterized by normal cardiac morphology and function. See text for references.
either a pathological stimuli (aortic banding) or physiological stimuli (exercise training by swim-training) [407]. Both stimuli induced cardiac hypertrophy, however aortic-banded mouse hearts presented with interstitial fibrosis, whilst no fibrosis was evident the hearts of the swimming mice. The molecular phenotypes of rat hearts undergoing physiological and pathological hypertrophy were examined by Iemitsu et al. Gene expression of β1 adrenergic receptor kinase was elevated in SHR (model of pathological hypertrophy), which was accompanied by increased expression of BNP, ACE and ET-1 [414]. The mRNA levels of these pathological hypertrophic markers were significantly lower in swim-trained rats (model of physiological hypertrophy). These observations were concordant with Kong et al.’s study, which reported increases in the mRNA expression of ANP and BNP in a rat model of pathological hypertrophy [419]. In addition, inflammatory-response genes (e.g. pancreatitis-associated protein) and stress-response genes (e.g. heat shock protein) were upregulated in pathological hypertrophy, but not physiological hypertrophy [419]. Together, these experimental models highlight the distinct molecular phenotypes accompanied by pathological and physiological hypertrophy, which are key to our understanding of the signaling pathways involved in their development.

1.3.2 IGF1-PI3K(p110α)-Akt signaling in heart disease

Various genetic mouse model studies have highlighted the significant role of IGF-1-PI3K-Akt signaling in regulating physiological cardiac growth. IGF-1 is a 70-amino acid basic peptide which shares many of the effects of growth hormone, including the ability to induce hypertrophy in the heart and other tissues [413]. PI3K activation involves the binding of peptide growth factors, including IGF-1 to their membrane tyrosine kinase receptors, whereby the PI3K subclass IA is activated [409]. Class IA PI3Ks are made up of a p85 regulatory subunit, which functions to stabilize the p110 catalytic subunit [408]. Three isoforms of the p110 catalytic subunit (p110α, p110β and p110δ) exist in both humans and mice, encoded by three separate genes. The class IA PI3Ks have a distinct role compared with class IB PI3Ks, which are activated by GPCRs [420]. Of the Class I p110 isoforms, the p110α and the p110δ isoforms are the most abundantly expressed in the heart [408]. The serine/threonine kinase Akt (also known as protein kinase B), is a well-known target of PI3K involved in various cellular processes including cell growth and survival, metabolism and cell cycle regulation [63].
1.3.2.1 Cardioprotective IGF-1 signaling in cardiovascular disease

Due to its prominent role in inducing physiological hypertrophy, attenuating cardiac contractile dysfunction, improving cardiac output, suppressing apoptosis and prolonging cell survival of cardiomyocytes [421], IGF-1 has been regarded as a cardiac survival factor, coordinating the interplay between cardiac growth and contractile function [422]. IGF-1 transgenic mouse models have demonstrated the benefit of IGF-1 overexpression in various cardiac pathologies, including MI and dilated cardiomyopathy [423-425]. IGF-1 transgenic mice subjected to coronary ligation (to induce MI) exhibited substantially reduced wall thickness, chamber diameter and cavity volume in comparison to their Ntg controls [423]. IGF-1 overexpression also blunted the MI-induced increase in LV/BW and diastolic wall stress, and prevented the activation of cell death and the development of pathological hypertrophy. In a study by Welch and coworkers, IGF-1 transgenic mice were selectively cross-bred with a mouse model of heart failure displaying severe dilation [425]. IGF-1 overexpression protected the diseased mice against myocyte hypertrophy, adverse changes in cardiomyocyte morphology and cell death, thus almost completely preventing cavity expansion and diastolic dysfunction characteristic of the dilated heart.

1.3.2.2 Cardioprotective PI3K(\(p110\alpha\)) signaling in cardiovascular disease

The role of PI3K signaling on cardiac growth in an intact mammalian system was first examined by Shioi et al. [408], with the generation of two transgenic mouse models. Constitutively active PI3K (caPI3K) mice expressed PI3K constitutively in the heart, whilst dominant negative PI3K (dnPI3K) mice expressed a catalytically inactivate p110\(\alpha\) molecule, resulting in reduced PI3K activity in the heart. CaPI3K signaling resulted in a larger heart, associated with increase in cardiomyocyte size, but devoid of pathological changes such interstitial fibrosis, myocyte necrosis or contractile dysfunction [408]. Conversely, PI3K activity in the hearts of dnPI3K transgenic mice was reduced by 77%, which coincided with a 17% reduction in HW/BW. DnPI3K transgenic hearts were also free of histopathological damage and impaired cardiac function. A later study by McMullen et al. demonstrated that PI3K(\(p110\alpha\)) signaling was critical for physiological, but not pathological cardiac hypertrophy [407]. Aortic banding of dnPI3K transgenic mice was associated with an exaggerated disease response, comprising significant hypertrophy (HW/BW was increased by >65% compared to non-banded dnPI3K mice), a marked increase in LV systolic pressure,
contractile dysfunction and increased interstitial fibrosis [407]. In contrast, dnPI3K mice subjected to chronic swim training (a stimulus of physiological hypertrophy) exhibited a significantly blunted hypertrophic response. These observations emphasized the role of PI3K(p110α) in the induction of exercise-induced physiological, but not pressure-overload-induced pathological hypertrophy.

In subsequent studies further assessing the critical role of PI3K(p110α) signaling in the induction of physiological hypertrophy in IGF-1R mice, McMullen et al. also cross-bred IGF-1R transgenic mice with dnPI3K(p110) transgenic mice [413]. The HW/BW of the double transgenic mice was not different from the dnPI3K transgenic mice alone, and was significantly lower than in the IGF-1R transgenic animals, suggesting that PI3K(p110α) is a critical effector for regulating physiological cardiac growth downstream of the IGF-1R. In a recent study by Weeks et al., swim training effectively attenuated pathological remodeling and systolic function induced by pressure-overload [426]. This exercise-mediated protection was mimicked by increased PI3K(p110α) activity, whereby caPI3K mice, whether swim-trained or untrained, displayed similar levels of protection against pathological hypertrophy induced by aortic banding. In contrast, swim-training failed to protect the hearts of dnPI3K mice against pressure-overload-induced pathological hypertrophy, cardiac dysfunction and fibrosis [426]. Collectively, these observations suggest that PI3K(p110α) is a key regulator of exercise-induced cardiac protection and physiological hypertrophy.

The central role of PI3K(p110α) in mediating physiological cardiac growth has made it an attractive target for combating various cardiac disorders. In a study by Lin and coworkers, cardiomyocyte-specific caPI3K and dnPI3K mice were subjected to 8 weeks of MI, via occlusion of the left coronary artery [427]. dnPI3K MI mice had severely impaired systolic function, whilst caPI3K MI mice exhibited improved systolic function compared to their Ntg counterparts. A subsequent study demonstrated that PI3K(p110α)’s cardioprotective properties in a setting of MI was linked to its ability to regulate components of the Z-disc and cardiac structure [428]. Furthermore, observations that exercise-induced hypertrophy was not associated with the electrical abnormalities present in pressure-overload induced pathological hypertrophy [429] has also led to studies examining the role of PI3K(p110α) in a setting of abnormal myocardial electrical activity, such as atrial fibrillation. In both pathological hypertrophy and heart failure, elevated caPI3K expression was accompanied by the upregulation of K⁺ channel subunits, normalization of K⁺ current densities and preservation of LV function [430]. Furthermore, pathological hypertrophy (induced by transverse aortic
constriction) did not prolong ventricular action potentials or QT intervals in caPI3K mice in comparison to Ntg counterparts [431]. These observations were consistent with an earlier study looking at the relationship between reduced PI3K(p110α) activity and atrial fibrillation. DnPI3K mice were crossed with a mouse model of dilated cardiomyopathy (DCM), and subjected to morphological and functional ECG analysis [429]. DnPI3K-DCM double transgenic mice displayed atrial fibrillation associated with depressed cardiac function, enlarged atria and atrial fibrosis. Ntg-DCM mice did not develop atrial fibrillation, even with aging. In contrast, caPI3K-DCM mice exhibited reduced atrial fibrosis and enhanced cardiac conduction compared with Ntg-DCM mice [429]. Taken together, these studies demonstrate that augmented PI3K(p110α) signaling can preserve ventricular function and prevent arrhythmogenic electrical remodeling in a setting of pathological cardiomyopathy.

Diminished PI3K(p110α) signaling, in contrast, renders the heart susceptible to irregular myocardial electrical function and arrhythmic disorders. Evidence suggests that increased vulnerability to developing atrial fibrillation may be associated with a reduction in L-type Ca^{2+} current [432]. Loss of PI3K(p110α) signaling, via both genetic ablation and pharmacological inhibition, has been shown to directly reduce both inward Ca^{2+} current and voltage-dependent L-type Ca^{2+} channels, culminating in impaired cardiac contractility [433]. These studies suggest that activating the PI3K(p110α) signaling pathway may be an effective therapeutic approach to preserve normal cardiac contractility, morphology and electrical conduction in patients with heart failure, by strategies including exercise training, or by genetically increasing caPI3K levels via gene therapy [426].

**1.3.3 IGF1-PI3K(p110α)-Akt signaling in diabetic cardiomyopathy**

Due to its prominent role in inducing cardioprotective physiological hypertrophy, increased IGF-1R–PI3K(p110α)–Akt signaling has also been associated with an improvement in cardiac function in a setting of diabetes. Circulating IGF-1 levels are diminished in diabetes [434, 435]. It has been suggested that this loss of IGF-1 may contribute to the pathogenesis of diabetic cardiomyopathy [436]. IGF-1 has been shown to attenuate diabetes-mediated defects in intracellular Ca^{2+} homeostasis [153, 436-438]. The concentration-dependant inotropic effect could be inhibited by blocking IGF-1 receptors with the IR3 antibody or by blocking PI3K activity with wortmannin [438]. IGF-1 overexpression in STZ
mice also prevented abnormal excitation-contraction coupling in ventricular myocytes and restored the SERCA2a/PLB ratio, compensating for slowed Ca\(^{2+}\) clearing and lengthened duration of relaxation induced by diabetes [436].

Although growth hormone therapy may be beneficial for congestive heart failure by improving systolic function and normalizing left ventricular mass, prolonged elevation of IGF-1 levels may create unwanted side effects, as IGF-1 instigates a broad spectrum of actions [439, 440]. For example, exogenous IGF-1 administration has been linked to the development of renal hypertrophy and dysfunction, and cancer [441, 442]. To overcome these non-cardiac side effects of IGF-1, our laboratory used an IGF-1R transgenic model developed by McMullen et al. [413], where the IGF-1R is overexpressed specifically in cardiomyocytes. This provides a more direct means to examine the effect of increased IGF-1 signaling in settings of cardiac pathology. We demonstrated that IGF-1R overexpression imparted protection against diastolic dysfunction and adverse structural remodeling in a T1DM model of diabetic cardiomyopathy [51] (see Appendix 1). Ntg diabetic mice exhibited a reduced E/A ratio and prolonged deceleration time, indicative of diastolic impairment, accompanied by cardiomyocyte hypertrophy and interstitial fibrosis. Cardiac-specific IGF-1R transgenic animals were protected against diabetes-induced diastolic dysfunction and fibrosis. Although both Ntg and IGF-1R diabetic mice exhibited enlarged cardiomyocytes, the hearts of IGF-1R mice exhibited an upregulation in PI3K(p85) and phospho-Akt activity, and no increase in cardiac fibrosis compared Ntg diabetic animals. These observations were consistent with our hypothesis that the cardioprotective effects of IGF-1R overexpression are the result of physiological hypertrophy mediated by IGF-1R-PI3K(p110α)-Akt signaling.

Our lab subsequently sought to determine the impact of both increased and reduced PI3K(p110α) expression on diabetes-induced cardiomyopathy [52] (see Appendix 2). Using caPI3K and dnPI3K transgenic mice, we were able to show that increased activation of PI3K(p110α) protected the diabetic heart from pathological hypertrophy, cardiac fibrosis, apoptosis and diastolic dysfunction, associated with reduced oxidative stress and ROS suppression [52]. Conversely, the development of diabetic cardiomyopathy was exaggerated in diabetic mice with reduced PI3K(p110α) activity, where cardiac fibrosis, apoptosis and diastolic dysfunction were exacerbated compared to Ntg diabetic mice. A novel finding of the study was PI3K(p110α)’s ability to regulate \(\cdot O_2\^-\) generation in the heart, under both basal and diabetic settings. CaPI3K diabetic mice exhibited significantly less LV \(\cdot O_2\^-\) generation in comparison to both Ntg diabetic and dnPI3K diabetic mice. Interestingly, non-diabetic
dnPI3K mice also showed an upregulation in •O₂⁻ production. Furthermore, using cardiac-myoblast H9c2 cells and neonatal ventricular cardiomyocytes, A66 [a specific inhibitor of PI3K(p110α)] significantly increased •O₂⁻ production, both in the absence and presence of H₂O₂ and/or high glucose [52]. The addition of tempol (SOD mimic) reduced the levels of •O₂⁻ in both groups of cells. In the in vivo setting, •O₂⁻ generation was aggravated in both non-diabetic dnPI3K and diabetic dnPI3K mice. In contrast, the level of •O₂⁻-production was not different between non-diabetic caPI3K and non-diabetic Ntg mice; the caPI3K transgene protected the diabetic heart from increased ROS generation [52]. These in vivo and in vitro data together highlight the importance of PI3K(p110α) signaling in protecting the heart against diabetic damage, possibly through its ability to blunt hyperglycemia-induced oxidative stress.

Despite the compelling evidence in the literature supporting the importance of the IGF-1R-PI3K(p110α) pathway in preserving cardiac function under the diabetic setting, the mechanisms underlying this protection remains unclear. We have established a possible link between PI3K activity and oxidative stress; however it is not yet known whether the increase in •O₂⁻ production seen in mice with reduced PI3K(p110α) levels can be rescued by antioxidants. In neuronal cells, the antioxidant metallothionein-III inhibited the accumulation of ROS in a PI3K-dependent manner [443]. Whether this is also applicable in cardiomyocytes is unknown. Since the PI3K(p110α) pathway possesses many anti-apoptotic effects, it may prevent cell death via mechanisms which reduce oxidative stress. To investigate the mechanisms behind this anti-apoptotic property, cells were also treated with the PI3K inhibitor wortmannin. Wortmannin treatment abolished the anti-apoptotic effect of insulin, suggesting that PI3K protects cardiomyocytes by preventing oxidative-induced apoptosis. However, further studies are necessary to determine whether overexpression of PI3K is thus protective against oxidative stress-induced diabetic cardiomyopathy.

1.4 Experimental models of diabetic cardiomyopathy

Animal models are indispensable as a research tool for increasing our understanding of the underlying mechanisms implicated in diabetic cardiomyopathy. Rodent models are relatively resistant to developing atherosclerosis, thus effectively allowing for the specific study of changes in the diabetic heart independent of coronary artery disease [444]. Mouse
models are favoured in particular for the study of diabetic cardiomyopathy, due to the ease of generating transgenic models expressing specific gain-of-function and loss-of-function mutations, as well as the overexpression or deletion of specific genes to rescue or exacerbate disease. Despite the plethora of knowledge acquired from such studies, the appropriateness of using rodents to model diabetic heart disease has been debated. Different traits such as shorter cardiac cycle length and differential expression of electrophysiological and contractile protein isoforms suggests that murine cardiac physiology does not fully replicate all the facets of human cardiac physiology [445]. Despite this, there are many similar characteristics between human and rodent settings of diabetic cardiomyopathy. For instance, cardiac features of human diabetes and insulin resistance, including LV hypertrophy, diastolic dysfunction, reduced cardiac efficiency, enhanced fatty acid uptake, weakened mitochondrial energetics and impaired Ca\(^{2+}\) handling have also been observed in various rodent models of diabetes (Table 1.3). These models have facilitated numerous studies investigating the efficacy of various therapeutic agents for rescuing diabetic complications, many which have gone on to benefit diabetic patients.

Due to the similarities in cardiac phenotype, T1DM and T2DM mouse models have often been used interchangeably to assess pathophysiological mechanisms involved in diabetic cardiomyopathy. However, there are noteworthy variations between the two types of models, in particular due to their vastly dissimilar fatty acid metabolic profiles. Fatty acid-induced mitochondrial uncoupling, resulting in decreased cardiac efficiency, is a commonly observed trait in \(db/db\) mice [446, 447]. However, this trait does not seem to be present in mouse models of type 1 diabetes. Our lab has shown previously that both systolic and ventricular blood pressures tended to be reduced in type 1 diabetic mice compared to controls [51]. In contrast, T2DM animals are associated with increased systolic, diastolic and mean arterial pressures [448]. Furthermore, mitochondrial ROS production has been shown to increase in the hearts of T2DM mice; this is not always apparent in T1DM mice [447, 449]. These differences are of particular importance when evaluating the efficacy of antioxidants which function predominantly in the mitochondria (ie. coenzyme Q\(_{10}\)), and thus both T1DM and T2DM models must be examined in this setting.

Various aspects of diabetic cardiomyopathy including altered myocardial insulin signaling, oxidative stress and cardiac dysfunction have been modeled in rodents. Hsueh et al.’s review listed several criteria which needs to be satisfied when constructing a mouse model of diabetic cardiomyopathy, including evidence of LV systolic and/or diastolic
dysfunction (either by echocardiography or catheterization), increased interstitial fibrosis and LV hypertrophy [450]. Table 1.3 depicts a summary of the characteristics of various T1DM and T2DM models; some of these animal models are described in detail below. Table 1.4 summarises the advantages and disadvantages of each type of rodent model.

1.4.1 Common type 1 diabetic rodent models

1.4.1.1 Streptozotocin (STZ) model

STZ, an N-nitros derivative of glucosamine that is similar structurally to glucose, has been extensively utilized to induce insulin-dependent T1DM in experimental rodents [51, 87, 451]. The efficacy of STZ lies in its high specificity for destroying pancreatic β-islet cells following its uptake by the GLUT2 glucose transporter [452]. STZ induces alkylation of DNA within the β-cells and activates poly adenosine diphospho ribosylation, which results in pancreatic β-islet cell necrosis [452]. In mice, STZ is usually administered by i.p. or i.v. injections, either as a single large dose (e.g. 200mg/kg i.p. in mice, 50-60mg/kg i.p. in rats) or multiple small doses on consecutive days (e.g. 50mg/kg/day i.p. for 5 days). STZ-treated rodents develop hyperglycemia within 7-14 days post-injection, as well as arrested weight gain (or even weight loss) and increased serum fatty acid, triglyceride and cholesterol levels [453]. Hypoinsulinemia and glucose tolerance progressively worsens with the duration of diabetes, similar to diabetic patients. The biggest advantages of the STZ rodent model are the ease of inducing diabetes, and that it allows the study of diabetes on a numerous genetic backgrounds without the complexity of having to crossbreed mutant mouse strains with other genetic strains. The phenotype of STZ-induced diabetic rats and mice also includes cardiac dysfunction, cardiomyocyte hypertrophy and increased fibrosis, rendering the model useful for the study of diabetic cardiomyopathy in vivo [51, 53, 454]. Furthermore, as the STZ rodent model has been frequently used in studies of the diabetic heart, it is particularly beneficial for building upon and comparing data from other studies. A significant drawback of the model however is the possibility of extrapancreatic toxicity. Wold and Ren reported that cardiac contractile function may be directly impaired by STZ administration, via a p38 MAPK-dependent mechanism [290]. To further investigate this, our lab measured cardiomyocyte hypertrophy in rats treated with STZ (single dose, 55mg/kg i.v.), rats treated with STZ plus insulin (STZ single dose 55mg/kg i.v.; insulin administered for last 4 weeks of
Table 1.3 Rodent models of diabetic cardiomyopathy – advantages and disadvantages. Adapted from [455]

<table>
<thead>
<tr>
<th>Type of model</th>
<th>Advantages of the model</th>
<th>Disadvantages of the model</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chemically-induced diabetes</strong></td>
<td>Specific targeting of pancreatic β-islet cells leaves other cells in the pancreas intact</td>
<td>Possibility of extra-pancreatic toxicity; STZ may have toxic effects on the heart</td>
</tr>
<tr>
<td>e.g. STZ</td>
<td>Insulin treatment not required (in mice) due to residual insulin secretion§</td>
<td>Less stable diabetes due to possibility of spontaneous recovery from hyperglycemia; β cells may spontaneously regenerate</td>
</tr>
<tr>
<td></td>
<td>Ease of diabetes induction, relatively inexpensive and easy to maintain</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Allows study of diabetes on numerous genetic backgrounds without need to cross-breed</td>
<td></td>
</tr>
<tr>
<td><strong>Genetically derived diabetes</strong></td>
<td>Diabetes develops spontaneously due to genetic defects; animals develop a phenotype which closely resembles human diabetes</td>
<td>Due to homogeneity, monogenetic inheritability and highly inbred nature of these animals, development of diabetes is largely determined by genetics, unlike the heterogeneity observed in the human disease</td>
</tr>
<tr>
<td>(spontaneous development)</td>
<td>As the animal model is mostly inbred, genetic background is homogenous and external environmental factors can be controlled for</td>
<td>Limited availability, expensive, difficult to maintain</td>
</tr>
<tr>
<td>e.g. T1DM – Akita; T2DM – ob/ob, db/db, BB, Goto-Kakizaki, ZDF.</td>
<td>Severity of diabetes is consistent within transgenic strains, resulting in minimal variability of results</td>
<td>Animals often require insulin treatment for survival in later stages of diabetes</td>
</tr>
<tr>
<td>Diet-induced diabetes</td>
<td>Transgenic/KO genetically derived diabetes</td>
<td></td>
</tr>
<tr>
<td>------------------------</td>
<td>-------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>e.g. T2DM – high-fat fed, high-sucrose fed, high-fructose fed</td>
<td>e.g. Cardiomyocyte GLUT4 KO, OVE26</td>
<td></td>
</tr>
<tr>
<td>Clinically relevant model, as animals develop hyperglycemia and insulin resistance from chronic exposure of tissues to dietary nutrients, similar to the human disease</td>
<td>Allows assessment of the effect of a single gene overexpression or KO on development of diabetes</td>
<td></td>
</tr>
<tr>
<td>Avoids extrapancreatic toxicity induced by diabetogenic chemicals</td>
<td>Severity of diabetes is largely consistent within transgenic/KO strains, resulting in minimal variability of results</td>
<td></td>
</tr>
<tr>
<td>Relatively cheap and easy to maintain</td>
<td>Avoids extrapancreatic toxicity induced by diabetogenic chemicals</td>
<td></td>
</tr>
<tr>
<td>Animals have a long life span and thus can be used for long-term studies</td>
<td>Involves complex and expensive genetic manipulation procedures</td>
<td></td>
</tr>
<tr>
<td>Degree of hyperglycemia and obesity is varied between animals depending on % fat/sucrose of diet, and the duration</td>
<td>Use is limited to investigation of disease mechanism; may not be effective for pharmaceutical testing</td>
<td></td>
</tr>
<tr>
<td>Pronounced hyperglycemia is often not observed in high-fat fed obese animals</td>
<td>Genetic manipulation may reduce relevance of physiology with human diabetes</td>
<td></td>
</tr>
</tbody>
</table>

#Insulin treatment is required in STZ rats
Table 1.4 Cardiac phenotype of animal models of diabetes. ↓ Reduced; ↑ Elevated; ↔ No change; ND; not determined

<table>
<thead>
<tr>
<th>Models of T1DM</th>
<th>STZ</th>
<th>Akita</th>
<th>OVE26</th>
<th>Biobreeding (BB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of model</td>
<td>Chemically induced</td>
<td>Genetically derived</td>
<td>Genetically derived</td>
<td>Genetically derived</td>
</tr>
<tr>
<td>Species</td>
<td>rat/mouse</td>
<td>mouse</td>
<td>mouse</td>
<td>rat</td>
</tr>
<tr>
<td>Cardiac systolic function</td>
<td>↓/ ↔</td>
<td>↓/ ↔</td>
<td>↓/ ↔</td>
<td>↓</td>
</tr>
<tr>
<td>Cardiac diastolic function</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>LV mass: body weight</td>
<td>↔/ ↑</td>
<td>↑/ ↔</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Cardiomyocyte size</td>
<td>↑</td>
<td>ND</td>
<td>↑</td>
<td>ND</td>
</tr>
<tr>
<td>Myocardial fibrosis</td>
<td>↑</td>
<td>↔</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Systemic oxidative stress</td>
<td>↑</td>
<td>↔</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Cardiac oxidative stress</td>
<td>↑</td>
<td>↔</td>
<td>↑</td>
<td>ND</td>
</tr>
<tr>
<td>Cardiac Ca\textsuperscript{2+} handling</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Cardiac mitochondrial function</td>
<td>↓/ ↔</td>
<td>↓/ ↔</td>
<td>↓</td>
<td>ND</td>
</tr>
</tbody>
</table>

References
[45, 51, 52, 456-458] [433, 459-463] [153, 469-474]

<table>
<thead>
<tr>
<th>Models of T2DM</th>
<th>ob/ob</th>
<th>db/db</th>
<th>Zucker diabetic</th>
<th>Goto-Kakizaki</th>
<th>High fat-fed</th>
<th>High fructose</th>
<th>High sucrose</th>
<th>High milk fat-fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of model</td>
<td>Genetically derived</td>
<td>Diet induced</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>mouse</td>
<td>mouse</td>
<td>rat</td>
<td>rat</td>
<td>rat/mouse</td>
<td>rat/mouse</td>
<td>rat/mouse</td>
<td>mouse</td>
</tr>
<tr>
<td>Cardiac systolic function</td>
<td>↓/ ↔</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Cardiac diastolic function</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LV mass: body weight</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cardiomyocyte size</td>
<td>↑</td>
<td>↑</td>
<td>↔</td>
<td>↑</td>
<td>↑</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Myocardial fibrosis</td>
<td>↑</td>
<td>↔</td>
<td>ND</td>
<td>↑</td>
<td>↑</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Systemic oxidative stress</td>
<td>↑</td>
<td>↑</td>
<td>ND</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cardiac oxidative stress</td>
<td>↑</td>
<td>↑</td>
<td>ND</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>NT</td>
<td>ND</td>
</tr>
<tr>
<td>Cardiac Ca\textsuperscript{2+} handling</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>ND</td>
<td>↓</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cardiac mitochondrial function</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>ND</td>
<td>↓</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

References
[130, 446, 475-478] [54, 130, 479] [480-483] [78, 484-488] [489-493] [494-496] [126, 173, 497-499] [500]
study, 6IU/day) and citrate buffer-treated control mice. STZ administration alone induced significant hypertrophy, whilst STZ rats treated with insulin exhibited normal cardiomyocyte size [204]. These observations imply that the cardiac impairments seen in these rats were induced by diabetes as they were reversible with insulin, and were not a result of cardiotoxicity from STZ itself.

1.4.1.2 OVE26 mouse model

The OVE26 mouse model, a model of severe-onset type 1 diabetes, was first produced by Epstein et al. in 1989 in female FVB/N mice [501]. Overexpression of calmodulin, an intermediate messenger protein that mediates various actions of Ca\(^{2+}\), specifically in pancreatic β-islet cells, resulted in a phenotype characteristic of T1DM within the first few weeks of life, owing to pancreatic β-islet cell damage. Transgenic mice had significantly elevated blood glucose levels, coupled with reduced levels of serum insulin and increased levels of serum triglycerides. Hyperglycemia progressively worsened with age, and blood glucose levels remained significantly elevated for the remainder of the animal’s lifespan [502]. The OVE26 mouse model is useful for the study of diabetes-induced cardiomyopathy and nephropathy, as animals can survive up to 2 years without insulin therapy. Cardiac contractility has been explored in both OVE26 isolated cardiomyocytes and whole hearts. Impaired peak shortening, and prolonged time to peak shortening, both markers of contractile dysfunction were observed in cardiomyocytes from OVE26 hearts [310]. Contrary to this, contractile force was unchanged in OVE26 diabetic hearts when studied \textit{ex vivo} [468]. Furthermore, basal cardiac contractility was not reduced in OVE26 animals on echocardiography; indeed both ejection fraction and fractional shortening may be enhanced in OVE26 hearts compared to non-diabetic hearts under basal conditions [309].

The OVE26 model has not been as widely used as the STZ model. Relevant to this thesis however, the role of oxidative stress in the development of diabetic cardiomyopathy has been assessed in several studies using the OVE26 mouse model. Increased levels of ROS were observed in isolated OVE26 cardiomyocytes incubated in high glucose medium [310]. In addition, levels of glutathione were reduced in the hearts of OVE26 mice, whilst levels of MDA were elevated [310, 467]. The upregulation of ROS in these animals were likely to have originated from the mitochondria. Using a mitochondrial ROS detection reagent, Song \textit{et al.} demonstrated that high glucose and Ang-II treatments stimulated an increase in
mitochondrial fluorescence in OVE26 isolated cardiomyocytes, compared to control cardiomyocytes [309]. Overexpression of metallothionein or catalase protected the hearts of OVE26 transgenic mice from contractile dysfunction, mitochondrial ultrastructural abnormalities and aberrant gene expression [258, 468].

The OVE26 mouse model affords various advantages over traditional models of T1DM, such as STZ models, for the evaluation of diabetes-induced cell and tissue damage. The severity of diabetes presented by these OVE26 transgenic mice is consistent, and as suitable for long-term studies of diabetes [468]. This is in contrast to STZ models which are more useful for short-term studies, as they tend to succumb to diabetes much earlier. Importantly, potential extrapancreatic toxicity rendered by use of diabetogenic chemicals such as STZ are avoided by use of the OVE26 model [290]. Despite the obvious advantages, a major discrepancy in the development of diabetes is present between OVE26 mice and humans. Hyperglycemia and reduced insulin levels are present in OVE26 mice as early as 1 week postpartum [444]. This early onset of metabolic changes may influence the development of post-natal cardiac growth and result in myocardial adaptations which may not necessarily reflect the pathophysiology of the disease in humans. Furthermore, as the utility of OVE26 mice in the assessment of diabetic complications is relatively unexplored, it would be of value to confirm any novel observations in more commonly used diabetic models such as STZ or db/db models.

1.4.1.3 Biobreeding rat model

The Biobreeding (BB) rat develops spontaneous autoimmune diabetes, and has been frequently used as a model of T1DM since its discovery in 1974 in Ottawa from a colony of pathogen-free Wistar rats [503]. The onset of symptoms such as polydipsia, polyuria, glucosuria, ketonuria and hyperglycemia manifests at approximately 40-120 days of age, and are readily reversible with daily insulin therapy [503]. Disease progression in the BB rats closely resembles the human disease, whereby autoimmune responses against the pancreas leads to the selective abolishment of insulin-secreting β-islet cells, resulting in renal dysfunction and a reduced life expectancy [504]. This autoimmune response has since been attributed to the presence of autoantibodies to a 64kD islet cell protein [505]. Furthermore, much like the human disease, the BB rats exhibit an inherited predisposition to the disease
(possibly linked to genes in the major histocompatibility complex [506]), impaired immune response to pathogens and the requirement for daily insulin therapy [507].

Despite being one of the most favourable animal models to examine mechanisms involved in insulin-dependent diabetes, cardiomyopathy in the BB rat has not been examined in detail. Ren et al. assessed the in vitro contractile properties of cardiomyocytes isolated from BB diabetic-prone rats; myocytes exhibited reduced peak shortening and prolonged time-to-peak shortening, concomitant with a slowed Ca\textsuperscript{2+}-transient delay [153]. These observations indicate impairment in cardiac excitation-contraction coupling in BB rat cardiomyocytes. Consistent with these findings, isolated perfused working hearts from BB diabetic rats exhibited depressed LV developed pressure, LV relaxation rates and cardiac contractility compared with non-diabetic BB rats [469]. Abnormal contractile performance was also observed in isolated diabetic BB rat working hearts subjected to increased aortic afterload resistance [508]. Furthermore, heart rate, contractility and cardiac output were all depressed in BB rats undergoing reperfusion following ischemia [509]. In addition to systolic dysfunction, diabetic BB rats also exhibit diastolic dysfunction (decreased LV –dP/dt\textsubscript{min}) and increased interventricular septal thickness compared to their diabetic-resistant littermates [471].

The spontaneously diabetic BB rat model recapitulates many features of the human disease, in particular their autoimmune T cell mediated etiology [510]. It is therefore a very clinically relevant and invaluable model which allows for critical insights into mechanisms involved in the generation and release of self-reactive pancreatic \(\beta\)-cells. The development of a spontaneous diabetic phenotype in BB rats also eliminates the need for chemical induction of diabetes, thus preventing any extra-pancreatic toxicity, which may result from diabetogenic chemicals. Studies have also identified disease susceptibility genes which are present in both T1DM patients and BB rats; the identification of additional genetic risk factors or shared susceptibility genes is very possible, and may facilitate an improved understanding of autoimmune mechanisms in the pancreas [510]. Despite these obvious benefits, the BB rat model of T1DM is not without its flaws. A major difference between spontaneous development of diabetes in BB rats versus humans is that genetic risk is constant in BB rats and can be predicted. Therefore, early intervention (i.e., via lymphocyte transfusions or pancreatic islet grafts [511]) can lead to complete prevention of the disease. Due to complete or near-complete destruction of insulin-producing pancreatic \(\beta\)-islet cells, BB rats require daily intravenous insulin injections for survival. Furthermore, as the cardiac
phenotype of BB rats have not yet been comprehensively characterized, it thus may not be the most suitable animal model for investigating mechanisms for the prevention or treatment of diabetic cardiomyopathy.

1.4.1.4 Akita (Ins2^Akita) mouse model

The Akita mouse model of spontaneous T1DM was first discovered in the lab of Dr Koizuni only 15 years ago in Japan and is characterized by early age onset and an autosomal dominant mode of inheritance [512]. Their diabetic phenotype arises from a spontaneous heterozygous mutation in the Ins2^Akita gene, leading to hyperglycemia, hypoinsulinemia, polydipsia and polyuria, which typically develop around 4 weeks of age. The Ins2^Akita single base pair substitution prevents proper formation of disulfide bonds between insulin 2 chains, resulting in a mutant, misfolded form of proinsulin 2 in the pancreatic β-cell [513]. Progressive loss of pancreatic β-islet cells manifests as a consequence of Ins2^Akita expression, contributing to the development of hyperglycemia in the Akita mouse.

The cardiac phenotype of the Akita mouse has been well-characterized considering its recent discovery, and shows many similarities with the frequently used STZ-induced model of T1DM. An increase in LVEDP, tau and isovolumic relaxation time, accompanied by a reduction in LV –dP/dt_min collectively indicate diastolic dysfunction in Akita diabetic mice [459]. Importantly, these diastolic disturbances occurred alongside normal systolic function, which is often seen during the early stages of diabetic cardiomyopathy in patients [36]. Concomitant with diastolic dysfunction was a reduction in LV SERCA2a levels; no changes in cardiac fibrosis or hypertrophy were evident. Contrary to this, Hong et al. reported significant increases in ventricular thickness on echocardiography in Akita mice, accompanied by hepatic and peripheral insulin resistance [463]. Reduced cardiac contractility is also evident in Akita mice, together with decreased L-type Ca^{2+} current density and PI3K signaling [433]. The mechanisms underlying reduced cardiac function remain to be elucidated, however Bugger et al. have shown that this dysfunction is not associated with fatty acid-induced mitochondrial uncoupling, in contrast with STZ-induced T1DM rodents [460, 514].

In spite of its recent discovery, the Akita mouse is a promising model of diabetic cardiomyopathy. In comparison to the OVE26 mouse, the onset of hyperglycemia at
approximately 4-5 weeks of age parallels the juvenile development of T1DM in humans [444]. As well as displaying features typical of T1DM, including normal/reduced body weight and insulin deficiency, the Akita mouse also develop metabolic abnormalities characteristic of T2DM, such as systemic insulin resistance [460, 463]. The Akita mouse may thus be a potential model to examine the common etiology of complications present in both T1DM and T2DM. In addition to cardiac dysfunction and remodeling, Akita mice also exhibit various other diabetic complications present in the human disease, including retinopathy and neuropathy [515, 516]. Due to the spontaneous nature of the $\text{Ins}2^{\text{Akita}}$ mutation, Akita mice display a severe diabetic phenotype without the need for chemical induction. Despite these benefits, there have been conflicting reports on the cardiac phenotype of Akita mice, where Bugger et al. observed no changes in cardiac function, and a preserved inotropic response to $\text{Ca}^{2+}$-induced elevations in workload [460]. Furthermore, in contrast to the STZ and OVE26 mouse models of T1DM, oxidative stress levels were unchanged in Akita mice [460]; this indicates that there may be disparate mechanisms which underlie the development of diabetic cardiomyopathy in Akita mice. Needless to say, more studies utilizing the Akita mouse model are necessary to better validate their cardiac phenotype and redox profile.

**1.4.2 Common type 2 diabetic rodent models**

**1.4.2.1 ob/ob mouse model**

In 1978, Coleman discovered that the $\text{ob}/\text{ob}$ mouse model weighed three times as much as wild-type mice, with fat stores comprising over 80% of total body weight [517]. Linked to this morbid obesity was a deficiency in an unknown circulating factor in adipose tissue, which is responsible for decreasing food intake. More than two decades later, recessive mutations within the mouse $\text{ob}$ gene were discovered following an 8-year search by a team led by Jeffery Friedman [518]. The $\text{ob}$ gene codes for leptin, a hormone which relays signals to targets in the hypothalamus to maintain body weight and energy balance by appetite regulation. Recessive mutations in the $\text{ob}$ gene (and thus a deficiency in leptin) results in the development of diabetes, owing to long-term failure of hypothalamic appetite suppression [518]. At 4 weeks of age, $\text{ob}/\text{ob}$ mice are moderately obese, hyperglycemic, hyperinsulinemic and have abnormal glucose tolerance; this is their ‘pre-diabetes’ stage [130]. By 15 weeks of age, the $\text{ob}/\text{ob}$ mice have developed T2DM and are morbidly obese, coupled with disturbed thermoregulation, reduced physical activity and infertility [130, 519].
The increase in food consumption and reduction in energy expenditure is the direct result of leptin deficiency, leading to a self-perpetuating cycle of weight gain. The lifespan of an ob/ob animal is approximately 14 months of age [476].

The diabetic cardiac phenotype of ob/ob mice are in many ways reminiscent of the T2DM human disease. Diastolic dysfunction was observed in ob/ob mice on Doppler echocardiography, where E/A wave ratio was significantly reduced in diabetic ob/ob mice compared to non-diabetic ob/+ controls [520]. Histological analysis revealed an increase in the accumulation of lipid droplets in cardiomyocytes, however cardiac fibrosis was not pronounced. Indices of systolic function were also unchanged. Consistent with other models of T2DM, the ob/ob mouse model exhibits increased oxidative stress whereby MDA content, protein carbonyl formation and NADPH oxidase subunit expression in the heart were all elevated [521]. The Ca^{2+} regulating protein, SERCA2a exhibited reduced activity, diminished responsiveness to extracellular Ca^{2+}, prolonged intracellular Ca^{2+} decay and impaired sarcoplasmic reticulum Ca^{2+} reuptake in isolated ob/ob cardiomyocytes [521]. All these changes in Ca^{2+} handling translated to reduced peak shortening, and a prolongation in the maximal velocities of shortening and re-lengthening in the isolated myocytes. In contrast, contractile function was normal on both cardiac catheterization and in isolated working heart perfusions [130, 476].

The metabolic phenotype of obese, insulin-resistant humans and ob/ob mice share many common traits. As overt hyperglycemia and hyperinsulinemia develop by 4 weeks of age, the ob/ob model is useful for the assessment of early obesity and insulin-resistance on cardiac growth and function. Whilst the ob/ob animals remain insulin-resistant throughout their life, impaired glucose tolerance occurs exclusively during the post-weaning period of their life when rapid growth occurs [522, 523]. Furthermore, after peaking at 3-5 months of age, blood glucose gradually decreases until it almost normalizes at old age. Westman et al. observed a marked reduction in blood glucose in ob/ob mice 7 months or older, whereby blood glucose levels were similar to or lower than those of their lean ob/+ controls [522]. The ob/ob model thus is only useful for short-term studies of the acute effects of obesity and insulin-resistance. A significant flaw in using the ob/ob mice as a model of diabetic cardiomyopathy is the lack in the understanding of leptin deficiency on cardiac function. On its own, leptin has been proposed to both induce and reverse hypertrophy, increase ROS generation, and reduce cardiac contractile function both in vitro and in vivo [476, 524, 525]. Complete leptin deficiency may therefore produce confounding results due to the potential
effects which leptin may have on cardiac function and growth. Furthermore, whilst the leptin deficiency and impaired leptin activity present in *ob/ob* diabetic mice may mimic the leptin resistance observed in obese/diabetic patients with the metabolic syndrome, it is uncertain whether all organelles, in particular the heart, are resistant to leptin [444].

1.4.2.2 *db/db* mouse model

*db/db* mutations on the C57BLKS/J strain were first characterized in 1966 by the Jackson Lab as a syndrome resembling obesity and diabetes in humans [526]. The *db/db* diabetic mutant mice are indistinguishable from the leptin-deficient *ob/ob* mice in phenotype; both mice present with obesity, hyperphagia and hyperglycemia post-weaning. Unlike *ob/ob* mice however, *db/db* animals failed to respond to recombinant leptin therapy, suggesting that the defect is present at the level of receptor signaling [527]. In 1996, a point mutation (G to T) encoded by the *db* gene was identified, which due to abnormal splicing, replaced the long intracellular domain form of the mouse leptin receptor (Ob-Rb) with the short-form isoform (Ob-Ra) [528, 529]. As Ob-Rb receptors are crucial for initiating the signal transduction of leptin action in the hypothalamus, their absence results in persistent hunger, weight gain and reduced energy expenditure.

The T2DM *db/db* mouse model is well characterized, and shares similar metabolic derangements with the human disease. *db/db* mice become hyperinsulinemic at 2 weeks of age and become severely obese and hyperglycemic within 4-6 weeks of age, which progresses to T2DM by 8 weeks of age following pancreatic β-islet cell failure [530]. This sequence of disease progression closely parallels the development of T2DM in humans. Furthermore, *db/db* animals also displayed diabetic cardiomyopathy, diabetic nephropathy and ketosis after 2 months of age, which were similarly seen in patients with advanced T2DM [531]. Compared to *ob/ob* mice, *db/db* mice displayed a cardiac phenotype which more closely resembles diabetic patients, owing to the earlier onset and greater severity of hyperglycemia. On Doppler echocardiography, a reduction in E/A wave ratio, indicative of diastolic dysfunction, was observed in *db/db* diabetic mice compared to *db/+* non-diabetic controls [54]. Markers of contractile disturbance, such as reduced fractional shortening and diminished velocity of circumferential shortening were also observed on echocardiography. Langendorff®-perfused *db/db* hearts exhibited reduced LV +dp/dt\(_{\text{max}}\) and depressed LV developed pressure [172]. Apart from cardiac dysfunction, the hearts of *db/db* mice are also
characterized by adverse structural remodeling. Pathological cardiac hypertrophy, a common feature of diabetic cardiomyopathy, was detected on cardiac MRI in \(db/db\) animals as an increase in LV mass and wall thickness [31]. \(db/db\) mice also exhibited impaired substrate metabolism (e.g., decreased rates of glucose oxidation), enhanced fatty acid oxidation and mitochondrial uncoupling [450, 532], making it a particularly useful model to investigate mitochondrial dysfunction and oxidative stress in the diabetic heart.

Despite the extensive use of \(db/db\) mice in the study of various diabetes-related pathologies, it also has limitations. Like the \(ob/ob\) model, a shortcoming of the \(db/db\) spontaneous mouse model is the lack of understanding of the consequences involved in complete leptin deficiency directly on cardiac function and growth. A defect in the leptin receptor subjects \(db/db\) mice to severe lipotoxicity, more excessive than observed in human diabetic patients, and leptin defects are not the primary cause of T2DM in humans. Despite these limitations, the \(db/db\) mouse model is considered one of the more favourable models for the evaluation of potential mechanisms underlying diabetic heart disease, and maybe a useful model for the identification of potential cardiomyopathy-reducing therapeutic agents [444].

1.4.2.3 Zucker Fatty Rat and Zucker Diabetic Fatty Rat

The Zucker fatty rat is a spontaneous genetic model of obesity discovered by Drs Lois and Theodore Zucker in 1961, whereby a homozygous missense mutation in the \(fa\) gene encoding the rat leptin receptor results in the dysregulation of hypothalamic appetite suppression and hyperphagia [533]. Homozygous recessive (\(fa/\)) rats recapitulate many of the features observed in T2DM, including morbid obesity, insulin resistance, hyperlipidemia and impaired glucose tolerance [534]. Hyperglycemia is not always evident however [533], most likely due to sustained compensatory hyperinsulinemia, thus limiting the use of the Zucker fatty rat model to obesity or pre-diabetes. Selective breeding of Zucker fatty rats with high glucose gave rise to an inbred diabetic strain of Zucker rats, known as the Zucker diabetic fatty rat (ZDF rat) [535]. The ZDF rat is characterized by hyperinsulinemia and euglycemia at approximately 4 weeks of age, which progresses to a hyperglycemic insulin-deficient state by ~12 weeks of age [535]. An overt hyperglycemic profile develops only at 8 weeks of age, despite the onset of severe peripheral insulin resistance post-weaning. Like the Zucker fatty rat, the ZDF rats present with overt obesity, hyperphagia, elevated cholesterol and fatty acid levels, polyuria and polydipsia.
The cardiovascular changes in the ZDF rats have been well characterized. The ZDF rat exhibited a marked enlargement of cardiomyocyte width (correlated with serum glucose and HbA1c levels), a moderate increase in LV posterior wall thickness and an elevation in ANP gene expression compared with Zucker lean rat controls [536]. Accompanied by these markers of cardiac hypertrophy was an increase in pericellular laminitic thickness and perivascular fibrosis. Contrary to other models of T2DM however, no difference in myocardial interstitial fibrosis was evident between ZDF and Zucker lean rats [536]. Assessment of systolic function has yielded more consistent results. Fractional shortening was reduced in ZDF rats [537-539] which may be associated with myocardial triacylglycerol–induced lipotoxicity [537]. Several studies have shown that diastolic dysfunction tends not to manifest early in diabetes in this model (unlike in the human disease), however progressively worsens over the longer-term. Daniels et al. reported that ZDF rats exhibited mildly elevated LVEDP and relaxation time constant, tau, at 45 weeks of age, which was accompanied by a significant accumulation of interstitial collagen [482]. Furthermore, another study showed that end diastolic volume increased from 16% at 16 weeks of age to 37% by 36 weeks of age [481]. LVEDP and end-systolic volume were also markedly elevated. The development of cardiac dysfunction in the ZDF rat heart was associated with a reduction in insulin-mediated glucose utilization [538].

One of the major advantages of the ZDF rat model of T2DM is the close resemblance of its disease progression, from the hyperinsulinemic-euglycemic state to the hyperglycemic insulin-deficient state, with the human disease. The gradual step-wise change in diabetic phenotype over the first 12 weeks of life in the ZDF mice allows assessment of the alterations in their metabolic profile which contribute to the onset of hyperglycemia [535]. Like the leptin-resistant db/db mice, the ZDF rats are thus a clinically-relevant animal model of T2DM, sharing many metabolic and cardiomyopathic traits.

1.4.2.4 Diet-induced diabetic models

The past few years have seen an increasing number of studies using specially formulated diets to induce obesity and diabetes in animals, in an attempt to overcome potential confounding problems with using leptin-deficient or resistant spontaneously diabetic models, or diabetogenic agents, for the investigation of diabetic cardiomyopathy. The high-fat diet-fed C57BL/6J mouse model of diabetes was first introduced in 1988 [540],
and has been since used to evaluate mechanisms involved in the development of impaired glucose tolerance and type 2 diabetes [541]. The high-fat fed mouse is characterized by increased weight gain, stable hyperglycemia and gradual worsening of insulin resistance (as indicated by progressively increased hyperinsulinemia). Following 1 week on a high-fat diet (58% energy by fat), the mice exhibited significantly elevated glucose and insulin levels, accompanied by reduced glucose clearance and defective insulin secretion [542]. At 2 weeks after the commencement of a high fat diet, the animals exhibited changes in myocardial substrate utilization which lead to the development of obesity and insulin resistance, including reduced basal rates of glycolysis and glucose oxidation and suppressed levels of insulin-stimulated glucose uptake in cardiomyocytes [543]. The high-fat fed mouse model has also been used as a model of diabetic cardiomyopathy. Ouwens et al. demonstrated that high-fat diet (25% fat) induced cardiac dysfunction (systolic LV dilation, decreased fractional shortening and ejection fraction), cardiac hypertrophy and increased accumulation of triglycerides, accompanied by myocardial insulin resistance [544, 545].

High-sucrose diets are also increasingly being used to induce severe T2DM in rodent models. A high-sucrose diet induced whole body insulin resistance, hyperglycemia and hyperlipidemia in rats [173]. These metabolic changes were sufficient to induce a pathological cardiac phenotype which closely resembles diabetic cardiomyopathy in humans; sucrose-fed rats showed early abnormalities in diastolic function, as demonstrated by reduced E/A wave ratios on echocardiography [173]. Ejection fraction and fractional shortening were also significantly depressed, in addition to decreased uptake of Ca\textsuperscript{2+} in the hearts of 10 week-fed sucrose rats.

Diet-induced diabetic rodents are an important and clinically-relevant model of type 2 diabetes. High fat or high-sucrose-fed animals develop hyperglycemia and insulin resistance in much the same way as a majority of the human diabetic population; from chronic exposure of tissues to increased dietary nutrients and increased accumulation of harmful lipid-derived metabolites [494, 542, 546]. Furthermore, this model does not rely on monogenic mutations or toxic chemicals which destroy pancreatic β-islet cells to induce diabetes, and therefore avoids any confounding, non-specific influences of diabetogenic chemicals on cardiac function. Despite the obvious benefits, the degree of hyperglycemia and insulin resistance in these diet-induced diabetic rodents are not as severe as seen in leptin-resistant or deficient mice, and resembles a pre-diabetic or early diabetic phenotype. Furthermore, short-term high-fat diets are not sufficient to impair cardiac function; one study showed that cardiac
dysfunction had not developed in C57BL/6 mice fed a high-fat diet until after 20 weeks [547]. Another disadvantage of the model is the length of time required to maintain the animals on the high fat/sucrose diet in order to obtain a suitably diabetic phenotype. In addition, due to the variable effect of different diets on different strains of mice, a general consensus on what the most suitable fat/sucrose composition and duration of diet-feeding still needs to be established. Nevertheless, the diet-induced diabetic rodent model is a highly relevant model of type 2 diabetes, which accurately replicates the metabolic and cardiovascular symptoms observed in human patients.

1.4.2.5 KO mouse models of insulin resistance

Several KO models have also been developed to study defective insulin signaling in T2DM. An insulin-resistant mouse model can be achieved by the deletion of the gene for the insulin-sensitive GLUT4 transporter. The phenotype of the cardiac-specific GLUT4 KO model includes cardiac hypertrophy, increased cardiac fibrosis and decreased cardiac glucose utilization [68, 548]. These mice are hyperinsulinemic, and most go on to develop hypertension and diabetes. McQueen et al. also created a mouse model with a cardiomyocyte-restricted deletion of the insulin receptor (CIRKO) to evaluate the role of insulin signaling in the development of cardiovascular disease [234]. CIRKO mice are characterized by abnormalities in cardiac contractility and relaxation, as well as interstitial fibrosis and cardiomyocyte apoptosis in response to catecholamine-mediated myocardial injury [234]. Whilst these models are not true animal models of T2DM, they allow researchers to obtain important knowledge on insulin signaling pathways and the impact of glucose transporter deletions on cardiac function and structure.

1.4.3 Summary of rodent models of diabetic cardiomyopathy

The discovery, development and characterization of various rodent models of T1DM and T2DM have greatly advanced the knowledge of molecular mechanisms responsible for diabetic cardiomyopathy in humans. The plethora of diabetic rodent models available has allowed researchers to choose the model with characteristics which are most relevant for the diabetic complication under investigation. The STZ model of T1DM is currently the most commonly used model for diabetic cardiomyopathy, due to the ease of diabetes induction,
and the consistency of the cardiac phenotype. The db/db mouse model has been frequently used to examine the adverse changes evident in the T2DM heart, and possesses cardiac characteristics which closely resemble those seen in patients. The cardiac phenotype of both these diabetic rodent models has been well-characterized, and are thus extremely useful for the assessment of novel therapeutic strategies which can protect the heart against diabetes-induced cardiovascular complications.
1.5 Summary

The mechanisms potentiating the development and progression of diabetic cardiomyopathy remain unclear. Numerous clinical and experimental studies have indicated the role of increased oxidative stress in the pathogenesis of cardiac dysfunction and structural remodeling in the diabetic heart. Selected antioxidants have proven effective in protecting the heart in rodent models of diabetic cardiomyopathy but these have not translated to use in humans due to conflicting results. The efficacy of the lipophilic antioxidant coenzyme Q$_{10}$ in treating diabetic heart disease has not yet been established. This may represent a superior antioxidant choice to those previously used in humans due to its ability to also improve cardiac bioenergetics, which may translate to improved cardiac function. The efficacy of antioxidants such as coenzyme Q$_{10}$ compared to ACE-I, the gold-standard therapy for the treatment of cardiovascular complications in diabetic patients, is also unresolved. Mechanistic studies to elucidate the key effectors of ROS-induced cardiac changes would prove useful towards understanding how ROS upregulation affects, or is affected by various molecular signaling pathways. The well-characterized T1DM STZ mouse and T2DM db/db mouse models are thus utilized in this thesis to determine whether coenzyme Q$_{10}$ can attenuate diabetes-induced cardiac dysfunction and structural remodeling. Given that cardiac complications are a major contributor to morbidity and mortality in both T1DM and T2DM patients, studies such as these may identify possible new therapeutic approaches for managing diabetic cardiomyopathy.
1.6 Aims

Our lab and others have previously demonstrated that antioxidant administration may be protective against cardiac dysfunction and morphological abnormalities, including cardiomyocyte hypertrophy and cardiac fibrosis, in diabetic animal models. To date, the role of coenzyme Q₁₀ in protecting the diabetic heart against damage has not yet been sought. Thus, the specific aims of this study are:

- To investigate the role of ROS-induced damage in the development of cardiac dysfunction and adverse cardiac remodeling in a db/db spontaneous mouse model of T2DM, using the antioxidant coenzyme Q₁₀.
- To investigate the role of ROS-induced damage in the development of cardiac dysfunction and adverse cardiac remodeling in a STZ mouse model of T1DM using the antioxidant, coenzyme Q₁₀.
- To compare the efficacy of coenzyme Q₁₀ with the ACE-I, ramipril (conventional pharmacotherapy).
- To elucidate whether there is an association between downregulation of PI3K(p110α) signaling and enhanced ROS production in a setting of T1DM and thus determine if antioxidant administration can improve cardiac function and structure in diabetic mice with diminished PI3K(p110α) signaling.
CHAPTER TWO

Coenzyme Q\textsubscript{10} attenuates diastolic dysfunction, cardiomyocyte hypertrophy and cardiac fibrosis in the \textit{db/db} mouse model of type 2 diabetes
Declaration for thesis chapter 2

Declaration by candidate

In the case of Chapter Two, the nature and extent of my contribution to the work was the following:

<table>
<thead>
<tr>
<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Performed 80% of the experiments, and 100% of the data and statistical analysis.</td>
<td>80%</td>
</tr>
<tr>
<td>Wrote the manuscript with the assistance of co-authors</td>
<td></td>
</tr>
</tbody>
</table>

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

<table>
<thead>
<tr>
<th>Name</th>
<th>Nature of contribution</th>
<th>Extent of contribution (%) for student co-authors only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helen Kiriazis</td>
<td>Performed 10% of experiments (echocardiography and catheterization) and provided intellectual input</td>
<td>N/A</td>
</tr>
<tr>
<td>Xiao-Jun Du</td>
<td>Provided intellectual input and editing of manuscript</td>
<td>N/A</td>
</tr>
<tr>
<td>Jane Love</td>
<td>Performed 10% of experiments</td>
<td>N/A</td>
</tr>
<tr>
<td>Karin Jandeleit-Dahm</td>
<td>Provided intellectual input and editing of manuscript</td>
<td>N/A</td>
</tr>
<tr>
<td>Josephine Forbes</td>
<td>Assisted with study protocol, provided intellectual input</td>
<td>N/A</td>
</tr>
<tr>
<td>Julie McMullen</td>
<td>Provided intellectual input and editing of manuscript</td>
<td>N/A</td>
</tr>
<tr>
<td>Rebecca Ritchie</td>
<td>Provided intellectual input and editing of manuscript</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Candidate’s Signature

Date 11/04/13

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate’s contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

(4) there are no other authors of the publication according to these criteria;

(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

<table>
<thead>
<tr>
<th>Location(s)</th>
<th>Heart Failure Pharmacology Laboratory, BakerIDI Heart and Diabetes Institute, Melbourne, Victoria, Australia</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Signature</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helen Kiriazis</td>
<td></td>
<td>15/2/2013</td>
</tr>
<tr>
<td>Xiao-Jun Du</td>
<td></td>
<td>15/2/2013</td>
</tr>
<tr>
<td>Jane Love</td>
<td></td>
<td>5/11/13</td>
</tr>
<tr>
<td>Karin Jandeleit-Dahm</td>
<td></td>
<td>15/12/13</td>
</tr>
<tr>
<td>Josephine Forbes</td>
<td></td>
<td>5/11/13</td>
</tr>
<tr>
<td>Julie McMullen</td>
<td></td>
<td>15/12/13</td>
</tr>
<tr>
<td>Rebecca Ritchie</td>
<td></td>
<td>5/11/13</td>
</tr>
</tbody>
</table>

Steve Jane signing in lieu of Jane Love and Josephine Forbes.

**Professor Stephen M. Jane**

MBBS, PhD, FRACP, FFPRHA

Head, Central Clinical School, Monash University

Director of Research, The Alfred

Faculty of Medicine, Nursing & Health Sciences

The Alfred Centre, 90 Commercial Rd.

Melbourne, Victoria 3004, Australia
Chapter 2: Coenzyme Q\textsubscript{10} attenuates diastolic dysfunction, cardiomyocyte hypertrophy and cardiac fibrosis in the \textit{db/db} mouse model of type 2 diabetes

The development and progression of diabetic cardiac complications has been frequently attributed to an increase in oxidative stress. Previous studies utilizing antioxidant therapy have shown promising results in protecting the diabetic heart against damage [68, 275, 307]. To date however, the efficacy of coenzyme Q\textsubscript{10} in reducing cardiac dysfunction and structural remodeling is unclear. Therefore, the aim of this chapter is to examine the adverse cardiac changes mediated by hyperglycemia-induced ROS overproduction, and to determine whether coenzyme Q\textsubscript{10} is able to attenuate the functional and structural damage induced by type 2 diabetes. Given that diabetic patients exhibit lower levels of coenzyme Q\textsubscript{10} [338], I hypothesize that exogenous coenzyme Q\textsubscript{10} supplementation will reduce cardiac $\cdot$O\textsubscript{2}- production and lipid peroxidation, thereby preventing ROS-induced diastolic dysfunction, cardiomyocyte hypertrophy, cardiac fibrosis and apoptosis. This chapter forms a manuscript accepted for publication in January 2012 by \textit{Diabetologia}, titled “Coenzyme Q\textsubscript{10} attenuates diastolic dysfunction, cardiomyocyte hypertrophy and cardiac fibrosis in the \textit{db/db} mouse model of type 2 diabetes”.

Coenzyme Q₁₀ attenuates diastolic dysfunction, cardiomyocyte hypertrophy and cardiac fibrosis in the db/db mouse model of type 2 diabetes

K. Huynh · H. Kiriazis · X.-J. Du · J. E. Love · K. A. Jandeleit-Dahm · J. M. Forbes · J. R. McMullen · R. H. Ritchie

Received: 12 October 2011 / Accepted: 16 January 2012 / Published online: 29 February 2012 © Springer-Verlag 2012

Abstract

Aims/hypothesis An increase in the production of reactive oxygen species is commonly thought to contribute to the development of diabetic cardiomyopathy. This study aimed to assess whether administration of the antioxidant coenzyme Q₁₀ would protect the diabetic heart against dysfunction and remodelling, using the db/db mouse model of type 2 diabetes. Furthermore, we aimed to compare the efficacy of coenzyme Q₁₀ to that of the ACE inhibitor ramipril.

Methods Six-week-old non-diabetic db/+ mice and diabetic db/db mice received either normal drinking water or water supplemented with coenzyme Q₁₀ for 10 weeks. Endpoint cardiac function was assessed by echocardiography and catheterisation. Ventricular tissue was collected for histology, gene expression and protein analysis.

Results Untreated db/db diabetic mice exhibited hyperglycaemia, accompanied by diastolic dysfunction and adverse structural remodelling, including cardiomyocyte hypertrophy, myocardial fibrosis and increased apoptosis. Systemic lipid peroxidation and myocardial superoxide generation were also elevated in db/db mice. Coenzyme Q₁₀ and ramipril treatment reduced superoxide generation, ameliorated diastolic dysfunction and reduced cardiomyocyte hypertrophy and fibrosis in db/db mice. Phosphorylation of Akt, although depressed in untreated db/db mice, was restored with coenzyme Q₁₀ administration. We postulate that preservation of cardioprotective Akt signalling may be a mechanism by which coenzyme Q₁₀-treated db/db mice are protected from pathological cardiac hypertrophy.

Conclusions/interpretation These data demonstrate that coenzyme Q₁₀ attenuates oxidative stress and left ventricular diastolic dysfunction and remodelling in the diabetic heart. Addition of coenzyme Q₁₀ to the current therapy used in diabetic patients with diastolic dysfunction warrants further investigation.

Keywords Antioxidant · Cardiomyopathy · Diabetes · Remodelling · Superoxide

Abbreviations

ACE-I ACE inhibitor
AMREP Alfred medical research and education precinct
DBP Diastolic blood pressure
E:A Ratio of peak early to late transmitral blood flow velocities
FS Fractional shortening
H&E Haematoxylin and eosin
HW Heart weight
IGF1R IGF1 receptor
LV Left ventricular
LVEDD LV end-diastolic dimension
LVEDP LV end-diastolic pressure
LVESD   LV end-systolic dimension  
MDA   Malondialdehyde  
PI3K(p110α)   Phosphoinositide-3 kinase p110α isoform  
ROS   Reactive oxygen species  
SBP   Systolic blood pressure  
SERCA2a   Sarcoplasmic reticulum Ca2+-ATPase  
TL   Tibial length  

Introduction

The global incidence of diabetes mellitus has dramatically increased throughout the past two decades, firmly establishing the condition as one of the most significant health burdens of the 21st century. By the year 2025, 300 million individuals are projected to have diabetes, with the majority of these suffering from type 2 diabetes [1].

Coronary heart disease and peripheral vascular diseases are among the most common causes of mortality in diabetic individuals; however, a specific cardiomyopathy independent of these complications is also evident [2, 3]. Diabetic cardiomyopathy is typically characterised by left ventricular (LV) dysfunction, with diastolic dysfunction manifesting early in the disease [4]. Structural abnormalities such as LV hypertrophy and increased cardiac fibrosis occur together with the onset of diastolic dysfunction in the diabetic heart [5]. The presence of these characteristics acts as a predictive indicator of mortality in diabetic patients, necessitating the development of new pharmacological targets to treat LV dysfunction and remodelling in the diabetic heart.

Despite the importance of diabetic cardiomyopathy as a clinical entity, the molecular mechanisms potentiating the adverse changes in myocardial function and structure are poorly understood. The development and progression of diabetic complications is frequently attributed to an increase in the generation of reactive oxygen species (ROS), in particular superoxide, within susceptible tissues [6, 7]. The balance between the generation and elimination of ROS via antioxidant degradation is critical in maintaining cardiovascular health. As hyperglycaemia impairs the endogenous antioxidant defence system [8], numerous studies have examined the benefits of antioxidant supplementation in the setting of diabetes [9–11].

Coenzyme Q10, an endogenous component of the mitochondrial respiratory chain, functions as an antioxidant in its reduced form, ubiquinol-10. Coenzyme Q10 has previously been shown to inhibit cardiac oxidative stress [12, 13]. A protective role for coenzyme Q10 has been suggested in settings of heart failure outside diabetes [14–16]. However, the potential of coenzyme Q10 to attenuate the structural and functional defects specifically caused by diabetic cardiomyopathy still remains to be elucidated.

The aim of the present study was to assess the efficacy of the antioxidant coenzyme Q10 in protecting the diabetic heart against adverse changes using the db/db mouse model of type 2 diabetes. We hypothesised that the administration of coenzyme Q10 would protect against the impaired LV function and adverse structural changes induced by type 2 diabetes. As ACE inhibitors (ACE-Is) are the current gold standard in minimising cardiovascular risk and hypertension in clinical settings, we also aimed to evaluate the efficacy of coenzyme Q10 compared with the ACE-I ramipril in treating diabetic cardiomyopathy.

Methods

Animal model All animal research was conducted in accordance with the National Health and Medical Research Council of Australia guidelines, and was approved by the Alfred Medical Research and Education Precinct (AMREP) Animal Ethics committee.

Female C57BL/6 db/+ and db/db mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). The mice were housed in the AMREP Precinct Animal Centre and maintained under a 12 h light–dark cycle. Age-matched female non-diabetic db/+ mice and diabetic db/db mice received a standard diet and normal drinking water. At 6 weeks of age, the db/+ and db/db mice were allocated either to continue receiving regular water, to receive water supplemented with coenzyme Q10 (10 mg/kg; LiQsorb Liposomal CoQ10, a kind gift from R. K. Chopra, Tishcon Corp, NY, USA, using a dose previously shown to limit the renal complications of diabetes [17]) or to receive water supplemented with ramipril (3 mg kg−1 day−1; Sigma Aldrich, St Louis, MO, USA). The mice were followed for a further 10 weeks prior to killing and tissue collection.

Blood was collected at the endpoint for assessment of plasma glucose (Austin Pathology Service, Heidelberg, VIC, Australia). Animals with plasma glucose levels exceeding 28 mmol/l were considered diabetic. Blood was also retained for measurement of HbA1c by HPLC (CLC330 GHb Analyser; Primus, Kansas City, MO, USA) [18].

Analysis of LV function in vivo Endpoint LV function and chamber dimensions were assessed via two-dimensional targeted M-mode echocardiography and Doppler echocardiography. At 16 weeks of age, mice were anaesthetised with a cocktail of ketamine, xylazine and atropine (100, 10 and 1.2 mg/kg i.p.), and echocardiography was performed using a Philips (North Ryde, NSW, Australia) iE33 ultrasound machine with a 15 MHz linear array transducer.

Variables measured on M-mode echocardiography included LV end-systolic dimension (LVESD), LV end-diastolic dimension (LVEDD), LV mass (calculated as...
[external LV dimension $^{−3}$–LVEDD]×1.055] and fractional shortening (FS, calculated as [(LVEDD−LVEDD)/LVEDD]×100%). Doppler echocardiography allowed assessment of LV diastolic filling (LV E:A – the ratio of peak early, E, and late, A, transmitral blood flow velocities) and deceleration time of early (E) velocity [19].

LV pressure was also measured by cardiac catheterisation. A micromanometer-tipped catheter (1.4 F; Millar Instrument Co., TX, USA) was inserted through the right common carotid artery into the LV and the aorta of an anaesthetised mouse (ketamine 100 mg/kg, xylazine 10 mg/kg, atropine 1.2 mg/kg i.p.) as previously described [19]. Variables derived from catheterisation included systolic blood pressure (SBP) and LV end-diastolic pressure (LVEDP).

Tissue collection and histology Following catheterisation, with the animal still anaesthetised, cardiac puncture was performed to collect approximately 1 ml of blood in a heparinised syringe. The heart and lungs were then excised from the animals and their wet weights recorded. The tibia was removed and used to calculate the ratio of heart weight to tibial length (HW:TL). A portion of the ventricle was cut, fixed and embedded in paraffin for histology. Paraffin-embedded sections were cut and stained with haematoxylin and eosin (H&E) or 0.1% Picosirius Red and analysed as previously described [19]. The remaining portion of the ventricle was snap-frozen and stored at ~80°C.

Levels of apoptosis were assessed in paraffin-embedded ventricular sections using the CardioTACS In Situ Apoptosis Detection Kit ( Trevigen, Gaithersburg, MD, USA) [20]. This method detects nuclear DNA fragmentation by using a terminal deoxynucleotidyl transferase enzyme, which incorporates labelled nucleotides onto the free 3’ OH ends of DNA fragments. Positively stained apoptotic cells were distinguished by blue staining, while negatively stained cells were counterstained red with Nuclear Fast Red. Apoptotic cells were quantified as a percentage of non-apoptotic cells, and the results were expressed as fold levels detected in untreated db/db mouse heart.

Gene expression and protein analysis RNA and protein were extracted from frozen tissue samples as previously described [10]. cDNA was produced from DNase-treated RNA via reverse transcription (Taqman Reverse Transcription reagents; Applied Biosystems, Mulgrave, VIC, Australia) at template concentrations of 20 ng/μl, as previously published [10, 19, 21]. SYBR Green chemistry (Applied Biosystems) was employed to determine expression of both β-myosin heavy chain (Myh7) and sarco/endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA2a, also known as Atp2a2), using the Applied Biosystems ABI Prism 7700 Sequencing Detection System. Ribosomal 18S (also known as Rn18S) was used as the endogenous control. All primers were generated using murine-specific sequences derived from Genebank, at predetermined concentrations. The relative fold increases in the expression of the gene of interest compared with untreated non-diabetic mice were calculated using the comparative delta-delta Ct ($\Delta\Delta$Ct) method [10, 22].

Analysis of protein levels SDS-PAGE and western blotting were employed to measure the protein levels of phosphorylated Akt (Ser473) and total Akt. Both antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA), and used at a 1:1,000 dilution, according to manufacturer’s instructions. Akt protein bands were detected at 60 kDa, and results were analysed using the Image J 1.4 software (NIH, Bethesda, MD, USA) [19].

Detection of lipid peroxidation and superoxide generation The levels of lipid peroxidation in plasma samples were determined using the Malondialdehyde-586 kit (Bioxbyte MDA-586, Foster City, CA, USA), as per the manufacturer’s instructions. Superoxide generation was quantified using lucigenin (5 μmol/l)-enhanced chemiluminescence in fresh heart tissue, as previously described [10]. Results were normalised against tissue weight, and expressed as relative light units per second per milligram.

Statistical analysis All data are presented as means±SE (unless otherwise specified). Two-way ANOVAs were performed to identify differences between genotypes (db/+ and db/db) and treatments (untreated, coenzyme Q₁₀-treated, ramipril-treated). Interactions between groups were statistically analysed using the Student’s Newman–Keuls post hoc test. A value of $p<0.05$ was considered significant.

Results

Hyperglycaemia is evident in db/db mice untreated, coenzyme Q₁₀-treated and ramipril-treated db/db mice all exhibited marked hyperglycaemia compared with relative db/+ controls in terms of both plasma glucose and HbA₁c (Table 1). HbA₁c levels were modestly but significantly reduced in coenzyme Q₁₀-treated db/db mice vs untreated db/db mice. Body weight was greater in db/db diabetic animals compared with db/+ animals; neither coenzyme Q₁₀ nor ramipril treatment noticeably affected body weight in either db/+ or db/db mice compared with untreated littermates (Table 1). Lung weight was no different between db/+ and db/db animals (Table 1) and was unaffected by treatment. HW data are discussed below.

Coenzyme Q₁₀ preserves diastolic function Analysis of endpoint cardiac function via M-mode echocardiography
Cardiac fibrosis is reduced in coenzyme Q10-treated diabetic mice. On Sirius Red-stained sections, diabetes induced a 1.8-fold increase in collagen deposition in untreated db/db mice relative to non-diabetic db/+ mice (Fig. 2). Comparatively, no differences in collagen deposition were observed between the db/db and db/+ genotypes, in both coenzyme Q10 and ramipril-treated animals (Fig. 2). Diabetes-induced increases in collagen deposition were completely ameliorated by coenzyme Q10 and ramipril treatment.

Coenzyme Q10 limits diabetes-induced cardiomyocyte hypertrophy. Untreated db/db mice had significantly greater HW than db/+ controls, when normalised to TL (Table 1). Despite HW:TL being greater in ramipril-treated db/db mice than db/+ controls, ramipril-treated db/db mice had comparatively smaller hearts than untreated db/db mice; a similar trend was evident in coenzyme Q10-treated db/db mice, but this was not significant (p=0.1). On M-mode echocardiography, LV dimensions and function by M-mode and Doppler echocardiography and cardiac catheterisation in anaesthetised mice

Table 2

<table>
<thead>
<tr>
<th>Variable</th>
<th>db/+ untreated</th>
<th>db/db untreated</th>
<th>db/+ + CoQ</th>
<th>db/db + CoQ</th>
<th>db/+ + ramipril</th>
<th>db/db + ramipril</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (bpm)</td>
<td>38±3.1</td>
<td>364±3.1</td>
<td>379±1.1</td>
<td>370±1.2</td>
<td>38±1.8</td>
<td>360±1.4</td>
</tr>
<tr>
<td>External LV dimension (mm)</td>
<td>4.93±0.05</td>
<td>6.12±0.09</td>
<td>4.93±0.05</td>
<td>5.28±0.08</td>
<td>4.91±0.05</td>
<td>5.24±0.11</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.41±0.07</td>
<td>3.84±0.09</td>
<td>3.27±0.06</td>
<td>3.63±0.10</td>
<td>3.50±0.06</td>
<td>3.72±0.14</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.01±0.12</td>
<td>2.04±0.12</td>
<td>1.92±0.11</td>
<td>2.10±0.06</td>
<td>2.06±0.05</td>
<td>2.03±0.08</td>
</tr>
<tr>
<td>LV mass:TL (mg/mm)</td>
<td>5.16±0.15</td>
<td>7.77±0.40</td>
<td>5.57±0.14</td>
<td>6.75±0.28</td>
<td>5.05±0.20</td>
<td>6.31±0.34</td>
</tr>
<tr>
<td>LVPW (mm)</td>
<td>0.76±0.03</td>
<td>0.80±0.05</td>
<td>0.77±0.02</td>
<td>0.84±0.03</td>
<td>0.71±0.03</td>
<td>0.82±0.09</td>
</tr>
<tr>
<td>FS (%)</td>
<td>40±3</td>
<td>45±2</td>
<td>43±2</td>
<td>44±2</td>
<td>42±1</td>
<td>44±2</td>
</tr>
<tr>
<td>E:A ratio</td>
<td>1.73±0.08</td>
<td>1.48±0.05</td>
<td>1.74±0.09</td>
<td>1.65±0.08</td>
<td>1.85±0.09</td>
<td>1.75±0.09</td>
</tr>
</tbody>
</table>

*p<0.05 vs db/+ untreated, †p<0.05 vs db/db CoQ, ‡p<0.05 vs db/+ ramipril, §p<0.05 vs db/db untreated, §p<0.05 vs db/db CoQ

CoQ coenzyme Q; HW:TL (mg/mm) heart weight:total length; LV left ventricular posterior wall thickness
mass normalised to TL was reduced in both coenzyme Q\textsubscript{10}-treated and ramipril-treated \textit{db/db} mice compared with untreated \textit{db/db} controls (Table 2).

Untreated \textit{db/db} mice exhibited significantly hypertrophied cardiomyocytes on H&E-stained sections (Fig. 3a and b), accompanied by elevated gene expression of the hypertrophic marker \textit{Myh7} (Fig. 3c) compared with untreated \textit{db/+} mice. Although coenzyme Q\textsubscript{10}-treated \textit{db/db} mice also displayed increased cardiomyocyte size and \textit{Myh7} expression compared with \textit{db/+} mice, these parameters were significantly lower than in untreated \textit{db/db} mice. Ramipril-treated \textit{db/db} mice were similarly protected from diabetes-induced increases in cardiomyocyte width and \textit{Myh7} expression.

The cell survival kinase Akt, a downstream target of the IGF1-receptor-phosphoinositide-3 kinase p110\textsubscript{x} isoform (IGF1R-P13K[p110\textsubscript{x}]) pathway, is an important mediator of physiological heart growth [23]. In the present study, Akt phosphorylation showed a strong trend to decrease in untreated diabetic mice vs. untreated control mice (\(p=0.05\); Fig. 3d). Both coenzyme Q\textsubscript{10} and ramipril restored Akt phosphorylation back to levels observed in the non-diabetic mice (Fig. 3d).

Coenzyme Q\textsubscript{10} treatment prevents diabetes-induced cardiomyocyte death Diabetes induced a 3.9±0.4-fold increase in the proportion of apoptotic cells in untreated \textit{db/db} mice compared with \textit{db/+} mice (Fig. 4). Both coenzyme Q\textsubscript{10} and ramipril-treated \textit{db/db} mice also exhibited a greater number of apoptotic cells compared with their non-diabetic controls. Apoptosis was significantly reduced in both coenzyme Q\textsubscript{10}-treated \textit{db/db} mice (1.8±0.2-fold that detected in untreated \textit{db/+} mice) and ramipril-treated \textit{db/db} mice (2.3±0.5-fold untreated \textit{db/+} mice).

Coenzyme Q\textsubscript{10} attenuates oxidative stress in \textit{db/db} mice Plasma concentrations of MDA, a marker of systemic lipid peroxidation, were significantly upregulated in both untreated and ramipril-treated \textit{db/db} mice compared with \textit{db/+} controls (Fig. 5a). Importantly, MDA concentrations in coenzyme Q\textsubscript{10}-treated \textit{db/db} mice were markedly reduced compared with their untreated counterparts, such that they were no different from the values in coenzyme Q\textsubscript{10}-treated
Coenzyme Q₁₀ limits diabetes-induced cardiomyocyte hypertrophy. a Representative sections of the H&E stained LV sections; magnification ×400; scale bar 20 µm. The arrow indicates a representative cardiomyocyte width measurement. b Pooled data on cardiomyocyte width (n=10–11/group). c Gene expression of hypertrophic marker Myh7/analysed using real-time PCR and normalised against 18S expression. n=5–10/group. d Quantitative analysis of Akt phosphorylation (phospho-Akt) normalised to total Akt with representative western blot analysis in untreated, Coenzyme Q₁₀ and ramipril-treated hearts. Mean values for db/+ untreated were normalised to 1. n=6–8/group. *p<0.05 vs db/+ untreated. ***p<0.001 vs db/+ untreated. †p<0.05 vs db/+ Coenzyme Q₁₀. ‡p<0.05 vs db/+ ramipril. §p<0.05 vs db/db untreated. ¶p<0.05. White bars, db/+; black bars, db/db. CoQ₁₀, Coenzyme Q₁₀.

db/+ mice. On lucigenin chemiluminescence, untreated db/+ db mice exhibited greater superoxide generation in the myocardium compared with db/+ controls (Fig. 5b). Coenzyme Q₁₀ showed a strong tendency to reduce superoxide generation (p=0.05), whereas ramipril significantly lowered superoxide production in diabetic animals.

Coenzyme Q₁₀ reduces SBP. Diabetic db/db mice exhibited a significantly higher SBP than non-diabetic db/+ mice, regardless of treatment group (Fig. 6). Both coenzyme Q₁₀ and ramipril treatment resulted in a modest reduction of SBP (<10 mmHg), which remained significantly elevated compared with untreated diabetic mice. Neither coenzyme Q₁₀ nor ramipril treatment affected diastolic blood pressure (DBP) in the db/db mice, or either SBP or DBP in non-diabetic mice (results not shown).

Discussion

The pathophysiology of diabetic cardiomyopathy is multifactorial: cardiomyocyte hypertrophy, interstitial fibrosis and cardiomyocyte death further exacerbate diastolic dysfunction [24]. Hyperglycaemia-induced ROS generation has been implicated as a key stimulator of these cardiac impairments [25, 26]. Consequently, reducing oxidative stress, in particular excess cardiac superoxide generation, should be favourable in the management of diabetic cardiomyopathy. The major finding of the current study is that the antioxidant coenzyme Q₁₀ protected the heart in the db/db mouse model of type 2 diabetes from the development of adverse structural and functional changes, with efficacy comparable to the ACE-I ramipril.

LV diastolic dysfunction is often the earliest hallmark of diabetic cardiomyopathy, preceding the onset of systolic dysfunction [4]. In the current study, diabetes-induced diastolic dysfunction was evident on Doppler echocardiography as prolonged deceleration time, with a similar trend for E:A, indicative of impaired LV relaxation. Diabetes also significantly elevated LVEDP. Importantly, treatment with either coenzyme Q₁₀ or ramipril significantly reduced diastolic dysfunction in terms of all three variables. Previously, Chew and colleagues failed to observe significant effects of coenzyme Q₁₀ on LV diastolic dysfunction in diabetic patients, although a trend towards improved E:A was evident [27]. Importantly, differences in patient characteristics at baseline as well as concomitant medication (ACE-I, statins, angiotensin receptor blockers) were likely to have masked...
with previous findings significantly increased in untreated diabetic mice, consistent with systolic dysfunction manifested later in the disease. Coenzyme Q10 cardioprotection [27]. The absence of systolic dysfunction in db/db mice is consistent with previous reports in both experimental and clinical settings [19, 28] in which systolic dysfunction manifested later in the disease.

In the present study, cardiac collagen deposition was significantly increased in untreated diabetic mice, consistent with previous findings [29]. Diastolic dysfunction may thus be a secondary consequence of this increased myocardial stiffness. Coenzyme Q10 and ramipril treatment significantly prevented this upregulation of collagen deposition. We also present evidence that type 2 diabetes-induced downregulation of SERCA2a, a key modulator of cardiomyocyte contractile function, is significantly ameliorated by coenzyme Q10, suggesting a further role for coenzyme Q10 in protecting the heart against contractile dysfunction, possibly through improved cardiac bioenergetics [30, 31].

Pathological LV hypertrophy is a common structural hallmark of the diabetic heart, and is a strong predictor of myocardial infarction, heart failure and stroke [32]. Our findings show that HW:TL, cardiomyocyte width and Myh7 expression, all markers of hypertrophy, were upregulated in the hearts of untreated db/db mice. Although coenzyme Q10 did not significantly reduce HW:TL in db/db diabetic mice, it significantly reduced LV mass (on echocardiography), cardiomyocyte width and Myh7 expression compared with untreated diabetic mice. Ramipril elicited comparable reductions in LV mass, cardiomyocyte width and Myh7 expression, as well as decreasing HW:TL.

![Diabetes and Cardiac Function](image-url)

**Fig. 4** Coenzyme Q10 prevents diabetes-induced cardiomyocyte apoptosis. 
(a) Representative LV sections stained using the CardioTACS In Situ Apoptosis Detection Kit. Positively stained apoptotic cells appear dark blue (indicated by arrows); magnification ×200; scale bar 20 μm. 
(b) Quantification of positively stained apoptotic cells/negatively stained non-apoptotic cells, expressed as fold levels detected in untreated/db/+ untreated, AU. White bars, db/+; black bars, db/db. AU, arbitrary units.

- **Fig. 5** Coenzyme Q10 reduces oxidative stress in db/db mice. 
  - **a** Coenzyme Q10, but not ramipril, significantly reduced plasma concentrations of MDA, a marker of lipid peroxidation (n=6–8/group).
  - **b** Ramipril markedly reduced the cardiac generation of superoxide; similar trends were observed with coenzyme Q10 treatment (n=8–14/group). ***p<0.001 vs db/+ untreated, *p<0.05 vs db/+ ramipril, †p<0.05 vs db/db untreated. White bars, db/+; black bars, db/db. RLU, relative light unit.

- **Fig. 6** SBP is decreased in coenzyme Q10-treated diabetic mice. Both coenzyme Q10 and ramipril treatment marginally reduced SBP in hypertensive diabetic animals. n=7–11/group. ***p<0.001 vs db/+ untreated, *p<0.05 vs db/+ coenzyme Q10, †p<0.05 vs db/+ ramipril, ‡p<0.05 vs db/db untreated. White bars, db/+; black bars, db/db.
Coenzyme Q10 treatment has also been shown to elicit beneficial antihypertrophic effects in the setting of hypertrophic cardiomyopathy [33]. The net increase in HW:TL in diabetic mice occurred in the face of increased LV apoptosis, consistent with previous studies [9, 34]. Both hyperglycemia and oxidative stress can induce apoptosis, further impairing myocardial structure and function, which is implicated in the transition from compensated to decompensated hypertrophy in the diabetic heart [34, 35]. The ability of coenzyme Q10 to reduce diabetes-induced cell death may underlie its effectiveness in reducing myocardial dysfunction and structural remodelling.

Elevated oxidative stress plays a prominent role in the progression of diabetic cardiac complications [8]. Coenzyme Q10 levels are reduced in patients with heart disease and/or diabetes [36], perhaps due to fewer functional mitochondria. In the present study, replenishment of coenzyme Q10 reduced both systemic lipid peroxidation and ventricular superoxide production, in accordance with its ability to reduce oxidative stress and scavenge free radicals in its reduced state [37]. However, it still remains to be determined whether exogenously administered coenzyme Q10 is taken up by mitochondria and reincorporated into the electron transport chain, in addition to increasing circulating coenzyme Q10 [27, 38].

Coenzyme Q10 is detected in membranes other than mitochondria, suggesting that its antioxidant actions are widespread and of physiological importance [30]. Interestingly in the current study, ramipril failed to reduce systemic lipid peroxidation, but it effectively lowered cardiac superoxide production, as previously described [39, 40]. The reduced generation of ROS (and hence restored redox balance) achieved by coenzyme Q10 and ramipril treatment in the present study is likely to be a key mechanism by which they limited myocardial fibrosis and cardiomyocyte hypertrophy in this model of type 2 diabetes, as has been implicated in other settings of cardiac fibrosis [25, 41] and LV hypertrophy [22, 42, 43].

The downstream molecular signalling pathways implicated in ROS-induced cardiac remodelling have not been fully resolved. We demonstrated that cardiac Akt phosphorylation, a downstream target of IGF1R-P13K(p110α), was reduced in type 2 diabetes, but was restored with coenzyme Q10 and ramipril treatment. The IGF1R-P13K(p110α)-Akt pathway induces a physiological hypertrophic response associated with cardioprotection [19, 23, 44], in which cardiac structure and function are preserved. Based on our observations, we postulate that increased ROS depresses protective IGF1R-P13K(p110α)-Akt signalling. The preservation of Akt activity in the coenzyme Q10-treated diabetic myocardium may thereby confer protection against pathological cardiac hypertrophy. This relationship between LV ROS generation and IGF1R-P13K(p110α)-Akt signalling warrants further investigation.

Hypertension is a frequent cardiovascular complication in the diabetic population, which further limits patient prognosis [45]. In the current study, SBP was significantly elevated in diabetic mice (in line with previous studies [46]), which was evident in all treatment groups. Although this was modestly reduced by both coenzyme Q10 and ramipril, this reduction was not strongly correlated with cardiomyocyte width reduction; i.e. the effect of coenzyme Q10 and ramipril on reducing cardiomyocyte size was much greater than would be expected based on the modest lowering of SBP. The benefits of coenzyme Q10 on diabetic cardiomyopathy are thus likely to be largely independent of reduced afterload. The mechanism underlying the antihypertensive effect of coenzyme Q10 is inadequately understood, but a reduction in peripheral vascular resistance has been suggested [38, 47, 48]. The antihypertensive actions of ramipril are well known [39, 49].

**Limitations of the study** Despite the extensive use of db/db mice in the study of various diabetes-related pathologies, the db/db mouse model is not a perfect model of the human disease. A defect in the leptin receptor subjects db/db mice to severe lipotoxicity, more excessive than is observed in diabetes in humans. However, db/db mice display a cardiac pathological phenotype that closely mirrors human diabetic cardiomyopathy, including increased cardiac size, depressed cardiac function and efficiency, and impaired Ca2+ handling [50]. Despite its limitations, the db/db mouse model is considered one of the better animal models for studying the mechanisms underlying human diabetic heart disease and the identification of potential cardiomyopathy-reducing therapeutic agents [50].

Another limitation of our study was the route of coenzyme Q10 administration. The dosage of coenzyme Q10 in drinking water was designed in accordance with average daily water intake of db/+ and db/db mice, and small differences in total dose between animals probably occurred. We acknowledge that oral gavage is a more accurate rate of drug delivery, but this is not well-tolerated by db/+ or db/db mice.

**Conclusions**

In summary, this study demonstrates the benefit of treating diabetic cardiomyopathy with exogenous antioxidants. Coenzyme Q10 attenuated diabetes-induced diastolic dysfunction, cardiomyocyte hypertrophy, cardiac fibrosis and cell death. The cardioprotective effect of coenzyme Q10 may be mediated through its ability to inhibit systemic oxidative stress, improve cardiac bioenergetics and preserve Akt and SERCA2a regulation in the diabetic context. To our knowledge, our study is the first to establish the ability of
coenzyme Q10 to prevent diabetic cardiomyopathy, and to compare the efficacy of coenzyme Q10 with that of an ACE-I. Considering that ROS-mediated cardiac complications are a major contributor to morbidity and death in the diabetic population, coenzyme Q10 may be a clinically valuable adjunct therapy to standard care for preserving cardiac function in diabetic patients.

Acknowledgements The authors thank N. Jennings for her help with echocardiography, K. Gilbert for her help with animal monitoring and M. A. Arinstein for helpful advice on histological analysis (all from the Baker IDI Heart and Diabetes Institute). Parts of this study were presented in abstract form at the 2010 Scientific Sessions of the American Heart Association, Chicago, IL, USA, November 2010.

Funding This work was supported by the Diabetes Australia Research Trust and the National Health and Medical Research Council of Australia (NHMRC project ID526638 to RHR, JRM and KAJ-D), and supported in part by the Victorian Government’s Operational Infrastructure Support Program. KH is supported by an Australian Postgraduate Award. JRM is supported by an Australian Research Council Future Fellowship (FT0001657) and holds an Honorary NHMRC Research Fellowship (856604). X-JD holds an NHMRC Senior Research Fellowship (ID317808). RHR is the recipient of an NHMRC Senior Research Fellowship (ID472673).

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement KH, JRM and RHR contributed to the design of the study, data acquisition, analysis and interpretation of data and drafting of the manuscript. HK, JMF and JEL contributed to data acquisition and interpretation, and manuscript revision. XJD and KJD contributed to data interpretation and manuscript revision. All authors have approved the final version of the manuscript.

References


CHAPTER THREE

Targeting the Upregulation of Reactive Oxygen Species Subsequent to Hyperglycemia Prevents Type 1 Diabetic Cardiomyopathy in Mice
Declaration for thesis chapter 3

Declaration by candidate

In the case of Chapter Three, the nature and extent of my contribution to the work was the following:

<table>
<thead>
<tr>
<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Performed 80% of the experiments, and 100% of the data and statistical analysis.</td>
<td>80%</td>
</tr>
<tr>
<td>Wrote the manuscript with the assistance of co-authors</td>
<td></td>
</tr>
</tbody>
</table>

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

<table>
<thead>
<tr>
<th>Name</th>
<th>Nature of contribution</th>
<th>Extent of contribution (%) for student co-authors only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helen Kiriazis</td>
<td>Performed 10% of experiments (echocardiography and catheterization) and provided intellectual input</td>
<td>N/A</td>
</tr>
<tr>
<td>Xiao-Jun Du</td>
<td>Provided intellectual input and editing of manuscript</td>
<td>N/A</td>
</tr>
<tr>
<td>Jane Love</td>
<td>Performed 10% of experiments</td>
<td>N/A</td>
</tr>
<tr>
<td>Stephen Gray</td>
<td>Assisted with study protocol</td>
<td>N/A</td>
</tr>
<tr>
<td>Karin Jandeleit-Dahm</td>
<td>Provided intellectual input and editing of manuscript</td>
<td>N/A</td>
</tr>
<tr>
<td>Julie McMullen</td>
<td>Provided intellectual input and editing of manuscript</td>
<td>N/A</td>
</tr>
<tr>
<td>Rebecca Ritchie</td>
<td>Provided intellectual input and editing of manuscript</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Candidate's Signature

Date
11.04.13
Declaration by co-authors

The undersigned hereby certify that:

(7) the above declaration correctly reflects the nature and extent of the candidate’s contribution to this work, and the nature of the contribution of each of the co-authors.
(8) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(9) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(10) there are no other authors of the publication according to these criteria;
(11) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(12) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

<table>
<thead>
<tr>
<th>Location(s)</th>
<th>Heart Failure Pharmacology Laboratory, BakerIDI Heart and Diabetes Institute, Melbourne, Victoria, Australia</th>
</tr>
</thead>
</table>

Helen Kiriazis
Xiao-Jun Du
Jane Love
Stephen Gray
Karin Jandeleit-Dahm
Julie McMullen
Rebecca Ritchie

Signature

Date

15/2/2013
15/2/2013
15/12/13
15/12/13
15/12/13
15/12/13

Professor Stephen M. Jane
MBBS, PhD, FRACP, FRCPA
Head, Central Clinical School, Monash University
Director of Research, The Alfred
Faculty of Medicine, Nursing & Health Sciences
The Alfred Centre, 99 Commercial Rd,
Melbourne, Victoria 3004, Australia
Chapter 3: Targeting the Upregulation of Reactive Oxygen Species Subsequent to Hyperglycemia Prevents Type 1 Diabetic Cardiomyopathy in Mice

The previous chapter demonstrated that type 2 diabetic db/db mice exhibited impaired diastolic function and adverse structural remodeling, together with a significant increase in LV •O₂- generation and systemic lipid peroxidation. Exogenous coenzyme Q₁₀ supplementation was able to attenuate markers of functional and structural abnormalities, as well as blunt the increase in oxidative stress. Coenzyme Q₁₀ was also able to reduce systolic blood pressure and improve glycemic control, which may have indirectly contributed to the improvements seen in the treated diabetic mice. To elucidate whether coenzyme Q₁₀ is still able to protect the diabetic heart in the absence of hypertension, I sought to determine whether coenzyme Q₁₀ treatment can prevent functional and structural markers of diabetic cardiomyopathy in a non-obese, non-hypertensive STZ mouse model of type 1 diabetes. This chapter forms a manuscript that has recently been accepted by Free Radical Biology and Medicine, titled “Targeting the upregulation of reactive oxygen species subsequent to hyperglycemia prevents type 1 diabetic cardiomyopathy in mice”.

Targeting the upregulation of reactive oxygen species subsequent to hyperglycemia prevents type 1 diabetic cardiomyopathy in mice

Karina Huynh, Helen Kiriazis, Xiao-Jun Du, Jane E. Love, Stephen P. Gray, Karin A. Jandeleit-Dahm, Julie R. McMullen, Rebecca H. Ritchie

Abstract

Cardiac oxidative stress is an early event associated with diabetic cardiomyopathy, triggered by hyperglycemia. We tested the hypothesis that targeting left-ventricular (LV) reactive oxygen species (ROS) upregulation subsequent to hyperglycemia attenuates type 1 diabetes-induced LV remodeling and dysfunction, accompanied by attenuated proinflammatory markers and cardiomyocyte apoptosis. Male 6-week-old mice received either streptozotocin (55 mg/kg/day for 5 days), to induce type 1 diabetes, or citrate buffer vehicle. After 4 weeks of hyperglycemia, the mice were allocated to coenzyme Q10 supplementation (10 mg/kg/day), treatment with the angiotensin-converting-enzyme inhibitor (ACE-I) ramipril (3 mg/kg/day), treatment with olive oil vehicle, or no treatment for 8 weeks. Type 1 diabetes upregulated LV NADPH oxidase (Nox2, p22phox, p47phox and superoxide production), LV uncoupling protein UCP3 expression, and both LV and systemic oxidative stress (LV 3-nitrotyrosine and plasma lipid peroxidation). All of these were significantly attenuated by coenzyme Q10. Coenzyme Q10 substantially limited type 1 diabetes-induced impairments in LV diastolic function (E:A − dP/dt by micromanometry), LV remodeling (cardiomycyte hypertrophy, cardiac fibrosis, apoptosis), and LV expression of proinflammatory mediators (tumor necrosis factor-α, with a similar trend for interleukin IL-1β). Coenzyme Q10 supplementation may thus represent an effective alternative to ACE-Is for the treatment of cardiac complications in type 1 diabetic patients.

Introduction

The alarming escalation in the global incidence and prevalence of diabetes mellitus over the past 20 years, to its current estimates of almost 300 million, is projected to further increase to 440 million individuals by the year 2030. Of these, up to 70% will succumb to cardiovascular disease secondary to their diabetes [1,2]. Hyperglycemia-induced impairments in redox balance are considered a key trigger of diabetic complications, through upregulated generation of reactive oxygen species (ROS) [3–5], together with an impaired ability of the endogenous antioxidant defense system to remove them [6–8]. The heart is particularly susceptible to oxidative damage, as it possesses lower levels of endogenous antioxidants in comparison to other organs [9]. The predominant sources of ROS in the heart include NADPH oxidase (particularly Nox2 oxidase) and mitochondrial oxidative phosphorylation [10–12]. Recent evidence now suggests that NADPH oxidase-derived superoxide is a key trigger of mitochondrial dysfunction and upregulated mitochondrial superoxide generation [12,13]. Moreover, ROS upregulation is an important driver of cardiovascular inflammation, remodeling, and dysfunction [10,13–16]. Targeting this ROS upregulation is thus an.
attractive therapeutic approach for the cardiac complications of diabetes.

One of the potential candidate drugs targeting ROS is coenzyme Q₁₀. Coenzyme Q₁₀ is an important component of the mitochondrial respiratory chain, in which it functions primarily as an electron transfer intermediate [17]. In its reduced state, it acts as a potent antioxidant by preventing the reaction of hydroxyl and superoxide radicals with the lipid membrane (lipid peroxidation) [18]. Clinical evidence suggests coenzyme Q₁₀ supplementation attenuates cardiac dysfunction in congestive heart failure and may also ameliorate cardiovascular risk [19,20]. Our own evidence specifically in the diabetes context demonstrates that coenzyme Q₁₀ supplementation ameliorates renal remodeling and dysfunc-

tion, accompanied by improvements in renal mitochondrial func-
tion and renal mitochondrial coenzyme Q₁₀ content [4] in db/db mice. Coenzyme Q₁₀ also effectively attenuated cardiomyocyte hypertrophy, cardiac remodeling, and diastolic dysfunction in db/db mice [21]. These beneficial effects were accompanied by a reduction in blood pressure and improved glycemic control, which may have indirectly contributed to the improvements observed in this experimental model of type 2 diabetes.

Diabetic patients are more susceptible to coronary heart disease and peripheral vascular disease; independent of (but often coexistent with) these macrovascular abnormalities, diabetic patients are also at considerable risk of developing distinct impairments directly at the level of the myocardium, termed “diabetic cardiomyopathy” [22–24]. Diabetic cardiomyopathy is characterized by early impairments in cardiac relaxation (diastolic dysfunction, identifiable on echocardiography and magnetic resonance imaging) [24,25], accompanied by structural abnormalities such as cardiomyocyte hypertrophy, myocardial fibrosis, and increased cardiomyocyte apoptosis [26–28]. Patients with type 1 diabetes suffer the disease for several decades (more than those with type 2 diabetes) [29], probably further increasing their likelihood of developing diabetes-induced heart failure, even when adjusted for age and coronary artery disease [30,31]. There remains a lack of choice of therapies for managing the changes in cardiac structure and function specifically in the diabetic heart, and the “gold standard” therapy for treating this diabetic cardio-

mopathies, angiotensin-converting-enzyme inhibitors [32], are not well tolerated by a significant number of patients [32,33].

The objective of this study was to test the hypothesis that targeting cardiac ROS upregulation after hyperglycemia is established attenuates diabetes-induced remodeling and dysfunction, in a mouse model of type 1 diabetes, and this cardioprotection is associated with attenuation of proinflammatory markers and cardiomyocyte apoptosis. Chronic supplementation with the endogenous antioxidant coenzyme Q₁₀ was selected to target ROS in this study, as diabetic individuals are often coenzyme Q₁₀-deficient [34]. We now demonstrate in a male, nonobese mouse model of type 1 diabetes that chronic supplementation with coenzyme Q₁₀ commenced 4 weeks after the onset of hyperglycemia, effectively targets both the structural and the functional aspects of diabetic cardiomyopathy, inde-

pendent of glycemic control and blood pressure. Moreover we demonstrate for the first time that these cardioprotective effects of coenzyme Q₁₀ are associated with restoration of NADPH oxidase expression (Nox2, p22phox, and p47phox), oxidative stress, and proin-

flammatory signaling in the diabetic myocardium.

Material and methods

Animal model

All animal research was conducted in accordance with the National Health and Medical Research Council of Australia guidelines, and approval was obtained from the Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committee. Age-matched male FVB/N mice were bred and housed in the AMREP Precinct Animal Centre and maintained under a 12-h light/dark cycle with up to four littersmates per cage. At 6 weeks of age, mice were assigned to either nondiabetic (sham) or diabetic groups. Type 1 diabetes was induced in mice by five consecutive daily intraperitoneal injections of streptozotocin (STZ; 55 mg/kg body wt, in 0.1 mol/l citrate buffer, pH 4.5; Sigma–Aldrich, St. Louis, MO, USA) [35–37]. An equivalent volume of citrate buffer vehicle was administered to the sham group. Four weeks after the initial STZ/citrate buffer injection, the mice were further allocated into the untreated group, the coenzyme Q₁₀-treated group (10 mg/kg/day, dissolved in olive oil vehicle, administered daily via oral gavage; Sigma), the vehicle-treated group (olive oil alone, oral gavage), and the ramipril-treated group (3 mg/kg/day in drinking water; Sigma). Doses of both coenzyme Q₁₀ and ramipril were based on those previously effective in a mouse model of type 2 diabetes [21]. Mice were followed for a further 8 weeks before euthanasia and tissue collection. Blood glucose levels were moni-
tored fortnightly using a handheld glucometer (Accu-Check Advan-
tage; Roche, Basel, Switzerland). Animals with blood glucose levels exceeding 28 mmol/l were considered diabetic. Blood was also retained for measurement of glycated hemoglobin (GHB) by HPLC (CLC330 GHB Analyzer; Primus, Kansas City, MO, USA) [38].

Analysis of cardiac function in vivo

Two-dimensional M-mode echocardiography and Doppler echocardiography were employed to noninvasively assess end-
point LV function. At 18 weeks of age, mice were anesthetized with a cocktail of ketamine, xylazine, and atropine (100, 10, and 1.2 mg/ kg, ip). Echocardiographic images were obtained using a Philips IE33 ultrasound machine with a 15-MHz linear array transducer as previously described [21,35,36]. Parameters derived from M-mode echocardiography included LV end-diastolic dimension (LVEDD), LV end-diastolic dimension (LVEDD), and fractional shortening (calculated as ((LVEDD – LVEDD)/LVEDD) × 100%). LV filling (LV E/A ratio, the ratio of peak early, E, to late, A, transmural blood flow velocities across the mitral valve) and deceleration time (on E velocity) were assessed using Doppler echocardiography. As pre-

viously described [21,36], we measured by micrometerometry ventricular and aortic blood pressure parameters including LV end-diastolic pressure (LVEDP), aortic systolic (AoSBP) and dias-
tolic blood pressures, and LV ±dP/dt (maximal rate of LV pressure rise or fall during contraction or relaxation), in addition to tau.

Tissue collection and histology

The wet weight of the heart and lungs and the length of the tibia bone were recorded and used to normalize the heart weight (HW: TL). A portion of the ventricle was fixed and stained with hematox-

ylin and eosin (H&E) or 0.1% picrosirius red for determination of cardiomyocyte width and LV interstitial collagen deposition, as previously described [21,36]. Cardiomyocyte cross-sectional area was determined as a second measure of cardiomyocyte hypertrophy, from > 60 individual cardiomyocytes per mouse, calculated from cell outlines using Image-Pro Plus (Media Cybernetics, Bethesda, MD, USA). Apoptosis was examined in NBF-fixed ventricular sections using the CardiToACS In Situ Apoptosis Detection Kit (Trevigen, Gaithersburg, MD, USA). Fifty fields per ventricle section were randomly captured and used for the quantitation of apoptotic cells, which were expressed as a percentage of nonapoptotic cells [21,36]. For immunohistochemical assessment of the p22phox subunit of NADPH oxidase, ventricular sections were deparaffinized in xylene, rehydrated, and rinsed in phosphate-buffered saline (PBS), before proteolytic-induced epitope retrieval using proteinase K [36].
Endogenous peroxidases were quenched in 3% H$_2$O$_2$, and sections were blocked in goat serum (15 µl/ml in 1% bovine serum albumin/PBS). Sections were incubated for 1 h with p22 protein (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-20781, 1:100), followed by a biotinylated goat α-rabbit secondary IgG (Vector Laboratories, Burlingame, CA, USA; 1:100). After incubation with Vectastain Elite ABC avidin–biotinylated horseradish peroxidase complex (Vector Laboratories), sections were developed using DAB peroxidase substrate solution, and color development was monitored under a microscope. Positive p22$^{ABC}$ stained brown, and the intensity of brown staining was graded blindly and scored as follows: 0—negative stain, 1—weak, 2—moderate, 3—intense. 3-Nitrotyrosine content was similarly semiquantitatively assessed on immunohistochemistry, as previously described [38].

### Results

#### Systemic characteristics

STZ-untreated and STZ vehicle (olive oil)-treated mice did not exhibit differences in all parameters measured, and thus these groups were considered not to have differences between two groups, where appropriate.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Postmortem systemic analysis of citrate nondiabetic and STZ-diabetic mice.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Citrate sham</td>
</tr>
<tr>
<td>Blood glucose (mM)</td>
<td>14.2±1.2</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>31.7±0.7</td>
</tr>
<tr>
<td>GHB (%)</td>
<td>9.8±0.9</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>133.5±3.4</td>
</tr>
<tr>
<td>Lung weight (mg)</td>
<td>142.8±4.6</td>
</tr>
<tr>
<td>Tibia length (mm)</td>
<td>16.6±0.1</td>
</tr>
<tr>
<td>HW/LW (mg/g)</td>
<td>4.5±0.1</td>
</tr>
<tr>
<td>LW/TL (mg/mm)</td>
<td>7.7±0.2</td>
</tr>
</tbody>
</table>

* p < 0.05 vs citrate sham, one way ANOVA.

#### Detection of superoxide generation and lipid peroxidation

The malondialdehyde–586 kit (Bioxytech MDA–586; Oxis International, Foster City, CA, USA) was used as a marker of systemic lipid peroxidation in plasma samples, as per the manufacturer’s instructions. Generation of superoxide was assessed in fresh LV tissue using lucigenin (5 µmol/L)-enhanced chemiluminescence, as previously described [21,35]. All results were normalized against tissue dry weight and expressed as relative light units per second per milligram.

#### Statistical analysis

All data are presented as the mean±SE. One-way ANOVAs were conducted to compare and identify differences between all four groups. Interactions between groups were statistically analyzed using the Student Newman–Keuls post hoc test. p < 0.05 was considered significant. Unpaired t tests were also used to detect differences between two groups, where appropriate.
were analyzed and are presented together (referred to as STZ control). STZ control, coenzyme Q10-treated STZ, and ramipril-treated STZ mice all exhibited significantly increased blood glucose levels compared with nondiabetic citrate controls (Table 1, Fig. 1) within 2 weeks after STZ administration, which persisted for the duration of the study. GHb was elevated in all STZ-treated mice, regardless of experimental group (Table 1). STZ animals in all groups exhibited a slight, but significantly, lower body weight compared to nondiabetic controls at study end; coenzyme Q10 and ramipril treatment did not noticeably alter body weight in diabetic mice compared to their untreated littermates (Table 1). Lung weight was not different between diabetic and nondiabetic animals (Table 1); heart weight data are discussed below. Both heart rate and AoSBP remained comparable in diabetic untreated and diabetic coenzyme Q10-treated animals compared with nondiabetic controls under anesthesia (Table 2; both p nonsignificant by one-way ANOVA). Ramipril treatment induced a small, but significant, reduction in AoSBP in diabetic mice compared with sham counterparts.

**Coenzyme Q10 attenuates type 1 diabetes-induced oxidative stress**

Type 1 diabetes markedly upregulated LV NADPH oxidase expression of p47phox and Nox2 subunits (Fig. 2A and B), p22phox protein content (Fig. 2C), and NADPH-driven LV superoxide production (lucigenin chemiluminescence; ramipril elicited similar protection (n=7-9/group). All values are given as the mean±SEM. *p<0.05 vs citrate sham, **p<0.05 vs STZ control.
generation (via lucigenin chemiluminescence, Fig. 2D), compared to nondiabetic mice. Oxidative stress was similarly increased by type 1 diabetes, both in the myocardium (3-nitrotyrosine (3-NT) staining and expression of the mitochondrial UCP3, Fig. 3A and B) and systemically (plasma lipid peroxidation, Fig. 3C). None of these diabetes-induced markers of oxidative stress remained significantly attenuated after coenzyme Q₁₀ supplementation; ramipril was similarly effective, with the exception of UCP3 expression. Neither diabetes nor either treatment significantly affected the expression of the housekeeping gene 18S, on Ct (threshold cycle number, p nonsignificant by one-way ANOVA, results not shown).

Coenzyme Q₁₀ attenuates LV inflammation and apoptosis in type 1 diabetes

Expression of the proinflammatory marker TNFα (Fig. 4A) was significantly upregulated in the type 1 diabetic heart, with a similar trend evident for IL-1β (p = 0.08 by one-way ANOVA, Fig. 4B). This was accompanied by increased cardiomyocyte apoptosis (Fig. 4C) and a trend for an increase in BAX/BCL2 protein expression in untreated diabetic mice compared with citrate sham (Fig. 4D). Each of these markers were significantly ameliorated by supplementation with coenzyme Q₁₀ in diabetic mice; ramipril was similarly effective.

Diastolic function is improved with coenzyme Q₁₀ administration

Assessment of endpoint cardiac function via Doppler echocardiography and micromanometry revealed significant diabetes-induced impairment in LV diastolic dysfunction. A reduced E/A ratio, prolonged deceleration time (DT), elevated LVEDP, and impaired LV +dP/dt peak (Fig. 5A–D; indicators of impaired LV relaxation) were evident in STZ control mice. Coenzyme Q₁₀ administration ameliorated the diabetes-induced impairments in E/A ratio, DT, and LVEDP; there was also a trend for an improvement of LV −dP/dt peak (p = 0.07 vs STZ control). The ACE-I ramipril had a comparable beneficial effect, but did not significantly improve all parameters of diastolic function. Analysis of endpoint systolic function via micromanometry (Table 2) or M-mode echocardiography (Table 3) revealed no differences in fractional shortening, LV +dP/dt peak, or tau between nondiabetic and diabetic mice, regardless of treatment.

Cardiac remodeling is limited by coenzyme Q₁₀ administration

Diabetes induced a 3.4-fold increase in LV interstitial collagen deposition (assessed using Sirius red staining) compared to nondiabetic mice (Fig. 6A). Coenzyme Q₁₀ treatment was associated with lower LV fibrosis compared to diabetic control mice; ramipril

![Image](https://example.com/image.png)
elicited comparable anti-fibrotic effects. Gene expression of CTGF (Fig. 6B) was similarly increased in untreated diabetic mice and attenuated with coenzyme Q₁₀ and ramipril treatment. There was no significant change in HW:BW and HW:TL ratios in STZ control animals, consistent with previous observations [27,28] (Table 1). Despite this, cardiomyocyte width and area (on H&E-stained ventricular sections; Fig. 6C and D) and gene expression of the prohypertrophic markers β-myosin heavy chain and ANP (Fig. 6E) were significantly increased in diabetic mice. Coenzyme Q₁₀ administration significantly ameliorated the diabetes-induced increase in these markers of cardiomyocyte hypertrophy. Ramipril, for comparison, exhibited a similar reduction in cardiomyocyte size and hypertrophic gene expression in STZ mice compared with untreated diabetic controls.

Discussion

The mechanisms underlying the pathophysiology of diabetic cardiomyopathy are complex and multifaceted. Increased ROS production is a common result of prolonged hyperglycemia, and early onset oxidative stress and inflammation have been associated with the development of diabetic cardiomyopathy [3,7,10–12]. A critical component of the mitochondrial electron transport chain, coenzyme
Coenzyme Q₁₀ is best known for its role as an antioxidant in its reduced state. Coenzyme Q₁₀ is highly concentrated in the heart, probably because of the high-energy expenditure of functioning cardiomyocytes [40]. The severity of heart failure has been strongly correlated with the severity of coenzyme Q₁₀ deficiency [41]. Diabetic patients are also coenzyme Q₁₀-deficient, evidenced by overall reduced plasma total coenzyme Q₁₀/total cholesterol levels [34]. Coenzyme Q₁₀ supplementation reduces inflammatory mediators in ischemic heart disease [42], but whether it could also prevent ROS upregulation and inflammation in a model of type 1 diabetes with established hyperglycemia was previously unknown. In this study we hypothesized that targeting cardiac ROS upregulation with coenzyme Q₁₀ would attenuate type 1 diabetes-induced remodeling and dysfunction and that this cardioprotection would be associated with attenuation of proinflammatory markers and cardiomyocyte apoptosis. Here we demonstrate in a male, nonobese mouse model of type 1 diabetes that chronic supplementation with coenzyme Q₁₀, commenced 4 weeks after the onset of hyperglycemia, effectively targets both the structural and the functional aspects of diabetic cardiomyopathy, independent of glycemic control and blood pressure. Moreover we demonstrate for the first time that these cardioprotective effects of coenzyme Q₁₀ are associated with attenuation of NADPH oxidase expression (p47<sub>phox</sub>, p22<sub>phox</sub>, and Nox2), oxidative stress, and proinflammatory signaling in diabetic myocardium.

Fig. 5. Coenzyme Q₁₀ preserves diastolic function in diabetic hearts. Impact of coenzyme Q₁₀ treatment on the diabetes-induced increase in (A) E/A ratio (representative images shown on the right), (B) deceleration time, (C) LVEDP, as well as (D) LV −dP/dt max. Ramipril elicited similar changes in deceleration time and LVEDP (n = 9–13/group). All values are given as the mean±SEM; *p < 0.05 vs citrate sham, #p = 0.05 vs STZ control.
Hyperglycemia is a well-known stimulator of ROS production [3], which in turn triggers several detrimental processes, including inflammation, altered activity of protein kinase C isofoms, and advanced glycation end-product formation [10,12,43]. Our observations now confirm that elevated oxidative stress is a critical contributory mechanism in the adverse changes in myocardial structure and function specifically in type 1 diabetes and that LV expression of proinflammatory cytokines parallels the extent of LV oxidative stress. Supplementation of coenzyme Q10 prevented the diabetes-induced increase in NADPH-driven superoxide generation and expression of three subunits of NADPH oxidase, Nox2, p22phox, and p47phox. This was accompanied by reduced LV content of 3-NT and UCP3, in addition to plasma lipid peroxidation levels. We might thus predict that supplementation of coenzyme Q10 in this study was sufficient to overcome the low total antioxidant capacity of the myocardium, as well as that further suppressed by type 1 diabetes [9,34]. We have shown previously that this dose of coenzyme Q10 is sufficient to restore plasma and renal levels of coenzyme Q10 [4]. NADPH oxidase-derived superoxide is a key trigger of mitochondrial dysfunction and upregulated mitochondrial superoxide generation [10,12,13]. Our results suggest that euglycemic coenzyme Q10 scavenges extra-mitochondrial ROS. Further, the finding that coenzyme Q10 also attenuated type 1 diabetes-induced upregulation of LV UCP3 expression, a marker of mitochondrial uncoupling and redox stress [44], is consistent with actions of coenzyme Q10 at the level of the cardiac mitochondria. ROS are a major driver of both cardiac hypertrophy and profibrotic processes [14,45,46], largely via neurohormonal stimuli (e.g., angiotensin II, endothelin I) and/or mechanical stretch, which trigger the activation of NADPH oxidase, profibrotic/prohy- tropic genes, and matrix metalloproteinases [11,14]. Upregulated cardiac inflammation may further drive cardiac fibrosis [47]. In this study, ramipril elicited comparable protection against most parameters of redox imbalance, such as superoxide generation, 3-NT content, and NADPH oxidase expression, but was less effective on UCP3 expression. ACE-I may have antioxidant capabilities, as the renin–angiotensin system is a major stimulator of NADPH oxidase [48]. Suppressing ROS generation is thus an important therapeutic target for preventing or treating myocardial fibrosis in the hypertrophied heart.

Cardiomyocyte hypertrophy is characteristic of the diabetic heart and is of particular clinical significance, as LV hypertrophy is a prominent predictor of myocardial infarction, stroke, and death from heart failure [49]. In our model, cardiomyocyte hypertrophy is indicated by a significant increase in cardiomyocyte width and β-myosin heavy chain gene expression in diabetic mice. This was, however, not accompanied by an increase in HW/TL, an observation that is consistent with our previous work using the FVB/N STZ model [35,36]. Coenzyme Q10 completely prevented both the increase in cardiomyocyte size and the upregulation of β-myosin heavy chain, suggestive of protection against pathological myocyte hypertrophy, again equal to that achieved with ramipril. Although LV hypertrophy is commonly associated with settings of elevated pressure overload (i.e., in hypertension), it can occur independent of increased pressure loading [16,50].

Table 3

<table>
<thead>
<tr>
<th></th>
<th>Citrate sham</th>
<th>STZ control</th>
<th>STZ CoQ treated</th>
<th>STZ ramipril treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (bpm)</td>
<td>410±20</td>
<td>420±9</td>
<td>388±13</td>
<td>409±15</td>
</tr>
<tr>
<td>External LV dimension (mm)</td>
<td>5.5±0.1</td>
<td>5.4±0.0</td>
<td>5.2±0.0</td>
<td>5.5±0.1</td>
</tr>
<tr>
<td>LV end-diastolic dimension (mm)</td>
<td>3.4±0.1</td>
<td>3.5±0.0</td>
<td>3.3±0.1</td>
<td>3.5±0.1</td>
</tr>
<tr>
<td>LV end-systolic dimension (mm)</td>
<td>2.0±0.1</td>
<td>1.9±0.1</td>
<td>1.9±0.1</td>
<td>2.0±0.1</td>
</tr>
<tr>
<td>Fractional shortening (%)</td>
<td>41.9±1.5</td>
<td>46.2±1.4</td>
<td>42.8±1.2</td>
<td>44.0±1.0</td>
</tr>
</tbody>
</table>

STZ, streptozotocin; CoQ, coenzyme Q10; LV, left ventricular; external LV dimension, distance between the outer edges of the LV posterior and anterior walls.

Conclusions

In summary, our data demonstrates the efficacy of coenzyme Q10 in the treatment of diabetic cardiomyopathy, by prevention of diabetic dysfunction, cardiomyocyte hypertrophy, and apoptosis, as well as interstitial fibrosis. Furthermore, the efficacy of coenzyme Q10 as beneficial as an ACE-I, the current therapy of choice for managing cardiovascular complications in diabetic patients. The exact mechanisms via which coenzyme Q10 exerts this protection require further elucidation, but based on this study include attenuation of the expression of NADPH oxidase subunits (p22phox, p47phox, and Nox2), superoxide production, and inflammatory mediators in the heart (Fig. 7). ACE-I are generally well tolerated but their use can be associated with side effects/adverse drug reactions including hypoten- sion, cough, hyperkalemia, and renal failure in patients with impaired kidney function [48]. Although similar therapeutic effects were
Fig. 6. Diabetes-induced cardiac remodeling was attenuated by coenzyme Q₁₀. Impact of coenzyme Q₁₀ treatment on the diabetes-induced increase in (A) LV interstitial collagen content (representative Sirius red-stained sections shown on right, collagen appears red as indicated by arrow), (B) CTGF gene expression (fold citrate sham; n = 5 or 6/group), (C) cardiomyocyte width (n = 8–12/group), (D) cardiomyocyte cross-sectional area (right side shows representative H&E-stained sections; arrow indicates an example of a cardiomyocyte area measurement; n = 5/group), and (E) hypertrophic gene expression of β-myosin heavy chain and ANP (both normalized to 18S, fold citrate sham; n = 7–9/group). All values are given as the mean ± SEM. *p < 0.05 vs citrate sham, †p < 0.05 vs STZ control, ‡p < 0.05 vs STZ control (by t test).
observed after coenzyme Q0 and ramipril treatment in this study, further studies are warranted to address the similarities and differences in the mechanisms of both approaches and to determine whether their combination yields an additive effect. Furthermore, up to 20% of patients treated with ACE-Is develop intolerance [49]. Numerous studies have shown coenzyme Q0 to be safe and generally well tolerated in humans [19,20,55–60]. Indeed, coenzyme Q0 effectively attenuates diastolic dysfunction induced by chronic statin use in hypercholesterolemic patients [60]. Coenzyme Q0 appears to have fewer side effects than ACE-Is and animal studies indicate that it may also provide protection against renal dysfunction [36,54]. Our findings suggest that coenzyme Q0 may represent an alternative treatment strategy for type 1 diabetic patients to reduce cardiac morbidity and mortality. Prospective, randomized clinical trials of coenzyme Q0 efficacy for limiting type 1 diabetes-induced diastolic dysfunction may thus be warranted based on our findings.

Acknowledgments

The authors thank N. Jennings for technical assistance with echocardiography, B. Bernardo for assistance with immunohistochemistry, and K. Gilbert for assistance with oral gavage and animal monitoring (all from Baker IDI Heart and Diabetes Institute). Parts of this study were presented in abstract form at the 2012 Basic Cardiovascular Sciences Scientific Session—American Heart Association in New Orleans, Louisiana, USA, July 2012. This work was supported by the Diabetes Australia Research Trust and the National Health and Medical Research Council of Australia (NHMRC Project ID526638 to R.H.R., J.R.M., and K.A.J.-D.) and supported in part by the Victoria government’s Operational Infrastructure Support Program. K.H. is supported by an Australian Postgraduate Award. J.R.M. is supported by an Australian Research Council Future Fellowship (FT0001757) and NHMRC Senior Research Fellowship (588604). R.H.R. (ID472673) and X.J.D. (ID1043026) are recipients of NHMRC Senior Research Fellowships.

References


CHAPTER FOUR

Coenzyme Q\textsubscript{10} protects the heart against diastolic dysfunction and structural remodeling in mice with diminished PI3K(p110\(\alpha\)) signaling
4.1 Introduction

The role of oxidative stress in the pathophysiology of diabetic complications has been the focus of a multitude of clinical and experimental studies for over 2 decades. Baynes et al first drew attention to the link between the accumulation of glycoxidation products in tissue collagen and accelerated rates of diabetes [549]. Aberrantly high levels of damaging free radicals accompanied by a synchronous decline in antioxidant defence mechanisms may result in the damage and destruction of cellular organelles, proteins and lipids, widely accepted to contribute to the development and progression of diabetic cardiovascular disorders [186, 191]. The mechanisms underlying oxidative stress-induced diabetic complications however remain unclear. Several processes have been postulated, including increased activity of PKCβ [245], the formation of AGEs [550] and mitochondrial dysfunction [212]. We have previously shown in the STZ mouse model of type 1 diabetes that NADPH-driven \( \cdot \text{O}_2^- \) generation in the heart is elevated, accompanied by an increase in protein content and gene expression of NADPH oxidase subunits [51, 52]. In addition to this increase in ROS production, STZ mice also exhibited a significant increase in the gene expression of apoptosis signal-regulating kinase 1 (Ask1) and PKCβ protein content, alongside a concomitant elevation in cardiomyocyte apoptosis. Interestingly, a reduction in PI3K(p110α) expression exacerbated cardiac \( \cdot \text{O}_2^- \) upregulation, and further elevated Ask1 and PKCβ expression, whilst diabetic mice with increased expression of PI3K(p110α) were protected from the increase in markers of redox stress and apoptosis [52](refer to Appendix 2). Our study was the first to identify a link between reduced PI3K(p110α) signaling and increased LV \( \cdot \text{O}_2^- \)-production.

Constitutive activation of PI3K(p110α) has been shown to be protective in various cardiac pathologies. As an essential mediator of physiological (but not pathological) cardiac growth, constitutively active PI3K(p110α) signaling promotes physiological cardiac hypertrophy that is associated with preserved contractile function, but not interstitial fibrosis or myocyte loss [408]. Physiological hypertrophy is induced by chronic exercise training, and is beneficial for cardiac function. Mice expressing caPI3K(p110α) specifically in the heart showed comparable protection against aortic banding-induced pathological cardiac hypertrophy to swim-trained aortic-banded Ntg animals [426]. caPI3K transgenic animals were also protected from systolic dysfunction induced by MI, and from atrial fibrosis and impaired cardiac conduction induced by dilated cardiomyopathy [427, 429]. Conversely,
dominant negative PI3K(p110α) transgenic mice, which exhibit a 77% reduction in cardiac PI3K(p110α) signaling, displayed an exaggerated disease response following aortic banding-induced hypertrophy, including increased interstitial fibrosis and impaired contractile function compared to Ntg counterparts [426]. Furthermore, swim-training failed to protect the hearts of dnPI3K mice from aortic banding-induced hypertrophy, indicating that diminished PI3K(p110α) signaling translates to an inability of the heart to respond to exercise-induced cardioprotection. Under disease settings such as MI or dilated cardiomyopathy, dnPI3K(p110α) mice exhibit severely impaired systolic function, increased fibrotic deposits and arrhythmic irregularities [412, 429]. In our previous study, dnPI3K STZ diabetic mice exhibited increased cardiac \( \cdot \text{O}_2^- \) generation compared with Ntg STZ diabetic mice [52]. It is unknown whether this increase in oxidative stress was linked to the development of diabetic complications exacerbated by diminished PI3K(p110α) signaling, which included cardiac dysfunction and pathological cardiac remodeling. In order to determine whether enhanced ROS production as a result of downregulated PI3K(p110α) signaling in the diabetic animal is a major contributor to adverse changes in the diabetic heart, I aim to exogenously supplement dnPI3K(p110α) STZ diabetic mice with the antioxidant coenzyme Q\( _{10} \). I hypothesize that the exaggerated diabetes-induced cardiac remodeling and dysfunction observed in dnPI3K mice is attenuated by coenzyme Q\( _{10} \), in parallel with reduced ROS generation.
4.2 Methods

4.2.1 Animal Model

All animal research was conducted in accordance with the ‘National Health and Medical Research Council of Australia’ guidelines, and was approved by the Alfred Medical Research and Education Precinct (AMREP) Animal Ethics committee. All mice used in this study were bred and housed in the AMREP Precinct Animal Centre and maintained under a 12-hr light/dark cycle with up to 4 littermates per cage. Heterozygous dnPI3K transgenic mice were originally produced by cloning a truncated p110 mutant which lacks the active kinase domain into the αMHC promoter construct. The catalytically inactive p110 molecule competes with endogenous p110 for binding with the p85 regulatory subunit, therefore inhibiting the function of endogenous p110 in vivo [407, 408]. Age-matched Ntg and dnPI3K transgenic male mice on a FVB/N background were assigned to either the diabetic group or non-diabetic group. At 6 weeks of age, mice assigned to the diabetic group were administered STZ to induce T1DM, and the non-diabetic group was given a citrate buffer vehicle. Four weeks following administration of STZ, diabetic and non diabetic animals were assigned to either the untreated or the coenzyme Q₁₀-treated groups. Mice in the coenzyme Q₁₀-treated group received an intraperitoneal injection of 10mg/kg/day of coenzyme Q₁₀ (LiQsorb, Tishcon Corp, NY, United States) three times weekly. Mice were treated for 8 weeks prior to cull and collection of tissue.

4.2.2 Glucose measurements

Fortnight and endpoint collection of blood from the diabetic mice was used for confirmation of hyperglycemia. Saphenous vein bleeds were conducted every 2 weeks following STZ/citrate buffer administration. In this procedure, a 50ml centrifuge tube with an open end to allow airflow was used to restrain the mouse. The tail was tightly restrained through the open end to keep the mouse still and the hind leg was extended to reveal the saphenous vein. The vein was punctured carefully with a 25 gauge needle and the resultant drop of blood was placed on a glucometer test strip and read by the glucometer (Accu-check Advantage; Roche, Basel, Switzerland). A sterile gauze pad was applied on the punctured vein to cease blood flow. The blood glucose value was recorded in mmol/L [51].
4.2.3 Analysis of cardiac function in vivo

4.2.3.1 M-mode and Doppler baseline echocardiography

M-mode and Doppler echocardiography were performed at the study endpoint in all animals. Mice were first anaesthetized with a cocktail of ketamine, xylazine and atropine (100mg/kg, 10mg/kg and 1.2 mg/kg) via intraperitoneal injection, and ultrasonic gel was applied to the shaved chest of the mice to ensure optimal clarity of the cardiac tracings. All echocardiograph heart images were obtained using a Phillips iE53 ultrasound machine with a 15MHz linear array transducer [51]. On M-mode echocardiography, parameters measured include external LV diameter, anterior and posterior wall thicknesses, LV mass, LV end-diastolic dimension (LVEDD) and left ventricular end-systolic dimension (LVESD). Fractional shortening, a measure of systolic function, was calculated as follows:

\[
\% \text{ FS} = \frac{(\text{LVEDD} - \text{LVESD})}{\text{LVEDD}} \times 100
\]

Two-dimensional Doppler echocardiography permits measurement of early (E) and late (or atrial, A) peak filling velocities of blood flow across the mitral valve. Furthermore, mitral deceleration times of early filling also were derived from Doppler echocardiography. A reduction in overall E/A wave ratio and prolonged deceleration time indicates impaired LV relaxation and diastolic dysfunction. Following echocardiography, mice were administered saline eye drops to prevent eye dryness, placed on a 37°C heat pad and closely monitored until a full recovery was made [51].

4.2.3.2 Cardiac catheterization

Cardiac function was also examined via cardiac catheterization, in anaesthetized mice (intraperitoneal injection of ketamine (100mg/kg), xylazine (10mg/kg) and atropine (1.2mg/kg)) prior to cull and collection of tissues. A micro-manometer-tipped catheter (1.4F, Millar Instrument Co, Texas, United States) was inserted through the right carotid artery and introduced into the LV. The animal was allowed to stabilize for approximately 10-15 minutes, before pressure recordings were obtained and calculated for LV +dP/dt\text{max} (indicator of systolic function), LV -dP/dt\text{min} (indicator of diastolic function) and LVEDP. Aortic systolic (AoSBP) and diastolic (AoDBP) blood pressures were recorded with the catheter positioned in the ascending aorta [551].
4.2.4 Tissue collection

Following data acquisition, the heart and lungs were carefully removed, rinsed in saline, lightly dried with gauze and dissected for weighing. The lungs were weighed and disposed. The left and right atria were dissected, weighed individually and also disposed. The top third portion of the ventricles was cut and fixed in 10% neutral buffered formalin (NBF, Australian Biostain, Melbourne, Australia), and paraffin-embedded for histological analysis. A segment of the bottom of the ventricle (approximately 20mg) was removed and used for the lucigenin chemiluminescence assay (see below). The remainder of the ventricle was snap frozen in liquid nitrogen and stored at -80°C for determination of gene expression and protein content. A single hind leg containing the tibia bone of each mouse was removed and stored at 4°C until ready for tibia measurement. To digest the hind leg, approximately 5ml of 1M NaOH (MP Biomedicals, Ohio, United States) was added to each hind leg and incubated overnight in a 37°C oven to digest. The tibia was easily removed the following morning following the digestion of muscle, skin and fat, and its length measured using a vernier calliper [51].

4.2.5 Histology

4.2.5.1 H&E staining

Ventricular sections fixed in 10% NBF were paraffin-embedded, then microtome sectioned at 4µm (Leica Microsystems, Wetzlar, Germany). H&E staining was employed to measure cardiomyocyte width.Slides were dewaxed in xylene (2x 10 minutes), then washed in 100% ethanol (2x 5 minutes), 95% ethanol (1x 5 minutes), rinsed under running tap water (1x 5 minutes) then stained in 50M hematoxylin for 8 minutes. The slides were then rinsed under running water again (1x 5 minutes), submerged in Scott’s tap water (1x 1 minute), rinsed under running water (1x 5 minutes), dehydrated in 95% ethanol (1x 5 minutes) and counter-stained in eosin for 10 minutes. Slides were then quickly dipped in 100% ethanol then placed in xylene (2x 5 minutes). A drop of Depex medium (BDH Chemicals, Poole, United Kingdom) was used to attach a coverslip to the slide and dried in the fumehood for 48 hours. Images of the stained heart were viewed using a light microscope at 400X magnification, and 6-8 fields were randomly chosen and photographed. Images were analysed using Olympus Image Pro-plus software (Version 6.0, Media Cybergenetics, Bethesda, United States).
Cardiomyocyte width was determined by measuring across the shortest axis of each individual cardiomyocyte. For each heart, 100 individual myocytes were measured [51, 52].

4.2.5.2 Picrosirius red staining

Picrosirius red staining was employed to assess the level of collagen deposition. Slides were dewaxed in xylene (2x 10 minutes), then washed in 100% ethanol (2x 5 minutes), 95% ethanol (1x 5 minutes) and tap water (1x 3 minutes). Slides were then stained with 0.1% picrosirius red solution (Picric acid from Fluka, Buchs, Switzerland; pH 2) for 1 hour, followed by a brief rinse with 70% ethanol, 95% ethanol and 100% ethanol. Slides were placed in xylene (2x 2 minutes), a coverslip was affixed using Depex and left to dry in the fumehood for 48 hours. Images of the stained heart were viewed using a light microscope at 200X magnification. Ten fields were randomly chosen and photographed, taking care to avoid perivascular collagen deposition. Collagen stained an intense red colour, which was measured and analysed using Olympus Image Pro-plus as a ratio of area of collagen to total area of image [51, 52].

4.2.6 Detection of •O₂⁻ generation and lipid peroxidation

4.2.6.1 •O₂⁻ generation

•O₂⁻ production was measured in fresh LV tissue using lucigenin (5µmol/L)-enhanced chemiluminescence. The LV tissue was dissected into small portions (~1-2mm in diameter), and incubated in the dark for 1 hour at 37°C in NADPH (100µM)-containing Krebs buffer in 96-well optiplates. Lucigenin (N,N’-dimethyl-9-9’-biaacidianium; Sigma Aldrich) was added to each well, giving a final concentration of 5µM in NADPH-containing Krebs buffer. The photons released from the reaction between lucigenin and •O₂⁻ were counted using the MicroLumat Plus luminometer (Berthold Technologies GmbH, Germany). All results were normalized against tissue dry weight and expressed as relative light units per second per milligram [68].
4.2.6.1 MDA assay

The levels of systemic lipid peroxidation were measured in plasma samples using the Malondialdehyde-586 kit (Bioxytech MDA-586, Foster City, CA, USA), as per the manufacturer’s instructions.

4.2.7 Analysis of gene expression

RNA was extracted from frozen LV samples and reverse transcribed as previously described [51, 68]. Cardiac gene expression of the pro-hypertrophic markers β-myosin heavy chain and ANP (for primer sequences, see Table 4.1) were determined via real-time PCR using SYBR® Green reagents (Applied Biosystems). Relative expression of the target genes were normalized to the constitutively expressed housekeeping gene, 18S. All primers were generated from murine-specific sequences published on GeneBank (Table 4.1). Quantitative real-time analysis was performed using the ABI Prism® 7700 Sequence Detection System software, and the comparative delta-delta C\textsubscript{T} (\(ΔΔC\text{T}\)) method was used to detect changes in expression, as relative fold difference, between the target gene and 18S.

4.2.8 Statistical analysis

Results are presented as mean ± SE. One-way ANOVAs were performed on all raw data using SigmaStat (Version 3.5, Erkath, Germany) to identify differences between groups, unless otherwise stated. The Student Newman Keuls post-hoc test was run to determine statistical differences between specific groups. P < 0.05 was considered significant.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Concentration</th>
<th>cDNA final</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-MHC forward primer</td>
<td>TCTCCTGCTGTT TCCTTACTTGCTA</td>
<td>300nM</td>
<td>12.5ng</td>
</tr>
<tr>
<td>β-MHC reverse primer</td>
<td>GTACTCCTCTGCTAGGCTTCCT</td>
<td>300nM</td>
<td></td>
</tr>
<tr>
<td>ANP forward primer</td>
<td>GAACCTGCTAGACCACCT</td>
<td>300nM</td>
<td>12.5ng</td>
</tr>
<tr>
<td>ANP reverse primer</td>
<td>CCTAGTCCACTCTGGGCT</td>
<td>300nM</td>
<td></td>
</tr>
<tr>
<td>18S forward primer</td>
<td>TGTTCACCATGAGGCTGAATC</td>
<td>40nM</td>
<td>N/A</td>
</tr>
<tr>
<td>18S reverse primer</td>
<td>TGGTTGCCTGGGAAAATCC</td>
<td>60nM</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1. Oligonucleotide sequences (5’-3’), concentration of the primers and probes, and the final concentration of cDNA per reaction are depicted in the table below for each gene investigated. The primers and probes for β-MHC, ANP and the housekeeping gene 18S were generated upon murine-specific sequences published on GeneBank. Both β-MHC and ANP gene expression were determined using SYBR® Green chemistry. Both genes required a separate reaction for 18S control. β-MHC, β-myosin heavy chain; ANP, atrial natriuretic peptide.
4.3 Results

4.3.1 Systemic data

As expected, blood glucose levels were significantly elevated in all STZ groups (Table 4.2). Neither genotype nor drug treatment had an effect on blood glucose concentrations. Consistent with previous observations, body weight was slightly, but significantly reduced in diabetic animals, regardless of genotype or treatment. Tibia length, being similar in all groups, was used as a constant to normalize heart weight and lung weight. Lung weight to tibia length ratio (LW/TL) was not different between groups, irrespective of genotype, diabetes or treatment. HW/TL is described below.

4.3.2 Coenzyme Q$_{10}$ reduces oxidative stress in both Ntg and dnPI3K diabetic animals

The present study examined two markers of oxidative stress: systemic lipid peroxidation via measuring plasma MDA levels and cardiac •O$_2^-$ generation via lucigenin-enhanced chemiluminescence in LV sections. MDA levels were increased 2.7±0.2 fold in Ntg diabetic untreated mice, and 2.6±0.2 fold in dnPI3K diabetic untreated mice compared to their respective untreated controls (Figure 4.1A). There was a strong trend for plasma MDA concentration in untreated dnPI3K diabetic mice to increase (by ~28%) compared to untreated Ntg diabetic mice (p=0.05). Coenzyme Q$_{10}$ significantly attenuated the increase in MDA levels in both Ntg diabetic and dnPI3K diabetic mice. Similar trends were also observed in the measurements of ventricular •O$_2^-$ generation. Increased lucigenin-enhanced luminescence, indicative of elevated •O$_2^-$ levels, was detected in Ntg diabetic untreated mice (increased 53±6% vs Ntg citrate untreated) and in dnPI3K diabetic untreated mice (increased 80±5% vs dnPI3K citrate untreated; Figure 4.1B). Moreover, expression of the dnPI3K genotype in untreated diabetic mice further enhanced •O$_2^-$ levels by 32±4% in comparison to Ntg diabetic untreated mice. Although coenzyme Q$_{10}$ did not significantly reduce •O$_2^-$ generation in Ntg diabetic mice, it effectively reduced •O$_2^-$ levels in dnPI3K diabetic mice (p<0.05 vs dnPI3K citrate untreated).
Table 4.2: Postmortem systemic analysis of Ntg/dnPI3K, non-diabetic/diabetic and untreated/CoQ-treated mice

<table>
<thead>
<tr>
<th>Variable</th>
<th>Ntg</th>
<th>dnPI3K</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-diabetic</td>
<td>Diabetic STZ</td>
</tr>
<tr>
<td></td>
<td>Untreated</td>
<td>CoQ-treated</td>
</tr>
<tr>
<td>Blood glucose (mmol/L)</td>
<td>10.2 ± 0.3</td>
<td>10.1 ± 0.2</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>31.8 ± 1.3</td>
<td>32.3 ± 0.7</td>
</tr>
<tr>
<td>Tibia length (mm)</td>
<td>17.5 ± 0.2</td>
<td>17.2 ± 0.2</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>127 ± 3</td>
<td>124 ± 4</td>
</tr>
<tr>
<td>Lung weight (mg)</td>
<td>145 ± 3</td>
<td>150 ± 4</td>
</tr>
<tr>
<td>HW/TL (mg/mm)</td>
<td>7.37 ± 0.21</td>
<td>7.10 ± 0.19</td>
</tr>
<tr>
<td>LW/TL (mg/mm)</td>
<td>8.24 ± 0.32</td>
<td>8.21 ± 0.23</td>
</tr>
</tbody>
</table>

*p<0.05 vs same-genotype non-diabetic untreated mice, #p<0.05 vs same-genotype non-diabetic CoQ-treated mice, †p<0.05 vs Ntg non-diabetic same-treatment mice, ‡p<0.05 vs Ntg STZ same-treatment mice. CoQ, coenzyme Q10; HW, heart weight; TL, tibia length; LW, lung weight.
Figure 4.1: Both Ntg and dnPI3K diabetic mice exhibited significantly greater levels of lipid peroxidation (A; plasma MDA concentration) and LV $\cdot$O$_2^-$ generation (B; lucigenin chemiluminescence); n=5/group. $\cdot$O$_2^-$ generation in dnPI3K diabetic mice was additionally elevated compared with Ntg diabetic mice. Coenzyme Q$_{10}$ significantly attenuated both these markers of oxidative stress in dnPI3K diabetic mice. All values are given as mean ± SEM; *p<0.05 vs same-genotype citrate untreated, †p<0.05 vs same-genotype STZ untreated, ‡p<0.05 vs Ntg STZ untreated.
4.3.3 Attenuation of diastolic dysfunction with coenzyme Q$_{10}$ administration in Ntg and dnPI3K diabetic mice

Endpoint diastolic and systolic function was measured via both echocardiography and cardiac catheterization. Doppler echocardiography was employed to determine diastolic function. E/A ratio tended to decrease in Ntg STZ untreated animals compared with their Ntg non-diabetic counterparts (p=0.07), however this was not significant (Figure 4.2A). dnPI3K STZ untreated animals however, exhibited a significantly reduced E/A wave ratio compared with dnPI3K citrate untreated mice. Coenzyme Q$_{10}$ treatment failed to increase E/A ratio in both Ntg and dnPI3K diabetic animals significantly, despite a strong trend (p=0.08 and 0.1, respectively). Deceleration time, also measured on Doppler echocardiography, was prolonged in both Ntg and dnPI3K diabetic untreated mice compared with their respective non-diabetic controls (Figure 4.2B). Deceleration time in dnPI3K diabetic untreated mice was further prolonged in comparison to Ntg diabetic mice. Coenzyme Q$_{10}$ treatment effectively attenuated deceleration time in both Ntg and dnPI3K diabetic animals. Two additional markers of diastolic function, LVEDP and LV $-dP/dt_{\text{min}}$, were measured via cardiac catheterization. LVEDP was significantly elevated in both Ntg and dnPI3K diabetic untreated animals (Figure 4.2C). Coenzyme Q$_{10}$ treatment attenuated the increase in LVEDP in both Ntg and dnPI3K STZ animals. Similarly, LV $-dP/dt_{\text{min}}$ was significantly reduced in diabetic untreated animals of both genotypes (Figure 4.2D). There was a trend for LV $-dP/dt_{\text{min}}$ in dnPI3K STZ untreated mice to be further reduced in comparison with Ntg STZ untreated animals (p=0.1). Coenzyme Q$_{10}$ treatment prevented the fall in LV $-dP/dt_{\text{min}}$ in dnPI3K STZ animals. Markers of systolic function, including fractional shortening and LV $+dP/dt_{\text{max}}$, remained unchanged regardless of genotype, diabetes or treatment (Table 4.3).

4.3.4 Coenzyme Q$_{10}$ administration attenuates diabetes-induced cardiomyocyte hypertrophy in both Ntg and dnPI3K mice

Neither net HW, nor HW/TL were different between Ntg mice in all groups (Table 4.3). In contrast, expression of the dnPI3K transgene was associated with a reduction in HW/TL compared to their respective Ntg controls; all dnPI3K transgenic mice exhibited similar HW/TL regardless of diabetic status or treatment. External LV diameter, measured on
Figure 4.2

A) E wave:A wave ratio

B) Deceleration time (ms)

untreated CoQ-treated
Figure 4.2: Ntg STZ untreated mice exhibited prolonged deceleration time (B), elevated LVEDP (C) and reduced LV dP/dt_{min} (D) compared with Ntg citrate untreated mice. dnPI3K STZ untreated mice displayed similar trends in these markers of diastolic dysfunction, in addition to reduced E/A wave ratio (A). Coenzyme Q_{10} attenuated some markers of diastolic dysfunction. n=5/group. All values are given as mean ± SEM; *p<0.05 vs same-genotype citrate untreated, ^p<0.05 vs same-genotype STZ untreated, ^p<0.05 vs Ntg STZ untreated.
Table 4.3: Echocardiography and catheterization analysis of heart size and function

<table>
<thead>
<tr>
<th>Variable</th>
<th>Ntg</th>
<th>Diabetic STZ</th>
<th>dnPI3K</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-diabetic</td>
<td>Diabetic STZ</td>
<td>Non-diabetic</td>
</tr>
<tr>
<td></td>
<td>Untreated</td>
<td>CoQ-treated</td>
<td>Untreated</td>
</tr>
<tr>
<td>M-mode echocardiography, n</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>External LV diameter (mm)</td>
<td>5.42 ± 0.09</td>
<td>5.48 ± 0.15</td>
<td>5.42 ± 0.09</td>
</tr>
<tr>
<td>LVPW (mm)</td>
<td>0.92 ± 0.06</td>
<td>0.98 ± 0.08</td>
<td>0.86 ± 0.04</td>
</tr>
<tr>
<td>LVDD (mm)</td>
<td>3.52 ± 0.05</td>
<td>3.57 ± 0.23</td>
<td>3.59 ± 0.12</td>
</tr>
<tr>
<td>LVSD (mm)</td>
<td>1.96 ± 0.03</td>
<td>2.02 ± 0.18</td>
<td>1.77 ± 0.25</td>
</tr>
<tr>
<td>Fractional shortening (%)</td>
<td>44 ± 1</td>
<td>43 ± 4</td>
<td>51 ± 6</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>405 ± 23</td>
<td>366 ± 20</td>
<td>435 ± 11</td>
</tr>
<tr>
<td>Catheterization, n</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>333 ± 10</td>
<td>350 ± 18</td>
<td>372 ± 14</td>
</tr>
<tr>
<td>Aortic SBP (mmHg)</td>
<td>93 ± 5</td>
<td>95 ± 7</td>
<td>75 ± 5</td>
</tr>
<tr>
<td>LVSP (mmHg)</td>
<td>91 ± 3</td>
<td>94 ± 4</td>
<td>81 ± 4</td>
</tr>
<tr>
<td>LV +dP/dt_{max} (mmHg/s)</td>
<td>7250 ± 337</td>
<td>7058 ± 447</td>
<td>7710 ± 438</td>
</tr>
</tbody>
</table>

†p<0.05 vs Ntg non-diabetic same-treatment mice; ‡p<0.05 Ntg STZ same-treatment mice

LV, left ventricular; LVPW, left ventricular posterior wall thickness; LVDD, left ventricular diastolic dimension; LVSD, left ventricular systolic dimension; SBP, systolic blood pressure; LVSP, left ventricular systolic pressure; +dP/dt_{max}, maximum rate of LVP change.
echocardiography was similarly smaller in all dnPI3K animals compared to their respective controls (with the exception of dnPI3K STZ untreated mice, where p=0.06 vs Ntg STZ untreated). Diabetes did not have an effect on LV diameter (Table 4.3). Despite the absence of change in HW/TL induced by diabetes, cardiomyocytes were significantly enlarged as a result of diabetes in both Ntg and dnPI3K mice, consistent with previous studies [52]. This could be attributable to an increase in apoptosis, which can mediate a compensatory increase in the size of remaining viable cardiomyocytes [51]. In the present study, diabetes induced a ~24% increase in cardiomyocyte width in Ntg untreated mice, and a ~38% increase in dnPI3K untreated mice (Figure 4.3A). Coenzyme Q₁₀ administration significantly attenuated this increase in both Ntg and dnPI3K diabetic mice, in comparison to untreated diabetic controls. Associated with this increase in cardiomyocyte size was a significant upregulation of the hypertrophic markers ANP and β-myosin heavy chain in Ntg STZ untreated animals compared to Ntg citrate controls (Figure 4.A, B). Coenzyme Q₁₀ treatment was also associated with a reduction in both ANP and β-myosin heavy chain expression, in particular ANP, whose expression levels were not different to those observed in the non-diabetic controls.

Similar trends were seen in mice expressing the dnPI3K transgene; diabetes induced an increase in the expression of ANP and β-myosin heavy chain in untreated animals, which were lower in coenzyme Q₁₀-treated dnPI3K diabetic mice. Diminished PI3K(p110α) activity in diabetic mice was associated with an exaggerated increase in both ANP and β-myosin heavy chain expression compared with Ntg diabetic animals. dnPI3K STZ untreated mice exhibited a 12.5 ± 0.8 fold increase in ANP levels, and a 1.8 ± 0.9 fold increase in β-myosin heavy chain levels, in comparison to Ntg STZ untreated mice. Expression of the dnPI3K transgene, compared with Ntg mice, also induced an increase in ANP expression under the basal/non-diabetic setting, which has been previously demonstrated in this transgenic mouse model [52, 407].

4.3.5 Diabetes-induced cardiac fibrosis is reduced with coenzyme Q₁₀ treatment

In Ntg animals, collagen deposition was significantly elevated in diabetic untreated mice, compared to non-diabetic controls. Ntg diabetic animals treated with coenzyme Q₁₀ exhibited less fibrosis compared with their untreated counterparts (Figure 4.5A). The hearts of dnPI3K
Figure 4.3: Cardiomyocyte width was increased in STZ untreated mice of both genotypes (A); coenzyme Q$_{10}$ significantly attenuated this enlargement in cardiomyocyte width. The bottom panel depicts representative sections of H&E stained LV samples from each group (B); an example of a cardiomyocyte width measurement is indicated by an arrow. All values are given as mean ± SEM; *p<0.05 vs same-genotype citrate untreated, #p<0.05 vs same-genotype STZ untreated.
Figure 4.4: Gene expression of ANP (C) and β-myosin heavy chain (D) were increased in diabetic untreated animals of both genotypes; expression of these hypertrophic gene markers were exacerbated in dnPI3K STZ untreated animals compared to Ntg STZ controls. Coenzyme Q₁₀ significantly attenuated the expression of both these genes (n=5/group). All values are given as mean ± SEM; *p<0.05 vs same-genotype citrate untreated, †p<0.05 vs same-genotype STZ untreated, ‡p<0.05 vs Ntg citrate untreated, ‡p<0.05 vs Ntg STZ untreated.
Figure 4.4: An increase in collagen deposition was observed in both Ntg and dnPI3K diabetic mice compared to non-diabetic shams (A). Coenzyme Q$_{10}$ significantly attenuated the increase in collagen content in diabetic mice of both genotypes. The bottom panel depicts the representative sections of sirius-red stained LV samples from each group (B); the presence of collagen, which stains red, is indicated by an arrow. All values are given as mean ± SEM; *p<0.05 vs same-genotype citrate untreated, #p<0.05 vs same-genotype STZ untreated.
diabetic untreated animals showed a $3.0 \pm 0.3$ fold increase in collagen content compared to dnPI3K non-diabetic controls, and a $1.2 \pm 0.1$ fold increased compared to Ntg diabetic untreated mice. Coenzyme $Q_{10}$ was also able to attenuate the increase in collagen levels in dnPI3K diabetic mice.
4.4 Discussion

The mechanisms responsible for the distinct cardiomyopathy observed in diabetic patients, characterized by early diastolic dysfunction and detrimental structural remodeling of the heart, are complex and multifaceted, and may involve the activation of redox or protein signaling pathways. Chapters 2 and 3 have extensively examined the role of oxidative stress in the development of diabetic cardiomyopathy, using both type 1 and type 2 diabetic mouse models. Furthermore, in a separate study, our lab has also identified a possible link between increased cardioprotective PI3K(p110α) signaling and reduced •O2- generation [52]. In this chapter, I aimed to further investigate the relationship between PI3K(p110α) signaling and oxidative stress, by utilizing a transgenic mouse model with reduced PI3K(p110α) (dnPI3K) supplemented with the antioxidant coenzyme Q10. The results from this study provide further evidence that reduced PI3K(p110α) signaling in the heart is indeed associated with a more severe cardiomyopathy phenotype under the diabetic setting, and that this is related to an upregulation of oxidative stress. This was demonstrated by showing that coenzyme Q10 was effective in reducing oxidative stress and attenuating cardiomyopathy in a diabetic model with diminished PI3K(p110α) signaling.

Impaired relaxation of the heart, or diastolic dysfunction, is one of the earliest indicators of diabetic cardiomyopathy. Our group and others have previously demonstrated that IGF-1R-PI3K(p110α) signaling is protective against cardiac dysfunction under various pathological settings, including pressure-overload-induced hypertrophy and diabetes [51, 52, 426, 431]. Diabetes is associated with depressed E/A ratio, prolonged deceleration time, reduced LV -dP/dtmin and elevated LVEDP, all markers of impaired diastolic function [51]. Diabetic mice expressing a cardiac-specific constitutively active PI3K(p110α) transgene were protected from abnormal LV relaxation and filling, whilst diabetic mice with reduced PI3K(p110α) signaling exhibited worsened diastolic function [52]. Results from the present study are consistent with this; both Ntg and dnPI3K diabetic untreated mice exhibited lengthened deceleration time, increased LV EDP and reduced LV -dP/dtmin. In particular, deceleration time in dnPI3K transgenic diabetic mice was additionally prolonged compared with Ntg diabetic controls, supporting the hypothesis that diminished PI3K(p110α) signaling can further exacerbate cardiac dysfunction under the diabetic setting. There were also trends for other parameters to be worse in dnPI3K diabetic mice including fibrosis and LV – dP/dtmin. The relatively small numbers in each group (n=5) is likely to explain why these
trends failed to attain statistical significance. Ntg diabetic mice treated with coenzyme Q\textsubscript{10} were protected from diastolic impairments (deceleration time, LVEDP), an observation previously reported in both type 1 and type 2 mouse models of diabetes (refer to chapters 2 and 3). A novel finding of the study was that coenzyme Q\textsubscript{10} administration was also effective in protecting dnPI3K diabetic mice from impairments in LV relaxation and filling. This lends further support to the hypothesis that reduced PI3K(p110\textalpha) signaling is associated with an increase in oxidative stress, which can subsequently be reduced by antioxidant supplementation. Systolic function was not impaired in this diabetic model, in either Ntg or dnPI3K mice, as seen in the lack of change in fractional shortening and LV +dP/dt\textsubscript{max}. This is clinically relevant, as systolic function in diabetic patients is frequently described as developing at a later onset to the initial development of diastolic dysfunction [48].

Diastolic impairment in the diabetic heart at the early stage of the disease is intricately linked to adverse changes in cardiac morphology. Cardiomyocyte hypertrophy and cardiac fibrosis are frequently observed in both experimental models of diabetes and in diabetic patients [51, 238, 552]. Consistent with previous findings in our lab, Ntg diabetic untreated mice exhibited significantly enlarged cardiomyocytes compared with their non-diabetic controls [51]. This was accompanied by an upregulation in hypertrophic gene markers ANP and β-myosin heavy chain. The changes in cardiomyocyte size and gene expression occurred in the absence of changes in cardiac hypertrophy (i.e., on net HW or HW/TL) in the Ntg STZ animals, as previously observed [51, 52]. We have attributed this to a possible increase in cell death, which may result in a compensatory increase in the size of remaining viable cardiomyocytes. As PI3K(p110\textalpha) signaling is associated with an increased physiological hypertrophic response [408], non-diabetic mice expressing the dnPI3K transgene exhibited significantly reduced HW/TL, as well as diminished external LV diameter (measured on M-mode echocardiography). dnPI3K diabetic untreated mice also displayed reduced HW/TL, despite cardiomyocyte enlargement and elevated gene expression levels of ANP and β-myosin heavy chain. Their expression was further elevated in dnPI3K diabetic mice compared to Ntg diabetic mice. This is consistent with the hypothesis that diminished PI3K(p110\textalpha) signaling can further exacerbate hypertrophic responses under other pathological settings [407]. Coenzyme Q\textsubscript{10} administration significantly attenuated cardiomyocyte width and hypertrophic gene expression in Ntg diabetic animals, thus reinforcing the role of oxidative stress in the development of cardiomyocyte hypertrophy. Furthermore, coenzyme Q\textsubscript{10} was also effective in attenuating markers of hypertrophy in
dnPI3K diabetic mice, supporting the notion that reduced PI3K(p110α) signaling is associated with elevated oxidative stress, which can be reduced by enhancing antioxidant defence.

In addition to cardiomyocyte hypertrophy, both Ntg and dnPI3K diabetic mice presented with increased cardiac fibrosis in comparison to their respective non-diabetic controls. Coenzyme Q10 was also effective in attenuating collagen levels in both groups of diabetic mice. Collagen deposition measured on histology was not significantly exacerbated in the dnPI3K diabetic mice compared to the Ntg diabetic mice, in contrast to our previous observations [52]. I propose that this may be due to relatively low animal numbers in the current study (n=5/group). At present, there is a slight, but insignificant trend for collagen deposition to be increased in dnPI3K diabetic mice compared with Ntg diabetic mice; increasing animal numbers may confirm this relationship.

Oxidative stress has been implicated in the development and progression of diabetic cardiomyopathy [549, 553, 554]. Several studies have highlighted hyperglycemia-induced changes in redox state as a critical stimulator of cardiac dysfunction and remodeling. As the heart possesses lower antioxidant content relative to other organs, and generates a large amount of ROS due to the high concentration of mitochondria, it is particularly susceptible to oxidative damage [555]. The NADPH oxidase enzyme complex is a major source of •O2- in the heart [189]. In the present study, NADPH-drive •O2- generation was elevated in the Ntg diabetic untreated heart compared with non-diabetic controls, accompanied by a significant increase in systemic lipid peroxidation. Interestingly, dnPI3K diabetic untreated mice exhibited even higher levels of NADPH-driven •O2- generation in comparison to Ntg diabetic mice, and similarly elevated levels of lipid peroxidation. This finding was consistent with previous in vitro observations in neonatal ventricular cardiomyocytes and H9c2 cells (in which PI3K(p110α) activity was inhibited with A66), and also with in vivo findings [52]. A66-treated cells were associated with elevated levels of •O2- generation under high glucose conditions, whilst cells treated with IGF-1 displayed lower •O2- levels. Moreover, tempol, a SOD mimetic, was able to blunt the increase in •O2- generation induced by A66 treatment. Interestingly, dnPI3K non-diabetic mice also displayed an increase in •O2- levels in that study [52]; this was not apparent in the current study.

In the current study, I have also shown that exogenously administered antioxidants were effective in reducing •O2- generation and systemic lipid peroxidation in an in vivo
model of diminished PI3K(p110α) activity. Although not assessed in this study, our lab has
previously shown that the cardioprotection induced by increased PI3K(p110α) may partially
be attributed to an improvement in mitochondrial function, as depicted by an increase in
transcription factor A (Tfam) and mitochondrial uncoupling protein 3 (UCP3) expression.
This increase was absent in dnPI3K mice. As coenzyme Q₁₀’s main site of action is
considered to be at the mitochondria, where it functions primarily as an electron transporter
and a •O₂⁻ scavenger [556], coenzyme Q₁₀ treatment in dnPI3K diabetic animals may reduce
mitochondrial •O₂⁻ levels whilst simultaneously improving mitochondrial bioenergetics. This
may also explain why coenzyme Q₁₀ was not able to completely blunt the generation of •O₂⁻
in dnPI3K diabetic animals, despite being able to attenuate diastolic dysfunction and cardiac
remodeling; coenzyme Q₁₀ may be able to protect the diabetic heart beyond its antioxidant
capabilities. Exogenous coenzyme Q₁₀ administration has been shown to preserve normal
mitochondrial function following a cardiac ischemic insult [557]. Coenzyme Q₁₀
supplementation can also reverse mitochondrial dysfunction and consequently improve
endothelial function in patients with ischemic LV systolic dysfunction [558]. Thus, a possible
mechanism of coenzyme Q₁₀’s protection against diabetes-induced cardiac dysfunction in
mice expressing reduced PI3K(p110α) activity may be through its ability to improve
mitochondrial function. Antioxidant administration, in particular coenzyme Q₁₀, may be able
to compensate for the increased levels of oxidative stress and mitochondrial dysfunction
induced by diabetes, which contribute to both cardiac dysfunction and adverse morphological
changes. However, whether exogenously administered coenzyme Q₁₀ can taken up by the cell
and incorporated into the mitochondria requires further elucidation, and is beyond the scope
of the current study.
4.5 Limitations and future research

Due to limitations in myocardial tissue availability, we did not measure either plasma or tissue coenzyme Q\textsubscript{10} levels in the current study. As a result, the precise concentration of the exogenously administered coenzyme Q\textsubscript{10} was in the bloodstream or taken up by the heart remains unknown. Despite this shortcoming, we have previously shown in a separate study that coenzyme Q\textsubscript{10} levels were increased in the plasma and in renal tissue of coenzyme Q\textsubscript{10}-treated db/db mice, by approximately 61\% [559] (Refer to Appendix 3). Future studies are required to assess additional markers of diabetic cardiomyopathy, including apoptosis, mitochondrial dysfunction and upregulated NADPH oxidase subunit expression, and whether these can be reduced with coenzyme Q\textsubscript{10} administration in dnPI3K diabetic mice. Moreover, due to the large number of groups in the current study, time constraints limited the number of animals studied to only 5 per group. Increasing the numbers of animals in each group would increase statistical power.

The present study further supports the detrimental role of reduced PI3K(p110\textalpha) in exacerbating diabetic cardiomyopathy, suggesting that endogenous PI3K(p110\textalpha) is important for attenuating increased ROS generation and limiting diabetes-induced cardiac changes. Strategies to increase PI3K(p110\textalpha) may thus be useful in preventing LV •O\textsubscript{2}- generation and the development and progression of diabetic cardiomyopathy. Weeks et al. have recently developed a recombinant adeno-associated viral (rAAV) vector expressing caPI3K specifically in the heart, which significantly improved cardiac function in a mouse model of pressure-overload-induced hypertrophy [426]. rAAV6-caPI3K-based therapy, may also represent a novel strategy to combat heart disease in diabetic patients, by increasing cardioprotective signaling and reducing •O\textsubscript{2}- generation.
CHAPTER FIVE

General Discussion
The major goal of this PhD thesis was to assess whether the antioxidant, coenzyme Q\textsubscript{10}, could attenuate oxidative stress and pathological remodeling in a setting of T1DM and T2DM, and to assess the mechanisms responsible. Here I have shown that:

1) Coenzyme Q\textsubscript{10} attenuated cardiac and systemic oxidative stress in both the STZ T1DM model and \textit{db/db} T2DM model, and this was associated with attenuation of diastolic dysfunction, cardiomyocyte hypertrophy and cardiac fibrosis.

2) In the \textit{db/db} model, coenzyme Q\textsubscript{10} treatment attenuated the diabetes-induced increase in apoptosis and prevented a fall in the phosphorylation of Akt. Coenzyme Q\textsubscript{10} treatment was also accompanied by a reduction in blood pressure and improved glycemic control, which may have indirectly contributed to the improvements observed in the \textit{db/db} model.

3) In the STZ model, coenzyme Q\textsubscript{10} attenuated diabetes-induced upregulation of LV NADPH oxidase (Nox2, p22\textsuperscript{phox}, and p47\textsuperscript{phox} expression), LV UCP3 expression, and LV expression of pro-inflammatory mediators. These actions of coenzyme Q\textsubscript{10} actions were independent of glycemic control, body weight and blood pressure.

4) Coenzyme Q\textsubscript{10} showed comparable efficacy to an ACE-I in attenuating cardiac functional and structural abnormalities in T1DM and T2DM.

5) STZ diabetic mice with diminished expression of the cardioprotective kinase PI3K(p110\textalpha) in the heart exhibited worsened diastolic function and exaggerated cardiac remodeling compared with Ntg diabetic mice, which was accompanied by an exacerbated production of \textbullet{O}\textsubscript{2}. Coenzyme Q\textsubscript{10} was able to attenuate diastolic dysfunction and blunt the diabetes-induced remodeling of the heart in dnPI3K diabetic mice.

There remains a prevailing increase in the number of individuals diagnosed with T1DM and T2DM worldwide, many of whom will succumb to associated cardiovascular diseases [2]. New therapies for diabetic cardiomyopathy are greatly needed.

Hyperglycemia activates various signaling pathways, including PKC and RAAS, which culminates in increased production of damaging ROS [19, 245, 251, 384]. The diabetic heart is particularly susceptible to damage induced by enhanced ROS generation, due to its weakened antioxidant defence system [235, 560]. To determine whether exogenous antioxidant supplementation is effective in attenuating some of the functional and structural impairments in the T1DM and T2DM heart, I treated insulin-resistant \textit{db/db} mice and insulin-
deficient STZ mice with the antioxidant coenzyme Q\textsubscript{10}. I demonstrated that coenzyme Q\textsubscript{10} was able to reduce •O\textsubscript{2}- generation and attenuate cardiac dysfunction and structural remodeling in these diabetic animal models. As our previous studies had shown that increased PI3K(p110\alpha) signaling was protective, whilst reduced PI3K(p110\alpha) signaling was detrimental to the hearts of diabetic animals, I also performed studies to elucidate whether antioxidant administration was able to attenuate the impairments in the diabetic hearts of mice with diminished PI3K(p110\alpha).

5.1 Coenzyme Q\textsubscript{10} attenuated diabetes-induced oxidative stress

Hyperglycemia is a major etiological factor in the development and progression of diabetic cardiomyopathy, due to its involvement in the activation and dysregulation of various metabolic pathways [561]. Of particular interest to us is the stimulation of ROS generation, which in turns triggers pro-inflammatory processes, inducing cell damage and death [86, 562]. Insulin-resistant, obese \textit{db/db} mice exhibited significantly elevated levels of NADPH-driven cardiac •O\textsubscript{2}- generation as well as enhanced systemic lipid peroxidation. Insulin deficient, non-obese STZ mice similarly showed increased cardiac •O\textsubscript{2}- and lipid peroxidation levels, accompanied by an upregulation in the expression of the LV NADPH oxidase subunits Nox2, p47\textsubscript{phox} and p22\textsubscript{phox}. Studies in the past decade have identified NADPH oxidases as a major cardiovascular source of ROS [194, 260, 563]. NADPH oxidase activity was increased by a range of stimuli, many of which are relevant to the pathogenesis of diabetes and heart failure e.g. Ang II, ET-1, PKC and TNF-\alpha [194-197, 245]. An upregulation in NADPH subunit expression has been previously demonstrated in both experimental animal models of cardiovascular disorders and diabetes [194, 226, 563, 564].

In addition to NADPH oxidase activity, LV content of 3-NT was also elevated in our T1DM STZ mice. 3-NT protein formation is mediated by the production of ONOO\textsuperscript{-}, the reaction product of NO and •O\textsubscript{2}-; an increase in 3-NT in the myocardium thus suggests increased ONOO\textsuperscript{-} levels [565, 566] (Figure 5.1). Whilst it is well accepted that elevated 3-NT formation is involved in the pathogenesis of cardiovascular disease [566, 567] and diabetes [565, 568], 3-NT is also implicated in the development of pro-inflammatory disorders such as rheumatoid arthritis and systemic lupus erythemathodes, as well as in myocardial inflammation [569]. Indeed, in the hearts of my STZ animals, an upregulation of 3-NT was
coupled with augmented gene expression levels of the pro-inflammatory markers, TNFα and IL-1β. Both oxidative stress and inflammation are thus implicated in the development of diabetic cardiomyopathy in the hearts of the diabetic mice in the current study.

The perpetually high-energy demands of the heart are facilitated by mitochondrial metabolism, a tightly-regulated process that results in the coupling of oxygen to the phosphorylation of ADP into ATP. Hyperglycemia impairs mitochondrial function through disruption of the mitochondrial membrane and its membrane potential [570]. As a consequence, the mitochondria can be rendered less efficient, and there may be an increase in the leakage of single electrons to molecular oxygen to form •O₂-. Coenzyme Q₁₀ is an integral component of the mitochondrial electron transport chain, where it functions primarily to facilitate electron transfer from complexes I and II to complex III [556]. In its reduced state, known as ubiquinol, coenzyme Q₁₀ can act as an antioxidant, and reduce lipid peroxidation by preventing the production of lipid peroxyl radicals [556]. Indeed, in chapters 2 and 3, coenzyme Q₁₀ treatment in both diabetic db/db and STZ mice was able to significantly blunt the increase in systemic lipid peroxidation. Coenzyme Q₁₀ was similarly able to attenuate NADPH-driven LV •O₂− generation in treated db/db and STZ mice, as well as NADPH oxidase subunit expression, 3-NT levels and pro-inflammatory markers in STZ mice (Figure 5.1). Endogenous coenzyme Q₁₀ is concentrated in the heart, likely owing to high mitochondrial content as a result of high-energy expenditure required for cardiomyocyte contraction [328]. Diabetic patients exhibited reduced coenzyme Q₁₀ levels, as evidenced by overall diminished plasma total coenzyme Q₁₀/total cholesterol levels [338]. The severity of heart failure has also been closely correlated with the degree of coenzyme Q₁₀ deficiency. Through replenishing coenzyme Q₁₀ levels via exogenous supplementation, coenzyme Q₁₀ may protect the diabetic heart either through strengthening antioxidant defences [316, 571], or by improving mitochondrial bioenergetics and function [333, 557].

As noted previously, coenzyme Q₁₀ administration was able to reduce LV •O₂− generation in diabetic mice in our studies, reinforcing its role as a potent antioxidant. Interestingly, Lakomkin et al. demonstrated that coenzyme Q₁₀ protected the myocardium from an increased oxidative insult not only through its direct antioxidant mechanism, but also indirectly via increased protection of other antioxidant enzymes [571]. In our STZ mice, coenzyme Q₁₀ was also able to blunt the diabetes-induced increase in LV UCP3 expression (Figure 5.1). UCP3 is a marker of mitochondrial uncoupling and redox stress, and has previously been shown to be upregulated in the STZ diabetic heart [52, 572]. Our results thus
suggest that coenzyme Q_{10} is also protective at the level of the cardiac mitochondria; whether this protection is via its ability to reduce mitochondrial ROS or by improving mitochondrial bioenergetics still requires further investigation.

5.2 Coenzyme Q\textsubscript{10} attenuated diabetes-induced diastolic dysfunction and cardiac fibrosis

Several lines of evidence in both experimental and clinical settings have indicated diastolic dysfunction as one of the earliest and most consistent manifestations of diabetic cardiomyopathy [36, 46, 48, 164, 573]. In our studies, diastolic function, measured on several parameters using both Doppler echocardiography and cardiac catheterization, were impaired in both T1DM and T2DM mice. \textit{db/db} mice exhibited prolonged deceleration time and a trend for reduced E/A wave ratio, as well as elevated LVEDP, all indicative of impaired LV relaxation. These findings mirrored those observed in STZ mice, which additionally exhibited a reduction in LV \(-\text{dP/dt}\text{min}\). Importantly, coenzyme Q\textsubscript{10} was able to attenuate all these parameters in both treated \textit{db/db} and STZ mice. LV diastolic dysfunction has been linked to reduced antioxidant protection and decreased cardiopulmonary capacity in hypertensive patients with preserved systolic function [574]. In the current study, coenzyme Q\textsubscript{10} was also able to prevent the diabetes-induced reduction in SERCA2a expression in \textit{db/db} mice. The SERCA2a pump is responsible for the removal of cytosolic Ca\textsuperscript{2+} into the SR, which results in muscle relaxation. The rate of muscle relaxation is dependent upon reuptake of Ca\textsuperscript{2+} by SERCA2a. Diminished SERCA2a has been previously implicated in the development of impaired LV relaxation in diabetic cardiomyopathy [177, 178, 181]. Coenzyme Q\textsubscript{10}’s ability to attenuate diastolic dysfunction may also be linked to the preservation of normal SERCA2a expression levels.

Morphological changes in the heart, such as enhanced cardiac interstitial collagen and cardiomyocyte hypertrophy may be both a causative factor, and a secondary consequence of, diastolic impairment. Increased diastolic stiffness of the failing diabetic heart has been directly linked with excessive myocardial deposition of collagen [55, 59, 575]. Thus, coenzyme Q\textsubscript{10}’s ability to attenuate diastolic dysfunction may also be indirectly through reducing the generation of ROS which contributes to the hyperglycemia-induced accumulation of collagen. Several other studies using different antioxidants have also shown
a similar improvement in diastolic function with concomitant reduction in cardiac collagen deposition [576, 577]. However, due to its main site of action in the mitochondria, coenzyme Q\textsubscript{10} may have the additional benefit of improving mitochondrial bioenergetics, which translates to improved cardiac energetics and function [333, 557].

Myocardial fibrosis results from an increased deposition of ECM proteins, composed predominantly of fibrillar collagen, with smaller components of elastin, laminin and fibronectin [71]. In our study, LV collagen deposition was significantly elevated in db/db and STZ diabetic animals compared with non-diabetic shams, consistent with previous observations in the lab and clinic [55, 72, 238]. Hyperglycemia can directly stimulate collagen synthesis, and increase fibronectin and TGF-β gene expression in cultured cardiomyocytes [80, 82]. High glucose levels can also indirectly increase collagen levels by increasing ROS generation. Numerous studies have previously implicated oxidative stress as a critical regulator of pro-fibrotic processes [227, 236]. NADPH oxidase-dependent ROS production is a major contributor to the development of cardiac fibrosis. Ang II infusion in mice resulted in an increase in cardiac collagen content; this increase was absent in mice with targeted gene disruption of the Nox2 NADPH oxidase subunit [226]. Furthermore, a two-fold increase in NADPH oxidase activity was accompanied by a concomitant elevation in procollagen I and III expression in the LV of aldosterone-infused rats [225]. Indeed, in both STZ and db/db diabetic mice in the current study, coenzyme Q\textsubscript{10} was able to significantly attenuate cardiac collagen deposition levels, most likely through its ability to reduce NADPH-driven •O\textsubscript{2}− generation in the diabetic heart.

5.3 Coenzyme Q\textsubscript{10} reduced cardiomyocyte hypertrophy in diabetic mice

In addition to cardiac fibrosis, another structural hallmark of the diabetic heart is cardiomyocyte hypertrophy. LV hypertrophy in the clinical setting is a prominent risk factor for the development of MI, stroke and death from heart failure [63]. db/db untreated mice in my study exhibited numerous markers of LV hypertrophy, including increased HW/TL, enlarged cardiomyocytes and upregulated LV expression of the hypertrophic marker β-myosin heavy chain. STZ diabetic mice similarly expressed an increase in β-myosin heavy chain expression and cardiomyocyte width and area. Coenzyme Q\textsubscript{10} supplementation was able to attenuate all markers of cardiomyocyte hypertrophy in both our treated db/db and STZ
mice. This is consistent with previous studies that have implicated ROS as a driver of cardiac hypertrophy [188, 194, 224, 226].

Hyperglycemia is a major activator of pro-hypertrophic and pro-oxidant signaling processes including release of vasoactive peptides Ang II and ET-1, and activation of redox-sensitive protein kinases (i.e., MAPK) [192]. Antioxidant administration effectively attenuated both TNFα and Ang II-induced cardiomyocyte hypertrophy, through diminishing NADPH-dependent oxidase activity and reducing $\cdot$O$_2$- generation in cultured rat cardiomyocytes [194, 224]. Similarly, Ang II-induced cardiac hypertrophy was prevented in a Nox2$^{-/-}$ mouse model [226]. These studies collectively implicate the importance of ROS generation, in particular NADPH-driven $\cdot$O$_2$- generation, in the pathophysiology of cardiac hypertrophy. I propose that coenzyme Q$_{10}$’s ability to reduce NADPH oxidase-driven $\cdot$O$_2$- contributes to its protection against diabetes-induced cardiac hypertrophy in our studies.

Beyond neurohormonal activation of cardiac hypertrophy, pressure-overload is another common stimulator of hypertrophy in the diabetic heart [390]. Despite a significant elevation of systolic blood pressure in our $db/db$ mice, which could be reduced with coenzyme Q$_{10}$ administration, there was a very weak correlation between lowered blood pressure and diminished cardiomyocyte size. Furthermore, cardiomyocyte hypertrophy persisted despite an absence of hypertension in STZ mice, suggesting that the development of cardiomyocyte hypertrophy in these animal models were not the result of pressure- or volume-overload, and that coenzyme Q$_{10}$’s protection was merely secondary to its anti-hypertensive properties.

Importantly, the phosphorylation of Akt, a signaling molecule responsible for the modulation of numerous cellular processes including cell growth and survival, was reduced in the LV of untreated diabetic $db/db$ mice compared with non-diabetic shams. Akt lies downstream of cardioprotective IGF-1R-PI3K(p110$\alpha$) signalling. This signaling cascade is associated with physiological hypertrophy, which is beneficial for the heart, as opposed to pathological hypertrophy which is detrimental for cardiac function [63]. The fact that coenzyme Q$_{10}$ could prevent a fall in pAkt levels supports the theory that IGF-1R-PI3K(p110$\alpha$) may regulate oxidative processeses, and forms the basis on which my third experimental study is based. The role of coenzyme Q$_{10}$ in attenuating diabetic cardiomyopathy in dnPI3K mice is discussed below.
5.4 Beneficial effect of coenzyme Q_{10} in mice with reduced PI3K(p110α) activity

An extensive number of studies have examined the cardioprotective role of IGF-1R-PI3K(p110α) signaling in the failing heart. In separate studies, we have also demonstrated the benefits of increasing IGF-1R and PI3K(p110α) activity in attenuating the adverse functional and structural changes observed in the diabetic heart [51, 52]. IGF-1R-PI3K(p110α) signaling is associated with physiological hypertrophy, which is characterized by preserved or enhanced cardiac function, regular cardiac structure and a normal pattern of gene expression [407]. This is in contrast to pathological hypertrophy, which occurs in response to chronic pressure or volume overload in disease settings such as hypertension and MI, and is associated with increased fibrosis and reduced LV function. Recently, in a separate study, our lab identified a possible link between PI3K(p110α) and oxidative stress [52]. CaPI3K diabetic mice exhibited a significant attenuation in NADPH-driven LV •O_2^- generation with concomitant attenuation in fibrosis and apoptosis levels. In contrast, LV •O_2^- production was increased in dnPI3K diabetic mice compared to non-diabetic Ntg mice; this was accompanied by worsened cardiac function and increased fibrosis and apoptosis [52].

To determine whether coenzyme Q_{10} is able to attenuate LV •O_2^- generation, and prevent adverse remodeling and dysfunction even when PI3K(p110α) signaling is reduced, I administered coenzyme Q_{10} exogenously to dnPI3K mice. Coenzyme Q_{10}-treated dnPI3K STZ mice exhibited reduced cardiomyocyte size and collagen deposition, as well as better diastolic function compared with untreated diabetic dnPI3K mice. All these beneficial effects observed with coenzyme Q_{10} administration were associated with significantly reduced lipid peroxidation and LV •O_2^- generation in dnPI3K diabetic mice (Figure 5.1). These novel observations suggest that coenzyme Q_{10} is also effective in attenuating diastolic dysfunction and cardiac remodeling in a mouse model with diminished PI3K(p110α) activity compared with Ntg mice. Strategies to increase PI3K(p110α) signaling in the heart (ie. via caPI3K-based gene therapy [426]) may represent a novel strategy to improve cardiac function and structure in diabetic patients.
Figure 5.1 Summary of coenzyme Q\textsubscript{10}'s antioxidant actions in the cardiomyocyte

Elevated glucose levels can directly stimulate NADPH oxidase activity, resulting in increased generation of $\bullet{O}_2^-$. Ang II signaling can similarly increase $\bullet{O}_2^-$ production through activating NADPH oxidase. Mitochondrial $\bullet{O}_2^-$ production may also be increased in this setting, which subsequently increases the production of other ROS including H\textsubscript{2}O\textsubscript{2} and ONOO\textsuperscript{-}. Reduced PI3K(p110\textalpha) signaling under high glucose conditions can also exacerbate $\bullet{O}_2^-$ production. Coenzyme Q\textsubscript{10} can blunt the diabetes-induced increase in the activity of NADPH oxidase subunits, as well as reduce NADPH-driven $\bullet{O}_2^-$ generation. Furthermore, coenzyme Q\textsubscript{10} can reduce cardiac UCP3 levels and prevent the formation of 3NT. Coenzyme Q\textsubscript{10} is also effective in attenuating $\bullet{O}_2^-$ levels under a setting of diminished PI3K(p110\textalpha) in the diabetic heart. Consequently, coenzyme Q\textsubscript{10} administration is able to blunt diabetes-induced cardiac remodeling and diastolic dysfunction, and this appears to be directly related to its ability to reduce oxidative stress levels. Whether the mechanism differs in T1DM and T2DM will required further studies. All references in text.
5.5 Comparing the efficacy of coenzyme Q\textsubscript{10} with an ACE-I in preventing diabetic cardiomyopathy

Owing to the importance of the RAAS in regulating blood pressure, ACE-I were developed initially as anti-hypertensive agents [347]. This class of drug has since found use clinically for the treatment of various cardiovascular disorders, including AMI and congestive heart failure [348, 578, 579]. The HOPE trial also demonstrated the benefit of ACE-I treatment on the risk reduction of various cardiovascular events, including stroke, MI, congestive heart failure and cardiovascular death; this benefit was particularly striking in patients with diabetes [580]. ACE-I are thus considered the first-line therapy for patients with high cardiovascular risk [581]. In an attempt to compare the efficacy of coenzyme Q\textsubscript{10} with the efficacy of a clinically proven first-line ACE-I for the treatment of diabetic cardiomyopathy, I ran an additional group of ramipril-treated diabetic mice in the db/db and STZ studies. As expected, ramipril attenuated all the diabetes-induced adverse changes in the diabetic heart, including cardiomyocyte hypertrophy, cardiac fibrosis, cardiomyocyte apoptosis and diastolic dysfunction. This study shows, for the first time, that coenzyme Q\textsubscript{10} and ramipril exhibit comparable efficacy in attenuating both functional and structural impairments.

The mechanisms by which ramipril and coenzyme Q\textsubscript{10} exerts cardioprotection may be similar. A plethora of studies have described the role of Ang II in stimulating NADPH oxidase activity, thereby promoting the generation of \textbullet{O}_2\textsuperscript{-} [253, 582-584] (Figure 5.1). Inhibition of RAAS signaling may thus blunt the increase in \textbullet{O}_2\textsuperscript{-} generation, and protect the heart from oxidative damage. Indeed, ramipril-treated STZ and db/db diabetic mice exhibited a reduction in NADPH-driven LV \textbullet{O}_2\textsuperscript{-} generation. Despite the widespread use of ACE-Is clinically for the prevention and treatment of cardiovascular disorders, side effects amongst patients are relatively common. The most common side effect is cough, affecting 15-30\% of patients, resulting from an increase in bradykinin levels due to ACE inhibition [363, 364]. Although this side effect is non-life threatening to the patient, it may cause sufficient disturbance to justify discontinuation. Furthermore, a major contraindication to ACE-I therapy is pregnancy; prior studies have demonstrated that ACE-I use in the second and third trimester can cause fetal defects [585, 586]. In general, coenzyme Q\textsubscript{10} has been shown to be safe and well-tolerated in patients, even at high doses [587-589]. In addition to its cardioprotective properties, coenzyme Q\textsubscript{10} has also shown to provide protection against renal
dysfunction [559, 590]. These findings therefore suggest that coenzyme Q_{10} may be an effective alternative therapy to ACE-Is for the treatment of cardiomyopathy in diabetic patients who experience side effects related to ACE-I treatment. The goal of this study was not to delineate whether coenzyme Q_{10} administration was able to modulate the RAAS pathway. Further studies would be required to assess whether coenzyme Q_{10} provided any protection by regulating the RAAS pathway.

5.6 Future directions

Although this study has offered a comprehensive insight into the benefits of coenzyme Q_{10} in protecting the diabetic heart against diastolic dysfunction and remodeling, the mechanism and site of action which coenzyme Q_{10} exerts this protection still remains unclear. In my study, I have only indirectly shown that coenzyme Q_{10} executes its antioxidant actions both systemically and in cardiac tissue through its ability to reduce systemic lipid peroxidation and LV \cdot O_{2}^{-} generation. It would be important to confirm this by measuring the levels of coenzyme Q_{10} in plasma and in different organs, particularly the heart. Furthermore, in light of evidence that coenzyme Q_{10} levels are deficient in both diabetic and heart disease patients [334, 338], it would be of interest to assess whether exogenous administration can be taken up by the mitochondria and re-incorporated into the mitochondrial electron transport chain where it can scavenge \cdot O_{2}^{-} generated as a byproduct of ATP production. In a separate study, we have previously demonstrated that coenzyme Q_{10} levels were elevated in the plasma and renal tissue of coenzyme Q_{10}-treated mice by \sim 60\% [559]. In my three current studies, coenzyme Q_{10} was administered via three routes: dissolved in drinking water, orally gavaged and via intraperitoneal injection. Although it appears all three methods were effective in delivering sufficient drug to attenuate markers of diabetes-induced dysfunction and remodeling, it would be of interest to determine the method of delivery that results in the most efficient uptake of coenzyme Q_{10}, systemically and in cardiac tissue.

Another key characteristic of diabetic cardiomyopathy we are yet to examine is mitochondrial dysfunction. Mitochondrial dysfunction has been widely reported in the diabetic heart, proposed to be the result of fatty acid-induced mitochondrial uncoupling, mitochondrial ROS generation, impaired mitochondrial Ca^{2+}-handling and altered mitochondrial biogenesis [479, 591, 592]. Beyond its ability to reduce oxidative stress, coenzyme Q_{10} is also very well known for its role in improving mitochondrial bioenergetics.
Pre-treatment with coenzyme Q$_{10}$ prior to cardiac surgery in patients has been shown to improve the efficiency of mitochondrial respiration, alongside a significant increase in myocardial and cardiac mitochondrial coenzyme Q$_{10}$ level [333]. Chapter 3 has shown that cardiac expression of the mitochondrial uncoupling protein, UCP3, is upregulated in STZ diabetic mice, which was subsequently attenuated by coenzyme Q$_{10}$ administration. It would be of interest to further assess mitochondrial bioenergetics, via assessment of ATP production, proton leak rate, coupling efficiency, maximum respiratory rate, mitochondrial Ca$^{2+}$-handling etc., to determine whether exogenous coenzyme Q$_{10}$ supplementation can indeed improve mitochondrial respiration in the diabetic heart. Whether an improvement in mitochondrial energetics can translate to improved cardiac efficiency and enhanced function will also be important to determine.

My studies have thus far only examined the antioxidant properties of coenzyme Q$_{10}$ and its ability to prevent diastolic dysfunction and cardiac pathology. It will be important to assess whether coenzyme Q$_{10}$ treatment can reverse cardiac pathology and restore diastolic function in models of diabetic cardiomyopathy with pre-existing cardiac dysfunction and fibrosis.

5.9 Conclusion

Diabetes is one of the fastest growing chronic diseases of the 21st century, with more than 429 million adults proposed to be affected by the year 2030. Apart from an increased risk of developing microvascular and macrovascular conditions, diabetic patients are also susceptible to diabetic cardiomyopathy, characterized by early diastolic dysfunction and structural changes such as cardiomyocyte hypertrophy, cardiac fibrosis and apoptosis. Oxidative stress is frequently attributed to the development and progression of these adverse changes in the diabetic heart. Results presented in this thesis demonstrate that coenzyme Q$_{10}$ administration can attenuate the increase in oxidative stress, alongside attenuating cardiac dysfunction and structural defects. Considering that it is as effective as an ACE-I in protecting the heart against diabetes-induced damage, coenzyme Q$_{10}$ may be a clinically valuable alternative, or adjunct therapy, to standard care for the preservation of normal cardiac function in patients with diabetic cardiomyopathy.
REFERENCES


75. Yoon, Y.S., et al., *Progressive attenuation of myocardial vascular endothelial growth factor expression is a seminal event in diabetic cardiomyopathy - Restoration of


111. Putnam, K., et al., The renin-angiotensin system: a target of and contributor to dyslipidemias, altered glucose homeostasis, and hypertension of the metabolic


199. Qin, F.Z., M. Simeone, and R. Patel, *Inhibition of NADPH oxidase reduces myocardial oxidative stress and apoptosis and improves cardiac function in heart


288. Dakhale, G.N., H.V. Chaudhari, and M. Shrivastava, Supplementation of vitamin C reduces blood glucose and improves glycosylated hemoglobin in type 2 diabetes


Appendix 1: Cardiac-Specific IGF-1 Receptor Transgenic Expression Protects Against Cardiac Fibrosis and Diastolic Dysfunction in a Mouse Model of Diabetic Cardiomyopathy

Karina Huynh*, Julie R. McMullen*, Tracey L. Julius, Joon Win Tan, Jane E. Love, Nelly Cemerlang, Helen Kiriazis, Xiao-Jun Du and Rebecca H. Ritchie

*both authors contributed equally to the work
Cardiac-Specific IGF-1 Receptor Transgenic Expression Protects Against Cardiac Fibrosis and Diastolic Dysfunction in a Mouse Model of Diabetic Cardiomyopathy

Karina Huynh,* Julie R. McMullen,* Tracey L. Julius, Joon Win Tan, Jane E. Love, Nelly Cemerlang, Helen Kiriazis, Xiao-Jun Du, and Rebecca H. Ritchie

OBJECTIVE—Compelling epidemiological and clinical evidence has identified a specific cardiomyopathy in diabetes, characterized by early diastolic dysfunction and adverse structural remodeling. Activation of the insulin-like growth factor 1 (IGF-1) receptor (IGF-1R) promotes physiological cardiac growth and enhances contractile function. The aim of the present study was to examine whether cardiac-specific overexpression of IGF-1R prevents diabetes-induced myocardial remodeling and dysfunction associated with a murine model of diabetes.

RESEARCH DESIGN AND METHODS—Type 1 diabetes was induced in 7-week-old male IGF-1R transgenic mice using streptozotocin and followed for 8 weeks. Diastolic and systolic function was assessed using Doppler and M-mode echocardiography, respectively, in addition to cardiac catheterization. Cardiac fibrosis and cardiomyocyte width, heart weight index, gene expression, Akt activity, and IGF-1R protein content were also assessed.

RESULTS—Nontransgenic (Ntg) diabetic mice had reduced initial (E)-to-second (A) blood flow velocity ratio (E:A ratio) and prolonged deceleration times on Doppler echocardiography compared with nondiabetic counterparts, indicative markers of diastolic dysfunction. Diabetes also increased cardiomyocyte width, collagen deposition, and prohypertrophic and profibrotic gene expression compared with Ntg nondiabetic littermates. Overexpression of the IGF-1R transgene markedly reduced collagen deposition, accompanied by a reduction in the incidence of diastolic dysfunction. Akt phosphorylation was elevated ~15-fold in IGF-1R nondiabetic mice compared with Ntg, and this was maintained in a setting of diabetes.

CONCLUSIONS—The current study suggests that cardiac overexpression of IGF-1R prevented diabetes-induced cardiac fibrosis and diastolic dysfunction. Targeting IGF-1R–Akt signaling may represent a therapeutic target for the treatment of diabetic cardiac disease. Diabetes 59:1512–1520, 2010

Diabetes represents a major threat to human health, with global incidence projected to reach 300 million by 2025 (1,2). Cardiovascular complications including coronary heart disease and peripheral vascular disease are regarded as primary causes of morbidity and mortality in both type 1 and type 2 diabetes (3,4). In addition, clinical and experimental evidence supports the existence of a distinct diabetic cardiomyopathy, associated with adverse changes to the structure and function of cardiomyocytes, which can occur independent of macrovascular complications (5,6).

Left ventricular (LV) diastolic impairments (in LV filling, relaxation, and/or diastolic distensibility) are evident early in disease progression (7–9), often followed by later onset of systolic dysfunction (e.g., reduced LV ejection fraction and fractional shortening) (10). Functional alterations in the diabetic heart occur concomitantly with development of the structural abnormalities cardiomyocyte hypertrophy and cardiac fibrosis (11–13). In both type 1 and 2 diabetes, this cardiomyopathy is a prognostic indicator, particularly for mortality (14). New approaches to rescue LV remodeling and dysfunction specifically in diabetic myocardium are thus highly desirable.

Physiological heart growth or hypertrophy, which occurs during normal postnatal development and can be induced by exercise, is characterized by a normal cardiac structure and gene expression (15). In contrast, pathological hypertrophy is characterized by fibrosis, myofiber disarray, reduced cardiac output, and eventual heart failure (15). IGF-1, structurally and functionally related to insulin (16), plays a crucial role in stimulating physiological LV hypertrophy and conferring protection against cardiac dysfunction (17–19). The therapeutic potential of IGF-1 has thus been extensively examined in an array of cardiac pathologies, including heart failure and diabetes (18,19). In dilated cardiomyopathy, eccentric hypertrophy, and myocardial infarction (20–22), transgenic IGF-1 expression limited structural abnormalities such as myocyte necrosis and fibrosis (21). IGF-1 upregulation may also improve systolic function, via restoration of normal Ca2+ handling and increased cardiomyocyte contractility (17,20). Similar benefits have been proposed in diabetic myocardium (18,23), although in vivo cardiac functional studies have been limited. Furthermore, chronic IGF-1 may represent a flawed therapeutic approach for diabetes-induced LV dysfunction and remodeling, as both transgenic and pharmacological IGF-1 approaches significantly elevate...
systemic plasma IGF-1 concentrations and thus can have undesirable effects on nonmyocytes and other tissues. For instance, IGF-1 may induce fibroblast proliferation (24) and increase size of other organs including brain and kidney (17,25,26).

The current study seeks to circumvent the problem of potential noncardiac IGF-1 effects by using a cardiomyocyte-specific IGF-1R transgenic (Tg) mouse (27), which develops physiological cardiac hypertrophy and enhanced systolic function, without histopathology (27). Despite compelling evidence for IGF-1 preservation of cardiac function, the role of cardiac IGF-1R specifically in protecting LV function and structure in the context of diabetes-induced cardiomyopathy in vivo remains unexplored.

Thus, the present study tested the hypothesis that cardiomyocyte-specific IGF-1R protects against diabetes-induced LV diastolic dysfunction and remodeling, using a streptozotocin (STZ)-induced mouse model of diabetes in vivo.

RESEARCH DESIGN AND METHODS

Animal model. This study was conducted in accordance to National Health and Medical Research Council of Australia guidelines, and approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee. Heterozygote 7-week-old IGF-1R Tg mice and their age-matched nontransgenic (Ntg) male littermates (FVB/N background) were used. These mice were generated by cloning a cDNA insert for the human IGF-1R gene into a NcoI-digested α-myosin heavy chain promoter construct (originated from the laboratory of Dr. Seigo Izumo, Beth Israel Deaconess Medical Center and Harvard Medical School). The bacterial sequence was removed from the construct and injected into the male pronucleus of fertilized single-cell FVB/N embryos on an FVB/N background (no. 7) exhibiting a 20-fold increase in IGF-1R expression specifically in the myocardium, without changes in IGF-1 plasma or tissue levels, as previously described (27). Diabetes was induced by five consecutive daily intraperitoneal STZ injections (55 mg/kg, in 0.1 mol/l citrate buffer, pH 4.5, Sigma Aldrich, St. Louis MO); sham mice were administered an equivalent volume of citrate buffer (28). Saphenous vein blood glucose levels were measured every 2 weeks using a handheld glucometer (Accu-Check Advantage; Roche, Basel, Switzerland), with blood glucose levels exceeding 28 mmol/l considered diabetic. Diabetes then progressed for 8 weeks (13), i.e., to 15 weeks of age, and submandibular Saphenous vein blood glucose levels were measured every 2 weeks using a OneTouch安稳 meter (LifeScan, Stockholm, Sweden).

RESULTS

Induction of diabetes. STZ-induced diabetic Ntg and IGF-1R Tg mice exhibited marked hyperglycemia, on both plasma glucose and GHB in comparison with age-matched nondiabetic sham (Table 1), without genotype-dependent differences. Plasma insulin decreased in both Ntg and IGF-1R STZ mice (P < 0.001 on two-way ANOVA, n = 5–8, Table 1). Furthermore, plasma free fatty acids increased from basal (~0.4 mmol/l in Ntg sham mice, n = 2) to 1.90 ± 0.14 and 1.57 ± 0.15 mmol/l in Ntg STZ and IGF-1R Tg STZ mice (P < 0.001 on two-way ANOVA, both n = 3), respectively. Body weight and tibia length were comparable across the four groups at the end of the study. IGF-1R Tg mice exhibited greater heart mass than Ntg mice, by ≥30% whether normalized to body weight or tibia length (Table 1), as previously observed in this strain (27). Diabetes however did not significantly change net heart weight-to-body weight or heart weight-to-tibia length ratio, in either Ntg or Tg mice.

The basal portion of the ventricle was fixed in 10% neutral buffered formalin (Australian Biostain, Melbourne, Australia), and paraﬁn-embedded for histo-
IGF-1R prevents diabetes-induced cardiomyocyte hypertrophy. Cardiomyocyte width was significantly greater in Ntg diabetic mice versus Ntg sham (Fig. 1A and B), despite no net increase in heart weight–to–body weight or heart weight–to–tibia length ratios. IGF-1R Tg develop physiological cardiac hypertrophy with a 40% increase in myocyte volume (27). Consistent with the initial characterization of IGF-1R Tg mice, IGF-1R sham mice had enlarged cardiomyocytes versus Ntg sham. In contrast to the diabetes-induced increase in myocyte width in Ntg, diabetes failed to elicit any further increase in cardiomyocyte width in IGF-1R Tg mice (Fig. 1). In IGF-1R diabetic mice, IGF-1R sham mice had increased cardiomyocyte volume (27). Consistent with the initial characterization of IGF-1R Tg mice, IGF-1R sham mice had increased cardiac collagen deposition by 2.2 ± 0.3-fold in Ntg mice (P < 0.05; Fig. 3A and B). Cardiac collagen content in IGF-1R sham mice was similar to Ntg sham mice. Expression of the IGF-1R transgene significantly reduced the extent of cardiac fibrosis in diabetic mice. In contrast to Ntg animals, diabetes failed to increase collagen content in IGF-1R Tg hearts, where collagen deposition of IGF-1R diabetic mice was 1.2 ± 0.1-fold IGF-1R sham (P = not significant). Gene expression of the fibrotic marker pro–collagen III tended to increase in Ntg diabetic mice (2.4 ± 0.6-fold Ntg sham, P = 0.06; unpaired t test). Diabetes failed to upregulate pro–collagen III expression in IGF-1R Tg mice (pro–collagen III expression in IGF-1R diabetic mice was 0.87 ± 0.3-fold that of IGF-1R sham mice, P = not significant).

IGF-1R limits diabetes-induced cardiac fibrosis. On Sirius red–stained sections, diabetes significantly increased cardiac collagen deposition by 2.2 ± 0.3-fold in Ntg mice (P < 0.05; Fig. 3A and B). Cardiac collagen content in IGF-1R sham mice was similar to Ntg sham mice.

**TABLE 1**
Postmortem systemic analysis of Ntg and IGF-1R sham and diabetic mice (15 weeks of age)

<table>
<thead>
<tr>
<th></th>
<th>Ntg sham</th>
<th>Ntg diabetic</th>
<th>IGF-1R sham</th>
<th>IGF-1R diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose (mM)</td>
<td>8.5 ± 0.8</td>
<td>35.5 ± 1.1*</td>
<td>10.5 ± 1.0</td>
<td>35.4 ± 1.7*</td>
</tr>
<tr>
<td>GHb (%)</td>
<td>2.4 ± 0.2</td>
<td>9.0 ± 0.6*</td>
<td>2.3 ± 0.1</td>
<td>8.7 ± 0.5*</td>
</tr>
<tr>
<td>Plasma insulin (ng/ml)</td>
<td>1.3 ± 0.2</td>
<td>0.4 ± 0.0*</td>
<td>1.1 ± 0.2</td>
<td>0.6 ± 0.1*</td>
</tr>
<tr>
<td>BW (g)</td>
<td>30.1 ± 1.4</td>
<td>28.7 ± 0.6</td>
<td>30.7 ± 0.9</td>
<td>29.4 ± 0.8</td>
</tr>
<tr>
<td>HW (mg)</td>
<td>122.4 ± 6.3</td>
<td>116.2 ± 3.2</td>
<td>165.5 ± 3.3†‡</td>
<td>154.8 ± 5.2†‡</td>
</tr>
<tr>
<td>LW (mg)</td>
<td>154.7 ± 4.2</td>
<td>154.8 ± 4.9</td>
<td>151.0 ± 3.5</td>
<td>145.8 ± 7.4</td>
</tr>
<tr>
<td>TL (mm)</td>
<td>16.3 ± 0.2</td>
<td>16.5 ± 0.1</td>
<td>16.5 ± 0.6</td>
<td>16.5 ± 0.2</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>4.06 ± 0.08</td>
<td>4.05 ± 0.05</td>
<td>5.46 ± 0.10†‡</td>
<td>5.27 ± 0.09†‡</td>
</tr>
<tr>
<td>LW/BW (mg/g)</td>
<td>5.31 ± 0.25</td>
<td>5.11 ± 0.24</td>
<td>5.27 ± 0.15</td>
<td>5.01 ± 0.32</td>
</tr>
<tr>
<td>HW/TL (mg/mm)</td>
<td>7.48 ± 0.32</td>
<td>7.04 ± 0.18</td>
<td>9.97 ± 0.13†‡</td>
<td>9.38 ± 0.24†‡</td>
</tr>
<tr>
<td>LW/TL (mg/mm)</td>
<td>9.41 ± 0.32</td>
<td>9.39 ± 0.32</td>
<td>9.15 ± 0.23</td>
<td>9.13 ± 0.47</td>
</tr>
</tbody>
</table>

BW, body weight; HW, heart weight; LW, lung weight; TL, tibial length. *P < 0.05 vs. sham of the same genotype; †P < 0.05 vs. Ntg sham; ‡P < 0.05 vs. Ntg diabetic.

**FIG. 1.** Cardiomyocyte width is increased by diabetes and IGF-1R overexpression on histological analysis of ventricular cross-sections stained with H-E. A: Representative sections from the LV of sham and diabetic Ntg and IGF-1R mice (H-E stain; magnification ×400). Scale bars show 20 µm. B: Cardiomyocyte width pooled data. n = 8–12 in each group; *P < 0.001 vs. Ntg sham; †P < 0.001 vs. Ntg diabetic. (A high-quality digital representation of this figure is available in the online issue.)
and diabetic mice. After 8 weeks of diabetes in Ntg mice, diastolic dysfunction was evident compared with Ntg sham mice (decreased E wave velocity and E/A ratio, increased A wave velocity and a trend for decreased E wave velocity, on Doppler flow echocardiography, Fig. 4A–E). Diastolic function was similar in IGF-1R sham and Ntg sham. A fall in the E:A ratio was also observed in IGF-1R diabetic mice compared with IGF-1R sham mice. Diabetes reduced the E:A ratio by 44% in Ntg mice, and by 35% in IGF-1R Tg mice. However, the number of mice with normal diastolic function (arbitrarily defined as E/A > 1.5; Fig. 4C) appeared greater in IGF-1R diabetic mice compared with Ntg diabetic mice. Diabetes-induced prolongation in deceleration time was significantly ameliorated by IGF-1R upregulation (Fig. 4F). LV catheterization performed after 8 weeks of diabetes further confirmed the presence of diastolic dysfunction in Ntg diabetic mice compared with sham mice (LV-dP/dt decreased by $-20\%$, $P = 0.06$ on two-way ANOVA; Fig. 4G). In IGF-1R mice however, diabetes failed to significantly impact on either LV-dP/dt or LVEDP. Together, these data suggest that transgenic IGF-1R expression exhibited some protection against diabetes-induced diastolic dysfunction.

**Preserved Akt activity in diabetic IGF-1R transgenic mice.** It has previously been shown that the physiological hypertrophy observed in IGF-1R is associated with activation of the phospho-inositide 3-kinase (PI3K) (p110α)–Akt pathway (27) and Akt is a critical mediator of physiological heart growth (32). In the current study, Akt phosphorylation was elevated in ventricles of IGF-1R sham mice by more than 15-fold versus Ntg sham, from 0.27 ± 0.04 to 4.26 ± 0.54 arbitrary units (AU) in Ntg and IGF-1R sham mice, respectively (Fig. 5A), which was maintained in diabetes. Akt phosphorylation was 3.92 ± 0.82 AU in IGF-1R Tg STZ mice ($P$(genotype) < 0.001 on two-way ANOVA). Interestingly, a modest increase in phospho-Akt (approximately fourfold) was also observed in Ntg diabetic mice compared with Ntg sham; $P$ < 0.05 vs. Ntg sham; $P$ < 0.05 vs. Ntg diabetic. This elevation in phospho-Akt was also observed in IGF-1R sham mice compared with Ntg sham, with a 4.26 ± 0.54 AU increase in Akt phosphorylation in IGF-1R sham mice versus Ntg sham (from 0.27 ± 0.04 to 4.26 ± 0.54 AU), which was maintained in diabetes. This increase in Akt phosphorylation in IGF-1R sham mice compared with Ntg sham was not statistically significant, but it suggests that IGF-1R expression may play a role in promoting Akt phosphorylation in diabetic mice.

**FIG. 2.** A: Gene expression of β-myosin heavy chain (hypertrophic marker) was analyzed on real-time PCR and normalized against 18S expression. $n = 8–11$ for each group. B: Representative Northern blot showing total RNA. GAPDH was used to normalize for RNA loading (insert). Gene expression of ANP ($n = 9–10$ per group) and BNP ($n = 4–5$ per group) was assessed. Mean values for Ntg sham were normalized to 1. *$P < 0.05$ vs. Ntg sham; †$P < 0.05$ vs. Ntg diabetic. Sh, sham; D, diabetes.

**FIG. 3.** A: Representative sections from the LV of sham and diabetic Ntg and IGF-1R mice. Collagen deposition appears red (sirius red stain; magnification ×200). Scale bars show 40 µm. B: Quantitation of collagen area/total ventricular area pooled data. $n = 8–12$ in each group; *$P < 0.05$ vs. Ntg sham; †$P < 0.05$ vs. Ntg diabetic. A high-quality digital representation of this figure is available in the online issue.
gene expression of hypertrophy, including cardiomyocyte width as well as that diabetes increased several markers of cardiomyocyte growth. Our results show that diabetes is characterized by adverse structural changes to the heart. Key findings from the study provide evidence that IGF-1R specifically protects against diabetes-induced diabetic mice.

DISCUSSION

Diabetic cardiomyopathy is typically associated with diabetic dysfunction, myocardial fibrosis, and cardiomyocyte hypertrophy. The primary aim of the present study was to elucidate whether cardiac-specific upregulation of IGF-1R prevented cardiac dysfunction and adverse structural remodeling associated with pathophysiology of the diabetic heart. Key findings from the study provide evidence that IGF-1R specifically protects against diabetes-induced diabetic hypertrophy and diabetic fibrosis. IGF-1R-induced activation of the cell survival kinase Akt was maintained in a setting of diabetes, suggesting that beneficial actions of IGF-1R may be linked to promotion of physiological hypertrophy. The attenuation of diabetes-induced structural and functional cardiac damage mediated by IGF-1R signaling was independent of the degree of hyperglycemia and body weight.

IGF-1R promotes physiological hypertrophy and is protective in a setting of diabetes. Physiological cardiac growth occurs during postnatal development, and in response to long-term exercise training (15). This form of hypertrophy is associated with normal or enhanced cardiac function and normal cardiac structure. In contrast, pathological cardiac hypertrophy may arise as a compensatory mechanism against increased myocyte stress in many disease states. Any initial benefit is however over-riden by eventual derangement of myocardial architecture and deterioration of function (15). Diabetic cardiomyopathy is characterized by adverse structural changes to the heart, including increased cardiac fibrosis (pathological in nature) and cardiomyocyte hypertrophy. Our results show that diabetes increased several markers of cardiomyocyte hypertrophy, including cardiomyocyte width as well as gene expression of β-myosin heavy chain, ANP, and BNP as previously described (13). No changes in heart weight-to-body weight or heart weight-to-tibia length ratios were observed in Ntg diabetic mice, indicating no net increase in heart size. Diabetes-induced cardiomyocyte hypertrophy in the absence of gross LV mass increase may result from loss of cardiomyocytes via hyperglycemia-induced apoptosis and necrosis (23,33). Compensatory hypertrophy of the remaining viable cardiomyocytes, in addition to fibrosis, may then occur, and therefore any increase in overall size of each cardiomyocyte is counter-balanced by a reduction in the total number of myocytes (33). The upregulation of hypertrophic gene expression observed in this study is consistent with this. The causes underlying the diabetes-induced hypertrophic response is multifaceted, and may involve metabolic derangements such as impaired glucose handling (34) and oxidative stress (23,31).

Adaptive physiological hypertrophy in both humans (35) and animal models (27,36) has been associated with increased IGF-1 formation and/or activation. This hypertrophy is characterized by normal heart structure and absence of myocardial fibrosis (17,27). In contrast, pathological hypertrophy (15) is accompanied by cardiac fibrosis and detrimental changes in LV function (such as that observed in diabetes in the present study). Consistent with previous findings (27), we demonstrated significant enlargement in cardiomyocyte size in IGF-1R Tg mice, accompanied by increased heart weight-to-body weight and heart weight-to-tibia length ratios, increased LV wall thicknesses, and enhanced systolic function compared with Ntg mice. Surprisingly, markers commonly associated with pathological hypertrophy (β-myosin heavy chain, ANP, and BNP) were also increased in IGF-1R Tg compared with Ntg sham mice. However, this is consistent with both the original characterization and the recent contention as to whether fetal gene expression alone is an indicator of whether hypertrophy is “pathological” or “physiological” in nature (27,29). Histological analysis of cardiac structure and analysis of LV function might thus be more appropriate markers of the type of hypertrophy present. Importantly, diabetes failed to elicit further increases in any parameter of hypertrophy studied in IGF-1R mice, suggesting that the IGF-1R-induced physiological hypertrophic response was able to blunt the pathological hypertrophic response induced by diabetes. Expression of the IGF-1R transgene was previously shown to blunt pressure-overload induced pathological growth (27). Together, these results show that the IGF-1R-induced physiological hypertrophic response is associated with the preservation of normal cardiac structure and function in the setting of diabetes.

IGF-1R overexpression inhibits diabetes-induced cardiac fibrosis. Increased cardiac fibrosis is a common hallmark of the diabetic heart (11,37). The resultant increased LV stiffness further compromises the diabetic heart’s ability to contract and relax efficiently (14). In the present study, Ntg diabetic mice exhibited increased cardiac collagen deposition, consistent with previous findings in rats (38) and there was a tendency for elevated procollagen III gene expression compared with Ntg sham mice.

<table>
<thead>
<tr>
<th>TABLE 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Echocardiographic analysis of heart dimensions and function</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
</tr>
<tr>
<td>LV posterior wall thickness (mm)</td>
</tr>
<tr>
<td>LV end diastolic dimension (mm)</td>
</tr>
<tr>
<td>LV end systolic dimension (mm)</td>
</tr>
<tr>
<td>Fractional shortening (%)</td>
</tr>
</tbody>
</table>

*P < 0.05 vs. Ntg sham (baseline measurement); †P < 0.05 vs. Ntg sham and Ntg diabetic; ‡P < 0.05 vs. IGF-1R sham.
controls. IGF-1R overexpression alone was not associated with increased cardiac fibrosis on either marker (in line with its "physiological" phenotype), as collagen deposition in IGF-1R sham mice was similar to Ntg sham mice. Furthermore, in contrast to Ntg mice, no difference in collagen deposition was detectable between IGF-1R sham and IGF-1R diabetic mice. This suggests that IGF-1R overexpression confers protection against cardiac fibrosis induced by diabetes. Procollagen gene expression of the IGF-1R diabetic mice also tended to decrease compared with Ntg diabetic mice; however this change was not significant. The lack of cardiac fibrosis in diabetic IGF-1R Tg mice lends further support to the existing body of evidence that IGF-1R–induced hypertrophy is of a physiological nature, providing protection against adverse cardiac remodeling (27).

IGF-1R expression attenuates diastolic dysfunction. In the present study, diastolic and systolic function was measured both noninvasively (echocardiography) and invasively (LV catheterization). LV diastolic dysfunction is considered one of the earliest hallmarks of diabetic cardiomyopathy, occurring prior to the onset of systolic dysfunction (8,39). Doppler echocardiography revealed that Ntg diabetic mice exhibited a pronounced decrease in the transmitral E:A ratio, 60% of the mice had an E:A ratio >1.5, and deceleration time was prolonged, all indicative of diastolic dysfunction. This was further supported by LV catheterization, on which diabetes reduced LV-dP/dt, with a trend for increased LVEDP. Taken together, these results support the presence of diabetes-induced diastolic dysfunction, as previously described (8,30,39). Our observation is consistent with increased collagen deposition observed in Ntg diabetic mice; the onset of LV diastolic dysfunction may be secondary to changes in the extracellular matrix scaffold of the heart, resulting in increased cardiac stiffness (37). Although transgenic IGF-1R expression itself did not enhance diastolic function in the absence of diabetes, it prevented the diabetes-induced prolongation of LV deceleration time and increased the number of diabetic mice with normal diastolic function.
Diabetes also failed to elevate LVEDP in IGF-1R Tg mice. To our knowledge, we provide the first evidence that IGF-1R signaling specifically ameliorates diabetes-induced diastolic dysfunction and fibrosis in a mouse model in vivo.

We chose our 8-week study period of STZ-induced diabetes in mice to specifically assess the impact of IGF1-R on diastolic function in isolation. As such, no difference was detected in systolic function (assessed by both fractional shortening and LV+dP/dt) of Ntg diabetic mice compared with Ntg sham mice. The duration of diabetes is important, as systolic dysfunction in humans often manifests with a later onset than diastolic dysfunction (8,39). Our observations of diastolic dysfunction accompanied by a lack of systolic dysfunction in the diabetic heart are consistent with previous reports (40,41). However, species and strain clearly are important in dictating the presence of systolic dysfunction. Eight weeks of hyperglycemia is sufficient to induce systolic dysfunction in Wistar rats (9) and C57/BL6J mice (42). In contrast, 8 weeks of STZ-induced diabetes was not associated with impaired systolic function in our mice, which were generated on an FVB background. Importantly, FVB and C57 backgrounds show comparable cardiac (diastolic dysfunction, cardiomyocyte hypertrophy, and fibrosis) and blood glucose sensitivities to the STZ mouse model of diabetes (43,44). Consistent with previous reports (27), mice over-expressing IGF-1R exhibited enhanced systolic function (on echocardiography-derived fractional shortening) compared with Ntg mice, both prior to and after induction of diabetes (6 and 15 weeks of age, respectively). Because systolic function was preserved in our model of diabetes, IGF-1R Tg mice had increased systolic function compared with Ntg mice that was not impacted by the presence of diabetes.

**Cardioprotective signaling in the diabetic heart.** P3K (p110α) and Akt are important downstream effectors of IGF-1R signaling, critical for regulation of cardiac growth and survival (27,32,45,46). We observed a marked increase in ventricular phospho-Akt in IGF-1R Tg mice in the current study, in both the absence and presence of diabetes. Contrary to a previous report (47), Akt phosphorylation was increased in Ntg diabetic animals. This

![Image](diabetes.diabetesjournals.org)
effect was relatively modest, and may be a compensatory response to the functional and structural derangements in the diabetic heart, thus warranting further investigation. The IGF-1R-PI3K(p110α)–Akt pathway plays a crucial role in inducing physiological (but not pathological) hypertrophy (19,27,46). Maintained elevated Akt activity in hearts of IGF-1R, together with improvements in both diastolic function and cardiac structure compared with Ntg diabetic mice, suggest the protective effects of IGF-1R signaling in diabetes may be attributable to IGF-1R induction of physiological hypertrophy. These findings may provide an additional mechanism by which regular exercise (which also activates this cardioprotective cascade [15]) improves morbidity and mortality in patients with diabetes (48–50).

A previous study reported that IGF-1 ligand transgenic expression in an STZ mouse model of diabetes protected the heart against diabetes-induced diastolic dysfunction (23). However, collagen deposition was not examined, and mice were studied only for up to 30 days after STZ treatment. The authors acknowledged that limitations of their study included higher circulating IGF-1 levels, which may have contributed to their results, in addition to the relatively short period of diabetes. Kajstura et al. attribute some of the IGF-1–mediated protection to inhibition of apoptosis, necrosis, and angiotensin II–mediated oxidative stress (23). Here, we demonstrate that IGF-1R overexpression is beneficial even in the absence of elevated circulating IGF-1 levels, and over a longer period of time (8 weeks vs. 30 days). Furthermore, we provide another potential mechanism to explain the protective properties of the IGF-1R, i.e., activation of Akt.

To our knowledge, this study is the first to assess the cardioprotective effect of IGF-1R overexpression on diabetes-induced cardiac dysfunction and remodeling. We have demonstrated that overexpression of IGF-1R specifically in the heart attenuates diabetes-induced cardiac fibrosis, and prevented diastolic dysfunction. This cardioprotective effect may be mediated via maintenance of enhanced IGF-1R–PI3K(p110α)–Akt signaling, although further investigation is required. Given that cardiac complications are a major contributor to morbidity and mortality in both type 1 and type 2 diabetic patients, studies such as this may identify possible new therapeutic approaches toward reducing cardiac structural and functional damage induced by diabetes.

ACKNOWLEDGMENTS

This work was supported by the Diabetes Australia Research Trust and the National Health and Medical Research Council of Australia (NHMRC project ID526638). K.H. is supported by an Australian Postgraduate Award. J.R.M. was the recipient of a Career Development Award cofunded by the NHMRC and National Heart Foundation of Australia (317835/CR 04M1716) and is supported by an Australian Research Council Future Fellowship (FT0991657). X.-J.D. holds an NHMRC Senior Research Fellowship (ID472673). X.-J.D. holds an NHMRC Senior Research Fellowship (ID472673). X.-J.D. holds an NHMRC Senior Research Fellowship (ID472673). X.-J.D. holds an NHMRC Senior Research Fellowship (ID472673). X.-J.D. holds an NHMRC Senior Research Fellowship (ID472673). X.-J.D. holds an NHMRC Senior Research Fellowship (ID472673).

No potential conflicts of interest relevant to this article were reported.

Parts of this study were presented in abstract form at the 20th Scientific Sessions of the American Heart Association, Orlando, Florida, November 14–18, 2009.

We thank Keith Buxton for assistance with animal studies and MaryAnn Arnstein for helpful advice on histological analysis.

REFERENCES

IGF-IR LIMITS DIABETIC CARDIOMYOPATHY


Appendix 2: Enhanced phosphoinositide 3-kinase (p110α) activity prevents diabetes-induced cardiomyopathy and superoxide generation in a mouse model of diabetes


*both authors contributed equally to the work
Enhanced phosphoinositide 3-kinase (p110α) activity prevents diabetes-induced cardiomyopathy and superoxide generation in a mouse model of diabetes

R. H. Ritchie · J. E. Love · K. Huynh · B. C. Bernardo · D. C. Henstridge · H. Kiriazis · Y. K. Tham · G. Sapra · C. Qin · N. Cemerlang · E. J. H. Boey · K. Jandeleit-Dahm · X.-J. Du · J. R. McMullen

Received: 27 May 2012 / Accepted: 10 August 2012 / Published online: 22 September 2012
© Springer-Verlag 2012

Abstract

Aims/hypothesis Diabetic cardiomyopathy is characterised by diastolic dysfunction, oxidative stress, fibrosis, apoptosis and pathological cardiomyocyte hypertrophy. Phosphoinositide 3-kinase (PI3K) (p110α) is a cardioprotective kinase, but its role in the diabetic heart is unknown. The aim of this study was to assess whether PI3K (p110α) plays a critical role in the induction of diabetic cardiomyopathy, and whether increasing PI3K (p110α) activity in the heart can prevent the development of cardiac dysfunction in a setting of diabetes.

Methods Type 1 diabetes was induced with streptozotocin in adult male cardiac-specific transgenic mice with increased PI3K (p110α) activity (constitutively active PI3K [p110α], caPI3K) or decreased PI3K (p110α) activity (dominant-negative PI3K [p110α], dnPI3K) and non-transgenic (Ntg) mice for 12 weeks. Cardiac function, histological and molecular analyses were performed.

Results Diabetic Ntg mice displayed diastolic dysfunction and increased cardiomyocyte size, expression of atrial and B-type natriuretic peptides (Anp, Bnp), fibrosis and apoptosis, as well as increased superoxide generation and increased protein kinase Cβ2 (PKCβ2), p22^phox and apoptosis signal-regulating kinase 1 (Ask1) expression. Diabetic pnPI3K mice displayed an exaggerated cardiomyopathy phenotype compared with diabetic Ntg mice. In contrast, diabetic caPI3K mice were protected against diastolic dysfunction, pathological cardiomyocyte hypertrophy, fibrosis and apoptosis. Protection in diabetic caPI3K mice was associated with attenuation of left ventricular superoxide generation and increased protein kinase Cβ2 (PKCβ2), p22^phox and apoptosis signal-regulating kinase 1 (Ask1) expression. Diabetic dnPI3K mice displayed an exaggerated cardiomyopathy phenotype compared with diabetic Ntg mice. In contrast, diabetic caPI3K mice were protected against diastolic dysfunction, pathological cardiomyocyte hypertrophy, fibrosis and apoptosis. Protection in diabetic caPI3K mice was associated with attenuation of left ventricular superoxide generation, attenuated Anp, Bnp, PKCβ2, Ask1 and p22^phox expression, and elevated Akt. Further, in cardiomyocyte-like cells, increased PI3K (p110α) activity suppressed high glucose-induced superoxide generation and enhanced mitochondrial function.

Conclusions/interpretation These results demonstrate that reduced PI3K activity accelerates the development of diabetic cardiomyopathy, and that enhanced PI3K (p110α) activity can prevent adverse cardiac remodelling and dysfunction in a setting of diabetes.

Keywords Diabetes · Fibrosis · Hypertrophy · Left ventricular function · Myocardium · NADPH oxidase · PI3K · PKCβ2 · Reactive oxygen species
Introduction

The incidence of diabetes is rising globally and diabetes is predicted to affect 439–472 million adults by 2030 [1, 2]. Diabetes is associated with increased mortality and morbidity, attributed largely to cardiovascular and kidney disease [3, 4]. Diabetic cardiomyopathy, both independent of and exacerbated by coexistent atherosclerosis, coronary disease and renal dysfunction, is characterised by structural and functional remodelling, including diastolic dysfunction, fibrosis, apoptosis and pathological cardiomyocyte hypertrophy [3, 4]. Oxidative stress, an imbalance between reactive oxygen species (ROS) generation and endogenous antioxidant capacity, is triggered in cardiomyocytes by hyperglycaemia. This contributes to the development and progression of diabetic cardiomyopathy [5–8]. The superoxide-generating enzyme NADPH oxidase is considered a major source of ROS in the heart [9].

Current glucose-lowering agents do not consistently reduce cardiovascular risk, and some may even increase it [10, 11]. Thus, new therapeutic strategies to protect the heart in the setting of diabetes are greatly needed. We have previously demonstrated that increased activation of phosphoinositide 3-kinase PI3K(p110α) in the heart is protective in pathological settings in which the heart is subjected to a localised cardiac insult such as aortic constriction (pressure overload) or coronary artery ligation (myocardial infarction) [12–14]. However, whether PI3K (p110α) can protect the heart against diabetic cardiomyopathy caused by global hyperglycaemia is unknown. Furthermore, PI3K has previously been linked to ROS inhibition in the kidney [15], but whether PI3K can regulate cardiac ROS has not been elucidated.

Multiple signalling proteins/cascades are dysregulated in the diabetic heart, including extracellular signal-regulated kinase 1 (ERK1), signal transducer and activator of transcription 3 (STAT3) and AKT (a key downstream target of PI3K) [4, 16–19]. A critical step in developing better therapeutics is to understand which signalling events represent key mechanisms in the development of cardiomyopathy in a setting of hyperglycaemia. The goal of the current study was to assess whether PI3K(p110α) plays a critical role in the induction of diabetic cardiomyopathy, and whether increasing PI3K(p110α) activity in the mouse heart prevents the development of diabetes-induced left ventricular (LV) remodelling and dysfunction.

Methods

Mouse models The Alfred Medical Research and Education Precinct Animal Ethics Committee approved animal care and experimentation. Cardiomyocyte-specific transgenic (Tg) mice (FVB/N background) with increased PI3K (p110α) activity (constitutively active PI3K [p110α], caPI3K) or decreased PI3K(p110α) activity (dominant-negative PI3K [p110α]), dnPI3K were originally generated and provided by S. Izumo (Beth Israel Deaconess Medical Center, Boston, MA, USA) [20]. Compared with non-transgenic (Ntg) mice, caPI3K-Tg mice have elevated cardiac PI3K(p110α) activity, normal cardiac function and hearts that are 20% larger (physiological hypertrophy) [20], while dnPI3K-Tg mice have reduced cardiac PI3K(p110α) activity, normal cardiac function and hearts that are 20% smaller.

Male caPI3K-Tg, dnPI3K-Tg and Ntg mice were randomly allocated into diabetic and non-diabetic groups. At 6–7 weeks of age, mice received five consecutive daily i.p. injections of streptozotocin (STZ, 55 mg/kg body weight, in 0.1 mol/l citrate buffer, pH4.5; Sigma-Aldrich, St Louis, MO, USA) to induce diabetes or five consecutive daily i.p. injections of citrate buffer vehicle of equivalent volume (non-diabetic group) [21]. Diabetes was confirmed by measuring blood glucose every 2 weeks from saphenous vein whole blood using a glucometer (ACCU-CHEK Advantage; Roche, Basel, Switzerland). The upper limit of detection for
blood glucose readings was 33.3 mmol/l; readings at this level were entered as 33.3 mmol/l and confirmed by subsequent submandibular bleed from conscious mice for assessment of the final plasma glucose level (Austin Pathology Service, Heidelberg, VIC, Australia). Mice with blood glucose levels exceeding 28 mmol/l were considered diabetic. Diabetes progressed untreated for 12 weeks. At the endpoint of the study (18–19 weeks of age), whole blood was collected by cardiac puncture after cardiac catheterisation for analysis of HbA1c (Austin Pathology Service).

**LV function** Echocardiography (two-dimensional M-mode and Doppler flow) was performed in anaesthetised mice (ketamine/xylazine/atropine: 100/10/1.2 mg/kg i.p.) prior to catheterisation and mouse dissection (18–19 weeks of age) utilising a Philips iE33 ultrasound machine (North Ryde, NSW, Australia) with a 15 MHz linear array transducer, as previously described [21]. LV posterior wall thickness, LV chamber dimensions and fractional shortening were assessed. LV filling was assessed on transmitral Doppler echocardiography, the ratio of initial (E) and second (A) blood flow velocities (E/A ratio), E velocity deceleration time and isovolumic relaxation time (IVRT).

Haemodynamic variables were examined after 12 weeks of diabetes by catheterisation. Arterial pressures, LV systolic pressure, LV end-diastolic pressure (LVEDP), maximum and minimum (dp/dt_min) rates LV pressure change and heart rate were measured in anaesthetised mice (ketamine/xylazine/atropine: 100/10/1.2 mg/kg i.p.) using a 1.4 Fr Millar MIKRO-TIP catheter and a PowerLab System (AD Instruments, Bella Vista, NSW, Australia) [21].

**Tissue collection and histological and immunohistochemical analyses** Following cardiac catheterisation, heart, lung and tibia were collected [21]. Ventricular sections (4 μm) were stained with haematoxylin and eosin for assessment of cardiomyocyte width or with 0.1% Picrosiris Red for assessment of collagen deposition, as previously described [21]. Apoptosis was detected in paraffin-embedded ventricular sections (4 μm) by TUNEL staining (CardioTACS In Situ Apoptosis Detection Kit; Trevigen, Gaithersburg, MD, USA). Apoptotic nuclei were stained blue and quantified as a percentage of non-apoptotic nuclei (counterstained red with Nuclear Fast Red) and expressed as a fold-change of non-diabetic Ntg mice.

For immunohistochemical examination of p22-phox, paraffin-embedded ventricular sections (4 μm) were deparaffinised and rehydrated, and antigen retrieval was induced by heat (100°C) in a citrate buffer (10 mmol/l citric acid, 0.05% Tween-20, pH 6.0). Endogenous peroxidase activity was quenched (3% H2O2 [vol./vol.]) and sections were blocked (15 μl/ml normal goat serum in 1% BSA) and stained with p22-phox (sc-20781, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight, followed by biotinylated secondary goat α-rabbit IgG (1:200;Vector Laboratories, Burlingame, CA, USA). After exposure to VECTASTAIN Elite ABC avidin-biotinylated horseradish peroxidase complex (Vector Laboratories), the chromogenic reaction was carried out with Sigma-FAST diaminobenzidine tablets (Sigma-Aldrich). Sections were counterstained with haematoxylin. Positive p22-phox stained brown and was graded by a blinded observer as follows: score 0, negative stain; score 1, weak; score 2, moderate; score 3, strong/intense.

**Superoxide generation** Generation of superoxide was assessed in fresh LV tissue, neonatal rat ventricular myocytes (NRVM) or H9c2 cells using lucigenin-enhanced chemiluminescence, as previously described [22]. Primary NRVM [22] or H9c2 cells (Cell Bank Australia, Westmead, NSW, Australia) were plated for 72 or 18 h, respectively, in 96 well OptiView plates (50,000 and 5,000 cells/well, respectively, 37°C, 5% CO2), serum-deprived for approximately 18 h and pretreated with or without a specific PI3K(p110α) inhibitor (A66 [23]), 1 μmol/l), IGF1 (10 nmol/l) and/or tempol (100 μmol/l) for 24 h prior to 24 h treatment with or without H2O2 (100 μmol/l) in high glucose (HG, 25 mmol/l) or low glucose (LG, 5.56 mmol/l) media, as specified.

**Mitochondrial function** H9c2 cells were seeded into Seahorse plates (10,000 cells/well in HG: 25 mmol/l), serum-deprived overnight and treated with IGF1 (10 nmol/l) and/or A66 (10 μmol/l) for 24 h, followed by assessment of mitochondrial function (Seahorse Bioscience XF Analyser, North Billerica, MA, USA). Following basal respiration measurements, cells were sequentially treated with (all 1 μmol/l) oligomycin (ATP synthase inhibitor), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP, proton ionophore) and antimycin A/rotenone combination injection (inhibitors of complex I and III), and changes in respiration were recorded. Treatments were analysed as ten replicates over two independent experiments (plates) and data were pooled to give average values for each treatment. Basal respiration, proton leak, ATP turnover, spare respiratory capacity and maximal respiratory capacity were calculated from these analyses.

**RNA and protein extraction** RNA and protein were extracted from frozen ventricle samples as previously described [21].

**Northern blot analysis** Northern blot analysis was performed as previously reported [20]. Total RNA (10 μg) was electrophoresed in 1.3% denaturing formaldehyde-agarose gels and blotted onto Hybond–N membranes (GE Healthcare, Rydalmere, NSW, Australia). Membranes were probed with Anp (also known as Nppa), Bnp (also known as Nppb), p22-phox (also known as Cyba), Ask1 (also known as
Western blotting For pAKT, blots were probed with anti-phospho (p)AKT antibody (Ser473, Cell Signaling Technologies, Danvers, MA, USA: 9271, 1:500) followed by anti-AKT antibody (Cell Signaling: 9272, 1:1,000). For BAX (Bcl-2-associated X protein) and BCL2 (B cell lymphoma 2), blots were probed with anti-BAX antibody (Cell Signaling: 2772, 1:1,000) followed by anti-BCL2 antibody (Cell Signaling: 2876, 1:1,000). For protein kinase C β2 (PKCβ2) and uncoupling protein 3 (UCP3), blots were probed with anti-PKCβ2 (sc-210, 1:200; Santa Cruz Biotechnology) and anti-UCP3 antibody (PA1-065, 1:1,000; ABR Affinity BioReagents, Golden, CO, USA), both followed by anti-GAPDH antibody (sc-32233, 1:5,000; Santa Cruz Biotechnology).

Statistical analysis Results are presented as mean ± standard error. Differences between groups were compared using one-way ANOVA followed by the Fisher’s protected least significant difference, unless otherwise specified. p<0.05 was considered significant.

Results

Induction of diabetes in Ntg and PI3K Tg mice STZ induced a comparable degree of diabetes in Ntg, dnPI3K and caPI3K mice. Blood glucose, plasma glucose and HbA1c levels increased to a similar extent in STZ-treated mice from each genotype compared with citrate-treated non-diabetic controls (Table 1). No genotype-dependent differences in glycaemia, body weight or tibial length (TL) were observed (Table 1).

Increased PI3K(p110α) activity prevents diabetes-induced increases in cardiomyocyte size, fibrosis and apoptosis

Cardiomyocyte hypertrophy, fibrosis and apoptosis all contribute to the pathogenesis and progression of diabetic cardiomyopathy [24, 25]. Induction of diabetes in Ntg mice for 12 weeks had no impact on heart weight (HW; see HW/TL; Table 1) but was associated with increased cardiomyocyte width (≥13%; Fig. 1a–c), increased Anp and Bnp cardiac gene expression (markers of pathological hypertrophy and/or cardiac stress; Fig. 1d), cardiac fibrosis (Fig. 2a) and increased apoptosis (Fig. 2b). A diabetes-induced increase in cardiomyocyte size in the absence of a parallel increase in heart size is consistent with previous findings [21] and can be attributed to increased cell death in the diabetic heart (Fig. 2b) [26].

Under basal/non-diabetic conditions, caPI3K-Tg mice displayed physiological cardiac hypertrophy associated with larger cardiomyocytes compared with Ntg, but no increase in Anp or Bnp expression, as previously reported (Table 1, see HW, HW/TL; Fig. 1a, b, d) [20]. In contrast, non-diabetic dnPI3K-Tg mice had smaller hearts, smaller cardiomyocytes and elevated Anp and Bnp expression (Table 1, Fig. 1a, b, d) [20]. Neither caPI3K-Tg nor dnPI3K-Tg mice showed evidence of fibrosis or apoptosis under basal/non-diabetic conditions (Fig. 2a, b). Diabetes had no effect on HW/TL in caPI3K-Tg or dnPI3K-Tg mice (Table 1). However, PI3K activity had a significant impact on cardiomyocyte size, fibrosis and apoptosis in a setting of diabetes that differed from diabetic Ntg mice. Expression of the dnPI3K transgene was associated with an exaggerated diabetes-induced increase in cardiomyocyte size compared with diabetic Ntg mice.

**Table 1** Systemic and morphological characteristics of Ntg and PI3K non-diabetic and diabetic mouse models

<table>
<thead>
<tr>
<th>Variable</th>
<th>Ntg Non-diabetic</th>
<th>Ntg Diabetic</th>
<th>caPI3K Non-diabetic</th>
<th>caPI3K Diabetic</th>
<th>dnPI3K Non-diabetic</th>
<th>dnPI3K Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose (mmol/l)</td>
<td>11.3±0.3</td>
<td>12.3±0.1*</td>
<td>11.3±0.5</td>
<td>33.3±0.0*</td>
<td>11.0±0.7</td>
<td>32.8±0.6*</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>8.6±0.4 (n=6)</td>
<td>33.3±1.7* (n=10)</td>
<td>7.9±0.9 (n=5)</td>
<td>34.1±1.7* (n=6)</td>
<td>8.4±0.3</td>
<td>31.2±2.1*</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>3.8±0.5 (n=6)</td>
<td>9.3±0.3* (n=10)</td>
<td>3.9±0.7 (n=5)</td>
<td>8.9±0.3* (n=5)</td>
<td>3.2±0.3</td>
<td>9.9±0.4*</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>17.7±5.3 (n=6)</td>
<td>78.0±3.7* (n=10)</td>
<td>18.7±7.3 (n=5)</td>
<td>74.2±3.6* (n=5)</td>
<td>11.8±3.7</td>
<td>84.2±3.9*</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>32.9±1.3</td>
<td>29.8±0.8</td>
<td>31.2±1.6</td>
<td>29.3±0.8</td>
<td>30.2±1.3</td>
<td>28.7±1.0</td>
</tr>
<tr>
<td>TL (mm)</td>
<td>16.7±0.1</td>
<td>16.5±0.1</td>
<td>16.6±0.2</td>
<td>16.2±0.2</td>
<td>16.3±0.3</td>
<td>16.2±0.2</td>
</tr>
<tr>
<td>HW (mg)</td>
<td>133.1±3.4</td>
<td>122.3±4.2</td>
<td>105.0±7.2*</td>
<td>98.9±2.3*</td>
<td>147.2±6.0*</td>
<td>149.3±6.4*</td>
</tr>
<tr>
<td>Lung weight (mg)</td>
<td>148.0±3.8</td>
<td>155.3±4.6</td>
<td>155.6±8.8</td>
<td>153.0±6.9</td>
<td>140.7±4.5</td>
<td>139.5±5.9</td>
</tr>
<tr>
<td>HW/TL (mg/mm)</td>
<td>7.96±0.18</td>
<td>7.41±0.23</td>
<td>6.31±0.38*</td>
<td>6.10±0.10*</td>
<td>9.05±0.31*</td>
<td>9.19±0.31*</td>
</tr>
</tbody>
</table>

* p<0.05 vs non-diabetic mice of the same genotype; † p<0.05 vs Ntg non-diabetic mice; ‡ p<0.05 vs dnPI3K non-diabetic mice; § p=0.06 vs Ntg non-diabetic mice

© Springer
with Ntg mice (≈35% in dnPI3K vs ≈13% in Ntg mice), whereas expression of the caPI3K transgene prevented any significant increase in cardiomyocyte size in a setting of diabetes (Fig. 1a–c). There was no increase in Anp or Bnp expression in the hearts of diabetic caPI3K-Tg mice, consistent with the suggestion that increased PI3K activity prevents pathological cardiomyocyte growth (Fig. 1d). Furthermore, fibrosis, apoptosis and the BAX/BCL2 ratio (apoptotic marker) were significantly increased in the hearts of diabetic dnPI3K-Tg but not diabetic caPI3K-Tg mice (Fig. 2a–c). Increased apoptosis in diabetic dnPI3K-Tg mice is likely to account for the absence of an increase in HW/TL despite the increase in cardiomyocyte size in diabetic dnPI3K-Tg mice. Protection against fibrosis and apoptosis in caPI3K diabetic hearts was associated with enhanced AKT phosphorylation (Fig. 2d). pAKT/total AKT was not elevated in Ntg or dnPI3K hearts under non-diabetic or diabetic conditions. Gene expression of Pim1 (a kinase that mediates protection downstream of AKT) was elevated in Ntg and caPI3K hearts in response to diabetes, but not in dnPI3K hearts (Fig. 2e).

Enhanced PI3K(p110α) activity protects the heart against diabetes-induced cardiac dysfunction. Non-diabetic caPI3K-Tg mice had thicker LV walls than non-diabetic Ntg and dnPI3K-Tg mice, reflecting the development of physiological hypertrophy (Table 2, see LV posterior wall thickness), as previously shown [20]. In contrast, dnPI3K-Tg mice had thinner walls than Ntg mice (Table 2). At comparable heart rates, there were no significant differences in systolic or diastolic function in non-diabetic Ntg, caPI3K-Tg or dnPI3K-Tg mice (Table 2). The model of diabetes used in the current study has previously been shown to induce diastolic but not systolic dysfunction [21], consistent with the clinical context of the diabetic heart [3, 4]. In the current study, diabetes did not suppress fractional shortening in Ntg mice; interestingly fractional shortening was significantly higher in diabetic caPI3K-Tg than in diabetic Ntg and diabetic dnPI3K-Tg mice (Table 2). The maximum rate of LV pressure change (a marker of LV systolic function) was not different between the six groups (Table 2).

Diabetic Ntg mice displayed diastolic dysfunction, as shown by reduced LV dP/dtmin (~10%, Fig. 3a) and elevated LVEDP (~87%, Fig. 3b). The increased LVEDP was exaggerated in diabetic dnPI3K-Tg compared with non-diabetic dnPI3K-Tg mice (~119%), and was greater than that found in diabetic Ntg mice (Fig. 3b). There was also a fall in dp/dtmin in diabetic dnPI3K-Tg compared with non-diabetic...
Diabetic conditions. By contrast, superoxide generation was assessed in NRVM and caPI3K-Tg hearts, but was not significantly increased in diabetic caPI3K-Tg mice (Fig. 3c). A large body of evidence has demonstrated that hyperglycaemia leads to activation of PKCβ2 in the heart and subsequent activation of NADPH oxidase [29]. PKCβ2 protein production was increased in diabetic Ntg hearts compared with non-diabetic Ntg hearts (Fig. 4a) and increased further in diabetic dnPI3K hearts, but was not significantly increased in diabetic caPI3K hearts (Fig. 4b). Gene expression of \( p22^{phox} \) and Ask1 was also elevated in hearts of diabetic Ntg vs non-diabetic Ntg mice (Fig. 4c). As observed with LV superoxide generation, \( p22^{phox} \) and Ask1 expression levels were elevated in hearts of diabetic caPI3K-Tg mice (Fig. 4c). Immunochemistry analysis of \( p22^{phox} \) production was consistent with gene expression data (Fig. 4d).

Superoxide generation was also assessed in NRVM and the cardiomyoblast H9c2 cell line to assess whether acute activation of PI3K(p110α) with IGF1 (an upstream regulator) would inhibit HG-induced superoxide (as occurred in the caPI3K heart), and whether acute inhibition of PI3K

Increased PI3K(p110α) activity prevents superoxide generation in a setting of diabetes and can improve mitochondrial function Hyperglycaemia promotes ROS production in many tissues, contributing to the pathogenesis of multiorgan damage in diabetes [7]. The superoxide-generating NADPH oxidase is a key source of ROS in the heart [27, 28]. Diabetes induced an increase in cardiac superoxide generation by approximately 90% in Ntg compared with non-diabetic Ntg mice (Fig. 4a). Interestingly, superoxide was increased to a similar degree in non-diabetic dnPI3K-Tg mice and there was no further increase in a setting of diabetes. By contrast, superoxide generation was similar in hearts from non-diabetic Ntg and caPI3K-Tg mice, and there was no increase in hearts of diabetic caPI3K-Tg mice (Fig. 4a). A large body of evidence has demonstrated that hyperglycaemia leads to activation of PKCβ2 in the heart and subsequent activation of NADPH oxidase [29]. PKCβ2 protein production was increased in diabetic Ntg hearts compared with non-diabetic Ntg hearts (Fig. 4a) and increased further in diabetic dnPI3K hearts, but was not significantly increased in diabetic caPI3K hearts (Fig. 4b). Gene expression of \( p22^{phox} \) and Ask1 was also elevated in hearts of diabetic Ntg vs non-diabetic Ntg mice (Fig. 4c). As observed with LV superoxide generation, \( p22^{phox} \) and Ask1 expression levels were elevated in hearts of diabetic caPI3K-Tg mice (Fig. 4c). Immunochemistry analysis of \( p22^{phox} \) production was consistent with gene expression data (Fig. 4d).

Superoxide generation was also assessed in NRVM and the cardiomyoblast H9c2 cell line to assess whether acute activation of PI3K(p110α) with IGF1 (an upstream regulator) would inhibit HG-induced superoxide (as occurred in the caPI3K heart), and whether acute inhibition of PI3K
(p110α) with a specific inhibitor (A66) would increase superoxide (as occurred in the dnPI3K heart). In NRVM, HG induced an increase in superoxide compared with LG, which was blunted with IGF1 (Fig. 4e), whereas A66 in a setting of HG increased superoxide, which was prevented with tempol (a superoxide dismutase mimetic; Fig. 4f). The more robust H9c2 cell line was studied under HG conditions with and without H2O2. The addition of H2O2 was designed to mimic the chronic impact of HG causing mitochondrial dysfunction and the subsequent release of mitochondrial ROS (e.g. H2O2). Under HG conditions, H2O2 and A66 (alone or in combination) all increased superoxide generation and this was blunted by tempol (Fig. 4g). Furthermore, IGF1 treatment suppressed superoxide generation induced by HG with H2O2 (Fig. 4h).

Differential NADPH-driven superoxide generation in PI3K-Tg (Fig. 4a) indicates that PI3K regulates extramitochondrial ROS. To assess whether PI3K has the potential to regulate mitochondrial function and mitochondrial ROS production, we examined expression of Tfam (essential for mitochondrial transcription and replication) and Ucp3/UCP3 (a mitochondrial anion carrier protein that can limit mitochondrial ROS production [30]). Tfam gene expression was lower in hearts of non-diabetic dnPI3K compared with non-diabetic Ntg mice and there was no change with diabetes (Fig. 5a). By contrast, Tfam expression was higher in caPI3K than dnPI3K mice in non-diabetic and diabetic conditions (Fig. 5a). Ucp3 gene expression and UCP3 protein production tended to increase in a setting of diabetes in each of the genotypes, but were only significantly

### Table 2 Echocardiographic and catheterisation analyses of heart size and function

<table>
<thead>
<tr>
<th>Variable</th>
<th>Ntg</th>
<th>dnPI3K</th>
<th>caPI3K</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-diabetic</td>
<td>Diabetic</td>
<td>Non-diabetic</td>
</tr>
<tr>
<td>M-mode echocardiography, n</td>
<td>11</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>LVPW (mm)</td>
<td>0.88±0.02</td>
<td>0.80±0.03</td>
<td>0.74±0.03*</td>
</tr>
<tr>
<td>LVDD (mm)</td>
<td>3.79±0.07</td>
<td>3.83±0.07</td>
<td>3.74±0.11</td>
</tr>
<tr>
<td>LVSD (mm)</td>
<td>2.35±0.05</td>
<td>2.36±0.07</td>
<td>2.47±0.12</td>
</tr>
<tr>
<td>Fractional shortening (%)</td>
<td>38±1</td>
<td>38±2</td>
<td>34±2</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>426±13</td>
<td>450±13</td>
<td>410±20</td>
</tr>
<tr>
<td>Catheterisation, n</td>
<td>8</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>327±7</td>
<td>345±5</td>
<td>309±10</td>
</tr>
<tr>
<td>Aortic SBP (mmHg)</td>
<td>98±3</td>
<td>84±3*‡</td>
<td>101±8</td>
</tr>
<tr>
<td>LVSP (mmHg)</td>
<td>99±3</td>
<td>85±2*‡</td>
<td>95±6</td>
</tr>
<tr>
<td>dP/dt max (mmHg/s)</td>
<td>6,610±249</td>
<td>7,266±200</td>
<td>6,784±762</td>
</tr>
<tr>
<td>dP/dt min (mmHg/s)</td>
<td>5,330±163</td>
<td>4,686±185†</td>
<td>5,196±434</td>
</tr>
</tbody>
</table>

*p<0.05 vs Ntg non-diabetic mice; †p<0.05 vs non-diabetic mice of the same genotype; ‡p<0.05 vs Ntg diabetic mice; §p<0.05 vs dnPI3K diabetic mice

dP/dt max, maximum rate of left ventricular pressure change; LVDD, left ventricular diastolic dimension; LVPW, left ventricular posterior wall; LVSD, left ventricular systolic dimension; LVSP, left ventricular systolic pressure; SBP, systolic blood pressure

---


---

![Figure 3](image-url) **Fig. 3** PI3K(p110α) provides protection against diastolic dysfunction. (a) dP/dt max and (b) LVEDP assessed by catheterisation; n=5–11 per group. (c) Representative mitral flow patterns from pulsed-wave Doppler echocardiography. (d) E/A wave ratio, (e) deceleration time and (f) IVRT. (c–f) n=4–10 per group. White bars, non-diabetic; black bars, diabetic. †p<0.05 vs non-diabetic mice of the same genotype; ‡p<0.05 vs diabetic Ntg mice; †p<0.05 vs non-diabetic and diabetic Ntg mice
higher in caPI3K diabetic mice (Fig. 5b, c). To more directly examine whether activation of PI3K can affect mitochondrial function, we measured the respiratory profile of IGF1-treated H9c2 cells under HG conditions with or without the specific PI3K(p110α) inhibitor, A66. IGF1 treatment was associated with increased basal respiration, uncoupled respiration, ATP

Fig. 4 PI3K(p110α) protects against diabetes-induced superoxide generation. (a) LV superoxide generation assessed by lucigenin-enhanced chemiluminescence expressed as relative light units per second per mg (RLU s⁻¹ mg⁻¹). (b) Western blot showing PKCβ2 and GAPDH, and quantitative analysis of PKCβ2 normalised to GAPDH (n=4–5 per group). (c) Representative northern blot showing gene expression of p22phox (n=4–6 per group) and Ask1 (n=5–6 per group) from Ntg, dnPI3K (dnP) and caPI3K (caP) mice. Gapdh was used to normalise for RNA loading. Quantitative analyses. Mean values for non-diabetic Ntg mice were normalised to 1. (d) Immunohistochemical (IHC) examination of p22phox in LV sections. Representative staining (p22phox stains brown; magnification ×100; scale bars, 80 μm) and semi-quantitative analysis (IHC score; n=3 per group). (a–d) White bars, non-diabetic; black bars, diabetic; *p<0.05 vs non-diabetic Ntg mice; †p<0.05 vs non-diabetic Ntg mice (unpaired t test); ¶p<0.05 vs non-diabetic dnPI3K mice; ‡p<0.05 vs diabetic Ntg mice; §p<0.05 vs diabetic dnPI3K mice. Superoxide generation in NRVM under LG and HG conditions treated with IGF1 (e), A66 (PI3K[p110α] inhibitor) and tempol (superoxide dismutase mimetic) (f), n=4–5 per group. *p<0.05 vs LG; †p<0.05 vs HG; ‡p<0.05 vs LG and HG; §p<0.05 vs HG+A66. Superoxide generation in H9c2 cells under HG conditions treated with H₂O₂, A66, tempol (g) and IGF1 (h) (n=5–7 per group). *p<0.05 vs control; †p<0.05 vs A66+H₂O₂; ‡p<0.05 vs H₂O₂. (e–h) Statistics performed using one-way repeated measures ANOVA followed by pairwise multiple comparison (Student–Newman–Keuls). Values relative to LG or control (100%) as shown.
turnover and a trend towards increased maximal respiration \((p<0.06)\); these changes were all prevented by the PI3K \((p110\alpha)\) inhibitor (Fig. 5d, e).

**Discussion**

The development of improved therapeutics for patients with diabetic cardiomyopathy will require a comprehensive understanding of the critical mechanisms responsible for the induction and transition to cardiomyopathy in a setting of hyperglycaemia. To our knowledge, this is the first study to show a direct causal role of decreased PI3K activity in exacerbating diabetic cardiomyopathy and increased PI3K activity in preventing diabetic cardiomyopathy. Another key finding from this study is in vivo evidence that PI3K \((p110\alpha)\) regulates superoxide generation in the mouse heart under basal and diabetic settings. The cardiac protection observed in a setting of enhanced PI3K \((p110\alpha)\) activation was associated with increased AKT phosphorylation, \(Tfam\) and \(Ucp3\)/UCP3 expression, and suppression of LV superoxide generation, \(PKC\beta2\) production, \(p22\text{phox}\) and \(Ask1\) expression.

Utilising dnPI3K-Tg mice with depressed PI3K \((p110\alpha)\) activity, we have demonstrated that PI3K \((p110\alpha)\) is critical for protecting the heart against dysfunction and remodelling in a setting of type 1 diabetes. In contrast, increasing PI3K \((p110\alpha)\) activity utilising caPI3K-Tg mice provided protection.
against diastolic dysfunction and myocardial remodelling in a diabetes model. LV diastolic dysfunction is one of the earliest manifestations of diabetic cardiomyopathy, presenting prior to the onset of systolic dysfunction [26, 31, 32]. Diastolic dysfunction is characterised by abnormal LV relaxation and filling, associated with elevated LVEDP, a depressed E/A ratio and increased deceleration time [25]. Contributing factors include pathological cardiomyocyte hypertrophy, increased cell death and fibrosis. Previous studies have reported each of these features in the STZ-induced type 1 diabetes mouse model [21, 26, 33]. In the current study, diabetes in Ntg and dnPI3K-Tg mice was associated with a depressed E/A ratio and increased LVEDP, deceleration time, IVRT, myocyte size, apoptosis and fibrosis compared with non-diabetic mice. LVEDP, fibrosis and apoptosis were further elevated in diabetic dnPI3K-Tg compared with diabetic Ntg mice. By contrast, diabetes was not associated with changes in LVEDP, E/A ratio, IVRT, myocyte size, fibrosis or apoptosis in caPI3K-Tg mice.

Oxidative stress contributes to the cardiovascular complications associated with diabetes [34]. NADPH oxidase generates superoxide and is considered a major source of ROS in the heart [9, 35]. In the current study, NADPH-driven superoxide generation was increased in diabetic Ntg hearts compared with non-diabetic Ntg hearts, and this was accompanied by increased production of a membrane-associated subunit of NADPH oxidase (p22phox). The p22phox NADPH subunit was the focus of this study because it was the only subunit that was differentially regulated in the hearts of PI3K-Tg mice under control or sham conditions based on previous microarray data [12]. Interestingly, superoxide generation and p22phox in the current study were higher in hearts from non-diabetic dnPI3K compared with non-diabetic Ntg mice, and remained elevated in a setting of diabetes. By contrast, there was no increase in superoxide production or p22phox expression in hearts of non-diabetic and diabetic caPI3K-Tg mice. Findings in the diabetic setting were consistent with those obtained in NRVM and H9c2 cells under HG conditions. In a setting of HG, superoxide generation was enhanced in cells when PI3K(p110α) was inhibited with A66, and blunted when PI3K(p110α) was activated with IGF1. Tempol, a superoxide dismutase mimetic, attenuated A66-induced superoxide generation. Collectively, these data suggest that increased PI3K(p110α) activity mediates protection, at least in part, via attenuation or prevention of superoxide generation.

Our assessment of NADPH-driven superoxide generation indicates that PI3K regulates extra-mitochondrial ROS. Tissue availability constraints precluded direct measurements of mitochondrial ROS. However, to assess the potential contribution of mitochondria, we examined cardiac Tlam and Ucp3 expression/UCP3 production, and performed a mitochondrial function test in H9c2 cells in HG conditions. Tlam and Ucp3 expression/UCP3 production were elevated in hearts of diabetic caPI3K mice, and acute activation of the IGF1–PI3K(p110α) pathway in H9c2 cells enhanced mitochondrial function in a setting of HG. Collectively, this could contribute to the protection observed in diabetic caPI3K mice. UCP3 can protect muscle cells against mitochondrial ROS and oxidative damage [30], and overexpression of Tlam protected the heart against mitochondrial respiratory defects and cardiac dysfunction in a setting of myocardial infarction [36]. In addition, it was previously shown that isolated mitochondria from caPI3K mice (basal conditions) had increased mitochondrial enzymatic activity, associated with increased fatty acid oxidative capacity [37].

Under basal/non-diabetic conditions, dnPI3K-Tg mice have normal cardiac function despite increased cardiac expression of p22phox and Ask1, decreased Tlam and increased LV superoxide generation. We recently reported that genes encoding key components of the Z-disc are also depressed in hearts of dnPI3K mice [38]. This suggests that the dnPI3K heart can compensate for these abnormalities under normal conditions but not under conditions of stress such as diabetes, resulting in accelerated cardiomyopathy. Consistent with this hypothesis, cardiac-specific Ask1-Tg mice show no evidence of pathology under basal conditions, but display a more severe pathological phenotype (cardiac dysfunction, increased fibrosis and apoptosis) in response to pressure overload or ischaemia–reperfusion injury [39]. Furthermore, basal blood pressure was normal in Nox2-Tg mice despite increased vascular ROS production and increased p22phox protein. Differences in blood pressure compared with wild-type mice were only apparent in response to an insult (angiotensin II infusion) [40].

Based on data presented in the current study, together with previous reports in the literature, we have assembled a schematic highlighting mechanisms via which increased PI3K(p110α) could mediate protection in a setting of diabetes (Fig. 6). It is well recognised that hyperglycaemia causes activation of PKCβ2 in the heart and subsequent activation of NADPH oxidase [29]. In the current study, PKCβ2 production and p22phox expression were increased in diabetic Ntg hearts but not diabetic caPI3K hearts. PKCβ2 can phosphorylate p22phox [41], and we previously demonstrated that the caPI3K transgene can blunt cardiac pathology and PKCβ2 protein production in PKCβ2-Tg mice [42]. Superoxide generation, Ask1 expression and apoptosis were elevated in hearts of diabetic Ntg and dnPI3K-Tg mice, but not in diabetic caPI3K-Tg mice. Increased pAKT was maintained in hearts of diabetic caPI3K-Tg mice, and AKT was previously shown to directly inhibit apoptosis.
of mitochondria from PI3K-Tg mice under diabetic conditions, and in vivo studies, as mechanisms may differ. The isolation of NRVM is different from adult cells. Caution should be taken when comparing results from in vitro, ex vivo and in vivo studies, as mechanisms may differ. The isolation of mitochondria from PI3K-Tg mice under diabetic conditions will be important to comprehensively examine the role of PI3K(p110α) in regulating mitochondrial function and mitochondrial ROS.

The current study suggests that increasing PI3K(p110α) activity in the heart could prevent LV superoxide generation and diabetic cardiomyopathy. We recently generated a recombinant adenovirus-associated viral (rAAV) vector containing caPI3K that selectively transduced cardiac muscle, minimising the concern of PI3K's tumorigenic potential in other cell types [48]. rAAV6–caPI3K improved cardiac function in a mouse model of pressure overload and AAV-based therapies have entered clinical trials in heart failure patients [49, 50]. Future studies will be required to assess whether rAAV6–caPI3K can improve function in a setting of diabetic cardiomyopathy.

In summary, we have shown that increasing PI3K (p110α) in the hearts of STZ-induced type 1 diabetic mice provided protection against diastolic dysfunction, cardiomyocyte hypertrophy, cardiac fibrosis and apoptosis. By contrast, decreasing PI3K(p110α) in type 1 diabetic mice was associated with accelerated cardiomyopathy. Enhanced PI3K(p110α) activity prevented a diabetes-induced increase in LV superoxide generation, PKCβ2 production, and p22phox and Ask1 expression. Furthermore, activation of PI3K(p110α) in cardiomyocyte-like cells was able to suppress HG-induced superoxide generation and enhance mitochondrial function. Thus, therapies that target the cardiac PI3K(p110α) pathway may represent a potential strategy for the prevention and treatment of cardiomyopathy in diabetic patients.

Acknowledgements The authors thank T. Julius, N. Jennings, A. Matsumoto, J.W. Tan, P. Chew and L. Lim (all from the Baker IDI Heart and Diabetes Institute) for technical assistance with echocardiography, immunohistochemistry and molecular analyses.

Funding This work was supported by a National Health and Medical Research Council (NHMRC) of Australia project grant (ID526638 to RHR, JRM and KJD), and supported in part by the Victoria Government’s Operational Infrastructure Support Program. KH is supported by an Australian Postgraduate Award. DCH is supported by a National Heart Foundation Fellowship (PF 10M 5347). RHR, KJD, XJD and JRM are NHMRC Senior Research Fellows (IDs 472673, 526605, 317808 and 586604). JRM is supported by an Australia Research Council Future Fellowship (FT0001657).

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement RHR contributed to the design of the study, data acquisition, analysis and interpretation of data, and revising it critically for important intellectual content. JEL, KH, BCB and DCH contributed to the design of some experiments, data acquisition and interpretation, and manuscript revision. HK, YKT, GS, NC, CQ and EJHB contributed to data acquisition and interpretation, and manuscript revision. KJD and XJD contributed to design of the study, data interpretation and revising it critically for important intellectual content. JRM contributed to the design of the study, data acquisition, analysis and interpretation of data, and drafting the article. All authors approved the final version of the manuscript.

Springer
References

Appendix 3: Ubiquinone (Coenzyme Q\textsubscript{10}) Prevents Renal Mitochondrial Dysfunction in an Experimental Model of Type 2 Diabetes

Ubiquinone (coenzyme Q10) prevents renal mitochondrial dysfunction in an experimental model of type 2 diabetes

Karly C. Sourris, Brooke E. Harcourt, Peter H. Tang, Amy L. Morley, Karina Huynh, Sally A. Penfold, Melinda T. Coughlan, Mark E. Cooper, Tuong-Vi Nguyen, Rebecca H. Ritchie, Josephine M. Forbes

Abstract

Cardiovascular benefits of ubiquinone have been previously demonstrated, and we administered it as a novel therapy in an experimental model of type 2 diabetic nephropathy. db/db and dbH mice were followed for 10 weeks, after randomization to receive either vehicle or ubiquinone (CoQ10; 10 mg/kg/day) orally. db/db mice had elevated urinary albumin excretion rates and albumin:creatinine ratio, not seen in db/db CoQ10-treated mice. Renal cortices from db/db mice had lower total and oxidized CoQ10 content, compared with dbH mice. Mitochondria from db/db mice also contained less oxidized CoQ10 (ubiquinone) compared with dbH mice. Diabetes-induced increases in total renal collagen but not glomerulosclerosis were significantly decreased with CoQ10 therapy. Mitochondrial superoxide and ATP production via complex II in the renal cortex were increased in db/db mice, with ATP normalized by CoQ10. However, excess renal mitochondrial hydrogen peroxide production and increased mitochondrial membrane potential seen in db/db mice were attenuated with CoQ10. Renal superoxide dismutase activity was also lower in db/db mice compared with dbH mice. Our results suggest that a deficiency in mitochondrial oxidized CoQ10 (ubiquinone) may be a likely precipitating factor for diabetic nephropathy. Therefore CoQ10 supplementation may be renoprotective in type 2 diabetes, via preservation of mitochondrial function.

ARTICLE INFO

Article history:
Received 4 August 2011
Revised 9 November 2011
Accepted 12 November 2011
Available online 21 November 2011

Keywords:
Coenzyme Q
Diabetic nephropathy
Mitochondrial dysfunction
Free radicals

It has become increasingly apparent that not only is a cure for the current worldwide diabetes epidemic required, but also one for its vascular complications. Even with the best available management, which includes tight control of blood pressure and glycemic control, in addition to inhibition of the renin–angiotensin system, it is possible to achieve only a 30% improvement in declining kidney function as the result of diabetes [1], revealing a significant treatment gap. There has been a plethora of new therapies tested in clinical trials for diabetic kidney disease, with most failing to improve and some actually worsening disease progression above what is currently achieved. Indeed, we are yet to fully understand which changes in the diabetic kidney are protective rather than pathological responses to the disease process, which is essential to rationally design new treatment regimens.

Abnormalities in mitochondrial function, including the excess production of reactive oxygen species and deficiencies in antioxidant enzymes, are known contributors to diabetic renal disease. Indeed, excesses in mitochondrial superoxide [2] and hydrogen peroxide generation [3] have been associated with the development and progression of diabetic nephropathy. In addition, a decline in antioxidant capacity within mitochondria, such as that seen in SOD2-deficient mice [4] and as a result of polymorphism in the SOD2 gene, is also associated with renal dysfunction in diabetes. It remains unclear, however, as to whether restoration of mitochondrial function can prevent the onset of diabetic nephropathy.

Coenzyme Q can exist in two isoforms, Q9 and Q10; both exist within the mouse, Q9 being more abundant than Q10. Coenzyme Q10 (CoQ10; also known as Q10, vitamin Q10, ubiquidecarenone) can exist in two forms and is a benzoquinone compound synthesized naturally by the human body. The oxidized form of CoQ10, ubiquinone, is an electron carrier during mitochondrial respiration, but the reduced form of CoQ10, ubiquinol, is also used by the body as an endogenous antioxidant [5]. CoQ10 is the only component of the mitochondrial respiratory chain that is lipid rather than protein, and it is not anchored to the inner mitochondrial membrane. CoQ10 picks up reducing
equivalents from mitochondrial complex I and complex II and shuttles these "electrons" (as ubiquinol) to complex III, where it is recycled to ubiquinone to repeat the reduction process. CoQ10 is present in most tissues, but the highest concentrations are found in the heart, liver, kidneys, and pancreas [6-8].

Mutations in the genes that encode the CoQ10 pathway result in an inherited mitochondrialopathy in which there is primary renal involvement, not necessarily associated with neurological signs [9]. CoQ10 has also shown efficacy in treating experimental models of acute renal disease [5]; however, its effects on chronic kidney disease such as that seen as the result of diabetes remain to be determined. Therefore, in this study, we examined the effects of exogenous ubiquinone (CoQ10) in an experimental model of type 2 diabetes, the db/db mouse.

**Materials and methods**

**Experimental mouse model of diabetes**

Female lepr+/- C57BL/Ksj (db/db) mice were randomized at 8-10 weeks of age, to receive ubiquinone (coenzyme Q10, 10 mg/kg; LipoGard Liposomal CoQ10, a kind gift from Raj K. Chopra, Tischcon Corp., NY, USA), which was administered in their drinking water. This mouse strain develops diabetic nephropathy in the context of severe metabolic syndrome (hypertension, hyperlipidemia, obesity, insulin abnormalities) similar to that seen in type 2 diabetes in humans. Female lepr+/- C57BL/Ksj (db/db) littermates were also randomized to either coenzyme Q or vehicle, followed concurrently (n = 10/group), and served as the appropriate controls. All mice were obtained from colonies maintained at the Alfred Medical Research and Education Precinct Animal Services (Melbourne, Australia). All groups were followed for 10 weeks and at the completion of the study, mice were anesthetized with Lethabarb (10 mg/kg; Virbac, Animal Health, Australia), followed by exsanguination by cardiac puncture. Kidneys were rapidly isolated and snap-frozen in liquid nitrogen or fixed in 10% neutral-buffered formalin for further analysis. All procedures were approved and performed in accordance with the guidelines set out by the Alfred Medical Research and Education Precinct Animal Ethics Committee and the National Health and Medical Research Council of Australia.

**Measurement of physiological and biochemical parameters**

During week 9 of the study, mice were individually placed in metabolic cages (Tecniplast; VA, Italy) for a period of 24 h. A blood sample was taken, body weight and food and water intake were monitored, and urine was collected. Glycated hemoglobin (GHB) was measured in whole blood by high-performance liquid chromatography (HPLC) as per the AMDCC protocol [10]. Measurement of kidney function

Kim-1 (kidney injury molecule-1) is a recognized marker of renal tubular injury and was measured by commercially available ELISA (USCN, China) in the urine (1:2 dilution) samples collected from each group. Cystatin C, another well-described biomarker of renal dysfunction, was quantified by ELISA (Biovendor, Czech Republic) in plasma. All assays were performed according to the manufacturer’s instructions.

**Histological assessment of kidney injury**

Kidney sections were stained with periodic acid Schiff stain for quantitation of glomerulosclerosis. The degree of glomerulosclerosis, which was defined as thickening of the glomerular basement membrane and mesangial expansion, was evaluated by a semiquantitative method as described previously [14,15]. Tubulointerstitial collagen deposition was assessed in 20 fields (x40) after Masson’s trichrome staining using Image-Pro Plus as previously described [16].

**Renal fractionation**

In total, 50 mg of renal cortex was homogenized (Polytron PF-MR2100; Kinematica, Switzerland) in extraction buffer (20 mM Hepes buffer, pH 7.2, 1 mM EGTA, 210 mM mannitol, 70 mM sucrose) to isolate nuclear, cytosol, mitochondrial, and membranous fractions as previously described [12,15]. Total protein for all fractions was determined by the bicinchoninic acid method (Pierce, Rockford, IL, USA), according to the manufacturer’s protocol [13,17].

**Renal superoxide production**

Fresh kidney cortex pieces (1 mm in size) were placed in oxygen-saturated Krebs buffer (containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4·7H2O, 1.2 mM KH2PO4, 11 mM g-glucose, 0.03 mM EDTA, and 2.5 mM CaCl2, pH 7.4). The rate of cytosolic and mitochondrial superoxide anion production was determined by lucigenin, as described previously [13,17]. Briefly, approximately 15 μg of mitochondria was added to each well and incubated with NADH in the presence or absence of the complex I substrate 10 mM glutamate/malate or complex II substrate 10 mM succinate and the complex I inhibitor rotenone. The rate of superoxide production was determined by lucigenin chemiluminescence as measured on the Victor 3 plate reader.

**Mitochondrial membrane potential**

Mitochondrial membrane potential was assessed using the MitoProbe JC-1 assay kit (Molecular Probes, Eugene, OR, USA) by flow cytometry, according to the manufacturer’s instruction. Briefly, 10 μg of renal mitochondria was stained with 2 mM JC-1 probe for 15 min in isolation buffer (250 mM sucrose, 10 mM Tris–HCl, 1 mM EGTA) at 37 °C. Mitochondria were then washed in excess PBS and pelleted by centrifugation. After being washed with PBS, the mitochondria were analyzed on a FACSCalibur (Becton–Dickinson, USA) flow cytometer using 488 nm excitation. Red fluorescence emissions were collected on the FL2 channel and a minimum of 10,000 cells per sample was acquired. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP)-treated cells were used as a positive control, as CCCP is a mitochondrial membrane potential disrupter. Data were analyzed using WIN MDI version 2.9 (http://facs.scripps.edu.au/software.html) [13].

**H2O2 production**

Hydrogen peroxide in cytosolic extracts was measured using a commercial kit (Amplex red: Molecular Probes) following the manufacturer’s instructions as previously described [13,18,19].
Superoxide dismutase activity

The activities of manganese and copper superoxide dismutase in isolated renal mitochondrial and cytosol were measured using a commercially available kit (Cayman Chemical Co., Ann Arbor, MI, USA). The assay was run according to the manufacturer’s instructions.

Citrate synthase activity

Citrate synthase activity was measured using a method derived from Sree and Brooks [20]. Briefly, mitochondrial preparations (approximately 15 mg/ml total protein), permeabilized by three freeze–thaw cycles, were diluted 1 in 20 in 100 mM Tris buffer, pH 8.3. Ten microliters of diluted mitochondria, or a blank (Tris buffer, pH 8.3), was added to the wells of a 96-well microtiter plate (Sarstedt, Nümbrecht, Germany) in duplicate. A reaction mixture was prepared containing 1 mM 5,5′-dithiobis(2-nitrobenzoic acid), 3 mM acetyl coenzyme A (CoA), 10% Triton-X in Tris buffer, pH 8.3. The reaction mixture was pipetted into each well. To initiate the reaction, 20 μl of a 7.5 mM oxaloacetate solution was added to each well. Absorbance at 412 nm was then measured on a Victor 3 plate reader every minute for a 10-min period to calculate rate of activity. The colorimetric changes are indicative of the hydrolysis of acetyl-CoA to CoA. The change in absorbance per minute was calculated, in the linear range of the plot. After correction for the blank, citrate synthase activity was calculated using the molar extinction coefficient of 5-thio-2-nitrobenzoic acid at 412 nm of 13.6 mM−1 cm−1 and a path length of 0.552 cm and was standardized per renal cortical wet weight.

Active TGF-β1

Renal cortices were fractionated as previously described [18]. Biologically active TGF-β1 was measured in plasma membrane fractions using the TGF-β1 Emax ImmunoAssay System (Promega, Madison, WI, USA). Before measurement, samples were acid activated with 1 M HCl followed by neutralization to pH 7.0 with 1 M NaOH. Values were expressed as pg/ml and assay was run according to the manufacturer’s instructions.

Glutathione peroxidase (Gpx) ELISA

Gpx activity was measured in the isolated mitochondrial fraction from the renal cortices using the Glutathione Peroxidase Activity kit (703102; Cayman Biochemicals, Ann Arbor, MI, USA). Isolated mitochondria were diluted 1:4 and assay was run according to the manufacturer’s instructions.

Measurement of reduced and oxidized coenzyme Q9 and coenzyme Q10 in renal tissue and subcellular fractions

Reduced and oxidized coenzymes Q9 and Q10 were quantitated by HPLC with coulometric detection in kidney tissue and renal mitochondrial and cytosolic subcellular fractions [21]. Quantitation was run on homogenized kidneys and subcellular fractions, which were isolated by ultracentrifugation. HPLC analysis was performed with an ESA Model 582 solvent delivery module with AS3000 autosampler and ESA CouloChem II Model 5200A detector as previously described [21].

Table 1

Biochemical and physiological parameters at week 20 in db/db and db/db mice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>db/db</th>
<th>CoQ10</th>
<th>db/db</th>
<th>CoQ10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>22±2</td>
<td>23±2</td>
<td>56±2</td>
<td>53±3</td>
</tr>
<tr>
<td>Plasma glucose (mmol/L)</td>
<td>9.0±0.6</td>
<td>8.9±1.1</td>
<td>17.0±7.1</td>
<td>19.8±9.9</td>
</tr>
<tr>
<td>Glycated hemoglobin (%)</td>
<td>3.4±0.5</td>
<td>3.2±0.2</td>
<td>8.0±2.9</td>
<td>6.4±2.4</td>
</tr>
<tr>
<td>Total kidney weight (g)</td>
<td>0.39±0.02</td>
<td>0.25±0.01</td>
<td>0.22±0.08</td>
<td>0.31±0.14</td>
</tr>
<tr>
<td>Food intake (g/24 h)</td>
<td>3.2±0.9</td>
<td>3.2±0.6</td>
<td>2.0±1.5</td>
<td>3.1±1.2</td>
</tr>
<tr>
<td>Urine output (ml/24 h)</td>
<td>1.2±1.1</td>
<td>0.8±0.2</td>
<td>3.4±1.9</td>
<td>4.3±2.5</td>
</tr>
<tr>
<td>Water intake (ml/24h)</td>
<td>3.6±2.0</td>
<td>3.6±2.5</td>
<td>5.7±3.7</td>
<td>5.0±3.6</td>
</tr>
<tr>
<td>CoQ10 consumption (mg/24 h)</td>
<td>0</td>
<td>36±25</td>
<td>0</td>
<td>50±36</td>
</tr>
</tbody>
</table>

All data are expressed as means±SD. *P<0.05 vs db/db.

Statistical analysis

Data are expressed as means±SD, unless otherwise stated. Analyses of data were performed by ANOVA followed by post hoc analysis using Tukey’s test or Student’s t test. Data for albuminuria were not normally distributed and therefore analyzed after logarithmic transformation. A P value of less than 0.05 was considered statistically significant.

Results

Biochemical parameters

All db/db mice were obese by week 20 of age (Table 1) and required 1–2 IU/kg insulin daily by the end of the study, which commenced when β cell failure was diagnosed in each mouse (from approximately week 14 of age). However, db/db mice did not consume more food or water per day at week 20, but did have a greater daily urinary output (Table 1). There was no effect of CoQ10 treatment on any of these parameters. Plasma glucose concentrations and glycated hemoglobin were increased in the diabetic db/db mice, but were not attenuated with CoQ10 therapy (Table 1).

Renal function and structural parameters

Urinary albumin excretion over 24 h was increased in db/db mice, which was ameliorated by CoQ10 (Fig. 1A). Urinary albumin:creatinine ratios were also elevated in db/db mice, which were attenuated with CoQ10 intervention (Fig. 1B). db/db mice also had lower plasma cystatin C (Fig. 1C) concentrations, and they showed increased urinary Kim-1 excretion. There were, however, no effects of CoQ10 treatment on cystatin C or Kim-1 concentrations (Fig. 1D). Glomerulosclerosis was increased by diabetes in db/db mice and this remained unaffected by CoQ10 (Figs. 1E and 1–K). Total kidney-to-body weight ratios were lower in all db/db mice compared with db/db mice (Fig. 1F). Indeed, total kidney mass was reduced in db/db mice (Table 1). There were no effects of CoQ10 therapy seen on kidney weight or kidney-to-body weight ratios. Tubulointerstitial collagen deposition in renal cortices was elevated in db/db mice, which was ameliorated by administration of CoQ (Fig. 1G). CoQ10 therapy did ameliorate diabetes-induced renal cortical activation of TGF-β1 in db/db mice (Fig. 1H).
Renal oxidized and reduced coenzymes Q9 and Q10

Renal cortical homogenates from db/db mice had lower content of total CoQ10 and the oxidized forms of both CoQ9 and CoQ10 (ubiquinone), compared with dbH mice (Table 2). Treatment of db/db mice with exogenous CoQ10 restored deficiencies in total renal cortical CoQ10 but did not affect renal cortical oxidized CoQ9 (ubiquinone) or CoQ10 concentrations (Table 2). Reduced CoQ9 and reduced CoQ10 in addition to total CoQ9 concentrations remained unchanged in kidney cortices from db/db mice compared with those from dbH (Table 2) and were not altered after CoQ10 therapy.

Within mitochondria extracted from renal cortices, there were no differences in the concentrations of oxidized, reduced, or total CoQ9 among groups (Table 3). There was, however, a significant decrease in mitochondrial content of oxidized CoQ10 (ubiquinone) seen in db/db mice compared with dbH mice (Table 3). In addition, db/db mice also had a significant increase in the ratio of total CoQ9 to total CoQ10 seen in mitochondria compared with dbH mice (Table 3). These parameters were unaffected by exogenous CoQ10 therapy. No changes among groups were noted for reduced or total CoQ10 in renal cortical mitochondria (Table 3).

Pooled renal cytosolic fractions from dbH and db/db mice had almost undetectable levels of CoQ9 and CoQ10, with most samples below the lower limits of the HPLC detection system (data not shown).

Renal mitochondrial function

The production of renal cortical ATP within isolated mitochondria in the presence of glutamate/malate, representative of ATP production via complex I substrates, was not significantly different among all groups studied (Fig. 2A). Conversely, administration of the complex II substrate succinate in the presence of rotenone induced significant elevations in renal ATP production, which were completely abrogated in those mice that received CoQ10 (Fig. 2B). Mitochondrial membrane potential was increased in db/db mice, indicative of hyperpolarization, which was ameliorated with CoQ10 and dissipated in the presence of the uncoupling agent CCCP (Fig. 2C). There was also an elevation in mitochondrial membrane potential with CoQ10 administration seen in dbH mice (Fig. 2D). These changes in ATP production and mitochondrial membrane potential were seen in the absence of alterations in mitochondrial citrate synthase activity, a housekeeping enzyme for mitochondrial function (Fig. 2D).

Renal production of reactive oxygen species

There was significant generation of mitochondrial superoxide in the presence of NADH (Fig. 3A) and glutamate/malate (Fig. 3B), and succinate/rotenone (Fig. 3B). This excess superoxide production was moderately attenuated with CoQ10 in the presence of NADH (Fig. 3A) and glutamate/malate (Fig. 3B), but CoQ10 had no effect on mitochondrial superoxide production in the presence of succinate (Fig. 3C).

Renal cortical cytosolic hydrogen peroxide concentrations were not different among groups (Table 4). Renal mitochondrial hydrogen peroxide generation was increased in db/db mice in the presence of both glutamate/malate (Fig. 4A) and succinate/rotenone (Fig. 4B), suggesting production via both complex I and complex III of the mitochondrial respiratory chain. Intervention with CoQ10 ameliorated all hydrogen peroxide production within the mitochondria of db/db mice (Figs. 4A and B).

Renal antioxidant activities

Renal cortical CuZnSOD activity was decreased in db/db mice compared with dbH (Table 4), but this remained unaffected by CoQ10 therapy. Within isolated mitochondria, the activities of the mitochondrial antioxidant MnSOD was also lower in db/db mice (Table 4). There was no change seen in the activity of mitochondrial GPx among groups (Table 4).

Discussion

In this study, we examined the exogenous administration of oxidized CoQ10, ubiquinone, as a therapeutic intervention to retard the development and progression of diabetic nephropathy in an experimental model of type 2 diabetes, db/db mice. Overall, there were significant beneficial effects of chronic exogenous CoQ10 therapy on albuminuria, mitochondrial function, renal ATP production, and tubulointerstitial fibrosis seen in our diabetic db/db mice. It must be noted that we did not see improvements in cystatin C and glomerular sclerotic index with exogenous CoQ10 administration. The reasons for this dissociation are unclear, however, and this requires further investigation.

After administration, exogenously administered ubiquinone is thought to be rapidly converted to ubiquinol, its reduced form, although there is debate about which forms of CoQ10 can be absorbed through the gastric lumen. This conversion of ubiquinone to ubiquinol is known to be effective in many diseases [22]. Therefore the renal benefits of CoQ10 seen in previous acute studies have been attributed to ubiquinol's (reduced CoQ10) potent antioxidant characteristics [5,23]. However, in contrast, db/db mice had no changes in total or mitochondrial concentrations of renal ubiquinol, despite evidence of reactive oxygen species (ROS) production and reduced antioxidant activity. Furthermore, there were no elevations in renal ubiquinol concentrations demonstrated with exogenous CoQ10 administration, as per a previous study [24].
suggests that boosting antioxidant defenses via ubiquinol was probably not the mechanism of action responsible for the renal benefits of CoQ10 seen in our db/db mice.

In contrast to ubiquinol, significantly lower concentrations of ubiquione (oxidized CoQ10) were seen in both renal cortices and mitochondria taken from db/db mice compared with non-diabetic dbH mice. These were restored in db/db mice that received ubiquinol therapy. In addition, Pdds2 (the first enzyme of the CoQ synthesis pathway) mutant mice, and humans with a mutation in the Pdds2 gene, have a profound loss of CoQ10 [25,26] and documented loss of CoQ9 [27] within the kidney, which losses are dramatically improved by supplementation with ubiquinone. Indeed, these findings suggest that a loss of renal CoQ10, most likely ubiquinone, is responsible for the resultant renal disease. Therefore from this study, we suggest for the first time that CoQ10 deficiency, specifically ubiquinone, is a precipitating factor for diabetic nephropathy. Although beyond the scope of this study, the capacity of exogenous ubiquinone to restore mitochondrial ubiquinone concentrations warrants further investigation in the future. One previous study has, however, suggested that exogenous CoQ10 administration does not influence renal concentrations of CoQ10 [24].

Dysfunction of mitochondria is commonly postulated as a major contributor to diabetic renal disease [3,28]. Not surprisingly, db/db mice showed abnormalities in respiratory chain function in the presence of excessive mitochondrial ROS production, which were attenuated with exogenous ubiquinone administration. Ubiquinone is a known lipophilic molecule involved in electron transport by the mitochondrial respiratory chain. The effects of diabetes on the mitochondrial respiratory chain, however, were most prominent at the level of complex I in the kidneys of db/db mice, which is thought to be a major site of superoxide production [29]. Indeed, no elevations in ATP production in the presence of complex I substrates were seen despite excessive superoxide and hydrogen peroxide generation at this site, in addition to mitochondrial membrane hyperpolarization. This was not the case after administration of the complex II substrate succinate in the presence of the complex I inhibitor rotenone. One could speculate that restoration of mitochondrial ubiquinone concentrations by exogenous ubiquinone therapy may directly protect against mitochondrial dysfunction in the diabetic kidney, most probably at the level of the mitochondrial respiratory chain. We have previously shown that complex I can be altered in diabetic kidneys [13], and given that Leigh disease, an inherited mitochondriopathy that also leads to a deficiency in complex I in kidneys, can be effectively treated with ubiquinol [25], we postulate that this agent confers at least some of its protective effects via preservation of electron transport in mitochondria, possibly at the level of complex I.

Further support for the postulate that ubiquinone is renoprotective via effects on the mitochondrial respiratory chain comes from findings in this study. Exogenous ubiquinol had the greatest beneficial effects on the tubular portion of the kidney, which contains the highest proportion of mitochondria owing to its many functional roles, including the energetically dependent active resorption of glucose from the urinary filtrate. Indeed, renal function and tubulointerstitial fibrosis including collagen deposition and activation of TGF-β1 were normalized in db/db mice that received exogenous ubiquinone therapy. This is supported by previous studies in which tubulointerstitial nephritis as the result of mitochondrial abnormalities was ameliorated by exogenous CoQ10 administration [25,26]. Given that tubulointerstitial fibrosis is the most accurate predictor of progressive renal damage [30], it is likely that this therapy may be effective in other forms of chronic kidney disease.

Fig. 2. Mitochondrial functional parameters. ATP production in mitochondria using (A) substrates for complex I (glutamate and malate) and (B) substrate for complex II (succinate) and the complex I inhibitor rotenone. (C) Mitochondrial membrane potential using the potentiometric dye JC-1. (D) Citrate synthase activity. All data are expressed as means±SD.

*P<0.05 vs dbH; †P<0.05 vs db/db; ‡P<0.05 vs dbH-CoQ10.
Overall, this group of studies has shown the efficacy of exogenous oxidized CoQ10, namely ubiquinone, administration as a treatment for experimental diabetic nephropathy. Because ubiquinone is thought to be safe for human consumption, it would be prudent to suggest that this agent should be further investigated as a potential therapy for nephropathy in diabetic individuals. Although beyond the scope of this study, the capacity of exogenous ubiquinone to restore mitochondrial ubiquinone concentrations and therefore mitochondrial respiratory chain function in the diabetic kidney also warrants further investigation.

Acknowledgments

The authors thank Kylie Gilbert and Jade Moselle for their expert care of the animals throughout the study and Maryann Arnstein and Edward Grixti for their technical expertise, within the Baker IDI Heart and Diabetes Institute. We also acknowledge the contribution of Dr. Darren Henstridge for his assistance with the citrate synthase protocol. This work was completed with support from the Juvenile Diabetes Research Foundation, the Victorian Government’s OIS Program, and the National Health and Medical Research Council of Australia (NHMRC). J.M.F. and R.H.R. are recipients of NHMRC Senior Research Fellowships.

References
