Novel therapeutic interventions that blunt hyperglycemia-induced cardiac contractile dysfunction

by

Rudo Fiona Mapanga

Thesis presented in fulfilment of the requirements for the degree of Doctor of Philosophy (Physiological Sciences) in the Faculty of Science at Stellenbosch University

Supervisor: Professor M Faadiel Essop

March 2013
DECLARATION

By submitting this thesis/dissertation, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

March 2013

Copyright © 2013 Stellenbosch University

All rights reserved
Abstract

Introduction

Diabetes constitutes a major health challenge. Since cardiovascular complications are common in diabetic patients, this will further increase the overall burden of disease. Furthermore, stress-induced hyperglycemia in non-diabetic patients with acute myocardial infarction is associated with higher in-hospital mortality. Hyperglycemia-induced oxidative stress results in DNA damage and subsequent activation of poly-ADP-ribose polymerase (PARP) as a restorative mechanism. However, PARP attenuates glyceraldehyde–3-phosphate dehydrogenase (GAPDH) activity, thereby diverting upstream glycolytic metabolites into damaging non-oxidative glucose pathways (NOGP). For example, hyperglycemia-induced stimulation of four NOGP, i.e. the polyol pathway, hexosamine biosynthetic pathway (HBP), advanced glycation end products (AGE), and PKC activation elicit cardiovascular complications. The current thesis examined the regulation of NOGP in the setting of ischemia and reperfusion under hyperglycemic conditions.

Here we hypothesized that administration of two unique therapeutic interventions, i.e. oleanolic acid (OA; clove extract) and benfotiamine (BFT; vitamin B1 derivative), can blunt oxidative stress and NOGP-induced cardiac dysfunction under hyperglycemic conditions following ischemia and reperfusion. Our choice for these agents was based on the principle that OA possesses antioxidant properties; and BFT stimulates transketolase (pentose phosphate pathway [PPP] enzyme) thereby shunting flux away from the NOGP pathways. Additionally, hyperglycemia-induced oxidative stress can also result in dysregulation of the ubiquitin-proteasome system (UPS) that removes misfolded proteins. There are conflicting data whether increased/decreased UPS is detrimental with hyperglycemia and/or in response to ischemia and reperfusion. In light of this, we also hypothesized that BFT and OA act as novel cardio-protective agents by diminishing myocardial UPS activity in response to ischemia and reperfusion under acute hyperglycemic conditions.

Materials and Methods

For the first part of the study, we employed several experimental systems: 1) H9c2 cardiac myoblasts were exposed to 33 mM glucose for 48 hr vs. controls (5 mM glucose); and subsequently treated with two OA doses (20 and 50 µM) for 6 and 24 hr, respectively; 2) Isolated rat hearts were perfused ex vivo with Krebs-Henseleit buffer containing 33 mM glucose vs. controls (11 mM glucose) for 60 min, followed by 20 min global ischemia and 60 min reperfusion ± OA treatment; 3) Infarct size was
determined using Evans Blue dye and 1% 2,3,5-triphenyl tetrazolium chloride (TTC) staining with 20 min regional ischemia and 2 hr reperfusion 4) In vivo coronary ligations were performed on streptozotocin-diabetic rats ± 0.45 mg/kg OA administration within the first two minutes of reperfusion; and 5) Effects of long-term OA treatment (2 weeks) on heart function were assessed in streptozotocin (STZ)-diabetic rats. Here, STZ was dissolved in citrate buffer (pH 6.3) and diabetes was induced by administering 60 mg/kg i.p. Tissues were collected at the end of the global ischemia experiments and analyzed for oxidative stress, apoptosis, UPS activity and HBP activation.

For the second part of the study we employed several experimental systems: 1) Isolated rat hearts were perfused ex vivo with Krebs-Henseleit buffer containing 33 mM glucose vs. controls (11 mM glucose) for 90 min, followed by 30 min global ischemia and 60 min reperfusion ± 25, 50 and 100 μM BFT treatment, respectively, added during the first 20 min of reperfusion; 2) Infarct size determination as in #3 above but with 30 min regional ischemia and 2 hr reperfusion ± 100 μM BFT treatment; and 3) In vivo coronary ligations performed on streptozotocin-diabetic rats ± 0.50 mg/kg BFT treatment within the first two min of reperfusion. In parallel experiments, NOGP inhibitors were added during the first 20 min of reperfusion. The following inhibitors were individually employed: AGE pathway (100 μM aminoguanidine); PKC (5 μM chelerythrine chloride); HBP (40 μM 6-diazo-5-oxo-L-norleucine); and polyol pathway (1 μM zopolrestat); Infarct size determination as in #2) with 30 min regional ischemia and 120 min reperfusion ± similar treatments.

Results

Our data show decreased cardiac contractile function in response to ischemia and reperfusion under hyperglycemic conditions. This was linked to increased PARP and attenuated GAPDH activities, together with higher activation of the NOGP. Moreover, we found elevated myocardial oxidative stress, UPS and cell death under these conditions. OA treatment resulted in cardio-protection, i.e. for ex vivo and in vivo rat hearts exposed to ischemia and reperfusion under hyperglycemic conditions. In parallel, OA decreased oxidative stress, apoptosis, HBP flux and UPS activity following ischemia and reperfusion. Long-term OA treatment also improved heart function in streptozotocin-diabetic rats. Our data also reveal that acute BFT treatment significantly decreased myocardial oxidative stress and apoptosis, and provided cardio-protection in response to ischemia and reperfusion under hyperglycemic conditions. In parallel, BFT blunted hyperglycemia-induced activation of four NOGP in the rat heart.
Acute administration of each of the NOGP inhibitors decreased PARP and enhanced GAPDH activities, while diminishing oxidative stress and myocardial apoptosis. Moreover, each of the NOGP inhibitors (individually) employed blunted activation of the other three pathways here examined. Hearts treated with NOGP inhibitors also displayed improved functional recovery and smaller infarct sizes following ischemia and reperfusion. Interestingly, NOGP inhibitors resulted in the same degree of change (for all above-mentioned parameters evaluated) when compared to each other.

Conclusions

This study shows that acute and chronic hyperglycemia trigger myocardial oxidative stress that eventually results in NOGP activation and contractile dysfunction following ischemia and reperfusion. Moreover, our findings establish - for the first time as far as we are aware - that there is a convergence of downstream NOGP effects in our model, i.e. increased myocardial oxidative stress, further NOGP pathway activation, apoptosis, and impaired contractile function. Thus a vicious metabolic cycle is established whereby hyperglycemia-induced NOGP further fuels its own activation by generating even more oxidative stress, thereby exacerbating damaging effects on the heart under these conditions. We also found that both OA and BFT treatment blunted high glucose-induced detrimental effects and provided robust cardio-protection in response to ischemia and reperfusion under hyperglycemic conditions (acute and chronic). These findings suggest that the UPS may be a unique therapeutic target to treat ischemic heart disease in individuals that present with stress-induced, acute hyperglycemia. Moreover, BFT exhibited its cardio-protective effects by NOGP inhibition after ischemia and reperfusion under acute and chronic high glucose conditions. A similar effect was observed at baseline although the underlying mechanisms driving this process still need to be elucidated. In summary, the findings of this thesis are highly promising since it may eventually result in novel, cost-effective therapeutic interventions to treat acute hyperglycemia (in non-diabetic patients) and diabetic patients with associated cardiovascular complications.
Uitreksel

Inleiding

Diabetes skep ’n groot gesondheidsuitdaging. Omrede kardiovaskulêre komplikaseis algemeen onder diabetiese pasiënte is, sal dit oorkoopelend die las van hierdie siekte verder laat toeneem. Verder word stresgeïnduseerde hiperglukemie in nie-diabetiese pasiënte met akute miokardiale infarksie geassosieër met ’n hoër binne-hospitaalmortaliteit. Hiperglukemies-geïnduseerde oksidatiewe stres veroorsaak DNA skade, en gevolglike aktivering van poli-ADF-ribose polimerase (PARP), as ’n herstelmeganisme. Nietemin, PARP verminder gliseraldehyd–3-fosfaatdehidrogenase (GAPDH) aktiwiteit om sodoende die opstroom glikolitiese metaboliete te herlei na skadelike nie-oksidatiewe glukose weë (NOGW). Byvoorbeeld, hiperglukemie-geïnduseerde stimulasie van vier NOGW, i.e. die poliolweg, heksosamienbiosintetiese weg, (HBW), gevorderde glukasie eindprodukte (GGE), en PKC aktivering, lei tot kardiovaskulêre komplikasies. Die huidige tesis ondersoek die regulering van NOGW in ’n isgemiese-reperfussie onder hiperglukemiese toestande.

Ons hipotetiseer dat die toediening van twee unieke terapeutises intervensies, i.e. oleanoliëse suur (OS, naaltjie ekstrak), en benfotiamien (BFT, vitamien B1 derivaat) oksidatiewe stress kan versag, en NOGW geïnduseerde kardiale disfunksie onder hiperglukemiese toestande na ischemie en reperfussie. Ons keuse vir hierdie middels is gebaseer op die beginsel dat OS anti-oksidantiewenskappe bevat, en dat BFT transketolase (pentosefosfaat weg (PFW) ensiem) stimuleer en sodoende die fluks weg van die NOGW weg veroorsaak. Addisioneel kan hiper-glukemie-geïnduseerde oksidatiewe stres ook tot wanregulering van die ubikwitien-proteosoomsisteem (UPS) wat wangevoude protëine verwyder, aanleiding gee. Daar bestaan kontrasterende data oor ’n verhoogde/verlaagde UPS, tesame met hiper-glukemie en/of in reaksie tot isgemies-reperfussie. In die lig hiervan, hipotetiseer ons dat BFT en OS as ’n nuwe kardiobeskermingsmiddel kan optree deur miokardiale oksidatiewe stres en UPS aktiviteit in reaksie op isgemies-reperfussie tydens akute hiper-glukemiese toestande kan verlaag.

Materiale en Metodes

Vir die eerste deel van die studie het ons van verskeie eksperimentele sisteme gebruik gemaak: 1) H9c2 kardiale mioblaste is aan 33 mM glukose vir 48 uur vs. kontrole (5 mM glukose) blootgestel; en gevolglik met twee OS dosisse (20 en 50 µM) vir 6 en 24 hr, onderskeidelik behandeld; 2) geïsoleerde rotharte is ex vivo met Krebs-Henseleit buffer, wat, 33 mM glukose vs. kontrole (11 mM glukose)
bevat, vir 60 min geperfuseer, daarna is dit deur 20 min globale isgemie gevolg en 60 min reperfussie ± OS behandeling; 3) Infarkgrootte is bepaal deur Evans bou kleursel en 1% 2. 3-5 tripfeniel tetrazoloimcholierd (TTC) kleuring met 20 minute regionale ischemie, en 2 uur reperfussie 4) In vivo koronêre liggasies is op streptozotosien-diabetiese rotte uitgevoer ± 0.45 mg/kg OS toediening binne die eerste twee minute van reperfussie; en 5) effekte van langtermyn OS behandeling (2 weke) op hartfunksie is in hierdie streptozotosien-diabetiese rotte ondersoek. Hier is STZ opgelos in ‘n sitraatbuffer (pH 6.3), en diabetes is geinduseer deur 60mg/kg i.p. toe te dien. Weefsels is aan die einde van die globale isgemie eksperimente versamel, en vir oksidatiewe stres, apoptose, UPS aktiwiteit en HBW aktivering, ontleed.

Vir die tweede deel van die studie het ons van verskeie eksperimentele sisteme gebruik gemaak: 1) geïsoleerde rotharte is ex vivo met Krebs-Henseleit buffer, wat 33 mM glukose vs. kontrole (11 mM glukose) bevat, vir 90 min geperfuseer. Daarna is dit gevolg met 30 min globale isgemie en 60 min reperfussie ± 25, 50 en 100 μM BFT behandeling onderskeidelik, gevolg, bykomend, gedurende die eerste 20 min reperfussie; 2) Infarkgrootte is bepaal soos in #3 hierbo, maar met 30 minute regionale ischemie en 2 uur reperfussie ± 100 μM BFT behandeling; en 3) In vivo koronêre liggasies is op streptozotosien-diabetiese rotte uitgevoer ± 0.50 mg/kg BFT behandeling binne die eerste twee minute van reperfussie. Met parallele eksperimente is NOGW inhibeerders bygevoeg binne die eerste 20 min van reperfussie. Die volgende inhibeerders is individueel ontplooi: GGE weg (100 μM aminoguanidien); PKC (5 μM chelleritrienchloried); HBW (40 μM 6-diazo-5-oxo-L-nor-leusien); en poliolweg (1 μM zopolrestaat); 2) Infarkgrootte is bepaal soos in #2) met die uitsondering van 30 min regionale isgemie en 120 min reperfussie ± identiese behandels.

Resultate

Ons data toon aan dat kardiale kontraktiele funksie, in reaksie op isgemie-reperfussie onder hiperglukemiese toestande, verlaag. Dit is verwant aan verhoogde PARP en verminderde GAPDH aktiwiteit, tesame met ‘n hoër aktivering van die NOGW. Verder het ons bevind dat verhoogde miokardiale oksidatiewe stres, UPS en seldood onder die toestande voorkom. OS behandeling lei tot kardiale beskerming, i.e. vir ex vivo en in vivo rotharte wat aan isgemie-reperfussie onder hiperglukemiese toestande blootgestel is. Parallel hiermee het OS oksidatiewe stres, apoptose, HBW invloed, en UPS aktiwiteit na isgemie-reperfussie, verlaag. Langtermyn OS behandeling het ook hartfunksie in streptozotosien-diabetiese rotte verbeter. Ons data vertoon verder dat akute BFT behandeling, miokardiale oksidatiewe stres en apoptose, betekenisvol verlaag het in reaksie op
isgemie-reperfussie onder hiperglukemiese toestande. Parallel hiermee het BFT hiperglukemie-geïnduseerde aktivering van vier NOGWë in die rothart, verminder.

Akute toediening van die elk van die NOGW inhibeerders het PARP verlaag, en GAPDH aktiwiteite verhoog, terwyl oksidatiewe stres, en miokardiale apoptose verminder. Verder het elk van die NOGW inhibeerders wat (individueel) toegedien is, aktivering van die ander drie weë, hier ondersoek, verlaag. Die harte wat met NOGW inhibeerders behandel is het ook ‘n verbeterde herstel en kleiner infarkgrootte na isgemie-reperfussie getoon. Interessant is hoe die NOGW inhibeerders tot dieselfde graad verandering (vir al die bogemelde parameters wat geevalueer is) indien dit vergelyk word teen mekaar, geleli het.

Gevolgtrekking

Hierdie studie het bevind dat akute en chroniese hiperglukemie, miokardiale oksidatiewe stres ontlok, en dat dit geleidelik tot NOGW aktivering en kontraktiele wanfunksionering na isgemie-reperfussie lei. Verder het ons bevindinge vir die eerste keer, volgens ons wete, bewys dat daar ‘n ineenlooping is van afstroom NOGW effekte in ons model, i.e. verhoogde miokardiale oksidatiewe stres, verdere NOGW weg aktivering, apoptose, en ingeperkte kontraktiele funksie. Dus, ‘n gebrekkige metaboliese siklus word verkry waarby hiperglukemies-geïnduseerde NOGW verder sy eie aktivering aanvuur deur meer oksidatiewe stres, en sodoende die skadelike effekte op die hart onder hierdie toestande verder versleg. Ons het verder bevind dat beide OS en BFT behandeling, hoë glukose-geïnduseerde skadelike effekte onderdruk, en krachtige kardiale-beskerming in reaksie op isgemie-reperfussie onder hiperglukemiese toestande (akuut en chronies), teweeg bring. Hierdie bevindinge dui moontlik daarop dat die UPS ‘n unieke terapeutiese teiken kan wees vir die behandeling van isgemiese hartsiekte in individue wat presenteer met stres-geïnduseerde, akute hiperglukemie. BFT het ook sy kardiale beskermende effekte getoon deur NOGW inhibering na isgemie-geïnduseerde reperfussie onder aktute en chroniese hoë glukose toestande. ‘n Soorgelyke effek is tydens die basislyn waargeneem, alhoewel die onderliggende mekanisme wat hierdie proses dryf verder ondersoek moet word. Opsommend is ons bevindinge baie belowend omrede dit daartoe kan aanleiding gee tot ‘n nuwe, meer koste-effektiewe terapeutiese intervensie vir die behandeling van akute hiperglukemie (in nie-diabetiese pasiënte) en diabetiese pasiënte met geassosieërde kardiovaskulêre kompleksies.
ACKNOWLEDGEMENTS

First and foremost I thank God Almighty for granting me such an opportunity to reach this level and make it through. I also want to thank the following people whose input made this work achievable:

To my supervisor and mentor, Professor M. Faadiel Essop words in this space are not enough to express my gratitude. You have made attaining this PhD possible in every way, allowing growth of myself as a human being in a holistic approach. I hope I have attained some of your qualities as a leader and an intellectual. With your open door policy I have received constant and committed guidance, constructive criticisms and financial support for all the requirements of this research all the way through. I truly appreciate all that I learnt from him;

I am very grateful to Dr Meiring and Dr Kelly-Laubscher for the training supervision, advice and training during my ex vivo and in vivo studies in this study;

Dr Theo Nell thank you for all the assistance, guidance, mentorship and positive criticisms throughout the course of this study and translating my abstract;

I wish to acknowledge the assistance rendered to me by staff members of the Department of Physiological Sciences, especially Noel Markgraff, Katrina, Grazelda and Jonnifer not forgetting the Animal Unit staff at the University of Cape Town, Health Sciences;

I also wish to acknowledge the contribution I received on some of the work from Dr U Rajamani, Dr M Zungu-Edmondson and her student (N Dlamini), Prof E Bourdon and his colleague (Dr P Rondeau), Prof MA Fahim and his students (M Shafiullah and A Wahab).

I pay my sincere thanks to my family, particularly to my mother, for the constant support and a warm heart to accommodate all my doings (right and wrong) and also my daughter, Makatendeka for being an inspiration and always there to cheer me up; to my sisters (Lucy and Angie) and brother Ian for all the support, love and care throughout, hope I have been a good role model; to Munya Mavhunga thanks for the love and support;

To my colleagues in CMRG and PhD room (Danzil and Charlene) thank you for your sense of humor, all I learnt from you and being true friends always, same goes for the rest of CMRG group, thank you
for being a family closer to me away from home. Last but not least I would like to thank the Oppenheimer, Beit Trust and Harry Crossley for granting me the funds to pursue my studies.
DEDICATION

This is especially dedicated to my family; I love you guys for being there for me at all times. I am glad to grow up as part of you; in all these years as a student you made everything possible. To Makatendeka, my daughter, from the day you came in this world you have brightened all my days. To my mother, you are the backbone of my life and have been a good role model to all of us. Maka, grow wiser, with a God fearing character and personality, loving others more. All things are possible my dear if you believe. Fly high in all that you do and aim to achieve more……love you all.
# TABLE OF CONTENTS

Declaration ii  
Abstract (English) iii  
Uittreksel (Afrikaans) vi  
Acknowledgements ix  
Dedication xi  
Table of contents xii  
List of Tables xvii  
List of Figures xviii  
List of Conference Proceedings xxii  
List of Publications xxiii  
List of Abbreviations xxiv  
Units of Measurements xxxv  

## CHAPTER 1  Cardiac metabolism and function under homeostatic conditions

1.1 Background 1  
1.2 Cardiac energy metabolism 4  
1.2.1 Substrate utilization by the heart 4  
1.2.2 Mechanisms regulating cardiomyocyte glucose uptake 5  
1.2.3 Role of insulin in substrate utilization 6  
1.2.4 Fate of glucose after uptake by cardiomyocytes 9  
1.2.4.1 Glycolysis 9  
1.2.5 Myocardial fatty acid (FA) uptake 12  
1.2.5.1 Fate of FAs in cardiomyocytes 13  
1.2.6 The tricarboxylic acid (TCA)/ Krebs/ citric acid cycle 16  
1.2.7 The role of the mitochondria in oxidative phosphorylation 18  
1.2.7.1 The electron transport chain (ETC)/ respiratory chain 19  
1.2.8 The creatine kinase system of the heart 21  
1.2.9 Inter-relationship between glucose and fatty acid metabolism (Randle cycle) 22  
1.2.10 Summary and conclusion 25  
1.2.11 References 26  

## CHAPTER 2 The effects of hyperglycemia on cardiac metabolism and function with ischemia and reperfusion

2.1 Introduction 41
2.2  Diabetes mellitus
2.3  How does hyperglycemia occur with diabetes?
2.4  The link between hyperglycemia and the onset of CVDs
2.4.1  Chronic hyperglycemia and CVD
2.4.2  Acute (stress-induced) hyperglycemia and CVD
2.4.3  Acute post-prandial and fasting hyperglycemia
2.5  Mechanisms for adverse cardiovascular effects of hyperglycemia
2.5.1  Hyperglycemia-induced oxidative stress
2.5.1.1  Sources and types of ROS in diabetes and/or ischemia and reperfusion
2.5.2  Mitochondrial antioxidant defense mechanisms
2.6  Glucose flux through the non-oxidative pathways
2.6.1  Polyl pathway
2.6.1.1  Contribution of the polyl pathway to diabetic oxidative stress and complications
2.6.2  Advanced glycosylation end products
2.6.2.1  Contribution of AGE pathway to diabetic oxidative stress and complications
2.6.3  Protein kinase C
2.6.3.1  Contribution of PKC activation to diabetic oxidative stress and complications
2.6.4  The hexosamine biosynthetic pathway
2.6.4.1  Contribution of the HBP to diabetic oxidative stress and complications
2.6.5  Pentose phosphate pathway
2.6.5.1  Non-oxidative branch of the PPP
2.6.5.2  Oxidative branch of the PPP
2.6.6  Non-oxidative glucose pathways and cardiac function
2.7  Diabetic complications
2.7.1  Micro-vascular complications
2.7.1.1  Diabetic neuropathy
2.7.1.2  Diabetic retinopathy
2.7.1.3  Diabetic nephropathy
2.7.2  Macro-vascular complications
2.7.2.1  Arterial diseases
2.7.2.2  Atherosclerosis
2.7.2.3  Diabetic cardiomyopathy
2.8  Diabetes mellitus management
2.8.1  Insulin
2.8.2  Insulin sensitizers
CHAPTER 3 Oleanolic acid: a novel cardio-protective agent that blunts hyperglycemia-induced contractile dysfunction

3.1 Introduction 186
3.2 Materials and Methods 188
3.2.1 Isolation of oleanolic acid from clove extract 188
3.2.2 Cell culture and oleanolic acid treatments 189
3.2.3 Measurement of intracellular ROS levels and apoptosis 190
3.2.4 Animals and ethics statement 192
3.2.5 Ex-vivo global ischemia during (simulated acute hyperglycemia) 192
3.2.6 Ex-vivo regional ischemia and reperfusion during simulated acute hyperglycemia 194
3.2.6.1 Determination of infarct size 195
3.2.7 In vivo regional ischemia and reperfusion in streptozotocin-treated rats during chronic hyperglycemia 195
3.2.8 Effects of long-term OA treatment on heart function in streptozotocin-treated rats (chronic hyperglycemia) 197
3.2.9 Western blot analysis 198
3.2.10 Measurement of superoxide dismutase 198
3.2.11 Myocardial superoxide levels 199
3.2.12 Isolation of proteins for carbonylation and proteasome activity experiments 199
3.2.13 ELISA carbonyl protocol 200
3.2.14 Proteasome activity measurements 201
3.2.15 Statistical analysis 201
3.3 Results 202
3.3.1 Isolation of oleanolic acid from clove extract 202
3.3.2 Structural elucidation of OA 202
3.3.3 Effects of OA treatment on ROS levels and apoptosis in heart cells 206
3.3.4 Evaluation of ex vivo heart function during global ischemia and reperfusion (simulated acute hyperglycemia) 212
3.3.5 In vivo coronary artery ligations in streptozotocin-treated rats (chronic hyperglycemia) 218
3.3.6 Effects of long-term OA treatment on heart function in streptozotocin-treated rats (chronic hyperglycemia) 221
3.3.7 Effects of OA treatment on ex vivo myocardial ROS levels and apoptosis 222
3.3.8 Evaluating the effects of OA treatment on myocardial proteasomal activity in hearts subjected to ischemia and reperfusion under high glucose conditions 228
3.4 Discussion 231
3.5 References 237

CHAPTER 4 Acute benfotiamine treatment counteract hyperglycemia-mediated contractile dysfunction following ischemia and reperfusion 245
4.1 Introduction 246
4.2 Materials and methods 251
4.2.2 Animals and ethics statement 251
4.2.3 Ex-vivo global ischemia and reperfusion during simulated acute hyperglycemia 251
4.2.4 *Ex vivo* regional ischemia and reperfusion during simulated acute hyperglycemia

254

4.2.5 Determination of infarct size

255

4.2.6 *In vivo* regional ischemia and reperfusion in streptozotocin-treated rats (chronic hyperglycemia)

255

4.2.7 Myocardial superoxide levels

257

4.2.8 Measurement of superoxide dismutase (SOD) activity

257

4.2.9 Isolation of proteins for carbonylation and proteasome activity experiments

258

4.2.10 ELISA carbonyl protocol

258

4.2.11 Proteasome activity measurements

259

4.2.12 Evaluation of myocardial apoptosis

260

4.2.13 PARP assay

261

4.2.14 GAPDH assay

261

4.2.15 Determination of non-oxidative pathway activation

262

4.2.15.1 AGE

262

4.2.15.2 PKC assay

263

4.2.15.3 Hexosamine biosynthetic pathway (HBP)

264

4.2.15.4 Polyol pathway

264

4.2.15.5 Non-oxidative pentose phosphate pathway (PPP) assay

265

4.2.16 Statistical analysis

266

4.3 Results

267

4.3.1 Acute high glucose exposure impairs contractile heart function following ischemia and reperfusion

267

4.3.2 Acute BFT treatment enhances non and post-ischemic contractile function

270

4.3.3 Acute BFT administration decreases infarct size and attenuates high glucose induced oxidative stress and apoptosis

275

4.3.4 BFT treatment decreased chymotrypsin proteasomal activity

281

4.3.5 BFT blunts high glucose-induced metabolic dysfunction and activation of non-oxidative glucose utilizing pathways

282

4.3.6 Acute inhibition of damaging non-oxidative glucose pathways blunts high glucose-induced contractile dysfunction following ischemia and reperfusion

287

4.3.7 Hyperglycemia-induced oxidative stress and apoptosis is blunted by acute inhibition of flux through the damaging non-oxidative glucose pathways

291

4.3.8 The interlink between the damaging non-oxidative glucose pathways in attenuating hyperglycemia-induced metabolic dysfunction

292

4.4 Discussion

297
4.4.1 Acute hyperglycemia triggers oxidative stress, the coordinated induction of non-oxidative glucose metabolic pathways and impaired contractile function 297

4.4.2 Acute BFT treatment attenuates the detrimental effects of hyperglycemia with ischemia and reperfusion 300

4.4.3 Individual acute inhibition of NOGP attenuates the detrimental effects of hyperglycemia with ischemia and reperfusion 303

4.5 References 308

CHAPTER 5 Final Conclusions, limitations and recommendations 320

5.1 Conclusions 320

5.2 Limitations and future recommendations 321

5.3 References 322

APPENDICES 323
LIST OF TABLES

CHAPTER 1
Table 1.1: Examples of studies that show the effect of substrate utilization on cardiac function

CHAPTER 2
Table 2.1: Epidemiological studies showing the link between glycemic control and CVDs

CHAPTER 3
Table 3.1: $^{13}$C (100.64 MHz) Bruker Avance III NMR spectral data of plant-derived and reported OA
Table 3.2: Effects of OA on ex vivo coronary flow and end-diastolic pressure during the first ten min of stabilization and at the end of reperfusion
Table 3.3: Body weight and blood glucose levels after 1 week of STZ injection.
Table 3.4: Effects of OA on in vivo heart rate, ST height, systolic and diastolic blood pressures during early reperfusion

CHAPTER 4
Table 4.1: Coronary flow and end-diastolic pressure under high vs. baseline glucose conditions hearts
Table 4.2: Body weight and blood glucose levels after 1 week of STZ injection.
Table 4.3: Coronary flow, end-diastolic pressure, heart rate and velocity of contraction for hearts under high glucose (33 mM) vs. control (11 mM) ± NOGP inhibitors at the end of reperfusion
LIST OF FIGURES

CHAPTER 1

Figure 1.1: The role of insulin on glucose and fatty acid uptake
Figure 1.2: Cardiomyocyte glucose uptake and metabolism by glycolysis
Figure 1.3: Fatty acid uptake and regulation of metabolism by the cardiomyocyte under normal conditions
Figure 1.4: The tricarboxylic acid cycle/ Krebs cycle/ citric acid cycle
Figure 1.5: Schematic diagram of the mitochondrial electron transport chain
Figure 1.6: The Randle cycle showing the interrelationship between myocardial fatty acid and glucose metabolism

CHAPTER 2

Figure 2.1: Mechanism of FA-induced insulin resistance as proposed by Randle et al
Figure 2.2: Proposed alternative mechanism of FA-induced insulin resistance
Figure 2.3: Mechanisms of formation of hyperglycemia-induced ROS
Figure 2.4: Role of polyol pathway in hyperglycemia-induced oxidative stress
Figure 2.5: Simplified biochemistry of advanced glycation end product formation
Figure 2.6: Activation and downstream targets of the hexosamine biosynthetic pathway in diabetes
Figure 2.7: The non-oxidative reactions of the pentose phosphate pathway
Figure 2.8: Effect of hyperglycemia on atherosclerosis
Figure 2.9: ATP balance in the heart mitochondria
Figure 2.10: Ionic imbalances during myocardial ischemia and reperfusion
Figure 2.11: Protein ubiquitination process and 26S proteasome structure

CHAPTER 3

Figure 3.1: Schematic diagrams showing the perfusion protocol for assessment of effects of OA
Figure 3.2: Syzygium aromaticum (cloves)-derived OA 1D and 2D $^1$H and $^{13}$C- NMR spectra
Figure 3.3: Structure and numbering of oleanolic acid according to IUPAC.
Figure 3.4: OA treatment attenuates oxidative stress in H9c2 cells.
Figure 3.5: Representative images on the effects of OA treatments on in vitro ROS levels.
Figure 3.6: Decreased apoptotic cell death in H9c2 cells treated with OA (caspase activity)
Figure 3.7: Diminished apoptosis in OA-treated H9c2 cells (flow cytometry).
Figure 3.8: OA treatment does not affect pre-ischemic cardiac function
Figure 3.9: OA treatment blunts high glucose-induced cardiac dysfunction following ischemia and reperfusion.
Figure 3.10: OA treatment blunts high glucose-induced cardiac dysfunction following ischemia and reperfusion.

Figure 3.11: OA administration decreases infarct size under high glucose perfusion conditions.

Figure 3.12: OA treatment decreases infarct size following coronary artery ligation in streptozotocin-diabetic rats.

Figure 3.13: Long-term OA treatment improves cardiac function in STZ-diabetic rats.

Figure 3.14: OA treatment does not affect superoxide dismutase and caspase activities without ischemia.

Figure 3.15: Anti-oxidant effects of OA in hearts subjected to ischemia and reperfusion under high glucose perfusion conditions.

Figure 3.16: OA treatment decreases carbonylation levels in hearts subjected to ischemia and reperfusion under high glucose conditions.

Figure 3.17: Anti-apoptotic effects of OA in hearts subjected to ischemia and reperfusion under high glucose conditions.

Figure 3.18: OA treatment attenuates O-GlcNAcylation in hearts subjected to ischemia and reperfusion under high glucose conditions.

Figure 3.19: Increased proteasomal activity in hearts under high glucose conditions without ischemia and reperfusion.

Figure 3.20: Increased proteasomal activity in hearts subjected to ischemia and reperfusion under high glucose conditions.

Figure 3.21: OA attenuates high glucose-induced proteasomal activity following ischemia and reperfusion.

CHAPTER 4

Figure 4.1: Hyperglycemia activates non-oxidative pathways of glucose metabolism.

Figure 4.2: Schematic diagrams showing protocols for assessment of effects of BFT.

Figure 4.3: Target sites for inhibition of non-oxidative glucose pathways.

Figure 4.4: High glucose-induced cardiac contractile dysfunction.

Figure 4.5: High glucose-induced cardiac contractile dysfunction.

Figure 4.6: Acute BFT treatment increases cardiac contractile function under baseline glucose and non-ischemic conditions.

Figure 4.7: Acute BFT treatment blunts cardiac dysfunction following ischemia and reperfusion at baseline glucose conditions.

Figure 4.8: Acute BFT treatment blunts high glucose-induced cardiac dysfunction following ischemia and reperfusion.
Figure 4.9: Acute BFT treatment blunts high glucose-induced cardiac dysfunction following ischemia and reperfusion

Figure 4.10: Acute BFT treatment decreases infarct size following ischemia and reperfusion *ex vivo*

Figure 4.11: Acute BFT treatment decreases infarct size following ischemia and reperfusion *in vivo*

Figure 4.12: Acute BFT treatment blunts high glucose-induced oxidative stress

Figure 4.13: Myocardial apoptosis in response to high glucose conditions

Figure 4.14: Anti-apoptotic effects of BFT in hearts subjected to ischemia and reperfusion under high glucose conditions

Figure 4.15: BFT attenuates proteasomal activity under baseline glucose conditions following ischemia and reperfusion

Figure 4.16: BFT attenuates high glucose-induced proteasomal activity following ischemia and reperfusion

Figure 4.17: BFT treatment attenuates O-GlcNAcylation in hearts subjected to ischemia and reperfusion

Figure 4.18: Acute BFT administration attenuates high glucose-induced metabolic dysfunction and activation of non-oxidative pathways

Figure 4.19: Activation of non-oxidative pathways in high glucose perfusions relative to baseline conditions ± BFT treatment

Figure 4.20: High-glucose induced cardiac dysfunction blunted by acute non-oxidative pathway inhibition following ischemia and reperfusion.

Figure 4.21: Acute inhibition of non-oxidative glucose pathways decreases infarct size

Figure 4.22: Effects of pathway inhibitors on oxidative stress and myocardial apoptosis

Figure 4.23: Cardiac metabolic function at baseline glucose conditions with acute non-oxidative pathway inhibition following ischemia and reperfusion

Figure 4.24: High-glucose induced cardiac metabolic dysfunction blunted by acute non-oxidative pathway inhibition following ischemia and reperfusion

Figure 4.25: Activation of non-oxidative pathways in high glucose perfusions with pathway inhibitors relative to high glucose conditions without treatment.
LIST OF CONFERENCE PROCEEDINGS

International


National


- Mapanga RF, Rajamani U, Dlamini IN, Zungu M and Essop MF. Clove extract: able to blunt hyperglycemia-induced contractile dysfunction? 39th PSSA Annual conference, University of Western Cape (29th -31st August 2011). Abstract accepted as a poster presentation.

- Mapanga RF and Essop MF. Exploring novel ways to blunt hyperglycemia-induced contractile dysfunction. 39th PSSA Annual conference, University of Western Cape (29th -31st August 2011). Runner-up oral presentation

- Meiring JJ, Mapanga RF and Essop MF. Hyperglycemia-induced flux through the hexosamine biosynthetic pathway impairs cardiac contractile function. 38th PSSA Annual Conference, Walter Sisulu University (6th -8th September 2010). Accepted for poster presentation.
LIST OF PUBLICATIONS


- **Mapanga RF**, Kelly-Laubscher R, M Faadiel Essop. Acute benfotiamine treatment counteracts hyperglycemia-mediated contractile dysfunction following ischemia and reperfusion. (Manuscript in preparation for submission to Antioxidant Redox & Signaling).
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>absorbance</td>
</tr>
<tr>
<td>AAR</td>
<td>area at risk</td>
</tr>
<tr>
<td>ACBP</td>
<td>acyl-CoA binding protein</td>
</tr>
<tr>
<td>ACC1/2</td>
<td>acetyl-CoA carboxylase 1/2</td>
</tr>
<tr>
<td>ACCORD</td>
<td>Action to Control Cardiovascular Risk in Diabetes</td>
</tr>
<tr>
<td>ADA</td>
<td>American Diabetes Association</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ADVANCE</td>
<td>Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation</td>
</tr>
<tr>
<td>AGE(s)</td>
<td>Advanced glycation end-product(s)</td>
</tr>
<tr>
<td>AIF</td>
<td>apoptosis inducing factor</td>
</tr>
<tr>
<td>AKR</td>
<td>aldo-keto reductase</td>
</tr>
<tr>
<td>ALEs</td>
<td>advanced lipoxidation end products</td>
</tr>
<tr>
<td>AMG</td>
<td>aminoguanidine</td>
</tr>
<tr>
<td>AMI</td>
<td>acute myocardial infarction</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>adenosine monophosphate protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>one way analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>adaptor protein apoptotic protease activating factor-1</td>
</tr>
<tr>
<td>AR</td>
<td>aldose reductase</td>
</tr>
<tr>
<td>ARE</td>
<td>antioxidant response elements</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>Bad</td>
<td>Bcl-2 associated death promoter</td>
</tr>
<tr>
<td>Bak</td>
<td>Bcl-2 homologous antagonist /killer</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2-associated X protein</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid assay</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B cell leukemia/lymphoma-2</td>
</tr>
<tr>
<td>Bcl-x&lt;sub&gt;L&lt;/sub&gt;</td>
<td>B cell leukemia/lymphoma-x-isoform</td>
</tr>
<tr>
<td>BFT</td>
<td>benfotiamine</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>BH3</td>
<td>Bcl-2 homology domain 3</td>
</tr>
<tr>
<td>BH₄</td>
<td>tetrahydro biopterin</td>
</tr>
<tr>
<td>Bid</td>
<td>BH3-only interacting protein domain</td>
</tr>
<tr>
<td>Bim</td>
<td>Bcl-2 like protein 11</td>
</tr>
<tr>
<td>Bok</td>
<td>Bcl-2 related ovarian killer</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CA</td>
<td>California state</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium</td>
</tr>
<tr>
<td>CACT</td>
<td>carnitine/acylcarnitine transferase</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CD36</td>
<td>cluster of differentiation 36</td>
</tr>
<tr>
<td>CHE</td>
<td>chelerythrine chloride</td>
</tr>
<tr>
<td>CHS</td>
<td>Cardiovascular Health Study</td>
</tr>
<tr>
<td>CML</td>
<td>carboxy-methyl lysine</td>
</tr>
<tr>
<td>CO₃⁻</td>
<td>carbonate radical</td>
</tr>
<tr>
<td>CPT-I/II</td>
<td>carnitine palmitoyl transferase I/II</td>
</tr>
<tr>
<td>CuSOD</td>
<td>copper superoxide dismutase</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DCCT</td>
<td>Diabetes Control and Complications Trial</td>
</tr>
<tr>
<td>DCFDA</td>
<td>2',7'-dichlorodihydrofluorescein diacetate</td>
</tr>
<tr>
<td>DECODE</td>
<td>Diabetes Epidemiology Collaborative Analysis of Diagnostic Criteria in Europe</td>
</tr>
<tr>
<td>DHAP</td>
<td>dihydroxy acetone phosphate</td>
</tr>
<tr>
<td>DIS</td>
<td>Diabetes Intervention Study</td>
</tr>
<tr>
<td>DISC</td>
<td>death-inducing signaling complex</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxy-ribonucleic acid</td>
</tr>
<tr>
<td>DNPH</td>
<td>dinitrophenylhydrazine</td>
</tr>
<tr>
<td>DON</td>
<td>6-diazo-5-oxo-L-norleucine</td>
</tr>
<tr>
<td>dP/dtmax</td>
<td>maximal velocity of contraction</td>
</tr>
</tbody>
</table>
E
E1    ubiquitin-activating enzymes
E2    ubiquitin-conjugating enzymes
E3    ubiquitin–protein ligases
EAS   ethyl acetate solubles
ECG   electrocardiogram
EDC   Epidemiology of Diabetes Complications
EDIC  Epidemiology of Diabetes Interventions and Complications
EDP   end-diastolic pressure
EHDF  endothelium-derived hyperpolarizing factor
eNOS  endothelium nitric oxide synthase
EPIC  European Prospective Investigation of Cancer and Nutrition
ER    endoplasmic reticulum
ERK   extracellular regulated kinase
ESRD  end-stage renal disease
ET-1  endothelin-1
ETF   electron transfer flavoprotein
ETRA  endothelin receptor A

F
F2,6 BP  fructose 2,6 biphosphate
FA(s)   fatty acid(s)
FABPpm  fatty acid binding protein (plasma membrane)
FAD    flavin adenine dinucleotide
FADD   Fas-associated death domain protein
FADH2  reduced flavin adenine dinucleotide
FAO    fatty acid oxidation
FAT    fatty acyl translocase
FATP1/6 fatty acid transporter 1/6

G
G 3-P  glyceraldehyde 3-phosphate
GADPH  glyceraldehyde-3-phosphate dehydrogenase
GBM    glomerular basement membrane
GDH    glycerophosphate dehydrogenase
GDP  guanosine diphosphate
GFAT  glutamine:fructose-6-phosphate amidotransferase
GFR  glomerular filtration rate
GIK  glucose insulin potassium
GLP  glucagon like peptide
GLUT-1  Glucose transporter-1
GLUT-2  Glucose transporter-2
GLUT-3  Glucose transporter-3
GPAT  glycerol-3-phosphate acyltransferase
G6PD  glyceraldehyde phosphate dehydrogenase
GSH  glutathione
GSK  glycogen synthase kinase
GSSG  glutathione disulfide
GTP  guanosine triphosphate

H
HbA\textsubscript{1C}  glycosylated hemoglobin
HBP  Hexosamine biosynthetic pathway
HCl  hydrochloric acid
HDL  high density lipoprotein
HF  heart failure
H-FABP\textsubscript{c}  cytoplasmic heart-type fatty acid binding protein
HK  hexokinase
HMQC  Heteronuclear multiple quantum coherence
HR  heart rate
HRP  horse radish peroxidase

I
i.p  intraperitoneal
IA  infarct area
IAP  inhibitor of apoptosis
ICAM-1  intracellular cell adhesion molecule-1
IFM  interfibrillar mitochondria
IFN-\gamma  interferon gamma
IGT  impaired glucose tolerance
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMS</td>
<td>mitochondrial inter-membrane space</td>
</tr>
<tr>
<td>IR</td>
<td>insulin receptor</td>
</tr>
<tr>
<td>IRS 1/2</td>
<td>insulin receptor substrate 1/2</td>
</tr>
<tr>
<td>J</td>
<td></td>
</tr>
<tr>
<td>JAK</td>
<td>janus kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun NH₂ terminal kinase</td>
</tr>
<tr>
<td>K</td>
<td></td>
</tr>
<tr>
<td>K⁺</td>
<td>potassium</td>
</tr>
<tr>
<td>KATP</td>
<td>Adenosine-5' triphosphate sensitive potassium channels</td>
</tr>
<tr>
<td>L</td>
<td></td>
</tr>
<tr>
<td>LCFAs</td>
<td>long chain fatty acids</td>
</tr>
<tr>
<td>LDA</td>
<td>lipid peroxidation-derived aldehydes</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>LLE-NA</td>
<td>N-Cbz-Leu-Leu-Glu-b-naphthylamide</td>
</tr>
<tr>
<td>LLVY-MCA</td>
<td>Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin</td>
</tr>
<tr>
<td>LPL</td>
<td>lipoprotein lipase</td>
</tr>
<tr>
<td>LSTR-MCA</td>
<td>N-t-Boc-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin</td>
</tr>
<tr>
<td>LTD</td>
<td>limited</td>
</tr>
<tr>
<td>LVDP</td>
<td>left ventricular developed pressure</td>
</tr>
<tr>
<td>M</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MA</td>
<td>Massachusetts state</td>
</tr>
<tr>
<td>MAP</td>
<td>mean arterial pressure</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MCD</td>
<td>malonyl-CoA decarboxylase</td>
</tr>
<tr>
<td>MCT-1</td>
<td>monocarboxylic acid transporter-1</td>
</tr>
<tr>
<td>MD</td>
<td>Maryland state</td>
</tr>
<tr>
<td>MG</td>
<td>methyglyoxal</td>
</tr>
<tr>
<td>MnSOD</td>
<td>manganese superoxide dismutase</td>
</tr>
<tr>
<td>MnTBAP</td>
<td>manganese (III) tetrakis (4-Benzoic acid) porphyrin chloride</td>
</tr>
<tr>
<td>MO</td>
<td>Missouri state</td>
</tr>
</tbody>
</table>
mPTP  mitochondrial permeability transition pore
mTOR  mammalian target of rapamycin
NaCl  sodium chloride

N
NAD+  oxidized nicotinamide dinucleotide
NADH  nicotinamide adenine dinucleotide hydrogen
NADPH  nicotinamide adenine dinucleotide phosphate hydrogen
NEI  National Eye Institute
NF-κβ  Nuclear factor-kappa beta
NHANES II/III  Second/Third National Health and Nutrition Examination Survey
NMR  Nuclear Magnetic Resonance
NO  nitric oxide
NO2•  nitrogen dioxide
NOGP  non-oxidative glucose pathways
Nox  NADPH oxidase
NPDR  non-proliferative diabetic retinopathy
Nrf2  nuclear factor erythroid 2 p45-related factor 2
NSW  New South Wales
3-NT  3-nitrotyrosine
NY  New York state

O
O2•  superoxide radical
OA  oleanolic acid
OGTT  oral glucose tolerance test
OH•  hydroxyl radical
OMM  outer mitochondrial membrane
ONCOO•  nitrosoperoxycarbonate
ORIGIN  Outcome Reduction with an Initial Glargine Intervention
ox-LDL  Oxidized low density lipoprotein

P
PA  phosphatidic acid
PAGE  polyacrylamide gel electrophoresis
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PARP</td>
<td>poly(ADP)ribose polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCO</td>
<td>protein carbonyl content</td>
</tr>
<tr>
<td>PDH</td>
<td>pyruvate dehydrogenase</td>
</tr>
<tr>
<td>PDK4</td>
<td>pyruvate dehydrogenase kinase 4</td>
</tr>
<tr>
<td>PFK 1/2</td>
<td>phosphofructokinase ½</td>
</tr>
<tr>
<td>6PGD</td>
<td>6-phosphogluconate dehydrogenase</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PI3-K</td>
<td>Phosphatidyl inositol-3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PKC-θ</td>
<td>protein kinase C-theta</td>
</tr>
<tr>
<td>PKC-α</td>
<td>protein kinase C-alpha</td>
</tr>
<tr>
<td>PKC-β</td>
<td>protein kinase C-beta</td>
</tr>
<tr>
<td>PKC-γ</td>
<td>protein kinase C-gamma</td>
</tr>
<tr>
<td>PKC-δ</td>
<td>protein kinase C-delta</td>
</tr>
<tr>
<td>PKC-ε</td>
<td>protein kinase C-epsilon</td>
</tr>
<tr>
<td>PKC-ξ</td>
<td>protein kinase-xi</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PMNs</td>
<td>polymorphonuclear leukocytes</td>
</tr>
<tr>
<td>PPAR-α</td>
<td>peroxisome proliferator alpha</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>peroxisome proliferator gamma</td>
</tr>
<tr>
<td>PRKAG2</td>
<td>gene encoding for 5'-AMP-activated protein kinase subunit gamma-2</td>
</tr>
<tr>
<td>ProACTIVE</td>
<td>PROspective pioglitAzone Clinical Trial In macroVascular Events</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>ribose 5-phosphate</td>
</tr>
<tr>
<td>R 5-P</td>
<td>ribose 5-phosphate</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation end product</td>
</tr>
<tr>
<td>RAS</td>
<td>renin angiotensin system</td>
</tr>
<tr>
<td>RECORD</td>
<td>Rosiglitazone evaluated for cardiovascular outcomes in oral agent combination therapy for type 2 diabetes</td>
</tr>
<tr>
<td>RFC</td>
<td>reduced folate carrier-1</td>
</tr>
</tbody>
</table>
RIAD  Risk factors in Impaired Glucose Tolerance for Atherosclerosis and Diabetes
RIPA  radio-immunoprecipitation assay buffer
RLU  relative luminescence units
RNS  reactive nitrogen species
ROI  reactive oxygen intermediates
ROS  reactive oxygen species
RPP  rate pressure product

S
SDH  sorbitol dehydrogenase
SDS  sodium dodecyl sulfate
SEM  standard error of means
SERCA  sarco(endo)plasmic reticulum Ca2+-ATPase
SGLT  Sodium glucose transporter
SGLT-1  Sodium glucose transporter-1
SGLT-2  Sodium glucose transporter-2
SH2  Src homology 2
SLC  solute carrier family
Smac/DIABLO  second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI
SQR  succinate: ubiquinone oxidoreductase
SSM  subsarcolemmal mitochondria
STAT  signal transducers and activators of transcription
STZ  Streptozotocin
SU  Sulphonylurea
SUR-1  Sulphonylurea receptor-1
SUR-2  Sulphonylurea receptor-2

T
T1DM  type 1 diabetes mellitus
T2DM  type 2 diabetes mellitus
TAG  triacylglycerides
TBARS  thiobarbituric acid reactive substances
TCA  tricarboxylic acid
TDP  thiamine diphosphate
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>TK</td>
<td>transketolase</td>
</tr>
<tr>
<td>TKTL 1/2</td>
<td>transketolase like 1/2</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TMB</td>
<td>3',5',5' Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TPI</td>
<td>triophosphate isomerase</td>
</tr>
<tr>
<td>TPP</td>
<td>triphenylphosphonium</td>
</tr>
<tr>
<td>TPP</td>
<td>thiamine pyrophosphate</td>
</tr>
<tr>
<td>TTC</td>
<td>2,3,5-triphenyl tetrazolium chloride</td>
</tr>
<tr>
<td>tBID</td>
<td>truncated BID</td>
</tr>
<tr>
<td>TZD</td>
<td>Thiazolidinediones</td>
</tr>
<tr>
<td>Ub</td>
<td>ubiquitin</td>
</tr>
<tr>
<td>ucNOS</td>
<td>uncoupling nitric oxide synthase</td>
</tr>
<tr>
<td>UDP</td>
<td>uridine diphosphate</td>
</tr>
<tr>
<td>UDP-GlcNac</td>
<td>uridine diphosphate acetylglucosamine</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>UKPDS</td>
<td>United Kingdom Prospective Diabetes Study</td>
</tr>
<tr>
<td>UPS</td>
<td>ubiquitin proteasome system</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>VADT</td>
<td>Veterans Affair diabetes trial</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
</tr>
<tr>
<td>VT</td>
<td>Vermont state</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>WST-1</td>
<td>water-soluble tetrazolium salt</td>
</tr>
</tbody>
</table>
X
X 5-P  xylulose 5-phosphate
XIAP  X-linked inhibitor of apoptosis protein

Z
ZnSOD  zinc superoxide dismutase
ZOPO  zopolrestat
UNITS OF MEASUREMENT

%    percent/percentage
AU   arbitrary units
cm   centimeter
ºC   degrees Celsius
g    gram
hr/s hour/s
kDa  kilodalton
Hz   Hertz
l/L  litre
IU   international units
M    molar
mg   milligram
mg/kg milligram per kilogram
min  minutes
ml   millilitres
mHz  milliHertz
ml/min millilitre per minute
mm   millimetre
mM   millimolar
mmol/l millimoles per liters
mm/Hg millimetres of mercury
ng/µl nanograms per microliter
nm   nanometre
nM   nanomolar
pmol picomole
RLU  relative light units
ppm  parts per million
sec  seconds
V    volt
µg   microgram
µg/ml microgram per millilitre
µmol/L micromoles per litre
µl   microlitre
µm   micrometre
μM  micromolar
v/v  volume per volume
w/v  weight per volume
CHAPTER 1
Cardiac metabolism and function under homeostatic state

1.1 Background

Diabetes mellitus poses a huge health burden that may have devastating effects if there are no approximate measures taken immediately (129). Currently, diabetes affects ~171 million people worldwide, and this number is predicted to increase to 366 million by the year 2030, thus making it an epidemic (2, 189). Moreover, diabetes is associated with long-term cardiovascular complications, particularly atherosclerosis, thus further contributing to the overall burden of disease (2, 68, 85). Diabetes is defined as a complex chronic metabolic disorder arising from absolute or relative insulin deficiency or due to insulin resistance (2, 175). This condition can be classified into four main categories: type 1, type 2, gestational diabetes, and secondary to other underlying disease or cause (123).

Type 1 diabetes occurs due to autoimmune destructive lesions of pancreatic β-cells leading to an absolute lack of insulin secretion. However, for type 2 diabetes mellitus (T2DM) (~ 90-95% of all diabetics) there is a combination of impaired insulin secretion and sensitivity (175, 189). T2DM also constitutes one of the phenotypes for the metabolic syndrome i.e. together with obesity, insulin resistance, dyslipidemia and high blood pressure (21). The increased prevalence of T2DM is alarming and poor lifestyle choices mean that it is now manifesting at a much younger age than before. Alarmingly ~ 20% newly diagnosed T2DM cases are from children and adolescents (145). Gestational diabetes is defined as the first onset of diabetes mellitus in women during pregnancy precipitated by an excess production of glucocorticoids (2). The fourth type of diabetes is presence of hyperglycemia.
in the existence of pancreatic or hormonal disease or secondary to drug or chemical exposure and in certain genetic syndromes (123).

Hyperglycemia is a common symptom for all types of diabetes and is the main risk factor predisposing to the development of microvascular (mostly in type 1 diabetic patients), and macrovascular (mostly in type 2 diabetic patients) complications (43, 179). Indeed, diabetic patients have a greater risk for fatal myocardial infarction than non-diabetic individuals with an impaired recovery of the myocardium from ischemic events (38, 90, 166). Moreover, stress-induced, acute hyperglycemia in non-diabetic patients with acute myocardial infarction is associated with increased in-hospital deaths (126, 189). In light of this, the current study focused on the role of acute and chronic hyperglycemia and its link to a well-known macro-vascular complication, i.e. myocardial infarction.

It is proposed that diabetic complications occur as a result of hyperglycemia-induced oxidative stress that increases flux through non-oxidative pathways of glucose metabolism (18, 19). These pathways are the main focus of this thesis, and include the hexosamine biosynthetic pathway (HBP), the polyol pathway, activation of protein kinase C (PKC), and formation of advanced glycation end products (AGEs). This is our focus since previous studies suggest that increased flux through the non-oxidative pathways can lead to altered protein function, thereby exacerbating oxidative stress and leading to pathologic cardio-metabolic sequelae (18, 93). Thus, the goal for the management of diabetes mellitus should be to achieve near normal or improved glycemic control thereby diminishing the risk of long-term pathophysiologic complications (39, 99, 142, 197). There are many standard anti-diabetic drugs used in the management of diabetes mellitus, including various insulin formulations and oral anti-diabetic agents. These can be used as monotherapy, or in combination, in order to achieve improved glycemic regulation (83, 142).
Evidence from epidemiological and clinical trials on glycemic control indicate that management of blood glucose (moderately or intensively) still predisposes diabetic patients to development of both micro- and macrovascular complications. This implies that the burden of diabetes still requires much attention, and necessitates a comprehensive understanding of the underlying molecular mechanisms since it will enable the development of effective, novel therapies that aim to reduced development of the diabetic associated complications. Therefore, this thesis was designed to investigate the role of novel therapeutic interventions to help improve cardiac function following ischemia and reperfusion under hyperglycemic conditions. These include a) S-benzoyl-thiamine monophosphate or benfotiamine (BFT), and b) oleanolic acid (3β-hydroxy-olea-12-en-28-oic acid [OA]). BFT is a lipid soluble vitamin B1 analog that activates the enzyme transketolase (pentose phosphate pathway), thereby shunting glucose away from the four non-oxidative pathways mentioned earlier. Our approach was to identify feasible cardio-protective agents that will allow for easy translation into the clinic, especially within the developing world context. BFT was investigated as previous studies reported beneficial effects in preventing/reducing complications that occur with diabetes mellitus. Moreover, OA is a triterpenoid found in many medicinal plants, food spices, and vegetables and is also implicated in cardio-protection (104). OA possesses various beneficial medicinal effects, we were particularly focusing on its ability to decrease oxidative stress (antioxidant) for example, it has been reported to quench superoxide (1, 158). However, the efficacy of these compounds has not been investigated in the hyperglycemic context associated with myocardial ischemia.

Chronic hyperglycemia that occurs with diabetes is mainly due to derangements in carbohydrate, lipid and protein metabolism (137, 189). Such metabolic perturbations may impair cardiac function with ischemia and reperfusion by triggering a range of biochemical and electrophysiological changes (174). In diabetes there is shift in substrate utilization in the heart with fatty acids prominent. This leads to increased fatty acid oxidation (FAO), transport and metabolism. This shift consequently results in both diastolic and systolic cardiac function (3). The first chapter of this dissertation will review cardiac
energy metabolism of the normal adult mammalian heart and aims to set a platform for better understanding the biochemical and molecular mechanisms involved in cardiac pathophysiology under hyperglycemic conditions (to be reviewed in detail in Chapter 2). Cardiac energy metabolism is complex, and to simplify matters this chapter will focus on its three basic components, i.e. substrate utilization; oxidative phosphorylation, and the creatine kinase system. These are discussed in the sections below.

1.2 Cardiac energy metabolism

1.2.1 Substrate utilization by the heart

The heart is an omnivorous organ that can rapidly switch its substrate selection to accommodate different physiologic and pathophysiologic conditions. Substrate utilization depends on the concentration of extracellular hormones, substrate availability, rate of oxidative phosphorylation, and energy demand (4, 63, 94, 124, 168, 173). Fatty acids (predominantly long-chain) provide the main source of energy for the normal adult mammalian heart, accounting for ~60-80% of its energetic requirements. The remainder is provided by the oxidation of lactate and carbohydrates (primarily glucose) in equal proportions by glycolysis and the tri-carboxylic acid cycle as guanidine triphosphate (GTP) (63, 86). However, the first step for effective substrate utilization involves its uptake by cardiomyocytes. Thus, in the following sections the mechanisms regulating transport, uptake and metabolism of glucose and free fatty acids are described in more detail.
1.2.2 Mechanisms regulating cardiomyocyte glucose uptake

Under normal physiological conditions, glucose is one of the major carbohydrates utilized by the heart. Sources of glucose supply include both exogenous (dietary intake) and endogenous sources (glycogenolysis or gluconeogenesis). The metabolism of glucose is regulated through multiple steps that include uptake, glycolysis and pyruvate decarboxylation (3). Extracellular glucose uptake by cardiac myocytes is regulated by the transmembrane glucose concentration gradient, and also the amount, subcellular distribution and activity of glucose transporters (108, 121, 195). For the mammalian heart three facilitative glucose transport proteins (GLUTs) have thus far been identified, i.e. GLUT1, GLUT3 (159) and GLUT4. These transporters are members of a family of 50 kDa integral proteins that contain 12 membrane-spanning domains and six extracellular loops (121).

There are no studies on the role of GLUT3 in the heart in the literature. However, most of myocardial glucose uptake is carried out by GLUT1 and GLUT4. GLUT1 is largely found in the plasma membrane and accounts for glucose uptake under basal conditions (17, 120). GLUT4 is the most abundant transporter in the adult heart and is mainly located in intracellular storage vesicles under basal conditions (~90%) compared to the cell membrane (~3-10%) (77). GLUT4 is the classic insulin-stimulated glucose transporter that ensures increased trafficking from intracellular locations to the cell membrane (8, 53, 108, 118, 187). More recently, sodium-dependent glucose co-transporters I and II (SGLTI and II) were identified in the heart (5, 198), but their precise functional roles in the heart requires further elucidation. However, early studies showed the importance of SGLTI in a glycogen storage cardiomyopathy in transgenic mice (TG$^{400N}$) due to mutations in the PRKAG2 gene that encodes the $\gamma$-2 subunit of 5’ AMP-activated protein kinase (AMPK). These mice have been shown to exhibit inappropriate activation of AMPK with consequent increased glucose uptake, glycogen synthesis and storage as a result of increased SGLTI mRNA levels and protein expression (5).
Since intracellular glucose levels are usually very low (117), high extracellular glucose concentrations drive glucose transport and *vice versa* e.g. occurring during hypoglycemia or ischemia (95). GLUT transporters have distinct structural components and immune-adsorption studies show that they reside in different subcellular pools, and travel to the membrane independently (53, 169). Increased availability of transporters on the cell membrane involves four processes namely; signaling, translocation, docking and fusion (84, 87). The primary mechanism involved in the acute activation of GLUT transporters from the intracellular pool to the cell membrane is known as translocation (150). This primarily involves outward movement of transporters to the cell membrane, and to some extent it may also be a reflection of the decreased degree of recycling of glucose transporters by endocytosis (150). Insulin increases the incorporation of GLUT1 and GLUT4 into the cell membrane although GLUT4 is the main insulin-dependent isoform in the heart (mechanism is explained in more detail below). Although the cell membrane is the primary site for glucose transporter translocation, glucose transporters can also translocate to the T-tubules (162). In addition to insulin several other stimuli also lead to the translocation of myocardial glucose transporters and these include, ischemia (refer to Chapter 2), exercise and catecholamines.

### 1.2.3 Role of insulin in substrate utilization

Insulin is a potent anabolic hormone that promotes synthesis and storage of carbohydrates, lipids and proteins, thereby inhibiting their degradation and release into circulation (96). This hormone activates two major signaling events, the mitogen activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI 3-K)-dependent pathway (34, 96, 183). The MAPK pathway plays a prominent role in transcriptional and mitogenic processes such as alterations in growth and regulation of gene expression, while the PI 3-K pathway is crucial in metabolic signaling, i.e. glucose and fatty acid transport (Figure 1.1). Indeed, PI 3-K inhibitors (class Ia) or transfections with dominant negative PI 3-K constructs inhibit the effects of insulin on glucose transport, glycogen and lipid synthesis (160).
The proximal part of the insulin signal transduction pathway is common to all its metabolic effects. The insulin receptor is a tetrameric enzyme comprising of two extracellular α-subunits and two transmembrane β-subunits. The sequence begins by insulin binding to the extracellular part of the alpha subunits on the insulin receptor. This in turn causes a conformational change in the receptor that induces activation of the intrinsic tyrosine kinase activity on the β-subunits of the receptor. The activated kinase domain leads to an auto-phosphorylation where one β-subunit phosphorylates the other on several tyrosine residues of the receptor as well as in the interacting protein; insulin receptor-substrate 1 (IRS1) (10, 36, 73). Once activated IRS1 then bind with Src homology two proteins (SH2-phosphotyrosine binding sites), including PI 3-K, Ras GTPase-activating protein, phospholipase C and others (122, 148). PI 3-K catalyzes the formation of phospho-inositol triphosphate which activates protein kinase B (PKB) (see Figure 1.1). PKB phosphorylates glycogen synthase kinase 3β (GSK 3β) thereby inactivating it; hence resulting in increased glycogen stores (50, 167, 169). In addition PKB also facilitates vesicle fusion, resulting in an increase in glucose and fatty acid transporters (Figure 1.1) (113).

After the signal has been produced its termination occurs mainly by endocytosis and degradation. Signaling can also be terminated by de-phosphorylation of the receptor and IRS tyrosine residues by tyrosine phosphatases (PTPases). Most attention thus far has focused on protein tyrosine phosphatase 1B (PTP 1B) also known as tyrosine-protein phosphatase non-receptor type 1 (PTPN1), e.g. transgenic mice lacking PTP 1B showed greater insulin sensitivity with increased tyrosine phosphorylation of both the receptor and IRS1 (50). The activity of the insulin receptor may be attenuated by serine/threonine kinases that may decrease insulin-stimulated tyrosine phosphorylation and promote interaction with 14-3-3 proteins (36, 73). Such inhibitory phosphorylation provides negative feedback to insulin signaling and serve as a cross-talk mechanism with other pathways that may lead to insulin resistance. Several kinases have been implicated in this process, including PI 3-K, Akt/PKB, glycogen synthase kinase (GSK)-3β and mammalian target of rapamycin (mTOR) (148).
As discussed before, with insulin stimulation there is elevated myocardial uptake of both LCFAs and glucose due to increased translocation of FAT/CD36 transporters (49, 59, 101, 165) and GLUT1/4 (8, 49, 117, 150, 169, 182, 186), respectively (see Figure 1.1 below). More recent data show that insulin can also increase myocardial glucose uptake by SGLTI acting via the PI 3-K pathway (103). Furthermore, studies in isolated rat hearts found that insulin causes the release of hexokinase from the outer mitochondrial membrane of the mitochondria, thereby increasing glucose uptake and phosphorylation (174). Insulin-mediated glucose phosphorylation is the rate-limiting step for insulin-stimulated glucose utilization.

**Figure 1.1. The role of insulin on glucose and fatty acid uptake.** FAT/CD36 (fatty acid translocase), GLUT4 (glucose transporter 4), IRS1 (insulin receptor substrate 1), PI 3-K (phosphoinositide 3-kinase), PKB (protein kinase B/Akt), ADP (adenosine diphosphate), ATP (adenosine triphosphate, P (phosphate residue).
1.2.4 Fate of glucose after uptake by cardiomyocytes

1.2.4.1 Glycolysis

Upon entering the cardiac myocytes glucose is rapidly phosphorylated by hexokinase II (in adult hearts) into glucose-6-phosphate in the presence of magnesium ions. Thus, it is trapped in the cell since the membrane is impermeable to phosphate esters (14):

\[
\alpha-D\text{-glucose} + ATP^4- \rightarrow \alpha-D\text{-glucose-6-phosphate}^2- + ADP^3- + H^+.
\]

The Mg\(^{2+}\) is complexed with ATP\(^4-\) and is present as MgATP\(^2-\). Several steps are involved in controlling the glycolytic rate in the heart, including glucose transport, phosphorylation, and the reaction catalyzed by phosphofructokinase-1 (PFK-1) (140). PFK-1 catalyzes the generation of fructose 1,6-bisphosphate from fructose-6-phosphate (42, 140), and is a multi-modulated enzyme inhibited by low pH, high intracellular citrate or ATP (Figure 1.2). Conversely it is activated by ADP, AMP, phosphate and fructose 2,6-bisphosphate (F2,6-BP) (41, 140) (refer to Figure 1.2). F2,6-BP is formed from fructose-6-phosphate by phosphofructokinase-2 (PFK-2) (75). The production of F2,6-BP itself is highly regulated; here PFK-2 activity can be increased by phosphorylation mediated by hormones such as insulin, glucagon (166), epinephrine and thyroid hormone (75), or kinases e.g. 5’ adenosine monophosphate-activated protein kinase (AMPK) and increased levels of citrate (Figure 1.2). Additionally activation of protein kinase C (PKC), protein kinase A (PKA) and PI 3-K can increase PFK-2 activity (3, 40, 111) (Figure 1.2). Stimulation of PFK-2 through these mechanisms increases F2,6-BP generation, activates PFK-1, and subsequently promotes glycolysis. The second rate-limiting enzyme of glycolysis is glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which catalyzes the conversion of glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate, with production of NADH (see Figure 1.2). Since the factors regulating GAPDH activity become prominent during ischemia, this will be discussed within the context of ischemia and reperfusion (114).
Figure 1.2: Cardiomyocyte glucose uptake and metabolism by glycolysis. Key regulatory enzymes; PFK-1 and GAPDH with their respective modulators are shown. PFK-1 or 2 (phosphofructokinase 1/2); GAPDH (glyceraldehyde-3-phosphate dehydrogenase); AMP (adenosine monophosphate); AMPK (AMP-activated kinase); MCT (monocarboxylic translocase); PDH (pyruvate dehydrogenase); ADP (adenosine diphosphate); ATP (adenosine triphosphate); LDH (lactate dehydrogenase); PDK4 (pyruvate dehydrogenase kinase 4).

During conditions when oxygen is not limiting, glycolysis contributes ~10% of total myocardial ATP, i.e. two ATPs generated per glucose molecule (127). Glycolytic ATP is thought to play a critical role in the maintenance of ion pump function due to the proximity of glycolytic enzymes and the ATPases (67, 127). In support, studies in isolated heart tissues found that glycolytically-derived ATP can be employed for calcium re-uptake by the sarcoplasmic reticulum, and also for optimal diastolic relaxation.
Additionally key glycolytic enzymes are associated with the cardiac ATP-sensitive K\(^+\) channels that are inhibited by glycolytic ATP (185). Glycolysis is also important for optimal function of the Na\(^+/\)K\(^+\) ATPase and prevention of Na\(^+\) accumulation during ischemia (185).

The end products of glycolysis; reduced nicotinamide adenine dinucleotide (NADH) and pyruvate, can thereafter be shuttled into the mitochondrial matrix to eventually generate carbon dioxide and oxidized nicotinamide dinucleotide (NAD\(^+\)), or in some instances converted to lactate and NAD\(^+\) in the cytosol (non-oxidative glycolysis) (166). The latter is especially important in conditions where oxygen is limiting and pyruvate is converted to lactate by lactate dehydrogenase while oxidizing NADH to NAD\(^+\).

Lactate transport across the cardiac cell membrane is facilitated by the monocarboxylic acid transporter-1 (MCT-1) (see Figure 1.2). Lactate, ketone bodies and amino acids are not major contributors to ATP production under normal aerobic conditions since it is found in relatively low circulating concentrations (167). Normally the heart is a net consumer of lactate, however, if glycolysis is accelerated (e.g. during ischemia), it becomes a net producer of lactate (167).

Inside the mitochondrial matrix, pyruvate may either be decarboxylated to acetyl-CoA or carboxylated to oxaloacetate or malate. The latter reaction is anaplerotic and acts to maintain the tricarboxylic acid cycle (TCA) pool size of intermediates, and TCA function by counteracting the small constant loss of intermediates through efflux of citrate, succinate and fumarate (32, 51, 58, 131, 180). Pyruvate carboxylation accounts for \(~2-6\%\) of the citric acid cycle under normal aerobic conditions (32, 180).

The conversion of pyruvate to acetyl-CoA requires the action of pyruvate dehydroxylase, dihydrolipoyl transacetylase, dihydrolipoyl dehydrogenase and five coenzymes, i.e. thiamine pyrophosphate, lipoic acid, coenzyme A, flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NAD); collectively referred to as the pyruvate dehydrogenase complex (PDH) located within the mitochondrial matrix (173). PDH is phosphorylated on the E\(_1\) subunit and inactivated by PDH kinase 4 (PDK4).
PDK4 in turn is inhibited by pyruvate and decreases in acetyl-CoA/free CoA and NADH/NAD⁺ ratios (15, 72). At another regulatory level, high circulating lipid and intracellular accumulation of long-chain fatty acid moieties (e.g. with diabetes and fasting) enhance peroxisome proliferator activated receptor-alpha (PPAR-α) gene pathways thereby attenuating PDH and pyruvate oxidation (91, 190). The role of PPAR-α on fatty acid uptake will be discussed in detail below. PDH activity is also regulated by a specific PDH phosphatase (131), and can also be enhanced by calcium and magnesium ions that are increased by adrenergic stimulation (88, 91). The product of this reaction (acetyl Co-A) then enters the TCA cycle (Figure 1.2) where it is converted to either malate or oxaloacetate (166). Thus, PDH activity is finely regulated depending on the degree of activities of upstream modulators, i.e. PDK4, and PDH phosphatase and various metabolites as discussed. In summary, this section has reviewed the mechanisms regulating glucose uptake and its fate in cardiomyocytes, with some emphasis on the importance of insulin in this regard. The next section of this chapter will focus on mechanisms regulating myocardial fatty acid uptake and utilization.

1.2.5 Myocardial fatty acid (FA) uptake

As mentioned earlier, the adult heart uses FAs as its main source of ATP production. Fatty acids are present in the circulation in several forms: complexed with albumin, esterified in the lipid core of very low density lipoproteins (VLDLs), and chylomicrons (163, 181). Normal free fatty acid concentrations in the circulation vary between 0.2 and 0.6 mM. However, this may vary depending on the physio/pathophysiologic states e.g. FA levels are relatively low in the fetus, while it can increase to over 2 mM during ischemia or uncontrolled diabetes (101, 166). Albumin-bound FAs easily dissociate from albumin while esterified FAs are hydrolyzed by lipoprotein lipase at the luminal surface before traversing the cell membrane and entering cells. Although some FAs traverse the cell membrane passively by diffusion, the majority (~90%) are transported via carrier proteins (164).
Several carrier proteins play a role in FA transport across the cell membrane, including the 88 kDa scavenger receptor CD36 (rat homologue called fatty acid translocase [FAT]; 43 kDa plasma membrane fatty acid binding protein (FABPpm); and two isoforms of the fatty acid transport proteins (FATP) i.e. FATP1 and FATP6 (31, 34, 61, 110, 165, 170). FATP6 is exclusively expressed in the heart and it is more abundant than FATP1 (59, 151). Since FATP is associated with the cell membrane and co-localizes with FAT/CD36, it is suggested that these two transporters may act in concert (59). However, the main putative long-chain fatty acid (LCFA) carrier protein is CD36/FAT. Moreover, CD36/FAT is found both in storage vesicles inside the cell and on the cell membrane (similar to GLUT4) (62). Insulin can increase expression of both FAT/CD36 and FATPs for LCFAs uptake (49, 59, 101, 165) (refer Figure 1.1 above) (mechanisms involved are as previously described in section 1.2.3). The in vivo translocation of FAT/CD36 upon insulin stimulation, however, has not been demonstrated in the heart (161).

1.2.5.1 Fate of FAs in cardiomyocytes

After uptake LCFAs bind to a 15 kDa cytoplasmic heart-type fatty acid binding protein (H-FABPc) (refer Figure 1.3) and employ it as a vehicle to migrate towards their site of metabolic conversion or action (60, 97, 152). Subsequently, LCFAs are converted to acyl-CoAs by acyl-CoA synthetase (ACS) whereafter acyl-CoAs bind to the cytoplasmic acyl-CoA binding protein (ACBP) (refer Figure 1.3). Acyl-CoAs can either be used for triacylglycerol synthesis, be incorporated into intracellular lipid pools or undergo mitochondrial β-oxidation. Transport of acyl-CoAs into the mitochondrion is mediated by the action of three carnitine-dependent enzymes (48). As shown in Figure 1.3, carnitine palmitoyl transferase I (CPT-I), located at the outer mitochondrial membrane, catalyzes formation of acylcarnitine, and is the key regulatory enzyme in LCFA mitochondrial uptake. Thereafter, carnitine/acylcarnitine transferase (CACT) transports acylcarnitine into mitochondria in a 1:1 ratio for carnitine or other acylcarnitines. Carnitine palmitoyl transferase II (CPT-II) is located at the inner
mitochondrial membrane, and generates acyl-CoA by transferring acyl residues from acylcarnitine to free CoA, and carnitine (reshuttled back to the intermembrane space by the CACT transporter) (92, 106, 157). The acyl-CoA formed is oxidized via β-oxidation to acetyl-CoA, NADH and FADH$_2$ (see Figure 1.3). The latter two compounds are reoxidized in the mitochondrial electron transport chain, whereas the acetyl-CoA is oxidized to carbon dioxide in the tricarboxylic acid cycle to regenerate free CoA (34, 157).

As with glucose oxidation, fatty acid oxidation is also enhanced in response to increased AMPK activation following higher intracellular AMP: ATP ratios (66). AMPK facilitates FA utilization through phosphorylation and inactivation of acetyl-CoA carboxylase (ACC) (100). ACC catalyzes the conversion of acetyl-CoA to malonyl-CoA that direct either the inhibition of beta oxidation or the activation of lipid biosynthesis. Two main isoforms of ACC are expressed in mammalian tissues with the heart expressing ACC2 (or β) in a higher ratio than ACC1 (or α) confirming a greater need for FA oxidation in the heart (vs. FA synthesis) (6). Malonyl-CoA is a potent inhibitor of CPT-I and AMPK activation decreases its levels thereby removing the inhibition of CPT-I and elevated FA oxidation as shown in Figure 1.3. AMPK may also regulate FAT/CD36 indirectly by inactivation of ACC, thus promoting increased fatty acid transport and subsequent oxidation (109). Furthermore, malonyl-CoA decarboxylase (MCD) can increase FA oxidation since it catalyzes the production of acetyl-CoA from malonyl-CoA thereby attenuating malonyl-CoA levels (161) (see Figure 1.3).

Another key regulatory component of FA oxidation occurs at the transcriptional level by the nuclear receptor peroxisome proliferator-activated receptor-α (PPAR-α) a nuclear receptor (66, 92, 100). They are activated by natural ligands such as fatty acids or numerous pharmacological ligands (54). PPAR-α forms a heterodimer retinoid–X-receptor, and subsequently binds to PPAR response elements on the promoter regions of target genes (16, 52, 54). These include for example, medium-chain acyl-CoA dehydrogenase, fatty acid binding proteins, fatty acid transporters, 3-ketoacyl-CoA thiolase (119),
CPT-I (16) and malonyl-CoA decarboxylase (22). FAs are endogenous ligands for PPAR-α, hence if levels are increased they promote expression of genes involved in fatty acid oxidation (FAO), uptake and PDK4 expression (74) (refer to Figure 1.3 below). Similar to PPAR-α, PPAR-β (or -δ) is expressed abundantly in cardiac tissue (7). Activated by elevated intracellular FA (29), PPAR-β (or -δ) augments expression of a group of genes that promote FA utilization (46, 188). Cardiac-specific knockout of PPAR-β also decreases FA oxidative gene expression and FAO (30). Although the targets of PPAR-α and PPAR-β are partially overlapping (188), their unique roles and interaction remains unclear in the heart.

The acetyl-CoA generated by FAO subsequently converges with the end products of glycolysis and thereafter enter the Krebs cycle and mitochondrial electron transport chain discussed in sections 1.2.6 and 1.2.7.1, respectively.
1.2.6 The tricarboxylic acid (TCA)/ Krebs/ citric acid cycle

The TCA cycle generates reducing equivalents for mitochondrial oxidative phosphorylation that eventually results in the generation of ATP, carbon dioxide, NADH, FADH₂ and guanosine triphosphate (GTP). In the TCA the most important sites of control are citrate synthase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase and malate dehydrogenase. Of these, isocitrate dehydrogenase, pyruvate dehydrogenase and α-ketoglutarate dehydrogenase activity is increased by calcium ions (Figure 1.3) (128). This is fueled by acetyl-CoA formed primarily from the oxidation of free fatty acids, carbohydrates and proteins (58, 124). Here acetyl-CoA transfers its acetyl group to
oxaloacetate, thereby generating citrate. Citrate provides the precursors (acetyl-CoA, NADH) for fatty acid synthesis, and is a positive allosteric modulator of ACC (112). Citrate exits from mitochondria via the tricarboxylate carrier (173) and regulates glycolysis by negative modulation of 6-phosphofructokinase activity (see above). In a cyclic series of reactions, citrate is subjected to two successive decarboxylations and four oxidative events, generating malate, a four-carbon compound. Only one ATP molecule is directly generated by a singular TCA cycle. Five of the TCA intermediates (malate, 2-oxoglutarate, succinyl-CoA, oxaloacetate and fumarate) are involved in anaplerotic reactions (refer to Figure 1.3) that act to replenish the cycle and are crucial for normal cardiac function (58, 146, 172).

Most studies have focused on α-ketoglutarate dehydrogenase which catalyzes the conversion of alpha ketoglutarate, CoA and NAD⁺ to succinyl-CoA, NADH and CO₂. The production of NADH by α-ketoglutarate dehydrogenase is crucial for mitochondrial respiration and oxidative phosphorylation (33, 76, 116). This enzyme requires thiamine pyrophosphate as a cofactor (discussed later in Chapter 2), and is highly regulated by modulators that include ATP/ADP and NADH/NAD⁺ ratios, calcium, and substrate availability in mitochondria. It is also a primary site of control for TCA flux (81, 172) e.g. it can be inhibited by its end products succinyl-CoA and NADH (57, 178).

All enzymes of the TCA are located within the mitochondrial matrix, except for succinate dehydrogenase that is embedded within the inner mitochondrial membrane. Thus the reducing equivalents formed (NADH, FADH₂) have easy access to the mitochondrial electron transport chain and thereby ensuring generation of mitochondrial ATP (76, 92).
Figure 1.4: The tricarboxylic acid cycle/ Krebs cycle/citric acid cycle. The enzymes pyruvate dehydrogenase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase can enhance flux into the Krebs cycle in response to increased cytosolic calcium. NADH (nicotinamide adenine dinucleotide hydrogen); FADH$_2$ (flavin adenine dinucleotide hydrogen); GTP (guanosine 5-triphosphate); GDP (guanosine 5-diphosphate). The dotted lines indicate anaplerotic reactions.

1.2.7 The role of the mitochondria in oxidative phosphorylation

Due to the heart’s high energy demands, cardiac cells have a high oxidative capacity with mitochondria making up ~25-35% of total cardiomyocyte volume (44). Two types of mitochondria have been described i.e. interfibrillar mitochondria (IFM) and subsarcolemmal mitochondria (SSM) (130). These types differ in terms of their cristae structure, rates of respiration, and expression of metabolic
proteins (130, 141). Under normal conditions, both SSM and IFM are efficient in meeting demands of the cellular ATP-dependent processes and maintaining ionic homeostasis of cells (71). Mitochondria produce ATP via the TCA and the respiratory chain (in the presence of oxygen), and hence this process is referred to as mitochondrial oxidative phosphorylation. The reducing equivalents generated by the TCA cycle provide electron energy for mitochondrial ATP production. However, glycolytically-derived NADH enters mitochondria via the malate-aspartate cycle, i.e. aspartate is extruded from mitochondria in exchange for malate (89). The process of mitochondrial oxidative phosphorylation occurs via protein complexes within the mitochondria as described in detail below.

1.2.7.1 The electron transport chain (ETC)/ respiratory chain

The protein components of the respiratory chain are oligomeric complexes that are located within the inner mitochondrial membrane. These complexes are referred to as multi-subunit electron transport complexes I, II, II and IV along with ATP synthase (complex V). Electrons enter the electron transport chain through NADH: ubiquinone oxidoreductase (complex I), the largest respiratory complex (molecular mass > than 900 kDa and contains at least 45 different subunits) (23, 24, 27). Complex I is the main rate-controlling steps of the electron transport chain (35), and catalyzes the electron transfer from NADH to ubiquinone (Q) through a series of redox centers that include a flavin mononucleotide moiety, seven to nine iron-sulfur centers, and up to three detectable ubisemiquinone species (55, 98, 125, 192). Complex I is one of three respiratory complexes where energy is conserved since four protons are translocated across the mitochondrial inner membrane coupled to electron transfer (see Figure 1.5). Therefore, it contributes to the proton-motive force that supports the synthesis of ATP, maintains the NAD⁺/NADH ratio in the mitochondrial matrix, and supplies ubiquinol to the complex III (69). Several studies have shown that cardiolipin (a phospholipid) is required for optimal functioning of complex I (47, 56, 132, 135). Cardiolipin also plays an important role in the functioning of other inner
mitochondrial complexes of the electron transport chain and anion carriers, though its precise mechanisms are not yet fully understood (70, 143, 153).

Succinate dehydrogenase (complex II; or succinate: ubiquinone oxidoreductase, SQR) is a functional member of both the Krebs cycle and the aerobic respiratory chain. Complex II couples the oxidation of succinate to fumarate in the mitochondrial matrix with the reduction of ubiquinone in the membrane as shown in Figure 1.5 (26, 191). Ubiquinol produced by the action of membrane-bound dehydrogenases such as complex I, II and electron transfer flavoprotein (ETF)-ubiquinone oxidoreductase (Q-reductase) is oxidized by complex III (ubiquinol-cytochrome c oxidoreductase or cytochrome bc1 complex). Complex III is located at the “crossroads” of the ETC, independently receiving electrons from complexes I and II (134). Complex III contains two subunits that include a membrane-bound diheme cytochrome b, a membrane-anchored cytochrome c1, and [2Fe-2S]-containing Rieske iron-sulfur protein. The electrons from ubiquinol are transferred to cytochrome c, and this reaction develops the proton motive Q cycle (27, 134). The Q cycle receives electrons from complex I and II, and cytochrome b acts as a bridge for electron transfer from the Q cycle to complex IV (136). Complex III is thus another mitochondrial respiratory complex where energy is conserved. In between complex II and complex IV is a mobile electron carrier called cytochrome c (see Figure 1.5) (65). A proton gradient is generated by the terminal cytochrome oxidase (complex IV) (contains 13 subunits) (9, 27, 65, 107, 193, 194) that has four redox metal centers where electrons are transferred, and subsequently reduce oxygen to two water molecules (27, 107, 149, 193, 194).

The final component of the mitochondrial oxidative phosphorylation system is ATP synthase (complex V or F_1F_0 ATPase). This is a functionally reversible enzyme that can use the proton gradient generated by the electron system to produce ATP. It can also hydrolyze ATP and pump protons against an electrochemical gradient (see Figure 1.5) (27). Thus, mitochondrial F_1F_0 ATP synthase regenerates ATP utilizing the potential energy produced in the respiratory chain. Myocardial ATP
generated is in close equilibrium with creatine phosphate which acts as a temporary store of high energy phosphate bonds. Creatine kinase plays a key role and this is further discussed below.

**Figure 1.5: Schematic diagram of the mitochondrial electron transport chain.** Electrons flow from complex I to complex V and subsequently reduce oxygen to water generating ATP via the ATPase. NADH (reduced nicotinamide adenine dinucleotide); ADP (adenosine 5’ diphosphate); ATP (adenosine 5’ triphosphate).

**1.2.8 The creatine kinase system of the heart**

Creatine kinase enzyme is located within both the mitochondrial intermembrane space and the cytosol (147). It catalyzes the transfer of a high energy phosphate bond from ATP to creatine forming creatine phosphate, acting as a buffer for ATP (82). Bessman *et al.* (1980) proposed the existence and function of the phosphorylcreatine shuttle whereby mitochondrial creatine phosphate/phosphocreatinediffuses to the contractile apparatus to serve as an energy source for myocardial contraction (11). Here creatine kinase binds to the myosin chain and transfers the high energy phosphate moiety from creatine phosphate to ADP thereby regenerating ATP. This is then hydrolyzed by myosin ATPase to
initiate the myocardial contractile processes (45). Free creatine formed by the removal of the high energy phosphate then diffuses back into mitochondria.

The bulk of the creatine is formed by the liver and kidneys, transported to the heart and actively taken up by a creatine phosphate transporter (64). The creatine kinase shuttle system plays a key role to regulate the high demands of the heart that is required to maintain normal contractile function, metabolic processes, and ionic homeostasis (126, 166). At rest, the rate of myocardial ATP hydrolysis is high (~30 µmol/g wet wt/min), and this result in a complete turnover of the entire myocardial ATP pool every 10 seconds (126, 174). When the heart’s energy demands outstrip energy production and supply, phosphocreatine levels decrease to maintain ATP at normal levels, thus fulfilling its role as a buffer system. However, free ADP levels increase in parallel and results in the inhibition of many intracellular enzymes ultimately impairing the heart’s contractile mechanisms.

1.2.9 Inter-relationship between glucose and fatty acid metabolism (Randle cycle)

The regulation of glucose and fatty acid metabolism does not occur independently, but rather are inter-linked (138). The preferential use of FAs by cardiomyocytes involves FA-mediated inhibition of carbohydrate utilization at the levels of glucose transport, phosphofructokinase, hexokinase, glycogen phosphorylase, pyruvate dehydrogenase, and stimulation of glycogen synthase (78, 133). The inhibition of glucose transport was first demonstrated by using isolated rat hearts where LCFA or ketone bodies inhibited glucose uptake (133). At another level, inhibition of phosphofructokinase by FAs results via increased tissue levels of cytosolic citrate derived from FAO (see Figure 1.6) (139, 166). This leads to elevated fructose-6-phosphate and glucose-6-phosphate levels that inhibit hexokinase thereby increasing intracellular myocardial glucose concentrations. This decreases the concentration gradient for glucose uptake hence leads to impaired glucose uptake and insulin
resistance in cases of increased dietary FA uptake and/or in diabetes. It is unclear, however, if citrate levels inhibit PFK \textit{in vivo} (167).

Increased glucose-6-phosphate levels also inhibit glycogen utilization through the inhibition of glycogen phosphorylase-\(\beta\). Glycogen phosphorylase-\(\beta\) phosphorylates glycogen synthase thereby attenuating glycogen synthesis. Therefore, the net effect of PFK inhibition is decreased utilization of glucose via glycolysis and increased/decreased glycogenesis (28, 176). However, the key effect of fatty acids on glucose oxidation is the inhibition of pyruvate dehydrogenase by increased intra-mitochondrial acetyl-CoA and NADH derived from fatty acids oxidation (see Figure 1.6) (166). Additionally, fatty acyl-CoA blunts insulin-mediated glucose uptake by inhibiting IRS substrates, PKB and hexokinase (154, 155). However, this proposition by Randle (1963) still remains controversial with some studies indicating that the FA-mediated decrease, in glucose oxidation constitutes less than a third of the overall decrease, and that the remainder may be attributed to impaired glucose metabolism in non-oxidative pathways of glucose metabolism (12, 13, 144).

Conversely, inhibition of FAO increases glucose and lactate uptake/oxidation thereby improving cardiac function in some pathological states. This occurs by decreasing citrate levels removing the inhibition on PFK, and also lowering acetyl-CoA and/or NADH levels in the mitochondrial matrix, and thereby relieving the inhibition of PDH (166). This effect was observed in several studies where lipid oxidation was inhibited at various parts. These include inhibition of CPT-I with etomoxir; inhibition of MCD that elevates malonyl-CoA content to decrease CPT-I activity (22); and inhibitors of lipolysis in adipocytes. Conversely, other studies have shown that deficiency of CPT-Ib (an isoform predominantly expressed in the heart) may lead to lipotoxicity and cardiac dysfunction (3). Nevertheless, substrate utilization under different physiological states plays a crucial role on regulating cardiac function. As such Table 1.1 gives examples of studies that show the effect of substrate utilization on cardiac function.
Table 1:1: Examples of studies that show the effect of substrate utilization on cardiac function.

<table>
<thead>
<tr>
<th>Experimental model</th>
<th>Nature of substrate utilization</th>
<th>Effect on function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex vivo neonatal rabbit hearts</td>
<td>Increased FA levels</td>
<td>Increased post-ischemic cardiac contractile function</td>
<td>(79)</td>
</tr>
<tr>
<td>Ex vivo Wistar rat hearts</td>
<td>Inhibition of FAO increased glucose oxidation</td>
<td>Improved cardiac functional recovery</td>
<td>(105)</td>
</tr>
<tr>
<td>Ex vivo obese Zucker rat hearts</td>
<td>Increased FA availability</td>
<td>Contractile dysfunction</td>
<td>(196)</td>
</tr>
<tr>
<td>In vivo ob/ob and db/db mice hearts</td>
<td>Increased FAO and reduced glucose oxidation</td>
<td>Reduced cardiac efficiency and contractile dysfunction</td>
<td>(20)</td>
</tr>
<tr>
<td>Ex vivo rat hearts</td>
<td>Hypertriglyceridemia and effect of FAO inhibition</td>
<td>Impaired post-ischemic recovery</td>
<td>(115)</td>
</tr>
<tr>
<td>In vivo human hearts</td>
<td>Increased glucose uptake</td>
<td>Improved post-ischemic cardiac function</td>
<td>(25, 37)</td>
</tr>
</tbody>
</table>

FA (fatty acid); FAO (fatty acid oxidation); ob/ob (strain of obese mice); db/db (strain of diabetic mice)

In summary, increased FAO inhibits glycolysis (Randle cycle) as occurs under both physiological (postprandial increase of plasma FA levels) and pathophysiological conditions (e.g. diabetes, ischemia) (171). It therefore, implies that high FA utilization in diabetes and ischemia and reperfusion impair glucose utilization thereby contributing to hyperglycemia and insulin resistance (28) (refer Chapter 2). As the heart adapts to use of fatty acids for ATP generation there is decreased efficiency since more oxygen will be consumed with less energy produced. This increases the risk of cardiac dysfunction especially during ischemia with reduced oxygen supply for FAO (177).
Figure 1.6: The Randle cycle showing the interrelationship between myocardial fatty acid and glucose metabolism. IRS/PKB (insulin receptor substrate/protein kinase B); PFK-1 (phosphofructokinase-1); PDH (pyruvate dehydrogenase); PDK4 (pyruvate dehydrogenase kinase 4); CPT-I (carnitine palmitoyl transferase-I); PPAR-α (peroxisome proliferator-activated receptor-α); NADH (nicotinamide adenine dinucleotide hydrogen); FA (fatty acids).

1.2.10 Summary and conclusion

This chapter briefly reviewed cardiac energy metabolism to set the scene for the focus of this dissertation, i.e. the effects of hyperglycemia on the heart. Such metabolic dysregulation may occur at any or all of the three main components discussed i.e. substrate utilization, mitochondrial energy production, and ATP transfer/utilization. The next chapter of this dissertation will now discuss cardiac energy metabolism, focusing on hyperglycemia within the context of diabetes and myocardial infarction.
1.2.11 References


57. **Garland PB.** Some kinetic properties of pig-heart oxoglutarate dehydrogenase that provide a basis for metabolic control of the enzyme activity and also a stoichiometric assay for coenzyme A in tissue extracts. *Biochem J* 92: 10C-12C, 1964.


71. Holmuhammero EL, Oberlin A, Short K, Terzic A, Jahangir A. Cardiac subsarcolemmal and interfibrillar mitochondria display distinct responsiveness to protection by diazoxide. *Plos One* 2012;7:.


100. **Kudo N, Barr AJ, Barr RL, Desai S, Lopaschuk GD.** High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in malonyl-CoA levels due to an increase in 5'-AMP activated protein kinase inhibition of acetyl-CoA carboxylase. *J Biol Chem* 270: 17513–17520, 1995.


CHAPTER 2
The effects of hyperglycemia on cardiac metabolism and function with ischemia and reperfusion

2.1 Introduction

As a follow up from the previous chapter that reviewed cardiac energy metabolism under normal conditions, this chapter’s focus is on the detrimental effects of hyperglycemia ± ischemia and reperfusion on the heart. It will highlight evidence that link hyperglycemia to cardiovascular diseases (CVDs), particularly myocardial infarction. Various mechanisms whereby hyperglycemia affects the pathogenesis of cardiovascular disease ± myocardial infarction will be discussed. Moreover, there will be focus on both chronic and acute hyperglycemia as evidence show that both forms have deleterious effects (484). Chronic hyperglycemia is a common symptom of diabetes and/or the metabolic syndrome, whereas acute hyperglycemia can occur in both non-diabetics and diabetics during stress conditions, e.g. following an acute myocardial infarction (AMI), or after eating (post-prandial). Finally, we will discuss ways how the heart adapts during such stressful, pathological conditions- focusing on cell death and protein degradation via the ubiquitin proteasomal system (UPS). Here the aim is also to highlight the need for the establishment of novel therapies to reduce the burden of hyperglycemia and its associated cardiovascular complications.

2.2 Diabetes mellitus

Diabetes is defined as a heterogeneous group of metabolic disorders arising from absolute or relative deficiency of insulin or due to insulin resistance characterized by high glucose levels (515, 765) (see section 1.1, Chapter 1 of this thesis). The prevalence and incidence of diabetes is on the rise globally
possibly due to rapidly uncontrolled urbanization, and changing lifestyle and/or greater aging of affected populations (476). The burden of the disease is also exacerbated by the increased association with cardiovascular complications that occur as a result of the chronic hyperglycemia. For example, diabetes causes a 2- to 4-fold increase in the risk of CVDs (50, 93, 655), including stroke (601), atrial fibrillation, flutter, coronary heart disease, and left ventricular hypertrophy (498). Several risk factors are implicated in the pathogenesis of diabetes-related complications and these include: glycemic control, hypertension, dyslipidemia, diet, and smoking (463). For this thesis the focus is exclusively on hyperglycemia, i.e. both chronic (as in diabetes mellitus) and acutely (as in stress situations). Before highlighting evidence that demonstrate the link between hyperglycemia per se to CVDs, and the mechanisms whereby complications occur, the next section briefly describes how chronic hyperglycemia occurs (as supported by various animal studies).

2.3 How does hyperglycemia occur with diabetes?

Hyperglycemia that manifests during diabetes onset occurs as a result of decreased glucose transport (2, 20, 444), glycolysis and glucose oxidation (Figure 2.1). Defective glucose transport observed in diabetic animal models and patients is attributed to abnormal fatty acid (FA) metabolism, a decrease in total GLUT4 mRNA and protein levels secondary to insulin resistance or lack of insulin (53, 227, 344, 346, 711). Furthermore, impaired recruitment of GLUT4 to the cell membrane upon insulin stimulation that may occur as a result of alterations in the insulin receptor substrates (IRS)/phosphoinositide-3 kinase (PI 3-K)/Protein kinase B (Akt) signaling pathway is also implicated (163, 380, 538, 643) (see Figure 2.1). This may manifest in various ways that include: impaired translocation, persistent docking without fusion (373), partial fusion or altered configuration (rendering them cryptic with inadequate exposure to the extracellular milieu), and reduced activation (133, 345).
Figure 2.1: Mechanism of FA–induced insulin resistance as proposed by Randle et al. (1963) (581). An increase in FA concentrations results in an elevation of the intra-mitochondrial acetyl-CoA/CoA and NADH/NAD⁺ ratios, with subsequent inactivation of pyruvate dehydrogenase. This causes an increase in citrate concentrations and inhibition of phosphofructokinase. Subsequent elevations in intracellular glucose-6-phosphate concentrations inhibit hexokinase II activity, which results in an increase in intracellular glucose concentration and a decrease in myocardial glucose uptake. GLUT4- glucose transporter 4; HK- hexokinase; G-6-P- glucose-6-phosphate; PFK- phosphofructokinase; PDH- pyruvate dehydrogenase.

The attenuated effects of insulin that occur during the metabolic syndrome and diabetes result in high circulating FA levels that alter glucose metabolism (120, 223, 274, 548, 592, 604, 623, 624, 745). Increased FAs may manifest due to 1) higher lipolysis and release from adipose tissue and liver (174, 256, 551, 582, 588, 599); 2) the augmented and permanent expression of the FAT/CD36 proteins on the cell membrane (139, 449). Enhanced delivery of lipids to the heart increases myocardial FA uptake, metabolism and storage. In support, increased cardiac triacylglycerides (TAGs) content together with greater mRNA levels of glycerol-3-phosphate acyltransferase (GPAT) (805), an enzyme known to direct exogenous FAs into TAGs (327), are found in diabetic patients and animals. Hence the combined elevation of FAT/CD36 and GPAT provides a mechanism for increased FA channeling towards intracellular storage. Indeed, both animal models and human studies of obesity, insulin resistance and diabetes, established that lipids accumulate within the heart (30, 131, 805). Consequently, the diabetes-induced elevation in plasma FA levels together with enhanced fatty acid oxidation (FAO) rates result in an elevation of intra-mitochondrial acetyl-CoA levels (see Figure 2.1),
which likely results in activation of pyruvate dehydrogenase (PDH) kinase 4 and phosphorylation of PDH to its inactive form. In addition, acetyl-CoA inhibits the rate of flux through active dephosphorylated PDH (286) (refer Figure 2.1). It is unclear how much of the decrease in glucose uptake in the diabetic heart is due to impaired pyruvate oxidation and feedback inhibition on the glycolytic pathway. However, the fraction of myocardial PDH in the dephosphorylated active form is significantly reduced in experimentally-induced diabetic rodents (274, 364, 403, 779).

Figure 2.2 Proposed alternative mechanism for FA–induced insulin resistance. Enhanced delivery of FAs to muscle or a decrease in intracellular metabolism of FAs lead to increased in intracellular FA metabolites such as DAG, fatty acyl-CoA, and ceramides. HK, hexokinase II; PFK, phosphofructokinase; PDH, pyruvate dehydrogenase; PKC θ, protein kinase C θ.

Additionally, increased FAs result in elevated lipid metabolite levels such as acyl-CoAs, ceramides and diacylglycerols (DAGs) originating from, or in equilibrium, with the intracellular TAG pool. This in turn is proposed to influence insulin-induced glucose uptake and GLUT4 translocation (Figure 2.2) (7, 333, 672, 717). Acyl-CoAs are precursors for ceramides which are generated via de novo synthesis or degradation of sphingomyelin (533). These metabolites activate a serine/threonine kinase cascade (possibly initiated by protein kinase C [PKC] θ) leading to phosphorylation of serine/threonine sites on IRS-1/2, which in turn reduces the ability of the IRS to activate PI 3-K (622) (see Figure 2.2). As a consequence, glucose transport activity, and other events downstream of insulin receptor signaling are diminished (174, 599).
The chronic activation of such pathways can lead to diabetic cardiomyopathy (refer section 2.7.2.3) where both systolic and diastolic dysfunction are present (20). As the heart adapts to high FA utilization for ATP generation there is decreased efficiency since more oxygen will be consumed with less energy produced. This increases the risk of cardiac dysfunction especially during ischemia when reduced oxygen supply is available for FAO. Moreover, under these conditions the heart also increases its dependence on glucose as fuel substrate (707). This section briefly discussed how chronic hyperglycemia can actually occur with diabetes mellitus, and also re-iterated mechanisms already highlighted in Chapter 1. The next part of this review will now focus on the link between hyperglycemia and the onset of CVDs.

2.4 The link between hyperglycemia and the onset of CVDs

Hyperglycemia consists of two forms, i.e. chronic hyperglycemia (diabetes) and acute hyperglycemia that can occur in both diabetic and non-diabetic individuals.

2.4.1 Chronic hyperglycemia and CVD

Chronic hyperglycemia as assessed by glycosylated hemoglobin $\text{A}_{1c}$ ($\text{HbA}_{1c}$) levels is associated with the development of micro- and macro-vascular complications of diabetes (463). There are a number of controlled clinical trials that demonstrate improved glycemic control reducing the risk for CVD, and epidemiologic studies that have established the link of various forms of hyperglycemia to greater risk for CVD (6, 8, 172, 179, 310, 536, 547, 697) (refer Table 2.1). The epidemiological link between diabetes mellitus and the development of CVD was established as early as 1979 (356). Two landmark studies; the Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) highlighted the direct association between chronic glycemic control and vascular complications in patients with diabetes (167, 722).
A similar observation was also made in the DCCT follow up study, the Epidemiology of Diabetes Interventions and Complications (EDIC) (189); hence these studies have established the link between glycemic control and the risk of developing micro- and macro-vascular complications with T1DM (these complications are further discussed in section 2.7). Acute myocardial infarction is the major contributor to cardiovascular deaths with diabetes and it often progresses into end-stage heart failure (HF) (167, 355, 722). HF is increased with obesity, insulin resistance, and T2DM (57, 363), with incidences 2.4-fold and 5-fold higher in diabetic men and women, respectively (722). This is not only limited to T2DM but also include T1DM. For example, data from the Pittsburgh Epidemiology of Diabetes Complications (EDC) Study indicated that the incidence of major coronary artery disease events was 0.98% per year in young adults (age 28–38 years) with T1DM (20–30 years duration) (542, 595). In part, this increased mortality arising from heart disease ~2 to 4-fold higher in diabetic patients when compared to non-diabetics with the same magnitude of vascular diseases (153, 663).
Table 3.1 Epidemiological studies showing the link between glycemic control and CVDs.

<table>
<thead>
<tr>
<th>Trial (year)</th>
<th>Patients (n)</th>
<th>Population characteristics</th>
<th>Age (years)</th>
<th>Diabetes duration (years)</th>
<th>Intervention</th>
<th>Follow up (years)</th>
<th>Endpoint/outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCCT (1993) (187)</td>
<td>1,441</td>
<td>T1DM</td>
<td>27</td>
<td>6</td>
<td>Intensive insulin vs. standard care</td>
<td>1-15 years</td>
<td>Reduced CV event and outcome Reduced risk of micro-vascular complications</td>
</tr>
<tr>
<td>DCCT-EDIC (2005) (697)</td>
<td>1,340</td>
<td>Follow-up of the DCCT cohort</td>
<td>45</td>
<td>24</td>
<td>Post interventional follow up</td>
<td>11</td>
<td>Reduced CV events</td>
</tr>
<tr>
<td>UKPDS 33 (1998) (722)</td>
<td>3,867</td>
<td>T2DM</td>
<td>53</td>
<td>Newly diagnosed</td>
<td>Intensive SU or insulin vs. diet</td>
<td>10</td>
<td>Reduced MI and micro-vascular complications</td>
</tr>
<tr>
<td>Kumamoto (1995)</td>
<td>110</td>
<td>Japanese with diabetes or 49 vs. 52 without diabetes</td>
<td>49</td>
<td>10</td>
<td>Multiple insulin injection treatment vs. conventional treatment</td>
<td>6</td>
<td>Reduced risk of micro-vascular complications</td>
</tr>
<tr>
<td>ProACTIVE (2005) (172)</td>
<td>5,238</td>
<td>T1DM with macrovascular disease</td>
<td>62</td>
<td>8</td>
<td>Pioglitazone added vs. placebo</td>
<td>3</td>
<td>Reduced primary CV end point and all cause mortality, non-fatal MI and stroke</td>
</tr>
<tr>
<td>STENO-2 (2003) (547)</td>
<td>160</td>
<td>T2DM with microalbuminuria</td>
<td>55</td>
<td>6</td>
<td>Multifactorial intervention vs. standard care</td>
<td>7.8</td>
<td>Reduced CV disease and micro-vascular complications</td>
</tr>
<tr>
<td>ACCORD (2008) (5)</td>
<td>10,251</td>
<td>T2DM</td>
<td>62</td>
<td>10</td>
<td>Intensive vs. standard therapy</td>
<td>3.5</td>
<td>Increased mortality due to severe hypoglycemia</td>
</tr>
<tr>
<td>ADVANCE (2009) (8)</td>
<td>11,140</td>
<td>T2DM (32% with CV events)</td>
<td>66</td>
<td>5</td>
<td>Intensive glucose control with gliclazide + other drugs vs. standard drugs</td>
<td>5</td>
<td>Reduced combined major macro-vascular and micro-vascular events Severe hypoglycemia</td>
</tr>
<tr>
<td>VADT (2009) (179)</td>
<td>1,791</td>
<td>T2DM (40% with CV events)</td>
<td>60</td>
<td>11.5</td>
<td>Intensive glucose control vs. standard care</td>
<td>5.6</td>
<td>No CV benefit</td>
</tr>
<tr>
<td>RECORD (2009) (310)</td>
<td>4,447</td>
<td>T2DM on met or SU</td>
<td>On met 57 on SU 60</td>
<td>On met 6 on SU 8</td>
<td>Add on rosiglitazone vs. combination met + SU</td>
<td>5.5</td>
<td>Increased HF</td>
</tr>
<tr>
<td>ORIGIN (2012) (536)</td>
<td>12,537</td>
<td>T1DM on glargine insulin</td>
<td>45</td>
<td>Newly diagnosed</td>
<td>Glargine insulin vs. standard care and placebo</td>
<td>6</td>
<td>No effect on macro-vascular complications Hypoglycemia and increased weight</td>
</tr>
</tbody>
</table>

DCCT-EDIC (Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications); UKPDS (United Kingdom Prospective Diabetes Study); ProACTIVE (PROspective pioglitAzone Clinical Trial In macroVascular Events); ACCORD (Action to Control Cardiovascular Risk in Diabetes); ADVANCE (Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation); VADT (Veterans Affair diabetes trial); RECORD (Rosiglitazone evaluated for cardiovascular outcomes in oral agent
Chronic hyperglycemia and HbA1c measurements reflect overall glycemic control without revealing much information on individual daily glucose fluctuations (70). However, several studies found that short-term fluctuations in glycemic control also play an important role in the pathogenesis of CVDs in both diabetic and non-diabetic AMI patients - detailed evidence is further elaborated on the section below.

2.4.2 Acute (stress-induced) hyperglycemia and CVD

The link between acute hyperglycemia and CVD risk was reported in several non-diabetic patients (39, 220, 270, 362, 673) in the absence of chronic hyperglycemia. This link was established in studies where hyperglycemia occurring secondary to stress-induced AMI was associated with increased in-hospital deaths, congestive heart failure or cardiogenic shock (104, 465, 537). High blood glucose levels in patients admitted for AMI are found in both diabetic and non-diabetic individuals (13, 71, 104, 210, 251, 460, 675), and is an independent risk for development of CVD (508). In addition to the elevated plasma glucose in non-diabetics on admission after AMI, glucosuria was also reported since the 1930s (146). Admission hyperglycemia in non-diabetic individuals during the acute phase of AMI probably represents a combination of undiagnosed diabetes, impaired glucose tolerance (IGT) (16, 528) and a severe response to acute stress (104, 414, 537). IGT is defined as a condition where fasting plasma glucose levels are below 7 mmol/L, and 2-hour post-prandial values are between 7.8 mmol/L and 11.0 mmol/L, and precedes the development of T2DM (486).

In the event of undiagnosed diabetes, acute hyperglycemia can add to the damaging effects of chronic hyperglycemia (463). How does this occur? Elevated plasma adrenaline concentrations during the early stages of infarction can stimulate the sympathetic nervous system (104) and increase glycogenolysis, thereby contributing to high plasma glucose levels. Moreover, the relative insulin deficiency (in the event of underlying diabetes), together with lipolysis and excess circulating FAs, limit
uptake of glucose by mechanisms discussed in section 2.3. Likewise, glucose dysregulation of metabolism is also observed in non-diabetic patients during the acute phase of ST-elevation AMI (414) possibly due to an impairment in pancreatic β-cell function and insulin resistance (395). A further point to consider is that since AMI is a stress reaction mediating hyperglycemia, the reverse may also occur i.e. where non-diabetic AMI patients become predisposed to new onset of diabetes (535). In fact, AMI is considered a risk factor for development of diabetes (499), and this is supported by data from a retrospective analysis of the GISSI-PREVENZIONE trial that showed the interrelatedness of AMI and hyperglycemia (311). Despite the robust evidence linking acute hyperglycemia and CVDs, others report on the lack of correlation between the two parameters (103). This discrepancy could be partly due to differences in parameters analyzed to indicate endothelial function, and also the extent duration and pattern of hyperglycemia (590).

### 2.4.3 Acute post-prandial hyperglycemia and fasting hyperglycemia

Post-prandial hyperglycemia is a 2-hour post-prandial elevation of blood glucose (> 7.8 mmol/L) in the presence of good glycemic control according to the American Diabetes Association (ADA) criteria, i.e. HbA$_{1c}$ < 40% with normal fasting plasma glucose (190). Post-prandial hyperglycemia occurs when the multiple homeostatic mechanisms that minimize glucose fluctuations to restore glucose levels after a meal are blunted. For example, in subjects with IGT insulin-mediated suppression of hepatic glucose production, enhancement of glucose uptake and reduction of FAs are impaired following food intake (39). The development of post-prandial hyperglycemia occurs mainly in the progression towards T2DM, i.e. due to loss of the first phase of insulin secretion (569). The major factor responsible for post-prandial hyperglycemia in individuals with IGT is likely to be impaired early insulin secretion by β-cells. This was observed in studies that reported a negative correlation between impaired post-prandial suppression of glucose and insulin release (486); development of IGT following somatostatin
treatment; and lastly increasing insulin concentrations by different treatments normalizing glucose tolerance (101, 451, 720).

By contrast, fasting hyperglycemia occurs predominantly as a result of increased glucose release by the liver and kidneys through gluconeogenesis (171, 481). Rates of glucose uptake are generally increased in individuals with fasting hyperglycemia, mainly because of the mass action effects of hyperglycemia. Moreover, fasting hyperglycemia may also occur due to antecedent post-prandial hyperglycemia, i.e. the higher the hyperglycemia after an evening meal, the greater the hyperglycemia in the morning and vice versa. Hence treatment should therefore aim to control both post-prandial and fasting hyperglycemia.

Post-prandial hyperglycemia is associated with a 2-fold increased risk of death from CVD (230). Some of the effects associated with the effects of acute hyperglycemia on the cardiovascular system include a) myocardial perfusion defects in T2DM patients secondary to micro-vascular function deterioration (628) and, b) alteration of myocardial ventricular repolarization in T1DM patients and increased stiffness/resistance of arteries (247, 248). Another way in which post-prandial hyperglycemia may lead to onset of CVDs may be via suppression of micro-circulation in young healthy adults (218) possibly as a result of micro-vascular dysfunction through leukocyte adhesion, enhanced platelet activation and advanced glycation end products (AGEs) (254, 466). In support of this, Crandall et al. (2009) showed that post-prandial hyperglycemia was accompanied by a pro-atherosclerotic and pro-thrombotic vascular profile in older adults with normal or elevated fasting glucose concentrations (141). Moreover, carotid intimal thickness (a marker of atherosclerosis) correlated to 2-hour post-prandial glucose in the Risk factors in Impaired Glucose Tolerance for Atherosclerosis and Diabetes (RIAD) Study (1999) with asymptomatic diabetes (693).
Several epidemiological studies reported the role of post-prandial hyperglycemia in the development of CVD. Examples include the Diabetes Epidemiology Collaborative Analysis of Diagnostic Criteria in Europe (DECODE) study that evaluated the relative risk of death from CVD, coronary heart disease, stroke and all-cause mortality in 22,514 individuals followed up for 8.8 years. It is from these studies that the converse relationship between given glucose loads, blood glucose level and fasting glucose was established. Using a Cox proportion of hazards it was found that risks for death from CVD was increased in individuals with a 2-hour post-prandial glucose of 7.8-11.0 mmmol/L and with T2DM, respectively (153). These results were corroborated in a different ethnic population (Funagata Diabetes Study) which showed that Japanese individuals have a 2-fold increased risk of mortality with post-prandial hyperglycemia, but no risk with impaired fasting blood glucose (709).

The Norfolk cohort of the European Prospective Investigation of Cancer and Nutrition (EPIC Norfolk) Study (1995 - 1999) evaluated 4,662 individuals (45 - 79 years) and found that the presence or absence of diabetes was not a risk factor, but instead that hyperglycemia per se was the key factor (369). Moreover, in the Cardiovascular Health Study (CHS), a prospective study of 4,515 individuals 65 years or older followed up for eight years, the presence of IGT resulted in an increased risk of 22% for CVD after correcting for other confounding factors such as age, ethnicity and known risk factors for CVD relative to that of normal glucose tolerance (215). These data are similar with those from the Second National Health and Nutrition Examination Survey (NHANES II) Mortality Study (618), a 12-16 year follow-up of a representative sample of the US population who underwent oral glucose tolerance testing between 1979 and 1980. Here relative risk for death was increased by 20% in individuals with IGT and 70% in individuals with previously undiagnosed diabetes.

Furthermore, the Hoorn Study (728), an 8-year follow up of 2,363 individuals (aged 50 to 70 years) without known diabetes, showed a 62% increased risk for death from CVD in individuals with an elevated 2-hour post-prandial hyperglycemia even after excluding those with pre-existing CVD and
other known risk factors. Similarly, a longitudinal population Polynesian study that followed up on 10,000 individuals after 5 to 12 years, found that those with isolated post-prandial hyperglycemia i.e. 2-hour levels > 11.1 mmol/L displayed an increased CVD mortality of 2.3 to 2.6-fold in men and women, respectively (636). Moreover, the CAPRI cross sectional study (901 patients) on T2DM patients (Italy) reported a link between dietary glycemic index and load with metabolic control in T2DM as it corresponded to the HbA\textsubscript{1c} values and post-prandial glucose levels (193). The Diabetes Intervention Study (DIS), a prospective population-based multicenter study from the University of Dresden with 1,139 patients with newly diagnosed T2DM found that 2-hour post-prandial glucose levels and not fasting glucose measurements or HbA\textsubscript{1c}, predicted mortality (283). A more recent study performed on 1,115 T2DM patients (aged 30-75 years) showed that insulin treatment post-AMI decreased fasting blood glucose with no effect on post-prandial hyperglycemia (587). From all these studies it therefore emerges that post-prandial glycemia in non-diabetic individuals carries a greater risk for cardiovascular complications than increasing fasting glycemia, and hence more emphasis should be placed on its early detection and treatment (31, 231, 271, 406, 549).

Despite evidence from several epidemiological studies, the role of post-prandial glycemia in increasing CVD risk has been questioned by some who emphasized the need for more clinical studies (19). In support of this, Suileman et al. (2005) showed that fasting glucose was a better predictor of mortality in non-diabetic patients following AMI than admission blood glucose (679). However, more recent work challenged this notion as data correlated with the NHANES III study, further proving the role of post-prandial glycemia in onset of CVDs (75, 106, 375). Further studies will help clarify this dilemma, but the available evidence strongly implicates post-prandial hyperglycemia as another serious risk factor for CVD. All the available data from clinical trials, epidemiologic studies and some \textit{in vivo} and \textit{in vitro} studies highlight the role of hyperglycemia as an independent risk factor for CVD; the risk is continuous without an apparent threshold and thus there is a great need for better glycemic control to prevent both micro- and macro-vascular diseases with diabetes. The question therefore emerges: how
does hyperglycemia (independently) lead to the development of CVD complications? The next section will discuss the postulated molecular mechanisms whereby hyperglycemia causes damage to the cardiovascular system.

2.5 Mechanisms for adverse cardiovascular effects of hyperglycemia

Hyperglycemia contributes to the development of vascular complications through several mechanisms, however, for this thesis we are focusing on the role of hyperglycemia-induced oxidative stress in the development of cardio-metabolic complications. Evidence in the literature supports the presence of oxidative stress with diabetes, and its role in mediating cardiovascular complications. Furthermore, the effects of hyperglycemia are basically the same irrespective of its nature (acute or chronic), since both activate similar metabolic and hemodynamic pathways from increased mitochondrial production of ROS to the downstream pathways mediating tissue damage (463). For example, both acute (478, 782) and chronic hyperglycemia can elicit endothelial dysfunction (37, 45, 142).

2.5.1 Hyperglycemia-induced oxidative stress

Studies carried out in diabetic individuals, using both in vitro and in vivo experimental models, show that oxidative stress emerges as the main culprit in the development of cardio-metabolic complications (48, 86, 87, 109, 235, 295, 371, 462, 530, 608, 609, 685, 738) with the heart being at a higher risk due to low levels of antioxidants (127). Oxidative stress occurs when the rate of oxidant production exceeds oxidant scavenging capacities of cells (74, 370, 372). Further support for these concepts come from usage of classic antioxidants e.g. vitamins E/C and α-lipoic acid that attenuated diabetes-related complications in human (337, 543, 635, 806), in vitro and in vivo experimental studies. For
example, overexpression of manganese superoxide dismutase (MnSOD) blunted superoxide production in experimental diabetes (127, 526).

2.5.1.1 Sources and types of ROS in diabetes and/or ischemia and reperfusion

The mitochondrial respiratory chain is the principal source of intracellular ROS in diabetes and other stress pathological conditions, e.g. ischemia and reperfusion. The role of cardiac mitochondria under normal circumstances was earlier explained (refer Chapter 1). Briefly, glucose is metabolized through the tricarboxylic acid (TCA) cycle (or FAs through β-oxidation), generating electron donors, four of which reduce oxygen in the final stages of the respiratory chain to water. To be precise, electrons from reduced substrates (NADH and FADH$_2$) move from complexes I and II of the electron transport chain through complexes III and IV to oxygen, forming water and causing protons to be pumped across the mitochondrial inner membrane with ATP synthesis (258, 544).

Previous studies show that there is an overlap in the factors that lead to damage or protection of mitochondria. Mitochondria respond to different signals by altering ATP production, calcium homeostasis, ROS production and membrane permeability. Functional integrity of cardiomyocytes requires an abundance of mitochondria capable of producing ATP and involved in calcium homeostasis. However, under stressful conditions mitochondria can contribute to cell death due to increased production of oxidants, activation of apoptotic and necrotic pathways (250). Experimental studies demonstrated that mitochondria are damaged with diabetes (201, 205, 638). Here, a major side effect is that electrons may leak from the respiratory chain and react with oxygen to form free radicals which are highly reactive because of unpaired electrons in their structure.

The primary factor governing mitochondrial ROS generation is the redox state of the respiratory chain (648). If the membrane potential across the inner mitochondrial membrane rises above a certain
threshold value, significant ROS generation occurs (381). Electrons mainly leak from complexes I (43, 44, 126, 229, 301, 396, 405, 409, 534) and complex III (76, 123, 660). Additionally, the stoichiometry of complex III can be altered to generate incompletely reduced forms of oxygen (79, 660). Thus, complex III is considered the major site of ROS production secondary to hyperglycemia and/or ischemia (86, 87, 123). In support, glycation of complex III proteins is attributed to the excess production of superoxide ions in diabetic rats (602).

How is ROS production via the mitochondrial respiratory chain a causal link between high glucose and the main pathways responsible for hyperglycemic damage? The prevailing hypothesis is that the hyperglycemia-induced increase in electron transfer donors (NADH and FADH$_2$) increases electron flux through the mitochondrial ETC. There may also be changes in the ETC stoichiometry that can lead to an increase in reverse flow of electrons. Consequently, there is an increase of the ATP/ADP ratio and hyperpolarization of the mitochondrial membrane potential (79, 381, 647, 648). This high electrochemical potential difference generated by the proton gradient leads to partial inhibition of the electron transport in complexes I and III, resulting in an accumulation of electrons to coenzyme Q. In turn, this drives the partial reduction of oxygen to generate superoxide (86, 176, 177). The accelerated reduction of coenzyme Q, and subsequent ROS generation is considered a crucial aspect of mitochondrial dysfunction and diabetes-related metabolic disorders and tissue histopathology.

ROS produced can also damage the respective producing complex itself thereby further decreasing its activity and exacerbating free radical production (544, 799). Superoxide overproduction in the organ systems is an important feature of diabetic complications (176, 177, 526, 773, 774) as it mediates various oxidative chain reactions (86, 112, 116, 117, 176, 177, 526, 773, 774). An example of this is the activation of major, interrelated, pathogenic mechanisms for diabetic complications as modeled in endothelial cells exposed to \textit{in vitro} hyperglycemia (526).
There is also supporting evidence that greater cytosolic generation of ROS may precipitate increased mitochondrial ROS overproduction, which underscores the pathogenic importance of ROS generation from non-mitochondrial sources (225, 804). Other sources for ROS generation include; glucose autoxidation, the membrane NADPH oxidase (Nox), lipoxygenases, cyclo-oxygenases, peroxidases, heme proteins, xanthine oxidase, peroxisomes, or the hepatic P-450 microsomal detoxifying system (see Figure 2.3) (86, 114, 161, 526, 772). Both mitochondrial and non-mitochondrial sources generate three main ROS types, i.e. the superoxide radical (O$_2^*$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (OH$^*$). The hydroxyl radical can be generated by the combination of superoxide radical and hydrogen peroxide in the presence of traces of iron or copper during the Fenton-Haber-Weiss reaction. Increased copper levels may result because of damaged copper binding properties of ceruloplasmin and albumin that occurs with diabetes (25, 181, 572, 783). Thus, hydrogen peroxide (although not a free radical) can generate the hydroxyl and other reactive radicals at multiple intracellular locations, thereby propagating oxidative damage (319, 320). Other ROS that are important for the cardiovascular system include singlet oxygen, nitric oxide (NO) and the perhydroxyl radical, located near membranes where local pH is lower than the rest of the environment as occurs during ischemia and reperfusion.
Figure 2.3 Mechanisms of formation of hyperglycemia-induced ROS. BH$_4$ - tetrahydro biopterin; NO- nitric oxide; O$_2^\cdot$- superoxide radical; CO$_3^\cdot$- carbonate radical; ONOO$^\cdot$- peroxynitrite; ucNOS- uncoupled nitric oxide synthase; ONCOO$^\cdot$- nitrosoperoxycarbonate; NO$_2^\cdot$- nitrogen dioxide; Cu$^{2+}$ - copper ions; OH$^\cdot$- hydroxyl radical; NAD$^+$- oxidized nicotinamide adenine dinucleotide; NADH- reduced nicotinamide adenine dinucleotide; FADH$_2^\cdot$- reduced flavin adenine dinucleotide; FAD- oxidized flavin adenine dinucleotide.

Mitochondrial NO production is much lower than superoxide production. However, it is still an important role player due to its interaction with superoxide and other radicals to produce potent reactive nitrogen species (RNS), e.g. peroxynitrite (86, 114, 117, 161, 275, 526, 772), that causes nitrosative stress in organ systems. A significant increase in serum and tissue 3-nitrotyrosine (3-NT), a by-product of the reaction between peroxynitrite and proteins, is found in diabetic patients (100, 114, 115, 216). Moreover, in vivo and in vitro studies demonstrated that peroxynitrite is an important causative agent in diabetes-mediated cardiovascular injury (100, 114) (see Figure 2.3), by modifying macromolecules (86, 87). Protonation of peroxynitrite can also yield peroxynitrous acid that can
decompose to yield the hydroxyl radical thus further adding to overall damaging effects (109, 111, 113).

The roles of ROS and RNS in the diabetic complications of multiple organ systems are extensively documented (98, 161, 176, 349, 526, 609, 772), although the exact mechanisms of ROS/RNS-induced pathogenesis are not fully understood yet. However, some of the proposed mechanisms are discussed below. It is now known that peroxynitrite can directly cause oxidative DNA damage and lipid peroxidation (27, 349, 527, 681), and indeed damaged DNA was found in both T1DM and T2DM individuals (135, 246, 390, 419, 439, 616). Furthermore, peroxynitrite can also cause covalent modification of an active thiol site of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (thus inhibiting its activity), and also able to attenuate creatine kinase activity (684, 736). Here, peroxynitrite-mediated nitration of myofibrillar creatine kinase activity may lead to contractile dysfunction and inhibition of ion pumps including calcium, calcium-activated potassium channels and the membrane $\text{Na}^+$/K$^+$ ATPase (684, 736). As discussed earlier, non-mitochondrial ROS sources include Nox, xanthine oxidase and reduced neuronal nitric oxide synthase (427). For example, Nox activity is high in vascular tissue from diabetic patients and may therefore contribute to superoxide production (268).

Various antioxidants have been used in clinical trials, to ameliorate oxidative stress but with mixed outcomes, i.e. beneficial effects versus none (269, 793). Several antioxidants showed beneficial effects in experimental animals, for example, $\alpha$-lipoic acid decreased oxidative stress when administered in vivo to diabetic animals (483). Additionally, antioxidant potential is also observed in vitro with known antihypertensive agents e.g. nifedipine (a calcium channel blocker) and olmesartan (an angiotensin receptor blocker). For example, olmesartan is a potent scavenger for hydroxyl and tyrosyl radicals and prevents glycoxidation and lipoxidation reactions whereas nifedipine is a potent scavenger of peroxynitrite (487). Interestingly, despite the evidence that superoxide is the major oxidant in hyperglycemia-induced oxidative stress (177, 526, 772), few studies have tested the effects
of decreasing superoxide levels in diabetes (2). Some approaches include the use of catalytic superoxide dismutase (SOD) mimetics, e.g. Mn (III) tetrakis (4-Benzoic acid) porphyrin chloride (MnTBAP), that reduced hepatic superoxide generation, and reversed steato-hepatitis in diabetic \(ob/ob\) mice (413). Furthermore, mitochondrial targeting of antioxidants by conjugation to cations such as triphenylphosphonium (TPP), leads to preferential mitochondrial uptake. The theory behind this is that these cations initially accumulate within the cytoplasm because of the cell membrane potential (30–60 mV, negative charge inside), then readily permeate the lipid bilayers and become concentrated within mitochondria, because of the large mitochondrial transmembrane potential (150–180 mV, negative charge inside). Eventually, membrane-permeable cations will be predominantly sequestrated by mitochondria with much less than 5% remaining outside mitochondria. For example, TPP conjugates of tocopherol (Mito-E) and coenzyme Q (Mito-Q) have successfully been administered to mice (in chow or drinking water) for up to 6 weeks with no evidence of toxicity (649). However, despite these studies, little is known about the impact of pharmacological reduction of mitochondrial superoxide production on cardiac function and substrate metabolism, and this represents new possibilities for future research.

In summary, mitochondrial and non-mitochondrial ROS are key players in the manifestation of cardio-metabolic pathophysiologies. Here the initiating event is mitochondrial superoxide production that triggers further ROS generation (other sources) thereby creating a vicious cycle. Subsequently, ROS can induce cellular damage by direct oxidation of proteins, by conversion of lipids to reactive lipid peroxidation products, by increasing protein tyrosine nitration by generation of RNS, and by interaction with DNA. Mitochondrial DNA is particularly susceptible to oxidative damage leading to more ROS production due to altered function (746). It is also important to remember that increased ROS levels not only result from its overproduction, but may also manifest due to decreased efficiency of inhibitory scavenger systems, e.g. SODs (copper/zinc-SOD [Cu/Zn-SOD] and Mn-SOD), catalase, and the
glutathione peroxidase system. These are examples of endogenous mitochondrial antioxidants that prevent tissue damage and dysfunction (361) under normal physiological conditions.

2.5.2 Mitochondrial antioxidant defense mechanisms

Mitochondria together with the other intracellular antioxidant systems have the capability of maintaining oxidative stress homeostasis by a balance between ROS production and its elimination (165). However, these endogenous oxidative stress defense mechanisms are overwhelmed during pathological conditions such as hyperglycemia and/or ischemia-induced oxidative stress. Glutathione reductase and peroxidase play a key role in the recycling of glutathione between its reduced (GSH) and oxidized (GSSG) forms. Glutathione peroxidase removes hydrogen peroxide through the oxidation of GSH to GSSG, while glutathione reductase acts as an antioxidant by converting GSSG to GSH. SOD provides further defensive core by conversion of superoxide to hydrogen peroxide that is then converted to water by catalase (another core antioxidant). SOD is present in three isoforms, i.e. SOD1; a dimer found in the cytoplasm, whereas SOD2 and SOD3 are tetramers found in the mitochondrion and extracellular matrix, respectively (477). Accumulating evidence supports the concept that decreased Cu/Zn-SOD activity leads to apoptotic cell death. Moreover, SOD glycation and reactive oxygen intermediates (ROI) produced from glycated proteins are also involved in many diseases, including diabetic complications (524, 687).

For the present study, we have a particular interest in hyperglycemia and/or ischemia and reperfusion-mediated superoxide generation (by the ETC). Here, superoxide generation leads to peroxynitrite production that subsequently results in the inhibition of the glycolytic enzyme GAPDH, thereby causing increased flux of glycolytic metabolites through the non-oxidative glucose pathways such as polyol pathway, formation of AGEs, the hexosamine biosynthetic pathway (HBP) and activation of protein kinase C (PKC). This will further exacerbate oxidative stress under hyperglycemic conditions and
hence contribute to micro- and macro-vascular complications of diabetes. **One of the aims of this thesis is to elucidate the relative contributions of non-oxidative glucose pathways to cardiac function during ischemia and reperfusion under hyperglycemic conditions.**

### 2.6 Glucose flux through the non-oxidative pathways

Multiple biochemical pathways are proposed to link the adverse effects of hyperglycemia with vascular complications. As discussed earlier, intracellular hyperglycemia causes overproduction of ROS by the mitochondrial ETC, thereby creating oxidative stress that leads to high glucose-induced superoxide generation. The resultant peroxynitrite causes DNA strand breaks thereby activating poly(ADP)-ribose polymerase (PARP). Poly(ADP-ribosyl)ation represents an immediate cellular response to DNA damage induced by oxidants (97, 136, 168). Indeed, DNA damage is observed in both T1DM and T2DM patients, and can be prevented by vitamin E supplementation (616).

Poly(ADP-ribosyl)ation is usually a very rare event, but it can increase over 100-fold upon DNA damage. Under these conditions, about 90% of poly(ADP-ribose) is synthesized by poly(ADP-ribose) polymerase 1 (PARP-1) (507). PARP-1 is constitutively expressed but enzymatically activated by DNA strand breaks (56, 97). Thus, it functions as a DNA damage sensor and signaling molecule binding to both single- and double-stranded DNA breaks. PARP-1 catalyzes the formation of ADP-ribose from NAD$^+$ by cleavage of the glycosidic bond between nicotinamide and ribose (56, 97). Glutamate, aspartate, and carboxyterminal lysine residues of target (“acceptor”) proteins are then covalently modified by the addition of an ADP-ribose subunit, via formation of an ester bond between the protein and the ADP-ribose residue. Here GAPDH is poly(ADP)-ribosylated in a covalent posttranslational modification linked with genome protection (56, 97). It is plausible to suggest that the inhibitory effect of ADP-ribosylation on GAPDH probably represents a feedback loop in order to reduce levels of glycolysis, and transiently block the subsequent flux of metabolites to mitochondria allowing a
decrease in the levels of reducing equivalents and the subsequent mitochondrial ROS production and oxidative cellular and molecular damage (517). However, this causes upstream accumulation of glycolytic metabolites resulting in increased activation of the polyol (531, 532), AGE (754), PKC (388, 389), HBP and enhanced ROS production and actions (86) as discussed below.

2.6.1 Polyol pathway

Under normoglycemic conditions in mammalian cells, intracellular glucose is mainly phosphorylated into glucose-6-phosphate by hexokinase thereby entering the glycolytic pathway. Only trace amounts (~3%) of glucose enter the polyol pathway. With hyperglycemia, however, flux through the polyol pathway accounts for more than 30% of glucose metabolism (180, 245, 637, 713, 771). Here the rate limiting step is the reduction of glucose to sorbitol catalyzed by aldose reductase (AR), a member of the aldo-keto reductase (AKR) family of proteins (589). AR is a monomeric oxidoreductase located in the cytosol and is able to catalyze the nicotinamide adenosine dinucleotide phosphate (NADPH)-dependent reduction of several carbonyl compounds, including glucose despite its low affinity for this compound (86, 208, 233). This reaction occurs at the expense of NADPH. Sorbitol is in turn converted to fructose by sorbitol dehydrogenase (SDH) with NAD$^+$ as a co-factor (86, 180, 445, 589, 637, 771) (see Figure 2.4). This pathway was first identified in the seminal vesicle by Hers (1956) who demonstrated the conversion of blood glucose into fructose as an energy source for sperm cells (303). AR has since been isolated and identified from several human and animal tissues such as various eye regions (289, 305, 652), ovary (334), kidney (143) and the brain (144).
2.6.1.1 Contribution of the polyol pathway to diabetic oxidative stress and complications

With diabetes, the polyol pathway induces oxidative stress through various ways. Firstly it depletes NADPH and consequently decreases reduced glutathione (GSH) levels (86, 445, 637, 694) (refer Figure 2.4). Intracellular depletion of NADPH also leads to lowered NO synthesis since NADPH is a cofactor for NO-synthase which synthesizes NO from L-arginine (694) thus increasing development of vascular complications under hyperglycemic conditions. The conversion of sorbitol into fructose increases NADH levels, a substrate for Nox (see Figure 2.4) and hence elevated superoxide production (180, 497). Furthermore, fructose (end-product), can be further metabolized into fructose-3-phosphate and 3-deoxyglucosone, which are more potent non-enzymatic glycation agents than glucose (278). This implies that flux through the polyol pathway would increase formation of AGEs and ultimately ROS. Studies also show that AR reduces a number of lipid peroxidation-derived aldehydes (LDAs), and their GSH conjugates thereby contributing to cellular toxicity, tissue and DNA damage leading to apoptosis, and necrosis and uncontrolled growth (659). Additional damage also comes from sorbitol (impermeable to biological membranes) that evokes osmotic vascular damage in cataracts of
the eyes as it accumulates inside cells (305, 652). The involvement of sorbitol in osmotic vascular damage, however, is often difficult to rationalize (86, 637).

Based on these putative mechanisms, inhibitors of AR have been used experimentally and in clinical trials for several decades (140), e.g. synthetic drugs such as hydantoins (sorbinil) and carboxylic acids (e.g. tolrestat, penalrestat, epalrestat and zopolrestat) (279, 329, 330, 694, 807). However, some synthetic drugs induced hypersensitivity in clinical trials (78) without much beneficial effect. Other therapeutic options such as supplementation with vitamin C have reduced sorbitol levels in erythrocytes of young T1DM patients (147). AR inhibitors also include flavonoids isolated from various plants such as luteolin, apigenin, linarin and acacetin (416, 417, 474). Moreover, reno-protective effects were observed with berberine, one of the main constituents of *Coptis chinensis* (Franch) [Ranunculaceae] and *Phellodendron amurense* (Ruprecht) [Rutaceae], and this was associated with a concomitant inhibition of oxidative stress (436). Other isolated bioactive compounds from plants that inhibit AR activity include quercetin, silymarin, and puerarin (78). Of note, decreased AR activity prevents production of sorbitol and its downstream stimulation of AGE formation, and PKC pathways which are described in the next sections. Together these data show that the polyol pathway and AR are important targets for therapeutic interventions to treat T1DM and T2DM.

### 2.6.2 Advanced glycosylation end products (AGEs)

The non-enzymatic glycation reactions were first described around the turn of the century by Louis Camille Maillard who predicted that it would have an important impact on biomedical science - he coined the term “Maillard reaction” (455). Protein glycation occurs through a series of reactions that can be divided into a) stressors, i.e. sources of carbonyl agents that can drive the reaction, b) propagators, i.e. the reactive dicarbonyl agents that arise from the precursor stressors, and c) end products that mark the formation of the AGEs resulting from the Maillard reaction (495). This
distinction is quite helpful in defining the various steps where interference should be targeted, especially since this thesis also focuses on preventing oxidative stress through the non-oxidative pathways.

The protein glycation process starts with a nucleophilic addition reaction between a free ε-amino group or N-terminal group of proteins, and the carbonyl group of a reducing sugar (normally glucose) to form a reversible Schiff base (10, 11) (see Figure 2.5). The latter can rearrange into a stable, irreversible ketoamine or Amadori product (721, 750). Major sources of the carbonyl group in the glycation reaction include glucose and glyceraldehyde (614). The Schiff base is highly prone to oxidation and free radical generation leading to formation of oxoaldehydes, glyoxal and methyglyoxal, in the so-called Namiki pathway of the Maillard reaction (513) (refer Figure 2.5) which occurs early in the glycation process. Further, methyglyoxal can arise by spontaneous β-elimination of phosphate from triose phosphates, which are increased during hyperglycemia from inhibition of glycolysis.
Figure 2.5 Simplified biochemistry of advanced glycation end product formation. Prolonged hyperglycemia and oxidative stress during diabetes result in the production and accumulation of advanced glycation end products (AGEs). G-glucose.

Another oxidative pathway suggested that metal-catalyzed auto-oxidation of reducing sugars could be involved in AGE formation (318, 319, 762, 763). For example, fructose-lysine can bind redox-active copper to produce N-carboxy-methyl (lysine) (CML) and generating hydrogen peroxide in the process thus contributing to oxidative stress from AGE formation (617). This is an example of the Fenton reaction where copper ions attached to glycated proteins become reactive or increase reactivity of the glycated protein (25). AGEs can be generated from the Amadori product either by auto-oxidation into reactive dicarbonyl products such as glucosones (for instance methyglyoxal and 1,4-deoxyglucosone) (90) (see Figure 2.5), or be fragmented by glycoxidation to produce CML or pentosidine from lipids (also called advanced lipoxidation end products [ALEs]). It therefore follows that CML is formed by both glycoxidation and lipoxidation (617). The glycation spontaneous reaction, however, depends on the degree and duration of hyperglycemia; turnover of proteins; and availability and reactivity of the
amino groups on proteins. Moreover, it is known that this process is accelerated with diabetes (86, 614, 637, 696, 786).

Since methyglyoxal, the major source of intracellular AGEs (357) is very cytotoxic, eukaryotic cells have developed a system to metabolize it once formed in physiological situations. Together with its two carbon analog (glyoxal), methyglyoxal is physiologically metabolized by the cytosolic glutathione-dependent glyoxalase 1 and 2 to D-lactate (706). Lower expression of glyoxalase was reported with diabetes (583) and glyoxalase 1 deficiency increases intracellular AGEs (3, 488). Furthermore, methyglyoxal can increase oxidative stress by causing glycation and inactivation of glutathione reductase and peroxidase (488). Methyglyoxal can also directly deplete GSH in various cell types so that the cell becomes more sensitive to oxidative stress. Moreover, reduced GSH availability will affect the glyoxalase system and impair methyglyoxal degradation thus establishing a vicious cycle that leads to a further increase of methyglyoxal levels (706).

2.6.2.1 Contribution of AGE pathway to diabetic oxidative stress and complications

AGEs damaging effects are achieved either directly where glycated proteins cause oxidative stress (502), or indirectly through their interaction with receptors - especially receptors for AGEs (RAGE) (520, 778). Examples of the direct effects of AGEs include altered enzyme activity, decreased ligand binding and modification of protein half-life (737). Additionally, glycation-derived free radicals cause protein fragmentation and oxidation of nucleic acids and lipids (47, 48). Furthermore, AGEs form crosslinks thereby altering the structure and function of proteins, such as serum albumin, lens crystallin, intracellular proteins and collagen in the extra cellular matrix (86, 156, 614, 637, 786). These crosslinkages also reduce matrix protein flexibility hence abnormal interaction with their respective receptors on cells (86, 291).
RAGEs are present on various cells, including endothelial cells, mesangial cells and macrophages (86, 156, 637, 786). The elucidation of RAGE modulatory roles and signal transduction pathways are areas of intensive investigation. Thus, recent evidence suggests that RAGE binding initiates signaling activation of PKC (480, 627), tyrosine phosphorylation of Janus kinase (JAK)/signal transducers and activators of transcription [STAT], (316), recruitment of PI 3-K to the ras-dependent mitogen-activated protein kinase (MAPK) (430) or PKC (159, 388, 410, 645), and induction of oxidative stress cascades which culminate in nuclear factor (NF)-κβ and activator protein-1 (AP-1) transcription (62, 86, 786, 808). One of the crucial pathways impaired in this process is cellular NO signaling (808) which eventually causes the development of atherosclerosis and CVD under hyperglycemic conditions. Such signaling pathways also lead to a tissue-specific pro-inflammatory environment. RAGE activation and stimulate the renin angiotensin system (RAS) leading to increased angiotensin II formation (182, 211, 489). In support, blockade of RAS pathway activation decrease levels of AGEs and prevent associated detrimental effects, e.g. retinopathy (182, 211, 489). AGE pathway is closely associated with the development of cardio-metabolic complications and its accumulation can predict the severity of micro- and macro-vascular in diabetic patients (632).

As such, various strategies have been developed to prevent the detrimental effects of AGEs. These include: trapping of reactive dicarbonyl species; use of antioxidants such as transition chelating metal ions and free radical scavengers; breaking of AGE cross-links; blocking RAGE and its downstream signaling pathways; glycemic control; and AR inhibition and shunting of trioses towards the pentose phosphate pathway by transketolase (TK) activation (553). The inhibitors have multiple sites of action and only a few agents will be discussed. Firstly, glycemic control is a key intervention since AGEs formation is greatly accelerated under high glucose conditions (494) and reduced glucose levels prevent the glucose-dependent first step in the Maillard reaction. To our knowledge there is not much information on the effect of anti-hyperglycemic drugs on non-oxidative glucose pathways. One is
metformin (52, 343). Other drugs such as thiazolidinediones (470) and aspirin formulations are also successfully employed in AGE attenuation (723, 776).

The importance of inhibiting non-oxidative pathways emerged from the DCCT study where CVD complications occurred in association with increased levels of AGEs despite the presence of good glycemic control (189). Thus, this observation is consistent with the hypothesis that other factors e.g. oxidative stress also contribute to the production and accumulation of AGEs (32, 48), and that metabolic memory predisposes diabetic and non-diabetic patients to CVDs even after glycemic control (280).

The inhibition of AGE formation opens several exciting possibilities for the prevention of organ damage with diabetes. Here various approaches were employed including the prevention of AGE-formation, and reducing interaction of AGE with RAGE, i.e. ligand interactions/ signaling pathways effects or break established AGE crosslinks. Interestingly, vitamins and derivatives also exhibit the potent ability to decrease AGEs in diabetic animals (357) and patients (559). Synthetic anti-AGE agents are another focus area and were employed both in experimental animals and clinical trials to combat AGE formation. This included pimagedine (also known as aminoguanidine) that prevents formation of irreversible AGEs by trapping of reactive dicarbonyl intermediates (84, 704, 705). This approach yielded positive outcomes, i.e. slowing progression of overt nephropathy and retinopathy. However, it did not demonstrate statistically significant effects on lowering serum creatinine and urine albumin with T1DM, possibly because of its increased renal clearance (72). Another agent ALT-711/alagebrium breaks pre-accumulated AGEs and showed beneficial effects in preventing diabetic cardiomyopathy (28). In summary, AGEs play a central role in the pathogenesis of diabetic complications. Part of these effects are mediated by generation of increased oxidative stress. One such mechanism involves increased Nox activity via PKC-βII (766); hence activation of this pathway in the development of diabetic complications is discussed below.
2.6.3 Protein kinase C

PKC, a family of at least twelve enzyme isoforms of the AGC (cAMP-dependent protein kinase/PKG/PKC) family, is a serine/threonine-related protein kinase that plays a key role in many cellular functions and affects multiple signal transduction pathways (521). It acts by catalyzing the transfer of a phosphate group from ATP to various substrate proteins, and PKC itself undergoes a series of complex phosphorylation steps before activation, during which time it translocates from the cytosol to the cell membrane. Approximately 12 isoforms have been identified to date which differ in structure and substrate requirements (668). Nine of the PKC isoforms (including PKC-α, βI, βII, γ, δ, ε and ξ) are activated by the second messenger DAG, a critical signaling molecule that regulates many vascular functions such as permeability, growth factor signaling, vasodilator release and endothelial activation (12, 86, 366, 637).

DAG levels are increased with hyperglycemia, and is formed by multiple pathways, including agonist-induced hydrolysis of phosphatidylinositol (PI) by phospholipase C (PLC) (12, 86, 196, 366, 637), or de novo synthesis from the glycolytic intermediates dihydroxyacetone phosphate (DHAP) and glycerol 3-phosphate (767). By contrast, several studies show that the PLC pathway does not contribute to the diabetes-induced increase in DAG levels. For example, exposure of rat aortic smooth muscle cells to elevated glucose concentrations increased DAG levels without changing levels of inositol 1,4,5-triphosphate, a derivative of PI hydrolysis (767). The de novo synthesis or direct metabolism of glucose to DAG involves the conversion of the glycolytic intermediate DHAP to lysophosphatic acid and then phosphatidic acid (PA). DAG kinase can convert PA to DAG and vice versa. The involvement of this pathway in glucose-induced DAG formation has been confirmed in labeling studies where 14C-labeled glucose levels revealed the incorporation of glucose into the glycerol backbone of DAG in aortic endothelial cells and smooth muscle cells (328, 767).
2.6.3.1 Contribution of PKC activation to diabetic oxidative stress and complications

There is a growing body of evidence for the central role of PKC in signal transduction pathways in hyperglycemia-induced complications (328, 767, 768). Increased flux through the other non-oxidative glucose pathways, partly involves activation of the PKC signaling pathway in eliciting their detrimental effects. Increased PKC levels during diabetes onset are found in a) vascular tissues, including the retina (639) aorta, heart (328) and renal glomeruli (332); and b) non-vascular tissues such as liver and skeletal muscles (300). Moreover, ROS itself e.g. hydrogen peroxide can also activate PKC further exacerbating oxidative stress (12, 86, 196, 366, 637, 732). For example, endogenously formed pro-oxidants from PKC activation promote formation of oxidized low density lipoprotein (ox-LDL) (653) that cause endothelial cell activation and injury (critical in the pathogenesis of atherosclerosis). Here, lysophosphatidylcholine a major constituent of the ox-LDL, further increases activation of PKC and consequently ROS formation (775).

PKC activation mainly results in ROS production through enhancing Nox activity (766). Indeed, lysophosphatidylcholine-induced PKC activation is a critical upstream activator of Nox via phosphorylation of p47phox in various cells (33, 424). Nox activity can be further elevated by increased endothelin-1 (ET-1) levels and associated with enhanced angiotensin II stimulation in endothelial cells that leads to p47phox phosphorylation (33, 424). These data confirm that increased Nox activation by PKC can enhance superoxide production as earlier discussed in section 2.5.1. Also, Quagliaro et al. (2003) demonstrated that an intermittent high glucose challenge in endothelial cells resulted in Nox activation that was sensitive to PKC inhibitors (573).

Enhanced PKC activity is associated with changes in blood flow, basement membrane thickening, extra cellular matrix expansion, vascular permeability, angiogenesis, cell growth and enzymatic activity alterations (196). During diabetes, activation of PKC may impair retinal and renal blood flow possibly
by increasing ET-1 levels (86). PKC activation also directly increases the permeability of macromolecules across endothelial or epithelial barriers by phosphorylating cytoskeletal proteins, or indirectly by regulating expression of various growth factors such as vascular endothelial growth factor (VEGF) (12, 86, 196, 366, 637, 732). The effects of PKC activation on NO are unclear, though there is evidence that it can reduce the production of NO (12, 86, 196, 366, 637, 732). All these factors can lead to accelerated atherosclerosis thus predisposing to the development of micro- and macro-vascular complications.

Most of the manifestations due to PKC activation in diabetes mellitus are reversed with the use of PKC inhibitors (12, 86, 196, 366, 637, 732), e.g. ruboxistaurin (PKC-β inhibitor) reverses hemodynamic changes that manifest with retinopathy, nephropathy and neuropathy (12, 86, 196, 366, 637, 732).

2.6.4. The hexosamine biosynthetic pathway

The HBP is a relatively minor branch of glycolysis. It involves conversion of glucose to fructose-6-phosphate, then fructose-6-phosphate to glucosamine-6-phosphate by the first rate-limiting enzyme glutamine: fructose-6-phosphate amidotransferase (GFAT) (see Figure 2.6). The major end-product is uridine diphosphate-\(N\)-acetylglucosamine (UDP-GlcNAc) (formed from glucosamine-6-phosphate) which along with other HBP generated amino-sugars provide essential building blocks for glycosyl side-chains of proteins and lipids (94) (see Figure 2.6). UDP-GlcNAc is further processed by the second rate-limiting enzyme, \(O\)-linked GlcNAc transferase (OGT), that transfers GlcNAc to the side-chain hydroxyls of serine and threonine residues to generate \(\beta\)-D \(O\)-linked glycosylated proteins (see Figure 2.6). Modification of serine and threonine residues of nuclear and cytoplasmic proteins was first identified in 1984 (710), and it occurs at essentially the same serine and threonine side-chains that are targeted by the phosphorylation process, thus \(O\)-GlcNAc and phosphorylation have potential reciprocity (392, 469). The reversal removal of \(O\)-GlcNAc residues is carried out by the enzyme \(O\)-
GlcNAc hydrolase/O-GlcNAcase. The addition of O-GlcNAc to proteins modulates behavior via different mechanisms that include: regulating protein phosphorylation and thus protein function; altering protein degradation; adjusting the localization of proteins; modulating protein-protein interactions and mediating transcription (794).

2.6.4.1 Contribution of the HBP to diabetic oxidative stress and complications

The HBP usually functions as a nutrient sensor under physiological conditions (285). However, during hyperglycemia excess glucose is shunted into the HBP, and this is linked with the development of
insulin resistance (468, 785). This implies that under hyperglycemic conditions increased amounts of fructose-6-phosphate are diverted from glycolysis that provide substrates for reactions that require UDP-N-acetylglucosamine, such as proteoglycan synthesis and the formation of O-linked glycoproteins (86). Shunting of excess intracellular glucose into the HBP may account for several manifestations of diabetic renal and vascular complications (94). At present there are very few studies done to show a causal link between HBP activation and high glucose-induced complications in diabetic patients. Recently, a study from our laboratory showed increased HBP activation and O-GlcNAc levels in leukocytes of pre- and full blown diabetic individuals (656). Moreover, an association of GFAT mRNA levels and enzyme activity with post-prandial hyperglycemia and oxidative stress was found in T2DM patients (658). Here, HBP activation correlated with thiobarbituric acid reactive substances (TBARS) and protein carbonyl content (PCO), both markers of oxidative stress. The end-product of this pathway, UDP-GlcNAc, is a substrate for the glycosylation of important intracellular modulators (86) including transcription factors, thereby affecting the expression of several genes, e.g. plasminogen activator inhibitor-1 (PAI-1) (see Figure 2.6). This in turn can lead to the development of diabetes-induced micro-vascular complications (221, 241, 242).

Increased protein O-linked GlcNAcylation can result in diminished expression of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) in the diabetic heart thereby impairing myocardial contractility (134, 315). Additionally, upregulation of the HBP with hyperglycemia may accelerate atherosclerosis (175, 198, 657) by decreasing eNOS levels (see Figure 2.6) in the vascular endothelium and thereby promoting endoplasmic reticulum stress, lipid accumulation and increased inflammatory gene expression (77, 612, 755) which predispose to AMI.

There is growing evidence (\textit{in vitro} and \textit{in vivo}) that highlight a pivotal role for the HBP in the onset of diabetic nephropathy (473) and (155). This may occur as a result of HBP-mediated induction of TGF-\(\beta\) and PAI-1 in vascular smooth muscle, mesangial (379, 753) and aortic endothelial cells (94).
Following TGF-β production, it is converted to its active form (TGF-β1) that causes the subsequent production of matrix components (heparan sulphate, proteoglycan, fibronectin). These (matrix components) in turn, alter protein structure and function thus leading to micro-vascular complications. This can result in kidney cellular hypertrophy as previously demonstrated (473). Moreover, PAI-1 induction is mediated by the transcription factor, Sp1 that can be regulated by the HBP. Here PKC βI and δ (241) activation was required, therefore suggesting a link between the HBP and PKC under hyperglycemic conditions.

When focusing on ischemia and reperfusion, there are contradictory reports whether an increase in protein O-GlcNAcylation is beneficial or detrimental. Chatham and colleagues report on the beneficial effects of increasing flux through the HBP (121, 122), whilst Essop and colleagues found that increased HBP flux under high glucose conditions followed by ischemia and reperfusion elicits detrimental effects on cardiac contractile function together with increased oxidative stress and apoptosis (462, 576). Consistent with these findings, others found that increased HBP can exert both an anti-inflammatory and pro-oxidative effect in endothelial cells under hyperglycemic conditions (578). It is likely that such differences stem from variations in experimental models, the nature of the stress condition (acute vs. chronic), and other unknown factors. This intriguing question is currently being pursued in our laboratory. Thus, it is clear that hyperglycemia-induced intra- and extracellular changes lead to alterations of signal transduction pathways, affecting gene expression and protein function to cause cellular dysfunction and damage. Moreover, data imply that the ultimate result is increased ROS formation and consequently diabetic complications (769). The next section will now discuss another non-oxidative glucose pathway – pentose phosphate pathway - that is altered with hyperglycemia.
2.6.5  Pentose phosphate pathway

The pentose phosphate pathway (PPP)/ phosphogluconate/ hexose monophosphate shunt is a multifunctional pathway and one of the two main biochemical pathways involved in glucose metabolism. It consists of two main branches, i.e. the oxidative and the non-oxidative branch, respectively. In addition to glucose oxidation, the PPP mainly generates reducing equivalents in the form of NADPH and pentoses (5-carbon sugars) such as ribose-5-phosphate and erythrose-4-phosphate used in the synthesis of nucleic acids and aromatic amino acids, respectively (747). In mammals this pathway occurs exclusively in the cytoplasm and is most active in the liver, mammary gland and adrenal cortex (440, 597). For the current thesis we have a great interest in the role of the non-oxidative PPP branch with diabetes (hyperglycemia), and in the setting of ischemia and reperfusion.

2.6.5.1  Non-oxidative branch of the PPP

Transketolase (TK) catalyzes several reactions in the non-oxidative PPP, and serves as a bridge between the oxidative PPP and the oxidative decarboxyl metabolism of glucose. This therefore allows the cell to adapt to a variety of metabolic needs under different environmental conditions (312). TK recognizes D-xylulose 5-phosphate, D-fructose 6-phosphate and D-sedoheptulose 7-phosphate as donors while D-ribose 5-phosphate, D-erythrose 4-phosphate, D-glyceraldehyde 3-phosphate and glycoaldehyde as receptor substrates (191). The main function of the non-oxidative PPP branch is to convert hexoses into pentoses, particularly glyceraldehyde 3-phosphate into ribose-5-phosphate that is required for nucleic acid synthesis.

TK relies on thiamine pyrophosphate (a thiamine pyrophosphate [TPP] ester) as cofactor for its function. Here the main source is from Vitamin B1 (thiamine) a water-soluble compound that has been
used for decades in the treatment of several disorders such as neurological, diabetic, and cardiovascular complications (23, 55, 560). More recently, studies found that thiamine can prevent diabetes and its associated complications (292, 560, 671, 703). The main sources of thiamine include various foods e.g. pork, poultry, fish, eggs (440). However, due to its water solubility, it has limited durable storage capacity in the body and there is rapid urine excretion, hence decreased bioavailability in comparison with its fat soluble analogs (36, 38, 66, 219, 253). A variety of lipid-soluble thiamine derivatives were discovered (as early as 1954); subsequently named “allithiamines” because they belong to the Allium family of vegetables e.g. crushed garlic and onions (219).

As earlier discussed (Chapter 1), a major focus of this thesis is on is the lipid soluble analog of thiamine, i.e. benfotiamine, that exhibits strong antioxidant properties (232, 625). Benfotiamine contains an open thiazole ring, which allows it to pass through the cell membrane (36). The classification of benfotiamine as a lipophilic agent is disputed by findings that showed its increased solubility in aqueous solutions e.g. water at pH < 7; and not in organic solvents e.g. benzene (740, 743). Thus, it is likely that benfotiamine is an amphipathic agent. Thiamine and its analogs are initially metabolized to thiamine monophosphate, then TPP/ thiamine diphosphate (TDP) that is required by TK. TK is a homodimer with two active sites that are localized at the interface between the monomers. Thus far one TK gene and two TK-like genes (TKTL1 and TKTL2) were identified within the human genome (431). TKTL1 may play a role in carcinogenesis and have implications in the nutrition and future treatment of cancer patients. Researchers have found TK variants and reduced activities of TK enzymes in patients with neurodegenerative diseases (e.g. beriberi), diabetes and cancer (440, 597). The role of TK as illustrated in Figure 2.7 is a two-way process; i.e. firstly converting xylulose-5-phosphate and ribose-5-phosphate to glyceraldehyde-phosphate and sedoheptulose 7-phosphate, respectively. These are then converted to erythrose 4-phosphate and fructose 6-phosphate by transaldolase. In the second TK pathway, xylulose 5-phosphate and erythrose 4-phosphate are
converted to glyceraldehyde 3-phosphate and sedoheptulose 7-phosphate. All the reactions are reversible and depend on substrate availability.

\[
\begin{align*}
\text{X-5-P + R-5-P} & \quad \xrightarrow{\text{Transketolase}} \quad \text{G-3-P + S-7-P} \\
\text{G-3-P + S-7-P} & \quad \xrightarrow{\text{Transaldolase}} \quad \text{E-4-P + F-6-P} \\
\text{X-5-P + E-4-P} & \quad \xrightarrow{\text{Transketolase}} \quad \text{G-3-P + S-7-P}
\end{align*}
\]

**Figure 2.7 The non-oxidative reactions of the pentose phosphate pathway.** X-5-P (xylulose-5-phosphate); R-5-P (ribose 5-phosphate); G-3-P (glyceraldehyde 3-phosphate); E-4-P (erythrose 4-phosphate); S-7-P (sedoheptulose 7-phosphate).

TPP is also a cofactor of other enzymatic reactions that cleave alpha-keto acids in the TCA cycle, i.e. pyruvate dehydrogenase and α-ketoglutarate (55, 253, 440, 560). This therefore implies that thiamine and its analogs can promote glucose oxidation. Thiamine is transported across the cell membrane by members of the solute carrier (SLC) family, mainly SLC19A2 and also SLC19A3 found ubiquitously in mammalian tissues (224, 509). Studies reported that benfotiamine is delivered into cells via the reduced folate carrier-1 (RFC-1), and then de-benzoylated to thiamine monophosphate by cellular and plasma esterases (66, 703). Various studies reported reduced levels of thiamin, activity of transketolase (32, 294, 339, 613, 724) and beneficial effects of benfotiamine treatment with diabetes (32, 54, 55, 292, 358, 360).

### 2.6.5.2 Oxidative branch of the PPP

For the oxidative PPP branch, glucose 6-phosphate is oxidized into 6-phosphogluconolactone (6PG) by the rate-limiting enzyme, glucose 6-phosphate dehydrogenase (G6PD). This reaction results in the production of the first NADPH molecule in the pathway. The unstable lactone ring of 6PG is opened up by a lactonase into 6-phosphogluconic acid which undergoes an oxidative decarboxylation by the 6
phosphogluconate dehydrogenase (6PGD) to yield a second NADPH molecule and carbon dioxide. The end product of this pathway, ribulose 5-phosphate, can then be converted into ribose 5-phosphate in the non-oxidative pathway to yield nucleotides via the TK reaction. From these reactions it can be deduced that the main role of the oxidative PPP branch is to produce NADPH which functions in detoxification processes, i.e. reduced glutathione regeneration and lipid biosynthesis (440, 597) as well as provision of ribulose 5-phosphate for nucleotide synthesis.

There are conflicting reports on whether G6PD activity is increased, decreased or not changed with insulin resistance/ diabetes. Some found that G6PD activity increases (263, 633) while others reported decreased activity (105, 107, 359, 678, 747, 800). Such conflicting data further extends to NADPH produced by this pathway, i.e. whether it contributes to or blunts oxidative stress. In fact, almost all the studies show that increased G6PD activity enhances Nox-induced ROS production via PKC activation. Similar findings were observed in various heart failure studies where G6PD activity is increased to provide more NADPH possibly as a compensation mechanism to counter oxidative stress (264, 265). However, the association of G6PD to increased Nox activity has been disputed by Balteau et al. (2011) who suggested that this effect is independent of glucose metabolism but relies on glucose uptake via sodium glucose transporter 1 (SGLT1) (40). The differences observed may be attributed to variations of experimental (in vivo and in vitro) and animal models used (humans, rats and mice), different tissues where G6PD was measured (liver, cardiac, pancreas and neurons). Moreover, G6PD activity varies with ischemia and reperfusion i.e. its activity can be increased (336) or decreased (358) resulting in either detrimental or protective effects. These outcomes imply that G6PD may act as a pro- or antioxidant depending on different ischemia and reperfusion methods and experimental systems (801). In summary, this section highlighted the pathophysiology of hyperglycemia-induced ROS production by increased flux through the non-oxidative glucose pathways. Furthermore, the effect of hyperglycemia on the PPP was considered. The next section will now discuss various complications that occur secondary to the increased activation of pathways discussed above.
2.6.6 Non-oxidative glucose pathways and cardiac function

The previous sections have extensively elaborated on the effects of the non-oxidative glucose pathways in general. However, since the focus of this thesis is to link these pathways to alterations in cardiac function, it’s essential to clarify how these pathways have shown regulation of cardiac function under various conditions. These will be discussed in the same order as they are above. Firstly, the polyol pathway activation increases three-fold during ischemia and reperfusion (347) and possibly mediates ischemia-reperfusion injury by opening of the mitochondrial permeability transition pore (mPTP) (21, 321, 322) independent of hyperglycemia. The increased activation of the polyol subsequently causes cardiac contractile dysfunction by enhancing tyrosine nitration of the SERCA and oxidation of ryanodine proteins thus impairing its functional role in cardiac contractility (347, 691). Furthermore, inhibition of the polyol pathway has shown improved cardiac energy metabolism under both normoglycemic and hyperglycemic conditions (579, 580), attenuation of oxidative stress and a restoration in electrolyte homeostasis (531, 691, 712, 759).

Several studies have shown that AGEs and RAGE do play a role on cardiac function through various mechanisms. AGEs increase following ischemia and reperfusion (88) and enhance myocardial injury through glycative inhibition of thioredoxin activity (748) resulting in contractile dysfunction. Furthermore, AGE-RAGE axis increases ischemia and reperfusion injury via the JNK and STAT pathways causing phosphorylation of GSK-3β (15, 88, 89, 634). In support inhibition of AGE formation (529) or interaction with RAGE (446) has shown an improvement in cardiac function in vivo studies.

PKC is increasingly recognized as a key regulatory cardiac enzyme in both normal and pathophysiological conditions. In these circumstances it plays a pivotal role in functional adaptations, with reports on increased activity during ischemia and reperfusion and hyperglycemia (674). This may either be cardio-protective or damaging depending on which isoform is activated and timing of
activation in the protocol (i.e. pre-ischemia, during ischemia, post-ischemia and reperfusion). Various studies highlight the role of PKCε and ζ in cardio-protection at both baseline glucose concentrations (770, 798) and hyperglycemic conditions (457) with/without ischemia and reperfusion. Hyperglycemia activates PKC-α, β and δ causing contractile dysfunction (132, 262, 328, 435).

The role of the HBP on cardiac function has been highlighted earlier as contradictory i.e. whether increased activation is beneficial (121, 122) or harmful (462, 576) to cardiac function. The discrepancy in these experimental findings likely stem from variations in experimental models, the nature of the stress condition (acute vs. chronic), and other unknown factors. Increased O-GlcNAcylation under hyperglycemic conditions has been previously reported to elicit similar detrimental effects on cardiac contractile function (134, 315, 462). It is likely that detrimental effects are mainly due to altered protein function, for e.g. increased protein O-linked GlcNAcylation diminished expression of cardiac SERCA and lead to impaired myocardial contractility (134, 315). Moreover, HBP-mediated induction of FAO may also blunt cardiac function under these conditions (407).

With regards to the PPP various studies have shown pivotal roles of both the oxidative and non-oxidative branches in cardiac function regulating in different conditions. Increased flux via the non-oxidative pathway under hyperglycemic conditions and with ischemia and reperfusion has shown cardio-protective effects in animal studies (336, 358, 801). In contrast, however there are contradicting findings on the outcome of increased activation of G6PD, the rate limiting enzyme of the oxidative PPP branch. The varying effects have been elaborated in section 2.6.5.2 above and clearly indicate that the PPP plays in the regulation of cardiac function.
2.7 Diabetic complications

As discussed, chronic hyperglycemia is one of the main perpetrators predisposing to the development of micro- and macro-vascular complications with diabetes (167, 722). The latter is more common with T2DM, whereas, micro-vascular complications occur mainly in T1DM. Chronic hyperglycemia that occurs with diabetes is mainly due to derangements in carbohydrate, lipid and protein metabolism (575). For the current study, the focus is on one of the macro-vascular complications, i.e. myocardial infarction, since diabetic patients have a greater risk for fatal AMI with an impaired recovery than non-diabetic patients (153, 363, 663). The following section reviews both micro- and macro-vascular complications with diabetes.

2.7.1 Micro-vascular complications

The pathogenesis of micro-vascular complications is similar in T1DM and T2DM (86, 637). Micro-vascular complications most common in T1DM include retinopathy, neuropathy, and nephropathy (47, 230, 744).

2.7.1.1 Diabetic neuropathy

Diabetic neuropathy is defined as signs and symptoms of peripheral nerve dysfunction in a diabetic patient where other causes of peripheral nerve dysfunction are excluded (41). It is one of the commonest complications of diabetes present in about half of patients (to varying degrees) and typically manifests as polyneuropathy or mononeuropathy (637, 695). The disease can develop in all types of diabetes mellitus, but more typically with T1DM compared to T2DM (433). Diabetic neuropathy leads to increased incidences of ulceration and limb amputations due to the irreversible progressive development of the disease (41, 83). Moreover, it accounts for silent myocardial infarction
and shortens the lifespan of diabetic patients. The prevalence of diabetic neuropathy increases with the duration of the diabetic state (41, 433).

The cause(s) of diabetic neuropathy may include any of the following: oxidative stress, ischemia, and inflammation, leading to dysfunction and loss of axons (433). Here oxidative stress can result due to increased PKC activity (as discussed before). Blood supply to neurons may be impaired by vascular damage and endoneural hypoxia due to oxidative stress (637, 732). Hypoxia further leads to capillary damage aggravating disturbances in axonal metabolism and nerve conduction (41). Distal symmetrical sensorimotor polyneuropathy characterized by thickening of axons of small myelinated and non-myelinated C-fibers is the most common type of diabetic neuropathy (41, 433, 637, 695), and is manifested by paresthesia, dysesthesia, pain, impaired reflexes and decreased vibratory sensation (41, 433, 637, 695).

Inhibition of the pathways involved in the etiology of diabetic complications, glycemic control, antidepressants and analgesics may be used to manage diabetic neuropathy (732, 764). Furthermore, studies indicate that plant derivatives such as α-lipoic acid, primrose oil and capsaicin offer potential in the management of diabetic neuropathy (272). Also, in this study extensive work on thiamine and its derivative (benfotiamine) show that both agents can attenuate neuropathy (23, 292, 671, 703).

2.7.1.2 Diabetic retinopathy

Diabetic retinopathy, due to damaged blood vessels of the retina, is the most common cause of blindness in diabetic patients (366, 387). Nearly all T1DM individuals and more than half of T2DM develop retinopathy ~ 15-20 years after diagnosis of diabetes (758). Large increases in glucose levels within the retina (if toxic) might damage retinal cells, particularly Müller cells. Because the Km of the GLUT1 transporter is 5 mM, it does not saturate with substrate except under pathological conditions
Retinal complications in chronic diabetes may be due to micro-vascular dysfunction, neuroglial abnormalities, and a toxic metabolic environment (366). Diabetic retinopathy is a duration-dependent disease that develops in stages and that may not be detected during the first few years of diabetes (387). It is classified as non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (366, 387, 758). Additionally, NPDR is further divided into progressive stages: mild, moderate and severe (366, 387) and characterized by capillary basement membrane thickening, pericyte loss, micro-aneurysms, increased permeability, exudates deposits, and retinal micro-infarcts. The earliest sign of retinal damage during NDPR results from abnormal permeability and non-perfusion of capillaries, leading to the formation of micro-aneurysms. Visual acuity is impaired by macular edema, following the leakage of fluid and solutes into the surrounding retinal tissue (758). The later stages, sometimes called pre-proliferative retinopathy, show greater retinal damage as evidenced by increased retinal vascular blockage and infarcts (366, 387, 758).

Proliferative retinopathy develops if the pre-proliferative retinopathy is not treated and is characterized by abnormal proliferation of retinal blood vessels. These vessels are, however, fragile and hemorrhage quite easily. The resulting accumulation of blood in the vitreous humor from hemorrhaging vessels impairs vision; this impairment can be permanent due to complications such as retinal detachment (758). Biochemical abnormalities associated with hyperglycemia and identified in diabetic retinas include increased activation of PKC (389), AGEs formation (85, 541, 669), polyol pathway (29), and HBP (297). These lead to increased production of ROS and activation of growth factors that promote apoptosis (512). There is growing evidence that ROS and RNS are present in excess in diabetic retinas (178, 236, 237, 384), and in vascular endothelial cells (176, 570). Both ROS and RNS are toxic to tissues because of high reactivity and ability to non-enzymatically form covalent bonds. The various mitochondrial and non-mitochondrial ways through which they (ROS and RNS) are produced (409, 574, 718) were discussed earlier in section 2.5.1.
The pathways previously described particularly the polyol pathway (see section 2.6.1 - 2.6.4), lead to the structural and functional changes that occur with diabetes mellitus (366). Diabetic retinopathy, like most other complications of diabetes mellitus, does not usually occur in isolation in the diabetic state. Therefore, drugs, medicinal plants and their derivatives that are used to inhibit the pathways involved in the development of diabetic complications may also prevent the onset of diabetic retinopathy. For example, AGE pathway inhibition resulted in beneficial effects i.e. preventing progression of retinopathy and inhibiting hyperglycemia-induced thickening of the retinal basement membrane (9, 226, 280).

2.7.1.3 Diabetic nephropathy

Diabetic nephropathy a leading cause of end-stage renal disorder (ESRD), accounts for significant morbidity and mortality with diabetes (68, 493, 615, 620). The pathophysiology of diabetic nephropathy involves interactions between metabolic and hemodynamic factors. Some metabolic factors include increased formation of AGEs, polyols and PKC activation (137, 138, 584). The involvement of these pathways in the development of diabetic nephropathy has been earlier described. Hemodynamic factors include systemic hypertension and the tone of both afferent and efferent arterioles (138). Diabetic nephropathy progresses from micro-albuminuria to overt proteinuria and then renal failure. During the initial stages of diabetes, there is enlargement of the kidneys and increased glomerular filtration rate (GFR), whereafter GFR progressively decreases (584, 700). The two main hypotheses that describe the initial events of diabetic nephropathy are the ‘vascular hypothesis’ and the ‘tubular hypothesis’ (795). The vascular hypothesis states that the initial hyperfiltration is due to a) an excessive production of vasodilator products like nitric oxide and prostaglandins, and b) increased osmolar load (734). Additionally, there is higher glomerular hydrostatic pressure associated with micro-albuminuria (402, 471). These features result in basement membrane thickening, mesangial proliferation, and glomerulosclerosis as a compensatory mechanism.
to prevent electrolyte loss. By contrast, the tubular hypothesis proposes that hyperglycemia induces increased production of growth factors and cytokines which cause hyperplasia and hypertrophy of the nephron, particularly the proximal tubule (795). As a result increased reabsorption of sodium occurs in the proximal tubule, consequently reducing the sodium load to the macula densa (795). Experimental animal work indicates that the vascular hypothesis is more applicable considering that the hyperfiltration is observed within 24 hours of diabetes induction (795).

Nephron hypertrophy with diabetic nephropathy occurs because of excessive deposition of extracellular matrix proteins involved in the architecture of glomerular basement membrane. These include multifunctional glycoproteins, laminin, fibronectin and type IV collagen. At the same time, there is decreased production and under-sulphation of heparan sulphate proteoglycan. This enhances the permeability to macromolecules since the glycoproteins and heparan sulphate proteoglycan normally interact to form a barrier to charged molecules (402). ET-1, which increases five-fold in diabetic animal models is implicated in glomerular hypertrophy mediated by TGF-β1. Indeed, experimental evidence indicates that inhibition of ET-1, TGF-β1 and the endothelin-1 receptor A with plant extracts improves renal function and ameliorates glomerular injury (290, 367, 511, 654). ET-1 production is augmented by vasoconstrictor, profibrotic and inflammatory substances, all of which are increased under hyperglycemic conditions. PKC activation also favors ET-1 production, as this is the signaling pathway leading to its (ET-1) upregulation (654). The effects of ET-1 are mainly directed at mesangial cells, and their proliferation is due to direct or indirect stimulation of mitogenesis (654).

Irrespective of all the other structural and functional changes, the mesangial alterations appear to be the main cause of declining renal function in experimental diabetic animal models (240, 402, 471). Hyper-filtration can be attributed to increased production of the vascular permeability factor in response to stretching of the mesangium (260). The decline in GFR is due to the expanded extracellular mesangial matrix which compresses the glomerular capillaries thereby reducing the
filtration surface area (240, 402, 471). The mesangial cells can also increase glucose uptake through increased expression of GLUT1 (239, 620). Higher glucose uptake exacerbates intracellular hyperglycemia and increases activity of previously described pathways. Various treatment modalities are employed to manage diabetic nephropathy, and these may target oxidative stress (e.g. lisinopril and benfotiamine) (32, 200), and inhibition of the RAS pathway (200).

2.7.2 Macro-vascular complications

Diabetes mellitus is associated with coronary, cerebral and peripheral arterial disease (650). Coronary and cerebral arterial diseases can result in AMI and stroke, respectively. These disorders, with peripheral arterial disease are defined as macro-vascular diseases (86, 733).

2.7.2.1 Arterial diseases

Arterial disease is strongly associated with T2DM rather than T1DM. This is the case since with T2DM the metabolic syndrome usually manifests, i.e. characterized by hypertension, dyslipidemia (increased triglycerides, decreased high density lipoproteins (HDL), and increased low density lipoproteins (LDL) resulting in inflammation and impaired fibrinolysis. All these factors precipitate changes in the vasculature and create an environment conducive for accelerated atherosclerosis (50, 596, 733). A greater risk to develop cardiovascular diseases, as well as increased morbidity and mortality, is thus observed in diabetic patients due to the toxic metabolic milieu found within the circulatory system (50, 596, 733, 734). The chief cause of cardiovascular diseases with diabetes is atherosclerosis which is described below.
2.7.2.2 Atherosclerosis

Diabetes is associated with impaired function of the endothelium which contributes to atherosclerosis (142). Endothelial dysfunction arises due to a disruption of the homeostatic factors, usually maintained through integrity of the endothelium barrier and the balance between vasodilators and vasoconstrictors (611, 733). Vasodilators include NO, prostacyclin and endothelium-derived hyperpolarizing factor (EDHF), while the vasoconstrictors ET-1 and angiotensin II are well-known ones (80, 142, 611, 733). However NO, is regarded as the key marker of vascular health (142).

With diabetes mellitus the endothelium barrier is disrupted by the oxidative stress and increased activity of the metalloproteinases resulting in the entrapment of excess atherogenic lipoproteins like VLDL, ox-LDL and lipoprotein (a) (194, 197, 733). This infiltration triggers an inflammatory response thereby attracting monocytes and T-cells (287, 288, 611), and also increased expression of adhesion molecules on the endothelium e.g. VCAM-1 and ICAM-1, P-selectin and E-selectin (194, 197, 514, 733). This in turn triggers adhesion of the attracted monocytes and T-cells to the endothelium (see Figure 2.6). After binding to the arterial wall the monocytes and T-cells can migrate into the sub-endothelial space and differentiate into macrophages and foam cells (287, 288, 611). This migration is attributed to chemo-attractants like ox-LDL, IL-1 and TNF-α produced as a result of activation of the transcription factors NF-κβ and AP-1 (50, 194, 197).
Figure 2.8 Effect of hyperglycemia on atherosclerosis. Hyperglycemia can promote several aspects of the atherosclerotic process, including monocyte recruitment and adhesion to the endothelium, penetration into the arterial intima, formation of oxidized LDL (ox-LDL), foam cell formation, vascular smooth muscle cell (SMC) migration and proliferation, leading to fatty streak formation. Modified from (603).

This inflammatory response together with reduced NO formation thus precipitate atherosclerosis with diabetes. Here, reduced NO formation may occur due to the deformed endothelium now exposing eNOS to uncoupling by peroxynitrite and nitrotyrosine. Thus, eNOS produces superoxide free radicals instead of NO (50, 540). NO production may also be inhibited by excessive liberation of FAs from adipose tissue due to PKC-induced inhibition of an eNOS agonist pathway involving PI 3-K (50). Thus, as NO bioavailability progressively decreases, concomitant increases in peroxynitrite further impair production of subsidiary vasodilators like the antiplatelet prostanoid, prostacyclin (50, 142, 733). It is therefore clear that the disordered endothelium exhibits an imbalance between vasodilators and vasoconstrictors, with the latter dominating e.g. increased vasoconstrictors ET-1 and angiotensin II. In
support, angiotensin II-induced increased voltage-gated calcium flux has been observed in vascular smooth muscle from diabetic rats possibly due to increased activity of the canonical transient receptor channels 1/4/5 (TPRC1/4/5) (195, 252). This was observed in association with increased vasoconstriction and accelerated atherosclerosis (195, 252).

Vasoconstriction is further exacerbated by the effects of acetylcholine on smooth muscle muscarinic receptors, thereby fueling the onset of atherosclerosis (50, 733, 734). The processes discussed above, together with hypertension and the impaired fibrinolytic capacity with the prothrombotic milieu, strongly contribute to the onset of atherosclerosis in the setting metabolic syndrome and diabetes mellitus (50, 197). Within the context of this thesis, it is a major cardiovascular risk factor for AMI in non-diabetic and diabetic individuals.

Prevention of atherosclerosis with diabetes mellitus is associated with a reduction in the development of micro- and macro-vascular complications. Some anti-atherosclerotic drugs e.g. statins are not limited to lowering atherogenic lipoprotein levels, but also possess beneficial anti-inflammatory effects (80). Likewise, oral anti-diabetic drugs can elicit similar outcomes (14, 391, 594). Moreover, plant derivatives also offer antioxidant effects, e.g. dietary polyphenols and flavonoids (quercetin) can reduce atherogenic lipoproteins and ameliorate the oxidative stress with diabetes mellitus (453, 670). Other targets implicated in this process include the AGE-RAGE axis. For example, blocking or genetically deleting the RAGE in diabetic experimental animals reverses atherosclerosis (324), while AGE inhibitors (e.g. aminoguanidine, OPB-9195, and pyridoxamine) lead to reno-protective effects in diabetic animals (291, 411). Unfortunately, clinical trials with OPB-9195 resulted in side effects i.e. trapping of pyridoxal leading to vitamin B6 deficiency (351, 644). Furthermore, inhibition of AGEs effects can also be achieved by breaking of the AGE cross links by employing drugs such as alagebrium, and also by attenuating AGE signal transduction e.g. by using incubadronate disodium and cerivastatin (291).
Despite all the efforts to control glucose and lipid levels during diabetes the occurrence of HF amongst diabetic patients is increasing. This is secondary to the diabetic cardiomyopathy (discussed below) that occur independent of any other existing pathological condition besides diabetes.

### 2.7.2.3 Diabetic cardiomyopathy

Diabetic cardiomyopathy is the presence of myocardial dysfunction in the absence of coronary artery disease and hypertension with diabetes, hyperglycemia considered the main trigger (261). This condition was first described by Rubler et al. (1972) who reported autopsy data from four patients with diabetic renal micro-angiopathy and dilated left ventricles in the absence of other common causes (606). It is characterized by systolic and diastolic function, with impaired systolic function manifesting as prolonged relaxation and reduced compliance (22). Several mechanisms are proposed for diabetic cardiomyopathy including oxidative stress, impaired glucose metabolism (444, 600), biochemical and physiological changes in hormonal signaling, a number of structural changes in the heart (444), and abnormalities of proteins that control ion movement (particularly calcium) (308, 408, 593).

With diabetes there is a shift in energy production to FAO, with glucose oxidation attenuated due to depleted GLUT1 and 4 levels (600). The end result is the onset of lipotoxicity (excess ceramides triggering apoptosis) and also accumulation FAO toxic intermediates that can impair myocyte calcium handling signaling (hence contractile dysfunction) (1, 183, 429, 458, 459, 600, 688). Excess lipids with diabetic cardiomyopathy enhance atherogenic mechanisms such as ox-LDL, endothelial dysfunction and vascular smooth muscle proliferation and migration (164). Furthermore, inhibited glucose oxidation results in accumulation of glycolytic intermediates that trigger the previously described non-oxidative glucose pathways (183, 429) signaling. Moreover, pathological changes in the diabetic heart are also linked to increased accumulation of ROS or RNS (99, 216).
With hyperglycemia various mechanisms increase myocyte death by both apoptosis and necrosis (216). Some of the mechanisms involved include increased ROS production by AGEs (452), phosphorylation of p53 (203), impaired sympathetic nervous system (65) resistance to IGF-I (124, 216, 261), and increased RAS activation (261). Additionally, these pathologies can result in structural changes of the heart during development and progression of the diabetic cardiomyopathy. Normally the myocardial fibrous tissue is interstitial, perivascular or both, however in pathology there is hypertrophy, interstitial fibrosis, capillary endothelial changes and capillary basal lamina thickening (204). These changes occur as a result of increased deposition of collagen I and II (in the epicardium, and perivascular tissue), and collagen IV (in the endocardium) (640). The collagen ultimately interacts with glucose to form AGEs contributing to the arterial stiffness, endothelial dysfunction and atherosclerotic plaque formation (26, 212). Furthermore, increased TGF-β1 occurs due to overstimulation of fibroblasts and this ultimately results in fibrous tissue deposition and extracellular matrix synthesis which eventually causes myocardial dysfunction (490).

Clearly it can be seen that hyperglycemia-induced toxicity plays an important role in the development of CVDs with diabetes. Since toxic effects are mainly as a result of hyperglycemia-induced oxidative stress that occurs during diabetes, attenuation of these effects may possibly be achieved by normalizing blood glucose levels. Hence, the next section discusses various drugs used during diabetes management, focusing on glycemic control in the diabetic heart.

2.8 Diabetes mellitus management

The importance of blood glucose control in preventing complications of diabetes mellitus is now well recognized and the treatment regimen incorporates a controlled-energy diet, regular aerobic exercises and weight loss (167, 309, 331, 391, 546, 598). However, since most patients fail to achieve adequate blood glucose control with lifestyle interventions alone this also requires pharmaco-therapeutic
approaches (214, 331). There are many standard anti-diabetic drugs used in the management of diabetes mellitus. These include various formulations of insulin and oral anti-diabetic agents which can either be employed as monotherapies or in combination to achieve improved glycemic regulation (342, 598). Here oral anti-diabetic agents such as metformin is more effective when used in combination than when singly administered, e.g. with insulin, sulphonylureas and thiazolidinediones. Indeed, single therapy is less effective in maintaining normoglycemia particularly as diabetes progresses (374, 391). Recently, a 'polypill' treatment was suggested as a potential overall remedy for diabetes and its complications (391, 412), that includes an anti-hyperglycemic, anti-inflammatory, anti-hypertensive, and anti-angiogenic agents. According to Krentz and Bailey (2005), anti-diabetic drugs can be classified as insulin secretagogues, insulin sensitizers and those that delay carbohydrate absorption (391). The following sections discuss the anti-diabetic mechanisms of synthetic drugs.

2.8.1 Insulin

Insulin, discovered in 1921, is the major hypoglycemic agent currently used in the management of T1DM and late stage T2DM (187). Patients who do not achieve effective glycemic control with oral agents, or for whom other oral agents are contraindicated, are also treated with insulin (391, 412). Robertson et al. (2003) (598) classify insulin as human insulin and insulin analogs, while another classification is based on the duration of action, i.e. rapid-acting, short-acting, intermediate-acting and long-acting (60, 742).

The short acting type is designed to mimic bolus insulin secretion, while intermediate or long acting insulin analogs are designed to mimic basal glycemic control (209, 742). Insulin is either administered subcutaneously using multiple daily injections, or an external pump for continuous delivery (306). Other delivery routes include oral, inhaled, nasal, rectal, ocular, intra-vaginal and transdermal (306, 689). Severe hypoglycemia is a major disadvantage of insulin usage with over-dosage observed with
several clinical trials such as the ACCORD, ADVANCE, UKPDS and DCCT (4, 8, 167, 722). Additionally Muis et al. (2005) reported increased occurrence of atherosclerosis with insulin usage as it promotes smooth muscle proliferation (501). However, here the combination of insulin with oral anti-diabetic drugs can reduce some of these disadvantages (154, 412, 472, 598, 689).

2.8.2 Insulin sensitizers

2.8.2.1 Biguanides

Phenformin (phenethylbiguanide) and metformin (1,1 dimethylbiguanide hydrochloride) are examples of biguanides. Metformin is derived from *Galega officinalis* (Linnaeus) [Fabaceae] (French lilac), a plant rich in biguanides (259, 391, 492). Metformin can be used alone or in combination with other drugs like sulphonylureas in diabetes management (34). Although the precise mechanisms whereby biguanides trigger hypoglycemia are less clear, the end result is that it can increase insulin sensitivity within the context of T2DM (34). This may occur by it enhancing insulin effects. Metformin’s blood glucose lowering effects not only involve suppression of gluconeogenesis and glycogenolysis, but also enhancement of insulin-stimulated glucose uptake by skeletal muscle tissues (14, 391, 594).

Studies indicate that metformin activates AMPK, a heterotrimeric enzyme composed of a catalytic subunit (α) and two regulatory subunits (β and γ) (391, 719). There are two isoforms of the catalytic subunit: AMPK α1, which is widely distributed, and AMPK α2, which is expressed in skeletal muscle, heart, and liver (217, 506). Previous studies found that AMPK is activated following depletion of cellular ATP, resulting in phosphorylation of AMPK by AMPK kinase at threonine-172 to prevent breakdown of carbohydrates (217, 506). The increase in AMPK activity results in the stimulation of glucose uptake, and the inhibition of hepatic glucose production, cholesterol and triglyceride synthesis, and lipogenesis. By contrast, the enzyme protein phosphatase 2C dephosphorylates AMPK and
results in its inactivation (391, 485, 506). Metformin also lowers blood glucose concentrations by decreasing intestinal absorption of glucose (374). Moreover, novel anti-hyperglycemic mechanisms of metformin may involve enhancement of β-endorphin secretion from adrenal glands and stimulating opioid μ-receptors located in peripheral tissues. Here opioid μ-receptors stimulation can increase expression and activity of GLUT4 transporters (128).

The efficacy of glycemic control accomplished with metformin is similar to that achieved with sulphonylureas, although their modes of action differ, i.e. metformin mainly acts by decreasing overproduction of glucose in the fasting state (335, 676). For example, its usage also has several beneficial pleiotropic effects (391). Furthermore, it can result in weight reduction, improves lipid profiles, and enhances endothelial function. The use of metformin, however, is contraindicated in conditions such as hypoxia, reduced perfusion of the heart in respiratory insufficiency and impaired renal function (391).

### 2.8.2.2 Thiazolidinediones

Thiazolidinediones (TZDs) such as troglitazone, pioglitazone and rosiglitazone are selective and potent agonists of peroxisome proliferator-activated receptor (PPAR)-γ (129). PPAR-γ and its isoforms PPAR-α and PPAR-δ, are members of the nuclear hormone receptor family of a ligand-activated transcription factors (50, 129, 399). PPAR-γ receptors are found on insulin-sensitive tissues and act as lipid sensors to regulate carbohydrate and lipid metabolism. Upon activation, PPAR-γ results in an improvement in insulin sensitivity and increases glucose uptake by skeletal muscles, while also reducing hepatic glucose output (50, 129, 399). Other TZDs effects include its ability to protect pancreatic β-cell function (14, 129, 399), modulating most risk factors for CVDs (391, 399) and altering lipid profiles, lowering blood pressure and prevent inflammation and atherosclerosis in vascular tissues (399).
2.8.3 Insulin secretagogues

2.8.3.1 Sulphonylureas (SUs)

The drugs in the SU group include glibenclamide, chlorpropamide, tolbutamide and gliclazide. Sulphonylureas (SUs) augment glucose-induced late insulin release from pancreatic β-cells (166, 391) by blocking the opening of potassium channels ($K_{\text{ATP}}$) by binding to the pancreatic β-cell SU receptor (SUR)1 and depolarize the membrane leading to calcium influx through opened voltage-gated calcium channels (391, 594). Increased intracellular calcium levels mobilize calcium-dependent insulin vesicles to fuse with the membrane and release insulin (14, 391). SUR1 is the regulatory subunit on β-cell $K_{\text{ATP}}$ channels whereas variants of SUR2 are on $K_{\text{ATP}}$ channels of cardiac (SUR2A), and vascular smooth muscles (SUR2B) (166). Tolbutamide and gliclazide block the SUR1 subunit, whereas glibenclamide and glimepiride have affinity for both SUR1 and SUR2 isoforms (166). The binding of SUs to $K_{\text{ATP}}$ channels of coronary vessels causes dilatation to enable the heart to adapt to ischemic conditions (14). However, SUs can cause hypoglycemia, since insulin release is initiated even when glucose concentrations are below the normal threshold required for insulin release (391).

2.8.3.2 Meglitinides

Meglitinides include repaglinide and nateglinide and can enhance the initial surge of insulin release in response to meals by binding to specific SUR1 sites on pancreatic β-cell membranes that inhibit potassium channels (230, 391). Activity of meglitinides depends on the concentration of the blood glucose and the dose of the drug used (230). Here medication is taken prior to meals to prevent post-prandial hyperglycemia and because they (meglitinides) relatively have a short half-life (391). Repaglinide has a rapid and short-lived insulinotropic action (539), and is effective in T2DM as reflected by the lower HbA$_{1C}$ levels (148, 243, 454). It is also associated with less weight gain and
hypoglycemia and may be used as monotherapy or in combination with metformin, TZDs and long-acting insulin. Nateglinide, is a phenylalanine-derivative that has a lower affinity than repaglinide as it dissociates more rapidly from its receptor (314). Hence, it is less effective in lowering blood glucose levels and HbA1c levels (243, 313, 350) but causes less hypoglycemia than repaglinide (539).

### 2.8.4 α-Glucosidase inhibitors

α-Glucosidase inhibitor drugs such as acarbose, miglitol and voglibose inhibit intestinal glucose absorption (14, 391, 492). α-Glucosidase a membrane-bound enzyme on the epithelium of the small intestine hydrolyzes disaccharides and oligosaccharides (14, 391, 492). Inhibition of α-glucosidase prevents a post-prandial increase in blood glucose concentration due to delayed carbohydrate absorption (14). Acarbose can also increase the secretion of glucagon-like peptide (GLP)-1, an insulinotropic hormone. GLP-1 is a hormone released from intestinal L cells into the circulation after meals (173, 708), whereafter it enhances glucose-dependent insulin secretion through activation of cAMP-dependent protein kinase in pancreatic β-cells (450, 701). GLP-1 is effective in reducing post-prandial hyperglycemia (708), enhancing insulin sensitivity and also in preserving β-cell function (516, 701) although the relative contribution to reduction in post-prandial hyperglycemia is unknown (230). However, many patients do not tolerate the α-glucosidase inhibitors due to flatulence, abdominal pain and diarrhea (284, 631), hence need for development of alternative agents.

### 2.8.5 Peptide analogues

This class of anti-diabetic drugs consists of injectable incretin mimetics; glucagon-like analogs/agonists; dipeptidyl peptidase-4 (DPP-4) inhibitors and amylin analogues. Incretins are insulin secretagogues that include glucagon inhibitory peptide (GIP) (702) and the previously mentioned GLP-1. GIP secretion is stimulated by the hyperosmolarity of glucose in the duodenum. GLP-1
promotes insulin secretion and suppression of glucagon levels after meal ingestion. It has beneficial glucose lowering effects in T2DM as it reduces gastric emptying and decreases calorie intake. However, both GIP and GLP-1 have a relatively short half-life and are rapidly inactivated by the enzyme dipeptidyl peptidase-4 (DPP-4), hence development of their agonists (701).

At present only GLP-1 agonists and not GIP agonists have been approved. These include exendin-4/exenatide (GLP-1 homolog) (701), liraglutide and taspoglutide. Exendin-4, a 39 amino acid peptide originally from Gila monster saliva that promotes release and prolongs half-life of GLP-1. It has a relatively longer half-life than GLP-1 and may be used to treat T2DM and diabetes as it delays gastric emptying and reduces calorie intake (184). Exendin-4 is not an analogue of GLP-1 rather an agonist that has increased to resistance to degradation by DPP-4, hence prolonged half-life (222).

Amylin is a pancreatic hormone co-secreted with insulin (from pancreatic β-cells) that reduces post-prandial hyperglycemia by delaying gastric emptying. Its half-life, is however too short for it to be sustained as a routine clinical agent (496). Pramlintide is the only human amylin analogue available clinically and although its administration can reduce post-prandial glucose excursions in diabetics, pramlintide use may be limited by the relatively low efficacy (698, 699) and its adverse effect of nausea. The next section reviews the outcome of agents used for glycemic control in clinical trials and epidemiological studies carried out in diabetic patients, with particular emphasis on cardiovascular diseases. The overall aim of the following section is to highlight the importance of evaluating novel agents that aim to preserve cardiac function under conditions of acute and chronic hyperglycemia.

### 2.8.6 Glycemic control and outcome

Despite the availability of drugs for diabetes and various treatment modalities to improve cardiac function in coronary artery disease, there is still an increased occurrence of complications secondary
to both pathophysiological conditions, i.e. diabetes and myocardial infarction. It is therefore imperative to investigate alternative treatment modalities that may remedy the status quo. Prevention rather than treatment of heart disease can significantly improve patients’ quality of life and reduce health care costs. This is even more important considering that the detrimental effects of hyperglycemia not only manifest within the context of diabetes, but also in non-diabetics following AMI and in cases of stress-related hyperglycemia (118). The emphasis of this thesis is therefore on evaluating novel cost-effective therapies to alleviate the damaging effects of acute and chronic hyperglycemia during AMI.

The UKPDS employed various regimens were used in approximately 5,000 newly-diagnosed diabetic patients that were followed up for ten years, and reported that intensive treatment reduced the risk for AMI by 16% compared with conventional treatment. Moreover, hyperglycemia emerged as an independent risk factor for CVD but with no apparent threshold, i.e. the lower the HbA1c the reduction in the risk. Also for every 1% reduction in HbA1c (for levels <6% and >10%) there were corresponding reductions in risk for AMI, stroke, HF and amputation or death from peripheral vascular disease (722). The DIGAMI Study was a prospective study of 620 diabetic patients with AMI who were randomly treated with either an intensive insulin regimen (to achieve near normoglycemia), or conventional treatment, and followed up for 3.4 years. Here, mortality was reduced by 27% in the intensively treated group despite high admission blood glucose and HbA1c (460). However, experiences from the DIGAMI 2 and CODE studies (82, 428) highlighted that achievement of the targeted fasting glucose level of 5-7 mmol/L with blood glucose lowering treatments is difficult to accomplish (461). The DIGAMI 2 study did, however, establish the role of blood glucose level as a predictor of long-term mortality despite absence of the beneficial effects from acute and long term insulin versus conventional treatment. Hence there is a need for other management routines and improved pharmacological agents for this large group of patients. In this regard there is need for the evaluation of novel therapies to improve outcome (e.g. benfotiamine and oleanolic acid for this study).
In summary, the highlighted clinical trials and epidemiological studies support the notion that intensive glycemic control correlates to a decrease in the incidence of cardiovascular complications. However, it is imperative to note that despite all these measures the incidence of diabetic complications is still on the rise, of especially diabetic cardiomyopathy and HF secondary to AMI. Moreover, the link between intensive glucose control and benefits on CVD prevention has been questioned by several investigators. Here intensive glycemic control can result in increased mortality, likely due to the detrimental effects of hypoglycemia (6, 382, 682) e.g. renal failure (63) and possibly increased CVDs (186).

On the contrary, CVD complications are still prominent despite glycemic control, particularly macro-vascular complications (trials only showed a decrease in micro-vascular complications) (505). The concept of the metabolic memory may help explain the latter phenomenon, i.e. it is linked to the persistent manifestation of complications despite treatment (51, 234, 244, 325, 383, 385, 386, 781). Here pioneering work by Engerman and Kern (1987) found that the increased incidence of retinopathy in dogs was dependent on duration of exposure before glycemic control was implemented (188). In essence the concept is that the longer cells are exposed to high glucose, the more it diminishes subsequent metabolic responses when returning to normal glucose levels at a later stage. This effect is attributed to mitochondrial and non-mitochondrial ROS (mainly superoxide and peroxynitrite) and also increased activation of the non-oxidative glucose pathways such as the AGE and PKC pathways (505). With this gap in the current management of diabetes and associated CVDs, it is important to consider the development of drugs that address the fundamental pathophysiologic abnormalities that link diabetes/hyperglycemia and CVD while ensuring that the risk for CVD is not inadvertently increased (162).

It is also important to understand the molecular mechanisms of events that occur during AMI under hyperglycemic conditions and how this can result in myocardial damage. This pathological aspect is
reviewed in the next section since it may be a putative target for various therapeutic agents to maximize cardio-protection in response to acute and chronic hyperglycemia.

2.9 Metabolic changes during ischemia and reperfusion

As discussed previously, the heart is a highly aerobic tissue that obtains most of its energy from the oxidative phosphorylation of metabolic fuel substrates. Mild or severe impairment of coronary blood supply in large arteries to the myocardium causes myocardial ischemia usually as a result of thrombosis or atherosclerotic plaques. This deprives the myocardium of oxygen, fuel substrates and leads to accumulation of metabolites (545) consequently causing cell death within the infarcted area (491, 651). Myocardial ischemia results in many alterations in cellular function, the reduction in oxygen supply, and its impact on myocardial energetics (150). Studies over the years showed that myocardial cells can compensate for reduced ATP production by lowered metabolic flux and hence able to survive rather relatively long periods, i.e. 60 minutes or more of akinetic oxygen deprivation (provided coronary flow is adequate to remove metabolic products). The ATP balance of cardiac mitochondria under normal conditions and during ischemia and reperfusion is shown in Figure 2.9 below.
2.9.1 Effects of ischemia on substrate utilization

Myocardial substrate utilization is highly dependent on the severity of the ischemia (i.e. mild to moderate and severe ischemia) (665). The changes that are discussed in this section apply to ischemia with/without underlying hyperglycemia i.e. during chronic hyperglycemia as in diabetes or in non-diabetic individuals. As discussed earlier in this chapter, in non-diabetics with AMI, there is an elevation of stress hormone levels, and impaired insulin signaling (104, 118, 395) hence AMI is characterized by hyperglycemia and the metabolic dysfunction that occurs during ischemia is similar in
non-diabetic and diabetic individuals. The main difference is that AMI during chronic hyperglycemia may result in heightened ischemic metabolic changes due to the presence of altered metabolic dysfunction (356). This implies that an excess of FAs (and intermediates e.g. fatty acyl-CoAs, DAG and ceramides) and attenuated glucose oxidation seen with diabetes or metabolic syndrome (422, 429, 441, 442, 610, 665) is further exacerbated during AMI since it manifests in the same way (422, 429, 442, 610, 664). Here increased FA supply occur as a result of ischemia-induced myocardial lipolysis (degradation of endogenous TAGs) as reflected by release of glycerol from ischemic zones thus further increasing the amount of circulating FAs (64, 92, 340, 731). With increased FA levels in diabetes, PPAR-α activation is enhanced further promoting FA uptake, metabolism and FAO (397, 398) and consequently impairing cardiac function with ischemia and reperfusion. For example, overexpressing PPAR-α mice showed decreased functional recovery following global ischemia in comparison to the wild type mice (614). Furthermore, reduced cardiac efficiency occurred due increased CPT-I activity (58, 434) and fatty acyl-CoA-induced inhibition of adenine nucleotide translocase on the inner mitochondrial membrane (exchanges ATP for ADP) thereby uncoupling contractile function in the post-ischemic heart (64).

During ischemia glucose oxidation is also decreased due to lowered GAPDH activity as a result of increased accumulation of NADH, lactate and hydrogen ions (491). Interestingly however, recent studies have reported a more detrimental outcome in non-diabetics compared to diabetics following AMI (104, 118, 119, 460) indicating that there is a need for therapeutic interventions to minimize damage due to acute hyperglycemia. The variations for these observations are not clearly elucidated, however, various studies have reported on the variation of exposure duration to hyperglycemic conditions i.e. more chronic hyperglycemia reducing sensitivity of diabetic myocardium to AMI (456) possibly attenuating ischemia and reperfusion injury (585, 586).
During ischemia there is an elevation of glucose uptake by the myocardial cell membrane (680) that occurs from increased GLUT1/4 translocation and activity (81, 125, 185, 607, 756, 788, 789) due to increased AMPK activation (485). The increase in glucose transport with decreased oxygen availability may be of physiological importance, i.e. allowing the heart to obtain more energy from glycolysis under conditions where FAO is predominant (665). This occurs despite decreased glucose delivery and lower interstitial glucose concentrations because glucose transport is determined by the number of glucose transporters present in the cell membrane and the trans-membrane glucose concentration gradient (448, 500, 789). One of the main mechanisms through which this occurs is through AMPK activation (789). The increased ratio of AMP to ATP activates AMPK kinase (AMPKK) leading to production of AMPK. AMPKK is also activated by a decrease in the creatine phosphate to creatine ratio that occurs during ischemia (404, 561). Additionally AMPK promotes glycolysis by increasing activity of PFK-1 and PFK-2 (317). It is, however, important to note that glycolysis varies with the degree of ischemia. For example, during severe ischemia, glycolysis is inhibited by low delivery rates of glucose, glycogen depletion and intracellular acidosis, which all inhibit PFK-1. Moreover, PFK-1 is inactivated by translocating from cytosol to the cell membrane during severe ischemia (296).

The effects of ischemia on PDH, however, have not been clearly elucidated with studies reporting contradictory effects (286, 377, 662, 726) possibly due to differences in ischemia-protocols and experimental models used i.e. in vivo and ex vivo. The TCA cycle is attenuated and there is less energy production from oxidative phosphorylation (150) thereby decreasing ATP levels and impairing myocardial function in this pathophysiological state (see Figure 2.9) (18). Changes are also found in the process of oxidative phosphorylation and the creatine kinase shuttle. In this instance mitochondrial number may be increased but are structurally abnormal (323); the activity of ETC complexes are reduced (467); and the number of uncoupling proteins are increased (504). Moreover, the low enzyme activity in the creatine kinase shuttle result in a decline in ATP delivery (up to 71% ) to the myofibrils (58, 434).
In summary, FAO increases during reperfusion in association with impaired pyruvate oxidation and accelerated non-oxidative glycolysis (626). Increased FAO inhibits glucose oxidation, resulting in an imbalance between glycolysis and glucose oxidation (610). Prolonged ischemia can also suppresses DNA and protein synthesis (108), although there may be induction of some specific ones e.g. heat shock protein 70 (HSP 70), PKC-ε and inducible nitric oxide synthase (iNOS) (149, 554, 555). In the next section the ionic imbalances that occur during ischemia and reperfusion are discussed.

2.9.2 Ionic imbalances during ischemia and reperfusion

The ionic content of the cytosol changes markedly during ischemia as a result of decreased ATP levels. The Na⁺/K⁺-ATPase and the SERCA become ineffective, thereby causing increases in the cytoplasmic sodium and calcium ions (see Figure 2.10) (556–558). Prolonged inhibition of mitochondrial oxidative phosphorylation during ischemia dampens the proton gradient leading to decreased activity of the mitochondrial calcium uniport and reversed operation of the ATPase possibly contributing to the ATP loss observed during ischemia (130, 150, 151) (see Figures 2.9 and 2.10). The continued production of hydrogen ions during reperfusion has the potential to exacerbate injury to the myocardium (434, 443).
Figure 2.10  Ionic imbalances during myocardial ischemia and reperfusion. ATP dissipation during ischemia leads to an increase in resting cytosolic free calcium (Ca$^{2+}$). Reperfusion leads to excessive mitochondrial Ca$^{2+}$ uptake. Mitochondrial Ca$^{2+}$ overload together with oxidative stress and the prevailing low ATP provoke mPTP opening. These events initiate a vicious cycle, i.e. inner-membrane depolarization, ATP hydrolysis by the mitochondrial ATP synthase, further increases in cytosolic Ca$^{2+}$, finally leading to cell death. Ca$^{2+}$ (calcium ions); ATP (adenosine triphosphate); ADP (adenosine diphosphate); Na$^{+}$/H$^{+}$ (sodium hydrogen exchanger); Na$^{+}$/HCO$_3^-$ (sodium hydrogen carbonate exchanger); Na$^{+}$/Ca$^{2+}$ (sodium calcium exchanger); F$_0$-F$_1$ ATPase (ATP synthase); mPTP (mitochondrial permeability transition pore).

The hydrogen ions in the severely ischemic myocardium result from the hydrolysis of glycolytically-derived ATP and is the major contributor of acidosis in the myocardium (158, 619). Ion pumps on the cell membrane respond to remove excess hydrogen in exchange for sodium. In response to elevated levels of intracellular sodium, the Na$^{+}$/Ca$^{2+}$-exchanger (operating in reverse mode) is less capable of removing intracellular calcium culminating in increased cytoplasmic calcium levels (503). The mitochondrial calcium uniporter then transports calcium into mitochondria, that induces calcium-dependent dehydrogenase activation, declines in NADH and electron flux through the ETC, increased ROS, and decreased ATP levels (see Figure 2.10). Calcium uptake into mitochondria dissipates the
mitochondrial membrane potential but its increase in the mitochondrial matrix reaches a plateau under hypoxic conditions (due to limitation of proton gradient). Upon reperfusion, however, restoration of oxygen and ATP-generating capabilities rapidly restores ATP levels and mitochondrial membrane potential, together with marked ROS production (which damage cellular membranes and further induce oxidative stress). These changes regenerate the required ion gradient for more calcium entry into mitochondria, which causes long-lasting opening of mPTP (145, 757).

The mPTP is a non-specific multimeric protein channel located on the inner mitochondrial membrane, initially proposed to consist of a voltage-dependent anion channel, the adenine nucleotide translocase and a cyclophilin D. However, its structure has not yet been completely defined. Under normal physiological conditions (mPTP in closed conformation) the inner mitochondrial membrane is impermeable to almost all metabolites and ions. As the mPTP opens during reperfusion (257, 432), the mitochondrial membrane potential is abolished allowing transition of molecules smaller than ~1500 daltons. These molecules generate an osmotic force causing mitochondrial swelling and leading to rupture of the outer membrane and release of proapoptotic factors such as cytochrome c (see section 2.10.2 on apoptosis) (273). Experimental and clinical investigations show that although reperfusion does indeed salvage the ischemic myocardium, it can paradoxically also induce some detrimental effects, i.e. a phenomenon referred to as “ischemia/lethal reperfusion injury” (213, 293, 545, 780, 803). Here, myocardial reperfusion also results in cardiac myocyte death (of cells that were viable immediately before reperfusion) (557, 558). The exact and detailed mechanisms of ischemia/reperfusion injury are described in the section below.

2.9.3 Myocardial ischemia/reperfusion injury

Myocardial reperfusion injury was first described by Jennings et al. (1960) after observing histological features of the reperfused canine myocardium (338). In essence myocardial reperfusion injury is a
complex pathophysiological event, resulting in serious acute and chronic myocardial damage. It is characterized by a cascade of acutely initiated local inflammatory responses, metabolic disorder, and cell death that leads to myocardial ultra-structural changes/remodeling and subsequent myocardial systolic/diastolic dysfunction. The major mediators of lethal reperfusion injury are oxygen radicals, calcium loading and neutrophils (213, 545, 780, 802). The injury to the heart during reperfusion causes four main types of cardiac dysfunction, namely: myocardial stunning, no-reflow phenomenon, reperfusion arrhythmias and lethal reperfusion injury (780).

During the earliest phase of reperfusion (minutes) there is development of cardiomyocyte contracture which seems to be the main cause for cardiomyocyte necrotic injury (557). Contracture is a sustained shortening and stiffening of myocardium that occurs as a rigor-type mechanism within the ischemic myocardium. It mainly results from increased intracellular calcium ions and reduced ATP levels that occur during ischemia and reperfusion (see Figure 2.10). Studies on skinned cardiac cells found that a force-generating cross bridge is initiated when cytosolic ATP is reduced to low levels (17, 523). Although ATP levels are quickly exhausted with ischemia, onset of contracture does not cause major structural damage, but instead render the cardiomyocytes more fragile and susceptible to mechanical damage (621). When energy depletion is rapidly relieved then the ischemic rigor contracture is usually reversible (556–558). However, if ischemia is prolonged there may be irreversible structural damage to cardiomyocytes leading to increased end diastolic pressure and ventricular compliance (556–558). Reperfusion-induced contractures are caused by: 1) calcium overload during ischemia followed by rapid re-energization and 2) rigor contracture. With calcium-induced contracture, high cytosolic calcium availability leads to uncontrolled activation of the contractile machinery with damaging effects. Of interest in the context of this thesis is that increased flux though the non-oxidative glucose pathways may elevate the degree of ischemia and reperfusion injury (326, 353, 462, 691, 692). It is now appreciated that lethal myocardial injury caused by ischemia/reperfusion accounts for ~ 50% of the final size of a myocardial infarct (784). In essence altered cardiac energy metabolism leads to
activation of a cascade of events that result in oxidative stress and the next section therefore, focuses on the role of ROS in myocardial ischemia-reperfusion.

2.9.4 ROS in reperfusion injury

Most of the changes that occur to the redox balance during ischemia and reperfusion are similar to what was earlier discussed (refer section on hyperglycemia-induced oxidative stress) particularly regarding the role of mitochondria. Here superoxide anion is the principal ROS produced by several sources e.g. polymorphonuclear leukocytes (PMNs), Nox (255), incomplete oxidative phosphorylation in mitochondria by complex I (409) and III (298, 552), xanthine oxidase (59) and eNOS (562). Nox is a distinct ROS source since its role is to solely produce superoxide, whereas other enzymes produce ROS as by-products of specific catalytic pathways (341). Endothelial dysfunction occurs due to attenuation of NO bioavailability through formation of peroxynitrite after combining with superoxide (199, 558, 741). Such endothelial dysfunction promotes the upregulation of endothelial adhesion molecules (e.g. ICAM-1) thus facilitating adherence of PMNs and infiltration. The ROS and proteolytic enzymes produced by activated leukocytes cause damage to myocytes and vascular cells through lipid peroxidation thereby altering their permeability to ions such as calcium (447, 605). Thus the transmigrated PMNs are mainly responsible for compromising cardiac contractile function during reperfusion (418, 714). The early phase of reperfusion represents an important target for strategies protecting ischemic myocardium (558) as described below.

2.9.4.1 Interventions to ameliorate myocardial ischemia and reperfusion injury

Early and successful reperfusion with the use of thrombolytic therapy or primary percutaneous coronary intervention is the most effective treatment strategy to salvage the ischemic myocardium from inevitable death, thereby reducing infarct size and improving clinical outcome. Additionally, there
are various approaches used to protect the ischemic tissue and surrounding muscle at risk of the detrimental effects of ischemia. Firstly, promotion of glycolysis by use of glucose/insulin/potassium (GIK) stimulates glucose uptake thereby increasing glycolysis and suppressing blood FA supply. Findings of GIK trials did, however, result in contradictory findings and this may be due to differences in glucose concentrations in patients receiving treatment and the timing of GIK administration (110, 460). Another approach is to better link glycolysis to glucose oxidation, possibly by increasing activity of PDH to enhance glucose entry into the TCA cycle using pharmacologic agents (e.g. dichloroacetate). The rationale in this instance is to blunt the effects of the Randle cycle, i.e. high FAO blunting glucose oxidation during ischemia and reperfusion (discussed earlier; refer section 2.3). Finally a third approach is to directly limit FAO during ischemia and reperfusion to attenuate fat-mediated effects on glucose oxidation e.g. employing drugs such as trimetazidine (443).

Targeting oxidative stress during ischemia and reperfusion is an attractive therapeutic option but the simplistic approach of administering antioxidants to reduce ROS-induced injury has not always yielded beneficial results (735). The protective potential of an antioxidant depends on the scavenging of specific ROS species and its access to strategic intracellular sites (49, 790). Alternatively, “programming” the heart to either generate less ROS or to increase strategic ROS removal by endogenous mechanisms may yield success in attenuating reperfusion injury. Under these pathophysiological conditions (hyperglycemia and ischemia) the heart undergoes various stress responses discussed below.

2.10 The cardiomyocyte stress response

Adult heart cells endure a wide range of stress during their life span and in the process acquire numerous adaptations. The heart is an organ with limited capacity for regeneration and repair; hence it is susceptible to numerous stresses and must respond to these insults in order to adapt to ever-
changing workload demands. We have discussed the damaging effects of hyperglycemia and ischemia and reperfusion on the heart as examples of stress confronting cardiomyocytes. The heart’s adaptations occur in the context of continuous mechanical contraction and relaxation and include changes in signaling events (receptor and adaptor proteins), transcriptional events and the replacement of contractile proteins in response to wear and tear (646).

Moreover, cardiomyocytes respond to stress by the accumulation of protective proteins that may counteract damage, thereby temporarily increasing tolerance to such damage. Alternatively, it may trigger programmed cell death (apoptosis) to remove terminally damaged cells (24) which can be eliminated by the UPS and lysosomal-autophagal system. Cell death (progressive or acute) is a hallmark characteristic of various cardiac diseases that include HF, diabetic cardiomyopathy, AMI, and ischemia and reperfusion. All three types of cell death, i.e. autophagy, apoptosis, and necrosis are present during the progression of heart diseases (757). However, since this dissertation mainly focuses on apoptosis, it will be discussed in detail, whereas the other modes of cell death are only briefly discussed.

2.10.1 Necrosis

Necrosis is marked by distinct morphological changes; including cell and organelle swelling, cell membrane damage, and ATP loss. Disruption of cell integrity and release of cellular contents trigger a secondary inflammatory response, with potential pathological consequences (757). Necrosis is mainly caused by physical or chemical trauma to the cell and has long been considered as passive and accidental cell death (725). Recently, however, emerging evidence suggests that part of the necrotic process is regulated by serial signaling events in a controlled and orchestrated manner (757). Several terms have been introduced to describe this form of necrosis, e.g. programmed necrosis, caspase-independent cell death, and necroptosis (157, 299).
A number of proposed mechanisms may explain the initiation and execution of necrosis, including death receptors, ROS, calcium, and mPTP opening (394, 725). Necrosis can occur in at least four ways: (1) direct damage to the cell membrane induced by certain toxic chemicals e.g. products of activated leukocytes and osmotic fluctuations, such as the calcium paradox or disruption of the membrane cytoskeleton; (2) damage to the respiratory apparatus of mitochondria, with inhibition of oxidative phosphorylation leading to decreased ATP production and an increase in hydrogen ions (decrease in pH, intracellular acidosis); (3) proteolysis of membrane-associated cytoskeletal proteins, including dystrophin, dystrophin-associated protein complex and (4) unregulated membrane phospholipid degradation due to activation of phospholipases and membrane lipid peroxidation due to the generation of ROS (757).

Thus necrosis occurs under normo-/hyperglycemic conditions (216) and also ± ischemia and reperfusion (757). Based on these observations, three stages of membrane injury emerge during the progression from reversible to irreversible necrotic injury: (1) discrete alterations in ionic transport systems (reversible); (2) increase in permeability of the phospholipid bilayer (potentially reversible); and (3) physical disruption of the cell membrane (irreversible). These stages of necrotic membrane injury are accompanied by progressive morphologic changes including organelle and cell, membrane blebbing/rupture and cell rupture (73). Leakage of intracellular constituents from cells undergoing necrosis provokes inflammation (299, 420, 757).

However, with myocardial infarction there remains controversy regarding the predominant mode of cell death i.e. amongst necrosis, apoptosis, and autophagy (35, 249, 348, 475, 525). The discrepancy may be due to variations in timing, incidence, and prevalence of the different cell death markers. Moreover, the various cell death markers have different sensitivities and windows of detection during ischemia and reperfusion. Some suggested, however, that apoptosis becomes maximal within the first
four hours of permanent coronary occlusion, whereas necrosis peaks at 24 hours (348). To gain a better understanding of the differences between these two processes, apoptosis is discussed below.

2.10.2 Apoptosis

Apoptosis is an active evolutionarily conserved form of cell self-destruction (programmed cell death Type I) that is precisely regulated by a genetic program resulting in cell death (365, 393, 438). Apoptotic cells are characterized by specific morphological changes, including chromatin condensation and fragmentation and cell membrane blebbing (73, 393). During the late stages of apoptosis the cell becomes fragmented into vesicles called apoptotic bodies, which contain cytosolic, nuclear and organelle material (393). Apoptotic bodies are subsequently recognized by macrophages and cleared from the tissue to avoid inflammatory responses. Furthermore, cells undergoing apoptosis lose the normal phospholipid asymmetry of the cell membrane and accumulate large amounts of phosphatidylserine in the outer cell membrane leaflet. However, the semi-permeable property of the membrane is maintained, preventing leakage of intracellular constituents (unlike necrosis). The exposed phosphatidylserine moieties can be recognized by receptors on adjacent cells leading to rapid phagocytosis of apoptotic fragments, thereby avoiding a stimulus for exudative inflammation. All these features are found in atherosclerotic tissue which can be an underlying cause of AMI (67, 228, 281, 378). Oxidative stress (mitochondrial and non-mitochondrial sources) is the main cause of apoptosis in cardiac tissues under hyperglycemic conditions. The latter mainly include increased activation of non-oxidative glucose pathways which mainly converge on the activation of PKC and TNF-α (169, 170, 192, 207, 425, 683) as earlier discussed.

Apoptosis induction involves activation of specific enzymes, the caspases (cytosolic aspartate residue-specific cysteine proteases) that are a family of proteases thought to be the most important effector molecules of this process (69, 727). A catalytic cascade, much resembling the complement or clotting
cascade, is suggested for caspase activation and it can be initiated by several factors. Data indicate that the route for caspase activation differs depending on the pro-apoptotic stimuli and that not all caspases are active in all mechanisms (69, 727). In addition to caspases, the B cell leukemia/lymphoma-2 (Bcl-2) family member proteins also take part in the process. These are divided into three subfamilies according to their function and degree of homology shared within four Bcl-2 homology domains (BH1-4). In general, the anti-apoptotic Bcl-2 family members contain BH domains 1–4 (e.g. Bcl-2 and B cell leukemia/lymphoma-x-isoform [Bcl-xL]) (757) and need to be neutralized or down-regulated by other Bcl-2 family members for apoptosis to progress. The pro-apoptotic members can be subdivided into two groups, i.e. the first group is often referred to as the multidomain pro-apoptotic members that contain BH domains 1–3 and includes Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist /killer (Bak) and Bcl-2 related ovarian killer (Bok). The second group is made up of the Bcl-2 homology domain 3 (BH3)-only proteins (BH3-only interacting protein [Bid], Bcl-2 like protein 11 [Bim], Bcl-2 associated death promoter [Bad]) (591). Apoptosis in cardiomyocytes is mediated by the activation of the extrinsic or intrinsic pathways (see below).

2.10.2.1 Extrinsic /external/death receptor pathway

The external pathway is activated by binding of death ligands to cell surface receptors (endonucleases). The best known receptors are those for TNF-α and Fas (also called APO-1) ligand receptor (550). The activated death receptor subsequently conveys signals to the Fas-associated death domain protein (FADD). Both Fas and TNF-α receptors are expressed in cardiac myocytes and implicated in cardiovascular pathology. Since TNF-α signaling is more complex as it can promote survival or death; events following binding of Fas ligand are described. The resultant macromolecular complex constitutes a death-inducing signaling complex (DISC) that activates initiator caspases, including the conversion of procaspase-8 or -10 to active caspase-8 or -10 which in turn activates downstream procaspases (42, 69). Furthermore, the active caspase-8 can also activate the intrinsic
pathway by the proteolysis of BID to truncated BID (t-BID) leading to translocation of its carboxyl portion to the mitochondrion triggering apoptotic events (423).

2.10.2.2 The intrinsic/mitochondrial pathway

The intrinsic pathway is activated in response to a wide variety of extra- and intracellular stimuli, including loss of survival or trophic factors, toxins, radiation, hypoxia, oxidative stress, ischemia and reperfusion, and DNA damage. These stimuli converge at the mitochondrion to trigger a conformational change, characterized by mPTP opening and release of apoptogenic proteins (629). In addition, it causes the ER to release calcium and activate procaspase-12 to caspase-12 (522, 630). Death signals to mitochondria and ER are transduced by two classes of proapoptotic proteins, i.e. Bax and BH3-only proteins (160, 206, 365). Here Bax is proposed to undergo a conformational change and then translocates to mitochondria and the ER. However, the precise mechanisms mediating its activation are not completely understood (160, 206, 365). In contrast to the general involvement of Bax, BH3-only proteins transduce death signals in a stimulus-specific manner (591, 787). Moreover, death signals regulate abundance, activity and localization of these proteins through various transcriptional mechanisms, post-translational modifications (e.g. phosphorylation and cleavage). Deficiency of certain survival factors results in dephosphorylation of Bad, releasing it from 14-3-3 protein and translocation to the mitochondrion (796).

The key event leading to the release of apoptogens and execution of cell death is permeabilization of the outer mitochondrial membrane (OMM) and the subsequent release of apoptogenic factors from the mitochondrial inter-membrane space (IMS) (i.e. cytochrome c, second mitochondria-derived activator of caspase/direct inhibitor of apoptosis [IAP]-binding protein with low PI [Smac/DIABLO], Endonuclease G, and apoptosis inducing factor (AIF) (629). OMM permeabilization is tightly regulated by the Bcl-2 family of proteins, Bax and Bak (751). The specific mechanism by which these respective
Bcl-2 proteins induce OMM permeabilization remains controversial; however, it is known that they both undergo a complex pattern of BH3-induced homo- and hetero-oligomerization at the mitochondria (522, 630). Apoptogen release is opposed by antiapoptotic Bcl-2 proteins, i.e. Bcl-2 and Bcl-xL (368, 376).

Once released from mitochondria, cytochrome c binds the adaptor protein apoptotic protease activating factor-1 (Apaf-1) in an ATP-dependent oligomerization causing recruitment of procaspase-9 and ultimately formation of the ‘apoptosome’ (5, 426). This facilitates the clustering and activation of caspase-9 which, in turn, cleaves and activates caspase-3 and caspase-7 leading to many of the morphological changes associated with apoptosis (757). Activated caspase-3 mediates cleavage of a number of protein substrates and activation of calcium-dependent endonucleases, leading to the characteristic double-stranded inter-nucleosomal DNA fragmentation (180–200 base pairs) (757).

Some or all of these discussed mechanisms are involved in high-glucose and/or ischemia and reperfusion-induced apoptosis (depending on the cell type or tissues studied). Of note, cardiac myocytes are naturally resistant to apoptosis due to their low-level expression of Apaf-1, caspases, and high expression of X-linked inhibitor of apoptosis protein (XIAP). However, apoptosis could be the major form of cell death during myocardial infarction, preceding necrosis (24, 206) and could also be the major determinant of infarct size (348). With hyperglycemia oxidative stress is proposed as the main cause of apoptosis, especially linked with mitochondrial dysfunction (61, 96, 307, 727, 749). Hyperglycemia may also cause apoptosis through the activation of non-oxidative glucose pathways. Our laboratory demonstrated that increased HBP activation causes apoptosis by increased BAD O-GlcNAcylation thereby reducing its phosphorylation (510, 576, 577). Similarly, p53 activity increased following its O-GlcNAcylation under hyperglycemic conditions thereby causing angiotensin-induced apoptosis (203).
High glucose can also initiate oxidative stress-induced apoptosis via Bax-mediated mitochondrial permeability and cytochrome c release (mesangial cells) that could be prevented by insulin-like growth factor-I (IGF-I), which results in phosphorylation of Bad (354). Furthermore, p38 mitogen-activated protein kinases (MAPK) can also play a role in high glucose-induced apoptosis (510). Here high glucose caused sustained phosphorylation of p38 MAPK, caspase-3 activation and Bax-mediated apoptosis in endothelial cells.

2.9.3 Autophagy

One of the key cellular pathways that mediate stress-induced adaptation and damage control is autophagy (also called macro-autophagy). Autophagy is a highly conserved process of delivery of intracellular components, including mitochondria and long-lived macromolecules, via a double membrane structure (autophagosome) to lysosomes for degradation (525). Autophagy plays a crucial role in the turnover of organelles in cardiac cells at baseline conditions (250, 266, 267, 421, 525). Furthermore, it is increased under stressful conditions (e.g. with starvation/nutrient deprivation, hypoxia, ROS, and damaged organelles) in a mTOR-dependent process.

The functional role of autophagy with ischemia and reperfusion is complex and it is unclear whether increased or decreased autophagy results in cardio-protective or detrimental effects on the heart. Most studies show increased autophagy with ischemia and reperfusion in vitro and in vivo studies (152, 276, 475, 777). Activation of autophagy with ischemia and reperfusion showed cardio-protection in association with activation of Bcl-2 adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) (277) or AMPK (95, 686). This outcome, however, depends on the duration of ischemia, i.e. if prolonged the autophagic response becomes dysfunctional, as evidenced by the existence of impaired autolysosomes (421, 482). Interestingly, metformin can also activate mTOR thereby blunting cardiac remodeling and improving cardiac function after an AMI (95). Autophagy is further up-regulated during
reperfusion, even though the delivery of oxygen and nutrients is restored, and AMPK rapidly inactivated (276, 475). The continued activation of autophagy is qualitatively different during reperfusion versus ischemia with the involvement of oxidative stress, mitochondrial damage, ER stress, and calcium overload having more important roles in maintaining autophagy at a higher level. By contrast, a decline in autophagy with ischemia and reperfusion as evidenced by reduced levels of lysosomal-associated membrane protein 2 (LAMP2) (a protein critical for autophagosome–lysosome fusion) thus impairing autophagosome processing, culminates in increased ROS generation, mitochondrial permeabilization, and cardiomyocyte death (352). Although autophagy is strongly involved in lysosomal protein degradation, our interest here is the non-lysosomal UPS and its role in protein degradation in the heart under hyperglycemic conditions with ischemia and reperfusion.

2.10 The UPS

Cellular homeostasis and function require properly folded proteins. Proteins can be misfolded or damaged by factors such as thermal, osmotic and oxidative stress leading to exposure of hydrophobic amino acid residues normally located inside the protein. This leads to aggregation or impairment of protein and cellular function and potentially cell death (567, 715). Eukaryotic cells have developed the UPS in order to maintain protein quality by removal of damaged, oxidized and/or misfolded proteins (238, 304, 642). This is particularly important for non-dividing cells such as neurons and cardiomyocytes, where cells are not diluted during cell proliferation. Indeed, proteasomal dysfunction and cell death are found in many neurodegenerative diseases and the initiation/progression of cardiac diseases (568, 715, 716, 791, 792, 809). Also, recent studies indicate that alterations in the UPS contribute to pathogenesis and progression of a variety of cardiac diseases (479, 568, 715, 716, 791, 792, 809).
The UPS plays a central role in the non-lysosomal degradation of most intracellular proteins (80-90%) as well as regulation of cellular processes such as the cell cycle, transcriptional control, immune response, cell signaling, apoptosis antigen presentation, cellular mass regulation and sarcomere quality control (519, 760). The UPS regulates these processes by rapid ATP-dependent proteolysis of poly-ubiquinated (through lysine 48) targeted intracellular proteins by the 26S proteasome (479, 518, 519, 715, 792).

The cardiac proteasome (26S), a large multi-catalytic multi-subunit protease complex, constitutes the central proteolytic machinery of the UPS. It is composed of a barrel-shaped 20S proteolytic core and one or two 19S regulatory caps. The 19S complex recognizes, unfolds and removes the ubiquitin from the so-marked proteins and facilitates entry into the 20S for degradation. It binds ubiquitinated proteins via the ubiquitin-interacting motifs of the S5a subunit (Rpn10) (238, 667). The 20S catalytic core complex is composed of four axially stacked rings. Each outer ring consists of seven different non-proteolytic α subunits that allow conformational flexibility and substrate translocation into the central cavity of the 20S complex; the two inner rings are formed by seven different but related β-subunits giving the complex the general stoichiometry of α1–7β1–7α1–7β1–7 (46, 518, 519, 739). The two internal rings (β-rings) contain the catalytic units whereas the outer α-rings form a gated channel through which polypeptides enter the central chamber to be exposed to multiple proteolytic sites. Of the 14 subunits, three are catalytically active i.e. harbor proteolytic sites formed by N-terminal threonine residues that face the central cavity of the 20S complex. These are defined (based on oligopeptidyl specificity) as follows: β1/post glutamyl peptide hydrolyzing/caspase like/LLE (cleaves after acidic residues), β2/trypsin like/LSTR (cleaves after basic residues), and β5/chymotrypsin like/LLVY (cleaves after hydrophobic residues) (46, 304, 716, 739, 760, 761). The chymotrypsin subunit is of utmost importance for overall proteasome function since it is the main one involved in removal of oxidized proteins (238, 304, 642).
Protein ubiquitination, takes place in a multistep reaction and requires three classes of enzymes: ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin–protein ligases (E3). E1 activates ubiquitin by forming a high-energy thiol ester bond between an E1 active site-located cysteine residue and the C-terminal glycine residue of ubiquitin in a reaction that requires the hydrolysis of ATP. This activated ubiquitin moiety is then transferred to E2 via the formation of an
additional thiol ester bond, and finally transferred to E3 which catalyzes the covalent attachment of ubiquitin to the target protein by the formation of isopeptide bonds. Multiple cycles of ubiquitination finally result in the synthesis and attachment of poly-ubiquitin chains that serve as a recognition signal for the degradation of the target protein by the 26S proteasome (238, 304). The 20S subunits can be observed in a variety of forms as the constitutive catalytic subunits may be substituted by inducible β subunits forming immunoproteasomes with additional proteolytic activities. Interferon gamma (IFN-γ) or TNF-α can result in the up-regulation of the immunoproteasomes (β1i, β2i and β5i) and in turn alter the specificity and selectivity of the proteasome for substrates under various conditions (715, 716).

2.10.1 The role of the UPS in response to ischemia and reperfusion and under hyperglycemic conditions

There is controversy regarding the role of the UPS in CVDs. For example, some studies report increased proteasomal activity in atherosclerosis due to greater oxidative stress (302, 415, 690, 729, 730). Moreover, with ischemia and reperfusion various studies found decreased UPS activity (particularly 26S proteasome) possibly as a result of ATP depletion, direct inhibition by protein aggregates and oxidative damage to the proteasome and/or regulatory subunits (91, 563, 564). By contrast, inhibition of UPS is linked to cardio-protective effects (by attenuating NF-κβ inflammatory and apoptotic effects) with ischemia and reperfusion (91, 102, 462, 571, 666). Decreased UPS may also protect by ensuring increased levels of heat shock proteins in the heart (661).

What about the effects of the UPS during hyperglycemia? Depending on the distinct proteasome inhibitors and experimental systems employed, the role of the UPS with hyperglycemia is controversial and it is not clear whether decreased/increased activity is beneficial or not. Some found that UPS inhibition elicits beneficial effects while others observed that it caused detrimental effects on the heart (400, 464, 566–568, 715, 716, 752). 26S ATP-dependent activity decreased in response to chronic
hyperglycemia, whereas 11S activity increased together with higher oxidative stress and impaired cardiac function. This shows that increased 11S activity may be a compensatory mechanism to reduce effects of oxidative stress in response to chronic hyperglycemia. The decreased 26S activity may occur secondarily to hyperglycemia-induced alterations e.g. O-GlcNAc modification (282, 641, 677, 797) and 4-hydroxynonenal (202). Furthermore, others found that elevated levels of ubiquitinated proteins indicated a dysfunctional UPS (565, 566). However, the notion of reference to levels of ubiquitinated proteins as an indicator of UPS activity was questioned by Liu et al. (2000) who suggested that the rate-limiting step of enhanced protein degradation in diabetic rat heart/skeletal muscle may be located at ubiquitin conjugation and/or its binding to the proteasome, and not at the level of ubiquitin availability or the proteasome itself (437).

Conversely, hyperglycemia can initially increase UPS activity. However, this appears to occur in a temporal fashion since with chronic exposure it is inhibited to subsequently cause oxidative stress. The differences observed for UPS activity may be attributed to variations in experimental models used (in vivo versus in vitro), experimental protocols (i.e. time grading for acute and chronic periods), proteasomal components assessed and the degree of oxidative stress. Thus, further studies are required to determine the regulation of the myocardial UPS in response to ischemia and reperfusion under hyperglycemic conditions.

2.11 Conclusion

This chapter has elaborated on the causal link between high glucose (acute or chronic) in the development and prognosis of cardiovascular diseases (specifically AMI). There are several shortfalls in the current medications for glycemic control (e.g. onset of hypoglycemia) and also the issue of prevailing occurrences of cardiovascular complications despite glycemic control. This demonstrates the strong need for the development of novel therapies that are more accessible (particularly in
developing nations) that may help to combat the increased burden of high glucose-induced cardiovascular complications. The main aims of this thesis were to:

1. Determine the antioxidant and anti-apoptotic effects of OA in an *in vitro* model (H9c2 myoblasts) of hyperglycemia
2. Assess functional recovery of *ex-vivo* perfused rat hearts in response to ischemia-reperfusion under baseline & hyperglycemic conditions
3. Evaluate whether OA and BFT improve functional recovery of *ex-vivo* perfused hearts and *in vivo* STZ-diabetic rat hearts after ischemia and reperfusion (baseline & hyperglycemic conditions)
4. To evaluate the anti-oxidant and anti-apoptotic effects of OA and BFT in ex vivo perfused hearts under baseline & hyperglycemic conditions
5. Determine mechanisms of OA cardio-protection by evaluating its impact on the HBP pathway flux
6. Evaluate the relative contributions of non-oxidative glucose pathways (HBP, polyol, AGE and PKC) to oxidative stress under baseline & hyperglycemic conditions in *ex vivo* hearts
7. To determine the mechanisms of BFT-induced cardio-protection by evaluating its effect on flux of pathways mentioned in 6. above under baseline & hyperglycemic conditions in *ex vivo* hearts
8. Determine activity of UPS after ischemia and reperfusion under both baseline and hyperglycemic conditions in *ex vivo* hearts
9. Evaluate the effects of ischemia and reperfusion on protein carbonylation under both baseline and hyperglycemic conditions in *ex vivo* hearts
10. Investigate the effects of both OA and BFT on UPS activity after ischemia and reperfusion under baseline and hyperglycemic conditions in *ex vivo* hearts

The next chapter will therefore, discuss evaluation of OA as a potential therapy for cardiac function under hyperglycemic conditions following ischemia and reperfusion.
2.12 References


106. **Caputo S, Pitocco D, Ruotolo V, Ghirlanda G.** What is the real contribution of fasting plasma glucose and postprandial glucose in predicting HbA(1c) and overall blood glucose control? *Diab Care* 24: 2011, 2001.


117. **Ceriello A.** New insights on oxidative stress and diabetic complications may lead to a "causal" antioxidant therapy. *Diab Care* 26: 1589–1596, 2003.


227. Garvey WT, Maianu L, Huecksteadt TP, Birnbaum MJ, Molina JM, Ciaraldi TP. Pretranslational suppression of a glucose transporter protein causes insulin resistance in


256. **Griffin M, Marcucci M, Cline G.** Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade. *Diabetes* 48, 1999.


176


Chapter 3

Oleanolic acid: a novel cardio-protective agent that blunts hyperglycemia-induced contractile dysfunction

Diabetes constitutes a major health challenge. Since cardiovascular complications are common in diabetic patients this will further increase the overall burden of disease. Furthermore, stress-induced hyperglycemia in non-diabetic patients with acute myocardial infarction is associated with higher in-hospital mortality. Previous studies implicate oxidative stress, excessive flux through the hexosamine biosynthetic pathway (HBP) and a dysfunctional ubiquitin-proteasome system (UPS) as potential mediators of this process. Since oleanolic acid (OA; a clove extract) possesses antioxidant properties, we hypothesized that it attenuates acute and chronic hyperglycemia-mediated pathophysiologic molecular events (oxidative stress, apoptosis, HBP, UPS), and thereby improves contractile function in response to ischemia and reperfusion. We employed several experimental systems: 1) H9c2 cardiac myoblasts were exposed to 33 mM glucose for 48 hr vs. controls (5 mM glucose); and subsequently treated with two OA concentrations (20 and 50 µM) for 6 and 24 hr, respectively; 2) Isolated rat hearts were perfused ex vivo with Krebs-Henseleit buffer containing 33 mM glucose vs. controls (11 mM glucose) for 60 min, followed by 20 min global ischemia and 60 min reperfusion ± OA treatment; 3) In vivo coronary ligations were performed on streptozotocin treated rats ± OA administration during reperfusion; and 4) Effects of long-term OA treatment (2 weeks) on heart function was assessed in streptozotocin-treated rats. Our data demonstrate that OA treatment blunted high glucose-induced oxidative stress and apoptosis in heart cells. OA treatment also resulted in cardio-protection, i.e. for ex vivo and in vivo rat hearts exposed to ischemia and reperfusion under hyperglycemic conditions. In parallel, we found decreased oxidative stress, apoptosis, HBP flux and proteasomal activity following ischemia and reperfusion. Long-term OA treatment also improved heart function in streptozotocin-diabetic rats. These findings are promising since it may eventually result in novel therapeutic interventions to treat acute hyperglycemia (in non-diabetic patients), and diabetic patients with associated cardiovascular complications.
3.1 Introduction

The dramatic surge in diabetes during the past few decades constitutes a major threat to human health in developed and developing nations (6, 84). Since cardiovascular complications and mortalities are common in diabetic patients (5, 58), this will further increase the overall burden of disease. These alarming projections therefore necessitate a comprehensive understanding of the underlying molecular mechanisms orchestrating the onset of cardiovascular diseases (CVD) in diabetic individuals.

Diabetes is characterized by perturbed metabolic pathways usually resulting in hyperlipidemia, hyperinsulinemia and hyperglycemia. Cardiovascular complications frequently present in diabetic patients and chronic hyperglycemia is proposed to be an important risk factor for myocardial infarction (12, 77). Moreover, stress-induced, acute hyperglycemia in non-diabetic patients with acute myocardial infarction is associated with increased in-hospital deaths (56, 62). Acute and chronic hyperglycemia triggers biochemical and electrophysiological changes that may result in impaired cardiac contractile function (15). Furthermore, hyperglycemia generates reactive oxygen species (ROS) and cell death in the myocardium, thereby contributing to the onset of CVD (11, 31, 57, 65). For example, we previously found that hyperglycemia-induced ROS increased flux through the hexosamine biosynthetic pathway (HBP) leading to greater O-GlcNAcylation of target proteins and myocardial apoptosis (65, 66). Hyperglycemia-induced oxidative stress can also result in the formation of misfolded or damaged proteins that may be eliminated by the ubiquitin-proteasome system (UPS). Previous studies revealed dysfunctional UPS with hyperglycemia, linked to greater inflammation and attenuated cardiac function at baseline and in response to ischemia and reperfusion (57, 63). However, it remains unclear whether increased or decreased UPS is detrimental with hyperglycemia and/or in response to ischemia and reperfusion. Pye et al. (2003) (64) found that myocardial reperfusion injury is reduced by proteasomal inhibitors, while others determined that UPS over-activity
may enhance the risk of complication during myocardial ischemia in diabetic patients (57). Conversely, others established that proteasomal impairment may contribute to the detrimental effects of myocardial ischemia (10). Additional studies are therefore required to determine the mechanisms underlying dysfunctional UPS in the heart under these conditions.

Despite the prevalence of commercially-available drugs used to treat diabetes, the use of alternative, plant-derived medicines is gaining momentum (33). For example, earlier studies evaluated the anti-diabetic therapeutic potential of *Syzygium aromaticum* [(Linnaeus) Merrill & Perry], belonging to the family Myrtaceae (commonly referred to as cloves). Here research workers established that its active constituent is the triterpenoid, oleanolic acid (OA) that exists in a very wide range of foods, medicinal herbs and plants (38). This triterpene is hydrophobic hence has reduced aqueous solubility (18). In relation to its pharmacokinetics the mean steady-state maximum plasma concentrations (C_{max}) and time C_{max} was reached (T_{max}) was reported to be 12.1 ng/ml and 5.2 hr, respectively after oral administration of an OA capsule in humans, indicating delayed in vivo absorption. Additionally absolute oral bioavailability of OA was only 0.7 % for oral concentrations of 25 and 50 mg/kg possibly due to poor solubility and extensive metabolic clearance (42). OA has been reported to be relatively non-toxic with reports of a mean lethal dose (LC50) of 0.10 mg/ml (75).

OA medicinal use was reported since the 1960s when its anti-inflammatory properties were reported (32). It is commonly used in traditional medicine for its hepatoprotective effects (49, 51, 52, 79). In addition to the mentioned effects, many of its therapeutic effects have been confirmed by contemporary research and ,these also include anti-hyperglycemic properties (52, 55, 71); antioxidant (74, 75); anti-tumor (39, 50) and anti-fungal (52). This wide range of effects is mainly attributed to its aglycone structure capable of forming crosslinkages with several molecules leading to molecular structures with different health effects. Furthermore, OA exhibited cardio-protective properties in response to ischemia and reperfusion by up-regulation of myocardial anti-oxidant defenses (24, 75). Commercially OA is now available as capsules or tablets in lipid based formulations to improve its
poor bioavailability and in China for example, it is used clinically used in the treatment of hepatitis B (52).

In light of this, we hypothesized that OA possesses anti-oxidant and anti-apoptotic properties and is thus able to blunt acute and chronic hyperglycemia-mediated pathophysiologic sequelae within the rat heart. Additionally, we proposed that OA attenuates the myocardial UPS and HBP, and thereby improves cardiac contractile function in response to ischemia and reperfusion under hyperglycemic conditions.

3.2 Materials and Methods

3.2.1 Isolation of oleanolic acid from clove extract

We employed *Syzgium aromaticum* [(Linnaeus) Merrill & Perry] (Myrtaceae) cloves (Africa International Food and Cosmetics Technologies, Durban, South Africa) to isolate and purify OA for this study. This approach was adopted since it generates sufficient amounts of OA in a cost-effective manner compared to purchasing purified OA on a regular basis. Cloves (1 kg) were extracted at room temperature for 24 hr sequentially in 3 L of each, dichloromethane and ethyl acetate. This step was repeated 3 times to yield residues of dichloromethane-solubles and ethyl acetate-solubles (EAS), respectively. Previous studies demonstrated that OA is mostly concentrated in the latter fraction (55). Subsequently, filtration was performed with 30 cm filter paper (Whatman International Ltd, Maidstone, England) where after filtrates were concentrated *in vacuo* using a rotary evaporator (Boeco, Hamburg, Germany) at 60°C. This procedure resulted in the isolation of a crude ethyl-acetate extract.

To identify chemical constituents, crude ethyl-acetate extracts were thereafter analyzed by thin layer chromatography (TLC) on pre-coated aluminium plates using Silica Gel 60 F254 (Merck, Darmstadt,
Germany). Here, we spotted a diluted portion of the isolated, crude extract and compared this with commercially obtained OA (Sigma-Aldrich, St Louis, MO). After developing the TLC plate with ethyl acetate/hexane (7:3), it was exposed to ultraviolet light (254-366 nm), sprayed with anisaldehyde/sulphuric acid/alcohol solution and the TLC plate subsequently dried with hot air. The appearance of a blue/violet-blue colouration indicated the presence of triterpenoids (37, 75).

Since the EAS fraction of _S. aromaticum_ contained triterpenoids, it was subjected to further purification processes. We fractionated 2 g of EAS on silica gel (70-230 mesh, 3.5 x 45 cm) by open column chromatography with a ratio of 7:3 ethyl acetate and hexane, respectively. An aliquot of each collected fraction was then subjected to TLC as before, and compared to commercially obtained OA. This allowed us to pool the remainder of collected fractions according to TLC profiles (i.e. similar to OA), which was thereafter concentrated _in vacuo_ using a rotary evaporator (Boeco, Hamburg, Germany) at 55°C. Concentrates were reconstituted using minimal amounts of chloroform and crystallized OA allowed to air dry. We re-crystallized OA with ethanol and its structure was confirmed by spectroscopic analysis using 1D and 2D $^1$H and $^{13}$C nuclear magnetic resonance techniques to a purity of ~98%. For a small part of this study we also employed commercially available OA (Sigma-Aldrich, St. Louis, MO) due to logistic reasons.

### 3.2.2 Cell culture and oleanolic acid treatments

H9c2 rat cardiomyoblasts (ECACC No. 88092904) were maintained at 37°C (5% CO$_2$ and 95% humidity) in low glucose (5.5 mM) Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) as described before by us (65). On the first day, H9c2 cells were split, sub-cultured and allowed to plate for 24 hr. Cells were thereafter cultured in DMEM containing: 5.5 mM glucose (control group), or 33 mM glucose (high glucose). With the high glucose exposure we attempted to simulate chronic hyperglycemia in our cell-
based studies. H9c2 cells were cultured for an additional 48 hr under these conditions followed by treatment with various concentrations of OA i.e. 0, 20, 50 µM OA for 6 and 24 hr, respectively. The concentrations were selected based on literature (29, 60). Since these studies were in vivo the concentration was initially titrated lower.

3.2.3 Measurement of intracellular ROS levels and apoptosis

Intracellular ROS levels were determined by immunofluorescence microscopy as previously described (65). Briefly, cells were grown in special chamber slides and treated with OA as described above. Subsequently, live cells were incubated with 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) stain (1:200; Invitrogen, Carlsbad, CA) for 10 min at 37°C (in the dark). The cells were then further stained with Hoechst dye in PBS at a ratio of 1:200 for 3-5 min. Stains were then washed off, and cells were visualized using an Olympus CellˆR fluorescence 1 x 81 inverted microscope (Olympus Biosystems, Planegg, Germany) with an F-view II camera for image acquisition and CellˆR software for processing images. The temperature of the microscope was maintained at 37°C for live cell imaging using a Solent Scientific microscope incubator chamber (Solent Scientific, Segensworth, UK). Three independent experiments were conducted and at least 3 images per experiment analyzed.

We also measured ROS levels by flow cytometry. After treatment, cells were trypsinized and centrifuged (2 min at 20,000 g), and the cell pellet treated with 2',7'-dichlorodihydrofluorescein diacetate stain (1:200), resuspended, and incubated at 37°C for 20 min in the dark. Cells not treated with 2',7'-dichlorodihydrofluorescein diacetate acted as negative controls, and stained cells treated with 100 µL hydrogen peroxide (30% w/v hydrogen peroxide) incubated for 10 min served as positive controls. ROS levels were measured using a flow cytometer (Becton-Dickinson, Franklin Lakes, NJ) and quantified by determining the mean of fluorescence for each treatment. Three independent experiments were conducted for each condition investigated, with typically 5,000 – 10,000 cells analyzed per experiment.
In a separate set of experiments, we evaluated apoptosis by employing a caspase glow assay (Promega, Madison, WI) (see Appendix 4 for the detailed protocol). Briefly, H9c2 myoblasts were trypsinized, counted in a hemocytometer and ~ 1 x 10^4 cells seeded per well of a 96-well plate (Greiner, Kremsmünster, Austria). Cells were seeded with 300 µL DMEM. DMEM was removed from cells and 100 µL of the reconstituted assay reagent added into each well and gently mixed. Cells were subsequently incubated for ~2 hr at room temperature and the degree of luminescence measured in white walled 96-well luminometer plates (Amersham, Buckinghamshire, UK).

We further confirmed our apoptosis data by employing an Annexin V-FITC kit (Macs, Miltenyi Biotec, Germany) according to the manufacturer’s instructions. The principle of the assay is that Annexin V can specifically bind to phosphatidyl serine residues exposed outside the membrane of early apoptotic cells. This allows the fluorescein (Annexin V) conjugated stained apoptotic cells to be counted and quantified. Propidium Iodide (PI) is a dye for nucleic acid but can only penetrate into the later stages of apoptotic cells after cells have been permeabilized. Therefore, using both Annexin V and PI, apoptosis at different stages can be distinguished. Samples were gated based on analysis of the physical parameters - forward scatter. Electronic compensation required for Annexin was done in three stages: (1) by analyzing a sample without staining to determine the level of auto fluorescence, (2) by analyzing Annexin-V labeled cells and (3) PI-labeled population. All experiments were performed with these settings. For this experiment the concentration of OA used corresponded to the concentration used in perfusion experiments.

In brief, 100 µM OA was administered for 6 and 24 hr, respectively, as before (refer ‘Cell culture and OA treatments’ section). After the completion of our experimental protocol, cells were washed with sterile PBS (calcium free), trypsinized and centrifuged at 300 g for three min. The pellet was washed with 1 ml of 1x binding buffer per 10^6 cells and re-centrifuged at 300 g for three min. The resulting pellet was resuspended in 100 µl of 1x binding buffer per 10^6 cells and thereafter incubated with 10 µl
of Annexin V-FITC for 15 min in the dark at room temperature. Cells were subsequently washed with 1 ml of 1x binding buffer and centrifuged at 300 g for 3 min. The pellet was resuspended in 500 µl of 1x binding buffer and 5 µl of PI solution immediately added, prior to analysis by flow cytometry. Viable cells (Annexin V−PI−); non-viable, including late apoptotic or necrotic cells (Annexin V−PI+) or Annexin V−PI+; and apoptotic cells (Annexin V−PI+) were detected by the binding of Annexin V to externalized phosphatidylserine in conjunction with PI. Results are presented as the percentage of apoptosis normalized to control (ratio of early apoptotic, Annexin+/PI− cells to the total population).

3.2.4 Animals and ethics statement

All animals were treated in accordance with the Guide for the Care and use of Laboratory Animals of the National Academy of Sciences (NIH publication No. 85-23, revised 1996). Studies were performed with the approval of the Animal Ethics Committees of Stellenbosch University, and the University of Cape Town (South Africa) (refer Appendices 1 and 2), and the United Arab Emirates University (United Arab Emirates).

3.2.5 Ex-vivo global ischemia during (simulated acute hyperglycemia)

These studies were carried out at Stellenbosch University (South Africa). Male Wistar rats weighing 180-220 gr were used throughout the study. Rats were anesthetized (pentobarbitone, 100 mg/kg i.p) and hearts rapidly excised and perfused in a modified Langendorff model with Krebs-Henseleit buffer (refer Appendix 3 for preparation) equilibrated with 95% O2-5% CO2 (37°C, pH 7.4) at a constant pressure (100 cm). The Krebs-Henseleit buffer contained (in mM) 11 Glucose, 118 NaCl, 4.7 KCl, 1.2 MgSO4.7H2O, 2.5 CaCl2.2H2O, 1.2 KH2PO4, 25 NaHCO3. Hearts were randomly distributed into four experimental groups: 1) control (11 mM glucose), untreated; 2) control (11 mM glucose), OA treated; 3) high glucose (33 mM glucose), untreated and 4) high glucose (33 mM glucose), OA treated – n=9
for each group. The buffer mimics the key ionic content of rat plasma or blood (36, 73) and it does not result in any hemodynamic dysfunction in the \textit{ex vivo} heart perfusion system (20). With the high glucose perfusions we are attempting to simulate acute hyperglycemia within the clinical setting. Moreover, since \textit{ex vivo} Langendorff perfusions are typically performed with 11 mM glucose at baseline, we are of the opinion that the 33 mM concentration is representative of a three-fold elevation of glucose levels (above normal) within the clinical setting.

The protocol was divided into two parts, i.e. perfusions a) without ischemia and b) with ischemia and reperfusion. For the non-ischemic protocol, we stabilized for 60 min whereafter 100 µM OA was added to the perfusate for an additional 20 min period (Figure 3.1A). The concentration of OA used was chosen based on literature (55). Subsequently, we returned the buffer used in the stabilization period and perfused for a further 20 min (total perfusion time: 100 min). For the ischemic protocol, 100 µM OA was added during the first 20 min of reperfusion (for OA experimental groups only; refer details below; see Figure 3.1B). OA was dissolved in a small volume of DMSO and less than 0.0005% (v/v) DMSO was present during perfusion experiments.

\textbf{A}

\begin{figure}[h]
\begin{center}
\begin{tikzpicture}
\node [rectangle, fill=blue!20] (A) {stabilization (60 min)};
\node [rectangle, fill=green!30] (B) [right of=A] {OA (100 µM) (20 min)};
\node [rectangle, fill=blue!20] (C) [right of=B] {perfuse with initial buffer (20 min)};
\end{tikzpicture}
\end{center}
\caption{Schematic diagrams showing the perfusion protocols for assessment of effects OA on heart contractile function without ischemia (A), with ischemia (B) and on infarct size (C).}
\end{figure}
During perfusion, a latex balloon attached to a pressure transducer (Stratham MLT 0380/D, AD Instruments Inc., Bella Vista, NSW, Australia) compatible with the PowerLab System ML410/W (AD Instruments Inc., Bella Vista, NSW, Australia), was inserted into the left ventricle and inflated to produce a diastolic pressure of 4-12 mm Hg. The protocol had a 60 min stabilization period, 20 min of global ischemia, followed by reperfusion for 60 min. Additional experiments were carried out in order to rule out the effects of osmotic pressure on heart function. Here hearts were perfused with 22 mM mannitol plus 11 mM glucose (total molarity = 33 mM) and subjected to ischemia and reperfusion as before. Contractile parameters assessed included heart rate (HR), left ventricular developed pressure (LVDP), and rate-pressure product (RPP; RPP = HR x LVDP). Left ventricular tissues were collected at four time points, i.e. a) within the first two minutes after ischemia, b) 20 and c) 40 min after ischemia, respectively, and d) also after 60 min of reperfusion. Collected tissues were freeze-clamped in liquid nitrogen and stored at -80°C for further analysis.

3.2.6  *Ex-vivo* regional ischemia and reperfusion during simulated acute hyperglycemia

These studies were carried out at Stellenbosch University (South Africa). To further strengthen our Langendorff perfusion data we also evaluated the effects of OA by infarct size determination. This was performed as described before but with slight modifications (47), i.e. we employed regional ischemia with a reperfusion time of 2 hr (see Figure 3.1C). Here a 3/0 silk suture was placed on the proximal portion of the left anterior descending coronary artery and passing the ends through a plastic tube. For induction of regional ischemia, the ends were tightened by pressing the plastic tube against the surface of the heart (above the artery) for 20 min. The snare was released during the reperfusion period. The efficacy of ischemia was confirmed by regional cyanosis and a substantial decrease in coronary flow.
3.2.6.1 Determination of infarct size

After completion of each regional ischemia and reperfusion experiment the snare was re-tightened and 2.5% Evans Blue dye (in Krebs buffer) was perfused through the hearts for infarct development. Hearts were subsequently removed from the Langendorff apparatus, blotted dry, suspended within 50 ml plastic tubes (using suture) and frozen at -20°C for 3 days. Thereafter, frozen hearts were sliced into 2 mm transverse sections and incubated with 1% 2,3,5-triphenyl tetrazolium chloride (TTC) in phosphate-buffered saline for 20 min at 37°C to identify non-infarcted (stained) from infarcted (non-stained) tissues. The area that was not stained with Evans Blue was defined as the area at risk (AAR). The area which demonstrated neither blue nor red was defined as the infarct site. Slices were then fixed in 10% formalin for 24 hr at room temperature before being placed between glass plates for scanning (both sides). The infarct area (IA) size and the area at risk (AAR) were calculated using Image J software (v1.46p, National Institutes of Health, USA) (see Appendices 11 and 12). Values of tissue slices were added together in order to obtain the total IA and AAR for each heart analyzed. We expressed the infarct size as the ratio of IA versus the AAR (%IA/AAR).

3.2.7 In vivo regional ischemia and reperfusion in streptozotocin-treated rats during chronic hyperglycemia

We next tested the effects of OA on diabetic rats (chronic hyperglycemia) subjected to an ischemic insult. These experiments were completed at the University of Cape Town (South Africa). Hyperglycemia was induced in Wistar rats as previously described (55, 60). In brief, male Wistar rats weighing 250-300 gr were injected (intraperitoneally) with a single dose of streptozotocin (STZ) (60 mg/kg dissolved in freshly prepared 0.1 M citrate buffer, pH 6.2; see Appendix 13). Control rats were injected with the vehicle (citrate buffer). Blood glucose concentrations of ≥ 20 mmol/l after 1 week were considered as a stable diabetic state before experimental procedures. We also determined body
weights and non-fasting blood glucose levels before STZ induction and after the one week of diabetes induction.

For the in vivo coronary artery ligation experiments, rats were divided into control (citrate-treated) and diabetic (STZ-treated) groups. Each group was subjected to coronary artery ligations ± OA treatment (0.45 mg/kg i.v) (as described in detail below). The OA was dissolved in <0.001% DMSO and deionized water; freshly done for each treatment period.

Ligation experiments were performed one week after STZ intraperitoneal injection and confirmation of a stable diabetic state. Rats were anesthetized with sodium pentobarbital (60 mg/kg i.p), intubated, and thereafter ventilated with room air (2.5 ml/stroke) at a rate of 75 strokes per min via a rodent ventilator (Model 681, Harvard Apparatus, USA). Body temperature was monitored by a rectal temperature probe, and a constant temperature was maintained throughout the surgical procedure by placing rats on a custom-made heating block. The depth of anesthesia was checked by assessing the pedal withdrawal reflex and by monitoring heart rate. Maintenance doses of anesthetic (6 mg/kg i.p) were administered as required. Lead II electrocardiogram (ECG) was recorded via an Animal Bio Amplifier (ML136, AD Instruments, Australia). Carotid arterial blood pressure was recorded via a custom-made cannula attached to a pressure transducer (MLT0670, ADInstruments, Australia). Since formation of clots around intra-arterial cannulae poses a potential risk for arterial thrombosis, heparin (1000 IU/kg i.p) was injected concurrently with anesthetic (26, 80).

A left thoracotomy was performed through the 4th intercostal space and the left lung collapsed using a damp swab. The left anterior descending coronary artery was thereafter ligated as previously described (22). A 6/0 silk suture was placed around the left anterior descending coronary artery and its ends passed through a plastic tube. For induction of regional ischemia the ends of the suture were used as a snare to occlude the artery by applying it gently onto the ventricular surface for 30 min. The
efficacy of ischemia was confirmed by regional cyanosis and ECG changes. We employed S-T elevation (ECG) to confirm coronary artery ligation. The snare was released during reperfusion.

Rat hearts were subjected to 30 min ischemia followed by 2 hr of reperfusion. The penile vein was cannulated for OA administration, while the vehicle solution was administered to control animals. For the OA rats, a bolus concentration of 0.45 mg/kg i.v (55) was injected immediately on reperfusion (within 1 min of releasing the snare) and thereafter reperfused for 2 hr as before. After the reperfusion period, the heart was flushed with saline and the coronary artery was re-occluded with the suture that had been left in place. The heart was then stained with 2.5% Evans blue to reveal the AAR. TTC staining and infarct sizes were determined as described for the ex vivo model.

3.2.8 Effects of long-term OA treatment on heart function in streptozocin-treated rats (chronic hyperglycemia)

We also ascertained the effects of chronic OA treatment within the diabetic context and its effects on cardiac function. These studies were completed at the United Arab Emirates University (Al-Ain, United Arab Emirates) and the candidate not involved with actual experiments; but did analyze all data generated. Here diabetes was induced in male Sprague-Dawley rats by a single intraperitoneal injection of STZ (60 mg/kg body weight) dissolved in citrate buffer. For OA-treated rats daily oral gavage was performed with a dose of 60 mg/kg for the entire 2-week period. The OA was made up freshly on each day and dissolved in DMSO and water. At the end of the two week period, body weights and fasting blood glucose levels for all experiments were determined.

Following STZ treatment, rats were killed by decapitation and hearts rapidly removed, mounted in Langendorff mode and perfused at a constant flow of 8 ml (g heart)\(^{-1}\) min\(^{-1}\) at physiological temperature (36–37°C) with Tyrode solution containing: 140 mM NaCl; 5 mM KCl; 1 mM MgCl\(_2\); 10
mM glucose; 5 mM HEPES; and 1.8 mM CaCl$_2$; adjusted to pH 7.4 with NaOH and continuously bubbled with oxygen. When the heart rate stabilized, force was recorded from the left ventricle in spontaneously beating hearts with a purpose-built extracellular suction electrode as previously described (38). Here a clip is fixed at the apex of the hanging heart and a thread tied to the clip. The thread was guided through pulleys and tied to the force transducer that was connected to the Power Lab system. Signals from the electrode were collected at 400 Hz, amplified (ML136 Bioamp, ADInstruments, Castle Hill, New South Wales, Australia), and conveyed via a Powerlab (PL410, ADInstruments, Castle Hill, New South Wales, Australia) to a personal computer. Heart functional data are expressed as force generated (grams).

3.2.9 Western blot analysis

Protein isolation was performed as previously described (65). Briefly, collected heart tissues were homogenized with modified RIPA buffer (see Appendix 5), the supernatant was centrifuged twice at 4,300 $g$ for 10 min at 4°C then stored at -80°C until further use. Protein expression was determined by Western blotting as described before by us (65, 66) for the following antibodies: BAD, phosphorylated-BAD (Ser 136), caspase 3 (Cell Signaling, MA, USA), and O-GlcNAc (HBP marker; CTD110.6, Santa Cruz Biotechnology Inc.). We employed β–actin (Cell Signaling, MA, USA) as a loading control.

3.2.10 Measurement of superoxide dismutase (SOD) activity

We assessed the total SOD activity (cytosolic and mitochondrial components) as detailed in the instructions of a commercially obtained kit (Biovision K335-100, Mountain View, CA 94043 USA). The assay depends on utilizing a highly water-soluble tetrazolium salt, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium, monosodium salt), which produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of WST-1 reduction by superoxide anion is linearly related to the xanthine oxidase activity and is inhibited by SOD. Formazan levels can
be measured by absorption with a spectrophotometer at 450 nm. Briefly, collected heart tissues were homogenized with modified ice cold RIPA buffer, the supernatant was centrifuged twice at 4, 300 g for 10 min at 4°C. The samples were incubated with the enzyme and WST working solutions at 37°C for 20 min in a 96 well-microtiter-plate (Corning, New York, USA) in an orbital shaker incubator. Absorbance was read at 450 nm with a microplate reader (EL 800 KC Junior Universal Microplate reader, Bio-Tek Instruments Inc, Vermont, USA). The assay was optimized by a negative control and a positive control provided with the kit. SOD activity was calculated according to the following formula: 

\[
\% \text{ inhibition} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100.
\]

### 3.2.11 Myocardial superoxide levels

The heart tissue was pulverized and homogenized in 100 volumes of perchloric acid (10% v/v) and centrifuged for 20 min at 13, 000 g (44). Protein-free supernatant (0.1 ml) was subsequently incubated with 0.25 mM lucigenin (Sigma-Aldrich, St. Louis, MO) at room temperature for 5 min in the dark and chemiluminescence measured in a white-walled luminometer 96 well-microtiter plate (Corning, New York, USA). Superoxide levels were expressed as chemiluminescence (RLU) per mg tissue.

### 3.2.12 Isolation of proteins for carbonylation and proteasome activity experiments

Heart tissues were cut into small slices and homogenized in 1 ml of Tris-HCl buffer (pH 7.4) using an IKA Ultra Turrax T25 homogenizer (IKA Labortechnik, Staufen, Germany) and incubated on ice for 10 min before centrifugation at 9, 000 g for 15 min to remove cell debris. The supernatant was used for protein quantification using the BCA assay.
3.2.13 ELISA carbonyl protocol

Protein carbonyls are formed by a variety of oxidative mechanisms and are sensitive indices of oxidative injury. Protein carbonylation was determined by the carbonyl ELISA assay developed in the GEICO laboratory (Université de La Réunion, Saint Denis de La Réunion, France) based on recognition of protein-bound DNPH in carbonylated proteins with an anti-DNP antibody (67). Here 5 µl of protein from tissue lysates (0.2-0.6 µg) was denatured by adding 10 µl 12% SDS solution. Subsequently, proteins were derivatized to DNP hydrazone with 10 µl of DNPH solution (10 mM in 6 M guanidine hydrochloride, 0.5 M potassium phosphate buffer, pH 2.5). DNPH is a chemical compound that specifically reacts and binds to carbonylated proteins. Samples were incubated at room temperature for 30 min and the reaction was neutralized and diluted in coating buffer (10 mM sodium carbonate buffer, pH 9.6) to yield a final protein concentration of 0.2 - 0.6 ng/µl.

Diluted samples were added to wells of a Nunc Immuno Plate Maxisorp (Dutscher, Brumath, France) and incubated at 37°C for 3 hr, and thereafter washed 5x with PBS/Tween (0.1%) between each of the following steps: blocking the wells with 1% BSA in PBS/Tween (0.1%) overnight at 4°C; incubation with anti-DNP antibody (Sigma-Aldrich, St Louis, MO) (1:2000 dilution in PBS/Tween [0.1%]/BSA [1%]) at 37°C for 3 hr; incubation with horse radish peroxidase-conjugated polyclonal anti-rabbit immunoglobulin (GE Healthcare, Mannheim, Germany) (1:4000 dilution in PBS/Tween [0.1%]/BSA [1%]) for 1 hr at 37°C; addition of 100 µl of TMB substrate solution and incubation for 10 min before stopping the coloration with 100 µl of 2 M sulphuric acid. Absorbances were read at 490 nm against the blank (DNPH reagent in coating buffer without protein) with a FluoStar microplate reader (BMG Labtech, Ortenberg, Germany). Results are expressed as percentage of absorbance compared to control cells (treatment of samples with 11 mM glucose) after normalization with protein concentrations.
3.2.14 Proteasome activity measurements

Chymotrypsin-like, trypsin-like, and caspase-like activities of the proteasome were assayed using fluorogenic peptides (Sigma-Aldrich, St Louis, MO): Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY-MCA at 25 µM), N-t-Boc-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin (LSTR-MCA at 40 µM) and N-Cbz-Leu-Leu-Glu-b-naphthylamide (LLE-NA at 150 µM), respectively (28). Assays were performed with ~50 µg of protein lysate (in 25 mM Tris–HCl, pH 7.5) and the appropriate substrate that were incubated together for 0-30 min at 37°C. Aminomethylcoumarin and β-naphthylamine fluorescence were measured at excitation/emission wavelengths of 350/440 and 333/410 nm, respectively, using a Fluostar fluorometric microplate reader (BMG Labtech, Ortenberg, Germany). Peptidase activities were measured in the absence/presence of 20 µM of the proteasome inhibitor, MG132 (N-Cbz-Leu-Leu-leucinal), and the difference between the two values was attributed to proteasome activity. Data were normalized to protein concentrations.

3.2.15 Statistical analysis

Data are presented as mean ± standard error of mean (SEM). Differences between treatment groups and time points were analyzed using one way analysis of variance (ANOVA). Mann-Whitney unpaired t-test was used when comparisons were made between two groups. Significant changes between groups were further assessed by means of the Tukey – Kramer post hoc. All statistical analysis were performed using GraphPad Prism version 5.01 (Graphpad Software, Inc, CA, USA). Values were considered significant when p<0.05.
3.3 Results

3.3.1 Isolation of oleanolic acid from clove extract

Evaluation of one-dimensional $^1$H- and $^{13}$C-NMR spectra of the isolated OA compound confirmed the presence of the 48 hydrogen and the 30 carbon atoms present in the molecule. For the determination of complex components within the molecule, two-dimensional $^1$H- and $^{13}$C-NMR spectra was conducted and confirmed the chemical structure of OA.

3.3.2 Structural elucidation of OA

The *S. aromaticum* crude leaf extract was sequentially extracted with ethyl acetate to give ethyl acetate-solubles (EAS). The percentage yield of OA from EAS varied from 0.79% to 1.72%. Spectroscopic analyses of the white powder obtained after recrystallization with ethanol carried out using $^1$H- and $^{13}$C-NMR (1D and 2D) are shown in Figure 3.2. Figure 3.2A is the 1D $^1$H NMR spectra showing all the hydrogens in the molecule. Figure 3.2B shows the 1D $^{13}$C NMR spectra of all carbons in the molecule with two carbon signals at 143.6 and 122.7 ppm corresponding to the carbon-carbon double bond at carbon 12 and 13. Figure 3.2C and 3.2D are the two dimensional $^1$H and $^{13}$C NMR spectra of OA. In Figure 3.2C the bottom line shows all the carbons attached to hydrogen atoms; the middle line shows carbons that are attached to one hydrogen atom (CH groups), and in the uppermost line, signals pointing downwards are CH$_2$ upwards are CH and CH$_3$. Table 3.1 compares the relative resonance frequencies of all the carbon atoms in the *S. aromaticum*-derived OA with literature data (54) while Figure 3.3 shows the OA structure as elucidated by $^1$H- and $^{13}$C-NMR.
**Figure 3.2**: *Syzygium aromaticum* (cloves) derived OA one dimensional 1H and 13C- NMR spectra (A-B) and two dimensional 1H and 13C- NMR spectra by Distortion Enhancement Proton Testing (DEPT) (C) and Heteronuclear multiple quantum coherence (HMQC) (D).
Table 3.1: $^{13}$C (100.64 MHz) Bruker Avance III NMR spectral data of plant-derived and reported OA (54)

<table>
<thead>
<tr>
<th>Carbon Position</th>
<th>Plant-derived OA $^{13}$C</th>
<th>Reported OA $^{13}$C (54)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38.4</td>
<td>38.5</td>
</tr>
<tr>
<td>2</td>
<td>27.2</td>
<td>27.4</td>
</tr>
<tr>
<td>3</td>
<td>79.0</td>
<td>78.7</td>
</tr>
<tr>
<td>4</td>
<td>38.8</td>
<td>38.7</td>
</tr>
<tr>
<td>5</td>
<td>55.2</td>
<td>55.2</td>
</tr>
<tr>
<td>6</td>
<td>18.3</td>
<td>18.3</td>
</tr>
<tr>
<td>7</td>
<td>32.6</td>
<td>32.6</td>
</tr>
<tr>
<td>8</td>
<td>39.3</td>
<td>39.3</td>
</tr>
<tr>
<td>9</td>
<td>47.6</td>
<td>47.5</td>
</tr>
<tr>
<td>10</td>
<td>37.1</td>
<td>37.1</td>
</tr>
<tr>
<td>11</td>
<td>23.0</td>
<td>22.9</td>
</tr>
<tr>
<td>12</td>
<td>122.7</td>
<td>122.5</td>
</tr>
<tr>
<td>13</td>
<td>143.6</td>
<td>143.5</td>
</tr>
<tr>
<td>14</td>
<td>41.6</td>
<td>41.6</td>
</tr>
<tr>
<td>15</td>
<td>27.7</td>
<td>27.7</td>
</tr>
<tr>
<td>16</td>
<td>23.4</td>
<td>23.4</td>
</tr>
<tr>
<td>17</td>
<td>46.5</td>
<td>46.5</td>
</tr>
<tr>
<td>18</td>
<td>41.0</td>
<td>40.9</td>
</tr>
<tr>
<td>19</td>
<td>45.9</td>
<td>45.9</td>
</tr>
<tr>
<td>20</td>
<td>30.7</td>
<td>30.6</td>
</tr>
<tr>
<td>21</td>
<td>33.8</td>
<td>33.8</td>
</tr>
<tr>
<td>22</td>
<td>32.4</td>
<td>32.4</td>
</tr>
<tr>
<td>23</td>
<td>28.1</td>
<td>28.1</td>
</tr>
<tr>
<td>24</td>
<td>15.5</td>
<td>15.5</td>
</tr>
<tr>
<td>25</td>
<td>15.3</td>
<td>15.3</td>
</tr>
<tr>
<td>26</td>
<td>17.1</td>
<td>17.1</td>
</tr>
<tr>
<td>27</td>
<td>25.9</td>
<td>25.9</td>
</tr>
<tr>
<td>28</td>
<td>182.2</td>
<td>183.5</td>
</tr>
<tr>
<td>29</td>
<td>33.07</td>
<td>33.1</td>
</tr>
<tr>
<td>30</td>
<td>23.6</td>
<td>23.6</td>
</tr>
</tbody>
</table>
Figure 3.3: Structure (A) and numbering (B) of oleanolic acid (International Union of Pure and Applied Chemistry, IUPAC).
3.3.3 Effects of OA treatment on ROS levels and apoptosis in heart cells

We employed both fluorescence microscopy and flow cytometric analysis to evaluate whether OA acts as an anti-oxidant under *in vitro* simulated chronic hyperglycemic conditions. Our data show significantly increased ROS levels in heart cells that were cultured under high glucose conditions (Figure 3.4). However, OA treatment (acute and chronic) significantly decreased myocardial ROS levels under control and high glucose conditions. Here, even the lower OA concentration (20 µM) blunted the increase in ROS levels under high glucose culturing conditions (p<0.05 vs. untreated high glucose cells) (Figure 3.4).

![Fluorescence microscopy and flow cytometry results showing the effects of OA treatment on ROS levels in heart cells.](image-url)

**Figure 3.4 OA treatment attenuates oxidative stress in H9c2 cells under both acute and chronic normoglycemic and hyperglycemic conditions.** Quantification of oxidative stress (DCFDA staining) in H9c2 cells in response to simulated chronic hyperglycemia (33 mM glucose) vs. control (5.5 mM glucose) ± treatment with 20 µM or 50 µM OA for 6 and 24 hr, respectively. (A) and (C) Fluorescence microscopy; (B) and (D) Flow cytometry. Values are expressed as mean ± SEM (n=9). *p<0.05, **p<0.01, ***p<0.001 vs. respective controls.
The effect of OA on ROS levels is also shown by the representative images in Figure 3.5.

Figure 3.5 Representative images on the effects of OA treatments on in vitro ROS levels. (A), (B), (C) correspond to control, ± 20 µM or 50 µM after 6 hours of treatment, respectively; (D), (E) and (F) show 33 mM (simulated acute hyperglycemia) 20 µM or 50 µM after 6 hours of treatment respectively. (G), (H) and (I) show the same control groups after 24 hours whereas (J), (K) and (L) show the same corresponding high glucose groups after 24 hours. Scale bar represents 20 µm (J), with the original magnification of 60x used for the image acquisition.
To examine whether OA exhibits anti-apoptotic effects, H9c2 cells were treated with 20 µM and 50 µM OA, respectively, for 24 hr and apoptosis determined by aminoluciferin luminescence with the caspase glow assay. Our data revealed increased caspase-3 activity in response to high glucose culturing conditions (p<0.05 vs. 5.5 mM glucose group) (Figure 3.6). The low glucose-treated cells also exhibited a concentration-dependent reduction in caspase-3 enzymatic activity, i.e. by 28.4 ± 2.2% and by 43.7 ± 1.5%, respectively, for the 20 µM and 50 µM OA concentrations (p<0.001 vs. 5.5 mM glucose group). Likewise, high glucose-treated cells displayed a decrease, i.e. by 19.9 ± 0.8% and by 43.4 ± 3.4%, respectively, for the 20 µM and 50 µM OA concentrations (p<0.001 vs. 33 mM glucose control) (Figure 3.6B). Our results at the 6 hr time point revealed similar findings (Figure 3.6A).
Figure 3.6 Decreased apoptotic cell death in H9c2 cells treated with OA as indicated by decreased caspase 3/7 activity measured by aminoluciferin luminescence under both normal and hyperglycemic conditions. Evaluation of caspase 3/7 activity using the caspase glow assay kit in H9c2 cells in response to simulated acute and chronic hyperglycemia vs. control ± treatment with 20 µM and 50 µM OA, respectively, for 6 and 24 hr. Values are expressed as mean ± SEM (n=9). ***p<0.001 vs. respective controls.
In support, flow cytometric analysis (Annexin V/FITC PI) showed decreased apoptosis in H9c2 cells exposed to high glucose and treated with 100 µM OA for 6 hr (p<0.01) (Figure 3.7). After 24 hr the decrease was observed under both low (p<0.001 vs. control) and high glucose (p<0.001 vs. control; p<0.001 vs. high glucose without OA treatment) culturing conditions (Figure 3.7).
Figure 3.7 Diminished apoptosis in OA-treated H9c2 cells (flow cytometry) in acute hyperglycemic conditions and both chronic normoglycemic and hyperglycemic conditions. Flow cytometric analysis using the Annexin V/FITC apoptosis assay kit to evaluate the effects of 100 μM OA treatment under control and high glucose culturing conditions (24 hr). (A), (B), (C) and (D) Representative FACS analyses of four individual experiments corresponding to control and high glucose ± OA treatment, respectively. (E) and (F) Quantification of OA treatment after 6 and 24 hr, respectively. Values are normalized to the control and expressed as mean ± SEM (n=4). ***p<0.001 vs. controls and, ##, ### p<0.01, p<0.001 vs. high glucose exposure without OA treatment.
3.3.4 Evaluation of *ex vivo* heart function during global ischemia and reperfusion (simulated acute hyperglycemia)

The functional data for both low and high glucose perfusions (without ischemia) showed no significant differences with OA treatment on LVDP and RPP (Figure 3.8).

![NON-ISCHEMIA](image)

Figure 3.8 OA treatment does not affect pre-ischemic cardiac function as there was no change in LVDP and RPP between control and OA treated groups. Isolated rat hearts were perfused under simulated hyperglycemic conditions (33 mM glucose) vs. controls (11 mM glucose) ± 100 µM OA treatment. We initially perfused for 60 min, whereafter OA was added for a further 20 min. Subsequently, the buffer initially used was returned and hearts perfused for an additional 20 min. (A) Left ventricular developed pressure at baseline (11 mM) and (B) high glucose conditions (33 mM). Rate pressure product (RPP) at baseline (C) and (D) high glucose levels. Values are expressed as mean ± SEM (n=9).
However, perfusion data with global ischemia and reperfusion show that LVDP recovery for hearts perfused with high glucose was markedly lower compared to the control group (p<0.01 vs. 11 mM control), ranging between 12.7 ± 3.2% and 23.1 ± 0.9% of the baseline pre-ischemic values during reperfusion (Figure 3.9). By contrast, the control group’s functional recovery during reperfusion ranged from ~28 to 50% of the baseline pre-ischemic value. Our perfusion data revealed that OA treatment markedly improved functional recovery of high glucose perfused hearts during reperfusion (p<0.001 vs. untreated high glucose), i.e. to 62 ± 8% of the baseline pre-ischemic value (Figure 3.9B). In agreement, OA treatment enhanced RPP and dP/dt\textsubscript{max} for the high glucose perfused group while it resulted in no significant effects on baseline treated hearts (Figures 3.9 and 3.10).
Figure 3.9 OA treatment during reperfusion blunts high glucose-induced cardiac dysfunction following ischemia and reperfusion as shown by significant post-ischemic recovery of LVDP and RPP under hyperglycemic perfusion conditions. Isolated rat hearts were perfused under simulated hyperglycemic conditions (33 mM glucose) vs. controls (11 mM glucose) and subjected to 20 min of global ischemia, followed by 60 min of reperfusion. For OA treatments groups, 100 µM OA was added during the first 20 min of reperfusion. (A) Left ventricular developed pressure (% recovery) at baseline glucose levels (11 mM), and (B) with high glucose (33 mM). Rate pressure product (RPP) at baseline glucose levels (C), and (D) under high glucose conditions. Values are expressed as mean ± SEM (n=9). *p<0.05, ***p<0.001 vs. respective controls.
Figure 3.10 OA treatment blunts high glucose-induced cardiac dysfunction (improved velocity of contraction) following ischemia and reperfusion. Isolated rat hearts were perfused under simulated hyperglycemic conditions (33 mM glucose) vs. controls (11 mM glucose) and subjected to 20 min of global ischemia, followed by 60 min of reperfusion. For OA treatments groups, 100 µM OA was added during the first 20 min of reperfusion. (A) Velocity of contraction (dP/dt_{max}) at baseline glucose levels (11 mM), and (B) with high glucose (33 mM). Values are expressed as mean ± SEM (n=9).*p<0.05 vs. respective controls.
Furthermore, OA treatment increased coronary flow vs. untreated group (p<0.01) under high glucose perfusion conditions. This was associated with a decrease in end diastolic pressure in hearts exposed to high glucose conditions, though both coronary flow and end diastolic pressure were not affected by OA treatment (Table 3.2).

**Table 3.2.** Effects of OA on *ex vivo* coronary flow and end-diastolic pressure during the first ten min of stabilization and at the end of reperfusion.

<table>
<thead>
<tr>
<th></th>
<th>Coronary Flow (ml/min)</th>
<th>EDP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre Ischemia</td>
<td>Post Ischemia</td>
</tr>
<tr>
<td>Control (11 mM)</td>
<td>9 ± 1</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>+ 100 µM OA</td>
<td>10 ± 1</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>High Glucose (33 mM)</td>
<td>11 ± 3</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>+ 100 µM OA</td>
<td>10 ± 1</td>
<td>13 ± 1**</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. *p<0.05; **p<0.01 vs. respective control. (n=6 in each group)

Moreover, the regional ischemia and reperfusion experiments (Figure 3.11A) show that OA treatment of high glucose perfused hearts decreased the infarct size from 59.6 ± 3.1% vs. 38.7 ± 1.6% (p<0.01 vs. high glucose untreated) with no changes on the area at risk amongst the groups (Figure 3.11B). We also performed additional experiments with 11 mM and 33 mM mannitol, and found no significant effects on functional recovery of hearts following global ischemia and reperfusion, thus ruling out any osmotic effects (data shown in Chapter 4).
Figure 3.11 OA administration showed cardio-protective effects as it decreases infarct size under high glucose perfusion conditions following regional ischemia and reperfusion. Isolated rat hearts were perfused under high glucose conditions vs. controls and subjected to regional ischemia. For OA treated groups, 100 µM OA was added during the first 20 min of the two hr reperfusion period. (A) infarct size/area at risk (%) and (B) area at risk under baseline (simulated normoglycemia) vs. high glucose perfusion conditions (simulated acute hyperglycemia). Evans blue dye and TTC staining enabled visualization of viable tissue (blue), infarcted area (white) and the area at risk (red). Values are expressed as mean ± SEM (n=6). *p<0.05, ***p<0.001 vs. respective controls.
3.3.5  *In vivo* coronary artery ligations in streptozotocin-treated rats (chronic hyperglycemia)

We next evaluated the cardio-protective effects of OA treatment within the *in vivo* context, i.e. by employing coronary artery ligations in STZ-treated hyperglycemic rats. Our data revealed markedly elevated blood glucose levels in STZ-diabetic rats compared to matched controls (p<0.001) (Table 3.3). This was associated with decreased weight gain in the STZ-diabetic rats.

<table>
<thead>
<tr>
<th></th>
<th>% Body weight change</th>
<th>Non-fasting blood glucose (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-diabetic</td>
<td>8.1 ± 0.8</td>
<td>6.6 ± 0.5</td>
</tr>
<tr>
<td>STZ-diabetic</td>
<td>-4.3 ± 1.2</td>
<td>27.1 ± 1.6***</td>
</tr>
</tbody>
</table>

Data are expressed as mean ±SEM, n ≥ 4 in each group. ***p<0.001 vs. non-diabetic control group.

In agreement with our *ex vivo* perfusion data, we found that infarct size was significantly increased for the STZ-treated rats versus controls (47.5 ± 2.9% vs. 27.9 ± 5.2%) following 30 min coronary artery ligation (Figure 3.12A), and similarly there were no changes in the area at risk amongst the groups (Figure 3.12B).
Figure 3.12 OA treatment decreases infarct size following coronary artery ligation in streptozotocin-diabetic rats. Wistar rats were injected with STZ and followed for a 1-week period. Subsequently, 0.45 mg/kg OA was injected via the penile vein within the first two min of reperfusion. (A) infarct size/area at risk (%) and (B) area at risk under baseline vs. high glucose conditions. Evans blue dye and TTC staining enabled visualization of viable tissue (blue), infarcted area (white) and the area at risk (red). Values are expressed as mean ± SEM (n=6). *p<0.05, **p<0.01 vs. respective controls.
OA administration also decreased systolic and diastolic blood pressures in STZ-diabetic rats vs. untreated STZ-diabetic controls during the early reperfusion period i.e. within the first twenty minutes of reperfusion after the one week of STZ-induction (Table 3.4). There were no significant differences on heart rate and systolic/diastolic blood pressures before the regional ischemia amongst all the groups (Table 3.4).

**Table 3.4** Effects of OA on *in vivo* heart rate, ST height, systolic and diastolic blood pressures during early reperfusion.

<table>
<thead>
<tr>
<th></th>
<th>Heart rate (beats/minute)</th>
<th>Systolic BP (mmHg)</th>
<th>Diastolic BP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PI</td>
<td>Reperfusion</td>
<td>PI</td>
</tr>
<tr>
<td>ND</td>
<td>404.7 ± 6.01</td>
<td>411.7 ± 8.5</td>
<td>130.9 ± 8.3</td>
</tr>
<tr>
<td>ND + OA</td>
<td>411.6 ± 12.4</td>
<td>399.7 ± 15.1</td>
<td>113.6 ± 13.9</td>
</tr>
<tr>
<td>STZ</td>
<td>394.3 ± 35.0</td>
<td>429.6 ± 20.4</td>
<td>127.4 ± 8.9</td>
</tr>
<tr>
<td>STZ + OA</td>
<td>386.3 ± 14.7</td>
<td>391.0 ± 17.8</td>
<td>117.4 ± 5.8</td>
</tr>
</tbody>
</table>

ND (non-diabetic); STZ (STZ-diabetic); BP (blood pressure), PI (pre-ischemia). Data are expressed as mean ±SEM, n ≥ 4 in each group) *p<0.05 vs. respective control.
3.3.6 Effects of long-term OA treatment on heart function in streptozotocin-treated rats (chronic hyperglycemia)

To assess the effects of long-term OA treatment on heart function, rats were injected with STZ and followed for 2 weeks ± OA treatment (daily). STZ treatment markedly increased fasting blood glucose (16.3 ± 0.8 mmol/L) versus matched controls (5.4 ± 0.3 mmol/L) (p<0.01). Our data show that the force generated by STZ-diabetic rat hearts was significantly lower compared to non-diabetic controls (p<0.01) (Figure 3.13). However, chronic OA treatment for two weeks significantly improved cardiac function in STZ-diabetic rats.

Figure 3.13 Long-term oral OA treatment improves cardiac function in ex vivo STZ-diabetic rats. Sprague-Dawley rats were injected with STZ and followed for a 2-week period ± daily OA treatment. Subsequently, isolated hearts from STZ-diabetic and matched controls were perfused and action potentials recorded via a force transducer. Values are expressed as mean ± SEM (n=6) **p<0.05 vs. non-diabetic control, and ***p<0.01 vs. respective controls.
3.3.7 Effects of OA treatment on ex vivo myocardial ROS levels and apoptosis

To confirm our in vitro data, we next evaluated whether OA exhibits anti-oxidant and anti-apoptotic properties within an ex vivo context. Under pre-ischemic conditions OA treatment did not significantly affect myocardial SOD activity or apoptosis (Figure 3.14). However, OA administration blunted high glucose-induced myocardial superoxide levels and concomitantly up-regulated SOD activity (Figure 3.15A and 3.15B) following ischemia and reperfusion. To gain further insight into temporal effects, we also performed analyses immediately after ischemia and for several time points thereafter. These data show that OA exerts its main anti-oxidant effects within the first 20 min following ischemia, whereafter it is sustained (Figure 3.15C).
Figure 3.14 OA treatment does not affect superoxide dismutase and caspase activities without ischemia. Isolated rat hearts were perfused under simulated hyperglycemic conditions (33 mM glucose) vs. controls (11 mM glucose) ± 100 µM OA treatment. We initially perfused for 60 min, whereafter OA was added for a further 20 min. Subsequently, the buffer initially used was returned and hearts perfused for an additional 20 min. (A) Superoxide dismutase activity (% inhibition) in response to high glucose vs. control ± OA treatment; (B) Caspase activity. Values are expressed as mean ± SEM (n=6).
Figure 3.15 Anti-oxidant effects of OA in hearts subjected to ischemia and reperfusion under high glucose perfusion conditions. Isolated rat hearts were perfused under high glucose conditions vs. controls and subjected to ischemia and reperfusion. For OA treatments groups, 100 µM OA was added during the first 20 min of reperfusion. (A) Superoxide levels under high glucose conditions vs. control ± OA treatment; (B) Superoxide dismutase activity (% inhibition) in response to high glucose vs. control ± OA treatment; (C) Time course for SOD activity following ischemic insult. Values are expressed as mean ± SEM (n=9). *p<0.05, **p<0.01, ***p<0.001 vs. respective controls.
We largely confirmed these data by evaluating the degree of protein carbonylation as an additional marker of oxidative stress (Figure 3.16). However, the OA-induced decrease in protein carbonylation under high glucose conditions did not reach statistical significance (p=0.076 vs. untreated high glucose) (Figure 3.16C).

Figure 3.16 OA treatment decreases carbonylation levels in hearts subjected to ischemia and reperfusion under high glucose conditions. Isolated rat hearts were perfused under high glucose conditions vs. controls and subjected to ischemia and reperfusion. For OA treatment groups, 100 µM OA was added during the first 20 min of reperfusion. (A) Degree of carbonylation under high glucose conditions vs. control; (B) OA treatment at baseline (11 mM glucose) and (C) under high glucose conditions (33 mM). Values are expressed as mean ± SEM (n=9). ***p<0.001 vs. respective controls.
We next assessed anti-apoptotic effects of OA in ex vivo perfused heart tissues. Myocardial caspase activity levels were increased under high glucose perfusion conditions (p<0.001 vs. untreated control). This effect was attenuated by OA treatment (p<0.01 vs. untreated high glucose) (Figure 3.17A). In agreement, OA treatment significantly decreased cardiac caspase-3, and increased p-BAD/BAD peptide levels under high glucose perfusion conditions (Figure 3.17B and 3.17C). We also investigated the temporal nature of myocardial apoptosis following ischemia. Our data show that anti-apoptotic effects emerge at the 40 min time point, suggesting that these effects occur as a result of the earlier upstream reduction of oxidative stress (Figure 3.17D).

Figure 3.17 Anti-apoptotic effects of OA in hearts subjected to ischemia and reperfusion under high glucose conditions. Isolated rat hearts were perfused under high glucose conditions vs. controls and subjected to ischemia and reperfusion. For OA treatments groups, 100 µM OA was added during the first 20 min of reperfusion. (A) Caspase activity; (B) Caspase-3 peptide levels; (C) p-BAD/BAD peptide levels; (D) Time course of myocardial apoptosis following the ischemic insult. Values are expressed as mean ± SEM (n=9). *p<0.05, **p<0.01, ***p<0.001 vs. respective controls.
To evaluate whether OA mediates its anti-apoptotic effects via HBP modulation, we next determined the overall degree of O-GlcNAcylation in our experimental system. Our data show increased O-GlcNAcylation in response to high glucose exposure (p<0.05 vs. untreated high glucose) that was significantly decreased by OA treatment (Figure 3.18).

Figure 3.18 OA treatment attenuates O-GlcNAcylation in hearts subjected to ischemia and reperfusion under high glucose conditions. Isolated rat hearts were perfused under high glucose conditions vs. controls and subjected to ischemia and reperfusion ± OA treatment during reperfusion. Western blot analysis for overall O-GlcNAcylation is shown with β–actin as loading control. Densitometric analysis for O-GlcNAcylation is displayed below gel image (normalized to corresponding β–actin values). Values are expressed as mean ± SEM (n=6). *p<0.05, **p<0.01 vs. respective controls.
3.3.8 Evaluating the effects of OA treatment on myocardial proteasomal activity in hearts subjected to ischemia and reperfusion under high glucose conditions

We initially evaluated pre- and post-ischemic proteasomal activities under high glucose perfusion conditions. Here we found that trypsin-like activity was significantly decreased under baseline glucose concentrations (Figure 3.19A). However, there were no changes on the chymotrypsin- and trypsin-like activities (Figures 3.19B and 3.19C). In contrast hyperglycemia induced a marked increase in all proteasomal activities following ischemia and reperfusion (Figure 3.20).

![Graphs showing proteasomal activities](image)

**Figure 3.19 Increased trypsin-like proteasomal activity in hearts under high glucose conditions without ischemia and reperfusion.** Isolated rat hearts were perfused under high glucose conditions vs. controls and subjected to ischemia and reperfusion. A) Trypsin-like proteasomal, (B) chymotrypsin-like, and (C) caspase-like activities after 60 min of reperfusion under high glucose conditions (33 mM) vs. control (11 mM). Values are expressed as mean ± SEM (n=9). *p<0.05 vs. respective control.
Figure 3.20 Increased total proteasomal activity in hearts subjected to ischemia and reperfusion under high glucose conditions. Isolated rat hearts were perfused under high glucose conditions vs. controls and subjected to ischemia and reperfusion. A) Trypsin-like proteasomal, (B) chymotrypsin-like, and (C) caspase-like activities after 60 min of reperfusion under high glucose conditions (33 mM) vs. control (11 mM). Values are expressed as mean ± SEM (n=9). *p<0.05, **p<0.01 vs. respective controls.

For 11 mM glucose perfusions, OA treatment decreased chymotrypsin-like proteasomal activity compared to the untreated control (p<0.05), but did not result in any significant effects on trypsin-like and caspase-like proteasomal activities (Figure 3.21A-C). Furthermore, OA treatment significantly diminished trypsin-like and chymotrypsin-like proteasomal activities in high glucose exposed rat hearts (Figure 3.21D and 3.21E). However, it had no effect on caspase-like activity in these hearts (Figure 3.21F).
Figure 3.21 OA attenuates high glucose-induced trypsin-like and chymotrypsin-like proteasomal activity following ischemia and reperfusion. Isolated rat hearts were perfused under high glucose conditions vs. controls and subjected to ischemia and reperfusion. For OA treatment groups, 100 µM OA was added during the first 20 min of reperfusion. (A) Trypsin-like proteasomal, (B) chymotrypsin-like, and (C) caspase-like activities after 60 min of reperfusion at baseline (11 mM glucose). (D) Trypsin-like proteasomal, (E) chymotrypsin-like, and (F) caspase-like activities after 60 min of reperfusion under high glucose conditions (33 mM glucose). Values are expressed as mean ± SEM (n=9). *p<0.05 vs. respective controls.
3.4 Discussion

The damaging alliance between hyperglycemia and myocardial infarctions necessitates the development of novel therapeutic interventions that offer cardio-protection within this context. Moreover, stress-induced hyperglycemia in non-diabetic patients suffering an acute myocardial infarction also results in damaging outcomes, e.g. increased in-hospital mortality (56, 62). For the current study we explored whether OA acts as a novel cardio-protective factor in response to ischemia and reperfusion under hyperglycemic conditions. We tested our hypothesis by employing cell culture studies, *ex vivo* and *in vivo* rat heart perfusion models, and long-term OA treatment of diabetic rats as experimental systems. The main findings of this study are that OA treatment results in cardio-protection for rat hearts by attenuating hyperglycemia-induced oxidative stress, apoptosis, and proteasomal activity following ischemia and reperfusion.

We initially investigated our hypothesis by exposing isolated rat hearts to ischemia and reperfusion and found a marked decline in contractile function under high glucose perfusion conditions (simulated acute hyperglycemia). However, OA treatment resulted in a striking increase in functional recovery and demonstrates, for the first time as far as we are aware, that it triggers cardio-protection in high glucose perfused rat hearts. Post-ischemic increased coronary flow, decreased end diastolic pressure and our infarct size data further corroborated these findings. The increased coronary flow may be as a result of the vasodilatory effects of OA due to its nitric oxide production properties (68, 72). It may be that it prevents post-ischemic damage by increasing supply of oxygen to the myocardium, thus promoting oxidative phosphorylation and improving post-ischemic function (45). Moreover, OA administration following *in vivo* coronary artery ligations in hyperglycemic rats also decreased infarct sizes in the STZ-diabetic rats. Although OA-mediated cardio-protection was previously reported following ischemia and reperfusion, such studies were performed on hearts isolated from rats that were pre-treated with OA for several days (17, 24). Furthermore, these experiments were completed
under normoglycemic perfusion conditions. We also performed experiments for long-term OA treatment and found that it restored heart function in isolated rat heart perfusions. Collectively, our data therefore demonstrate that OA treatment initiated after an ischemic insult elicited cardio-protection, thus meaning that our findings may offer therapeutic value to actual clinical settings (versus pre-treatments).

To gain further insight into underlying mechanisms responsible for OA-mediated cardio-protection, we next evaluated whether it displays anti-oxidant and anti-apoptotic properties in our experimental systems. Here we found that OA diminished high glucose-induced myocardial oxidative stress and apoptosis in both our in vitro and ex vivo experimental models. Here the speculation is that the anti-apoptotic effects of OA in vitro are concentration-dependent with a lower ED50 vs. the anti-oxidant effects that were significant with a higher dose and not the lower dose. Moreover, our ex vivo data show significantly elevated superoxide levels together with decreased SOD activity in simulated acute hyperglycemic hearts following an ischemic insult. The superoxide ions may be coming from several sources in the heart some of which include the mitochondria via the ETC, glucose auto-oxidation and uncoupled nitric oxide synthase (ucNOS). The bulk of these free radicals were derived from the mitochondria (8, 9, 16), glucose auto-oxidation and ucNOS (8, 14, 21, 61, 86) under both hyperglycemic conditions with ischemia and reperfusion. How does OA exert its anti-oxidant effects in hyperglycemic hearts exposed to ischemia and reperfusion? Although the precise mechanisms of OA-mediated anti-oxidant effects are unclear, earlier data support a direct scavenging role, i.e. by decreasing superoxide and hydrogen peroxide levels together with reduced lipid peroxidation (1, 71). Here OA (a lipophilic compound) is proposed to intercalate into the lipid matrix and stabilize myocardial cell membranes under stressful conditions (34). In addition, OA contains a single phenolic hydroxyl group that is likely to be the bioactive group for scavenging free radicals (23). Our time course studies suggest that this likely occurs quite rapidly after an ischemic insult, peaking at about 20 minutes and thereafter sustained up to 60 minutes post-ischemia.
Previous studies established that OA treatment also results in transcriptional effects that increase expression of several anti-oxidant defense genes, including α-tocopherol, glutathione peroxidase, catalase, thioredoxin peroxidase and superoxide dismutase (17, 23, 69, 76, 82, 83). Higher gene expression in this case is probably mediated by MAP kinases (JNK and ERK) that may regulate target transcriptional modulators (83). The basic leucine zipper transcription factor, nuclear factor erythroid 2 p45-related factor 2 (Nrf2), is strongly implicated in this process (83). Nrf2 plays a key role in protecting cells from oxidative stress by binding anti-oxidant-response elements (ARE) of gene promoters to induce expression of numerous target genes, e.g. glutathione peroxidase, heme oxygenase, and thioredoxin (35, 41, 48). Earlier work determined that OA upregulated Nrf2 expression in hepatocytes, with MAPK activation implicated in this process (83). Furthermore, Ichikawa et al. (40) found that dh404 (novel OA derivative) increased Nrf2 translocation to the nucleus to enhance Nrf2-driven transcription and suppress oxidative stress in H9c2 cardiomyoblasts. In support of its anti-oxidant properties OA attenuated protein carbonylation in hearts exposed following ischemia and reperfusion. Protein carbonylation is one of the more prominent ways through which proteins can be oxidized (53). The process of protein carbonylation is irreversible and often leads to loss of function and the need for degradation of damaged proteins mainly by the UPS. The exact mechanisms on how it occurs are unknown, however it involves non-enzymatic introduction of carbonyl groups to proteins by (i) direct oxidation of protein side chains or oxidative cleavage of the protein backbone, (ii) introduction of 4-hydroxy-2-noneal (HNE), 2-propenal or malondialdehyde from lipid peroxidation to a Cys, His or Lys residue and (iii) formation of advanced glycation end-products. Whether the probability and mechanism of carbonylation are constant or differ with increasing oxidative stress are still open questions. Collectively these data show that OA acts as an anti-oxidant and that its effects are mediated both by a direct scavenging role and also by induction of various anti-oxidant defense genes.

We propose that the improved functional effects detected with OA treatment stems from its anti-apoptotic effects observed in both our experimental models, that likely occur downstream of elevated
oxidative stress. There are several ways how this may happen. Firstly, rat hearts exposed to high glucose displayed increased superoxide and nitric oxide generation that favors the production of peroxynitrite and nitrotyrosine with damaging consequences (14). For example, others found that peroxynitrite inhibits the mitochondrial respiratory chain (7, 85) and triggers myocardial apoptosis (2). Our time course studies support the concept that myocardial apoptosis occurs downstream of the initial post-ischemic oxidative stress burst. Here we found that myocardial apoptosis in high glucose perfused hearts only increased from the 40 minute after the ischemic insult.

Secondly, our data show that OA exerts cardio-protective effects by attenuating HBP flux in hyperglycemic rat hearts. These data are in agreement with previous work from our laboratory that established that hyperglycemia results in greater oxidative stress, increased BAD O-GlcNAcylation and BAD-Bcl-2 dimer formation, thereby mediating HBP-induced myocardial apoptosis in H9c2 cardiomyoblasts (65). Thirdly, it is also possible that the OA-mediated decrease in oxidative stress may also limit hyperglycemia-induced inactivation of the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) (78) and electrophysiological alterations (arrhythmias, QT prolongation) (14, 25, 27). Thus our study shows that OA exerts anti-oxidant effects that attenuate myocardial apoptosis and thereby improve contractile functional recovery following ischemia and reperfusion of hyperglycemic rat hearts. Our ex vivo pre-ischemic data suggest that the cardio-protective effects of OA are not dependent on any inherent inotropic effects, but rather involve anti-oxidant and anti-apoptotic mechanisms. The potent hypotensive properties of OA confirm previous observations in Dahl salt sensitive (75) and STZ-diabetic rats (55). These data therefore offer significant clinical promise considering that hypertension is a robust risk factor for diabetes-induced CVD and non-diabetic heart diseases. The mechanisms underlying this interesting phenomenon were, however, not elucidated in this study. It is possible that OA treatment may also act to modulate the nervous system, but further studies are required to prove this.
We next investigated the role of the UPS in hyperglycemic hearts exposed to ischemia and reperfusion since it is the major non-lysosomal pathway for degradation of ubiquinated proteins (87). We reasoned that since UPS dysregulation occurs during ischemia and reperfusion, it may be an important contributing factor to reduced contractile function found under these conditions (87). However, conflicting data have been published with both inhibition and activation of the UPS linked to improved heart function in response to ischemia–reperfusion (reviewed in (3, 81)). It is likely that the discordant data may be due to differences in experimental models and protocols employed. We detected diminished proteasomal activities in 11 mM perfused rat hearts following ischemia (versus pre-ischemic hearts) (Figure 3.19). By contrast, high glucose perfused hearts exhibited a robust upregulation of trypsin-like, chymotrypsin-like and caspase-like proteasomal activities following ischemia (Figure 3.20). We propose that ischemia and reperfusion together with hyperglycemia trigger greater oxidative stress that may result in increased misfolded proteins that are targeted for removal by UPS degradation. In support of this notion, the increased protein carbonylation observed under hyperglycemic conditions also result in increased UPS to remove the misfolded proteins (46). However, excessively high UPS activation may result in the activation of damaging signaling pathways, e.g. nuclear factor-kappa B (NF-κB) (57). Here greater UPS activity will degrade IκB (NF-κB inhibitor) and release unbound NF-κB to the nucleus to induce expression of various genes that may exacerbate inflammation and reperfusion injury (4, 30). In support of this concept, Pye et al. (2003) (64) ascertained that proteasomal inhibition blunted NF-κB activation during reperfusion, thereby resulting in decreased reperfusion injury. Moreover, hyperglycemic rats treated with bortezomib (protease inhibitor) exhibited diminished UPS, inflammation and myocardial damage in response to ischemia and reperfusion (57). In agreement, we found that OA treatment attenuated proteasomal activities in high glucose hearts following ischemia and reperfusion. Together our data show that hearts exposed to high glucose levels and subjected to ischemia and reperfusion display UPS over-activity that may impair the heart’s functional recovery. Moreover, we established that OA treatment inhibits the UPS and that this is linked with improved cardiac contractile function following ischemia and reperfusion under simulated hyperglycemic conditions.
How does our data differ from previous studies that implicate glucose-insulin-potassium (GIK) in cardio-protection? We propose that the protective effects of GIK may largely depend on the actions of insulin i.e. via a) its direct cardio-protective (anti-apoptotic) effects (4, 19, 43, 70) and b) its glucose lowering abilities. It is well established that insulin administration should increase plasma glucose clearance thus limiting damaging effects of acute and chronic hyperglycemia (‘glucotoxicity’). Moreover, insulin promotes glucose uptake and the generation of glycolytic ATP that is linked with cardio-protection (19). GIK treatment has resulted in mixed success, e.g. GIK treatment for patients with acute myocardial infarction (CREATE-ECLA trial) did not result in any significant cardio-protective effects (59). Here it is proposed that the potential protective effects of GIK may have been abolished by higher blood glucose levels in GIK-treated patients (4). Finally, a recent study investigating glucose-insulin treatment in patients undergoing coronary artery bypass grafting (while maintaining normoglycemia) found that it is cardio-protective and improves myocardial function (13). Together these data strongly indicate that insulin acts as the major protective component of GIK, and that higher blood glucose levels are damaging to the cardiovascular system.

In summary, this study demonstrates that OA acts as a novel cardio-protective agent in hearts exposed to high glucose levels by decreasing oxidative stress, reducing apoptosis and the UPS, leading to cardio-protection following ischemia and reperfusion. These data are promising since it may eventually result in novel therapeutic interventions to treat stress-induced acute hyperglycemia (in non-diabetic patients) and also diabetic patients with associated CVD complications. Furthermore, in additional to its anti-inflammatory properties OA (32) use may be applicable in other cardiac diseases where inflammation is present e.g. in heart failure. We are of the opinion that this is particularly relevant within the developing world context, where it may provide a cost-effective therapeutic intervention for the treatment of acute myocardial ischemia in these individuals. The next chapter describes the second therapeutic agent benfotiamine addressing its effect on some aspects already highlighted in this chapter moreso on flux via the non-oxidative glucose pathways.
3.5 References


CHAPTER 4

Acute Benfotiamine Treatment Counteracts Hyperglycemia-mediated Contractile Dysfunction following Ischemia and reperfusion

Hyperglycemia (chronic and acute) is an important risk factor for acute myocardial infarction. With hyperglycemia, activation of non-oxidative glucose metabolic circuits i.e. the polyol pathway, hexosamine biosynthetic pathway (HBP), advanced glycation end products (AGE), and protein kinase C (PKC) activation can elicit the onset of cardiovascular complications. Previous studies found that benfotiamine (BFT) – vitamin B1 derivative - stimulates transketolase (pentose phosphate pathway [PPP] enzyme) thereby shunting flux away from these four pathways. We therefore hypothesized that acute BFT treatment activates PPP leading to cardio-protection following ischemia and reperfusion under hyperglycemic conditions. We employed several experimental systems: 1) Isolated rat hearts were perfused *ex vivo* with Krebs-Henseleit buffer containing 33 mM glucose vs. controls (11 mM glucose) for 90 min, followed by 30 min global ischemia and 60 min reperfusion ± BFT treatment added during the first 20 min of reperfusion; 2) Infarct size determination as in #1) but with 20 min regional ischemia and 2 hr reperfusion ± acute BFT treatment; and 3) *In vivo* coronary ligations performed on streptozotocin-treated rats ± acute BFT treatment during early reperfusion. Our data show that acute BFT treatment significantly decreased myocardial oxidative stress and apoptosis, and provided cardio-protection in response to ischemia and reperfusion under hyperglycemic conditions. In parallel, BFT blunted hyperglycemia-induced activation of four non-oxidative glucose pathways in the rat heart. This study therefore demonstrates that acute BFT treatment initiated after an ischemic insult offers significant potential as a novel therapeutic agent for acute myocardial infarctions under conditions of acute and chronic hyperglycemia.
4.1 Introduction

Diabetes poses a huge health burden that may result in devastating human and economic costs if no effective measures are implemented (104). Cardiovascular diseases frequently manifest in diabetic patients, and here chronic hyperglycemia presents as an important risk factor for acute myocardial infarction (AMI) (15, 125). Furthermore, stress-induced, acute hyperglycemia in non-diabetic individuals with AMI is associated with increased in-hospital deaths (86, 105). Thus, both acute and chronic hyperglycemia are able to trigger biochemical and electrophysiological perturbations that contribute to contractile dysfunction (19).

Under hyperglycemic conditions, enhanced flux through non-oxidative glucose metabolic circuits - polyol pathway, hexosamine biosynthetic pathway (HBP), formation of advanced glycation end products (AGE), protein kinase C (PKC) activation - may trigger reactive oxygen species (ROS) production and the development of micro- and macrovascular complications. Moreover, a positive feedback loop also exists where hyperglycemia-mediated oxidative stress activates non-oxidative glucose metabolic pathways. We previously found that hyperglycemia-induced ROS activated the HBP leading to greater O-GlcNAcylation (HBP end-product) of target proteins and myocardial apoptosis (109, 110). Moreover, we recently demonstrated that increased oxidative stress, HBP activation and apoptosis contribute to impaired contractile function in response to ischemia and reperfusion under acute and chronic hyperglycemic conditions (84). The underlying mechanisms responsible for hyperglycemia-mediated cardiac damage are, however, not precisely clear. An emerging paradigm suggests that hyperglycemia-induced ROS result in DNA damage and the subsequent activation of poly-ADP-ribose polymerase (PARP) as a restorative mechanism (12). However, PARP activation may also attenuate glyceraldehyde–3-phosphate dehydrogenase (GAPDH), a key glycolytic enzyme, thereby diverting upstream glycolytic metabolites into certain non-oxidative glucose pathways, with maladaptive consequences (Figure 4.1) (35, 47).
Ongoing research work is therefore focusing on ways to blunt this damaging sequence of events and to limit activation of certain non-oxidative glucose pathways under these conditions. In addition to oleanolic acid, this chapter investigates the potential use of another alternative therapeutic agent, benfotiamine (S-benzoylthiamine O-monophosphate, BFT). BFT is a synthetic S-acyl derivative of thiamine is emerging as a putative therapeutic agent since it stimulates transketolase, a key enzyme of the non-oxidative branch of the pentose phosphate pathway (PPP), thereby shunting flux away from the four non-oxidative glucose pathways earlier discussed and resulting in beneficial outcomes (Figure 4.1) (1, 82). Thiamine-derived compounds were discovered from plants on Allium genus such as onions, shallots and leeks and named as allithiamines (43). BFT was identified as the most effective compound with anti-AGEs properties. It is a lipid soluble molecule with an open thiazole ring that enables its entry directly through the cell membrane, hence it possesses increased bioavailability approximately five times that of thiamine (8, 81, 147). The thiazole ring closes once it is absorbed producing biologically active thiamine. After oral administration, BFT is first dephosphorylated to S-benzoylthiamine by the ecto-alkaline phosphatase in the brush borders of intestinal mucosal cells and enters the circulation. Studies reported that benfotiamine is delivered into cells via the reduced folate carrier-1 (RFC-1), and then de-benzoylated to thiamine monophosphate by cellular and plasma esterases (8, 130). Evidence suggest that BFT is an effective and safe compound to use. The classification of benfotiamine as a lipophilic agent is disputed by findings that showed its increased solubility in aqueous solutions e.g. water at pH < 7; and not in organic solvents e.g. benzene (135, 136). Thus, it is likely that benfotiamine is an amphipathic agent. Thiamine and its analogs are initially metabolized to thiamine monophosphate, then TPP/ thiamine diphosphate (TDP) that is required by TK.

Chronic BFT treatment of diabetic rodents prevented experimental retinopathy (47), and also enhanced post-myocardial infarction survival and functional recovery (70). Moreover, others emphasized the protective role of angiogenesis and pro-survival pathways (e.g. Akt) as potential mechanisms whereby BFT may offer cardio-protection within the setting of ischemia and reperfusion.
(70). However, as far as we are aware, no-one has previously tested the effects of acute BFT administration in response to ischemia and reperfusion (under acute and chronic hyperglycemic conditions), and its impact on the four non-oxidative glucose pathways earlier discussed. In light of this, we hypothesized that acute BFT treatment will enhance myocardial PPP flux, thereby attenuating hyperglycemia-mediated activation of four non-oxidative glucose pathways (AGE, HBP, polyol pathway, PKC activation) and resulting in cardio-protection following ischemia and reperfusion. We believe this research question is highly relevant within the clinical setting since, if our hypothesis is indeed correct, BFT could be rapidly administrated to both diabetic and non-diabetic patients after suffering an AMI.

Figure 4.1 Hyperglycemia activates non-oxidative pathways of glucose metabolism. Under hyperglycemic conditions there is elevated glycolytic flux and increased mitochondrial ROS production. PARP is subsequently activated to restore DNA damage but also attenuate GAPDH activity leading to pooling of upstream glycolytic metabolites (refer red arrows). The latter subsequently fuels activation of non-oxidative pathways of glucose metabolism: polyol pathway, HBP, PKC and AGE (refer blue arrows and text). By contrast, BFT activates transketolase and the non-oxidative branch of the pentose phosphate pathway (PPP), thereby shunting flux away from the polyol, HBP, AGE and PKC pathways (refer green arrows and text). AR: aldose reductase; SDH: sorbitol dehydrogenase; Glc: glucosamine; Glu: glutamate; GFAT: glutamine: fructose-6-phosphate amidotransferase; UDP-GlcNAc: uridine diphosphate-N-acetylglucosamine; DHAP: dihydroacetone phosphate; DAG: diacylglycerol; ROS: reactive oxygen species.
Flux through the polyol, AGE, PKC and HBP can be inhibited by various inhibitors at different targets. In this study we used zopolrestat (ZOPO), aminoguanidine (AMG), chelerythrine chloride (CHE) and 6-diazo-5-norleucine (DON). It is imperative therefore to describe the pharmacology of these drugs. Firstly ZOPO / Alond/ CP-73,8503/ 4-dihydro-4-oxo-3-[5-(trifluoromethyl)-2-benzothiazolyl] methyl]-1-phthalazineacetic acid is a carboxylic acid that inhibits AR. ZOPO has been shown to normalize sorbitol, fructose and myo-inositol levels in sciatic nerve, retina and kidney in diabetic rats (6) and to normalize kidney function in galactosemic rats (100). The half maximal inhibitory concentration (IC50) of ZOPO against AR and its median effective dose (ED50) in the acute in vitro and in vivo test of diabetic complications were 3.1 x 10(-9) M and 3.6 mg/kg, respectively. Under chronic test conditions the ED50s of ZOPO in reversing already elevated sorbitol accumulation in rat sciatic nerve, retina, and lens was reported to be 1.9, 17.6, and 18.4 mg/kg, respectively.

ZOPO is well absorbed in diabetic patients, resulting in high blood levels, and a highly favorable plasma half-life (27.5 h), and is undergoing further clinical evaluation (95). Furthermore, the mean steady-state maximum plasma concentration (C_{max}) was reported to be 127 and 144 μg/ml, respectively, for normal and diabetic rats. The time of maximal plasma concentrations for nearly all tissues was 4 hr after oral dosing with 50 mg/kg, and the half-life of radioactivity in most tissues (8-10 hr) was similar to the half-life in plasma (63, 116). In rats less than 2% of ZOPO is excreted unchanged in urine after 48 hr following dosing (orally 50 mg/kg) with no accumulation in the liver and plasma; however it accumulates in nerve, kidney and lens (63). By contrast in humans after dosing with ZOPO (50-1200 mg/kg orally) 34 to 45 % is excreted in urine after 48 hr with renal clearance ranging from 2.6 to 5.6 mL/min, and appeared to decrease as the dose was increased. In a 2-week multiple dose study, the mean steady-state minimum and maximum plasma concentrations, C_{min} and C_{max}, were 91.5 and 196 micrograms/mL for subjects administered 800 mg/day, and 131 and 281 micrograms/mL for subjects administered 1200 mg/day. Steady-state AUC(0-24) was also dose proportional. The mean steady state half-life of about 30.3 hours was consistent with the observed
2.2-fold accumulation in plasma. Apparent oral clearance (Clpo) was 5.2 mL/min, and apparent volume of distribution (Vdss/F) was 12 L (62, 63).

Aminoguanidine/pimagedine is a diamine oxidase inhibitor that prevents formation of irreversible AGEs by trapping dicarbonyl compounds such as 3-deoxyglucosone (11, 131, 132). AMG was introduced in 1986, however, results from the phase III clinical trials showed that it had a high toxicity, low efficacy and increased urinary clearance (9, 41); therefore its development and use has been stopped. In addition to inhibition of AGEs formation, AMG is an inhibitor of NOS, more potent on iNOS, (IC50 = 31 μM) than on nNOS (IC50 = 170 μM) and eNOS (IC50 = 330 μM) (23). Because the IC50 of AMG for inhibition of protein glycation in humans is 203 μM (80), it is likely that it also inhibits iNOS and nNOS (132). AMG has shown antioxidant effects in various experimental models, for example by quenching hydroxyl radicals and lipid peroxidation (45, 60, 107). In several models of diabetes, AMG has shown an improvement of renal (39, 124), cardiac (99) and nerve function (48).

CHE is a benzophenanthridine alkaloid that interacts with the catalytic domain of PKC (52). It is considered a highly specific PKC inhibitor (IC50 of 0.66 μM) with a number of other effects such as activation of caspase 3 leading to induction of apoptosis, induction (127) with cyclic nucleotide phosphodiesterases (38) and inositol metabolism (24). It also inhibits other kinases such as protein kinase A, calcium/calmodulin dependent protein kinase with IC50 ranging 100 to 170 μM (52). Several other effects of CHE have been reported and these include: inhibition of thymidine incorporation into DNA (3), inhibition of alanine aminotransferase (IC50 of 4 μM) (137) and Na⁺/K⁺ –ATPase (IC50 of 30-50 μM (22). Additionally it has anti-platelet activity (71) and is cytotoxicity in L-1210 tumor cells (IC50 of 0.53 μM) (52).

Lastly, 6-diazo-5-oxo-L-norleucine (DON) is a non-standard amino acid antibiotic that antagonizes glutamine and was first isolated from Streptomyces in a sample of Peruvian soil (31). It has been shown to possess antitumor properties in different animal models (149) and clinical trials but was
never approved (92). DON is a water soluble yellowish powder which can also be dissolved in aqueous solutions of methanol, acetone or ethanol. It is used as an inhibitor of various glutamine utilizing enzymes due to its similarity to glutamine by covalently binding to their catalytic centres (103, 106). DON is reported to be a cytotoxic inhibitor of many nucleotide synthesis enzymes in vitro leading to apoptosis (53, 142). More recently we have shown beneficial effects of DON under hyperglycemic conditions through inhibition of GFAT an amidotransferase. This effect was associated with decreased oxidative stress and apoptosis (53, 109, 110, 142).
4.2 Materials and Methods

4.2.2 Animals and ethics statement

All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals of the National Academy of Sciences (NIH publication No. 85-23, revised 1996). The study was carried out with the approval of the Animal Ethics Committees of Stellenbosch University and the University of Cape Town (South Africa) (see Appendices 1 and 2, respectively).

4.2.3 Ex vivo global ischemia during simulated acute hyperglycemia

Male Wistar rats weighing 180-220 gr were used throughout the study. Rats were anesthetized (pentobarbitone, 100 mg/kg i.p) and hearts rapidly excised and perfused in a modified Langendorff model as described before (84). Briefly, Krebs-Henseleit buffer containing (in mM) 11 Glucose, 118 NaCl, 4.7 KCl, 1.2 MgSO_4\cdot7H_2O, 2.5 CaCl_2\cdot2H_2O, 1.2 KH_2PO_4, 25 NaHCO_3 (refer to Appendix 3 for preparation of the buffer) was equilibrated with 95% O_2-5% CO_2 (37ºC, pH 7.4) at a constant pressure (100 cm). Buffer was not recirculated and the hearts were allowed to beat at their natural rate. During perfusion, a latex balloon attached to a pressure transducer (Stratham MLT 0380/D, ADInstruments Inc, Bella Vista NSW, Australia) compatible with the PowerLab System ML410/W (ADInstruments Inc, Bella Vista NSW, Australia) was inserted into the left ventricle via the mitral valve and inflated to produce a systolic pressure of 80-120 mmHg and a diastolic pressure of 4-12 mm Hg. The temperature of the heart was maintained at 37ºC by suspending it in a heated water jacket. Contractile parameters assessed throughout the experiment included heart rate (HR), left ventricular developed pressure (LVDP), end diastolic pressure, end systolic pressure, coronary flow and rate-pressure product (RPP = HR x LVDP).
Hearts were randomly distributed into three initial experimental groups for perfusions: 1) baseline control (11 mM glucose) ± BFT; and 2) high glucose (22 mM glucose) ± BFT (n=6 for each group) and high glucose (33 mM glucose) ± BFT (n=6 for each group). High glucose perfusions were used to simulate acute hyperglycemia within the clinical setting. The buffer mimics the key ionic content of rat plasma or blood (51, 123) and it does not result in any hemodynamic dysfunction in the ex vivo heart perfusion system (25). Moreover, since ex vivo Langendorff perfusions are typically performed with 11 mM glucose at baseline, we are of the opinion that the 22 mM and 33 mM concentrations are representative of a 2-fold and 3-fold elevation of glucose levels (above normal), respectively, within the clinical setting. After these initial experiments further work was done using only 11 mM and 33 mM ± and the highest concentration of BFT as these concentrations showed a more clear significant difference.

Figure 4.2 Schematic diagrams showing the perfusion protocols for assessment of effects BFT on heart contractile function without ischemia (A), with ischemia (B) and on infarct size (C).

Our initial experiments were carried out to determine the BFT concentration to use, and here we employed a non-ischemic protocol, where hearts exposed to baseline glucose (11 mM) were allowed
to stabilize for 90 min whereafter 25 or 50 μM BFT was added to the perfusate for 45 min (refer Figure 4.2A). Subsequently, hearts were perfused for a further 45 min with the same buffer used in the stabilization period (total perfusion time: 180 min). The ischemia and reperfusion protocol comprised a 90 min stabilization period, 30 min of global ischemia and 60 min of reperfusion. The cardio-protective effects of various concentrations of BFT (25, 50 and 100 μM), added during the first 20 min of reperfusion, were evaluated (see Figure 4.2B). Additional experiments were carried out in order to rule out the effects of osmotic pressure on heart function. Here hearts were perfused with 22 mM mannitol plus 11 mM glucose (total molarity = 33 mM) and subjected to ischemia and reperfusion as before.

To assess the contribution and activation of each of the hyperglycemia-induced pathways (AGE, PKC, HBP and polyol), specific pathway inhibitors were added to the perfusate (similar to BFT treatments). These experiments were carried out for both control and hyperglycemic groups using the ischemia reperfusion protocol, as outlined above. The following inhibitors (purchased from Sigma Aldrich, St Louis MO) were employed: AGE pathway - AMG (100 μM); PKC - CHE (5 μM); HBP – DON (40 μM); and polyol pathway - ZOPO (1 μM) (see Figure 4.3). All doses used were selected based on previously published studies (29, 30, 134, 146). At the end of each experiment hearts were removed and ventricular tissue was freeze-clamped with pre-cooled Wollenberger tongs whereafter it was stored at -80°C for further molecular and biochemical analysis.
Figure 4.3 Target sites for inhibition of non-oxidative glucose pathways. Under hyperglycemic conditions there is elevated glycolytic flux and increased mitochondrial ROS production. PARP is subsequently activated to restore DNA damage but also attenuate GAPDH activity leading to pooling of upstream glycolytic metabolites (refer red arrows). The latter subsequently fuels activation of non-oxidative pathways of glucose metabolism: polyol pathway, HBP, PKC and AGE (refer blue arrows and text) which can be inhibited by (1) zopolrestat; (2) 6-diazo-5-oxo-L-norleucine; (3) chelerythrine chloride and (4) aminoguanidine, respectively. AR: aldose reductase; SDH: sorbitol dehydrogenase; Glc: glucosamine; Glu: glutamate; GFAT: glutamine: fructose-6-phosphate amidotransferase; UDP-GlcNAc: uridine diphosphate-N-acetylglucosamine; DHAP: dihydroacetone phosphate; DAG: diacylglycerol; ROS: reactive oxygen species.

4.2.4 Ex vivo regional ischemia and reperfusion during simulated acute hyperglycemia

Perfusion experiments were carried out by employing male Wistar rats (180-220 gr) to determine the effect of BFT treatment on infarct size in our experimental system. This was performed using regional ischemia with a reperfusion time of 2 hr, as described previously (84). A 3/0 silk suture was placed around the proximal portion of the left anterior descending coronary artery. The ends of the suture were passed through a plastic tube to form a snare. For induction of regional ischemia, the snare was
occluded. After 20 min, the snare was released to initiate reperfusion. The efficacy of ischemia was confirmed by regional cyanosis and a substantial decrease in coronary flow.

4.2.5 Determination of infarct size

After completion of each regional ischemia and reperfusion experiment the snare was re-tightened and 2.5% Evans blue dye (in Krebs buffer) was perfused through the hearts for identification of the area at risk of ischemia. Hearts were subsequently removed from the Langendorff apparatus, blotted dry, suspended (using suture) within 50 ml plastic tubes and frozen at -20ºC for 3 days. Thereafter, frozen hearts were sliced into 2 mm transverse sections and incubated with 1% 2,3,5-triphenyl tetrazolium chloride (TTC) in phosphate-buffered saline for 20 min at 37ºC to distinguish non-infarcted (stained) from infarcted (non-stained) tissues. The area that was not stained with Evans Blue was defined as the area at risk (AAR). The area which demonstrated neither blue nor red was defined as the infarct site. Slices were then fixed in 10% formalin for 24 hr at room temperature before being placed between glass plates for scanning for preparation of the phosphate buffered saline). The infarct size (IS) and the area at risk (AAR) were determined using Image J software (v1.46p, National Institutes of Health, USA) as described in Appendices 11 and 12. Infarct size was expressed as a percentage of the AAR.

4.2.6 In vivo regional ischemia and reperfusion in streptozotocin-treated rats (chronic hyperglycemia)

The effects of BFT on infarct size in diabetic rats (chronic hyperglycemia) were assessed using an in vivo model of ischemia and reperfusion injury. These experiments were completed at the University of Cape Town (South Africa). Hyperglycemia was induced in Wistar rats as previously described (84, 85, 94). In brief, age-matched male Wistar rats weighing 250-300 gr were injected with a single dose of streptozotocin (STZ) (60 mg/kg, i.p). Control rats were injected with the vehicle (0.1 M citrate buffer,
pH 6.2; see Appendix 13). Blood glucose concentrations were measured using an Accu-Chek glucose monitor (Boehringer Mannheim Diagnostics, Indianapolis IN), and glucose levels of ≥ 20 mmol/l after one week were considered as a stable diabetic state. Body weights and non-fasting blood glucose levels before STZ induction and after a week of diabetes induction were also recorded.

For the in vivo coronary artery ligation experiments, rats were divided into control (citrate-treated) and diabetic (STZ-treated) groups. Each group was subjected to coronary artery ligations ± BFT treatment (0.5 mg/kg i.v) (as described in detail below). The BFT was dissolved in 0.9% saline immediately prior to administration. Ligation experiments were performed one week after STZ injection and confirmation of a stable diabetic state. Rats were anesthetized with sodium pentobarbital (60 mg/kg i.p.), intubated, and thereafter ventilated with room air (2.5 ml/stroke) at a rate of 75 strokes per min via a rodent ventilator (Model 681, Harvard Apparatus, Holliston MA). Body temperature was monitored by a rectal temperature probe and a constant temperature was maintained throughout the surgical procedure by placing rats on a custom-made heating block. The depth of anesthesia was checked by assessing the pedal withdrawal reflex and by monitoring heart rate. Maintenance doses of anesthetic (6 mg/kg i.p) were administered as required. Lead II electrocardiogram (ECG) was recorded via an Animal Bio Amplifier (ML136, ADInstruments, Bella Vista NSW, Australia). Carotid arterial blood pressure was recorded via a custom-made cannula attached to a pressure transducer (MLT0670, ADInstruments, Bella Vista NSW, Australia). Since formation of clots around intra-arterial cannulae poses a potential risk for arterial thrombosis, heparin (1000 IU/kg i.p) was injected concurrently with anesthetic (84).

A left thoracotomy was performed through the 4th intercostal space and the left lung collapsed using a damp swab. The left anterior descending coronary artery was thereafter ligated as previously described (84). A 5/0 silk suture was placed around the left anterior descending coronary artery and its ends passed through a plastic tube to form a snare. For induction of regional ischemia the snare was occluded for 30 min. The efficacy of ischemia was confirmed by regional cyanosis and ECG changes. S-T elevation (ECG) was used to confirm coronary artery ligation. After 30 min, the snare was
released to initiate reperfusion. Rat hearts were subjected to 30 min ischemia followed by 2 hr of reperfusion. The penile vein was cannulated for drug administration. BFT rats were given a bolus concentration of BFT (0.5 mg/kg i.v) (135) at the onset of reperfusion (within 1 min of releasing the snare) and therafter reperfused for 2 hr as before. Vehicle solution was administered to control rats. After the reperfusion period, the heart was removed, flushed with saline and the coronary artery was re-occluded with the suture that had been left in place. The heart was then stained with 2.5% Evans blue to reveal the AAR. TTC staining and infarct sizes were determined as described for the ex vivo model.

### 4.2.7 Myocardial superoxide levels

The heart tissue was pulverized and homogenized in 100 volumes of perchloric acid (10% v/v) and centrifuged for 20 min at 13, 000 g (65). Protein-free supernatant (0.1 ml) was subsequently incubated with 0.25 mM lucigenin (Sigma-Aldrich, St. Louis MO) at room temperature for 5 min in the dark and chemiluminescence measured in a white-walled luminometer 96-well microtiter plate (Corning Inc, Corning NY). Superoxide levels were expressed as chemiluminescence (RLU) per mg tissue.

### 4.2.8 Measurement of superoxide dismutase (SOD) activity

We assessed the total SOD activity (cytosolic and mitochondrial components) as detailed in the instructions of a commercially obtained kit (Biovision K335-100, Mountain View CA). The assay depends on utilizing a highly water-soluble tetrazolium salt, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium, monosodium salt), that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of WST-1 reduction by superoxide anion is linearly related to the xanthine oxidase activity and is inhibited by SOD. Formazan levels can be measured by absorption with a spectrophotometer at 450 nm.
Briefly, collected heart tissues were homogenized with modified ice-cold RIPA buffer (see Appendix 5), the supernatant centrifuged twice at 4, 300 g for 10 min at 4ºC. The samples were incubated with the enzyme and WST working solutions for 20 min at 37ºC in a 96-well microtiter plate (Corning Inc, Corning NY) in an orbital shaker incubator. Absorbance was read at 450 nm with a microplate reader (EL 800 KC Junior Universal Microplate reader, Bio-Tek Instruments, Winooski VT). Optimization of the kit was done using a negative and a positive control provided with the kit. SOD activity was calculated according to the following formula: % inhibition= \( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \). Lower absorbance of the reaction mixture indicated greater activity.

### 4.2.9 Isolation of proteins for carbonylation and proteasome activity experiments

Heart tissues were cut into small slices and homogenized in 1 ml of Tris-HCl buffer (pH 7.4) using an IKA Ultra Turrax T25 homogenizer (IKA Labortechnik, Staufen, Germany) and incubated on ice for 10 min before centrifugation at 9, 000 g for 15 min to remove cell debris. The supernatant was used for protein quantification using the BCA assay.

### 4.2.10 ELISA carbonyl protocol

Protein carbonyls are formed by a variety of oxidative mechanisms and are sensitive indices of oxidative injury. Protein carbonylation was determined by the carbonyl ELISA assay developed in the GEICO laboratory (Université de La Réunion, Saint Denis de La Réunion, France) based on recognition of protein-bound DNPH in carbonylated proteins with an anti-DNP antibody (115). Here 5 µl of protein from tissue lysates (0.2-0.6 µg) was denatured by adding 10 µl 12% SDS solution. Subsequently, proteins were derivatized to DNP hydrazone with 10 µl of DNPH solution (10 mM in 6 M guanidine hydrochloride, 0.5 M potassium phosphate buffer, pH 2.5). DNPH is a chemical compound that specifically reacts and binds to carbonylated proteins. Samples were incubated at room
temperature for 30 min and the reaction was neutralized and diluted in coating buffer (10 mM sodium carbonate buffer, pH 9.6) to yield a final protein concentration of 0.2 - 0.6 ng/µl.

Diluted samples were added to wells of a Nunc Immuno Plate Maxisorp (Dutscher, Brumath, France) and incubated at 37°C for 3 hr, and thereafter washed 5x with PBS/Tween (0.1%) between each of the following steps: blocking the wells with 1% BSA in PBS/Tween (0.1%) overnight at 4°C; incubation with anti-DNP antibody (Sigma-Aldrich, St Louis, MO) (1:2000 dilution in PBS/Tween [0.1%]/BSA [1%]) at 37°C for 3 hr; incubation with horse radish peroxidase-conjugated polyclonal anti-rabbit immunoglobulin (GE Healthcare, Mannheim, Germany) (1:4000 dilution in PBS/Tween [0.1%]/BSA [1%]) for 1 hr at 37°C; addition of 100 µl of TMB substrate solution and incubation for 10 min before stopping the coloration with 100 µl of 2 M sulphuric acid. Absorbances were read at 490 nm against the blank (DNP reagent in coating buffer without protein) with a Fluostar microplate reader (BMG Labtech, Ortenberg, Germany). Results are expressed as percentage of absorbance compared to control cells (treatment of samples with 11 mM glucose) after normalization with protein concentrations.

4.2.11 Proteasome activity measurements

Chymotrypsin-like, trypsin-like, and caspase-like activities of the proteasome were assayed using fluorogenic peptides (Sigma-Aldrich, St Louis, MO): Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY-MCA at 25 µM), N-t-Boc-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin (LSTR-MCA at 40 µM) and N-Cbz-Leu-Leu-Glu-b-naphthylamide (LLE-NA at 150 µM), respectively (42). Assays were performed with ~50 µg of protein lysate (in 25 mM Tris–HCl, pH 7.5) and the appropriate substrate that were incubated together for 0-30 min at 37°C. Aminomethylcoumarin and β-naphthylamine fluorescence were measured at excitation/emission wavelengths of 350/440 and 333/410 nm, respectively, using a Fluostar fluorometric microplate reader (BMG Labtech, Ortenberg, Germany). Peptidase activities were measured in the absence/presence of 20 µM of the proteasome inhibitor,
MG132 (N-Cbz-Leu-Leu-leucinal), and the difference between the two values was attributed to proteasome activity. Data were normalized to protein concentrations.

### 4.2.12 Evaluation of myocardial apoptosis

We evaluated apoptosis by employing a caspase activity assay (Biovision, Mountain View CA). Briefly, tissues were homogenized with modified ice-cold RIPA buffer (see Appendix 5), the supernatant centrifuged twice at 13,000 g for 10 min at 4°C and protein levels determined by the Bradford assay. The caspase activity assay is based on spectrophotometric detection of the chromophore, p-nitroaniline (pNA), after cleavage of the labeled substrate amino acid sequence Asp-Glu-Val-Asp (DEVD)-pNA. The samples were added onto a 96-well microtiter plate (Corning Inc, Corning NY) and incubated with the reaction mixture and DEVD-pNA for 2 hr at 37°C for 60 min in an orbital shaker incubator. The color intensity was quantified with a microplate reader (EL 800 KC Junior Universal Microplate reader, Bio-Tek Instruments, Winooski VT) at 505 nm. The caspase activity was expressed as arbitrary units per mg protein.

We further ascertained myocardial apoptosis by performing Western blotting analysis for the pro-apoptotic factors, BAD and caspase-3, as described (109). The detailed protocol for SDS-PAGE, sample preparation and Western blotting is described in detail in Appendices 6-10. Briefly, collected heart tissues were homogenized with modified ice-cold RIPA buffer (see Appendix 5), the supernatant was centrifuged twice at 13,000 g for 10 min at 4°C then stored at -80°C until further use. Protein expression was determined by SDS-PAGE as described before by us (109, 110) for total BAD and caspase-3 (Cell Signaling, Danvers MA). Protein expression was quantified by densitometric analysis and β-actin (Cell Signaling, Danvers MA) employed as a loading control.
4.2.13 PARP assay

Poly ADP-ribosylation of nuclear proteins is a post-translational event that occurs in response to DNA damage. PARP catalyzes the NAD-dependent addition of poly(ADP-ribose) to itself and adjacent nuclear proteins such as histones. The assay was carried out as stipulated by the manufacturers (Trevigen, Gaithersburg MD). Briefly, collected heart tissues were homogenized with modified ice-cold RIPA buffer (see Appendix 5), the supernatant was centrifuged twice at 4, 300 g for 10 min at 4°C. The wells of the 96 well-plate were initially rehydrated with 50 μl of the 1x PARP buffer and incubated at room temperature for 30 min. For the standard curve the PARP enzyme was diluted such that the total activity ranged between 0.00 to 1 unit per 25 μl, with the 0/blank wells acting as negative controls. This was added to the first wells of the 96-well plate and 25 μl of each sample to be tested added to the remaining wells. Thereafter 25 μl of the 1x PARP cocktail (contains the PARP cocktail, activated DNA and PARP buffer) was added to the wells and the plate was incubated at room temperature for 60 min. The wells were then washed with 1x PBS (see Appendix 14) and 0.1% Triton X-100 (200 μl/well) followed by two washes with 1x PBS, and tapping wells onto paper towels. Strep-HRP was then added to the wells and the plate incubated for 60 min at room temperature (on a shaker). The washing and drying steps were repeated before addition of the pre-warmed TACS-Sapphire™ colorimetric substrate, followed by 15 min incubation in the dark at room temperature. The reaction was stopped by 5% phosphoric acid and readings done at 450 nm using a micro plate reader (EL 800 KC Junior Universal Microplate reader, Bio-Tek Instruments, Winooski VT).

4.2.14 GAPDH assay

We utilized a GAPDH activity assay according to the kit’s instruction manual (Biomedical Research Service Centre, University at Buffalo NY). The assay is based on the reduction of a water-soluble tetrazolium salt, 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride, to formazan
(NADH-coupled reaction) exhibiting an absorption maximum at 492 nm. The intensity of the red color produced corresponds to the degree of GAPDH activity.

Briefly, tissue samples were lysed with cell lysis buffer and protein concentrations diluted to fall within 0.2-2 mg/ml range as stipulated in the kit’s manual. Ten μl of each sample was thereafter pipetted onto the 96-well plate (Corning Inc, Corning NY) and the reaction initiated by adding 50 μl of the GAPDH assay solution to each well. The plate was incubated at 37°C for 60 min in an orbital shaker incubator. The reaction was stopped by addition of 50 μl of 3% acetic acid and the absorbance measured at 492 nm using a microplate reader (EL 800 KC Junior Universal Microplate reader, Bio-Tek Instruments, Winooski VT). GAPDH activity is expressed as the absorbance units per mg protein.

4.2.15 Determination of non-oxidative pathway activation

4.2.15.1 AGE

Methylglyoxal (MG) derivatives are formed from the non-enzymatic reaction of reducing carbohydrates such as glucose and carbonyl compounds (glyceraldehyde) in the Maillard reaction - products of this reaction are referred to as AGEs. Therefore, MG concentrations, measured using the OxiSelect™ Methylglyoxal (MG) ELISA Kit (Cell Biolabs, San Diego CA), were used as a marker for AGE pathway activation. The kit measures the quantity of MG adduct in protein samples by comparing its absorbance with that of a known MG-BSA standard curve.

Briefly, tissues were homogenized in modified ice-cold RIPA buffer (see Appendix 5) and the supernatant was centrifuged twice at 13, 000 g for 10 min at 4°C. Protein quantification was performed using the Bradford assay. Standards (including a negative and a positive control) and protein samples were pipetted onto the 96-well plate (Corning Inc, Corning NY). Thereafter the plate was incubated overnight at 4°C for the MG in the standard and protein samples to adsorb onto plate wells. This was
followed by probing with an anti-MG specific monoclonal antibody for 60 min at room temperature on an orbital shaker. After a series of washing steps, an HRP-conjugated secondary antibody was added to all wells and incubated for an hour at room temperature. A substrate solution was next added and incubated for 5 - 20 min at room temperature on an orbital shaker to allow for color development. The latter reaction was terminated by adding a ‘stop solution’ before reading absorbances at 450 nm with a microplate reader (EL 800 KC Junior Universal Microplate reader, Bio-Tek Instruments, Winooski VT). MG levels were calculated from the standard curve and are expressed as nmol per μg protein.

4.2.15.2 PKC assay

The PKC assay was carried out as detailed in the kit’s instruction manual (Enzo Life Sciences, Farmingdale NY). This is an ELISA-based method where a synthetic substrate is used for PKC activation that is measured by employing a polyclonal antibody that recognizes its phosphorylated form. Samples were homogenized in modified ice-cold RIPA buffer (see Appendix 5) and the supernatant was centrifuged twice at 13,000 g for 10 min at 4°C. The active PKC control provided was serially diluted and added onto a 96-well microtiter plate (Corning Inc, Corning NY) as positive controls whereas the blanks were the negative controls. The tissue samples were subsequently added to appropriate wells, followed by ATP addition to initiate the reaction. The plate was thereafter incubated for 90 min at 30°C and the kinase reaction then terminated by emptying contents of each well. We next added the phosphospecific substrate to wells followed by an incubation step for 60 min at room temperature. A peroxidase-conjugated secondary antibody was thereafter added and the assay was developed with tetramethylbenzidine substrate, where the color intensity is proportional to PKC phospho-transferase activity. Color development was stopped with an acid solution and color intensity recorded in a microplate reader (EL 800 KC Junior Universal Microplate reader, Bio-Tek Instruments, Winooski VT) at 450 nm. PKC activity was determined from the standard curve and expressed per volume of lysate per minute.
4.2.15.3. Hexosamine biosynthetic pathway (HBP)

We employed Western blotting analysis to determine myocardial HBP activation in response to ischemia and reperfusion under high glucose conditions. Briefly, collected heart tissues were homogenized with modified ice-cold RIPA buffer (see Appendix 5), the supernatant was centrifuged twice at 13,000 g for 10 min at 4°C then stored at -80°C until further use. Total O-GlcNAc expression was determined by SDS-PAGE as described before by us (84) (see Appendices 6-10) using an O-GlcNAc antibody (CTD110.6, Santa Cruz Biotechnology, Santa Cruz CA). Total O-GlcNAcylation (per lane) was quantified by densitometric analysis, and β-actin (Cell Signaling, Danvers MA) was utilized as a loading control for all blots.

4.2.15.4 Polyol pathway

D-sorbitol, an intermediate of the polyol pathway, was measured as an index of the activity of this pathway. Total D-sorbitol levels (cytosolic and mitochondrial components) were measured as detailed in the instructions of a commercially obtained kit (Biovision K 631-100, Mountain View CA). Briefly, collected heart tissues were homogenized with modified ice-cold RIPA buffer (see Appendix 5) and the supernatant was centrifuged twice at 13,000 g for 10 min at 4°C. Samples and optimizing standards with controls (negative and positive) were subsequently incubated with 50 μl of the reaction mixture (assay buffer, enzyme mix, developer and probe) for 30 min at 37°C in a 96-well microtiter plate (Corning Inc, Corning NY) in an orbital shaker incubator. Sorbitol levels were determined by evaluating the oxidation of sorbitol to fructose and the reaction’s absorbance was recorded at 560 nm with a microplate reader (EL 800 KC Junior Universal Microplate reader, Bio-Tek Instruments, Winooski VT). We calculated the sorbitol concentration (C) of samples by using the sample amount (nmol) from the standard curve (S_v), sample volume (μl) used (S_v) and the dilution factor (D); C=S_v/S_v*D.
4.2.15.5 Non-oxidative pentose phosphate pathway (PPP)

We used a modified protocol (EC 2.2.1.1) from Sigma Aldrich (St. Louis MO) to determine transketolase activity as a marker for non-oxidative PPP activation. The reagents used were of analytical grade and purchased from Sigma Aldrich (St. Louis MO) and included: 216 mM glycylglycine buffer, 3.3 mmol/l xylulose 5-phosphate (X-5-P), 1.7 mmol/l ribose 5-phosphate (R-5-P), 0.002% (w/v) cocarboxylase (thiamine pyrophosphate solution) (TPP), 0.14 mmol/l reduced β-nicotinamide adenine dinucleotide (β-NADH), 15 mmol/l magnesium chloride (MgCl₂), 20 units α-glycerophosphate dehydrogenase/triophosphate isomerase enzyme solution (α-GDH/TPI) and transketolase enzyme solution. The detailed protocol for the assay is in Appendix 16. The principle of the assay is based on the reactions below:

\[
\text{X-5-P} + \text{R-5-P} \xrightarrow{\text{Transketolase}} \text{G-3-P} + \text{sedoheptulose 7-phosphate} \\
\text{G-3-P} \xrightarrow{\text{TPI}} \text{dihydroxyacetone phosphate} \\
\text{Dihydroxyacetone phosphate} + \beta-\text{NADH} \xrightarrow{\text{α-GDH}} \text{α-glycerol phosphate} + \beta-\text{NAD}
\]

(G-3-P; Glyceraldehyde-3-phosphate)

Briefly, heart tissues were homogenized in ice-cold RIPA buffer (see Appendix 5) and protein concentrations determined by the Bradford assay. The chemical reagents were freshly prepared and pipetted into a 96-well plate (Corning Inc, Corning NY) and mixed on an orbital shaker at room temperature. Absorbance was monitored using a microplate reader (EL 800 KC Junior Universal Microplate reader, Bio-Tek Instruments, Winooski VT) until constant, whereafter the samples and enzyme solution were added and the relative decrease in absorbance monitored for 10 min. In the assay the blank was used as the negative control and the enzyme solution for standards as positive controls.
Transketolase activity was calculated as follows:

\[ \text{Units/ml of enzyme} = \frac{(\Delta A_{340nm} / \text{min test} - \Delta A_{340nm} / \text{min blank}) (0.3)(df)}{(6.22)(0.01)} \]

The values were presented as units/mg protein = \[ \frac{\text{units / ml enzyme}}{\text{mg protein / ml enzyme}} \]

(\(\Delta\) - change increment; \(A\)-absorbance; df-dilution factor; ml-milliliter; mg-milligram; 6.22 = millimolar extinction coefficient of \(\beta\)-NADH at 340 nm; 0.01 = volume (in milliliters) of enzyme used; 0.3 = total volume (in milliliters) of assay)

4.2.16 Statistical analysis

Data are presented as mean ± standard error of mean (SEM). Differences between treatment groups and time points were analyzed using one way analysis of variance (ANOVA). Mann-Whitney unpaired t-test was used when comparisons were made between only two groups. Significant changes between groups were further assessed by means of the Tukey –Kramer post hoc. All statistical analyses were performed using GraphPad Prism version 5.01 (Graphpad Software, Inc, San Diego, USA). Values were considered significant when \(p<0.05\).
4.3 Results

4.3.1 Acute high glucose exposure impairs contractile heart function following ischemia and reperfusion

Our results indicate that acute hyperglycemia during the onset of ischemia and reperfusion elicits detrimental effects on the heart as shown by the reduced LVDP (Figure 4.4A) and RPP recovery (Figure 4.4B, p<0.05 vs. control). In control hearts, LVDP improved to 42.2 ± 3.9% whereas high glucose perfused hearts recovered to only 20.8 ± 4.6% after 60 min of reperfusion. Likewise RPP recovered much better in control hearts compared to hyperglycemic hearts after 60 min of reperfusion (39.5 ± 3.7% vs. 11.0 ± 2.6%, p<0.05). To rule out the possibility of attributing the effects of hyperglycemia on heart function to changes in osmolarity, we performed separate experiments where we perfused with 11 mM glucose plus 22 mM mannitol (mannitol control- total molarity 33 mM). Our data demonstrate higher LVDP and RPP recovery for the osmotic control group compared to hearts perfused under high glucose conditions (p<0.05) (Figure 4.4C and 4.4D). LVDP of the osmotic control improved to 35.7 ± 6.7% of baseline and RPP to 37.5 ± 3.7% of baseline compared to the respective high glucose perfused hearts.
Figure 4.4 High glucose-induced cardiac contractile dysfunction indicated by the decrease in % recovery of both LVDP and RPP in rat hearts exposed to high glucose conditions vs. hearts at baseline glucose concentrations. Effect is independent of osmotic increase as hearts perfused with mannitol + glucose (33 mM) performed to the same extent as hearts perfused with 11 mM glucose. Isolated rat hearts were perfused under high glucose conditions (33 mM glucose) vs. controls (11 mM glucose) and subjected to 30 min of global ischemia, followed by 60 min of reperfusion. (A) Left ventricular developed pressure (% recovery) and (B) rate pressure product (RPP) (% recovery) between control and high glucose perfused groups. In parallel experiments, groups were perfused similarly under hyperosmotic conditions (mannitol control - 33 mM i.e. 22 mM mannitol + 11 mM glucose). (C) Left ventricular developed pressure (% recovery) and (D) rate pressure product (RPP) (% recovery) between high glucose and hyperosmotic perfused groups. Values are expressed as mean ± SEM (n=6). *p<0.05 vs. respective controls.
Changes in contraction (dP/dt\textsubscript{max}) reflected a similar trend (Figure 4.5A and 4.5C) as for LVDP recovery and RPP though there were no significant differences. There were no differences on the heart rate under high glucose conditions and with the osmotic control group (Figure 4.5B and 4.5D).

Figure 4.5 Velocity of contraction and heart rate showed the same trend at baseline, hyperglycemic and osmotic control conditions. Isolated rat hearts were perfused under high glucose conditions (33 mM glucose) vs. controls (11 mM glucose) and subjected to 30 min of global ischemia, followed by 60 min of reperfusion. (A) maximal velocity of contraction, dP/dt\textsubscript{max}(mmHg/s) and (B) heart rate (beats/min) between control and high glucose perfused groups. In parallel experiments, groups were perfused similarly under hyperosmotic conditions (mannitol control 33 mM i.e. 22 mM mannitol + 11 mM glucose). (C) maximal velocity of contraction, dP/dt\textsubscript{max}(mmHg/s) and (D) heart rate (beats/min) between high glucose and hyperosmotic perfused groups. Values are expressed as mean ± SEM (n=6). *p<0.05 vs. respective controls.
4.3.2 Acute BFT treatment enhances non- and post-ischemic contractile function

Our findings show that acute BFT treatment (40 min) significantly improved LVDP at baseline (11 mM glucose) (Figure 4.6A) and concomitantly decreased heart rate (Figure 4.6B). Overall, this did not translate into significant outcomes on the RPP (Figure 4.6C). There were no differences in $dP/dt_{\text{max}}$ (Figure 4.6D).

![Figure 4.6](http://scholar.sun.ac.za)

**Figure 4.6** Acute BFT treatment increases cardiac contractile function under baseline glucose and non-ischemic conditions reflected by increased LVDP, however HR is significantly decreased vs. untreated hearts. Isolated rat hearts were perfused under baseline conditions (11 mM glucose) ± 25 or 50 μM BFT treatment. We initially perfused for 90 min, whereafter BFT was added for a 40 min period. Subsequently, the buffer initially used was returned and hearts perfused for an additional 40 min. (A) Left ventricular developed pressure, (B) rate pressure product (RPP) and (C) heart rate (HR) and maximal velocity of contraction ($dP/dt_{\text{max}}$) at baseline ± BFT. Values are expressed as mean ± SEM (n=8). *p<0.05 vs. respective controls.
We next determined the effects of acute BFT administration within the setting of ischemia and reperfusion. Our data reveal that all BFT concentrations enhanced post-ischemic LVDP (Figure 4.7A) at baseline (11 mM glucose), with no differences in the three concentrations used. There was no effect on RPP (Figure 4.7B), however the low concentration of BFT improved $dP/dt_{max}$ vs. control (Figure 4.7C). All the three concentrations of BFT decreased heart rate in the initial period of reperfusion (Figure 4.7D).

![Figure 4.7](image.png)

**Figure 4.7** Acute BFT treatment blunts cardiac dysfunction following ischemia and reperfusion at baseline glucose conditions since both LVDP and velocity of contraction are higher than in untreated hearts. In contrast BFT treatment reduced HR. Isolated rat hearts were perfused under baseline glucose (11 mM glucose) and subjected to 30 min of global ischemia, followed by 60 min of reperfusion. For BFT treatment groups; 25, 50 or 100 μM was added during the first 20 min of reperfusion. (A) Left ventricular developed pressure (LVDP) and (B) rate pressure product (RPP), (C) maximal velocity of contraction ($dP/dt_{max}$) and (D) heart rate (HR). Values are expressed as mean ± SEM (n=6).*p<0.05 vs. respective controls.
Similarly all BFT concentrations improved LVDP in high glucose perfused hearts (22 mM) vs. untreated hearts (Figure 4.8A), however, there was no effect on RPP and dP/dt\textsubscript{max} (Figures 4.8B and 4.8C). As seen in the baseline hearts, heart rate was also decreased (p<0.05) in the early phase of reperfusion in BFT treated groups vs. control (Figure 4.8D).

Figure 4.8 Acute BFT treatment blunts high glucose-induced cardiac dysfunction following ischemia and reperfusion since LVDP recovery was higher in comparison to untreated hearts. Isolated rat hearts were perfused under simulated hyperglycemic conditions (22 mM glucose) and subjected to 30 min of global ischemia, followed by 60 min of reperfusion. For BFT treatment groups; 25, 50 or 100 μM was added during the first 20 min of reperfusion. (A) Left ventricular developed pressure (LVDP) and (B) rate pressure product (RPP), (C) maximal velocity of contraction (dP/dt\textsubscript{max}) and (D) heart rate (HR) with high glucose exposure (22 mM). Values are expressed as mean ± SEM (n=6). *p<0.05 vs. respective controls.
With our highest glucose concentrations in simulated acute hyperglycemia (33 mM) the data show that all BFT concentrations significantly improved post-ischemic LVDP with the highest concentration of 100 μM eliciting a robust effect during the early reperfusion period (Figure 4.9A), reaching ~71.1 ± 5.9% (p<0.01 vs. respective control). A similar pattern was found for post-ischemic RPP (Figure 4.9B), reaching a peak of ~12, 904.3 ± 2, 500.6 mmHg/sec vs. 3, 033.0 ± 1, 729.3 mmHg/sec. Additionally all the concentrations enhanced post-ischemic dP/dt\text{max} (Figure 4.9C) (p<0.05) by the end of the reperfusion period. As observed in hearts perfused with 11 mM and 22 mM glucose concentrations; heart rate was also reduced during the 20 min of reperfusion (Figure 4.9D).

![Figure 4.9](image)

**Figure 4.9** Acute BFT treatment blunts high glucose-induced cardiac dysfunction following ischemia and reperfusion indicated by increased LVDP, RPP and velocity of contraction vs. untreated hearts. Isolated rat hearts were perfused under simulated hyperglycemic conditions (33 mM glucose) and subjected to 30 min of global ischemia, followed by 60 min of reperfusion. For BFT treatment groups; 25, 50 or 100 μM was added during the first 20 min of reperfusion. (A) Left ventricular developed pressure (LVDP) and (B) rate pressure product (RPP), (C) maximal velocity of contraction (dP/dt\text{max}) and (D) heart rate (HR) with high glucose exposure (33 mM). Values are expressed as mean ± SEM (n=6).*p<0.05, **p<0.01 vs. respective controls.
Changes in coronary flow and end-diastolic pressure under baseline (11 mM) and with simulated hyperglycemia ± BFT concentrations are shown in Table 4.1. There were no differences in coronary flow between hearts exposed to high glucose (22 and 33 mM) conditions vs. hearts at baseline glucose levels. However, BFT increased coronary flow in high glucose (22 and 33 mM) exposed hearts vs. their respective untreated controls. End-diastolic pressure was significantly elevated in high glucose exposed rat hearts vs. hearts in the control group. Moreover, there was a significant increase in end diastolic pressure for all the groups during post-ischemia, i.e. ranging from ~30 mmHg to 90 mmHg, with values higher in the high glucose untreated groups (22 and 33 mM) vs. baseline glucose exposed hearts. With BFT treatment end-diastolic pressure was significantly reduced in the high glucose exposed rat hearts, though there were no differences in the control group.

Table 4.1. Coronary flow and end-diastolic pressure (EDP) under high (22 and 33 mM) vs. baseline (11 mM) glucose conditions during the first ten min of stabilization and at the end of reperfusion.

<table>
<thead>
<tr>
<th></th>
<th>Coronary Flow (ml/min)</th>
<th>EDP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre Ischemia</td>
<td>Post Ischemia</td>
</tr>
<tr>
<td>Control (11 mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 25 µM BFT</td>
<td>12 ± 1</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>+ 50 µM BFT</td>
<td>14 ± 2</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>+ 100 µM BFT</td>
<td>15 ± 3</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>High Glucose (22 mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 25 µM BFT</td>
<td>13 ± 3</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>+ 50 µM BFT</td>
<td>14 ± 2</td>
<td>11 ± 1*</td>
</tr>
<tr>
<td>+ 100 µM BFT</td>
<td>13 ± 1</td>
<td>10 ± 2*</td>
</tr>
<tr>
<td>High Glucose (33 mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 25 µM BFT</td>
<td>14 ± 2</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>+ 50 µM BFT</td>
<td>15 ± 4</td>
<td>13 ± 3*</td>
</tr>
<tr>
<td>+ 100 µM BFT</td>
<td>16 ± 2</td>
<td>12 ± 2*</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. *p<0.05; **p<0.01 vs. respective control; #p<0.05 vs 11 mM control. (n=6 in each group)
4.3.3 Acute BFT administration decreases infarct size and attenuates high glucose-induced oxidative stress and apoptosis

Our data demonstrate that BFT reduced infarct sizes at baseline (11 mM glucose) and under high glucose conditions following regional ischemia (Figure 4.10A). Treatment with 100 μM BFT diminished infarct size to 39.0 ± 2.5% and 49.7 ± 7.4% versus 52.3 ± 2.8% and 74.5 ± 4.9% at baseline and high glucose perfusions, respectively. There were no differences in the area at risk amongst all the groups (Figure 4.10B).

![Figure 4.10](image)

**Figure 4.10** Acute BFT treatment decreases infarct size following ischemia and reperfusion ex vivo. Isolated rat hearts were perfused under normal or high glucose conditions and subjected to regional ischemia. For BFT treated groups, 100 μM BFT was added during the first 20 min of the 2 hr reperfusion period. (A) infarct size/area at risk (%) and (B) area at risk under baseline (simulated normoglycemia) vs. high glucose perfusions. Evans blue dye and TTC staining enabled visualization of viable tissue, infarcted area and the area at risk. Values are expressed as mean ± SEM. *p<0.05; **p<0.01 vs. respective control. (n=6 in each group).
We next evaluated the cardio-protective effects of BFT treatment within the *in vivo* context, i.e. by employing coronary artery ligations in STZ-treated hyperglycemic rats. Our findings revealed markedly elevated blood glucose levels in STZ-diabetic rats compared to matched controls (p<0.01) (Table 4.2). This was associated with decreased weight gain in the STZ-diabetic rats (p<0.01 vs. matched controls).

**Table 4.2** Body weight and blood glucose levels after 1 week of STZ injection.

<table>
<thead>
<tr>
<th></th>
<th>% Body weight change</th>
<th>Non-fasting blood glucose (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non diabetic</td>
<td>9.7 ± 1.7</td>
<td>6.9 ± 0.4</td>
</tr>
<tr>
<td>STZ-diabetic</td>
<td>-7.0 ± 2.6**</td>
<td>23.8 ± 1.8**</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM, n ≥ 4 in each group. **p<0.01 vs. non-diabetic control group.

In agreement with our *ex vivo* perfusion data, we found that infarct size was significantly increased for the STZ-treated rats versus controls (59.6 ± 3.1% vs. 46.4 ± 3.3%) following 30 min coronary artery ligation and 2 hr reperfusion (Figure 4.11A). Moreover, this effect was blunted by BFT treatment in control and STZ-treated rat hearts. There were no differences in the area at risk amongst all the groups (Figure 4.11B).
Figure 4.11 Acute BFT treatment decreases infarct size following ischemia and reperfusion *in vivo*. Wistar rats were injected with STZ and followed for a 1-week period. Subsequently, 0.50 mg/kg BFT was injected via the penile vein within the first two min of reperfusion. (A) infarct size/area at risk (%) and (B) area at risk under baseline vs. high glucose conditions. Evans blue dye and TTC staining enabled visualization of viable tissue, infarcted area and the area at risk. Values are expressed as mean ± SEM. *p<0.05; ***p<0.01 vs. respective control. (n=6 in each group).
BFT attenuated high glucose-induced oxidative stress, i.e. reduced superoxide levels in high glucose perfused hearts following ischemia and reperfusion (Figure 4.12A), and concomitantly enhanced SOD activity (p<0.05 vs. untreated high glucose) (Figure 4.12B). To corroborate these antioxidant effects, 25 µM and 50 µM BFT concentration decreased protein carbonylation from 0.55 ± 0.03 carbonyl levels/µg protein in high glucose untreated hearts to 0.29 ± 0.02 carbonyl levels/µg protein and 0.37 ± 0.03 carbonyl levels/µg protein respectively, under high glucose conditions. This effect was also observed under high glucose conditions with the highest concentration of BFT (Figure 4.12C).

![Figure 4.12 Acute BFT treatment blunts high glucose-induced oxidative stress by decreasing superoxide levels, increasing SOD activity and attenuating protein carbonylation. Isolated rat hearts were perfused under normal or high glucose conditions following ischemia and reperfusion. For BFT treated groups, 100 µM BFT was added during the first 20 min of the 2 hr reperfusion period. (A) Superoxide levels under high glucose conditions vs. control ± BFT treatment; (B) Superoxide dismutase (SOD) activity (% inhibition) in response to high glucose vs. control ± BFT treatment and (C) carbonylation levels/µg protein in response to high glucose vs. control ± 25 µM and 100 µM BFT treatment. Values are expressed as mean ± SEM (n=6). *p<0.05, **p<0.01, ***p<0.001 vs. respective controls.](#)
Myocardial apoptosis was increased under high glucose conditions (both 22 and 33 mM) vs. baseline glucose conditions (Figure 4.13).

Figure 4.13. Increased myocardial apoptosis in response to high glucose conditions indicated by increased expression of caspase-3. Caspase-3 expression (uncleaved) in isolated rat hearts perfused under high glucose (22 and 33 mM) vs. baseline glucose (11 mM). Western blot analysis is shown with β–actin as loading control. Densitometric analysis for caspase-3 is displayed below gel image (normalized to corresponding β–actin values. Values are expressed as mean ± SEM (n=6). *p<0.05, **p<0.01 vs control.

BFT treatment significantly blunted apoptosis as shown by the decreased cleaved caspase-3 peptide levels (Figure 4.14A and 4.14B), caspase activity (Figure 4.14C) and total BAD expression (Figure 4.14D) in high glucose (33 mM) perfused hearts compared to respective controls.
Figure 4.14 Anti-apoptotic effects of BFT in hearts subjected to ischemia and reperfusion under high glucose conditions shown by decreased caspase-3 expression (un/cleaved), decreased caspase 3/7 activity and decreased total BAD expression. Isolated rat hearts were perfused under high glucose conditions vs. controls and subjected to ischemia and reperfusion. For BFT treatment groups, various concentrations were added during the first 20 min of reperfusion. (A) Caspase-3 (cleaved) peptide levels under high glucose conditions with 25, 50 or 100 μM BFT, respectively, and (B) with 100 μM BFT vs. controls. (C) Caspase activity and (D) total BAD (BAD) peptide levels. Values are expressed as mean ± SEM (n=9). *p<0.05, **p<0.01, ***p<0.001 vs. respective controls.
4.3.4 BFT treatment decreased chymotrypsin proteasomal activity

The effect of high glucose perfusion conditions vs. control on post-ischemia proteasomal activity is shown in Chapter 3 (see Figure 3.19). There was no effect on post-ischemic chymotrypsin-like and trypsin-like activities with both the low and high concentrations of BFT (Figures 4.15A and 4.15B). However, the highest concentration of BFT blunted post-ischemic caspase-like proteasomal activity under baseline conditions (Figure 4.15C).

Figure 4.15 The highest dose of BFT attenuates caspase-like proteasomal activity under baseline glucose conditions following ischemia and reperfusion. Isolated rat hearts were perfused under baseline glucose conditions vs. controls and subjected to ischemia and reperfusion. For the treatment groups, 25 µM and 100 µM BFT were added during the first 20 min of reperfusion. (A) Trypsin-like proteasomal, (B) chymotrypsin-like, and (C) caspase-like activities after 60 min of reperfusion at baseline (11 mM glucose). Values are expressed as mean ± SEM (n=9). *p<0.05 vs. respective controls.
A similar observation was observed with hearts exposed to high glucose conditions with no effect on post-ischemic trypsin-like and caspase-like activities (Figures 4.16A and 4.16C). However, the highest BFT concentration blunted post-ischemic chymotrypsin-like proteasomal activity (Figure 4.16B) (p<0.05 vs. untreated hearts).

Figure 4.16 The highest dose of BFT attenuates high glucose-induced chymotrypsin-like proteasomal activity following ischemia and reperfusion under high glucose conditions. Isolated rat hearts were perfused under high glucose conditions vs. controls and subjected to ischemia and reperfusion. For the treatment groups, 25 µM and 100 µM BFT were added during the first 20 min of reperfusion. (A) Trypsin-like proteasomal, (B) chymotrypsin-like, and (C) caspase-like activities after 60 min of reperfusion under high glucose conditions (33 mM glucose). Values are expressed as mean ± SEM (n=9). *p<0.05 vs. respective controls.
4.3.5 BFT blunts high glucose-induced metabolic dysfunction and activation of non-oxidative glucose utilizing pathways.

Hearts perfused under high glucose conditions (22 and 33 mM) showed increased \(O\)-GlcNacylation vs. control group (Figure 4.17A). Treatment with BFT attenuated HBP flux, however, only the highest concentration showed a significant effect (\(p<0.05\)) vs. untreated hearts under high glucose perfusion conditions (Figure 4.17B).

![Figure 4.17 BFT treatment attenuates \(O\)-GlcNacylation in hearts subjected to ischemia and reperfusion under high glucose conditions.](image)

Isolated rat hearts were perfused under high glucose conditions vs. controls and subjected to ischemia and reperfusion ± BFT treatment during reperfusion. Western blot analysis for overall \(O\)-GlcNacylation is shown with \(\beta\)-actin as loading control. Densitometric analysis for \(O\)-GlcNacylation is displayed below gel image (normalized to corresponding \(\beta\)-actin values). (A) \(O\)-GlcNAc levels under high glucose (22 and 33 mM) vs. control group (B) \(O\)-GlcNAc levels under high glucose (33 mM) conditions with varying concentrations of BFT. Values are expressed as mean ± SEM (n=6). *\(p<0.05\), **\(p<0.01\) vs. respective controls.
In line with our hypothesis, we next determined the impact of acute BFT treatment on non-oxidative pathways of glucose metabolism. BFT diminished hyperglycemia-mediated PARP activation (p<0.001 vs. respective control) (Figure 4.18A) and in parallel restored GAPDH activity (p<0.01 vs. respective control) (Figure 4.18B). As predicted, increased GAPDH activity within this context resulted in less activation of the four non oxidative pathways here investigated. We found attenuated AGE (p<0.01 vs. respective control), PKC activity (p<0.01 vs. respective control), HBP (p<0.01 vs. respective control), and polyol pathway activation following acute BFT treatment (Figure 4.18C- 4.18G). By contrast, acute BFT treatment restored transketolase activity in high glucose hearts following ischemia and reperfusion (Figure 4.18H).
Figure 4.18 Acute BFT administration attenuates high glucose-induced metabolic dysfunction and activation of non-oxidative glucose pathways. Isolated rat hearts were perfused under high glucose conditions vs. controls and subjected to ischemia and reperfusion. (A) PARP activity, (B) GAPDH activity, (C) AGE activation (methyglyoxal concentration), (D) PKC activity, (E) O-GlcNAc peptide levels, (F) quantification of total O-GlcNAc peptide levels, (G) polyol pathway activation (sorbitol levels), and pentose phosphate pathway activation (transketolase activity). Values are expressed as mean ± SEM (n=6). *p<0.05, **p<0.01, ***p<0.001 vs. respective controls.
Interestingly, these data reveal that the PKC and AGE pathways showed greatest activation under high glucose conditions and were most sensitive to acute BFT treatment (Figure 4.19).

**Figure 4.19 Activation of non-oxidative pathways in high glucose perfusions relative to baseline conditions ± BFT treatment.** (A) Relative pathway activation (without BFT treatment) in high glucose perfused hearts following ischemia and reperfusion (normalized to baseline glucose perfused hearts). (B) Changes in pathway activation in high glucose perfused hearts (with BFT treatment) following ischemia and reperfusion (normalized to high glucose perfused hearts). Values are expressed as mean ± SEM (n=6).
4.3.6 Acute inhibition of flux through the non-oxidative glucose pathways blunts high glucose-induced contractile dysfunction following ischemia and reperfusion

Our data shows that inhibition of flux through the non-oxidative glucose pathways had no effect on both LVDP (% recovery) and RPP (Figure 4.20A and 4.20B) following ischemia and reperfusion after 60 min of reperfusion at baseline with the exception of polyol pathway inhibition that showed a strong (p<0.05) increase in LVDP (% recovery). By contrast, inhibition of the PKC, polyol, AGE and HBP pathways in high glucose perfused hearts showed improved contractile function vs. the untreated high glucose perfused hearts (p<0.05) (Figure 4.20C). LVDP recovery for inhibitor-treated hearts reached 43.6 ± 4.5% vs. untreated high glucose perfused hearts that only recovered to 20.8 ± 4.6% after 60 min of reperfusion. There was no significant effect of pathway inhibition on RPP in high glucose perfused hearts (Figure 4.20D).
Figure 4.20 High-glucose induced cardiac dysfunction blunted by acute non-oxidative pathway inhibition following ischemia and reperfusion. Acute treatment with specific pathway inhibitors (100 μM aminoguanidine (AMG) (for AGE pathway), 5 μM chelerythrine chloride (CHE) (for PKC pathway), 40 μM 6-diazo-5-oxo- norleucine (DON) (for HBP), and 1 μM zopolrestat (ZOPO) (for polyol pathway) during the first 20 min of reperfusion in isolated rat hearts. (A) LVDP recovery and (B) post-ischemic RPP (mmHgsec⁻¹) under baseline conditions (11 mM glucose) and (C) LVDP recovery and (D) post-ischemic RPP (mmHgsec⁻¹) under high glucose conditions (33 mM glucose) at 60 min of reperfusion. Values are expressed as mean ± SEM (n=8). *p<0.05, **p< 0.01 vs. respective controls.
There were additional various effects on other parameters for contractile function following inhibition of flux through the individual non-oxidative glucose utilizing pathways. As shown in Table 4.3, no significant changes were found for coronary flow and heart rate in baseline and high glucose perfused rat hearts ± inhibitor treatment. End-diastolic pressure was decreased following inhibition of the AGE and PKC pathways under high glucose conditions vs. untreated group. Furthermore, \( \frac{dP}{dt_{\text{max}}} \) was lower in the high glucose untreated rat hearts (33 mM) vs. control group (11 mM). This was, however, blunted (\( p<0.05 \)) by specific inhibitors for the non-oxidative glucose pathways that resulted in elevated values to \( \sim 1,500 \text{ mmHg/s} \) vs. \( \sim 750 \text{ mmHg} \) for untreated high glucose perfused hearts (Table 4.3).

**Table 4.3** Coronary flow, end-diastolic pressure, heart rate and velocity of contraction for hearts under high glucose (33 mM) vs. control (11 mM) ± NOGP inhibitors at the end of reperfusion.

<table>
<thead>
<tr>
<th></th>
<th>Coronary Flow (ml/min)</th>
<th>EDP (mmHg)</th>
<th>Heart rate (beats/min)</th>
<th>( \frac{dP}{dt_{\text{max}}} ) (mmHg/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control (11 mM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8 ± 2</td>
<td>38 ± 6</td>
<td>278 ± 19</td>
<td>1385 ± 56</td>
</tr>
<tr>
<td>+ 5 μM CHE</td>
<td>11 ± 1</td>
<td>42 ± 11</td>
<td>248 ± 9</td>
<td>1345 ± 63</td>
</tr>
<tr>
<td>+ 1 μM ZOPO</td>
<td>7 ± 2</td>
<td>36 ± 5</td>
<td>274 ± 17</td>
<td>1452 ± 23</td>
</tr>
<tr>
<td>+ 40 μM DON</td>
<td>9 ± 2</td>
<td>32 ± 12</td>
<td>276 ± 16</td>
<td>1265 ± 35</td>
</tr>
<tr>
<td>+ 100 μM AMG</td>
<td>6 ± 3</td>
<td>43 ± 12</td>
<td>282 ± 12</td>
<td>1300 ± 47</td>
</tr>
<tr>
<td><strong>High Glucose (33 mM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 ± 1</td>
<td>87 ± 7*</td>
<td>310 ± 17</td>
<td>745 ± 91**</td>
</tr>
<tr>
<td>+ 5 μM CHE</td>
<td>9 ± 3</td>
<td>60 ± 6**</td>
<td>285 ± 14</td>
<td>1262 ± 126*</td>
</tr>
<tr>
<td>+ 1 μM ZOPO</td>
<td>10 ± 3</td>
<td>67 ± 4</td>
<td>283 ± 18</td>
<td>1310 ± 144*</td>
</tr>
<tr>
<td>+ 40 μM DON</td>
<td>11 ± 2</td>
<td>69 ± 4</td>
<td>309 ± 19</td>
<td>1350 ± 264*</td>
</tr>
<tr>
<td>+ 100 μM AMG</td>
<td>7 ± 3</td>
<td>61 ± 5*</td>
<td>281 ± 16</td>
<td>1500 ± 232*</td>
</tr>
</tbody>
</table>

NOGP- non-oxidative glucose pathways; EDP- end diastolic pressure; \( \frac{dP}{dt_{\text{max}} \)}- maximal velocity of contraction. (100 μM aminoguanidine (AMG) (for AGE pathway), 5 μM chelerythrine chloride (CHE) (for PKC pathway), 40 μM 6-diazo-5-oxo-norleucine (DON) (for HBP), and 1 μM zopolrestat (ZOPO) (for polyol pathway). Values are expressed as mean ± SEM. *\( p<0.05 \); **\( p<0.01 \) vs. respective control. \( n=6 \) in each group)
No changes were observed in infarct size/area at risk (%) under baseline glucose conditions (Figure 4.21A). However, infarct sizes decreased following inhibitor treatment – thus in line with our improved functional data (Figure 4.21C). There were no differences in the area at risk under baseline and high glucose perfusion conditions.

**Figure 4.21 Acute inhibition of non-oxidative glucose pathways decreases infarct size under high glucose conditions.** Isolated rat hearts were perfused under normal or high glucose conditions and subjected to regional ischemia. Acute treatment with specific pathway inhibitors (100 μM aminoguanidine (AMG) (for AGE pathway), 5 μM chelerythrine chloride (CHE) (for PKC pathway), 40 μM 6-diazo-5-oxo-norleucine (DON) (for HBP), and 1 μM zopolrestat (ZOPO) (for polyol pathway) during the first 20 min of reperfusion in isolated rat hearts of the 2 hr reperfusion period. (A) infarct size/area at risk (%) and (B) area at risk under baseline (simulated normoglycemia) vs. (C) infarct size/area at risk (%) and (D) area at risk under high glucose perfusions (simulated hyperglycemia). Evans blue dye and TTC staining enabled visualization of viable tissue, infarcted area and the area at risk. Values are expressed as mean ± standard error of means (SEM). *p<0.05; **p<0.01; ***p<0.001 vs untreated high glucose group.
4.3.7 Hyperglycemia-induced oxidative stress and apoptosis is blunted by acute inhibition of flux through the damaging non-oxidative glucose pathways

Our data shows that oxidative stress is blunted by inhibition of flux through PKC, AGE, HBP and polyol as shown by decreased superoxide levels (Figure 4.22A); increased SOD activity (Figure 4.22B); decreased caspase activity (Figure 4.22C).

![Figure 4.22](image)

Figure 4.22 Inhibition of the non-oxidative glucose pathways attenuate high glucose-induced oxidative stress (decreased superoxide levels and increased SOD activity) and apoptosis as shown by decreased caspase 3/7 activity. Isolated rat hearts were perfused under high glucose conditions vs. controls and subjected to global ischemia ± inhibitor treatments during the first 20 min of the 1hr reperfusion period (100 μM aminoguanidine (AMG) (for AGE pathway), 5 μM chelerythrine chloride (CHE) (for PKC pathway), 40 μM 6-diazo-5-oxo-norleucine (DON) (for HBP), and 1 μM zopolrestat (ZOPO) (for polyol pathway). NT: non-treated controls). (A) superoxide levels, (B) superoxide dismutase activity and (C) caspase activity. Values are expressed as mean ± SEM (n=6). *p<0.05, **p<0.01, ***p<0.001 vs. respective controls.
4.3.8 The interlink of non-oxidative glucose pathways in attenuating hyperglycemia-induced metabolic dysfunction

Specific inhibition of individual non-oxidative glucose pathways showed no effect on flux through the other respective pathways in hearts under baseline glucose conditions (Figure 4.23).
Figure 4.23 Cardiac metabolic function at baseline glucose conditions with acute non-oxidative pathway inhibition following ischemia and reperfusion. Acute treatment with specific pathway inhibitors (100 μM aminoguanidine (AMG) (for AGE pathway), 5 μM chelerythrine chloride (CHE) (for PKC pathway), 40 μM 6-diazo-5-oxo-norleucine (DON) (for HBP), and 1 μM zopolrestat (ZOPO) (for polyol pathway) during the first 20 min of reperfusion in isolated rat hearts. (A) PARP activity, (B) GAPDH activity, (C) AGE activation (methylglyoxal concentration), (D) PKC activity, (E) O-GlcNAc peptide levels, (F) quantification of total O-GlcNAc peptide levels, (G) polyol pathway activation (sorbitol levels), and (H) pentose phosphate pathway activation (transketolase activity). Values are expressed as mean ± SEM (n=6).
However, inhibition of non-oxidative glucose pathway flux under high glucose conditions blunted metabolic dysfunction as shown by attenuation of PARP activity and normalizing of GAPDH activity (Figures 4.24A and 4.24B). Interestingly, inhibition of individual pathways attenuated activation through the other respective pathways (Figure 4.24C-4.24G). There was no effect, however, of the individual pathway inhibition on the pentose phosphate pathway.
Figure 4.24 High-glucose induced cardiac metabolic dysfunction blunted by acute non-oxidative pathway inhibition following ischemia and reperfusion. Acute treatment with specific pathway inhibitors (100 μM aminoguanidine (AMG) (for AGE pathway), 5 μM chelerythrine chloride (CHE) (for PKC pathway), 40 μM 6-diazo-5-oxo-norleucine (DON) (for HBP), and 1 μM zopolrestat (ZOPO) (for polyol pathway) during the first 20 min of reperfusion in isolated rat hearts. (A) PARP activity, (B) GAPDH activity, (C) AGE activation (methyglyoxal concentration), (D) PKC activity, (E) O-GlcNAc peptide levels, (F) quantification of total O-GlcNAc peptide levels, (G) polyol pathway activation (sorbitol levels), and (H) pentose phosphate pathway activation (transketolase activity). Values are expressed as mean ± SEM (n=6). *p<0.05, **p<0.01, ***p<0.001 vs. respective controls.
Thus our data shows that the individual non-oxidative glucose utilizing pathways are interrelated, i.e. inhibition of AGE, PKC, HBP and polyol down-regulated activation through all the other non-oxidative glucose utilizing pathways (Figures 4.25A to 4.25D).

Figure 4.25 Activation of non-oxidative pathways in high glucose perfusions with pathway inhibitors relative to high glucose conditions without treatment. Relative pathway activation after inhibition of (A) AGE pathway, (B) PKC pathway, (C) HBP pathway and (D) polyol pathway. Values are expressed as mean ± SEM (n=6).
4.4 Discussion

The damaging alliance between hyperglycemia and myocardial infarctions necessitates the development of novel therapeutic interventions for both chronic (diabetic individuals) and acute hyperglycemia (non-diabetic patients) (86, 105). In light of this, we employed ex vivo and in vivo models to investigate whether acute BFT administration - immediately following an ischemic insult – offers cardio-protection in response to ischemia and reperfusion under hyperglycemic conditions. The main findings of this study are that: 1) acute hyperglycemia triggers oxidative stress, the coordinated induction of non-oxidative glucose metabolic pathways and impaired contractile function following an ischemic insult under hyperglycemic conditions; and 2) post-ischemic BFT treatment resulted in cardio-protection by attenuating the detrimental effects of hyperglycemia with ischemia and reperfusion.

4.4.1 Acute hyperglycemia triggers oxidative stress, the coordinated induction of non-oxidative glucose metabolic pathways and impaired contractile function

Our ex vivo perfusion experiments revealed that acute hyperglycemia resulted in decreased contractile function following ischemia and reperfusion (independent of osmotic effects). Moreover, this was associated with higher myocardial superoxide levels and a concomitant decrease in SOD activity. This is in agreement with others where oxidative stress increased with hyperglycemia while activities of antioxidant enzymes decreased (16, 17, 26). Increased superoxide production was previously found in ex vivo models of hyperglycemia following ischemia and reperfusion (84), and it may also be converted to the damaging free radical, peroxynitrite (18). Our data are in agreement with the unifying hypothesis put forward by the Brownlee laboratory, that hyperglycemia-induced oxidative stress plays a crucial role in triggering damaging sequelae that contribute to the onset of type 2 diabetes (12, 13). Potential sources for hyperglycemia-induced ROS include greater superoxide levels
due to impaired mitochondrial function (13, 98) and/or higher NADPH oxidase activity (120), and glucose auto-oxidation (140). Moreover, ischemia and reperfusion *per se* also elevates cardiac ROS levels (20, 91, 118) thus further contributing to the overall oxidative stress here observed.

We also discovered increased PARP and attenuated GAPDH activities following ischemia and reperfusion in hearts exposed to high glucose. These findings are consistent with higher PARP activation operating as a restorative mechanism to alleviate hyperglycemia-induced DNA damage (66). However, its activation also leads to diminished GAPDH activity (28, 35, 153). Furthermore, GAPDH itself is susceptible to hyperglycemia-generated ROS (57), e.g. S-thiolation by hydrogen peroxide (10, 117, 121) and nitroalkylation by nitroalkene derivatives (5). Such modifications can cause GAPDH to form aggregates that translocate to the nucleus where it may participate in the induction of apoptosis (96). Lower GAPDH activity may also be due to the effects of ischemia (90).

Previous research established that PARP-mediated blunting of GAPDH activity leads to greater flux into the non-oxidative pathways of glucose metabolism – AGE, HBP, PKC and the polyol pathway – with damaging outcomes (35, 47). In parallel, PPP flux decreases under these conditions (35, 47). For our study we demonstrate that this scenario occurs within the setting of ischemia and reperfusion in response to acute hyperglycemia. Moreover, we determined - for the first time as far as we are aware – the relative degree of non-oxidative pathway activation, with AGE and PKC showing a marked increase under hyperglycemic conditions. However, does pathway activation in this instance *actually* result in harmful outcomes? Our data strongly support this since individual pathway inhibitor administration resulted in reduced myocardial ROS levels and apoptosis. In parallel, individual pathway inhibitors also decreased infarct sizes. Interestingly, the functional effects of individual pathway inhibitors did not significantly differ from each other and provides additional support for the idea of a unifying hypothesis and the inter-relatedness of the pathways (13). We propose that higher activation of AGE and PKC may further fuel flux through the HBP and polyol pathways with harmful effects. Furthermore, AGE and PKC activation may also trigger alternate functional outcomes not
investigated in the present study. Thus our data show that certain non-oxidative glucose pathways can be rapidly induced in response to acute hyperglycemic exposure and leads to serious implications for the heart’s contractile abilities.

By what mechanisms do the non-oxidative pathways elicit its detrimental effects on the heart? Here the non-enzymatic attachment of proteins to glucose produces AGE that may eventually impair extracellular matrix and intracellular protein function (12, 49). Moreover, augmented MG levels (AGE precursor) may contribute to the pathogenesis of diabetic complications (114, 141). However, these findings were reported in response to chronic elevations in blood glucose and are therefore unlikely to play a role in our study. Since AGE accumulation may also trigger oxidative stress and myocardial apoptosis (77), we are of the opinion that this likely applies in our case.

Under hyperglycemic conditions excess glycolytic intermediates can lead to increased de novo synthesis of DAG, a well-known activator of the PKC pathway (reviewed in (44). Moreover, PKC may also be activated as a result of lower ATP levels that occur due to the ischemic insult itself, thus resulting in a compounding effect together with hyperglycemia (126). Which PKC is implicated here since there are multiple isoforms that are expressed in the rat heart? We are of the opinion that the PKC-α, -β1 and -β2 isoforms likely play a role since earlier work established its up-regulation in the hyperglycemic heart (reviewed in (44). However, further studies are required to delineate PKC isoforms activated in response to ischemia and reperfusion under acute hyperglycemic conditions. PKC activation may impair contractile function by decreasing activity of the Na⁺/K⁺ ATPase pump (145). Here PKC activation increases cytosolic phospho-lipase A2 activity that enhances production of arachidonate and prostaglandin E2, known inhibitors of the Na⁺/K⁺ ATPase pump (145). Moreover, PKC activation may also further elevate myocardial ROS production by activation of NADPH oxidases (4, 143).
Although we found relatively lower activation of the HBP and polyol pathways with high glucose exposure, its stimulation also contributes to higher myocardial oxidative stress, apoptosis and infarct sizes. This is supported by our data generated by respective HBP and polyol pathway inhibitors, demonstrating lower cardiac ROS levels and apoptosis together with cardio-protection. Moreover, previous research from our laboratory demonstrated that hyperglycemia results in greater oxidative stress that triggered HBP-induced myocardial apoptosis in cardiomyoblasts (109). Activation of the polyol pathway is also linked with detrimental effects e.g. increased oxidative stress, sorbitol-induced osmotic effects, and perturbations in Na⁺/K⁺ ATPase activity (reviewed in (129)).

We also established that myocardial transketolase activity decreased in rat hearts following ischemia and reperfusion under hyperglycemic conditions, and this may occur due to its modification by oxidative stress (154). Our findings are in agreement with previous work that reported lower transketolase and glucose-6-phosphate dehydrogenase (G6PD) activities in the diabetic heart (70). Since the PPP generates NADPH (via G6PD reaction in oxidative branch) it allows for regeneration of reduced glutathione (GSH), thus playing a pivotal role in the myocardium’s antioxidant defense system (70). Together our data therefore demonstrate that hyperglycemia-induced activation of non-oxidative glucose pathways together with reduced PPP activation trigger damaging effects (ROS and apoptosis) that contribute to contractile dysfunction in response to ischemia and reperfusion under hyperglycemic conditions.

### 4.4.2 Acute BFT treatment attenuates the detrimental effects of hyperglycemia with ischemia and reperfusion

We initially tested two BFT concentrations at baseline (11 mM glucose) under non-ischemic perfusion conditions. Our data show that BFT treatment enhanced contractile function, implying that it possesses intrinsic inotropic properties. Moreover, we found that BFT also decreased the heart rate under these conditions. We are unclear how precisely this occurs, but previous research work...
suggests that this may constitute an energy conserving mechanism (by promoting glucose oxidation (40) and/or a direct effect of BFT on neuro-hormonal modulation (70). However, these interesting possibilities require further investigation.

We next tested whether acute BFT treatment could mediate cardio-protection in response to ischemia and reperfusion under hyperglycemic conditions. Our results demonstrate that all three BFT concentrations significantly improved contractile function and decreased infarct sizes. The increase in coronary flow may contribute to post-ischemic neovascularization previously reported (70), thus promoting oxygen supply and removing damaging metabolites to improve post-ischemic recovery (46, 54, 87). Adequate blood flow during reperfusion therefore allows efficient relaxation of the ventricles (decreased workload) (69) as observed by the decrease in end diastolic pressure with BFT treatment.

We also investigated whether BFT administration would lead to similar effects in response to chronic glucose exposure. Here acute intravenous BFT administration to STZ-induced diabetic rats following in vivo coronary artery ligations resulted in smaller infarct sizes compared to respective controls. Together these data show that post-ischemic BFT administration following acute and chronic hyperglycemia effectively blunts the harmful effects of excess glucose supply on contractile function. Of note, these findings are distinct from previous studies that employed chronic BFT treatment before the ischemic insult as a therapeutic strategy to offer cardio-protection to diabetic animals that were subjected to ischemia and reperfusion (70).

Our experiments also revealed an unexpected finding, i.e. that BFT acted as a novel cardio-protective agent when ischemia and reperfusion was performed under baseline conditions (simulated normoglycemia). We are unclear how exactly this occurs; however, our data indicate that the mechanism is likely independent of shunting of glycolytic metabolites to the pentose phosphate pathway. Several possibilities emerge that include activation of the reperfusion injury signaling kinase
(50, 83) and JAK-STAT pathways (70), and/or by alternate mechanisms. These possibilities are currently being investigated in our laboratory.

How does acute BFT treatment exert its cardio-protective effects (under hyperglycemic conditions) following an ischemic insult? The present study established that BFT administration markedly elevated transketolase activity thereby activating the non-oxidative branch of the PPP. In parallel, we found decreased activation of four non-oxidative glucose pathways thus confirming that BFT effectively shunted flux away from these detrimental pathways. Moreover, these data emphasize that there are distinct differences between non-oxidative glucose pathways with activation of AGE, HBP, polyol pathway and PKC linked to detrimental cardio-metabolic effects, while stimulation of the non-oxidative PPP branch was associated with protective effects. Our data also show that individual pathway inhibition resulted in attenuated myocardial superoxide levels for each respective pathway. In parallel, BFT supply reduced cardiac superoxide levels and concomitantly restored SOD function. Together these data demonstrate that BFT decreases overall myocardial oxidative stress by blunting ROS generation via certain non-oxidative pathways of glucose metabolism. Furthermore, BFT may also a) increase G6PD activity (70) (feed forward mechanism) and thereby further enhance myocardial antioxidant capacity, and b) attenuate ROS generated as a result of ischemia and reperfusion.

Benfotiamine administration concomitantly elicited cardio-protection in high glucose perfused hearts together with reduced cardiac cell death i.e. apoptosis and necrosis (decreased infarct size). Cardio-protection in this instance, likely results from BFT’s upstream effects, i.e. by lowering myocardial oxidative stress and attenuating cell death. Moreover, by shunting flux away from the AGE, HBP, PKC and polyol pathway, BFT effectively blunts the other damaging effects that these pathways may have on the heart’s function (earlier discussed). It is possible that BFT may have inhibited inflammation that occurs with ischemia and reperfusion, thus it may have additional beneficial effects in cardiac pathologies with inflammation such as arthritis. One of our interesting novel findings was inhibition of chymotrypsin-like proteasomal activity by BFT under high glucose conditions following ischemia and
reperfusion. As been discussed earlier in chapter 3 UPS activity varies with hyperglycemia and following ischemia and regulation. Literature also shows that posttranslational modification of UPS plays a role in regulating its activity. For example UPS activity is inhibited by protein kinase A phosphorylation (150), O-GlcNAcylation (151, 152) and glycation by AGEs (67, 108). We speculate that BFT inhibition of NOGP flux under high glucose conditions relieved the UPS of HBP-induced inhibition of the 26S. However, considering that BFT-inhibition on the AGE pathway is incomplete it implies that inhibition of the UPS chymotrypsin-like activity may be due to AGEs (67, 108) and possibly BFT itself.

4.4.3 Individual acute inhibition of NOGP attenuates the detrimental effects of hyperglycemia with ischemia and reperfusion

We tested effects of NOGP inhibition by employing various inhibitors specific for each pathway. Interestingly, inhibition of the polyol pathway at baseline significantly improved cardiac contractile function following ischemia and reperfusion with a corresponding decrease in infarct size (though did not reach statistical significance). This outcome supports previous observations regarding the role of the polyol pathway in ischemia and reperfusion damage, independent of hyperglycemia (2, 58, 59). It has been reported that ischemia increases AR activity approximately 3-fold possibly due to increased ROS produced during ischemia (65). Increased AR further increases ROS by peroxynitrite production and depletion of an important antioxidant, i.e. glutathione (128). Furthermore, increased ROS secondary to AR activation can also occur due to opening of the mitochondrial permeability transition pore (mPTP) (2). The increased activation of the polyol subsequently causes cardiac contractile dysfunction by enhancing tyrosine nitration of the SERCA and oxidation of ryanodine proteins thus impairing its functional role in cardiac contractility (65, 128). Increased oxidative stress by the polyol pathway also involves its close relationship to the AGE pathway. For example, AR catalyzes the reversible reduction of MG to hydroxyacetone with consumption of NADPH (64). Additionally, MG is also a substrate for SDH (79).
Ischemia and reperfusion under hyperglycemic conditions further increases ROS production and its effects are further exacerbated by altered glucose metabolism (76, 102). The polyol pathway also provides potent substrates for AGE formation, hence further increasing ROS levels and leading to impaired cardiac contractile function (65, 74). Furthermore, GSH depletion implies a decrease in the detoxification of MG by the GSH-dependent glyoxal system (133). Our findings show that inhibition of the polyol pathway attenuates oxidative stress as indicated by decreased superoxide levels, increased SOD, restored GAPDH and PARP activities. Thus polyol pathway inhibition under normoglycemic and hyperglycemic conditions results in cardio-protection by improving energy homeostasis (112, 113), attenuation of ROS production and changes in intracellular sodium and calcium (101, 128, 134, 139). The decreased oxidative stress with zopolrestat implies restored glycolysis, GAPDH activity and consequently PARP activity (34, 35, 47) following polyol inhibition.

Attenuation of oxidative stress may also explain the decreased caspase activity since oxidative stress can directly induce apoptosis (14, 32, 37, 68). Indeed, AR inhibition attenuates TNFα and PKC activation, and p38 MAPK and c-Jun N-terminal kinase phosphorylation (93, 111).

We found that the inhibitor effects were much more pronounced under hyperglycemic perfusion conditions. Increased MG levels under hyperglycemic conditions in untreated hearts may impair contractile cardiac function by glycative inhibition of thioredoxin activity, thus further increasing oxidative stress (138). Moreover, various ROS are produced during AGE formation thus contributing to increased oxidative stress, apoptosis, decreased GAPDH and increased PARP activities as we and other investigators have previously reported (33–35, 138). MG can increase oxidative stress by causing glycation and subsequent inactivation of glutathione reductase and glutathione peroxidase (89). Furthermore, MG can directly deplete GSH and increase oxidative stress. This in turn will further impair degradation of MG and establish a vicious cycle that leads to even greater MG levels (133).
The damaging effects of AGE may occur directly or via its interaction with RAGE binding can initiate important signaling pathways involving activation of PKC (88, 119), tyrosine phosphorylation of JAK)/STAT, (56), recruitment of PI 3-K to MAPK (78) or PKC (27, 72, 75, 122) and induction of oxidative stress cascades which culminate in NF-κβ and AP-1 transcription (7, 12, 148). Thus by inhibiting AGE formation all these damaging effects are blunted and thereby resulting in improved cardiac function.

There has been a growing body of evidence in the central role of PKC in signal transduction pathways in hyperglycemia-induced complications (61, 144). We observed increased PKC activity in our untreated hearts exposed to high glucose, and this likely occurs due to elevated DAG levels (12, 61, 144). Of note, PKC activity and expression can increase during ischemia and reperfusion in acute ischemia/ reperfusion models (126). This may either be cardio-protective or damaging depending on which isoform is activated.

Lastly we also found greater HBP activation in association with decreased cardiac function, increased oxidative stress, apoptosis, attenuated GAPDH and enhanced PARP activity in untreated hearts under hyperglycemic conditions following ischemia and reperfusion. Increased O-GlcNAcylation under hyperglycemic conditions has been previously reported to elicit similar detrimental effects on cardiac contractile function (21, 55, 84). It is likely that detrimental effects are mainly due to altered protein function, for e.g. increased protein O-linked GlcNAcylation diminished expression of cardiac SERCA and lead to impaired myocardial contractility (21, 55). Increased HBP activation also causes PKC activation thus leading to more ROS and consequently apoptosis (36, 97). Moreover, HBP-mediated induction of FAO may also blunt cardiac function under these conditions (73).

In conclusion, our data demonstrate that acute BFT treatment initiated after an ischemic insult offers significant promise as a novel therapeutic agent for AMIs under conditions of acute and chronic hyperglycemia. This may be especially useful for the alarming number of diabetic individuals suffering cardiovascular complications, and also for non-diabetic patients that exhibit stress-induced
hyperglycemia within the clinical setting. Of note, acute BFT treatment also resulted in cardio-
protection in control hearts, thereby highlighting that it actually has a broader therapeutic utility than
what would be expected. Additionally, the current study demonstrates that acute NOGP inhibition
initiated after an ischemic insult offers significant potential as therapeutic agent(s) for myocardial
infarction under acute hyperglycemic conditions. Moreover, our findings establish for the first time that
there is a convergence of downstream NOGP effects in our experimental systems, i.e. increased
myocardial oxidative stress, further pathway activation, apoptosis, and impaired contractile function.
Thus we propose a unique model to explain our intriguing findings. Here a vicious metabolic cycle is
established whereby hyperglycemia-induced NOGP further fuels its own activation by generating even
more oxidative stress, thereby exacerbating damaging effects on the heart under these conditions
(Figure 4.26).
Figure 4.26 Model to explain metabolic vicious cycle whereby hyperglycemia-mediated activation of non-oxidative pathways fuels oxidative stress and detrimental outcomes. With acute hyperglycemia, ROS are produced by activation of non-oxidative pathways of glucose metabolism: polyol pathway, HBP, PKC and AGE (refer blue arrows). This results in a vicious metabolic cycle i.e. pathway activation further increasing ROS generation and again activating non-oxidative glucose pathways. AR: aldose reductase; SDH: sorbitol dehydrogenase; Glc: glucosamine; Glu: glutamate; GFAT: glutamine: fructose-6-phosphate amidotransferase; UDP-GlcNAc: uridine diphosphate-N-acetylglucosamine; DHAP: dihydroacetone phosphate; DAG: diacylglycerol; AR: aldose reductase; SDH: sorbitol dehydrogenase; ROS: reactive oxygen species; G 3-P: glyceraldehyde 3-phosphate; GSSG: oxidized glutathione; GSH: reduced glutathione.
4.5 References


40. Fraser DA, Hessvik NP, Nikolić N, Aas V, Hanssen KF, Bøhn SK, Thoresen GH, Rustan AC. Benfotiamine increases glucose oxidation and downregulates NADPH oxidase 4


52. **Herbert JM, Augereau JM, Gleye J, Maffrand JP.** Chelerythrine is a potent and specific inhibitor of protein kinase C. *Biochem Biophys Res Commun* 172: 993–999, 1990.


318


Chapter 5

Final conclusions, limitations and recommendations

5.1 Conclusions

This study examined the damaging effects of hyperglycemia in the setting of ischemia and reperfusion. Several conclusions can be drawn from our work. We initially demonstrated that significant metabolic dysregulation occurs with ischemia and reperfusion under hyperglycemic conditions (acute and chronic). Specifically, we found that oxidative stress plays a key role in this process by triggering activation of non-oxidative glucose pathways (NOGP) metabolism that results in damaging outcomes for the heart. This sequence of events were deduced by evaluating our hypothesis in multiple experimental systems, i.e. in vitro, ex vivo and in vivo models of hyperglycemia using H9c2 cells, Langendorff heart perfusions and STZ-induced diabetic rats, respectively.

Another key objective was to identify novel therapeutic agents that are able to blunt the detrimental effects of hyperglycemia in the context of ischemia and reperfusion. Our findings show that both oleanolic acid (OA) and benfotiamine (BFT) offer exciting prospects to treat acute and chronic hyperglycemia-induced cardiac contractile dysfunction. Of note, acute BFT treatment also resulted in cardio-protection in control hearts, thereby highlighting that it actually has a broader therapeutic utility than what would be expected. This therefore opens up significant opportunities to effectively translate our basic findings into the clinical setting. However, more pre-clinical and small-scale clinical studies are required to further test the cardio-protective effects of OA and BFT. Our data also indicate that the UPS may be a unique therapeutic target to treat ischemic heart disease in individuals that present with stress-induced, acute hyperglycemia. However, this interesting possibility requires further investigation. The current study also establishes for the first time – as far as we are aware - that there is a convergence of downstream NOGP effects in our model, i.e. increased myocardial oxidative stress, further NOGP pathway activation, apoptosis, and impaired contractile function.
There are various mechanisms we speculate the inhibitors of the NOGPs may have exerted their cardio-protective effects under hyperglycemic conditions following ischemia and reperfusion. Inhibition of the polyol pathway may have attenuated oxidative stress by prevention of activation of other NOGPs or opening of the mitochondrial permeability transition pore (mPTP) independent of hyperglycemia, restored calcium homeostasis by preventing tyrosine nitration of the sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA) and oxidation of ryanodine proteins. These same mechanisms may also have been involved in the improved function with polyol inhibition at baseline glucose levels. Furthermore, inhibition of the polyol pathway has shown improved cardiac energy metabolism under both normoglycemic and hyperglycemic conditions. AGE inhibition also attenuates oxidative stress and improves contractile function since glycation of thioredoxin and phosphorylation of glycogen synthase 3-beta do not occur. Inhibition of PKC implies that there is no downward activation of PKC-dependent damaging signaling pathways such as activation of nuclear factor kappa beta. With the use of an inhibitor for the HBP pathway there is less O-GlcNAcylation hence protein function maintained and restored expression of SERCA (calcium homeostasis).

In summary, our data demonstrate that both OA and BFT may be useful therapies in the setting of acute myocardial infarction, especially for the alarming number of non-diabetic and diabetic individuals saddled with acute and chronic hyperglycemia, respectively. We are of the opinion that this is particularly relevant within the developing world context, where it may provide cost-effective therapeutic interventions for the treatment of acute myocardial ischemia in such individuals.

5.2 Limitations and future recommendations

For the in vitro studies there were no parallel experiments carried out to rule out the osmotic effect as previously described in our laboratory (2). Furthermore superoxide levels were the major oxidant measured, however, we acknowledge that other ROS and RNS may also be implicated in the
damaging effects of hyperglycemia. Although lucigenin chemiluminescence was used to measure superoxide levels, this method has shortfalls since superoxide are short-lived and lucigenin auto-oxidizes to produce superoxide and may therefore decrease sensitivity of superoxide detection (1). For future studies it is recommended that a variety of alternative markers of oxidative stress be measured e.g. aconitase, reduced and oxidized glutathione levels, dihydroethidium and lipid peroxidation. The source of superoxide ions was not determined in all the studies and hence these may have to be determined in future studies for example: *in vitro* with various markers (e.g mitoSox for mitochondrial superoxide), using L-NG-Nitroarginine Methyl Ester (L-NAME) for inhibition of NOS; cyclosporine to determine mPTP opening contribution). Furthermore expression of the enzymes involved in superoxide production may be determined by Western blotting or immunohistochemistry. Another point to consider is that for NOGP activation we measured metabolite concentrations as markers of pathway stimulation. However, an assessment of actual metabolic flux will further strengthen our existing data (3). The effects of OA, BFT and the specific inhibitor drugs used may be due to their non-specific effects hence in order to rule out these effects dose-dependent experiments should be performed using *in vitro*, *ex vivo* and *in vivo* models to analyze NOGP activation, oxidative stress, apoptosis, and cardiac functional effects. For future studies it is recommended to investigate effects of standard anti-diabetic drugs (e.g. insulin, metformin) on flux via NOGP. Finally, we propose that additional studies are required to elucidate the protective mechanisms of BFT at baseline glucose levels (e.g. JAK/STAT pathways).

### 5.3 References


APPENDICES

Appendix 1

Prof. M. F. Essop
Physiological Sciences,
Stellenbosch University
Stellenbosch

Dear Prof. Essop

Application for Ethical Clearance:
BENFOTIAMINE ATTENUATES HYPERGLYCEMIA-MEDIATED DECREASE IN MYOCARDIAL FUNCTION IN RESPONSE TO ISCHEMIA-REPERFUSION
Ref: 13NP_ESS01

4 June 2010

Your application for ethical clearance has been approved by the SU ACU committee. Please note that this clearance is only valid for a period of twelve months. Ethical clearance of protocols spanning more than one year must be renewed annually through submission of a progress report, up to a maximum of three years.

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2006 document will be available on the Division for Research Development’s website www.sun.ac.za/research, shortly.

Please feel free to contact Mr. Winston Beukes if any additional information is needed.

Kind regards,

Debbie Fouche (Manager, Research Support)
29 June 2012

AEC REF NO: 012/030

Dr R Kelly-Laubscher
Human Biology
Anatomy Building

Dear Dr Kelly-Laubscher,

PROJECT TITLE: NOVEL AGENTS THAT BLUNT HYPERGLYCEMIA-INDUCED CARDIAC DYSFUNCTION IN THE RAT.

Thank you for submitting your study to the Faculty of Health Sciences Animal Research Ethics Committee for review.

It is a pleasure to inform you that the FHS AEC has authorised the above mentioned study specifically for the use of 170 Wistar rats for the period of 18 months.

Please note that the first annual progress report is due in June 2013.

Please quote the REC. REF in all your correspondence.

Yours sincerely,

PROF GRAHAM LOUVIN
CHAIR, HSF AEC
Appendix 3

Krebs-Henseleit stock solutions

1. NaCl (279g/ 2L)
2. NaHCO₃ (83.6g/ 2L)
3. KCl (17.6g) + K₂HPO₄ (8.1 g) in 2 L
4. MgSO₄. 7H₂O (7.4g) + NaSO₄ (4.2g) in 1L
5. CaCl₂.2H₂O (18 g) in 1L

To make up the 5 L working Krebs-Henseleit buffer mix 250ml of 1 and 2; add 100ml of 3 and 4 then add glucose and finally # 5. Filter and pH to 6.7 with HCl or NaOH. To make up 11 mM glucose add 9.9 g and increase twice or thrice for 22 mM and 33 mM buffer solutions, respectively.

Appendix 4

Caspase-Glo® 3/7 assay

H9c2 cells in a 96-well format

Preparation of working reagent solution and storage:

Mix the Caspase-Glo® 3/7 buffer reagent gently and allow to equilibrate at room temperature. Transfer the lyophilized substrate to the buffer and mix by swirling. Store at -20°C. (Note that reconstituted reagent that is freeze thawed will display diminished signal over time – approximately 60% compared to freshly prepared reagent after 4 weeks according to the manufacturer. However, little reduction in signal intensity was noticed over longer time periods of freeze thawing in our experiments.)

Assay protocol:

Allow the working buffer reagent to equilibrate at room temperature for at least 30 minutes. Remove plates containing cells from 37°C growing conditions to allow them to equilibrate at room temperature. (At least 10 minutes). Transfer 50 µl (1:1) of working reagent to each well containing cells. Mix plates on a shaker for 30 seconds. Incubate plates in the dark for 1 hour at constant room temperature. (Can incubate for up to 3 hours). Measure the luminescence in a luminometer.
Modified RIPA Buffer:

A 100 ml modified RIPA buffer contains:

- 50 mM Tris-HCl (790 mg of Tris in 75 ml distilled water and 900 mg of NaCl and pH made 7.4 using HCl)
- 10 ml of 10% NP-40 [final concentration 1%]
- 2.5 ml of 10% sodium deoxycholate [final concentration 0.25%]
- 1 ml of 100 mM EDTA pH 7.4 [final concentration 1 mM]
- Protease inhibitors
  - 500 µL of 200 mM phenylmethylsulfonyl fluoride (PMSF) [final concentration 1 mM]
  - 100 µL of Leupeptin (1 mg/ml water) [final concentration 1 µg/ml]
  - 80 µL of SBT1 (5 mg/ml water) [final concentration 4 µg/ml]
  - 100 µL of Benzamidine (1 M) [final concentration 1 mM]
- Protein phosphatase inhibitors
  - 500 µL of 200 mM activated sodium orthovanadate (Na$_2$VO$_3$) [final concentration 1 mM]
  - 500 µL of 200 mM NaF [final concentration 1mM]
- 1 ml Triton X-100

This buffer is then made up to a final volume of 100 ml with distilled water and stored at -20°C.
Appendix 6

Bradford protein quantification method:

Bradford reagent

- 500 mg of Coomassie Brilliant Blue G in 250 ml of 95% ethanol
- 500 ml of phosphoric acid

This is made up to 1 litre using distilled water, filtered and stored at 4°C.

Working solution:

The Bradford stock is diluted in 1:5 ratio using distilled water, filtered and used for protein quantification.

Bradford method:

BSA (1 mg/ml) is diluted in a 1:4 ratio using distilled water. A protein standard with varying protein concentrations is prepared as follows:

<table>
<thead>
<tr>
<th>BSA (µL)</th>
<th>BSA concentration (µg)</th>
<th>Volume of distilled water (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Blank</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>90</td>
</tr>
<tr>
<td>20</td>
<td>4</td>
<td>80</td>
</tr>
<tr>
<td>40</td>
<td>8</td>
<td>60</td>
</tr>
<tr>
<td>60</td>
<td>12</td>
<td>40</td>
</tr>
<tr>
<td>80</td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td>100</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Unknown protein sample</td>
<td>95</td>
</tr>
</tbody>
</table>
To all these protein and BSA standards, 900 µL of Bradford working solution is added and vortexed gently. Samples were allowed to stand for ~ 5 minutes. The absorbancies of each sample was read using a spectrophotometer at 595 nm. (If the protein absorbancies fall outside the protein standard, the proteins must be diluted with RIPA buffer and reading taken again). The absorbancies were then plotted to construct a linear plot for the standards. Thereafter the amounts of protein in unknown samples were quantified in relation to the linear standard plot.

**Appendix 7**

**3x Sample buffer (pre-made, bench)**

For a 0.5 M Tris solution, add 3.03 g to 50 ml distilled water and adjust the pH to 6.6. Take 33.3 ml and add 8.8 g SDS, 20 g Glycerol and a tiny bit Bromophenol blue. Fill up to 75 ml with dH2O.

**Sample preparation (tissue lysates) Western blotting- SDS-PAGE:**

Set heating block temperature to 99 °C and keep samples on ice, and allow to thaw. A stock solution of sample buffer containing 850 µL sample buffer and 150 µL mercaptoethanol is prepared. A volume of sample buffer equivalent to 1/3 final volume of the sample if added (under the fume hood). The appropriate amounts of proteins are added to each tube calculated previously. Tiny holes are punched on the lids of microfuge tubes (containing the prepared sample) and placed in boiling water for about 5 minutes. The tubes are then briefly spun in a table top centrifuge. Samples can now be used for Western blot analysis or alternatively be stored at -80°C for later use.

**SDS =** denature proteins, constant anionic charge-to-mass ration

**Glycerol =** give sample a higher density than buffer to “sink” to the bottom of the well

**Mercaptoethanol =** reduce disulfide bonds present in protein sample
Appendix 8

SDS-PAGE -Western blotting

Clean pairs of large and small glass plates with methanol and a paper towel. Place the small glass plate onto the large plate and slide these into the green assembly. Tighten the assembly by pushing the green clips outward. Place the assembly onto the rubber base, pushing down gently. Prepare two small beakers, two Pasteur pipettes and a small stirring bar. Fill one beaker with H₂O and prepare isobutanol.

Gel recipe for 10% 0.75mm gels:

<table>
<thead>
<tr>
<th></th>
<th>2gels:</th>
<th>4 gels</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>3850</td>
<td>7700</td>
</tr>
<tr>
<td>1.5M Tris-HCl pH 8.8 (68.1 g Tris base dissolved in 1 liter dH₂O)</td>
<td>2500</td>
<td>5000</td>
</tr>
<tr>
<td>10 % SDS (stock)</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>10 % APS (0.1 g/ml)</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>Acrylamide (40 %)</td>
<td>2500</td>
<td>5000</td>
</tr>
<tr>
<td>(carcinogenic)</td>
<td>(add at very end in fume hood)</td>
<td>5</td>
</tr>
</tbody>
</table>

Mix solution and pour between the glass plates using a Pasteur pipette leaving enough space for the stacking gel. Add a layer of isobutanol using a fresh Pasteur pipette. Allow to set for 45 minutes - 1 hour. In the meantime prepare running buffer in a 1:10 dilution.
After 30-45 minutes has passed begin to prepare the stacking gel (4% recipe):

<table>
<thead>
<tr>
<th>Component</th>
<th>2gels: µl</th>
<th>4 gels µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>3050</td>
<td>6100</td>
</tr>
<tr>
<td>0.5M Tris-HCl pH 8.8 (6.06 g Tris base dissolved in 1 liter dH₂O)</td>
<td>1250</td>
<td>2500</td>
</tr>
<tr>
<td>10 % SDS (stock)</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>10 % APS (0.1 g/ml)</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Acrylamide (40%)</td>
<td>500</td>
<td>1000</td>
</tr>
<tr>
<td>Temed</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Once the gels are set (after ~1 h), wash off the isobutanol and ensure the plates are dry. Add Temed to the stacking solution, quickly and add stack between the plates. Gently push the combs (of the correct width) into the stacking gel. Allow to set for 30 minutes. Retrieve prepared samples from the -80 C freezer and allow to thaw on ice. Once thawed, vortex each sample briefly before denaturing on the heating block for 5 minutes.

In the meantime, remove the combs carefully from the gels and wash gently with deionized water being careful not to damage the wells. Take the gel plates out of the assembly stand and place them in the U-shaped adaptor cassette with the small plates facing inward. Place the U-shaped adaptor into the loading system and push the latches closed, away from your body. Carefully pour running buffer into the middle compartment between the gel plates, allowing the buffer to flow over the wells. Add 10 µl of pre-stained weight marker (peqGOLD, PEQLAB Biotechnologie GMBH, Germany) into the first well on the left of each gel for orientation and electrophoretic determination of molecular weights of specific bands.
Hereafter add 50 µg of protein samples into each well in the desired order using a micropipette and clean loading tips for every sample. Place the system into the outer running chamber, add the running buffer until ~1cm below the wells. Place the green lid with electrical leads onto the cell system, making sure to attach the electrodes correctly i.e. red to red and black to black. Run gels were run for 60 minutes at 100 V (constant) and 400 mA (Mini Protean System, Bio-Rad, USA) for 10 minutes then the voltage was increased to 200 V with the same current for 60 minutes.

Appendix 9

Electrotransfer of proteins:

Cut two chromatography filter papers and one 0.2 micron polyvinylidene fluoride (PVDF) membranes (Immunibilon, Millipore, USA). Soak filter papers in transfer buffer and for the membrane first soak in methanol for 15 seconds, thereafter wash with distilled water before soaking in transfer buffer. Place on filter paper onto the semi-dry apparatus (Bio-Rad, USA) and carefully place the PVDF membranes on top. Roll with a wet tube to remove any bubbles. Hereafter place gels onto membranes making sure to get rid of any bubbles with a wet tube. To complete the sandwich place filter paper on top of the gel, close the system and run at limit 0.5 A and 15 V for ~ 1 h.
Detection of proteins (Western blots): Probing the membrane

In order to prevent non-specific binding membranes were blocked in 5 % (weight/volume) fat-free milk in 0.1 % Tris Buffered Saline-Tween 20 (TBS-T see Appendix 14) for 2 hours at room temperature. Membranes were then incubated with the respective primary antibody diluted in 5 % (w/v) fat-free milk in 0.1 % TBS-T (1: 1000), overnight at 4 °C. Hereafter on the following day, membranes were washed a further three times in TBS-T (3x5 minutes) before being incubated in the secondary anti-mouse/rabbit/sheep/goat HRP monoclonal antibody (1:4000) for 1 hour at room temperature with gentle agitation.

For detection of the antibodies, membranes were treated with 2 ml ECL LumiGLO Reserve™ chemiluminescent substrate kit (KPL, Inc., USA) as per manufacturer’s instructions. It was then dried on tissue paper, placed between transparencies and was then developed in a dark room where bands were exposed to autoradiography film (Hyperfilm, Amersham Biosciences, UK). The film was then developed by placing it in developing solution until bands appeared followed by 15 seconds in a fixative. This film was then visualised and quantified by densitometry using the UNSCAN-IT© densitometry software (Silk Scientific Corporation, Utah, USA). All bands were expressed as optical density readings relative to a control present on the same blot.
Appendix 11

Preparation of the triphenyl-tetrazolium chloride (TTC) solution

Make up a high pH phosphate buffer by dissolving 14.2 mg of Na$_2$HPO$_4$ in a liter of distilled water and a low pH phosphate buffer by dissolving 12 mg of NaH$_2$PO$_4$ in a liter of distilled water. Mix in a ratio of 8 parts of the high pH buffer to 2 for the low pH buffer. pH to 7.4. For every heart use 5 ml of the mixed phosphate buffers and weigh 10 mg of the TTC salt for every 1 ml of the solution. Incubate the cut heart slices at 37 °C for 20 min shaking at least once.

Appendix 12

Determination of infarct size and area at risk using Image J software

Select the heart slices using the third tool for polygon selections in the tools bar and pressing down on the Shift key of the keyboard. Determine the total area by clicking the Edit tab then select clear outside, thereafter click the Analyze tab and select Measure. A table with the first set of results shows and for infarct size use the area values. Thereafter click on the Image tab, select Split channels. Here 3 windows will open i.e. green, red, and blue channels. Close the blue channel, use values from the green channel will be used for calculating infarct size and from the red channel area at risk. Here, it is advisable to do the analysis with a picture of the heart alongside. In either of the green or red channel, click on the Image tab, select Adjust threshold and using the eighth tool select the respective highlighted area in the heart slices corresponding to the infarct size and area at risk, respectively. Thereafter click on the Analyze tab and select measure. At the end three readings should appear for total area, infarct size and area at risk. For the calculations substract the second and third readings from the total area to obtain infarct size and area at risk values, respectively. These are calculated for both sides of the heart slices and expressed as infarct size/area at risk.
Appendix 13

Preparation of citrate buffer for dissolving streptozotocin

To make the buffer prepare 0.1 M sodium citrate and 0.1 M citric acid as follows:

Weigh 0.192 g citric acid and dissolve in 10 ml dH$_2$O. Weigh 0.294 g sodium citrate and dissolve in 10 ml dH$_2$O.

Pipette 1.8 ml citric acid and add to 8.2 ml of sodium citrate. Bring final volume to 100 ml with dH$_2$O and adjust pH to 6.2 with sodium hydroxide or hydrochloric acid.

Appendix 14

Phosphate buffered saline (PBS) preparation

Dissolve the following in 800 ml of dH$_2$O:

- 8 g NaCl
- 0.2 g KCl
- 1.44 g Na$_2$HPO$_4$
- 0.24 g KH$_2$PO$_4$

Adjust pH as desired, add more dH$_2$O to make it 1L, sterilize and autoclave. Store at room temperature.
Appendix 15

Buffers used during Western blotting

**10 x TBS** (STOCK – store in fridge)

- 48.4 g Tris
- 160 g NaCl

Dissolve in 500 ml distilled water, set pH to 7.6 with concentrated HCl and then make up to 2 L.

**10X running buffer** (STOCK – store in fridge)

- Tris base 60.6 g
- Glycine 288 g
- 10 % SDS 20 g

Dissolve in 2 liter distilled water.

**Transfer buffer (ready to use)**

In a 1L bottle add:

- 100 ml Biorad 10X transfer buffer
- 200 ml methanol
- 700 ml dH₂O
Appendix 16

Transketolase assay protocol

Prepare:

- 250 mM of Glycylglycine buffer, pH 7.7 at 25°C in dH₂O using Glycylglycine, Free Base.
- 1 ml 100 mM Xylulose 5-Phosphate solution (X 5-P) in dH₂O using D-X 5-P, Sodium Salt.
- 1 ml 50 mM Ribose 5-Phosphate Solution (R 5-P) in dH₂O using D-Ribose 5-Phosphate, Disodium Salt.
- 1 ml 0.10% (w/v) Cocarboxylase (Thiamine Pyrophosphate) Solution in cold dH₂O using Cocarboxylase (PREPARE FRESH.)
- Dissolve the contents of one 5 mg vial of β-NADH, Disodium Salt (PREPARE FRESH.)
- 1 ml 300 mM Magnesium Chloride Solution (MgCl₂) in deionized water using Magnesium Chloride
- a solution containing 2000 TPI units/ml of α-Glycerophosphate Dehydrogenase/ Triosephosphate Isomerase (α-GDH/TPI) (Immediately before use) in cold dH₂O
- a solution containing 5.0 units/ml of Transketolase Enzyme Solution in cold Glycylglycine buffer
- (Immediately before use)

Pipette in a 96 well microtiter plate as follows:

<table>
<thead>
<tr>
<th></th>
<th>µl</th>
<th>µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycylglycine buffer</td>
<td>248</td>
<td>248</td>
</tr>
<tr>
<td>X 5-P</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>R 5-P</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Cocarboxylase solution</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>NADH solution</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>a-GDH/TPI solution</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Sample/dH₂O for blank</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Immediately mix and record decrease in Absorbance at 340nm for approximately 10 minutes.