Compounds specific to *Aspalathus linearis* protect the diabetic heart against oxidative stress: a mechanistic study

by

Phiwayinkosi Vusi Dludla

Dissertation presented for the degree of Doctor of Philosophy (Medical Physiology) in the Faculty of Medicine and Health Sciences at Stellenbosch University

Supervisor: Dr R. Johnson

Co-supervisors: Prof B. Huisamen and Prof M. F. Essop

December 2016

Declaration

By submitting this thesis, 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.

Signature: …… Date: ……

Summary

In diabetics, hyperglycemia, hyperlipidemia and inflammation potentiates the development of cardiovascular diseases (CVDs). These conditions provoke excessive generation of oxidative stress that has been implicated in the pathogenesis of diabetic cardiomyopathy (DCM). In the diabetic state, excessive generation of free radicals can cause oxidative damage to DNA and alter protein and lipids, leading to the activation of various death-induced signaling pathways. Activation of these death pathways result in structural and functional modifications to the myocardium. Current diabetic drug therapies do not protect the diabetic heart at risk from developing cardiovascular complications. Thus, in search for new therapeutics, we aim to unravel the molecular mechanisms associated with the protective effect of two major bioactive compounds from *Aspalathus linearis,* phenyl pyruvic acid-2-*O*-β-D-glucoside (PPAG) and aspalathin against hyperglycemia-induced oxidative stress and apoptosis in H9c2 cardiomyocytes.

The study showed that PPAG and aspalathin were able to decrease mitochondrial membrane depolarization and prevent hyperglycemia-induced myocardial apoptosis by increasing the Bcl2/Bax ratio. We revealed that while both compounds were able to reduce hyperglycemia-induced apoptosis, only aspalathin could ameliorate lipid toxicity and oxidative stress-associated with insulin resistance.

An important feature of the failing heart is the observed shift in mitochondrial substrate preference that precedes the onset of oxidative damage. The current study revealed that aspalathin improved glucose metabolism by decreasing fatty acid uptake and subsequent β-oxidation. This was achieved through decreasing the expression of adenosine monophosphate-activated protein kinase threonine 172 (pAMPK (Thr172)) and carnitine palmitoyltransferase 1 (Cpt1), while increasing that of acetyl-CoA carboxylase (Acc) and glucose transporter 4 (Glut4). Additionally, it is known that cardiomyocytes have a very low antioxidant capacity and a shift in mitochondrial substrate preference can result in accelerated oxidative damage. In this study, we showed that aspalathin ameliorated oxidative stress by increasing the antioxidant capacity of the cells through activation of the antioxidant response pathway, nuclear factor erythroid 2 (NF-E2)-related factor 2 (*Nrf2*) and its downstream target genes. Moreover, we showed that aspalathin was able to reverse lipid toxicity by increasing the expression of Adiponectin, C1Q and collagen domain containing (*Adipoq*) and concomitantly decreasing cluster of differentiation 36 (*Cd36*) and Cpt1 mRNA expression. We further observed that *Adipoq* negatively regulated sterol regulatory element binding transcription factor 1 (*Srebf1*), stearoyl-Coenzyme A desaturase 1 (*Scd1*) and solute carrier family 27 (fatty acid transporter), member 3/5 (*Slc27a3/5*). This led to reduced lipid accumulation in H9c2 cardiomyocytes, with an associated decrease in total cholesterol, triglycerides and low-density lipoprotein in leptin resistant db/db mice. This was accompanied by decreased mRNA expression of inflammation markers in H9c2 cells, including

interleukin 3 and 6 (*IL3* and *IL*6), tumor necrosis factor receptor superfamily, member 1b and 13 (*Tnfrsf1b* and *Tnfsf13*), Janus kinase 2 (*Jak2*) and mitogen-activated protein kinase 3 (*Mapk3*).

Together our results infer that aspalathin can slow down the progression of DCM, and thus protect the myocardium against causal factors associated with the development and progression of CVD.

Opsomming

In diabete, hiperglukemie, hiperlipidemie en inflammasie versnel die ontwikkeling van kardiovaskulêre siektes (CVDs). Hierdie toestande lei tot oormatige generasie van oksidatiewe stres wat betrokke is by die patogenese van diabetiese kardiomiopatie (DCM). In die diabetiese toestand, kan oormatige generasie van vrye radikale oksidatiewe skade aan DNA asook proteïne en vette verander, wat tot die aktivering van verskeie seldood seinpaaie kan lei. Aktivering van hierdie seldood seinpaaie veroorsaak strukturele en funksionele wysigings aan die miokardium. Huidige medikasie beskerm nie die diabetiese hart teen die ontwikkeling van kardiovaskulêre komplikasies nie. In die studie ondersoek ons die beskermende effek van twee hoof bioaktiewe verbindings van *Aspalathus linearis*, fenielpirodruiwesuur-2-*O*-β-D-glukosied (PPAG) en aspalatien, as nuwe terapeutiese middels, teen oksidatiewe stres en hiperglukemie-geïnduseerde apoptose in H9c2 kardiomiosiete en ontrafel ons die molekulêre meganismes wat betrokke is.

Die studie het getoon dat PPAG en aspalatien mitokondriale membraan depolarisasie verminder en hiperglukemie-geïnduseerde miokardiale apoptose voorkom deur die Bcl2/Bax verhouding te verhoog. Die studie toon verder dat alhoewel beide verbindings in staat was om hiperglukemie-geïnduseerde apoptose te verminder, net aspalatien lipiedtoksisiteit en oksidatiewe stres, wat verband hou met insulien weerstand, verbeter.

'n Belangrike kenmerk van hartversaking is die verskuiwing in mitokondrialesubstraat voorkeur wat die aanvang van oksidatiewe skade voorafgaan. Die huidige studie het getoon dat aspalatien glukose metabolisme verbeter deur vetsuuropname en daaropvolgende β-oksidasie te verminder. Dit word bereik deur die verlaagde uitdrukking van adenosienmonofosfaat geaktiveerde proteïenkinase threonien 172 (pAMPK (Thr172)) en karnitien palmitoyltransferase 1 (Cpt1), terwyl die ekspresie van asetiel-KoA karboksilase (Rek) en glukose transporter 4 (Glut4) verhoog word. Verder is dit bekend dat kardiomiosiete baie lae antioksidant kapasiteit het en gevolglik dat 'n verskuiwing in mitokondrialesubstraat oksidatiewe skade kan veroorsaak. Die studie wys dat aspalatien die antioksidantkapasiteit van die selle beskerm en oksidatiewe stres verlig deur aktivering van kernfaktor erythroïd-faktor (NF-E2)-verwante faktor 2 (*Nrf2*) antioksidant response element seintransduksie-pad. Aspalatien kon ook lipiedtoksisiteit verhoed deur adiponektien, C1Q en kollageen domein-verwante (*Adipoq*) ekspressie te verhoog met gepaardgaande afname van groepering van differensiasie 36 (*Cd36*) and *Cpt1* mRNA uitdrukking. Ons het verder opgemerk dat Adipoq sterol regulatoriese element bindende transkripsie faktor 1 (*Srebf1*), stearoyl-koënsiem A desaturase 1 (*Scd1*) en opgeloste stof-draer familie 27 vetsuur transporter lid 3/5 (*Slc27a3/5*) ekspresie onderdruk, wat lipied akkumulasie in H9c2 kardiomiosiete teenwerk. In leptien weerstandige db/db muise het aspalatien totale cholesterol, trigliseriede en lae-digtheid lipoproteïen verlaag. Dit het gepaard gegaan met 'n afname in mRNA

uitdrukking van inflammasie merkers, wat interleukin 3 and 6 (*IL3* and *IL6*), tumor nekrose faktor reseptor superfamilie, lede 1b and 13 (*Tnfrsf1b* and *Tnfsf13*), Janus kinase 2 (*Jak2*) en mitogen-activated protein kinase 3 (*Mapk3*) insluit.

In samevatting toon ons resultate dat aspalatien die ontwikkeling van DCM kan vertraag en daardeur die miokardium teen oorsaaklike faktore wat met die ontwikkeling en vordering van CVD verbind word, beskerm.

Acknowledgments

My sincere thanks and appreciation to the following people and institutions without whom this work could have not been a success:

My supervisor, Dr Rabia Johnson, thank you for your excellent guidance and motivation as a mentor. Thank you for your commitment and willingness to put in extra work and preparation of this manuscript.

Prof Elizabeth Joubert, Prof Barbara Huisamen, and Prof Faadiel Essop, thank you for your consistent, timely and thorough contributions in shaping this project and preparing manuscripts for publication purposes.

Dr Johan Louw and Dr Christo Muller, thank you for believing in me and your valuable support throughout this project and my stay at the Biomedical Research and Innovation Platform (BRIP).

The Primate Unit and Delft Animal Centre of the South African Medical Research Council (SAMRC) for granting us an opportunity to use their animal facility, special thanks to Joritha van Heerden for technical assistance.

My colleagues at BRIP, thank you for your support. Specific thanks to Charna Chapman, Desmond Linden, Kwazi Gabuza, Monique Rossler, Samira Ghoor and Sithandiwe Mazibuko for all technical assistance.

Institutes that supported this project financially. This work was funded by the National Research Foundation (NRF): Thuthuka Programme Grant 87836. The grant holders acknowledge that opinions, findings and conclusions or recommendations expressed herein by the NRF supported research are those of the authors, and that the NRF accepts no liability whatsoever in this regard. Funding from the SAMRC: Research Capacity Development and BRIP, Stellenbosch University, Ernst Ethel Erikson Trust, and Whitehead Scientific (travel grant) are also acknowledged.

To family and friends, thank you for your encouragement and constant belief in my potential during this journey and preparation of this manuscript.

To my mother Busisiwe Dludla, thank you for your prayers and unconditional love. I can never find words to describe the depth and value of the sacrifices you have made to ensure my dreams are fulfilled.

List of publications

Journal articles

P. Dludla, C. Muller, E. Joubert, J. Louw, K. Gabuza, B. Huisamen, F. Essop and R. Johnson. Phenylpyruvic Acid-2-*O*-β-D-glucoside attenuates high glucose-induced apoptosis in H9c2 cardiomyocytes. Planta Medica 2016; 82: 1–7.

R. Johnson, **P. Dludla**, E. Joubert, F. February, S. Mazibuko, S. Ghoor, C Muller and J. Louw. Aspalathin, a dihydrochalcone *C*-glucoside, protects H9c2 cardiomyocytes against high glucose induced shifts in substrate preference and apoptosis. Molecular Nutrition and Food Research 2016; 60: 922–934.

Presentations at scientific meeting

P. Dludla, E. Joubert, J. Louw, C. Muller, B. Huisamen, F. Essop and R. Johnson. Aspalathin protects H9c2 cardiomyocytes against hyperglycemia induced oxidative injury. International Diabetes Federation World Congress 2015 (IDF). Vancouver, Canada, November 2015.

P. Dludla, E. Joubert, J. Louw, C. Muller, B. Huisamen, F. Essop and R. Johnson. A phenylpropenoic acid glucoside (PPAG) of *Aspalathus linearis* protects H9c2 cardiomyocytes against hyperglycemia-induced cell apoptosis. 63rd International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA). Budapest, Hungary, August 2015.

P. Dludla, E. Joubert, J. Louw, C. Muller, B. Huisamen, F. Essop and R. Johnson. The cardio-protective properties of PPAG against diabetic induced cardiomyopathy: an *in vitro* study. 50th Congress of the Society for Endocrinology, Metabolism and Diabetes of South Africa (SEDMSA). Bloemfontein, South Africa, April 2015.

P. Dludla, E. Joubert, J. Louw, C. Muller, B. Huisamen, F. Essop and R. Johnson. Aspalathin ameliorates glucose induced oxidative stress and cell apoptosis in cardiac muscle cells. Stellenbosch University 58th Annual Academic Day. Cape Town, South Africa, August 2014.

P Dludla, E Joubert, J Louw, C Muller, B Huisamen, F Essop and R Johnson. The *in vitro* cardio-protective effect of aspalathin against diabetic induced cardiomyopathy. 42nd Annual Conference of the Physiology Society of Southern Africa (PSSA). Durban, South Africa, September 2014.

Table of contents

List of figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of tables

Chapter 4

Chapter 5

List of abbreviations

Abca1	ATP-binding cassette, subfamily A (ABC1), member 1
ACC	Acetyl-CoA carboxylase
Acsl	Acyl-CoA synthetase long-chain family
Acsm	Acyl-CoA synthetase medium-chain family
Adipoq	Adiponectin, C1Q and collagen domain containing
AGEs	Advanced glycation end products
Akt1	V-akt murine thymoma viral oncogene homolog 1
AMI	Acute myocardial infarction
Apo	Apolipoprotein
ARE	Antioxidant response element
ASP	Aspalathin
Bax	Bcl-2-like protein 4
Bbc3	Bcl-2 binding component 3
Bcl2	B-cell lymphoma 2
BW	Body weight
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
Casp3	Caspase 3
Cat	Catalase
CCL3	Chemokine (C-C motif) ligand 3
Cd36	Cluster of differentiation 36
Cd3e	CD3 antigen, epsilon polypeptide
Cd44	Cd44 molecule
Chuk	Conserved helix-loop-helix ubiquitous kinase

Cpt1	Carnitine palmitoyltransferase 1
CVDs	Cardiovascular diseases
DAG	Diacylglycerol
DCFH-DA	Dichloro-dihydro-fluorescein diacetate
DCM	Diabetic cardiomyopathy
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle's Medium
Dnm1l	Dynamin 1-like
DPBS	Dulbecco's phosphate buffered saline
ETC	Electron transport chain
Fabp3	Fatty acid binding protein 3
FAO	Fatty acid oxidation
Faslg	Fas ligand (TNF superfamily, member 6)
FAT	Fatty acid translocase
FAU	Fatty acid uptake
FBS	Fetal bovine serum
FFAs	Free fatty acids
FI	Food intake
FPG	Fasting plasma glucose
FW	Fat weight
Gclc	Glutamate-cysteine ligase catalytic subunit
Gclm	Glutamate-cysteine ligase modifier subunit
Glut1	Glucose transporter 1
Glut4	Glucose transporter 4
Gpx2	Glutathione peroxidase 2

GRE	Green rooibos extract
GSH	Glutathione
Gsr	Glutathione reductase
Gss	Glutathione synthetase
Gst	Glutathione S-transferase
H&E	Hematoxylin and eosin
HDL	High density lipoprotein
HF	Heart failure
HG	High glucose
Hmxo1	Heme oxygenase
HOMA-IR	Homeostasis model assessment: insulin resistance
HW	Heart weight
IDF	International Diabetes Federation
Igf1	Insulin-like growth factor 1
IGT	Impaired glucose tolerance
IHD	Ischemic heart disease
IL18	Interleukin 18
IL1β	Interleukin 1 beta
IL6	Interleukin 6
IL8	Interleukin 8
Jak2	Janus kinase 2
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide
JNKs	c-Jun N-terminal kinases
Keap1	Kelch-like ECH-associated protein 1
LCFAs	Long chain fatty acids

LDL	Low density lipoprotein
Lepr	Leptin receptor
LV	Left ventricular
LW	Liver weight
Mapk	Mitogen-activated protein kinase
MCD	Malonyl-CoA decarboxylase
MET	Metformin
MPT	Mitochondrial permeability transition
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NG	Normal glucose
NOS	Nitric oxide synthase
Nox	Nicotinamide adenine dinucleotide phosphate oxidase
Nqo1	Nicotinamide adenine dinucleotide phosphate quinone 1
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
$O_2^{\bullet -}$	Superoxide radical
Opa1	Optic atrophy 1 homolog (human)
P53	Tumor protein 53
PAL	Palmitate
pAMPK(Thr^{172})	Adenosine monophosphate-activated protein kinase threonine 172
Park7	Parkinson protein
PARP	Poly (ADP-ribose) polymerase
Pde3b	Phosphodiesterase 3B, cGMP-inhibited
PFK1	Phosphofructokinase-1
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC	Protein kinase C

PPAG	Phenyl pyruvic acid-2-*O*-β-D-glucoside
PPAG	Phenylpyruvic acid-2-*O*-β-D, glucoside
Pparɣ	Peroxisome proliferator activated receptor gamma
Prdx	Peroxiredoxin
Prkacb	Protein kinase, cAMP dependent, catalytic, beta
ROS	Reactive oxygen species
Scd1	Stearoyl-Coenzyme A desaturase 1
Sele	Selectine E
Serpine	Serpin peptidase inhibitor
Slc25	Solute carrier family 25
Socs	Suppressor of cytokine signaling
SOD	Superoxide dismutase
Srebf	Sterol regulatory element binding factor
STZ	Streptozotocin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TAGs	Triacylglycerides
TNF	Tumor necrosis factor
TRPV	Transient receptor potential cation channel subfamily V
Txn	Thioredoxin
Txnrd	Thioredoxin reductase
UCPs	Uncoupling proteins
Vegf	Vascular endothelial growth factor A
WHO	World Health Organization
WI	Water intake

Introduction

The prevalence of diabetes mellitus (DM) is increasing at an alarming rate worldwide. According to the International Diabetes Federation (IDF) the number of individuals living with DM is 415 million and this figure is estimated to reach 642 million by the year 2040 (IDF, 2015). The World Health Organization (WHO) lists three main types of diabetes: gestational DM, which can be defined as a degree of glucose intolerance during pregnancy; type 1 DM (T1DM), also known as juvenile or insulin dependent diabetes that is usually diagnosed in children and young adults; and type 2 DM (T2DM) which is related to obesity and represents approximately 90% of diabetes cases worldwide (WHO, 2012). DM is characterized by disturbances in insulin secretion and chronic hyperglycemia. Literature suggests a strong correlation between chronic hyperglycemia and micro- and macro- vascular complications, including retinopathy, nephropathy, neuropathy and cardiovascular diseases (CVDs) such as heart failure (HF) (Ginsberg and Mazze, 1994; Swan et al., 1997; Boudina and Abel, 2010).

Chronic hyperglycemia remains the leading risk factor associated with HF in a diabetic state. Through various pathways, it is responsible for augmented production of oxidative stress (Fiordaliso et al., 2004; Kukidome et al., 2006; Giacco and Brownlee, 2010; Mapanga and Essop, 2016). Oxidative stress may directly induce cardiac structural remodeling, a prominent sign of diabetic cardiomyopathy (DCM) (Watanabe, 1998). DCM is a distinct clinical entity that was first described about four decades ago (Rubler et al., 1972). The diagnosis of DCM remains nebulous and the precise mechanisms explaining DCM remain to be fully elucidated. Although therapeutic drugs certainly play a significant role in the treatment of DM and its co-morbidities, there is currently no treatment specific for DCM.

Insulin and metformin are the first line antidiabetic drugs with known cardioprotective properties (Jonassen et al., 2001; Eurich et al., 2005; Yin et al., 2011). However, the rise in CVD related deaths in people with DM is still a major concern (Boudina and Abel, 2010; American Diabetes Association, 2014). Furthermore, lifestyle interventions of caloric restriction and physical activity in patients with impaired glucose tolerance has been shown to improve CVD function. Nonetheless, most individuals do not adhere to such lifestyle interventions. On the other hand, antioxidants are among the leading therapies being investigated for their efficacy against various metabolic complications (Maritim et al., 2003; Rahimi et al., 2005; Ford, 2006). In the last decade, there has been much interest in the potential health benefits of plant polyphenols such as resveratrol and mangiferin as dietary antioxidants (Sellamuthu et al., 2009; Szkudelski and Szkudelska, 2011; Wang et al., 2014). In addition, our laboratory has increasingly investigated and reported on the antidiabetic properties of compounds found in *Aspalathus linearis* (rooibos) (Muller et al., 2012; Mazibuko et al., 2013; Sanderson et al., 2014). However, the use of rooibos and its efficacy as a cardioprotective agent have not yet been investigated.

For this study we hypothesized that compounds specific to *Aspalathus linearis* (rooibos) protect the myocardium against hyperglycemia-induced oxidative injury by activating nuclear factor (erythroid-derived 2)-like 2 (*Nrf2*) antioxidant response pathway. Thus, we aim to investigate the mechanism by which phenylpyruvic acid-2-*O*-β-D-glucoside (PPAG) and aspalathin are able to protect the myocardium against oxidative stress associated with diabetes mellitus.

This thesis is divided into the following themes which include:

I. Literature review (Chapter 1)

II. Phenylpyruvic acid-2-*O*-β-D-glucoside attenuates high glucose-induced apoptosis in H9c2 cardiomyocytes (Chapter 2)

III. Aspalathin, a dihydrochalcone *C*-glucoside, protects H9c2 cardiomyocytes against high glucose-induced shifts in substrate preference and apoptosis (Chapter 3)

IV. Aspalathin protects the heart against hyperglycemia-induced oxidative damage by up-regulating *Nrf2* expression (Chapter 4)

V. Transcription profile unveils the cardioprotective effect of aspalathin against lipid toxicity (Chapter 5)

VI. General conclusions (Chapter 6)

Each chapter is structured according to the journal in which the article was published and if the results in a particular chapter have not been published yet, the specifications prescribed for the journal to be submitted to have been used.

References

American Diabetes Association, 2014. National Diabetes Statistics Report, 2014 Estimates of diabetes and its burden in the epidemiologic estimation methods. Natl. Diabetes Stat. Rep. 2009–2012.

Boudina, S., Abel, E.D., 2010. Diabetic cardiomyopathy, causes and effects. Rev. Endocr. Metab. Disord. 11, 31–39.

Eurich, D.T., Majumdar, S.R., McAlister, F.A., Tsuyuki, R.T., Johnson, J.A., 2005. Improved clinical outcomes associated with metformin in patients with diabetes and heart failure. Diabetes Care 28, 2345–2351.

Fiordaliso, F., Bianchi, R., Staszewsky, L., Cuccovillo, I., Doni, M., Laragione, T., Salio, M., Savino, C., Melucci, S., Santangelo, F., Scanziani, E., Masson, S., Ghezzi, P., Latini, R., 2004. Antioxidant treatment attenuates hyperglycemia-induced cardiomyocyte death in rats. J. Mol. Cell. Cardiol. 37, 959–968.

Ford, E.S., 2006. Intake and circulating concentrations of antioxidants in metabolic syndrome. Curr. Atheroscler. Rep. doi:10.1007/s11883-006-0018-8.

Giacco, F., Brownlee, M., 2010. Oxidative stress and diabetic complications. Circ. Res. doi:10.1161/CIRCRESAHA.110.223545.

Ginsberg, B.J., Mazze, R., 1994. Clinical consequences of the diabetes control and complications trial. N J Med 91, 221–224.

International Diabetes Federation (IDF), 2015. IDF Diabetes Atlas 7th edition. idf.org. doi:10.1289/image.ehp.v119.i03.

Jonassen, A.K., Sack, M.N., Mjøs, O.D., Yellon, D.M., 2001. Myocardial protection by insulin at reperfusion requires early administration and is mediated via Akt and p70s6 kinase cell-survival signaling. Circ. Res. 89, 1191–1198.

Kukidome, D., Nishikawa, T., Sonoda, K., Imoto, K., Fujisawa, K., Yano, M., Motoshima, H., Taguchi, T., Matsumura, T., Araki, E., 2006. Activation of AMP-activated protein kinase reduces hyperglycemia-induced mitochondrial reactive oxygen species production and promotes mitochondrial biogenesis in human umbilical vein endothelial cells. Diabetes 55, 120–127.

Mapanga, R.F., Essop, M.F., 2016. Damaging effects of hyperglycemia on cardiovascular function: spotlight on glucose metabolic pathways. Am J Physiol Heart Circ Physiol. 310, :H153-H173.

Maritim, A.C., Sanders, R.A., Watkins, J.B., 2003. Diabetes, oxidative stress, and antioxidants: A review. J. Biochem. Mol. Toxicol. doi:10.1002/jbt.10058.

Mazibuko, S.E., Muller, C.J.F., Joubert, E., De Beer, D., Johnson, R., Opoku, A.R., Louw, J., 2013. Amelioration of palmitate-induced insulin resistance in C2C12 muscle cells by rooibos (*Aspalathus linearis*). Phytomedicine 20, 813–819.

Muller, C.J.F., Joubert, E., De Beer, D., Sanderson, M., Malherbe, C.J., Fey, S.J., Louw, J., 2012. Acute assessment of an aspalathin-enriched green rooibos (*Aspalathus linearis*) extract with hypoglycemic potential. Phytomedicine 20, 32–39.

Rahimi, R., Nikfar, S., Larijani, B., Abdollahi, M., 2005. A review on the role of antioxidants in the management of diabetes and its complications. Biomed. Pharmacother. 59, 365–73.

Rubler, S., Dlugash, J., Yuceoglu, Y.Z., Kumral, T., Branwood, A.W., Grishman, A., 1972. New type of cardiomyopathy associated with diabetic glomerulosclerosis. Am. J. Cardiol. 30, 595–602.

Sanderson, M., Mazibuko, S.E., Joubert, E., De Beer, D., Johnson, R., Pheiffer, C., Louw, J., Muller, C.J.F., 2014. Effects of fermented rooibos (*Aspalathus linearis*) on adipocyte differentiation. Phytomedicine 21, 109–117.

Sellamuthu, P.S., Muniappan, B.P., Perumal, S.M., Kandasamy, M., 2009. Antihyperglycemic effect of mangiferin in streptozotocin induced diabetic rats. J. Heal. Sci. 55, 206–214.

Swan, J.W., Anker, S.D., Walton, C., Godsland, I.F., Clark, A.L., Leyva, F., Stevenson, J.C., Coats, A.J., 1997. Insulin resistance in chronic heart failure: relation to severity and etiology of heart failure. J. Am. Coll. Cardiol. 30, 527–532.

Szkudelski, T., Szkudelska, K., 2011. Anti-diabetic effects of resveratrol. Ann. N. Y. Acad. Sci. 1215, 34–39.

Wang, B., Yang, Q., Sun, Y.Y., Xing, Y.F., Wang, Y. Bin, Lu, X.T., Bai, W.W., Liu, X.Q., Zhao, Y.X., 2014. Resveratrol-enhanced autophagic flux ameliorates myocardial oxidative stress injury in diabetic mice. J. Cell. Mol. Med. 18, 1599–1611.

Watanabe, M., 1998. Catechins as antioxidants from buckwheat (*Fagopyrum esculentum Moench*) Groats. J. Agric. Food Chem. 46, 839–845.

World Health Organization, W.H.O., 2012. World Health Statistics 2012, World Health Statistics 2012. doi:10.2307/3348165.

Yin, M., van der Horst, I.C.C., van Melle, J.P., Qian, C., van Gilst, W.H., Silljé, H.H.W., de Boer, R.A, 2011. Metformin improves cardiac function in a nondiabetic rat model of post-MI heart failure. Am. J. Physiol. Heart Circ. Physiol. 301, H459–468.

Chapter 1: Literature review

1.1. Cardiovascular diseases: a leading cause of death worldwide

Cardiovascular diseases (CVDs) are the leading cause of death worldwide (Fig. 1.) (World Health Organization [WHO], 2012). The continuous rise in non-communicable diseases such as diabetes mellitus (DM) and obesity remains a major burden causing an increase in CVDs worldwide (Boden and Salehi, 2013; WHO, 2014). Acute myocardial infarction (AMI) that may cause heart failure (HF) is often closely associated with risk factors such as DM, hypertension, obesity and smoking (Anand et al., 2000; Smith, 2007). Ischemic heart disease (IHD), also known as coronary artery disease (CAD), is the most common type of CVD (Reaven and Sacks, 2005; McCullough, 2007; Kealey, 2010). According to WHO, CAD was accountable for up to 42.3% of the 17.5 million CVD-related deaths reported in 2012, of which 9.2% was in the African region (Kengne et al., 2005; WHO, 2014). Moreover, diabetes can also affect cardiac structure and function independent of CAD, a condition identified as the diabetic cardiomyopathy (DCM) (Rubler et al., 1972; Boudina and Abel, 2010; Forbes and Cooper, 2013).

1.2. Diabetic cardiomyopathy

Cardiomyopathies are diseases of the myocardium that are characterized by a measurable deterioration of heart muscle function. As the condition worsens, less blood is pumped through the heart to maintain normal contractile function leading to a decrease in electrical rhythm and subsequent HF. According to a report by the American Heart Association (AHA), cardiomyopathies can be divided into 2 major groups based on predominant organ involvement (Maron et al., 2006). Primary cardiomyopathies are those exclusively or mainly limited to the heart muscle while secondary cardiomyopathies are a result of an underlying condition such as DM. Although they remain mainly non-ischemic, distinctively affecting the heart muscle, cardiomyopathies play a pre-dominant role in inducing HF and are one of the major causes of death in Africa (Kengne et al., 2008, 2005; Mbanya et al., 2010; Dalal et al., 2011).

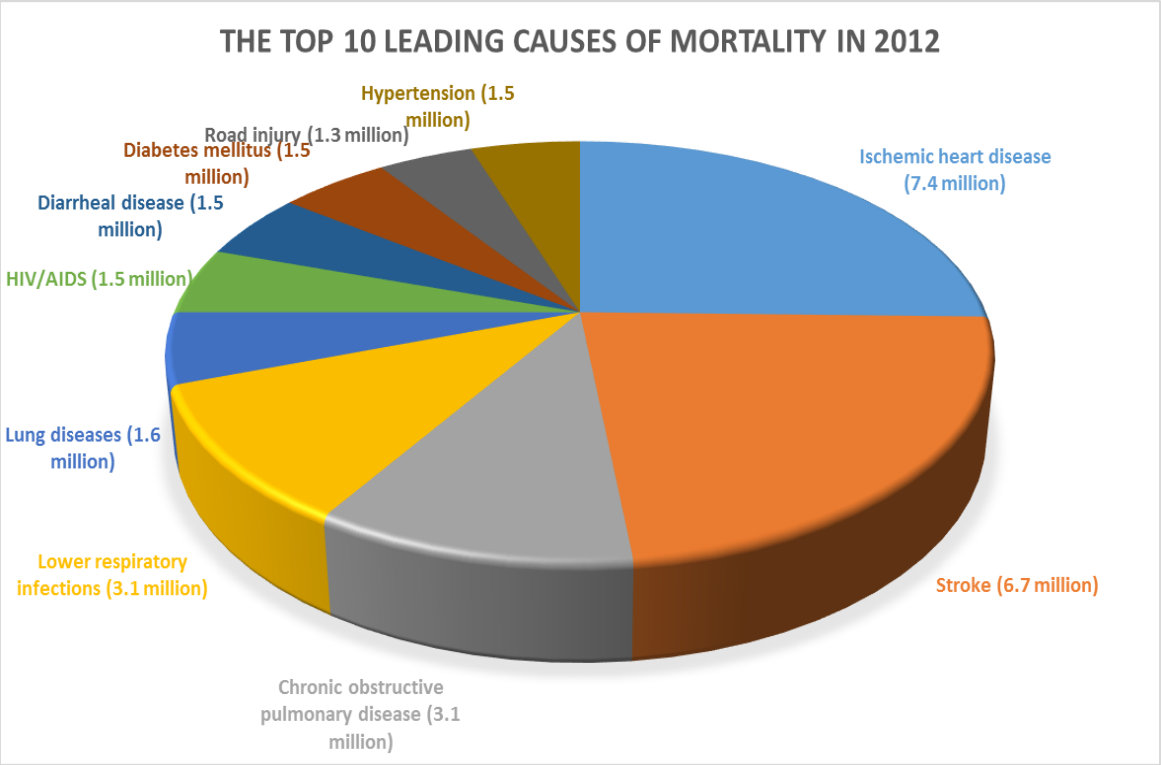


Figure 1. The top 10 leading causes of mortality in 2012 (compiled by author, based on statistics by World Health Organization, 2014). Heart diseases are the leading cause of death in the world, with ischemic heart disease being the most prominent, followed by stroke. Heart failure is responsible for 3 in every 10 deaths worldwide and approximately 17.5 million deaths globally.

DCM is a disease of the heart muscle in persons with DM. The prevalence of DCM is between 30-65% depending on the population under study (Miki et al., 2013; Dandamudi et al., 2014). DCM is characterized by left ventricular (LV) dysfunction accompanied by decreased systolic and diastolic function (Fonarow and Srikanthan, 2006). The precise mechanisms explaining LV impairment that is associated with systolic dysfunction in DCM remains to be fully elucidated. However, chronic hyperglycemia and hyperlipidemia, in the absence of CAD and hypertension is central in the etiology of DCM (Rubler et al., 1972; Poornima et al., 2006; Boudina and Abel, 2010). Epidemiological studies consistently report on a significantly increased LV wall thickness in both men and women with DM (Fig. 2.) (Devereux et al., 2000; de Simone et al., 2002). Moreover, these structural changes are distinctively identified in patients with DM but not in patients with impaired glucose tolerance (IGT) (Devereux et al., 2000; Adeghate and Singh, 2014; Cassidy et al., 2015). This indicates that myocardial modifications associated with a diabetic condition might not be an early defect but rather a result of prolonged exposure to hyperglycemia. In the diabetic heart, chronic exposure to

hyperglycemia can result in a shift in substrate preference leading to reduced cardiac efficiency (Stanley et al., 2005; Rijzewijk et al., 2010).

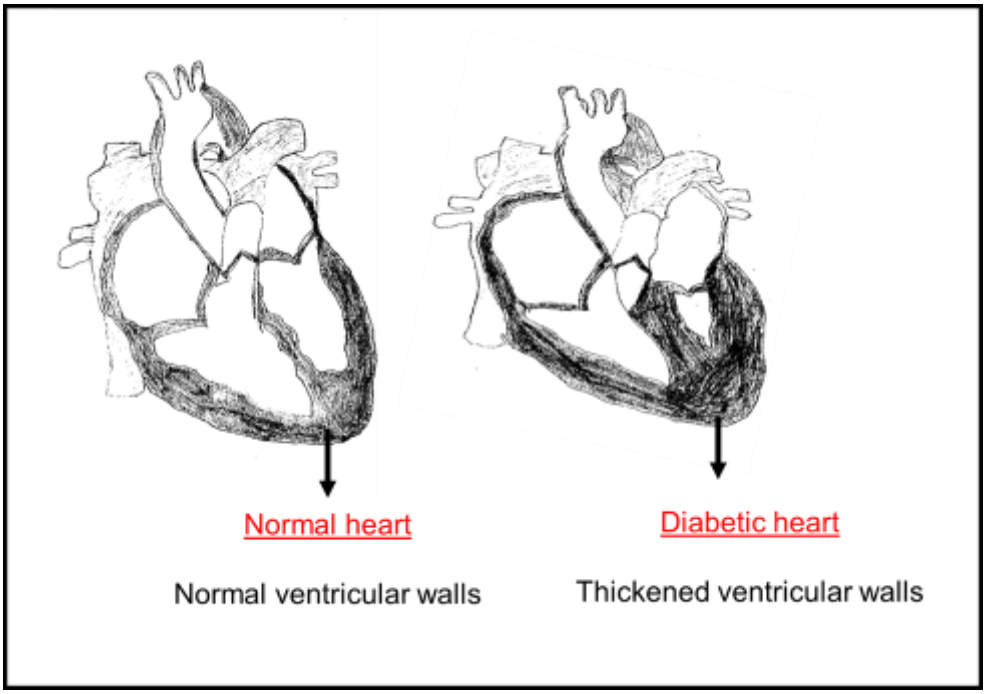


Figure 2. An illustration of a hypertrophic heart from a diabetic patient compared to a normal heart from a non-diabetic individual. Hearts of patients with diabetes mellitus display irregularly enlarged left ventricular mass, which is a conspicuous sign of diabetic cardiomyopathy.

1.3. Diabetic heart and energy substrate metabolism

The derangement of cardiac energy substrate preference plays an important role during HF. In the normal heart, free fatty acids (FFAs) are the predominant energy substrate and are accountable for up to 70% of the energy supply (Stanley et al., 2005). The remaining proportions are mainly from glucose (up to 40%) and lactate oxidation (up to 10%) (Lei et al., 2004). Glucose is the preferred energy substrate for metabolism and ATP generation during diabetic-induced HF and this is attributable to its greater efficiency to produce high-energy products per oxygen consumed compared to the use of FFAs (Nagoshi et al., 2011). Insulin resistance inhibits this adaptive response, by almost exclusively using FFAs as an energy source, further contributing to the progression of ventricular remodeling and cardiac contractile dysfunction (Marfella et al., 2009).

FFAs enter cardiac cells either passively or actively using fatty acid transporters such as fatty acid translocase (FAT)/CD36 and fatty acid binding protein (FABP) (Lopaschuk et al., 2010). Upon entry, long chain FFAs can be converted to triacylglycerides (TAGs) by glycerolphosphate acyltransferase, resulting in an intramyocardial TAG pool; and this is simultaneous to increased accumulation of diacylglycerol (DAG) levels within heart cells in a diabetic state (Inoguchi et al., 1939; Schmidt et al., 1999). DAG signaling may result in enhanced production of extracellular matrix proteins within a diabetic heart (Sharma and Ziyadeh, 1995; Zhao et al., 2014). Excess production of extracellular matrix proteins within the myocardium is closely associated with fibrosis, leading to adverse effects on cardiac structure and function (Mandavia et al., 2013).

Increased intracellular DAG favours synthesis of dihydroacetone, a triose carbohydrate that is associated with increased free radical damage as well as the activation of protein kinase C (PKC) (Connelly et al., 2009; Geraldes and King, 2010). PKC exists in various isoforms and those known and most investigated in diabetes research include beta, alpha, gamma and delta isoforms. The main function of PKC is to control the function of target proteins through phosphorylation (Mochly-Rosen et al., 2012). Its inhibition through *in vivo* study models of diabetes reveals that PKC mediates a variety of activities including inflammation (Cameron et al., 1999; Brownlee, 2001; Connelly et al., 2009). In addition, its overexpression in the heart is implicated in the development of hypertrophy, leading to decreased ventricular compliance (Sentex et al., 2006). Treatment with antioxidants and decreased FFA uptake appears to attenuate DAG-PKC induced diabetic complications (Beckman et al., 2002; Xu et al., 2009). However, more clinical studies are required to investigate the precise role PKC plays in a diabetic heart and whether its inhibition can prevent diabetic associated vascular complications (Mapanga and Essop, 2016).

1.3.1. Free fatty acid metabolism in the diabetic heart

Adenosine monophosphate-activated protein kinase (AMPK) is a heterotrimeric protein composed of a catalytic α, non-catalytic β and γ subunits. The main function of this kinase is to preserve ATP or to promote alternative methods of ATP generation. It functions as a sensor during low energy states such as ischemia to change substrate utilization and thereby increase or decrease ATP synthesis. Its activation is controlled by an increase in the AMP:ATP ratio (Carling, 2004; Zungu et al., 2011). Stimulation of AMPK leads to phosphorylation of many target proteins important for ATP synthesis and utilization while concurrently inhibiting ATP-consuming pathways such as fatty acid synthesis. In the diabetic heart, AMPK activation is linked to phosphorylation of both acetyl-CoA carboxylase (ACC) and malonyl-CoA decarboxylase (MCD); however its association with the latter in the heart remains to be fully elucidated (Park et al., 2002, 2002; Sambandam et al., 2004; Brownsey et al., 2006). ACC and

MCD are both important for the interconversion of acetyl-CoA to malonyl-CoA. Phosphorylation of ACC by AMPK reduces the generation of malonyl-CoA; thus prompting more FFAs to enter mitochondria through carnitine palmitoyltransferase I (CPT1) for β-oxidation (Fig. 3.) (Makaula et al., 2006). Resultant increased levels of ATP and citrate through β-oxidation are responsible for the allosteric inhibition of glycolysis through phosphofructokinase-1 (PFK1) (Randle et a., 1963, 1998). This causes accelerated reactive oxygen species (ROS) production and associated membrane peroxidation (Giacco and Brownlee, 2010; Rains and Jain, 2011). Hence, adequate control in the uptake and oxidation of FFAs remain crucial for optimal functioning of the myocardium, especially in a diabetic state.

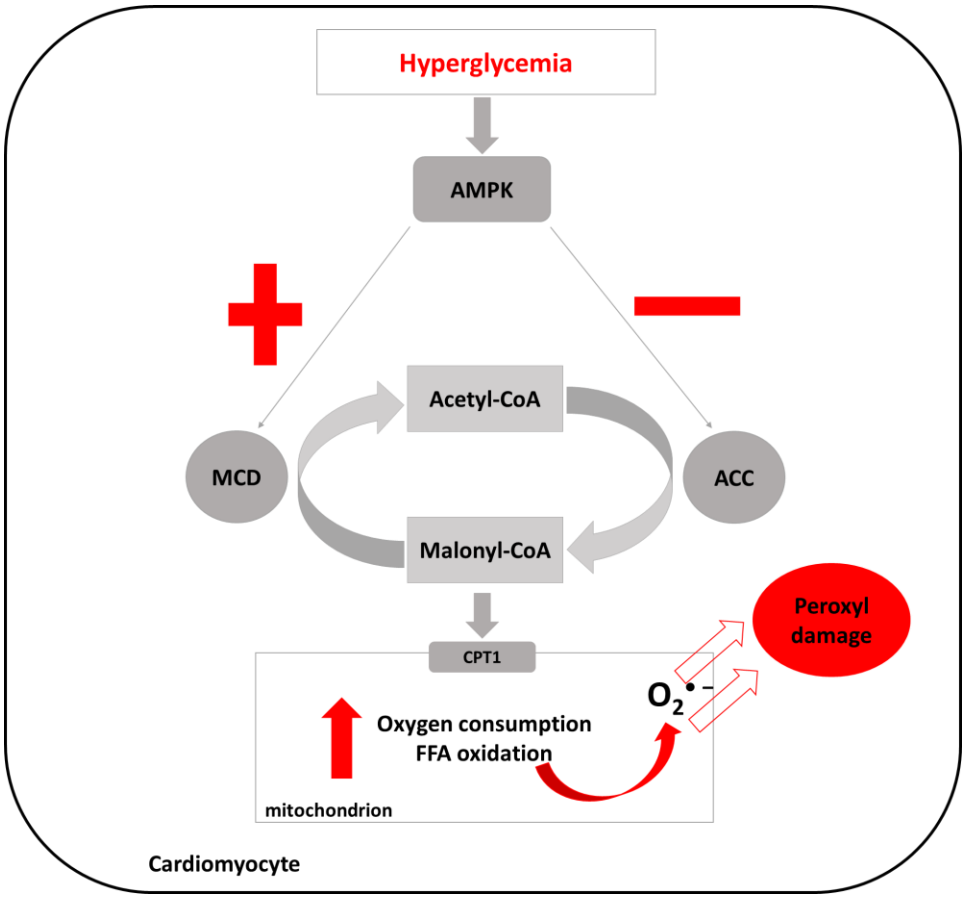


Figure 3. AMPK is associated with the phosphorylation of both ACC and MCD to regulate myocardial energy metabolism in the heart muscle. Chronic hyperglycemia activates AMPK, resulting in the phosphorylation of ACC, releasing the inhibitory effect of malonly-CoA on CPT1 and subsequently leading to enhanced entry of FFAs via CPT1 into the mitochondria for β-oxidation. Abnormally increased β-oxidation is further accountable for mitochondrial membrane damage through peroxyl radicals. **ACC:** acetyl-CoA carboxylase; **AMPK:** 5' adenosine monophosphate (AMP)-activated protein kinase; **CPT1:** carnitine palmitoyltransferase 1; **FFA:** free fatty acid; **MCD:** malonyl-CoA decarboxylase.

1.3.2. Glucose metabolism in the diabetic heart

Under specific pathological conditions glucose becomes the preferred substrate in the heart, as it can provide greater efficiency by producing high energy products per oxygen consumed compared to FFAs (Nagoshi et al., 2011). However, insulin resistance impedes this adaptive metabolic shift. In the diabetic heart, myocardial glucose uptake is suppressed due to increased uptake and oxidation of FFAs (Fig. 4.) (Aroor et al., 2012). This of course more complex as high glucose uptake still continues in endothelial cells of the heart (Mapanga and Essop, 2016). Concurrently, the rate of oxygen (O_2) consumption by the mitochondria is severely increased, causing the myocardium to be more susceptible to ischemic damage (How et al., 2006; Mandavia et al., 2013). Impaired myocardial glucose metabolism is observed in animal studies, where the rate of glycolysis was shown to be depressed by up to 48% in the hearts of untreated diabetic mice when compared to untreated lean control counterparts (Belke et al., 2000). A similar finding was reported in a study done on diabetic Zucker rats maintained on a high fat diet, showing an increased rate of fatty acid oxidation (FAO) with a concomitant decrease in glucose oxidation (Van den Brom et al., 2009, 2010).

As part of cardiac substrate homeostasis, glucose transportation and sensing plays an integral role in the disease pathology of T2DM. Glucose transport into cardiomyocytes is mediated by two members of the glucose transporter family of proteins, glucose transporter 1 (Glut1) and glucose transporter 4 (Glut4) (Fig. 4.). Glut1 is responsible for the basal uptake of glucose, whereas Glut4 is the main insulin-sensitive glucose transporter (Tsirka et al., 2001). For greater glucose demands, the stimulatory effect of insulin is responsible for the translocation of Glut4 to the plasma membrane for enhanced glucose uptake (Russell et al., 1999). When demand for glucose is lower Glut4 remains concealed in intracellular vesicles to a greater extent and less cycling to the plasma membrane. The potential association of Glut4 and increased glucose uptake is shown by various studies (Camps et al., 1992; Watson et al., 2004; Chen et al., 2007). Zisman and colleagues showed that mice lacking the Glut4 gene show signs of insulin resistance (Zisman et al., 2000). Furthermore, impaired myocardial substrate metabolism in the diabetic heart is associated with abnormally increased oxidative stress due to altered mitochondrial function (Lei et al., 2004; Joseph et al., 2014). This is characterized by morphological remodeling, occurring in parallel to contractile dysfunction (Boudina and Abel, 2007). Hence, the ideal ameliorative strategy to halt HF in DM should target moderately increasing intracellular glucose uptake while avoiding cardiac glucotoxicity.

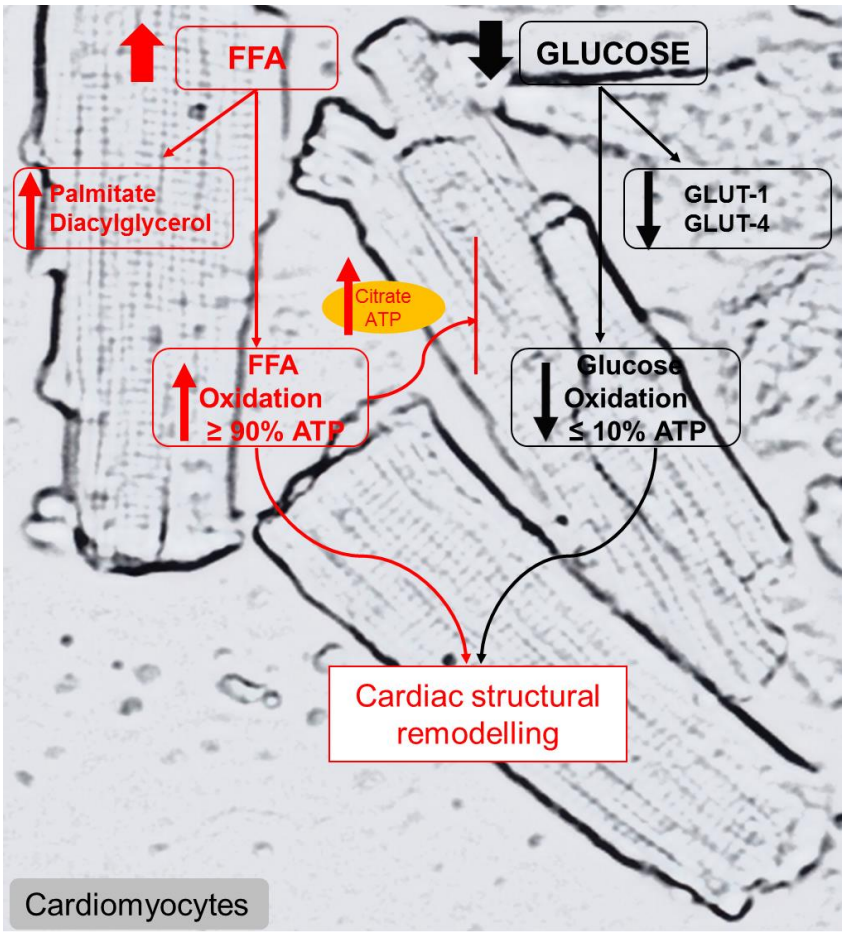


Figure 4. Cardiac structural remodeling as a consequence of fatty acid and glucose oxidation. While Glut1 is responsible for the basal uptake of glucose, Glut4 is the main glucose transporter in the heart. Impaired substrate preference due to abnormally increased oxidation of FFAs is associated with inhibition of glucose oxidation and subsequent cardiac remodeling. **ATP:** adenosine triphosphate; **FFAs:** free fatty acids; **Glut1:** glucose transporter 1; **Glut4:** glucose transporter 4.

1.4. Role of oxidative stress in a diabetic heart

Altered cardiac energy substrate utilization and increased oxidative stress are linked to the onset of cardiac hypertrophy (Ingwall, 2009). Chronic hyperglycemia enhances oxidative stress and exacerbates myocardium injury, subsequent to the development of DCM (Boudina and Abel, 2010; Tarquini et al., 2011; Joseph et al., 2014; Mapanga and Essop; 2016). This has been confirmed by various laboratory studies showing strong correlation between oxidative stress and matrix remodeling in cardiomyocytes isolated from diabetic heart tissue (Uemura et al., 2001; Soliman et al., 2008; Dludla et al., 2014). Oxidative stress is aggravated by enhanced levels of ROS within cardiomyocytes (Rajamani and Essop, 2010; Essick et al., 2011). Abnormal ROS production elicits an increased pro-

inflammatory response resulting in myocardial apoptosis (Sabri et al., 2003; Kim et al., 2005). Some of the well-known reactive oxygen substances, associated with myocardial damage include superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2). Generation of ROS is generally a cascade of reactions that starts with the formation of $O_2^{\bullet -}$ (Sharma et al., 2012). Superoxides rapidly dismutate to H_2O_2 either spontaneously or catalytically by superoxide dismutase (SOD), while H_2O_2 is decomposed to water (H_2O) and O_2 through catalase (Cat).

The mitochondrial electron transport chain (ETC) and the actions of the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) enzymes remain the foremost sources of stress in cardiomyocytes (Giacco and Brownlee, 2010; Kuroda et al., 2010; Sovari et al., 2013). Augmented activity of NADPH oxidase is demonstrated in the myocardium of a failing heart at the same rate as oxidative damage (Kuroda et al., 2010). Correspondingly, mitochondrial structural modification is affiliated with reduced endogenous antioxidant status in cardiomyocytes from diabetic heart tissue (Sivitz and Yorek, 2010; Ansley and Wang, 2013).

1.4.1. Mitochondrial stress

The mitochondrion remains an essential organelle for intracellular energy production. However, an increased capacity of the mitochondrion to produce energy is associated with increased ROS production. Given that a diabetic heart has a diminished mitochondrial antioxidant capacity (Huynh et al., 2014), it is therefore not surprising that minor alterations in mitochondrial structure or function induced by increased ROS are associated with major changes in the heart muscle (Marín-García et al., 2013; Zorov et al., 2014). Increased ROS and mitochondrial depolarization, subsequent to diastolic dysfunction has been reported in patients with metabolic disturbances (Sack, 2009; Montaigne et al., 2013). Though the precise role of mitochondrial dysfunction in a diabetic heart is still lacking, consensus is that defects in ETC and its oxidative status play a major role in impaired mitochondrial morphology and function.

Simultaneous to energy generation, there is constant generation of $O_2^{\bullet -}$ within mitochondria (Zamzami et al., 1995). Accumulation of these radical substances result in the induction of the mitochondrial permeability transition (MPT) pore (Baines, 2009). MPT occurs at the same time with altered redox state of the mitochondria (Crompton, 1999; Zorov et al., 2014). With diseased states, the MPT pore is unique for being nonselective and allowing for the accumulation of excessive calcium (Ca^{2+}) and other toxic compounds (Lemasters et al., 1998). Recent literature shows that the fate of the cell after an insult depends on the extent of the MPT pore formation (Orrenius et al., 2003; Kwong and Molkentin, 2015; Morciano et al., 2015). If MPT pore formation occurs to only a limited extent, the cell may

recover through cell recovery mechanisms such as activation of mitophagy/ubiquitination, whereas if pore formation is exacerbated it accelerates apoptosis (Galluzzi et al., 2014). If it occurs to an even larger degree, the cell is likely to undergo necrotic cell death. Thus prevention of mitochondrial membrane depolarization may play a role in reducing myocardial injury associated with chronic hyperglycemia.

1.4.2. NADPH oxidase

NADPH oxidase (Nox) is an important enzyme that plays a notable role in the generation of ROS in many cell types, including cardiomyocytes. Nox generates intracellular ROS by transferring electrons from NADPH across the cell membrane and coupling these to molecular oxygen to produce $O_2^{\bullet -}$. Nox exists as different isoforms, from Nox1 to Nox4 and those predominant in the heart muscle are Nox2 and Nox4 (Bedard and Krause, 2007; Mapanga and Essop, 2016). Nox1 has been identified to be the major source of ROS production in vascular tissues, resulting in low levels of nitric oxide (Paravicini and Touyz, 2008; Fu et al., 2014). Decreased levels of nitric oxide are associated with impaired endothelium-dependent vasodilatation of coronary arteries (Stockklauser-Färber et al., 2000). Human aortic endothelial cells exposed to high glucose, display upregulated expression of Nox1, concomitant to enhanced oxidative stress and pro-inflammatory markers (Gray et al., 2013). The same study showed that diabetic mice lacking Nox1 had a profound anti-atherosclerotic outcome related to reduced ROS and inflammation. Although Nox2 and Nox4 are predominant in the heart muscle, these ROS generating enzymes are also co-expressed in other cell types and are implicated in agonist-stimulated redox-sensitive signal transduction (Bedard and Krause, 2007). Nox2 has been shown to play a central role in insulin resistance-mediated oxidative damage in vascular tissue (Sukumar et al., 2013; Kowluru and Kowluru, 2014). Nox2 knockout transgenic mice with endothelial-specific insulin resistance present enhanced ROS production and vascular dysfunction (Guichard et al., 2006; Sukumar et al., 2013). On the other hand, the Nox4 isoform is specifically expressed in mitochondria of cardiomyocytes; and mice lacking Nox4 gene show accelerated free radical damage coupled by mitochondrial dysfunction (Kuroda et al., 2010; Maalouf et al., 2012). Its overexpression in the mouse heart deteriorates cardiac dysfunction by initiating apoptosis, leading to cytochrome-c release (Kuroda et al., 2010).

1.5. Inflammation as a precursor for apoptosis

Inflammation is the initial protective biological response of the body against harmful stimuli. There are various risk factors linked with the onset of inflammation; namely age, obesity, stress and smoking

(Willerson and Ridker, 2004; Sastre et al., 2011). However, hyperglycemia is one condition that has been shown to activate various pro-inflammatory transcription factors, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Esposito et al., 2002; Castello et al., 2010). NF-κB is the most significant regulator of pro-inflammatory gene expression and is strongly associated with fibrosis in heart tissue (Miguel-Carrasco et al., 2010; Lorenzo et al., 2011). Overexpression of NF-kB accelerates apoptosis in a diabetic state (Cameron and Cotter, 2008; Kumar et al., 2013). By contrast, its inhibition in diabetic mice ameliorates ROS-induced damage through improving mitochondrial structural integrity (Mariappan et al., 2010; Gordon et al., 2011).

Recent literature suggests that hyperglycemia-induced cardiomyocyte apoptosis is further deteriorated by the increased stimulation of inflammatory cytokines (Esposito et al., 2002; Pickup, 2004; King, 2008). Tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL6) are significantly increased in patients with uncontrolled hyperglycemia (Goyal et al., 2008). The activation of c-Jun N-terminal kinases (JNKs) pathway acts as a direct inducer of apoptosis in heart cells (Turner et al., 1998; Liu et al., 2004); and it triggers enhanced expression of pro-inflammatory cytokines (Lysiak et al., 2003; King, 2008). It is well known that inflammatory cytokines and chemokines are predictive makers for most cardiovascular events (Melter et al., 2001; Inoue et al., 2008). TNF-α is released by a variety of immune cells and is a prognostic marker for MI (Ridker et al., 2000). The reduced expression of TNF-α in the heart signals improved myocardial performance (Saini et al., 2005). Other well-known cytokines and chemokines include interleukin-1 beta (IL1β), Interleukin-8 (IL8), IL6, chemokine (C-C motif) ligand 3 (CCL3), and monocyte chemotactic protein 1 (MCP1) (Melter et al., 2001; Shanmugam et al., 2003). These cytokines not only stimulate adhesion molecules within vascular tissue in DM, but can indirectly cause AMI via the inhibition of the PKB pathway (Kidd et al., 2008). A number of studies support the concept that IL1β and IL18 might be implicated in the pathogenesis of DCM (Van Linthout et al., 2007; Westermann et al., 2007; Lorenzo et al., 2011). The major mechanism by which such pro-inflammatory cytokines may be associated with the development of DCM has been through initiating apoptosis (Wang et al., 2010; Wen et al., 2013). Inhibition of JNK phosphorylation, along with cytokines such as TNF-α and IL1β has been shown to deter high glucose-induced inflammation and apoptosis in cardiomyocytes from diabetic tissue (Thomas et al., 2014; Yu et al., 2016).

1.6. The role of apoptosis in the diabetic heart

Apoptosis is a regulated process of programmed cell death that forms a vital component in various biological systems. In a diabetic state, persistent hyperglycemia is understood to be the key mediator of myocardial apoptosis (Cai et al., 2002; Rajamani and Essop, 2010). Apoptosis signaling varies with different cell types and there are two main types of apoptotic pathways: intrinsic and extrinsic.

Literature has shown that both intrinsic and extrinsic apoptotic pathways in the diabetic myocardium are involved in HF and DCM (Li et al., 2007; Giacco and Brownlee, 2010; Kim and Kang, 2010). The intrinsic apoptotic pathway is mediated mainly through intracellular signals that involve the mitochondrion. Stimuli for the intrinsic pathway include generation of free radical species leading to alteration in the inner mitochondrial membrane resulting in loss of transmembrane potential, causing the release of pro-apoptotic proteins (Rajamani and Essop, 2010). This mitochondrial membrane depolarization is linked to a number of disturbances within the myocardium, including ATP depletion and bioenergetic failure (Johnson et al., 2016). In addition, deterioration of the proton-translocating ATPase and intracellular Ca^{2+} overload may occur concurrent the release of cytochrome-c (Webster, 2012).

Cytochrome-c is a vital component of the mitochondrial-ETC, acting as an electron shuttle during redox generation of ATP (Boekema and Braun, 2007). Its release and mitochondrial depolarization are considered key physiological events of apoptosis (Elmore, 2007). Earlier evidence shows that loss of cytochrome-c from mitochondria is one of the earliest changes in mitochondria during heart ischemia (Borutaite and Brown, 2003). In high glucose exposed cardiomyocytes, independent of mitochondrial depolarization status, cytochrome-c release is enough to cause apoptosis (Yu et al., 2008). However, once released into the cytosol, cytochrome-c may form an apoptosome by interacting with apoptotic protease activating factor 1 in the presence of deoxyadenosine triphosphate (dATP). This process results in the activation of pro-caspase-9 and caspase-3, respectively. Conversely, an extrinsic apoptotic pathway similarly lead to the cleavage of caspase-3, however the initial stimuli is usually external, such as pro-inflammatory cytokines (Elmore, 2007). Generally, myocardial remodeling is affiliated with increased activity of caspase-3, -7, and -9 (Schwarz et al., 2006). These observations are consistent with mitochondrial dysfunction found in heart cells of diabetic mice (Cai et al., 2002). Caspases are proteases that become incongruously activated as zymogens and may prompt apoptosis by activating poly (ADP-ribose) polymerase (PARP) proteins (Boulares et al., 1999). PARP activation plays a central role in the pathogenesis of diabetic cardiovascular dysfunction by inducing cardiac hypertrophy and fibrosis in the hearts of diabetic animals (Boulares et al., 1999; Joseph et al., 2014).

Pro-apoptotic proteins associated with accelerated myocardial damage are not only restricted to the cysteine-aspartic-specific caspases. B-cell lymphoma 2 (Bcl2) protein homologs are also needed for the modulation of apoptotic actions. These proteins can induce either pro-apoptosis or cellular survival depending on the cell's fate. This family of proteins consists of two classes: anti-apoptotic Bcl2 proteins or the pro-apoptotic proteins such Bax and Bad. Bax translocation and cytochrome-c release are regarded as two significant upstream molecular events of apoptosis (An et al., 2004). Bax is confined in the cytoplasm as a monomer and can be triggered by the BH3-only proteins such as Bim

and Puma, causing it to translocate into the mitochondria where it induces changes in mitochondrial membrane potential (An et al., 2004). Accumulative data suggests that drug agents that inhibit either Bax translocation or activation of caspases may alleviate MI induced by DM (Webster, 2012; Dludla et al., 2015; Johnson et al., 2016). Therefore, interventions that could inhibit pro-apoptotic proteins and mitochondrial cytochrome-c release could salvage myocardial injury.

1.7. Endogenous cardiac protective mechanisms

Free radicals within mitochondria are generally removed by mitochondrial SOD, thereby generating H_2O_2. This process allows H_2O_2 to be further reduced to water by glutathione (GSH)/thioredoxin (Txn) peroxidase or Cat enzymes. GSH remains an important intracellular antioxidant to prevent free radical damage. GSH and thioredoxins (Txns) can easily be oxidized to their disulfide form during oxidation reactions; thus, NADP transhydrogenase enzymes remain important to maintain the reduced form of these co-factors (Revollo et al., 2007). NADP transhydrogenase functions by transferring electrons from a reduced form of nicotinamide adenine dinucleotide (NADH) to $NADP^+$ in order to regenerate GSH and Txn (Rydström, 2006). Txns are proteins that act as antioxidants and they serve as electron donors for enzymes such as ribonucleotide reductases (Berndt et al., 2008). The antioxidant mechanism of Txns involves facilitating protein reduction through cysteine thiol-disulfide exchange. Thioredoxin reductase (Txnrd) is the only known enzyme to reduce Trxn and is crucial during embryogenesis (Jakupoglu et al., 2005). Reduced expression of both GSH and Txns is consistently reported in experimental models investigating a diabetic heart (Chen et al., 2009; Giacco and Brownlee, 2010; Dludla et al., 2014). This is confirmed when investigated in either type 2 diabetic subjects or mice that are chronically subjected to hyperglycemia and hyperlipidemia, respectively (Bhatt et al., 2013; Marnewick et al., 2011).

1.7.1. Activation of Nrf2 within the diabetic heart

Expression of most antioxidant response genes, including GSH and Txns is regulated by the redox-sensitive transcription factor, nuclear factor erythroid-2-related factor 2 (Nrf2) (He et al., 2009). Nrf2 is a transcriptional regulator that is activated in response to intracellular stress (Bryan et al., 2013). Genes activated by Nrf2 can be classified into numerous groups including phase II detoxifying and cytoprotective enzymes (Fig. 5.). Nrf2 resides in the cytoplasm, where it is subjected to continuous degradation by the ubiquitin-proteasome (Kobayashi et al., 2006). However, under stressful conditions such as ischemia or oxidative stress, it is activated by disassociating from its negative regulator Kelch-like ECH-associated protein 1 (Keap1) and translocate to the nucleus. Once in the nucleus, it forms a

heterodimer with Maf protein before binding to the antioxidant response element (ARE) to initiate and activate antioxidant defence genes (Bai et al., 2013). Activation of Nrf2 in epithelial cells has been shown to induce GSH synthesis and thus protect against oxidative stress (Kode et al., 2008). In addition to its negative regulation of Nrf2, Keap1 also acts as a sensor for a wide array of stressors that could activate Nrf2.

Antioxidant response components linked with Nrf2 activation include heme oxygenase-1 (Hmxo1/HO-1), Txnrd, glutathione-S-transferase (Gst), and NAD(P)H:quinone oxidoreductase (Nqo1) (Chen et al., 2009). Nrf2 also mediates gene expression of SOD, Cat, and nonenzymatic scavengers such as GSH and sulfiredoxin, which are vital for the reduction of oxidized protein thiols (Dinkova-Kostova and Abramov, 2015). Significant down-regulation of cardiac Nrf2 expression is concomitant to increased ROS and reactive nitrogen species damage in hearts of diabetic animals (Kode et al., 2008; He et al., 2009; Bai et al., 2013; Dinkova-Kostova and Abramov, 2015). Thus, agents that can significantly up-regulate Nrf2 expression have a potential to protect cardiomyocytes against high glucose induced apoptosis.

1.7.1.1. Nrf2 and mitochondrial function

Increased mitochondrial oxidative phosphorylation is associated with increased ROS production within the diabetic heart (Giacco and Brownlee, 2010). In a recent study, heart muscle cells exposed to H_2O_2 display increased uncoupling protein 3 (UCP3) expression simultaneous to nuclear accumulation of Nrf2 (Anedda et al., 2013). However, silencing of Nrf2 significantly reduced UCP3 expression in these cells resulting in enhanced oxidative damage. There are few studies linking activation of Nrf2 and reduction of ROS by transcriptional upregulating uncoupling proteins which influences mitochondrial biogenesis. Uncoupling proteins (UCPs) are a family of proteins that are located in the inner mitochondrial membrane of mammalian cells (Dinkova-Kostova and Abramov, 2015). They act as proton transporters within the mitochondrial matrix to disintegrate increased membrane potential (Kadenbach, 2003). These transporter proteins are expressed in a variety of cell types, whereas UCP1 and UCP2 are predominantly found in the heart muscle where they act as negative regulators of mitochondrial derived ROS production (Kadenbach, 2003; Essop et al., 2004). Although it has been shown that UCPs may ameliorate mitochondrial stress by modulating mitogen-activated protein kinases (MAPK) pathways and preserving Ca^{2+} homeostasis (Boudina and Abel, 2006; Valouskova and Modriansky, 2008), it has been reported that mitochondrial membrane potential is significantly increased in the absence of Nrf2 (Dinkova-Kostova and Abramov, 2015). Enhanced mitochondrial membrane potential and accelerated rate of oxidative respiration are constant with increased levels of mitochondrial free radical damage leading to increased MPT pore opening. However, this increased

ROS production is neutralized by the Nrf2-dependent transcriptional upregulation of UCP3 which improves proton conductance of the mitochondrial inner membrane and consequently decreases the production of $O_2^{\bullet -}$.

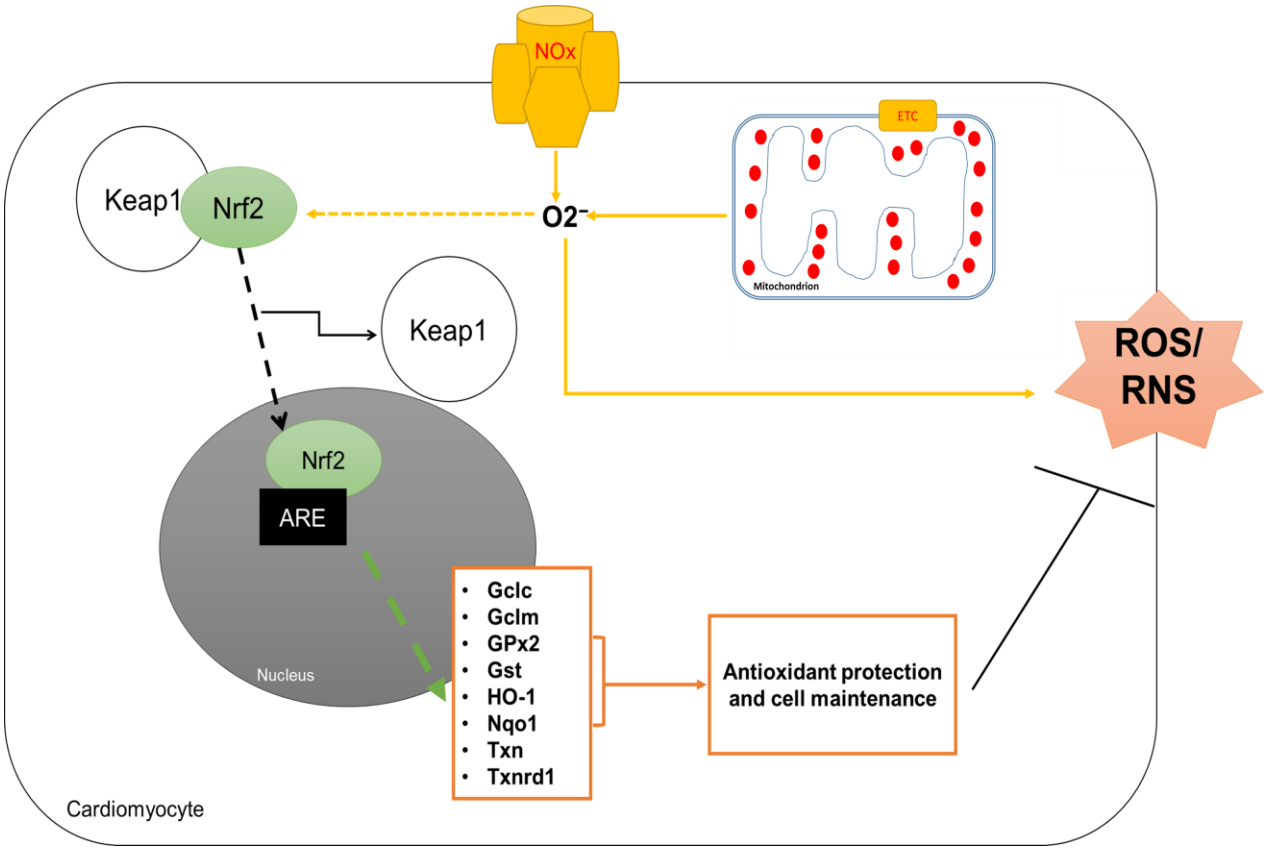


Figure 5. The role of Nrf2 in response to increased ROS within a diabetic heart. Nox and mitochondrial-ETC cause augmented production of O_2^-, which damages the cell through ROS/RNS. The cell reacts by activating the Nrf2-mediated antioxidant response system. Activated Nrf2 causes it to dissociate from Keap1 and migrate into the nucleus where it binds ARE and causes increased expression of cytoprotective genes and phase II detoxifying enzymes to combat ROS/RNS. **ARE**-antioxidant response element; **ETC**-electron transport chain; **GPx2**- glutathione peroxidase 2; **Gst**- glutathione S-transferase; **Gclc**- glutamate-cysteine ligase catalytic subunit; **Keap1**- Kelch-like ECH-associated protein 1; **Nqo1**- NADPH dehydrogenase [quinone] 1; **Nox**- NADPH oxidase; **O_2^-** superoxide ion; **Txn**- thioredoxin; **Txnrd1**- thioredoxin reductase 1; **HO-1**- Heme oxygenase 1; **Nrf2**- nuclear factor (erythroid-derived 2)-like 2; **RNS**-reactive nitrogen species; **ROS**-reactive oxygen species.

1.8. Current antidiabetic agents and cardiac protection

Primary interventions that may salvage a diabetic heart at risk from MI include lifestyle changes and maintaining blood sugar levels (American Diabetes Association, 2014). While such interventions are achievable and beneficial to the heart; adherence to lifestyle changes such as maintaining physical activity, remains a big challenge. Therefore, antidiabetic agents that could suppress postprandial and chronic hyperglycemia may be effective in decreasing the risk of HF. Although substantial work is required to confirm its long-term cardiac protective effect; metformin displays strong blood glucose lowering and potential cardiac protective effects (Kahn et al., 2006; Khurana and Malik, 2010; Yin et al., 2011).

Despite overwhelming evidence on the efficacy of other antidiabetic drugs such as beta statins and dipeptidyl peptidase-4 (DDP4) inhibitors (Magkou and Tziomalos, 2014), metformin remains the leading first line antidiabetic drug for T2DM individuals with known cardiac complications (Khurana and Malik, 2010; Messaoudi et al., 2011). In addition to its accomplished antidiabetic properties (Kahn et al., 2006; Yin et al., 2011), metformin is associated with improved clinical outcomes in diabetic patients with heart failure (Eurich et al., 2005). It enhances the efficacy of a number of synthetic drugs and novel medicinal compounds currently screened for metabolic benefits (Eurich et al., 2013; Dludla et al., 2016; Johnson et al., 2016). Metformin monotherapy or its use as an add-on effect to glibenclamide improves the intracellular antioxidant status of the myocardium in streptozotocin (STZ)-induced Sprague-Dawley rats (Erejuwa et al., 2010). Previous work also found that the benefit of metformin in the heart can be mediated through AMPK activation and improved antioxidative capacity (Gundewar et al., 2009; Johnson et al., 2016). However, there is an increasing toll of mortality due to cardiovascular complications within a diabetic population.

1.9. Polyphenols as a cardioprotective intervention

In recent years, more research has focused on the use of plant-derived products as a cardioprotective therapy (Marnewick et al., 2011; Muller et al., 2012; Chen et al., 2013; Mazibuko et al., 2013). Ideally, every plant has a potential medicinal use while for every disease there is likely a perfect natural cure. Medicinal plants have been extensively used in the pharmaceutical industry for the production of novel drugs to counteract various illnesses (Mukhtar and Ahmad, 2000; Pandey and Rizvi, 2009). Most medicinal plants contain a variety of compounds termed phytochemicals. Polyphenols are naturally occurring phytochemicals with variable phenolic structures. A polyphenol is classified according to the number and characteristics of the phenolic structure it contains. These functional phenol structures are unique to each class of polyphenols, giving it their distinct chemical and biological property.

Polyphenols are predominantly found in vegetables, fruits and teas. Furthermore, a profound relationship between a diet rich in polyphenols and health has given hope to long-term effective interventions that could prolong the onset of diabetes and it co-morbidities (Marnewick et al., 2011; Mazibuko et al., 2013; Sanderson et al., 2014).

1.10. The major polyphenolic compounds present in rooibos

Aspalathus linearis (Brum.f) Dahlg. (Fabaceae), commonly known as rooibos, is a popular South African plant that is used to make one of the leading herbal teas on the market. Rooibos tea is well known for its robust antioxidant activity and this has resulted in increased interest in its commercial value (Raynolds and Ngcwangu, 2010). Rooibos tea exists in two forms: a fermented one which is an oxidized form; and an unfermented one which is an unoxidized form. The fermentation process gives fermented rooibos its distinctive reddish-brown colour, while unfermented rooibos tea maintains its green color (Von Gadow et al., 1997). In addition to catechin, epicatechin, gallocatechin, and epigallocatechin; esters of gallic acid, catechin gallate, epicatechin gallate, gallocatechin gallate and epigallocatechin gallate are distinct polyphenolic compounds found in unfermented rooibos (Del Rio et al., 2013). Other bioactive compounds found in rooibos with ameliorative effect against various metabolic diseases, include its dihydrochalcones (aspalathin and nothofagin), flavones (orientin, isoorientin, luteolin, and apigenin), flavonols (quercetin, rutin), and the infrequently occurring phenylpyruvic acid-2-*O*-β-D-glucoside (PPAG) (Joubert et al., 2013). Therefore, due to their growing interest, it is important to assess the beneficial effects of major polyphenols from rooibos against oxidative-induced myocardial injury.

1.10.1. Cardioprotective potential of flavanols present in rooibos

The unfermented rooibos is unique from the fermented form by containing an additional group of flavonoids termed flavanols. This polyphenolic group is characterized by a 2-phenyl-3,4-dihydro-2H-chromen-3-ol skeleton and flavanols found in abundance in unfermented rooibos include epicatechin, epicatechin gallate and epigallocatechin gallate (Fig. 6.) (Snijman et al., 2009). Accompanying its well-documented antioxidant capacity (Von Gadow et al., 1997; Bramati et al., 2003; Joubert et al., 2004); unfermented rooibos has been shown to decrease body fat mass and levels of oxidized lipids in fat cells and human subjects, respectively (Marnewick et al., 2011; Sanderson et al., 2014). An aspalathin-enriched green rooibos extract (GRE) has been shown to display potential as an agent to induce hypoglycemia by inhibiting α-glucosidase *in vitro* and suppressing fasting plasma glucose (FPG) levels in Wistar rats; and ameliorate palmitate induced insulin resistance when tested in skeletal

muscle cells (Muller et al., 2012; Mazibuko et al., 2013; Mikami et al., 2015). In cultured skeletal muscle cells and pancreatic β-cells, GRE promotes GLUT-4 translocation to the plasma membrane and suppresses advanced glycation end products (AGEs)-induced ROS, respectively (Kamakura et al., 2015). In addition, this study shows that subchronic feeding with GRE for five weeks lowered the increased FPG levels found in a type 2 diabetic mouse model. In the heart, an aqueous extract of GRE protects against ischemic-induced reperfusion injury by modulating the phosphatidylinositol 3-kinase/protein kinase B (PI3K-Akt) pathway (Pantsi et al., 2011).

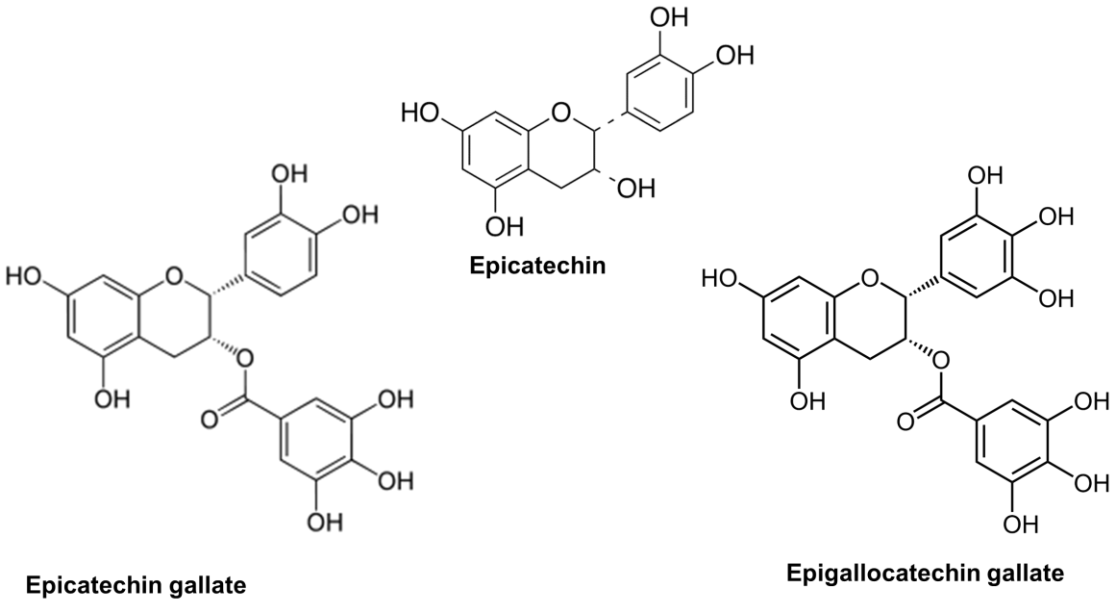


Figure 6. The chemical structure of major flavanols present in *Aspalathus linearis* (rooibos). Unfermented rooibos is distinct by containing an additional group of flavonoids termed flavanols that is characterized by a 2-phenyl-3,4-dihydro-2H-chromen-3-ol skeleton.

In addition to the aforementioned synergistic amelioration of DM-associated complications by GRE, 35 day treatment of STZ-induced diabetic rats with epigallocatechin gallate at doses of 15 and 30 mg/kg, respectively, significantly improved FPG levels and combats oxidative stress by increasing antioxidants such as GSH, Cat, SOD and glutathione peroxidase (Sabarimuthu Darlin Quine, 2005). In Wistar rats, administration of epigallocatechin gallate at a dose of 25 mg/kg for eight weeks displays a hypoglycemic effect in addition to attenuating oxidative stress (Roghani and Baluchnejadmojarad, 2009, 2010). This flavanol further protects type 2 diabetic rats against hyperglycemia-induced oxidative stress by reducing the degree of autophagy (Kim et al., 2013). Other mechanisms by which

unfermented rooibos/GRE and its flavanols may protect the heart against diabetes-induced damage include regulating cardiac lipid metabolism, directly increasing intracellular antioxidants, and modulating the activation of AMPK (Pantsi et al., 2011; Mazibuko et al., 2013; Kamakura et al., 2015;).

1.10.2. Cardioprotective potential of dihydrochalcones present in rooibos

Dihydrochalcones are a family of bicyclic flavonoids containing two distinct benzene rings joined by a saturated three-carbon bridge (Fig. 7.). Dihydrochalcones, aspalathin and nothofagin are the unique polyphenolic compounds found in rooibos with potent therapeutic properties. These two compounds primarily have known antioxidant properties (Von Gadow et al., 1997; Snijman et al., 2009; Van Der Merwe et al., 2010; Chen et al., 2013) and are found in abundance in GRE (Manley et al., 2006). The biological of activity of aspalathin and its structural analog, nothofagin, is associated with antithrombotic activity (Ku et al., 2015) and display an ameliorative effect against high glucose-induced inflammation when tested in endothelial cells from human umbilical vein and mice (Ku et al., 2015; Lee and Bae, 2015).

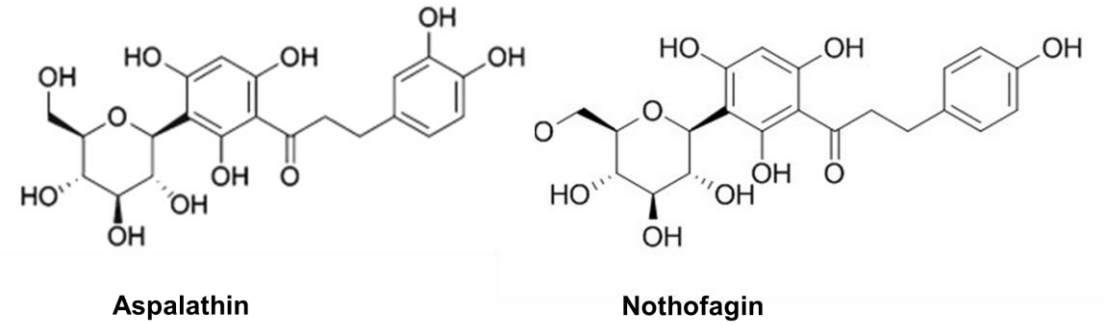


Figure 7. The chemical structure of major dihydrochalcones present in *Aspalathus linearis* (rooibos). Aspalathin and its structural analogue, nothofagin belong to a family of bicyclic flavonoids containing two distinct benzene rings joined by a saturated three-carbon bridge.

Aspalathin has demonstrated an even greater efficacy to prevent diabetes-associated complications. This is evident through its potential antidiabetic properties when tested in *in vitro* muscle cells and an *in vivo* type 2 diabetic mouse model (Kawano et al., 2009). There was further confirmation of its hypoglycemic activity after it was tested in obese mice (Son et al., 2013). Our laboratory has presented recent evidence that aspalathin reverses palmitate-induced insulin resistance in cultured

adipocytes; while it prevents apoptosis by improving substrate metabolism and recuing oxidative stress in H9c2 cardiomyocytes (Mazibuko et al., 2015; Johnson et al., 2016). Despite limited literature on the *in vivo* cardioprotective activity of aspalathin, the molecular mechanism of protection is associated with activation of AMPK (Mazibuko et al., 2015; Johnson et al., 2016). Optimal modulation of AMPK within the diabetic heart is crucial in the regulation of myocardial substrate preference (Johnson et al., 2016).

1.10.3. Cardioprotective potential of flavones present in rooibos

Flavones, in general, appear as crystalline compounds and they provide yellow or white pigments in certain plants. The group of flavones present in rooibos includes orientin, isoorientin, luteolin and apigenin (Fig. 8.) (Schloms and Swart, 2014). The number of functional hydroxyl groups on the structure of luteolin is directly correlated to its scavenging effect of hydroxyl radicals (Brown et al., 1998). In addition to its antidiabetic properties (Liu et al., 2013; Kim et al., 2014; Kwon et al., 2015), luteolin and its glycoside, luteolin-7-glucoside, attenuate myocardial oxidative damage and diabetic nephropathy by upregulating cellular antioxidants and inhibiting apoptosis (Fang et al., 2011; Qi et al., 2011; Hwang et al., 2013; Madhesh and Vaiyapuri, 2013). They further reverse early onset of DCM by hindering the destructive actions of aldose reductase and nitrogen oxide synthase (NOS) in diabetic animals (Wang et al., 2012, 2013). Transgenic mice broadly overexpressing the human aldose reductase gene have been shown to display enhanced ROS production concurrent to MPT pore opening and ischemic injury (Ramasamy and Goldberg, 2010). The effect of luteolin in preventing ischemia/reperfusion injury in diabetic rats has also been reported to be associated with upregulation of myocardial nitric oxide synthase (Sun et al., 2012). Furthermore, there is very limited on the cardioprotective effect of orientin and isoorientin; except for a few studies which have reported on the inflammatory properties of both compounds when tested in either cultured adipocytes or endothelial cells (Alonso-Castro et al., 2012; Ku et al., 2015).

Apigenin on the other hand has the unique ability to activate the transient receptor potential cation channel subfamily V member 4 (TRPV4) gene in endothelial cells (Ma et al., 2012). The expression of TRPV4 has been shown to be significantly reduced in the retinal microvascular endothelium (Monaghan et al., 2015). Apigenin inhibits lipopolysaccharide-associated inflammatory complications in mice by down-regulating increased mRNA expression of IL-6 and IL-1β via hindering actions of extracellular-signal-regulated kinases 1/2 (Zhang et al., 2014). Moreover, this flavone has recently been shown to combat diabetes-associated cognitive decline in rats by inhibiting oxidative stress and apoptosis in rats (Mao et al, 2015); and attenuates 2-deoxy-D-ribose-induced oxidative cell damage in pancreatic β-cells (Suh et al., 2012). Its hypoglycaemia-associated effect is linked with its capacity to

inhibit α-glucosidase activity and mediate glucose and lipid homeostasis when tested *in vitro* and diabetic animals (Panda and Kar, 2007; Cazarolli et al., 2012; Ohno et al., 2013).

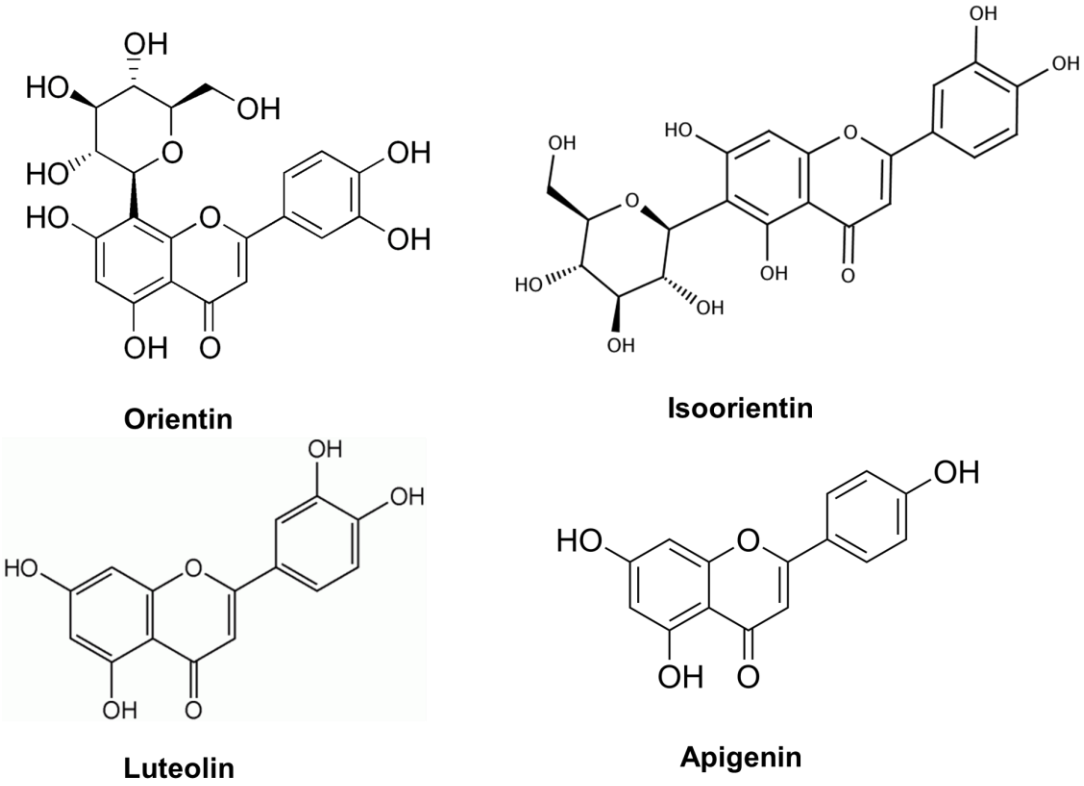


Figure 8. The chemical structure of major flavones present in *Aspalathus linearis* (rooibos). The group of flavones present in rooibos includes orientin, isoorientin and apigenin, which are classified by a basic chemical structure consisting of a chromone (1,4-benzopyrone) moiety connected to a phenyl ring in position 2-(2′-phenyl)-chromen-4-one or 2-phenyl-1,4-benzopyrone) .

1.10.4. Cardioprotective potential of flavonols present in rooibos

Quercetin and its glycoside, rutin are classified by a distinct 3-hydroxyflavone backbone and are the most explored group of flavonols in rooibos (Fig. 9.). Quercetin exhibits a broad range of pharmacological activities with the myocardium. It protects against atherosclerosis by inhibiting lipoxygenase-induced lipoprotein oxidation in endothelial cells and high fat-induced obese apolipoprotein E knockout mice (Reiterer et al., 2004; Russo et al., 2012; Shen et al., 2013). Its experimental use at a dose of 10 mg/kg body weight for 28 days protects against autoimmune myocarditis by suppressing oxidative stress in rats (Arumugam et al., 2012). Quercetin is thought to

exert its cardiac protection through quenching lipid peroxidation, as it is a known scavenger of peroxyl radicals (Terao et al., 1994; Da Silva et al., 1998).

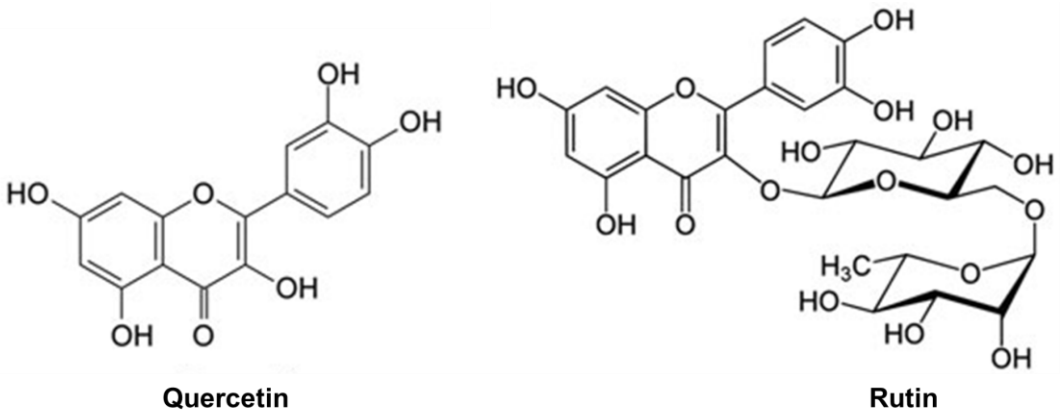
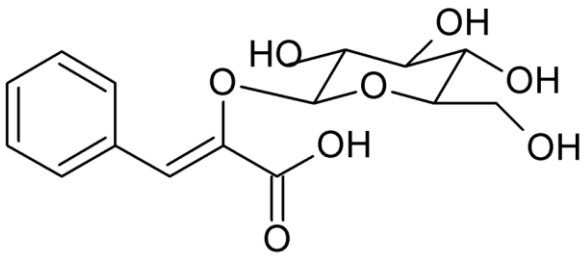


Figure 9. The chemical structure of major flavonols present in *Aspalathus linearis* (rooibos). Quercetin and its glycoside, rutin are classified by a distinct 3-hydroxyflavone backbone chemical structure.

Quercetin ameliorates hyperglycemia and dyslipidemia by improving the antioxidant status and inflammatory response in diabetic mice and rats (Cho et al., 2003; Oršolić et al., 2011; Maciel et al., 2013; Alam et al., 2014). In a double-blind randomized clinical trial done on women (n=72), quercetin supplementation (500 mg capsule daily) significantly reduced systolic blood pressure (Zahedi et al., 2013). In combination with rutin, it enhances glucose uptake in muscle cells subjected to oxidative stress (Dhanya et al., 2014). It also attenuates STZ-induced hepatocellular damage and kidney muscle dysregulations such as intracytoplasmic vacuolization and brush border loss (Elbe et al., 2015; Gomes et al., 2014). Quercetin has a high affinity to inhibit AGEs such as methylglyoxal and glyoxal in the bovine serum albumin system (Li et al., 2014). Its proposed potential mechanism against high glucose-induced damage is associated with the modulation of sirtuin-1-dependent endothelial nitric oxide synthase upregulation in endothelial progenitor cells (de Boer et al., 2006; Allard et al., 2009). It may also act by activating AMPK, causing increased GLUT-4 translocation associated with enhanced glucose uptake in rat muscle and liver cells (Eid et al., 2015). Likewise, rutin has been shown to display strong properties halt myocardial damage in a diabetic state decreasing postprandial hyperglycemia and slowing down formation of AGEs in various experimental models (Odetti et al., 1990; Stanley and Kamalakkannan, 2006; Fernandes et al., 2010; Wang et al., 2015).

1.10.5. The phenyl-propenoic acid glucoside present in rooibos

The occurrence of PPAG in rooibos was described for the first time about a decade ago (Fig. 10) (Marais et al., 1996). Phenylpyruvic acids apparently play a key role in the biosynthesis of a number of secondary metabolites, including PPAG (Joubert et al., 2013). Likewise, the biological activity of polyphenolic compounds with similar structures to PPAG such as 3-phenylpyruvate and β-phenylpyruvate have long been reported to display antidiabetic properties (Malaisse et al., 1983; Ben-Abraham et al., 2003). A fermented rooibos extract has been shown to contain abundant levels of PPAG compared to its unfermented form (Joubert et al., 2013). Exposure of cardiomyocytes isolated from diabetic rats to a low concentration of fermented rooibos that contains high levels of PPAG prevents oxidative stress and apoptosis (Dludla et al., 2014). Recent findings, studying PPAG have shown that this compound attenuates insulin resistance and protect beta cells from obese mice against endoplasmic reticulum stress-induced apoptosis by improving glucose tolerance (Muller et al., 2013; Mathijs et al., 2014; Himpe et al., 2016). Although an *in vitro* study on H9c2 cardiomyocytes recently showed that PPAG abolishes high glucose-induced altered myocardial substrate metabolism and apoptosis by increasing Bcl2/Bax ratio and reducing caspase 3/7 activity (Dludla et al., 2016), very limited data is available on the cardioprotective properties of PPAG.



Phenylpyruvic acid-2-*O*-β-D glucoside (PPAG)

Figure 10. The chemical structure of a phenylpropenoic acid-2-*O*-β-D-glucoside (PPAG) present in *Aspalathus linearis* (rooibos).

1.11. Problem statement

The World Health Organization is constantly reporting on the rise in the burden of non-communicable diseases. The increased percentage (up to 80%) in premature deaths resulting from non-communicable diseases in low and middle countries places a growing burden on healthcare resources in managing this worldwide epidemic. The largest proportion of non-communicable diseases are caused by complications associated with CVDs and DM. Furthermore, blood glucose lowering therapies such as insulin and metformin have played a major role in prolonging lives of diabetic patients. However, tight control of blood glucose still remains a challenge in such patients. By contrast, ameliorative therapies for oxidative stress, including polyphenols as an adjunct to current blood lowering drugs, show promise in protecting diabetic hearts in experimental models. In recent years rooibos has gained popularity due to its potential use as a dietary supplement that is rich in polyphenols. An increasing number of rooibos polyphenols present robust antioxidant properties that are associated with ameliorative effects on inflammation and apoptosis, leading to improved cardiac function in different animal models. However, the mechanisms by which rooibos and its polyphenolic compounds modulate hyperglycemia-induced oxidative stress are not fully elucidated, and the present dissertation aimed to improve on this understanding.

References

Adeghate, E., Singh, J., 2014. Structural changes in the myocardium during diabetes-induced cardiomyopathy. Heart Fail. Rev. 19,15–23.

Alam, M.M., Meerza, D., Naseem, I., 2014. Protective effect of quercetin on hyperglycemia, oxidative stress and DNA damage in alloxan induced type 2 diabetic mice. Life Sci. 109, 8–14.

Allard, J.S., Perez, E., Zou, S., de Cabo, R., 2009. Dietary activators of Sirt1. Mol. Cell. Endocrinol. 299, 58–63.

Alonso-Castro, A.J., Zapata-Bustos, R., Gómez-Espinoza, G., Salazar-Olivo, L.A., 2012. Isoorientin reverts TNF-α-induced insulin resistance in adipocytes activating the insulin signaling pathway. Endocrinology 153, 5222–5230.

American Diabetes Association, 2014. National Diabetes Statistics Report, 2014 estimates of diabetes and its burden in the epidemiologic estimation methods. Natl. Diabetes Stat. Rep. 2009–2012.

An, J., Chen, Y., Huang, Z., 2004. Critical upstream signals of cytochrome c release induced by a novel Bcl-2 inhibitor. J. Biol. Chem. 279, 19133–19140.

Anand, S.S., Yusuf, S., Vuksan, V., Devanesen, S., Teo, K.K., Montague, P.A., Kelemen, L., Yi, C., Lonn, E., Gerstein, H., Hegele, R.A., 2000. Differences in risk factors, atherosclerosis and cardiovascular disease between ethnic groups in Canada: the study of health assessment and risk in ethnic groups (SHARE). Indian Hear. J. 52, S35–S43.

Anedda, A., López-Bernardo, E., Acosta-Iborra, B., Saadeh Suleiman, M., Landázuri, M.O., Cadenas, S., 2013. The transcription factor Nrf2 promotes survival by enhancing the expression of uncoupling protein 3 under conditions of oxidative stress. Free Radic. Biol. Med. 61, 395–407.

Ansley, D.M., Wang, B., 2013. Oxidative stress and myocardial injury in the diabetic heart. J. Pathol. 229, 232–241.

Aroor, A.R., Mandavia, C.H., Sowers, J.R., 2012. Insulin resistance and heart failure: Molecular mechanisms. Heart Fail. Clin. 8, 609–617.

Arumugam, S., Thandavarayan, R.A., Arozal, W., Sari, F.R., Giridharan, V. V, Soetikno, V., Palaniyandi, S.S., Harima, M., Suzuki, K., Nagata, M., Tagaki, R., Kodama, M., Watanabe, K., 2012. Quercetin offers cardioprotection against progression of experimental autoimmune myocarditis by suppression of oxidative and endoplasmic reticulum stress via endothelin-1/MAPK signalling. Free Radic. Res. 46, 154–63.

Bai, Y., Cui, W., Xin, Y., Miao, X., Barati, M.T., Zhang, C., Chen, Q., Tan, Y., Cui, T., Zheng, Y., Cai,

L., 2013. Prevention by sulforaphane of diabetic cardiomyopathy is associated with up-regulation of Nrf2 expression and transcription activation. J. Mol. Cell. Cardiol. 57, 82–95.

Baines, C.P., 2009. The molecular composition of the mitochondrial permeability transition pore. J. Mol. Cell. Cardiol. 46, 850–857.

Beckman, J.A., Goldfine, A.B., Gordon, M.B., Garrett, L.A., Creager, M.A., 2002. Inhibition of protein kinase Cβ prevents impaired endothelium-dependent vasodilation caused by hyperglycemia in humans. Circ. Res. 90, 107–111.

Bedard, K., Krause, K.-H., 2007. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol. Rev. 87, 245–313.

Belke, D.D., Larsen, T.S., Gibbs, E.M., Severson, D.L., 2000. Altered metabolism causes cardiac dysfunction in perfused hearts from diabetic (db/db) mice. Am. J. Physiol. Endocrinol. Metab. 279, E1104–E1113.

Ben-Abraham, R., Gazit, V., Vofsi, O., Ben-Shlomo, I., Reznick, A.Z., Katz, Y., 2003. Beta-phenylpyruvate and glucose uptake in isolated mouse soleus muscle and cultured C2C12 muscle cells. J Cell Biochem 90, 957–963.

Berndt, C., Lillig, C.H., Holmgren, A., 2008. Thioredoxins and glutaredoxins as facilitators of protein folding. Biochim. Biophys. Acta - Mol. Cell Res. doi:10.1016/j.bbamcr.2008.02.003.

Bhatt, M.P., Lim, Y.C., Hwang, J., Na, S., Kim, Y.M., Ha, K.S., 2013. C-peptide prevents hyperglycemia-induced endothelial apoptosis through inhibition of reactive oxygen species-mediated transglutaminase 2 activation. Diabetes 62, 243–253.

Boden, G., Salehi, S., 2013. Why does obesity increase the risk for cardiovascular disease? Curr Pharm Des 19, 5678–5683.

Boekema, E.J., Braun, H.P., 2007. Supramolecular structure of the mitochondrial oxidative phosphorylation system. J. Biol. Chem. 282, 1–4.

Borutaite, V., Brown, G.C., 2003. Mitochondria in apoptosis of ischemic heart. FEBS Lett. 541, 1–5.

Boudina, S., Abel, E.D., 2010. Diabetic cardiomyopathy, causes and effects. Rev. Endocr. Metab. Disord. 11, 31–39.

Boudina, S., Abel, E.D., 2007. Diabetic cardiomyopathy revisited. Circulation. 115, 3213–3223.

Boudina, S., Abel, E.D., 2006. Mitochondrial uncoupling: a key contributor to reduced cardiac efficiency in diabetes. Physiology (Bethesda). 21, 250–258.

Boulares, A.H., Yakovlev, A.G., Ivanova, V., Stoica, B.A., Wang, G., Iyer, S., Smulson, M., Chem, M.J.B., 1999. Role of Poly (ADP-ribose) Polymerase (PARP) Cleavage in Apoptosis. J. Biol. Chem. 274, 22932–22940.

Bramati, L., Aquilano, F., Pietta, P., 2003. Unfermented rooibos tea: quantitative characterization of flavonoids by HPLC-UV and determination of the total antioxidant activity. J. Agric. Food Chem. 51, 7472–7474.

Brown, J.E., Khodr, H., Hider, R.C., Rice-Evans, C.A., 1998. Structural dependence of flavonoid interactions with Cu2+ ions: implications for their antioxidant properties. Biochem. J. 330 (Pt 3), 1173–1178.

Brownlee, M., 2001. Biochemistry and molecular cell biology of diabetic complications. Nature 414, 813–820.

Brownsey, R.W., Boone, A.N., Elliott, J.E., Kulpa, J.E., Lee, W.M., 2006. Regulation of acetyl-CoA carboxylase. Biochem. Soc. Trans. 34, 223–227.

Bryan, H.K., Olayanju, A., Goldring, C.E., Park, B.K., 2013. The Nrf2 cell defence pathway: Keap1-dependent and -independent mechanisms of regulation. Biochem. Pharmacol. doi:10.1016/j.bcp.2012.11.016.

Cai, L., Li, W., Wang, G., Guo, L., Jiang, Y., James Kang, Y., 2002. Hyperglycemia-induced apoptosis in mouse myocardium: Mitochondrial cytochrome c-mediated caspase-3 activation pathway. Diabetes 51, 1938–1948.

Cameron, N.E., Cotter, M.A., Jack, A.M., Basso, M.D., Hohman, T.C., 1999. Protein kinase C effects on nerve function, perfusion, Na(+), K(+)-ATPase activity and glutathione content in diabetic rats. Diabetologia 42, 1120–1130.

Cameron, N.E., Cotter, M.A., 2008. Pro-inflammatory mechanisms in diabetic neuropathy: focus on the nuclear factor kappa B pathway. Curr. Drug Targets 9, 60–67.

Camps, M., Castelló, A., Muñoz, P., Monfar, M., Testar, X., Palacín, M., Zorzano, A., 1992. Effect of diabetes and fasting on GLUT-4 (muscle/fat) glucose-transporter expression in insulin-sensitive tissues. Heterogeneous response in heart, red and white muscle. Biochem. J. 282 (Pt 3), 765–772.

Carling, D., 2004. The AMP-activated protein kinase cascade-a unifying system for energy control. Trends Biochem. Sci. 29, 18–24.

Cassidy, S., Hallsworth, K., Thoma, C., MacGowan, G.A., Hollingsworth, K.G., Day, C.P., Taylor, R.,

Jakovljevic, D.G., Trenell, M.I., 2015. Cardiac structure and function are altered in type 2 diabetes and non-alcoholic fatty liver disease and associate with glycemic control. Cardiovasc. Diabetol. 14, 23.

Castello, L., Froio, T., Maina, M., Cavallini, G., Biasi, F., Leonarduzzi, G., Donati, A., Bergamini, E., Poli, G., Chiarpotto, E., 2010. Alternate-day fasting protects the rat heart against age-induced inflammation and fibrosis by inhibiting oxidative damage and NF-kB activation. Free Radic. Biol. Med. 48, 47–54.

Cazarolli, L.H., Kappel, V.D., Pereira, D.F., Moresco, H.H., Brighente, I.M.C., Pizzolatti, M.G., Silva, F.R.M.B., 2012. Anti-hyperglycemic action of apigenin-6-C-β-fucopyranoside from *Averrhoa carambola*. Fitoterapia 83, 1176–1183.

Chen, C.H., Hsu, H.J., Huang, Y.J., Lin, C.J., 2007. Interaction of flavonoids and intestinal facilitated glucose transporters. Planta Med. 73, 348–354.

Chen, J., Cha-Molstad, H., Szabo, A., Shalev, A., 2009. Diabetes induces and calcium channel blockers prevent cardiac expression of proapoptotic thioredoxin-interacting protein. Am. J. Physiol. Endocrinol. Metab. 296, E1133–E1139.

Chen, W., Sudji, I.R., Wang, E., Joubert, E., Van Wyk, B.E., Wink, M., 2013. Ameliorative effect of aspalathin from rooibos (*Aspalathus linearis*) on acute oxidative stress in *Caenorhabditis elegans*. Phytomedicine 20, 380–386.

Chen, W., Sun, Z., Wang, X.J., Jiang, T., Huang, Z., Fang, D., Zhang, D.D., 2009. Direct interaction between Nrf2 and p21Cip1/WAF1 upregulates the Nrf2-mediated antioxidant response. Mol. Cell 34, 663–673.

Cho, S.Y., Park, S.J., Kwon, M.J., Jeong, T.S., Bok, S.H., Choi, W.Y., Jeong, W. Il, Ryu, S.Y., Do, S.H., Lee, C.S., Song, J.C., Jeong, K.S., 2003. Quercetin suppresses proinflammatory cytokines production through MAP kinases and NF-κB pathway in lipopolysaccharide-stimulated macrophage. Mol. Cell. Biochem. 243, 153–160.

Connelly, K.A., Kelly, D.J., Zhang, Y., Prior, D.L., Advani, A., Cox, A.J., Thai, K., Krum, H., Gilbert, R.E., 2009. Inhibition of protein kinase C-beta by ruboxistaurin preserves cardiac function and reduces extracellular matrix production in diabetic cardiomyopathy. Circ. Heart Fail. 2, 129–137.

Crompton, M., 1999. The mitochondrial permeability transition pore and its role in cell death. Biochem. J. 341 (Pt 2), 233–249.

Da Silva, E.L., Piskula, M.K., Yamamoto, N., Moon, J.H., Terao, J., 1998. Quercetin metabolites inhibit

copper ion-induced lipid peroxidation in rat plasma. FEBS Lett. 430, 405–408.

Dalal, S., Beunza, J.J., Volmink, J., Adebamowo, C., Bajunirwe, F., Njelekela, M., Mozaffarian, D., Fawzi, W., Willett, W., Adami, H.O., Holmes, M.D., 2011. Non-communicable diseases in sub-Saharan Africa: what we know now. Int. J. Epidemiol. 40, 885–901.

Dandamudi, S., Slusser, J., Mahoney, D.W., Redfield, M.M., Rodeheffer, R.J., Chen, H.H., 2014. The prevalence of diabetic cardiomyopathy: a population-based study in Olmsted County, Minnesota. J. Card. Fail. 20, 304–309.

De Boer, V.C.J., de Goffau, M.C., Arts, I.C.W., Hollman, P.C.H., Keijer, J., 2006. SIRT1 stimulation by polyphenols is affected by their stability and metabolism. Mech. Ageing Dev. 127, 618–627.

De Simone, G., Palmieri, V., Bella, J.N., Celentano, A., Hong, Y., Oberman, A., Kitzman, D.W., Hopkins, P.N., Arnett, D.K., Devereux, R.B., 2002. Association of left ventricular hypertrophy with metabolic risk factors: the HyperGEN study. J. Hypertens. 20, 323–331.

Del Rio, D., Rodriguez-Mateos, A., Spencer, J.P.E., Tognolini, M., Borges, G., Crozier, A., 2013. Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. Antioxid. Redox Signal. 18, 1818–92.

Devereux, R.B., Roman, M.J., Paranicas, M., O'Grady, M.J., Lee, E.T., Welty, T.K., Fabsitz, R.R., Robbins, D., Rhoades, E.R., Howard, B. V, 2000. Impact of diabetes on cardiac structure and function: the strong heart study. Circulation 101, 2271–2276.

Dhanya, R., Arun, K.B., Syama, H.P., Nisha, P., Sundaresan, A., Santhosh Kumar, T.R., Jayamurthy, P., 2014. Rutin and quercetin enhance glucose uptake in L6 myotubes under oxidative stress induced by tertiary butyl hydrogen peroxide. Food Chem. 158, 546–554.

Dinkova-Kostova, A.T., Abramov, A.Y., 2015. The emerging role of Nrf2 in mitochondrial function. Free Radic. Biol. Med. 88, 179–188.

Dludla, P.V., Muller, C.J.F., Louw, J., Joubert, E., Salie, R., Opoku, A.R., Johnson, R., 2014. The cardioprotective effect of an aqueous extract of fermented rooibos (*Aspalathus linearis*) on cultured cardiomyocytes derived from diabetic rats. Phytomedicine 21, 595–601.

Dludla, P.V., Muller, C.J.F., Joubert, E., Louw, J., Gabuza, K.B., Huisamen, B., Essop, M.F., Johnson, R., 2016. Phenylpyruvic acid-2-*O*-β-D-glucoside attenuates high glucose-induced apoptosis in H9c2 cardiomyocytes. Planta Med. 82, 1–7.

Eid, H.M., Nachar, A., Thong, F., Sweeney, G., Haddad, P.S., 2015. The molecular basis of the antidiabetic action of quercetin in cultured skeletal muscle cells and hepatocytes. Pharmacogn.

Mag. 11, 74–81.

Elbe, H., Vardi, N., Esrefoglu, M., Ates, B., Yologlu, S., Taskapan, C., 2015. Amelioration of streptozotocin-induced diabetic nephropathy by melatonin, quercetin, and resveratrol in rats. Hum. Exp. Toxicol. 34, 100–113.

Elmore, S., 2007. Apoptosis: a review of programmed cell death. Toxicol. Pathol. 35, 495–516.

Erejuwa, O.O., Sulaiman, S.A., Wahab, M.S.A., Sirajudeen, K.N.S., Md Salleh, M.S., Gurtu, S., 2010. Antioxidant protective effect of glibenclamide and metformin in combination with honey in pancreas of streptozotocin- induced diabetic rats. Int. J. Mol. Sci. 11, 2056–2066.

Esposito, K., Nappo, F., Marfella, R., Giugliano, G., Giugliano, F., Ciotola, M., Quagliaro, L., Ceriello, A., Giugliano, D., 2002. Inflammatory cytokine concentrations are acutely increased by hyperglycemia in humans: role of oxidative stress. Circulation 106, 2067–2072.

Essick, E.E., Ouchi, N., Wilson, R.M., Ohashi, K., Ghobrial, J., Shibata, R., Pimentel, D.R., Sam, F., 2011. Adiponectin mediates cardioprotection in oxidative stress-induced cardiac myocyte remodeling. Am. J. Physiol. Heart Circ. Physiol. 301, H984–H993.

Essop, M.F., Razeghi, P., McLeod, C., Young, M.E., Taegtmeyer, H., Sack, M.N., 2004. Hypoxia-induced decrease of UCP3 gene expression in rat heart parallels metabolic gene switching but fails to affect mitochondrial respiratory coupling. Biochem. Biophys. Res. Commun. 314, 561–564.

Eurich, D.T., Majumdar, S.R., McAlister, F.A., Tsuyuki, R.T., Johnson, J.A., 2005. Improved clinical outcomes associated with metformin in patients with diabetes and heart failure. Diabetes Care 28, 2345–2351.

Eurich, D.T., Weir, D.L., Majumdar, S.R., Tsuyuki, R.T., Johnson, J.A., Tjosvold, L., Vanderloo, S.E., McAlister, F.A., 2013. Comparative safety and effectiveness of metformin in patients with diabetes mellitus and heart failure: systematic review of observational studies involving 34,000 patients. Circ. Heart Fail. 6, 395–402.

Fang, F., Li, D., Pan, H., Chen, D., Qi, L., Zhang, R., Sun, H., 2011. Luteolin inhibits apoptosis and improves cardiomyocyte contractile function through the PI3K/Akt pathway in simulated ischemia/reperfusion. Pharmacology 88, 149–158.

Fernandes, A.A.H., Novelli, E.L.B., Okoshi, K., Okoshi, M.P., Muzio, B.P.D., Guimarães, J.F.C., Junior, A.F., 2010. Influence of rutin treatment on biochemical alterations in experimental diabetes. Biomed. Pharmacother. 64, 214–219.

Fonarow, G.C., Srikanthan, P., 2006. Diabetic Cardiomyopathy. Endocrinol. Metab. Clin. North Am. 35, 575–599.

Forbes, J.M., Cooper, M.E., 2013. Mechanisms of diabetic complications. Physiol. Rev. 93, 137–188.

Fu, X.J., Peng, Y.B., Hu, Y.P., Shi, Y.Z., Yao, M., Zhang, X., 2014. NADPH oxidase 1 and its derived reactive oxygen species mediated tissue injury and repair. Oxid. Med. Cell. Longev. 2014, 282854.

Galluzzi, L., Bravo-San Pedro, J.M., Vitale, I., Aaronson, S.A., Abrams, J.M., Adam, D., Alnemri, E.S., Altucci, L., Andrews, D., Annicchiarico-Petruzzelli, M., Baehrecke, E.H., Bazan, N.G., Bertrand, M.J., Bianchi, K., Blagosklonny, M. V., Blomgren, K., Borner, C., Bredesen, D.E., Brenner, C., Campanella, M., Candi, E., Cecconi, F., Chan, F.K., Chandel, N.S., Cheng, E.H., Chipuk, J.E., Cidlowski, J.A., Ciechanover, A., Dawson, T.M., Dawson, V.L., De Laurenzi, V., De Maria, R., Debatin, K.M., Di Daniele, N., Dixit, V.M., Dynlacht, B.D., El-Deiry, W.S., Fimia, G.M., Flavell, R.A., Fulda, S., Garrido, C., Gougeon, M.L., Green, D.R., Gronemeyer, H., Hajnoczky, G., Hardwick, J.M., Hengartner, M.O., Ichijo, H., Joseph, B., Jost, P.J., Kaufmann, T., Kepp, O., Klionsky, D.J., Knight, R.A., Kumar, S., Lemasters, J.J., Levine, B., Linkermann, A., Lipton, S.A., Lockshin, R.A., López-Otín, C., Lugli, E., Madeo, F., Malorni, W., Marine, J.C., Martin, S.J., Martinou, J.C., Medema, J.P., Meier, P., Melino, S., Mizushima, N., Moll, U., Muñoz-Pinedo, C., Nuñez, G., Oberst, A., Panaretakis, T., Penninger, J.M., Peter, M.E., Piacentini, M., Pinton, P., Prehn, J.H., Puthalakath, H., Rabinovich, G.A, Ravichandran, K.S., Rizzuto, R., Rodrigues, C.M., Rubinsztein, D.C., Rudel, T., Shi, Y., Simon, H.U., Stockwell, B.R., Szabadkai, G., Tait, S.W., Tang, H.L., Tavernarakis, N., Tsujimoto, Y., Vanden Berghe, T., Vandenabeele, P., Villunger, A., Wagner, E.F., Walczak, H., White, E., Wood, W.G., Yuan, J., Zakeri, Z., Zhivotovsky, B., Melino, G., Kroemer, G., 2014. Essential versus accessory aspects of cell death: recommendations of the NCCD 2015. Cell Death Differ. 1–16.

Geraldes, P., King, G.L., 2010. Activation of protein kinase C isoforms and its impact on diabetic complications. Circ. Res. 106, 1319–1331

Giacco, F., Brownlee, M., 2010. Oxidative stress and diabetic complications. Circ. Res. 107, 1058–1070.

Gomes, I.B.S., Porto, M.L., Santos, M.C.L.F.S., Campagnaro, B.P., Pereira, T.M.C., Meyrelles, S.S., Vasquez, E.C., 2014. Renoprotective, anti-oxidative and anti-apoptotic effects of oral low-dose quercetin in the C57BL/6J model of diabetic nephropathy. Lipids Health Dis. 13, 184.

Gordon, J.W., Shaw, J.A., Kirshenbaum, L.A., 2011. Multiple facets of NF-$_k$B in the heart: to be or not to NF-$_k$B. Circ. Res. 108, 1122–1132.

Goyal, R., Faizy, A.F, Islam, N., 2008. Effect of hyperglycemia on inflammatory markers in patients with type 2 diabetes, in: effect of hyperglycemia on inflammatory markers in patients with type 2 diabetes. Nature preceedings. doi:10.1038/npre.2008.1567.1.

Gray, S.P., Di Marco, E., Okabe, J., Szyndralewiez, C., Heitz, F., Montezano, A.C., De Haan, J.B., Koulis, C., El-Osta, A., Andrews, K.L., Chin-Dusting, J.P.F., Touyz, R.M., Wingler, K., Cooper, M.E., Schmidt, H.H.H.W., Jandeleit-Dahm, K.A., 2013. NADPH Oxidase 1 plays a key role in diabetes mellitus-accelerated atherosclerosis. Circulation 127, 1888–1902.

Guichard, C., Pedruzzi, E., Fay, M., Ben Mkaddem, S., Coant, N., Daniel, F., Ogier-Denis, E., 2006. [The Nox/Duox family of ROS-generating NADPH oxidases]. Med. Sci. (Paris). 22, 953–959.

Gundewar, S., Calvert, J.W., Jha, S., Toedt-Pingel, I., Ji, S.Y., Nunez, D., Ramachandran, A., Anaya-Cisneros, M., Tian, R., Lefer, D.J., 2009. Activation of AMP-activated protein kinase by metformin improves left ventricular function and survival in heart failure. Circ. Res. 104, 403–411.

He, X., Kan, H., Cai, L., Ma, Q., 2009. Nrf2 is critical in defense against high glucose-induced oxidative damage in cardiomyocytes. J. Mol. Cell. Cardiol. 46, 47–58.

Himpe, E., Cunha, D.A., Song, I., Bugliani, M., Marchetti, P., Cnop, M., Bouwens, L., 2016. Phenylpropenoic acid glucoside from rooibos protects pancreatic beta cells against cell death induced by acute injury. PLoS One 11, e0157604.

How, O.J., Aasum, E., Severson, D.L., Chan, W.Y.A., Essop, M.F., Larsen, T.S., 2006. Increased myocardial oxygen consumption reduces cardiac efficiency in diabetic mice. Diabetes 55, 466–473.

Huynh, K., Bernardo, B.C., McMullen, J.R., Ritchie, R.H., 2014. Diabetic cardiomyopathy: mechanisms and new treatment strategies targeting antioxidant signaling pathways. Pharmacol. Ther. 142, 375–415.

Hwang, Y.-J., Lee, E.-J., Kim, H.-R., Hwang, K.-A., 2013. Molecular mechanisms of luteolin-7-O-glucoside-induced growth inhibition on human liver cancer cells: G2/M cell cycle arrest and caspase-independent apoptotic signaling pathways. BMB Rep. 46, 611–616.

Ingwall, J.S., 2009. Energy metabolism in heart failure and remodelling. Cardiovasc. Res. 81(3):412–419.

Inoguchi, T., Li, P., Umeda, F., Yu, H.Y., Kakimoto, M., Imamura, M., Aoki, T., Etoh, T., Hashimoto, T., Naruse, M., Sano, H., Utsumi, H., Nawata, H., 1939. High glucose level and free fatty acid stimulate protein kinase C–dependent activation of NAD(P)H oxidase in cultured vascular cells.

medicine (Baltimore). 49, 1939–1945.

Inoue, T., Komoda, H., Nonaka, M., Kameda, M., Uchida, T., Node, K., 2008. Interleukin-8 as an independent predictor of long-term clinical outcome in patients with coronary artery disease. Int. J. Cardiol. 124, 319–325.

Jakupoglu, C., Przemeck, G.K.H., Schneider, M., Moreno, G., Mayr, N., Hatzopoulos, A.K., Hrabe, M., Angelis, D., Wurst, W., Bornkamm, G.W., Brielmeier, M., Conrad, M., Moreno, S.G., Mayr, N., Hatzopoulos, A.K., de Angelis, M.H., Wurst, W., Bornkamm, G.W., Brielmeier, M., Conrad, M., 2005. Cytoplasmic thioredoxin reductase is essential for embryogenesis but dispensable for cardiac development. Mol Cell Biol 25, 1980–1988.

Johnson, R., Dludla, P., Joubert, E., February, F., Mazibuko, S., Ghoor, S., Muller, C., Louw, J., 2016. Aspalathin, a dihydrochalcone C-glucoside, protects H9c2 cardiomyocytes against high glucose-induced shifts in substrate preference and apoptosis. Mol. Nutr. Food Res. 60, 922–934.

Joseph, D., Kimar, C., Symington, B., Milne, R., Faadiel Essop, M., 2014. The detrimental effects of acute hyperglycemia on myocardial glucose uptake. Life Sci. 105, 31–42.

Joubert, E., De Beer, D., Malherbe, C.J., Muller, N., Bonnet, S.L., Van Der Westhuizen, J.H., Ferreira, D., 2013. Occurrence and sensory perception of Z-2-(β-d-glucopyranosyloxy)-3- phenylpropenoic acid in rooibos (*Aspalathus linearis*). Food Chem. 136, 1078–1085.

Joubert, E., Winterton, P., Britz, T.J., Ferreira, D., 2004. Superoxide anion and α, α-diphenyl-β-picrylhydrazyl radical scavenging capacity of rooibos (*Aspalathus linearis*) aqueous extracts, crude phenolic fractions, tannin and flavonoids. Food Res. Int. 37, 133–138.

Kadenbach, B., 2003. Intrinsic and extrinsic uncoupling of oxidative phosphorylation. Biochim. Biophys. Acta - Bioenerg. 1604, 77–94.

Kahn, S.E., Haffner, S.M., Heise, M.A, Herman, W.H., Holman, R.R., Jones, N.P., Kravitz, B.G., Lachin, J.M., O'Neill, M.C., Zinman, B., Viberti, G., 2006. Glycemic durability of rosiglitazone, metformin, or glyburide monotherapy. N. Engl. J. Med. 355, 2427–2443.

Kamakura, R., Son, M.J., de Beer, D., Joubert, E., Miura, Y., Yagasaki, K., 2015. Antidiabetic effect of green rooibos (*Aspalathus linearis*) extract in cultured cells and type 2 diabetic model KK-Ay mice. Cytotechnology 67, 699–710.

Kawano, A., Nakamura, H., Hata, S. ichi, Minakawa, M., Miura, Y., Yagasaki, K., 2009. Hypoglycemic effect of aspalathin, a rooibos tea component from *Aspalathus linearis*, in type 2 diabetic model db/db mice. Phytomedicine 16, 437–443.

Kealey, A., 2010. Coronary artery disease and myocardial infarction in pregnancy: a review of epidemiology, diagnosis, and medical and surgical management. Can. J. Cardiol. 26, 185–189.

Kengne, A.P., Amoah, A.G.B., Mbanya, J.C., 2005. Cardiovascular complications of diabetes mellitus in sub-Saharan Africa. Circulation 112, 3592–3601.

Kengne, A.P., Dzudie, A., Sobngwi, E., 2008. Heart failure in sub-Saharan Africa: a literature review with emphasis on individuals with diabetes. Vasc. Health Risk Manag. 4, 123–130.

Khurana, R., Malik, I.S., 2010. Metformin: safety in cardiac patients. Postgrad. Med. J. 86, 371–373.

Kidd, L.B., Schabbauer, G.A., Luyendyk, J.P., Holscher, T.D., Tilley, R.E., Tencati, M., Mackman, N., 2008. Insulin activation of the phosphatidylinositol 3-kinase/protein kinase B (Akt) pathway reduces lipopolysaccharide-induced inflammation in mice. J. Pharmacol. Exp. Ther. 326, 348–353.

Kim, B.C., Kim, H.G., Lee, S.A., Lim, S., Park, E.H., Kim, S.J., Lim, C.J., 2005. Genipin-induced apoptosis in hepatoma cells is mediated by reactive oxygen species/c-Jun NH2-terminal kinase-dependent activation of mitochondrial pathway. Biochem. Pharmacol. 70, 1398–1407.

Kim, H.J., Lee, W., Yun, J.M., 2014. Luteolin inhibits hyperglycemia-induced proinflammatory cytokine production and its epigenetic mechanism in human monocytes. Phyther. Res. 28, 1383–1391.

Kim, H.S., Montana, V., Jang, H.J., Parpura, V., Kim, J.A., 2013. Epigallocatechin gallate (EGCG) stimulates autophagy in vascular endothelial cells: a potential role for reducing lipid accumulation. J. Biol. Chem. 288, 22693–22705.

Kim, N.H., Kang, P.M., 2010. Apoptosis in cardiovascular diseases: mechanism and clinical implications. Korean Circ. J. 40, 299–305.

King, G.L., 2008. The role of inflammatory cytokines in diabetes and its complications. J. Periodontol. 79, 1527–1534.

Kobayashi, A., Kang, M.I., Watai, Y., Tong, K.I., Shibata, T., Uchida, K., Yamamoto, M., 2006. Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. Mol. Cell. Biol. 26, 221–229.

Kode, A., Rajendrasozhan, S., Caito, S., Yang, S.R., Megson, I.L., Rahman, I., 2008. Resveratrol induces glutathione synthesis by activation of Nrf2 and protects against cigarette smoke-mediated oxidative stress in human lung epithelial cells. Am. J. Physiol. Lung Cell. Mol. Physiol. 294, L478–488.

Kowluru, A., Kowluru, R.A., 2014. Phagocyte-like NADPH oxidase [Nox2] in cellular dysfunction in

models of glucolipotoxicity and diabetes. Biochem. Pharmacol. 88, 275–283.

Ku, S.K., Lee, W., Kang, M, B.J., 2015. Antithrombotic activities of aspalathin and nothofagin via inhibiting platelet aggregation and FIIa/FXa. Arch. Pharm. Res. 38, 1080–1089.

Kumar, R., Yong, Q.C., Thomas, C.M., 2013. Do multiple nuclear factor kappa B activation mechanisms explain its varied effects in the heart? Ochsner J. 13, 157–165.

Kuroda, J., Ago, T., Matsushima, S., Zhai, P., Schneider, M.D., Sadoshima, J., 2010. NADPH oxidase 4 (Nox4) is a major source of oxidative stress in the failing heart. Proc. Natl. Acad. Sci. U. S. A. 107, 15565–15570.

Kwon, E.Y., Jung, U.J. u, Park, T., Yun, J.W. on, Choi, M.S., 2015. Luteolin attenuates hepatic steatosis and insulin resistance through the interplay between the liver and adipose tissue in mice with diet-induced obesity. Diabetes 64, 1658–1669.

Kwong, J.Q., Molkentin, J.D., 2015. Physiological and pathological roles of the mitochondrial permeability transition pore in the heart. Cell Metab. 21, 206-214.

Lee, W., Bae, J.S., 2015. Anti-inflammatory effects of aspalathin and nothofagin from rooibos (*Aspalathus linearis*) *in vitro* and *in vivo*. Inflammation 38, 1502–1516.

Lei, B., Lionetti, V., Young, M.E., Chandler, M.P., D'Agostino, C., Kang, E., Altarejos, M., Matsuo, K., Hintze, T.H., Stanley, W.C., Recchia, F.A., 2004. Paradoxical downregulation of the glucose oxidation pathway despite enhanced flux in severe heart failure. J. Mol. Cell. Cardiol. 36, 567–576.

Lemasters, J.J., Nieminen, A.L., Qian, T., Trost, L.C., Elmore, S.P., Nishimura, Y., Crowe, R.A., Cascio, W.E., Bradham, C.A., Brenner, D.A., Herman, B., 1998. The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. Biochim. Biophys. Acta - Bioenerg. 1366, 177–196.

Li, X., Zheng, T., Sang, S., Lv, L., 2014. Quercetin inhibits advanced glycation end product formation by trapping methylglyoxal and glyoxal. J. Agric. Food Chem. 62, 12152–12158.

Li, Z., Zhang, T., Dai, H., Liu, G., Wang, H., Sun, Y., Zhang, Y., Ge, Z., 2007. Involvement of endoplasmic reticulum stress in myocardial apoptosis of streptozocin-induced diabetic rats. J. Clin. Biochem. Nutr. 41, 58–67.

Liu, J., Minemoto, Y., Lin, A., 2004. c-Jun N-terminal protein kinase 1 (JNK1), but not JNK2, is essential for tumor necrosis factor alpha-induced c-Jun kinase activation and apoptosis. Mol. Cell. Biol. 24, 10844–10856.

Liu, Y., Tian, X., Gou, L., Sun, L., Ling, X., Yin, X., 2013. Luteolin attenuates diabetes-associated cognitive decline in rats. Brain Res. Bull. 94, 23–29.

Lopaschuk, G.D., Ussher, J.R., Folmes, C.D.L., Jaswal, J.S., Stanley, W.C., 2010. Myocardial fatty acid metabolism in health and disease. Physiol. Rev. 90, 207–258.

Lorenzo, O., Picatoste, B., Ares-Carrasco, S., Ramírez E., Egido, J., Tuñón J., 2011. Potential role of nuclear factor $_k$b in diabetic cardiomyopathy. Mediators Inflamm. 2011, 652097.

Lysiak, J.J., Nguyen, Q.A., Kirby, J.L., Turner, T.T., 2003. Ischemia-reperfusion of the murine testis stimulates the expression of proinflammatory cytokines and activation of c-jun N-terminal kinase in a pathway to E-selectin expression. Biol.Reprod. 69, 202–210.

Ma, X., He, D., Ru, X., Chen, Y., Cai, Y., Bruce, I.C., Xia, Q., Yao, X., Jin, J., 2012. Apigenin, a plant-derived flavone, activates transient receptor potential vanilloid 4 cation channel. Br. J. Pharmacol. 166, 349–358.

Maalouf, R.M., Eid, A.A., Gorin, Y.C., Block, K., Escobar, G.P., Bailey, S., Abboud, H.E., 2012. Nox4-derived reactive oxygen species mediate cardiomyocyte injury in early type 1 diabetes. Am J Physiol Cell Physiol 302, C597–C604.

Maciel, R.M., Costa, M.M., Martins, D.B., França, R.T., Schmatz, R., Graça, D.L., Duarte, M.M.M.F., Danesi, C.C., Mazzanti, C.M., Schetinger, M.R.C., Paim, F.C., Palma, H.E., Abdala, F.H., Stefanello, N., Zimpel, C.K., Felin, D. V., Lopes, S.T.A., 2013. Antioxidant and anti-inflammatory effects of quercetin in functional and morphological alterations in streptozotocin-induced diabetic rats. Res. Vet. Sci. 95, 389–397.

Madhesh, M., Vaiyapuri, M., 2013. Luteolin a dietary flavonoid attenuates isoproterenol-induced myocardial oxidative stress in rat myocardium: An in vivo study. Biomed. Prev. Nutr. 3, 159–164.

Magkou, D., Tziomalos, K., 2014. Antidiabetic treatment, stroke severity and outcome. World J. Diabetes 5, 84–88.

Makaula, S., Adam, T., Essop, M.F., 2006. Upstream stimulatory factor 1 transactivates the human gene promoter of the cardiac isoform of acetyl-CoA carboxylase. Arch. Biochem. Biophys. 446: 91–100.

Malaisse, W.J., Sener, A., Welsh, M., Malaisse-Lagae, F., Hellerstrom, C., Christophe, J., 1983. Mechanism of 3-phenylpyruvate-induced insulin release. Metabolic aspects. Biochem J 210, 921–927.

Mandavia, C.H., Aroor, A.R., Demarco, V.G., Sowers, J.R., 2013. Molecular and metabolic

mechanisms of cardiac dysfunction in diabetes. Life Sci. 92, 601–608.

Manley, M., Joubert, E., Botha, M., 2006. Quantification of the major phenolic compounds, soluble solid content and total antioxidant activity of green rooibos (*Aspalathus linearis*) by means of near infrared spectroscopy. J. Near Infrared Spectrosc. 14, 213–222.

Mao, X.Y., Yu, J., Liu, Z.Q., Z.H., 2015. Apigenin attenuates diabetes-associated cognitive decline in rats via suppressing oxidative stress and nitric oxide synthase pathway. Int. J. Clin. Exp. Med. 8, 15506–15513.

Mapanga, R.F., Essop, M.F., 2016. Damaging effects of hyperglycemia on cardiovascular function: spotlight on glucose metabolic pathways. Am J Physiol Heart Circ Physiol. 310, H153-173.

Marais, C., Steenkamp, J.A., Ferreira, D., 1996. The occurrence of phenylpyruvic acid in woody plants: Biosynthetic significance. Tetrahedron Lett. 37, 5763–5764.

Marfella, R., Di Filippo, C., Portoghese, M., Ferraraccio, F., Rizzo, M.R., Siniscalchi, M., Musacchio, E., D'Amico, M., Rossi, F., Paolisso, G., 2009. Tight glycemic control reduces heart inflammation and remodeling during acute myocardial infarction in hyperglycemic patients. J. Am. Coll. Cardiol. 53, 1425–1436.

Mariappan, N., Elks, C.M., Sriramula, S., Guggilam, A., Liu, Z., Borkhsenious, O., Francis, J., 2010. NF-kappaB-induced oxidative stress contributes to mitochondrial and cardiac dysfunction in type II diabetes. Cardiovasc. Res. 85, 473–483.

Marín-García, J., Akhmedov, A.T., Moe, G.W., 2013. Mitochondria in heart failure: The emerging role of mitochondrial dynamics. Heart Fail. Rev. 18, 439–456.

Marnewick, J.L., Rautenbach, F., Venter, I., Neethling, H., Blackhurst, D.M., Wolmarans, P., MacHaria, M., 2011. Effects of rooibos (*Aspalathus linearis*) on oxidative stress and biochemical parameters in adults at risk for cardiovascular disease. J. Ethnopharmacol. 133, 46–52.

Maron, B., Towbin, J., Thiene, G., Antzelevitch, C., 2006. cardiomyopathies an American heart association scientific statement from the council on clinical cardiology, heart failure and transplantation committee; quality of care. Circulation 113, 1807–1816.

Mathijs, I., da Cunha, D.A., Himpe, E., Ladriere, L., Chellan, N., Roux, C.R., Joubert, E., Muller, C., Cnop, M., Louw, J., Bouwens, L., 2014. Phenylpropenoic acid glucoside augments pancreatic beta cell mass in high-fat diet-fed mice and protects beta cells from ER stress-induced apoptosis. Mol. Nutr. Food Res. 58, 1980–1990.

Mazibuko, S.E., Joubert, E., Johnson, R., Louw, J., Opoku, A.R., Muller, C.J.F., 2015. Aspalathin

improves glucose and lipid metabolism in 3T3-L1 adipocytes exposed to palmitate. Mol. Nutr. Food Res. 59, 2199–2208.

Mazibuko, S.E., Muller, C.J.F., Joubert, E., De Beer, D., Johnson, R., Opoku, A.R., Louw, J., 2013. Amelioration of palmitate-induced insulin resistance in C2C12 muscle cells by rooibos (*Aspalathus linearis*). Phytomedicine 20, 813–819.

Mbanya, J.C.N., Motala, A.A., Sobngwi, E., Assah, F.K., Enoru, S.T., 2010. Diabetes in sub-Saharan Africa. Lancet. 375, 2254–2266.

McCullough, P.A, 2007. Coronary artery disease. Clin. J. Am. Soc. Nephrol. 2, 611–616.

Melter, M., Exeni, A., Reinders, M.E.J., Fang, J.C., McMahon, G., Ganz, P., Hancock, W.W., Briscoe, D.M., 2001. Expression of the chemokine receptor CXCR3 and its ligand IP-10 during human cardiac allograft rejection. Circulation 104, 2558–2564.

Messaoudi, S. El, Rongen, G.A., de Boer, R.A., Riksen, N.P., 2011. The cardioprotective effects of metformin. Curr. Opin. Lipidol. 22, 445–453.

Miguel-Carrasco, J.L., Zambrano, S., Blanca, A.J., Mate, A., Vázquez, C.M., 2010. Captopril reduces cardiac inflammatory markers in spontaneously hypertensive rats by inactivation of NF-kB. J. Inflamm. (Lond). 7, 21.

Mikami, N., Tsujimura, J., Sato, A., Narasada, A., Shigeta, M., Kato, M., Hata, S., Hitomi, E., 2015. Green rooibos extract from *Aspalathus linearis*, and its component, aspalathin, suppress elevation of blood glucose levels in mice and inhibit α-amylase and α-glucosidase activities in vitro. Food Sci. Technol. Res. 21, 231–240.

Miki, T., Yuda, S., Kouzu, H., Miura, T., 2013. Diabetic cardiomyopathy: Pathophysiology and clinical features. Heart Fail. Rev. 18, 149–166.

Mochly-Rosen, D., Das, K., Grimes, K. V, 2012. Protein kinase C, an elusive therapeutic target? Nat. Rev. Drug Discov. 11, 937–957.

Monaghan, K., McNaughten, J., McGahon, M.K., Kelly, C., Kyle, D., Yong, P.H., McGeown, J.G., Curtis, T.M., 2015. Hyperglycemia and diabetes downregulate the functional expression of TRPV4 channels in retinal microvascular endothelium. PLoS One 10, e0128359.

Montaigne, D., Marechal, X., Lefebvre, P., Modine, T., Fayad, G., Dehondt, H., Hurt, C., Coisne, A., Koussa, M., Remy-Jouet, I., Zerimech, F., Boulanger, E., Lacroix, D., Staels, B., Neviere, R., 2013. Mitochondrial dysfunction as an arrhythmogenic substrate: a translational proof-of-concept study in patients with metabolic syndrome developping post-operative atrial fibrillation. J. Am.

Coll. Cardiol. 62, 1466–1473.

Morciano, G., Giorgi, C., Bonora, M., Punzetti, S., Pavasini, R., Wieckowski, M.R., Campo, G., Pinton, P., 2015. Molecular identity of the mitochondrial permeability transition pore and its role in ischemia-reperfusion injury. J. Mol. Cell. Cardiol. 78, 142–153.

Mukhtar, H., Ahmad, N., 2000. Tea polyphenols: prevention of cancer and optimizing health. Am. J. Clin. Nutr. 71, 1698S–1702S.

Muller, C.J.F., Joubert, E., De Beer, D., Sanderson, M., Malherbe, C.J., Fey, S.J., Louw, J., 2012. Acute assessment of an aspalathin-enriched green rooibos (*Aspalathus linearis*) extract with hypoglycemic potential. Phytomedicine 20, 32–39.

Muller, C.J.F., Joubert, E., Pheiffer, C., Ghoor, S., Sanderson, M., Chellan, N., Fey, S.J., Louw, J., 2013. Z-2-(β-d-glucopyranosyloxy)-3-phenylpropenoic acid, an α-hydroxy acid from rooibos (*Aspalathus linearis*) with hypoglycemic activity. Mol. Nutr. Food Res. 57, 2216–2222.

Nagoshi, T., Yoshimura, M., M. C. Rosano, G., D. Lopaschuk, G., Mochizuki, S., 2011. Optimization of cardiac metabolism in heart failure. Curr. Pharm. Des. 17, 3846–3853.

Odetti, P.R., Borgoglio, A., De Pascale, A., Rolandi, R., Adezati, L., 1990. Prevention of diabetes-increased aging effect on rat collagen-linked fluorescence by aminoguanidine and rutin. Diabetes 39, 796–801.

Ohno, M., Shibata, C., Kishikawa, T., Yoshikawa, T., Takata, A., Kojima, K., Akanuma, M., Kang, Y.J., Yoshida, H., Otsuka, M., Koike, K., 2013. The flavonoid apigenin improves glucose tolerance through inhibition of microRNA maturation in miRNA103 transgenic mice. Sci. Rep. 3, 2553.

Orrenius, S., Zhivotovsky, B., Nicotera, P., 2003. Regulation of cell death: the calcium-apoptosis link. Nat. Rev. Mol. Cell Biol. 4, 552–565.

Oršolić, N., Gajski, G., Garaj-Vrhovac, V., Crossed D Signikić, D., Prskalo, Z.Š., Sirovina, D., 2011. DNA-protective effects of quercetin or naringenin in alloxan-induced diabetic mice. Eur. J. Pharmacol. 656, 110–118.

Panda, S., Kar, A., 2007. Apigenin (4',5,7-trihydroxyflavone) regulates hyperglycaemia, thyroid dysfunction and lipid peroxidation in alloxan-induced diabetic mice. J. Pharm. Pharmacol. 59, 1543–1548.

Pandey, K.B., Rizvi, S.I., 2009. Plant polyphenols as dietary antioxidants in human health and disease. Oxid. Med. Cell. Longev. 2, 270–278.

Pantsi, W.G., Marnewick, J.L., Esterhuyse, A.J., Rautenbach, F., Van Rooyen, J., 2011. Rooibos

(*Aspalathus linearis*) offers cardiac protection against ischaemia/reperfusion in the isolated perfused rat heart. Phytomedicine 18, 1220–1228.

Paravicini, T.M., Touyz, R.M., 2008. NADPH oxidases, reactive oxygen species, and hypertension: clinical implications and therapeutic possibilities. Diabetes Care 31 (Suppl 2), S170–180.

Park, H., Kaushik, V.K., Constant, S., Prentki, M., Przybytkowski, E., Ruderman, N., Saha, A.K., 2002. Coordinate regulation of malonyl-CoA decarboxylase, sn-glycerol-3-phosphate acyltransferase, and acetyl-CoA carboxylase by AMP-activated protein kinase in rat tissues in response to exercise. J. Biol. Chem. 277, 32571–32577.

Park, S.H., Gammon, S.R., Knippers, J.D., Paulsen, S.R., Rubink, D.S., Winder, W.W., 2002. Phosphorylation-activity relationships of AMPK and acetyl-CoA carboxylase in muscle. J. Appl. Physiol. 92, 2475–2482.

Pickup, J.C., 2004. Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes. Diabetes Care 27, 813–823.

Poornima, I.G., Parikh, P., Shannon, R.P., 2006. Diabetic cardiomyopathy: the search for a unifying hypothesis. Circ. Res. 98, 596–605.

Qi, L., Pan, H., Li, D., Fang, F., Chen, D., Sun, H., 2011. Luteolin improves contractile function and attenuates apoptosis following ischemia-reperfusion in adult rat cardiomyocytes. Eur. J. Pharmacol. 668, 201–207.

Rains, J.L., Jain, S.K., 2011. Oxidative stress, insulin signaling, and diabetes. Free Radic. Biol. Med. 50, 567–575.

Rajamani, U., Essop, M.F., 2010. Hyperglycemia-mediated activation of the hexosamine biosynthetic pathway results in myocardial apoptosis. Am. J. Physiol. Cell Physiol. 299, C139–147.

Ramasamy, R., Goldberg, I.J., 2010. Aldose reductase and cardiovascular diseases, creating human-like diabetic complications in an experimental model. Circ. Res. 106, 1449–1458.

Randle, P.J., 1998. Regulatory interactions between lipids and carbohydrates: the glucose fatty acid cycle after 35 years. Diabetes. Metab. Rev. 14, 263–283.

Randle, P. J., Garland, P. B., Hales, C. N., Newsholme, E.A., 1963. The glucose fatty-acid cycle its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. Lancet 281, 785–789.

Raynolds, L.T., Ngcwangu, S.U., 2010. Fair Trade Rooibos tea: Connecting South African producers and American consumer markets. Geoforum 41, 74–83.

Reaven, P.D., Sacks, J., 2005. Coronary artery and abdominal aortic calcification are associated with cardiovascular disease in type 2 diabetes. Diabetologia 48, 379–385.

Reiterer, G., Toborek, M., Hennig, B., 2004. Quercetin protects against linoleic acid-induced porcine endothelial cell dysfunction. J. Nutr. 134, 771–775.

Revollo, J., Grimm, A., Imai, S., 2007. The regulation of nicotinamide adenine dinucleotide biosynthesis by Nampt/PBEF/visfatin in mammals. Curr. Opin. 23, 164–170.

Ridker, P.M., Rifai, N., Pfeffer, M., Sacks, F., Lepage, S., Braunwald, E., 2000. Elevation of tumor necrosis factor-alpha and increased risk of recurrent coronary events after myocardial infarction. Circulation 101, 2149–2153.

Rijzewijk, L.J., Jonker, J.T., Van Der Meer, R.W., Lubberink, M., De Jong, H.W., Romijn, J.A., Bax, J.J., De Roos, A., Heine, R.J., Twisk, J.W., Windhorst, A.D., Lammertsma, A.A., Smit, J.W.A., Diamant, M., Lamb, H.J., 2010. Effects of hepatic triglyceride content on myocardial metabolism in type 2 diabetes. J. Am. Coll. Cardiol. 56, 225–233.

Roghani, M., Baluchnejadmojarad, T., 2010. Hypoglycemic and hypolipidemic effect and antioxidant activity of chronic epigallocatechin-gallate in streptozotocin-diabetic rats. Pathophysiology 17, 55–59.

Roghani, M., Baluchnejadmojarad, T., 2009. Chronic epigallocatechin-gallate improves aortic reactivity of diabetic rats: underlying mechanisms. Vascul. Pharmacol. 51, 84–89.

Rubler, S., Dlugash, J., Yuceoglu, Y.Z., Kumral, T., Branwood, A.W., Grishman, A., 1972. New type of cardiomyopathy associated with diabetic glomerulosclerosis. Am. J. Cardiol. 30, 595–602.

Russell, R.R., Bergeron, R., Shulman, G.I., Young, L.H., 1999. Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. Am. J. Physiol. 277, H643–649.

Russo, M., Spagnuolo, C., Tedesco, I., Bilotto, S., Russo, G.L., 2012. The flavonoid quercetin in disease prevention and therapy: Facts and fancies. Biochem. Pharmacol. 83, 6–15.

Rydström, J., 2006. Mitochondrial NADPH, transhydrogenase and disease. Biochim. Biophys. Acta-Bioenerg. 1757, 721–726.

Sabarimuthu Darlin Quine, P.S.R., 2005. Effects of (–)-epicatechin, a flavonoid on lipid peroxidation and antioxidants in streptozotocin-induced diabetic liver, kidney and heart. Pharmacol. Reports 57, 610–615.

Sabri, A., Hughie, H.H., Lucchesi, P.A., 2003. Regulation of hypertrophic and apoptotic signaling

pathways by reactive oxygen species in cardiac myocytes. Antioxid. Redox Signal. 5, 731–740.

Sack, M.N., 2009. Type 2 diabetes, mitochondrial biology and the heart. J. Mol. Cell. Cardiol. 46, 842–849.

Saini, H.K., Xu, Y.J., Zhang, M., Liu, P.P., Kirshenbaum, L.A., Dhalla, N.S., 2005. Role of tumour necrosis factor-alpha and other cytokines in ischemia-reperfusion-induced injury in the heart. Exp. Clin. Cardiol. 10, 213–222.

Sambandam, N., Steinmetz, M., Chu, A., Altarejos, J.Y., Dyck, J.R.B., Lopaschuk, G.D., 2004. Malonyl-CoA decarboxylase (MCD) is differentially regulated in subcellular compartments by 5'AMP-activated protein kinase (AMPK) studies using H9c2 cells overexpressing MCD and AMPK by adenoviral gene transfer technique. Eur. J. Biochem. 271, 2831–2840.

Sanderson, M., Mazibuko, S.E., Joubert, E., De Beer, D., Johnson, R., Pheiffer, C., Louw, J., Muller, C.J.F., 2014. Effects of fermented rooibos (*Aspalathus linearis*) on adipocyte differentiation. Phytomedicine 21, 109–117.

Sastre, M., Richardson, J.C., Gentleman, S.M., Brooks, D.J., 2011. Inflammatory risk factors and pathologies associated with Alzheimer's disease. Curr Alzheimer Res 8, 132–141.

Sentex, E., Wang, X., Liu, X., Lukas, A., Dhalla, N.S., 2006. Expression of protein kinase C isoforms in cardiac hypertrophy and heart failure due to volume overload. Can. J. Physiol. Pharmacol. 84, 227–238.

Schloms, L., Swart, A.C., 2014. Rooibos flavonoids inhibit the activity of key adrenal steroidogenic enzymes, modulating steroid hormone levels in H295R cells. Molecules 19, 3681–3695.

Schmidt, A.M., Yan, S.D., Wautier, J.L., Stern, D., 1999. Activation of receptor for advanced glycation end products: a mechanism for chronic vascular dysfunction in diabetic vasculopathy and atherosclerosis. Circ. Res. 84, 489–497.

Schwarz, K., Simonis, G., Yu, X., Wiedemann, S., Strasser, R.H., 2006. Apoptosis at a distance: Remote activation of caspase-3 occurs early after myocardial infarction. Mol. Cell. Biochem. 281, 45–54.

Shanmugam, N., Reddy, M.A., Guha, M., Natarajan, R., 2003. High glucose-induced expression of proinflammatory cytokine and chemokine genes in monocytic cells. Diabetes 52, 1256–1264.

Sharma, K., Ziyadeh, F.N., 1995. Hyperglycemia and diabetic kidney disease. The case for transforming growth factor-beta as a key mediator. Diabetes 44, 1139–1146.

Sharma, P., Jha, A.B., Dubey, R.S., Pessarakli, M., 2012. Reactive oxygen species, Oxidative

Damage, and antioxidative defense mechanism in plants under stressful conditions. J. Bot. 2012, 1–26.

Shen, Y., Ward, N.C., Hodgson, J.M., Puddey, I.B., Wang, Y., Zhang, D., Maghzal, G.J., Stocker, R., Croft, K.D., 2013. Dietary quercetin attenuates oxidant-induced endothelial dysfunction and atherosclerosis in apolipoprotein E knockout mice fed a high-fat diet: a critical role for heme oxygenase-1. Free Radic. Biol. Med. 65, 908–915.

Sivitz, W.I., Yorek, M.A., 2010. Mitochondrial dysfunction in diabetes: from molecular mechanisms to functional significance and therapeutic opportunities. Antioxid. Redox Signal. 12, 537–577.

Smith, S.C., 2007. Multiple risk factors for cardiovascular disease and diabetes mellitus. Am. J. Med. 120(3 Suppl 1), S3–S11.

Snijman, P.W., Joubert, E., Ferreira, D., Li, X.C., Ding, Y., Green, I.R., Gelderblom, W.C.A., 2009. Antioxidant activity of the dihydrochalcones aspalathin and nothofagin and their corresponding flavones in relation to other rooibos (*Aspalathus linearis*) flavonoids, epigallocatechin gallate, and Trolox. J. Agric. Food Chem. 57, 6678–6684.

Soliman, H., Craig, G.P., Nagareddy, P., Yuen, V.G., Lin, G., Kumar, U., McNeill, J.H., MacLeod, K.M., 2008. Role of inducible nitric oxide synthase in induction of RhoA expression in hearts from diabetic rats. Cardiovasc. Res. 79, 322–330.

Son, M.J., Minakawa, M., Miura, Y., Yagasaki, K., 2013. Aspalathin improves hyperglycemia and glucose intolerance in obese diabetic ob/ob mice. Eur. J. Nutr. 52, 1607–1619.

Sovari, A.A., Rutledge, C.A., Jeong, E.M., Dolmatova, E., Arasu, D., Liu, H., Vahdani, N., Gu, L., Zandieh, S., Xiao, L., Bonini, M.G., Duffy, H.S., Dudley, S.C., 2013. Mitochondria oxidative stress, connexin43 remodeling, and sudden arrhythmic death. Circ. Arrhythmia Electrophysiol. 6, 623–631.

Stanley, M.P.S., Kamalakkannan, N., 2006. Rutin improves glucose homeostasis in streptozotocin diabetic tissues by altering glycolytic and gluconeogenic enzymes. J. Biochem. Mol. Toxicol. 20, 96–102.

Stanley, W.C., Recchia, F. A., Lopaschuk, G.D., 2005. Myocardial substrate metabolism in the normal and failing heart. Physiol. Rev. 85, 1093–1129.

Stockklauser-Färber, K., Ballhausen, T., Laufer, A., Rösen, P., 2000. Influence of diabetes on cardiac nitric oxide synthase expression and activity. Biochim. Biophys. Acta 1535, 10–20.

Suh, K.S., Oh, S., Woo, J.T., Kim, S.W., Kim, J.W., Kim, Y.S., Chon, S., 2012. Apigenin attenuates 2-

deoxy-D-ribose-induced oxidative cell damage in HIT-T15 pancreatic β-cells. Biol. Pharm. Bull. 35, 121–126.

Sukumar, P., Viswambharan, H., Imrie, H., Cubbon, R.M., Yuldasheva, N., Gage, M., Galloway, S., Skromna, A., Kandavelu, P., Santos, C.X., Gatenby, V.K., Smith, J., Beech, D.J., Wheatcroft, S.B., Channon, K.M., Shah, A.M., Kearney, M.T., 2013. Nox2 NADPH oxidase has a critical role in insulin resistance-related endothelial cell dysfunction. Diabetes 62, 2130–2134.

Sun, D., Huang, J., Zhang, Z., Gao, H., Li, J., Shen, M., Cao, F., Wang, H., 2012. Luteolin limits infarct size and improves cardiac function after myocardium ischemia/reperfusion injury in diabetic rats. PLoS One 7.

Tarquini, R., Lazzeri, C., Pala, L., Rotella, C.M., Gensini, G.F., 2011. The diabetic cardiomyopathy. Acta Diabetol. 48:173–181.

Terao, J., Piskula, M., Yao, Q., 1994. Protective effect of epicatechin, epicatechin gallate, and quercetin on lipid peroxidation in phospholipid bilayers. Arch. Biochem. Biophys. 308, 278–284.

Thomas, C.M., Yong, Q.C., Rosa, R.M., Seqqat, R., Gopal, S., Casarini, D.E., Jones, W.K., Gupta, S., Baker, K.M., Kumar, R., 2014. Cardiac-specific suppression of NF-κB signaling prevents diabetic cardiomyopathy via inhibition of the renin-angiotensin system. Am. J. Physiol. Heart Circ. Physiol. 307, H1036–1045.

Tsirka, A.E., Gruetzmacher, E.M., Kelley, D.E., Ritov, V.H., Devaskar, S.U., Lane, R.H., 2001. Myocardial gene expression of glucose transporter 1 and glucose transporter 4 in response to uteroplacental insufficiency in the rat. J. Endocrinol. 169, 373–380.

Turner, N.A., Xia, F., Azhar, G., Zhang, X., Liu, L., Wei, J.Y., 1998. Oxidative stress induces DNA fragmentation and caspase activation via the c-Jun NH2-terminal kinase pathway in H9c2 cardiac muscle cells. J. Mol. Cell. Cardiol. 30, 1789–1801.

Uemura, S., Matsushita, H., Li, W., Glassford, A.J., Asagami, T., Lee, K.H., Harrison, D.G., Tsao, P.S., 2001. Diabetes mellitus enhances vascular matrix metalloproteinase activity: role of oxidative stress. Circ. Res. 88, 1291–1298.

Valouskova, E., Modriansky, M., 2008. Modulation of UCP2 expression by p38--a link to cardioprotection. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 152, 3–7.

Van den Brom, C.E., Bosmans, J.W.AM., Vlasblom, R., Handoko, L.M., Huisman, M.C., Lubberink, M., Molthoff, C.F.M., Lammertsma, A.A., Ouwens, M.D., Diamant, M., Boer, C., 2010. Diabetic cardiomyopathy in Zucker diabetic fatty rats: the forgotten right ventricle. Cardiovasc. Diabetol. 9,

25.

Van den Brom, C.E., Huisman, M.C., Vlasblom, R., Boontje, N.M., Duijst, S., Lubberink, M., Molthoff, C.F.M., Lammertsma, A.A., van der Velden, J., Boer, C., Ouwens, D.M., Diamant, M., 2009. Altered myocardial substrate metabolism is associated with myocardial dysfunction in early diabetic cardiomyopathy in rats: studies using positron emission tomography. Cardiovasc. Diabetol. 8, 1–12.

Van Der Merwe, J.D., Joubert, E., Manley, M., De Beer, D., Malherbe, C.J., Gelderblom, W.C.A., 2010. In vitro hepatic biotransformation of aspalathin and nothofagin, dihydrochalcones of rooibos (*Aspalathus linearis*), and assessment of metabolite antioxidant activity. J. Agric. Food Chem. 58, 2214–2220.

Van Linthout, S., Riad, A., Dhayat, N., Spillmann, F., Du, J., Dhayat, S., Westermann, D., Hilfiker-Kleiner, D., Noutsias, M., Laufs, U., Schultheiss, H.P., Tschöpe, C., 2007. Anti-inflammatory effects of atorvastatin improve left ventricular function in experimental diabetic cardiomyopathy. Diabetologia 50, 1977–1986.

Von Gadow, A., Joubert, E., Hansmann, C.F., 1997. Comparison of the antioxidant activity of aspalathin with that of other plant phenols of rooibos tea (*Aspalathus linearis*), α-tocopherol, BHT, and BHA. J. Agric. Food Chem. 45, 632–638.

Von Gadow, A., Joubert, E., Hansmann, C.F., 1997. Comparison of the antioxidant activity of rooibos tea (*Aspalathus linearis*) with green, oolong and black tea. Food Chem. 60, 73–77.

Wang, G., Li, W., Lu, X., Bao, P., Zhao, X., 2012. Luteolin ameliorates cardiac failure in type I diabetic cardiomyopathy. J. Diabetes Complications 26, 259–265.

Wang, Q.Q, Cheng, N., Zheng, X.W., Peng, S.M., Z.X., 2013. Synthesis of organic nitrates of luteolin as a novel class of potent aldose reductase inhibitors. Bioorg Med Chem 21, 4301–4310.

Wang, X., McLennan, S. V., Allen, T.J., Twigg, S.M., 2010. Regulation of pro-inflammatory and pro-fibrotic factors by CCN2/CTGF in H9c2 cardiomyocytes. J. Cell Commun. Signal. 4, 15–23.

Wang, Y.B., Ge, Z.M., Kang, W.Q., Lian, Z.X., Yao, J., Zhou, C.Y., 2015. Rutin alleviates diabetic cardiomyopathy in a rat model of type 2 diabetes. Exp. Ther. Med. 9, 451–455.

Watson, R.T., Kanzaki, M., Pessin, J.E., 2004. Regulated membrane trafficking of the insulin-responsive glucose transporter 4 in adipocytes. Endocr. Rev. 25, 177–1204.

Webster, K.A., 2012. Mitochondrial membrane permeabilization and cell death during myocardial infarction: roles of calcium and reactive oxygen species. Future Cardiol. 8, 863–84.

Wen, H.L., Liang, Z.S., Zhang, R., Yang, K., 2013. Anti-inflammatory effects of triptolide improve left ventricular function in a rat model of diabetic cardiomyopathy. Cardiovasc. Diabetol. 12, 50.

Westermann, D., Rutschow, S., Jager, S., Linderer, A., Anker, S., Riad, A., Unger, T., Schultheiss, H.P., Pauschinger, M., Tschope, C., 2007. Contributions of inflammation and cardiac matrix metalloproteinase activity to cardiac failure in diabetic cardiomyopathy: the role of angiotensin type 1 receptor antagonism. Diabetes 56, 641–646.

Willerson, J.T., Ridker, P.M., 2004. Inflammation as a cardiovascular risk factor. Circulation 109, II2–I10.

World Health Organization (WHO), 2014. Global status report on noncommunicable diseases 2014. World Health 176. doi:ISBN 9789241564854.

World Health Organization, W.H.O., 2012. World Health Statistics 2012, World Health Statistics 2012. doi:10.2307/3348165.

Xu, Q., Hao, X., Yang, Q., Si, L., 2009. Resveratrol prevents hyperglycemia-induced endothelial dysfunction via activation of adenosine monophosphate-activated protein kinase. Biochem. Biophys. Res. Commun. 388, 389–394.

Yin, M., van der Horst, I.C.C., van Melle, J.P., Qian, C., van Gilst, W.H., Silljé, H.H.W., de Boer, R.A, 2011. Metformin improves cardiac function in a nondiabetic rat model of post-MI heart failure. Am. J. Physiol. Heart Circ. Physiol. 301, H459–468.

Yu, W., Zha, W., Ke, Z., Min, Q., Li, C., Sun, H., Liu, C., 2016. Curcumin protects neonatal rat cardiomyocytes against high glucose-induced apoptosis via PI3K/Akt signalling pathway. J. Diabetes Res. 2016, 4158591.

Yu, X.Y., Song, Y.H., Geng, Y.J., Lin, Q.X., Shan, Z.X., Lin, S.G., Li, Y., 2008. Glucose induces apoptosis of cardiomyocytes via microRNA-1 and IGF-1. Biochem. Biophys. Res. Commun. 376, 548–552.

Zahedi, M., Ghiasvand, R., Feizi, A., Asgari, G., Darvish, L., 2013. Does quercetin improve cardiovascular risk factors and inflammatory biomarkers in women with type 2 diabetes: A double-blind randomized controlled clinical trial. Int. J. Prev. Med. 4, 777–785.

Zamzami, N., Marchetti, P., Castedo, M., Decaudin, D., Macho, a, Hirsch, T., Susin, S. a, Petit, P.X., Mignotte, B., Kroemer, G., 1995. Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. J. Exp. Med. 182, 367–377.

Zhang, X., Wang, G., Gurley, E.C., Zhou, H., 2014. Flavonoid apigenin inhibits lipopolysaccharide-induced inflammatory response through multiple mechanisms in Macrophages. PLoS One 9, e107072.

Zhao, J., Randive, R., Stewart, J.A., 2014. Molecular mechanisms of AGE/RAGE-mediated fibrosis in the diabetic heart. World J. Diabetes 5, 860–867.

Zisman, A., Peroni, O.D., Abel, E.D., Michael, M.D., Mauvais-Jarvis, F., Lowell, B.B., Wojtaszewski, J.F.P., Hirshman, M.F., Virkamaki, A., Goodyear, L.J., Kahn, C.R., Kahn, B.B., Division, E., Israel, B., Medical, D., Zisman, A., Peroni, O.D., Abel, E.D., Michael, M.D., Mauvais-Jarvis, F., Lowell, B.B., Wojtaszewski, J.F.P., Hishman, M.F., Virkamaki, A., Goodyear, L.J., Kahn, C.R., Kahn, B.B., 2000. Targeted disruption of the glucose transporter 4 selectively in muscle causes insulin resistance and glucose intolerance. Nat. Med. 6, 924–928.

Zorov, D.B., Juhaszova, M., Sollott, S.J., 2014. Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. Physiol. Rev. 94, 909–50.

Zungu, M., Schisler, J.C., Essop, M.F., McCudden, C., Patterson, C., Willis, M.S., 2011. Regulation of AMPK by the ubiquitin proteasome system. Am. J. Pathol. 178, 4–11.

Chapter 2: Phenylpyruvic acid-2-*O*-β-D-glucoside attenuates high glucose-induced apoptosis in H9c2 cardiomyocytes

Phiwayinkosi Vusi Dludla[1, 2], Christo John Frederick Muller[1], Elizabeth Joubert[3, 4], Johan Louw[1], Kwazi Bethuel Gabuza[1], Barbara Huisamen[1, 2], M. Faadiel Essop[5] and Rabia Johnson[1].

[1]Biomedical Research and Innovation Platform (BRIP), Medical Research Council (MRC), Tygerberg, South Africa.

[2]Division of Medical Physiology, Faculty of Health Sciences, Stellenbosch University, Tygerberg, South Africa.

[3]Post-Harvest and Wine Technology Division, Agricultural Research Council (ARC) Infruitec-Nietvoorbij, Stellenbosch, South Africa.

[4]Department of Food Science, Stellenbosch University, Stellenbosch, South Africa.

[5]Cardio-Metabolic Research Group (CMRG), Department of Physiological Sciences, Stellenbosch University, Stellenbosch, South Africa.

Published article: Planta Medica 2016; 82: 1–7.

My contribution:

Project conception and design

Performed experiments

Data analysis and interpretation

Wrote the manuscript

Abstract

Chronic hyperglycemia is closely associated with impaired substrate metabolism, dysregulated mitochondrial membrane potential and apoptosis in the diabetic heart. As adult cardiomyocytes display limited capacity to regenerate following an insult, it is essential to protect the myocardium against the detrimental effects of chronic hyperglycemia. This study therefore investigated whether phenylpyruvic acid-2-*O*-β-D-glucoside (PPAG), present in *Aspalathus linearis* (rooibos), is able to attenuate hyperglycemia-induced damage in H9c2 cardiomyocytes. H9c2 cardiomyocytes were exposed to a high glucose concentration (33 mM) prior to treatment with PPAG (1 μM), metformin (1 μM), or a combination of PPAG and metformin (both at 1 μM). Our data revealed that high glucose exposure increased cardiac free fatty acid uptake and oxidation, mitochondrial membrane potential and apoptosis (caspase 3/7 activity and TUNEL) and decreased the Bcl2/Bax protein expression ratio. PPAG treatment, alone or in combination with metformin, attenuated these glucose-induced perturbations, confirming its protective effect in H9c2 cardiomyocytes exposed to chronic hyperglycemia.

Key words: Phenylpyruvic acid-2-*O*-β-D-glucoside, hyperglycemia, fatty acid uptake and oxidation, myocardial apoptosis.

Introduction

Cardiovascular diseases (CVD) are a leading cause of death worldwide [1]. Moreover, the continuous rise in non-communicable diseases such as diabetes mellitus (DM) and obesity remains a major burden contributing to increased CVD onset [1]. There are currently ~415 million individuals burdened with DM and this is estimated to reach ~642 million by the year 2040 [2]. Patients with DM are at increased risk of developing CVD [2, 3]. In addition, the chronic hyperglycemia of DM contributes to the development of diabetic cardiomyopathy [4, 5]. Cardiomyopathies are a class of heart diseases that distinctly affect the structure of the heart muscle independently of coronary artery disease and hypertension [4, 5]. Although mainly non-ischemic of nature, cardiomyopathies play a predominant role to induce heart failure and are one of the major causes of death in southern Africa [5, 6].

The mechanisms by which chronic hyperglycemia induces myocardial injury are varied and have not been fully elucidated [7]; however, enhanced intracellular flux of free fatty acids (FFAs) is implicated as it can result in mitochondrial deficiencies and subsequently trigger myocardial apoptosis [8-10]. FFAs are the preferred substrate for energy generation within the normal mammalian heart, while glucose provides the majority of the remaining proportion [11]. The diabetic heart utilizes a high proportion of FFAs as an energy source that can elicit detrimental effects, e.g. the deterioration of heart function and a state of energy deficiency [11-13]. Furthermore, increased FFA uptake within cardiomyocytes subjected to elevated concentrations of glucose is accompanied by aberrant mitochondrial membrane potential, preceding myocardial apoptosis [14, 15].

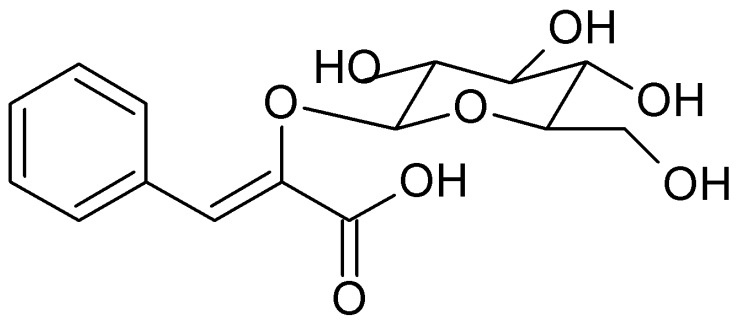


Figure 1. The chemical structure of phenylpyruvic acid-2-*O*-β-D-glucoside (PPAG).

Phytochemicals, especially those present in plant foods, are gaining increasing popularity due to potential health-promoting properties. Such compounds are therefore actively screened for novel antidiabetic and cardioprotective properties [16-19]. For example, *Aspalathus linearis* (Brum.f) Dahlg. (Fabaceae) used to brew a popular herbal tea known as rooibos, contains polyphenols such as the

novel dihydrochalcone, aspalathin, with robust antioxidant properties [17]. A precursor in the flavonoid biosynthesis pathway and a non-phenolic compound, phenylpyruvic acid-2-*O*-β-D-glucoside (PPAG) (Fig. 1) [20], is not unique to rooibos, but it occurs rarely in nature and only few studies have reported on its presence in plants [21]. Of note, *in vitro* and *in vivo* models of diabetes previously demonstrated that PPAG improved metabolic parameters such as glucose intolerance and it also protected pancreatic β-cells against endoplasmic reticulum stress and palmitate-induced apoptosis [19, 22]. However, as its effects within the mammalian heart are unknown, this study aimed to establish whether PPAG can protect heart cells exposed to chronic hyperglycemia.

Results

Compared to the experimental control (cells exposed to media only) (100.0 ± 8.2%), PPAG induced a concentration-dependent response with the highest ATP concentrations at 1 µM (119.4 ± 0.5%, p = 0.0001) (Fig. 2). We therefore employed this dosage for the remainder of experiments conducted in this study.

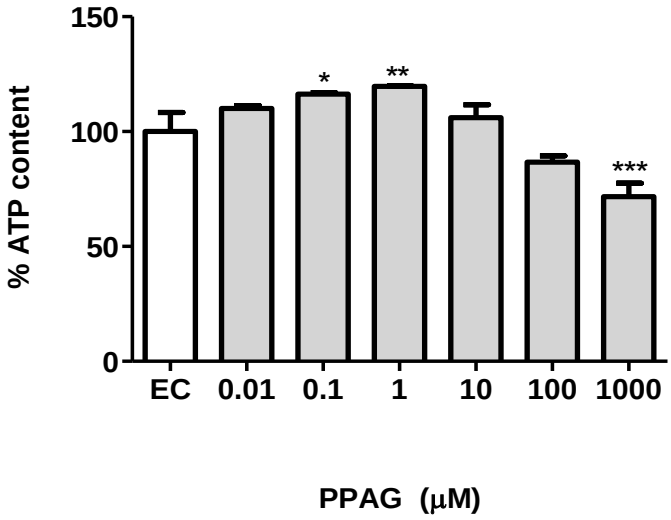


Figure 2. Determination of optimal PPAG treatment dose. H9c2 cardiomyocytes were treated for 12 hours with various concentrations of PPAG (0.01 to 1000 µM) to determine ATP content as a measurement of metabolic activity. Results are the mean ± SD of 3 independent biological experiments, each done in triplicate. *p ≤ 0.05, **p < 0.01, ***p < 0.001 compared to experimental control (EC; untreated cells).

High glucose (33 mM) induced a robust increase in fatty acid uptake (FAU) (135.2 ± 4.9%, p = 0.0001) and fatty acid oxidation (FAO) (140.0 ± 7.0%, p = 0.0002) in H9c2 cardiomyocytes when compared to

the normal glucose control (100.0 ± 3.9% and 100.0 ± 4.2%, respectively) (Fig. 3A, B). Post-treatment with metformin (123.2 ± 6.1%, p = 0.05 and 120.1 ± 8.2%, p = 0.05), PPAG (115.0 ± 4.7%, p = 0.001 and 112.1 ± 3.2%, p = 0.001), as well as a combination of metformin + PPAG (114.0 ± 2.4%, p = 0.001 and 113.2 ± 3.9%, p = 0.001) significantly reduced FAU and FAO, respectively (Fig. 3A, B).

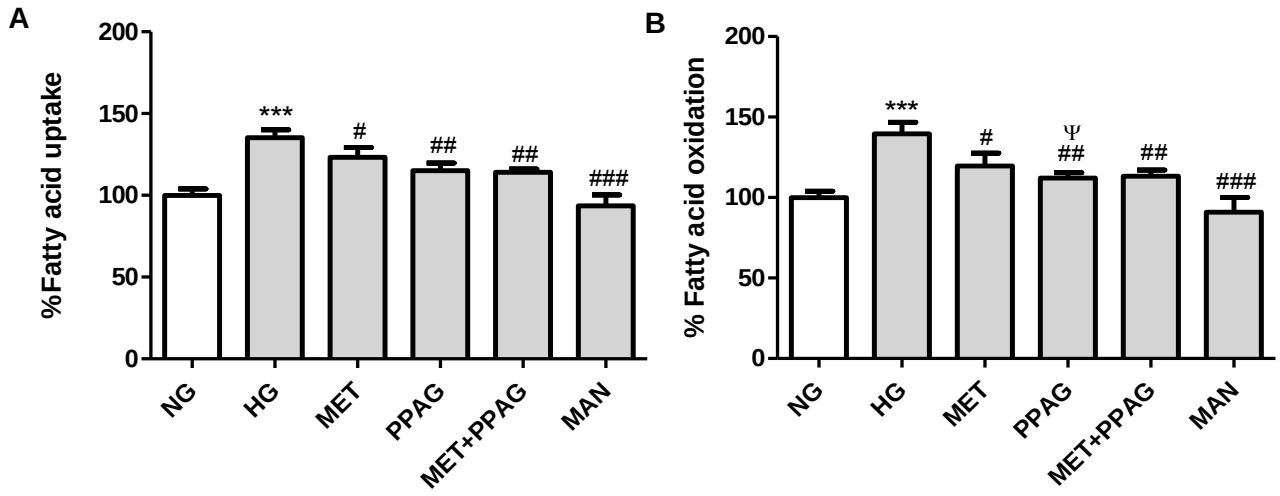


Figure 3. Effect of PPAG, metformin (MET) and MET + PPAG on high glucose-induced alteration of fatty acid uptake and oxidation in H9c2 cardiomyocytes. (A) Rate of fatty acid uptake (FAU) and (B) fatty acid oxidation (FAO). Mannitol (MAN) did not have an effect on either FAU or FAO and was comparable to the normal glucose (NG) control. Results are the mean ± SEM of 3 independent biological experiments relative to the NG control, each done in triplicate. ***$p < 0.0001$ versus NG; $^{\#}p < 0.05$, $^{\#\#}p < 0.001$, $^{\#\#\#}p < 0.0001$ versus high glucose (HG). $^{\Psi}p < 0.05$ versus MET.

2', 7'-dichlorfluorescein diacetate (DCFH-DA) fluorescence intensity as a representative of reactive oxygen species (ROS) formation was significantly increased (141.3 ± 4.9%, p = 0.0001) after chronically exposing H9c2 cells to 33mM glucose when compared to the normal glucose control (100.1 ± 3.1%) (Fig. 4A). Enhanced ROS in high glucose exposed cells occurred concomitant to the reduction of glutathione (GSH) content (CellTracker fluorescent intensity) (62.4 ± 1.6%, p = 0.0001) and superoxide dismutase (SOD) activity (59.4 ± 3.9%, p = 0.0001) (Fig. 4AB). Although PPAG was able to reduce enhanced ROS production (127.3 ± 5.0%, p = 0.05), it failed to improve decreased GSH content (68.4 ± 4.0%) and SOD activity (61.4 ± 4.8%). Interestingly, metformin and its combination with PPAG showed more potency in ameliorating ROS generation (123.4 ± 2.9%, p = 0.001 and 110.4 ± 5.0%, p = 0.0001) and improving GSH content (81.2 ± 4.2%, p = 0.0001 and 88.4 ± 4.4%, p = 0.0001) and SOD activity (86.0 ± 4.5%, p = 0.0001 and 75.2 ± 4.9%, p = 0.001) when compared to PPAG monotherapy (Fig. 4ABC).

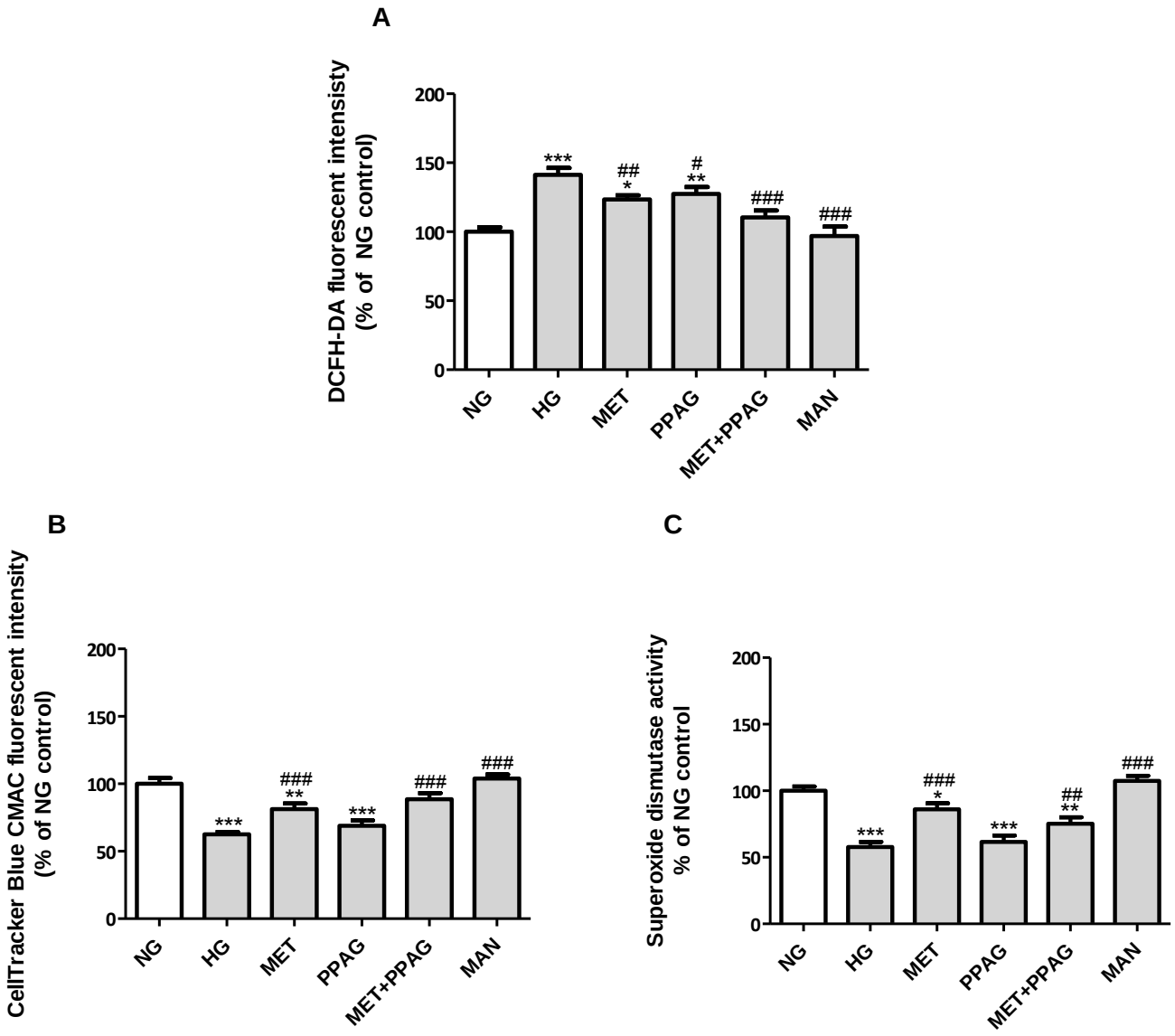


Figure 4. Effect of PPAG, metformin (MET) and MET + PPAG on oxidative stress markers. (A) Formation of reactive oxygen species (ROS), (B) glutathione (GSH) content and (C) superoxide dismutase (SOD) activity. Dichlorofluorescein diacetate (DCF-DA) fluorescent stain was used to detect the generation of ROS while CellTracker Blue CMAC fluorescent stain was used to detect the amount of GSH. Mannitol (MAN) did not have an effect on oxidative stress markers and was comparable to the normal glucose (NG) control. Results are the mean ± SEM of 3 independent biological experiments relative to the NG control, each done in triplicate. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$ versus NG; #$p < 0.05$, ##$p < 0.001$, ###$p < 0.0001$ versus high glucose (HG).

Exposure of H9c2 cardiomyocytes to high glucose increased mitochondrial membrane potential in cells stained with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) fluorescence stain (Figure 5A). Treatment with metformin, PPAG or metformin + PPAG ameliorated this effect (Figure 5A). Quantitative fluorescent analysis revealed that high glucose increased

mitochondrial membrane potential by 138.0 ± 4.1% (p = 0.0001) when compared to the normal glucose exposed cells (100.0 ± 3.6%) (Fig. 5B). PPAG, metformin and metformin + PPAG treatment attenuated this effect (110.4 ± 5.3%, p = 0.0001; 121.2 ± 4.1%, p = 0.006; and 115.3 ± 7.3%, p = 0.05, respectively) (Fig. 5A, B).

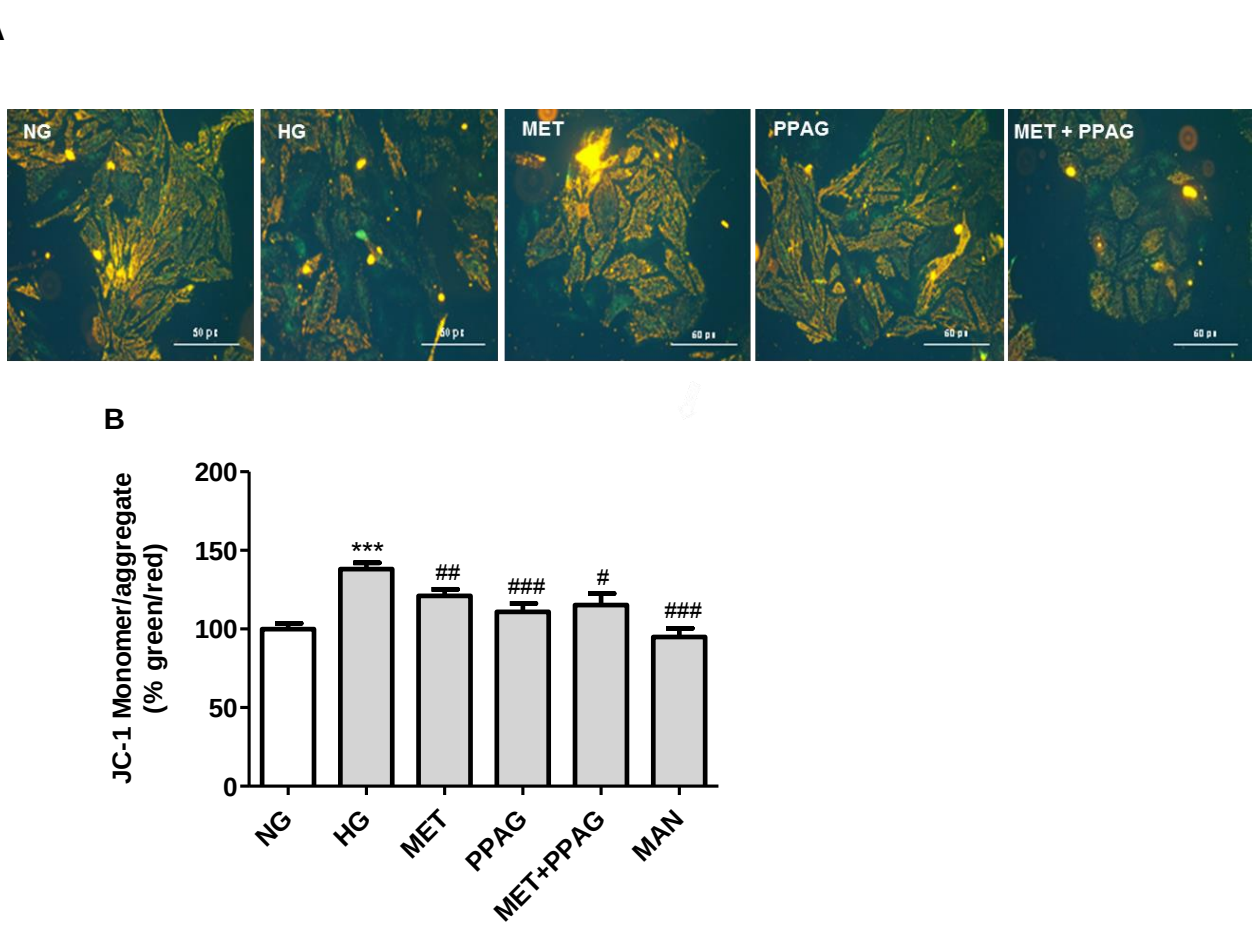


Figure 5. Effect of PPAG, metformin (MET) and MET + PPAG on mitochondrial transmembrane potential ($\Delta\Psi_m$) measured by JC-1 stain. (A) Representative images of H9c2 cardiomyocytes exposed to 33 mM glucose for 48 h before treatment with either PPAG, MET or MET + PPAG; cells with non-depolarized mitochondria exhibited an orange fluorescence while depolarized cell mitochondria displayed green fluorescence. Mannitol (MAN) did not have an effect on mitochondrial membrane potential and was comparable to the normal glucose (NG) control. (B) Quantitative analysis of JC-1 fluorescence staining. Results are the mean ± SEM of 3 independent biological experiments relative to the NG control, each done in triplicate. ***p < 0.0001 versus NG; #p < 0.05, ##p < 0.001, ###p < 0.0001 versus high glucose (HG).

High glucose-induced cells displayed increased levels of fluorescence intensity associated with late apoptosis (propidium iodide and TUNEL staining) when compared to the normal glucose controls (Fig. 6A). In support, H9c2 cardiomyocytes exposed to high glucose exhibited increased caspase 3/7

activity (132.2 ± 6.1%, p = 0.05), with a concomitant upregulation in TUNEL positive cells (8.4 ± 1.1, p = 0.001) when compared to normal glucose treated cells (101.0 ± 8.7% and 2.6 ± 0.7, respectively) (Fig. 6B, C). Treatment with PPAG and metformin + PPAG decreased both the activity of caspase 3/7 (109.3 ± 5.3%, p = 0.05 and 4.6 ± 0.4, p = 0.05), and TUNEL positive cells (108.2 ± 3.7%, p = 0.001 and 4.1 ± 1.0, p = 0.05) (Fig. 6A, B, C).

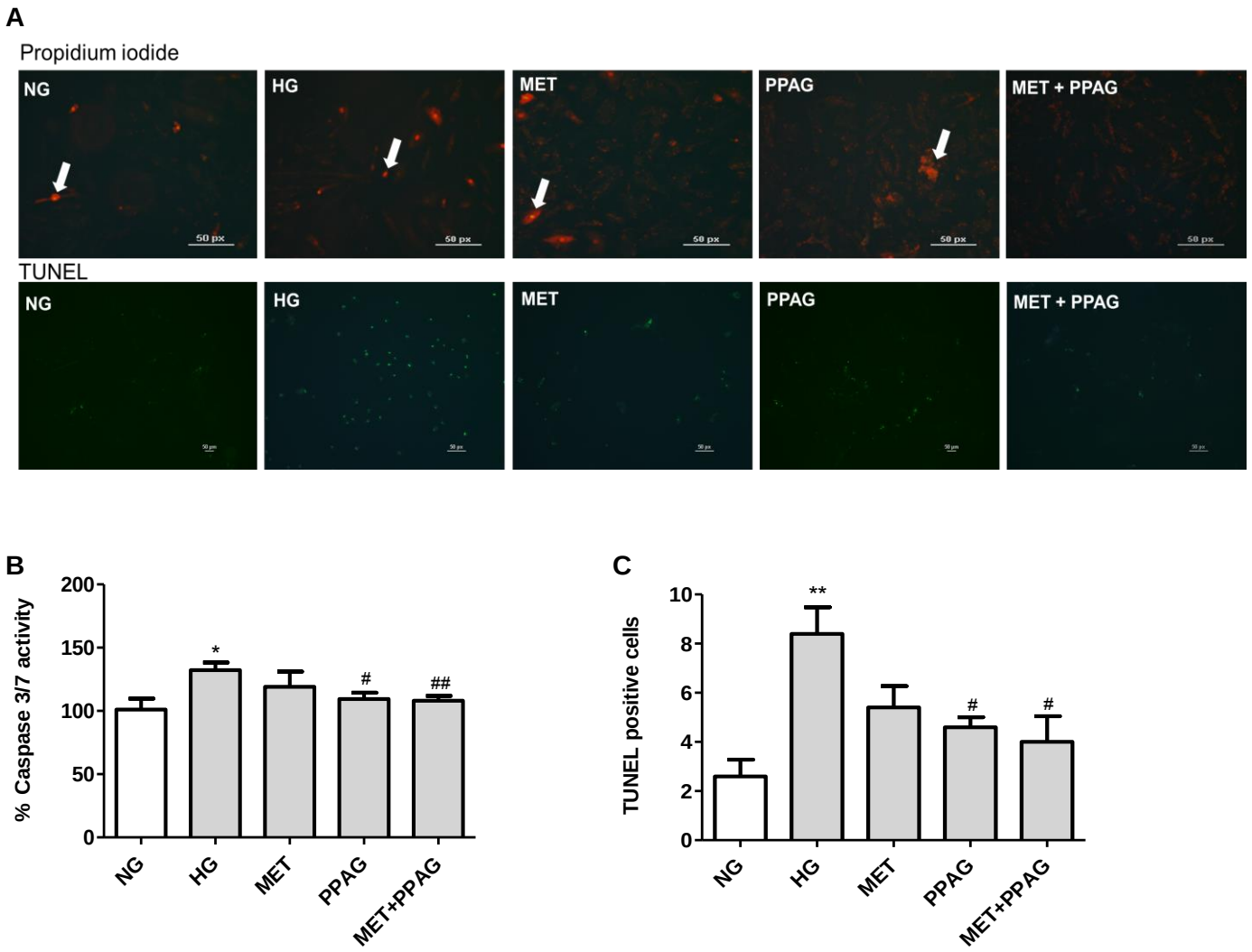


Figure 6. Effect of PPAG, metformin (MET) and MET + PPAG on high glucose-induced cell apoptosis in H9c2 cardiomyocytes. (A) Representative images of H9c2 cardiomyocytes exposed to 33 mM glucose for 48 h before treatment with either PPAG, MET or MET + PPAG; cells positive for propidium iodide nuclear stain exhibited red fluorescence, while those positive for TUNEL, displayed green fluorescence. (B) The anti-apoptotic potential of PPAG to reduce caspase 3/7 activity, (C) and TUNEL fluorescent staining was comparable to an additive effect of MET and PPAG. Results are the mean ± SEM of 3 independent biological experiments, each done in triplicate. *p < 0.05, **p < 0.001 versus normal glucose (NG); #p < 0.05, ##p < 0.001 versus high glucose (HG).

Bcl2/Bax protein expression ratio was robustly diminished in H9c2 cells exposed to high glucose (80.1 ± 8.8%, p = 0.03) when compared to normal glucose treated cells (100.0 ± 1.7%) (Fig. 7). Treatment with PPAG (107.4 ± 4.8%, p = 0.009) and metformin (104.4 ± 3.6%, p = 0.01) was able to ameliorate this effect (Fig. 7). However, treatment with metformin + PPAG (95.2 ± 2.5%) was ineffective (Fig. 7).

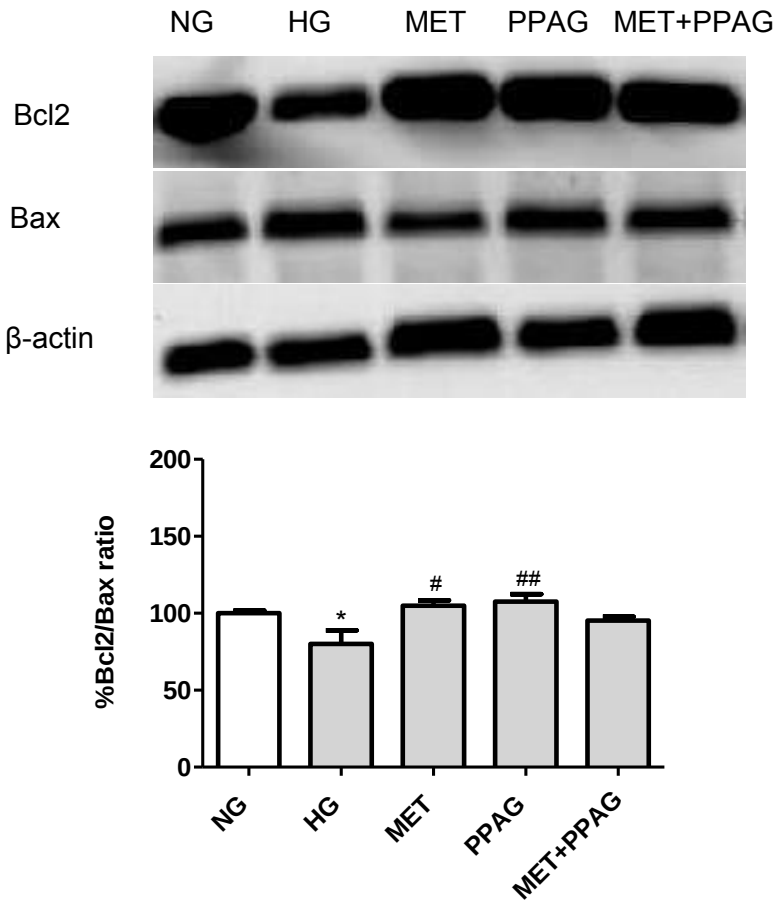


Figure 7. Effect of PPAG, metformin (MET) and MET + PPAG on Bcl2 and Bax protein expression. While high glucose (HG) reduced Bcl2/Bax expression ratio, treatment with PPAG was able to improve the survival ratio when compared to MET alone or in combination with PPAG. Results are the mean ± SEM of 3 independent biological experiments relative to the normal glucose (NG) control, each done in triplicate. *p < 0.05 versus NG; #p < 0.05, ##p < 0.001 versus HG.

Discussion

Previous studies found that rooibos can trigger a peripheral hypoglycemic effect, improve glucose uptake and relieve endoplasmic reticulum stress-induced cell apoptosis in muscle, liver and pancreatic β-cells of diabetic animals [19, 22]. Focusing on the heart, selective rooibos compounds can attenuate

myocardial dysfunction by improving endothelial function and limiting ischemia/reperfusion injury [23, 24]. However, it remains unknown whether PPAG is able to protect the myocardium against hyperglycemia-mediated apoptosis. The current study therefore focussed specifically on this phenylpropenoic acid glucoside, present in rooibos, by employing an *in vitro* cardiac model of chronic hyperglycemia. Our findings demonstrate that relatively low PPAG doses attenuated high glucose-induced apoptosis in H9c2 cardiomyocytes, thus offering early promise as a novel cardioprotective agent.

The initial experiments revealed that low PPAG doses increased metabolic activity of H9c2 cardiomyocytes. However, higher PPAG concentrations significantly reduced cell viability. These data are in agreement with earlier work demonstrating that a lower PPAG dose stimulated glucose uptake in Chang cells [19], unlike the high dose that resulted in reduced uptake.

Chronic hyperglycemia can result in a number of pathophysiologic disturbances that contribute to myocardial injury [7, 9]. Such perturbations include derangements in both glucose and fatty acid metabolic pathways, with increased ROS generation emerging as a central, damaging outcome in this process. This study found that high glucose availability resulted in altered cardiac FAU and FAO concomitant to increased ROS and reduced intracellular antioxidant capacity of the cells. In parallel, mitochondrial membrane potential was higher together with increased myocardial apoptosis. This is consistent with the findings of others [15, 25], where high glucose exposure induced mitochondrial membrane hyperpolarization and apoptosis in undifferentiated H9c2 cells compared to controls. It is likely that in our model elevated fuel substrate availability (e.g. fatty acids) results in increased flux through the mitochondrial electron transport chain leading to enhanced proton extrusion into the inter-mitochondrial membrane space [11, 12, 14]. Higher intracellular glucose levels will also lead to increased mitochondrial membrane potential due to greater availability of reducing equivalents for the mitochondrial electron transport chain [7]. Subsequently, this will generate increased mitochondrial ROS levels (due to impaired electron flow at electron transport complexes) and contribute to myocardial apoptosis [15, 26]. For example, others found that chronic hyperglycemia and associated mitochondrial deficiency are key factors that trigger myocardial apoptosis in a diabetic state [10, 11]. Increased mitochondrial membrane potential can also cause the release of pro-apoptotic proteins into the cytosol [27]. In agreement, our data show lower Bcl2/Bax protein expression together with increased caspase activity and higher propidium iodide and TUNEL staining. These observations are consistent with enhanced propidium iodide and TUNEL staining found in isolated heart cells of diabetic mice [28, 29]. Higher intracellular glucose and fatty acid levels can also trigger multiple, non-mitochondrial effects that can also contribute to the onset of myocardial cell death [30, 31].

Our data revealed that 1 μM PPAG treatment for 12 h attenuated several of the high glucose-induced perturbations earlier discussed. For example, PPAG treatment limited cardiac FAU and FAO, decreased mitochondrial membrane potential and lowered cardiac apoptosis. These findings are in agreement with the other results showing that PPAG ameliorated apoptosis by increasing Bcl2 expression and inhibiting Bax translocation to the mitochondrion. Similar findings were also reported in pancreatic β-cells of mice fed a high-fat diet [22]. In agreement with this, fermented rooibos extract with abundant levels of PPAG protected primary rat cardiomyocytes against oxidative stress-induced myocardial injury [16]. We have previously demonstrated that aspalathin, the major phenolic compound in rooibos protected against shifts in substrate preference in H9c2 cardiomyocytes exposed to high glucose concentrations and that this protective effect was enhanced if aspalathin was combined with metformin [32]. PPAG also decreased ROS production; interestingly, this effect was enhanced by metformin.

The potential mechanism by which PPAG inhibits hyperglycemia induced myocardial injury remains to be fully elucidated. However, recent research is highlighting a strong connection between activation of tumor protein p53 (p53) and increased expression of Bax or inhibition of Bcl2 in various disease models [33-35]. In hearts of diabetic mice, upregulated expression of p53 is associated with enhanced lipid accumulation and mitochondrial generated ROS, leading to apoptosis [35]. Thus, we propose that PPAG prevents hyperglycemic induced cardiac apoptosis through modulating increased FFA substrate flux into mitochondrial respiration complexes with a concomitant amelioration of the Bcl2/Bax ratio. However, further studies are required to assess the involvement of the p53 tumor suppressor gene in our model.

In summary, this study reveals the cardioprotective potential of PPAG against hyperglycemia-induced cell injury and provides impetus for future investigations into its long-term effects on CVD in diabetic individuals.

Materials and Methods

Materials

The ViaLight plus ATP kit, cell culture tested water, Dulbecco's modified Eagle's medium, Hank's balanced salt solution and Dulbecco's phosphate-buffered saline (DPBS) were obtained from Lonza; ^{14}C-palmitate from American Radiolabelled Chemicals; fetal bovine serum and horse serum from Biochrom; Bcl2 antibody from Cell Signaling Technology; secondary antibodies, Bax and β-actin from Santa Cruz Biotechnology; and the Caspase-Glo 3/7 kit from Promega. PPAG (99% purity; batch: MC1(2)-248-91D) was synthesized by High Force Research. H9c2 rat derived cardiomyoblasts

(ECACC No. 8809294) were purchased from European Collection of Cell Cultures. All consumables and reagents, including metformin (99% purity) and mannitol (99% purity) were purchased from Sigma-Aldrich Corp., unless otherwise specified.

Preparation of PPAG solution

PPAG (MW: 326) at 10 mg was dissolved in 1 mL cell culture-tested water to give a final concentration of 30.67 mM stock solution which was stored at –80°C. Working solutions of 1 mM were made up from the initial stock using Dulbecco's modified Eagle's medium to give dilutions ranging from 0.01 to 1000 µM.

H9c2 cell culture and treatment with PPAG

Embryonic heart-derived H9c2 cardiomyoblasts were cultured in supplemented Dulbecco's modified Eagle's medium (10% fetal bovine serum) for 48 h under standard tissue culture conditions (37°C in humidified air and 5% CO_2). Depending on the assay performed, 60-80% confluent cells were seeded at a density of 2 x 10^4 or 0.8 x 10^4 cells /mL in 6-well or 96-well multi-plates, respectively. After 48 h of culture, H9c2 cardiomyoblasts were differentiated into cardiomyocytes by substituting growth media with differentiation media consisting of Dulbecco's modified Eagle's medium supplemented with 1% horse serum and 10 nM all-trans-retinoic acid for 6 days [36]. All experiments were initiated on day 7. H9c2 cells were exposed to a range of PPAG log concentrations (0.01 to 1000 µM) for 3, 6, 12, 24 and 48 h, respectively. Thereafter, the effect of PPAG on hyperglycemia-induced cell apoptosis was tested. Here H9c2 cells were exposed to 33 mM glucose for 48 h prior to treatment with either PPAG (1 µM), metformin (1 µM) or metformin + PPAG (both at 1 µM) for an additional 12 h. Cells exposed to either 5.5 mM glucose, 33 mM glucose or 33 mM mannitol served as controls for normal glucose, high glucose and osmotic stress, respectively [25].

Measurement of metabolic activity

ATP production as a measurement of metabolic activity in H9c2 cells was determined using the ViaLight plus ATP kit, according to the manufacturer's instructions. Briefly, treated cells were lysed and incubated for 10 min at room temperature. Thereafter, ATP monitoring reagent was added and the luminescence determined after 2 min, using a BioTek FLx800 plate reader with data acquisition using Gen 5 software (Bio-Tek Instruments, Inc.).

Fatty acid uptake and oxidation

FAU and FAO were determined as previously described [32, 37]. Following treatment, H9c2 cells were washed twice with warm DPBS and re-cultured in high glucose Dulbecco's modified Eagle's medium (without phenol red) containing 0.5 µCi/mL final concentration of palmitate-D-[^{14}C (U)] in clear 6-well multi-plates. Each well was covered with filter paper wetted with 0.1 M NaOH for collection of $^{14}CO_2$ released from ^{14}C-palmitate. The filter paper was replaced every 3 h and at the end of the experiment, ^{14}C activity was measured by liquid scintillation (2220 CA, Packard Tri-Carb series, PerkinElmer) to calculate FAO. The remaining cells were rinsed in ice cold DPBS to stop metabolism, then lysed with 0.3 M NaOH + 1% sodium dodecyl sulfate buffer. The cell lysate was used to assess ^{14}C-palmitate by liquid scintillation, as well as protein content using the Bradford's protein assay [38]. The counts per minute (CPM) over specific activity (pre-determined using GraphPad radioactivity calculator: http://www.graphpad.com/quickcalcs/radcalcform.cfm) was used to determine fmol/mg protein.

Measurement of oxidative stress markers

Intracellular production of ROS was detected using DCFH-DA fluorescent dye (Cell Biolabs Inc.) as previously described [32]. Briefly, 100 µL of a 1 µM DCFH-DA final solution prepared in Hank's balanced salt solution was added to H9c2 cells in a multi well plate and incubated at 37°C for 30 min in humidified air with 5% CO_2. After 30 min, the dye was aspirated and cells rinsed in Hank's balanced salt solution. DCFH-DA fluorescent intensity (Ex 485 ± 20 nm; Em 528 ± 20 nm) was measured using a BioTek FLx800 plate reader.

GSH content was determined using 7-amino-4chloromethylcoumarin (CellTracker Blue CMAC) (Invitrogen). Briefly, 100 µL of 2.5 µM CellTracker solution was added to H9c2 cells and incubated at 37ºC for 30 min. Thereafter, media containing CellTracker solution was removed and cells rinsed in DPBS before fluorescence intensity (Ex 360 ± 20 nm; Em 460 ± 40 nm) was measured using a BioTek FLx800 plate reader.

SOD activity was quantified using a Biovision kit. Briefly, following treatment, H9c2 cells were lysed by the addition of 100 µL lysis buffer. Ten-microliter cell lysate was then transferred to a new 96 well plate to which 200 µL tetrazolium working solution was added. SOD activity was measured at an absorbance of 450 nm using a BioTek ELx800 plate reader.

Determination of mitochondrial membrane potential ($\Delta\Psi_m$)

Membrane depolarization was assessed by staining H9c2 cardiomyocytes with JC-1 according to a previously described method [15]. Briefly, treated H9c2 cells were washed twice with warm DPBS, 100 µL of 2 µM JC-1 solution (made up in Dulbecco's modified Eagle's medium without phenol red) was added and the cells then incubated at 37°C in humidified air and 5% CO_2 for 30 min the dark. After JC-1 exposure, cells were rinsed in DPBS before fluorescence (Ex 485 ± 20 nm; Em 530 ± 25 nm and 590 ± 35 nm) was measured using a BioTek FLx800 plate reader and Gen 5 software. In addition, fluorescent photomicrographs were taken at 10x magnification using a Nikon Eclipse Ti inverted microscope and NIS-Elements imaging software.

Propidium iodide fluorescent stain

Propidium iodide staining assay was performed following a published protocol [39]. Briefly, treated cells were exposed to 1 µg/mL propidium iodide solution for 30 min at 37°C in humidified air with 5% CO_2. After 30 min, cells were rinsed in DPBS and analyzed as described for determination of membrane depolarization. Fluorescent photomicrographs were taken at 10x magnification using a Nikon Eclipse Ti inverted microscope and NIS-Elements imaging software.

Caspase 3/7 activity assay

In this assay, Caspase-Glo 3/7 reagent is cleaved by caspases producing a luminescent signal proportional to caspase activity. Briefly, treated cells were washed twice with warm DPBS before being lysed. Caspase-3/7 Glo reagent was mixed with cell lysates and the mixture was incubated in the dark at 37°C in humidified air with 5% CO_2 for 30 min. Luminescence was measured using an integration time of 1 s and equipment described for measurement of metabolic activity. Results were normalized to the protein content determined according to the method of Bradford [38].

TUNEL assay

DNA fragmentation, as a measure of apoptosis in H9c2 cells, was detected using a DeadEnd Fluorometric TUNEL kit, according to the manufacturer's instructions. Briefly, treated cells were washed twice in DPBS and permeabilized with 0.2% Triton X-100. Thereafter, the equilibration buffer and TdT reaction mix (supplied with the kit) were added before incubation at 37°C in humidified air with 5% CO_2 incubator for 60 min. The apoptotic rate was calculated as the average number of

condensed TUNEL-positive cells in non-overlapping fields of 1 mm^2 under 10x magnification field (average of at least 5 fields per well) using a Nikon Eclipse Ti inverted fluorescent microscope and NIS Elements imaging software.

Western blot analysis

Protein extracts were isolated from different treatment groups and Western blot analysis performed according to an already described method [40]. Membranes were immunoblotted for 16 h at 4°C with the following primary antibodies: anti-Bax (1:250 dilution), anti-Bcl2 (1:1,000 dilution) and anti-β-actin (1:1,000 dilution) antibody, included as a loading control. The relevant horseradish peroxidase conjugated secondary antibodies were applied the following day for 90 min at room temperature. The protein signal was detected using a chemiluminescence solution and quantified using a Chemidoc-XRS imager (Bio-Rad).

Statistical analysis

Results were expressed as the mean ± SEM of 3 independent biological experiments. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc.). Comparisons between treatment groups were performed using a one way ANOVA followed by a Tukey post hoc test or unpaired Student t-test where appropriate. P-value of ≤ 0.05 was deemed as statistically significant.

Acknowledgements

This research was funded in part by the National Research Foundation (NRF) Thuthuka Programme Grant 87836 and the South Africa Medical Research Council's Biomedical Research and Innovation Platform. The grantholders acknowledge that opinions, findings and conclusions or recommendations expressed in any publication generated by the NRF supported research are those of the authors, and that the NRF accepts no liability whatsoever in this regard. Funding from Stellenbosch University and Ernst Ethel Erikson Trust is also acknowledged.

Competing interests

Potential conflict of interest statement: Co-authors Christo John Frederick Muller, Elizabeth Joubert, and Johan Louw declare their interest as inventors in PCT patent applications, dealing with the antidiabetic activity of RX-1 and analogues (WO2011/120576 A1, WO2012/045363 A1). RX-1 is the

code used for PPAG. The other authors have declared no conflict of interest.

References

1 World Health Organization (WHO). World health statistics 2012. Available at: http://www.who.int/gho/publications/world_health_statistics/WHS2012_IndicatorCompendium.pdf? Accessed December 9, 2015

2 International Diabetes Federation (IDF). IDF Diabetes atlas 7th edition. idf.org [Internet]. Accessed at: http://www.diabetesatlas.org/. Accessed December 12, 2015

3 Zhou L, Deng W, Zhou L, Fang P, He D, Zhang W, Liu K, Hu R. Prevalence, incidence and risk factors of chronic heart failure in the type 2 diabetic population: systematic review. Curr Diabetes Rev 2009; 5: 171–184

4 Boudina S, Abel ED. Diabetic cardiomyopathy revisited. Circulation 2007; 115: 3213–3223

5 Damasceno A, Cotter G, Dzudie A, Sliwa K, Mayosi BM. Heart failure in Sub-Saharan Africa: time for action. J Am Coll Cardiol 2007; 50: 1688–1693

6 Sliwa K, Damasceno A, Mayosi BM. Heart disease in Africa: epidemiology and etiology of cardiomyopathy in Africa. Circulation 2005; 112: 3577–3583

7 Mapanga RF, Essop MF. Damaging effects of hyperglycemia on cardiovascular function: spotlight on glucose metabolic pathways. Am J Physiol Heart Circ Physiol 2016; 310: H173–153

8 Kapur A, De Palma R. Mortality after myocardial infarction in patients with diabetes mellitus. Heart 2007; 93: 1504–1506

9 Giacco F, Brownlee M. Oxidative stress and diabetic complications. Circ Res 2010; 107: 1058–1070

10 Opie LH. Metabolic management of acute myocardial infarction comes to the fore and extends beyond control of hyperglycemia. Circulation 2008; 117: 2172–2177

11 Dyntar D. Diabetic cardiomyopathy: effects of fatty acids and glucose on adult rat cardiomyocytes [dissertation]. ETH Zürich, Nr. 15175; 2003

12 Carlsson M, Wessman Y, Almgren P, Groop L. High levels of nonesterified fatty acids are associated with increased familial risk of cardiovascular disease. Arterioscler Thromb Vasc Biol 2000; 20: 1588–1594

13 Naidu PB, Ponmurugan P, Begum MS, Mohan K, Meriga B, RavindarNaik R, Saravanan G. Diosgenin reorganises hyperglycaemia and distorted tissue lipid profile in high-fat diet-streptozotocin-induced diabetic rats. J Sci Food Agric 2015; 95: 3177–3182

14 Bugger H, Abel ED. Mitochondria in the diabetic heart. Cardiovasc Res 2010; 88: 229–240

15 Sun X, Chen RC, Yang ZH, Sun GB, Wang M, Ma XJ, Yang LJ, Sun XB. Taxifolin prevents diabetic cardiomyopathy *in vivo* and *in vitro* by inhibition of oxidative stress and cell apoptosis. Food Chem Toxicol 2014; 63: 221–232

16 Dludla PV, Muller CJ, Louw J, Joubert E, Salie R, Opoku AR, Johnson R. The cardioprotective effect of an aqueous extract of fermented rooibos (*Aspalathus linearis*) on cultured cardiomyocytes derived from diabetic rats. Phytomedicine 2014; 21: 595–601

17 Snijman PW, Joubert E, Ferreira D, Li XC, Ding Y, Green IR, Gelderblom WC. Antioxidant activity of the dihydrochalcones aspalathin and nothofagin and their corresponding flavones in relation to other rooibos (*Aspalathus linearis*) flavonoids, epigallocatechin gallate, and trolox. J Agric Food Chem 2009; 57: 6678–6684

18 Chellan N, Joubert E, Strijdom H, Roux C, Louw J, Muller CJ. Aqueous extract of unfermented honeybush (*Cyclopia maculata*) attenuates STZ-induced diabetes and β-cell cytotoxicity. Planta Med 2014; 80: 622–629

19 Muller CJ, Joubert E, Pheiffer C, Ghoor S, Sanderson M, Chellan N, Fey SJ, Louw J. *Z*-2-(*β*-D-glucopyranosyloxy)-3-phenylpropenoic acid, an α-hydroxy acid from rooibos (*Aspalathus linearis*) with hypoglycemic activity. Mol Nutr Food Res 2013; 57: 2216–2222

20 Marais C, Steenkamp JA, Ferreira D. Occurrence of phenylpyruvic acid in woody plants: biosynthetic significance and synthesis of an enolic glucoside derivative. J. Chem Soc Perkin Trans 1 1996; 24: 2915–2918

21 Joubert E, de Beer D, Malherbe CJ, Muller N, Bonnet SL, van der Westhuizen JH, Ferreira D. Occurrence and sensory perception of *Z*-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid in rooibos (*Aspalathus linearis*). Food Chem 2013; 136: 1078–1085

22 Mathijs I, Da Cunha DA, Himpe E, Ladriere L, Chellan N, Roux CR, Joubert E, Muller C, Cnop M, Louw J, Bouwens L. Phenylpropenoic acid glucoside augments pancreatic beta cell mass in high-fat diet-fed mice and protects beta cells from ER stress-induced apoptosis. Mol Nutr Food Res 2014; 58: 1980–1990

23 Kwak S, Han MS, Bae JS. Aspalathin and nothofagin from rooibos (*Aspalathus linearis*) inhibit endothelial protein C receptor shedding *in vitro* and *in vivo*. Fitoterapia 2015; 100: 179–186

24 Pantsi WG, Marnewick JL, Esterhuyse AJ, Rautenbach F, van Rooyen J. Rooibos (*Aspalathus linearis*) offers cardiac protection against ischaemia/reperfusion in the isolated perfused rat heart. Phytomedicine 2011; 18: 1220–1228

25 Rajamani U, Essop MF. Hyperglycemia-mediated activation of the hexosamine biosynthetic pathway results in myocardial apoptosis. Am J Physiol Cell Physiol 2010; 299: C139–C147

26 Di Paola M, Lorusso M. Interaction of free fatty acids with mitochondria: coupling, uncoupling and permeability transition. Biochim Biophys Acta 2006; 1757: 1330–1337

27 Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol 2007; 35: 495–516

28 Schwarz K, Simonis G, Yu X, Wiedemann S, Strasser RH. Apoptosis at a distance: remote activation of caspase-3 occurs early after myocardial infarction. Mol Cell Biochem 2006; 281: 45–54

29 Clyne AM, Zhu H, Edelman ER. Elevated fibroblast growth factor-2 increases tumor necrosis factor-α induced endothelial cell death in high glucose. J Cell Physiol 2008; 217: 86–92

30 Behl Y, Krothapalli P, Desta T, DiPiazza A, Roy S, Graves DT. Diabetes-enhanced tumor necrosis factor-α production promotes apoptosis and the loss of retinal microvascular cells in type 1 and type 2 models of diabetic retinopathy. Am J Pathol 2008; 172: 1411–1418

31 Yu W, Wu J, Cai F, Xiang J, Zha W, Fan D, Guo S, Ming Z, Liu C. Curcumin alleviates diabetic cardiomyopathy in experimental diabetic rats. PLoS One 2012; 7: e52013

32 Johnson R, Dludla P, Joubert E, February F, Mazibuko S, Ghoor S, Muller C, Louw J. Aspalathin, a dihydrochalcone C-glucoside, protects H9c2 cardiomyocytes against high glucose-induced shifts in substrate preference and apoptosis. Mol Nutr Food Res 2016; 60: 922–934

33 Heinzelmann-Schwarz V, Fedier A, Hornung R, Walt H, Haller U, Fink D. Role of p53 and ATM in photodynamic therapy-induced apoptosis. Lasers Surg Med 2003; 33: 182–189

34 Hemann MT, Lowe SW. The p53–Bcl-2 connection. Cell Death Differ 2006; 13: 1256–1259

35 Nakamura H, Matoba S, Iwai-Kanai E, Kimata M, Hoshino A, Nakaoka M, Katamura M, Okawa Y, Ariyoshi M, Mita Y, Ikeda K, Okigaki M, Adachi S, Tanaka H, Takamatsu T, Matsubara H. p53 promotes cardiac dysfunction in diabetic mellitus caused by excessive mitochondrial

respiration-mediated reactive oxygen species generation and lipid accumulation. Circ Heart Fail 2012; 5: 106–115

36 Karagiannis TC, Lin AJ, Ververis K, Chang L, Tang MM, Okabe J, El-Osta A. Trichostatin A accentuates doxorubicin-induced hypertrophy in cardiac myocytes. Aging (Albany NY) 2010; 2: 659–668

37 Nugent C, Prins JB, Whitehead JP, Wentworth JM, Chatterjee VK, O'Rahilly S. Arachidonic acid stimulates glucose uptake in 3T3-L1 adipocytes by increasing GLUT1 and GLUT4 levels at the plasma membrane. Evidence for involvement of lipoxygenase metabolites and peroxisome proliferator-activated receptor gamma. J Biol Chem 2001; 276: 9149–9157

38 Bradford M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248–254

39 Chellan N. The effect *of Cyclopia maculata* extract on β-cell function, protection against oxidative stress and cell survival [dissertation]. Stellenbosch University; 2014

40 Mahmood T, Yang P. Western blot: technique, theory, and trouble shooting. N Am J Med Sci 2012; 4: 429–434

Chapter 3: Aspalathin, a dihydrochalcone *C*-glucoside, protects H9c2 cardiomyocytes against high glucose-induced shifts in substrate preference and apoptosis

Rabia Johnson[1*], Phiwayinkosi Dludla[1,2], Elizabeth Joubert[3,4], Faghri February[5], Sithandiwe Mazibuko[1], Samira Ghoor[1], Christo Muller[1] and Johan Louw[1]

[1]Biomedical Research and Innovation Platform Cape Town, South Africa.

[2]Division of Medical Physiology, Faculty of Health Sciences, Stellenbosch University, Tygerberg, South Africa.

[3]Post-Harvest and Wine Technology Division, Agricultural Research Council (ARC) Infruitec-Nietvoorbij, Stellenbosch, South Africa.

[4]Department of Food Science, Stellenbosch University, Stellenbosch, South Africa.

[5]Department of Biotechnology, University of Western Cape, Bellville, South Africa.

Published article: Molecular Nutrition and Food Research 2016; 60: 922–934.

My contribution:

Planning of manuscript outline

Performed experiments

Data analysis and interpretation

Edited the manuscript

Abstract

Scope: Energy deprivation in the myocardium is associated with impaired heart function. This study aims to investigate if aspalathin (ASP) can ameliorate hyperglycemic-induced shift in substrate preference and protect the myocardium against cell apoptosis.

Methods and results: H9c2 cells were exposed to, either normal (5.5 mM) or high (33 mM) glucose concentrations for 48 hours. Thereafter, cells exposed to 33 mM glucose were treated with metformin (1 μM) or ASP (1 μM), as well as a combination of metformin and ASP for 6 hours. *In vitro* studies revealed that ASP improved glucose metabolism by decreasing fatty acid uptake and subsequent β-oxidation through the decreased expression of adenosine monophosphate-activated protein kinase threonine 172 (pAMPK (Thr^{172})) and carnitine palmitoyltransferase 1 (*Cpt1*), while increasing acetyl-CoA carboxylase (*ACC)* and glucose transporter 4 (*Glut4*) expression. ASP inhibited high glucose-induced loss of membrane potential in H9c2 cells as observed by an increase in 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) ratio (orange\red fluorescence) and decreased apoptosis by reducing intracellular reactive oxygen species and DNA nick formation, while increasing glutathione, superoxide dismutase, uncoupling protein 2 (*UCP2*) and Bcl-2\Bax ratio.

Conclusion: Our study provides evidence that ASP increases glucose oxidation and modulates fatty acid utilization producing a favorable substrate shift in H9c2 cardiomyocytes exposed to high glucose. Such a favorable shift will be of importance in the protection of cardiomyocytes in the diabetic heart.

Keywords

Apoptosis; diabetes; cardiomyopathy; fatty acid oxidation.

1. Introduction

In the last two decades, the incidence of diabetes mellitus (DM) has increased worldwide with a concomitant increase in cardiovascular complications [1]. This is not surprising as diabetes is a known contributor to the development of cardiovascular disease (CVD), with diabetic individuals having a 2-3 times higher risk of developing cardiovascular complications compared to the non-diabetic population [2]. This makes CVD the most common cause of mortality and morbidity in the diabetic population [2, 3]. However, the underlying pathogenesis of diabetic-induced cardiomyopathy (DICM) is still unclear. Current consensus is that exposure to chronic hyperglycemia enhances the production of reactive oxygen species (ROS) which may contribute to the development of DICM [4-6].

Hyperglycemia, dyslipidemia, and inflammation increase the generation of ROS or nitrogen species (RNS), key factors associated with the pathogenesis of DICM. The diabetic heart is particularly vulnerable to increased oxidative stress due to a deficient endogenous antioxidant defense mechanism [5, 7-9]. This impaired equilibrium between production and elimination of reactive species associated with the diabetic state results in structural remodeling of the myocardia and left ventricular dysfunction, which is the hallmark of DICM [3, 4, 10].

The mitochondria can be described as “the power house of the cell” and apart from increased ROS production, mitochondrial dysfunction and impaired energy metabolism have been associated with many forms of heart disease including DICM [11]. An increased shift in fatty acid β-oxidation (FAO) relative to carbohydrate oxidation has the potential to decrease cardiac efficiency and increase contractile dysfunction as observed in DICM. Increasing evidence suggests that therapies aimed at decreasing FAO, or directly stimulating glucose oxidation would be of benefit to the diabetic heart during and following myocardial ischemia [12, 13].

Metformin is a first-line antidiabetic drug known to reduce the risks of heart disease in people with diabetes. To date, several studies have shown that metformin is able to preserve left ventricular function by reducing endogenous glucose production and hepatic gluconeogenesis through the stimulation of AMP-activated protein kinase activity [14]. However, the efficacy of metformin to protect the diabetic heart at risk from increased chronic hyperglycemia decreases over time [15].

The antioxidant efficacy of dietary flavonoids and their beneficial effect on health have been reported on previously [16-18]. The use of plant-based products as a means to increase the antioxidant defense in the body is gaining popularity. The balance between oxidation and the body’s antioxidant system is critical in maintaining optimal cellular function. Flavonoids as antioxidants have the ability to scavenge and reduce free radical formation [19]. In the human body, flavonoids function as antioxidants and exert their protective effect through their ability to increase levels of endogenous

antioxidant defenses such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and glutathione (GSH) which allow for the elimination of excessive ROS in the body [20, 21]. Therefore, preventing redox disequilibrium caused by increased oxidative stress.

Aspalathin (ASP) a dietary flavonoid present in *Aspalathus linearis* (rooibos) has known antioxidant and antidiabetic properties. Muller et al (2012) reported that ASP has a sustained glucose lowering effect comparable to metformin in STZ-induced diabetic rats [22]. Furthermore, ASP was shown to suppress ROS induced by advanced glycation end products (AGEs) *in vivo* [23]. Therefore, ASP, as a dietary supplement, through its beneficial effect on glycemia and associated oxidative stress may protect the diabetic heart. A systematic review of literature led us to believe that a combination of ASP with metformin may have an additive effect on glycemia, reduce oxidative stress and lower the risk for DICM [22-28]. The aim of our study was to investigate the ameliorative effect of ASP as a monotherapy or in combination with metformin on oxidative stress and FAO in H9c2 cardiac muscle cells exposed to a high glucose (HG) concentration.

2. Materials and Methods

2.1. Reagents list

Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco`s phosphate buffered saline (DPBS) and penicillin/streptomycin were from Lonza BioWhittaker (Verviers, Belgium). All other cell culture reagents were obtained from Sigma-Aldrich (Saint Louis, Missouri, MO, USA). Protease and phosphatase inhibitor tablets were purchased from Roche Diagnostics (Mannheim, Germany). Radio-immunoprecipitation assay (RIPA) buffer and all primary antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA). Secondary antibodies and β-actin were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Fetal bovine serum (FBS) and horse serum (HS) were from Biochrom (Berlin, Germany). Aspalathin (ca. 98%, batch SZI-356-54) was synthesized according to the method of Han et al (2014) [29] by High Force Research LTD (Durham, UK). Two-deoxy-[^{3}H]-D-glucose and ^{14}C palmitate were obtained from American Radiolabeled Chemicals (St Louis, MO, USA). Palmitic acid (C18:O) and all other chemicals (analytical grade) were obtained from Sigma-Aldrich (Saint Louis, MO, USA).

2.2. *In vitro* culturing of embryonic ventricular rat heart derived cells (H9c2)

Embryonic rat heart-derived cardiac myoblasts H9c2 (ATCC, CRL-1446) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (supplemented with 10% (v/v) FBS, 100 U/ml of penicillin and 100

mg/ml of streptomycin) under standard tissue culture conditions (37°C in a water-saturated atmosphere of 5% CO_2) in 75 cm^2 culture flasks at a seeding density of 1 x 10^6. After 48 hours, sub-confluent (60%) cells were split and plated in either 6-, 24- or 96-well plates in DMEM (containing 10% FBS) at seeding densities of 2 x 10^4, 1 x 10^4 or 0.8 x 10^4, respectively for 48 hours. Thereafter, cells were differentiated for 6 days in DMEM supplemented with 1% horse serum and 10 nM retinoic acid (RA). The culture medium was replaced daily and experiments were initiated on day 7. To assess the effect of normal glucose (NG; 5.5 mM) compared to HG (HG; 33 mM), H9c2 cells were first washed in glucose free DMEM before exposure to DMEM containing 5.5 mM or 33 mM glucose for 24 and 48 hours, respectively. Control cells were treated with 33 mM mannitol to rule out the effect of osmolarity.

2.3. Effect of aspalathin on H9c2 treatment

A stock solution of ASP was prepared according to Muller et al (2012) [22]. Following pre-treatment of cardiomyocytes, with either 5.5 mM or 33 mM glucose for 48 hours, H9c2 cells were treated with or without either ASP (1 μM), metformin (MET) (1 μM) or a combination of ASP and MET (both at 1 μM). Experimental groups consisted of (1) NG control (5.5 mM); (2) HG control (33 mM); (3) HG + MET (1 μM); (4) HG + ASP (1 μM) and (5) HG + MET+ ASP (both at 1 μM).

2.4. ATP assay

Cellular ATP content used as a measure of metabolic activity was performed using a ViaLight™ plus ATP kit (Lonza, Basel, Switzerland), following the manufacturer's instructions. Luminescence was quantified using a BioTek® FLx800 plate reader and Gen 5® software (BioTek Instruments Inc., Winooski, VT, USA).

2.5. Fatty acid uptake and oxidation

Fatty acid uptake (FAU) and FAO were determined using a modified method of Nugent (2001) [30]. Briefly, ASP treated cells were cultured in DMEM (without phenol red) containing a final 0.5 μCi/mL of palmitate-D-[^{14}C (U)] for 6 hours in 6-well tissue culture plates, overlaid with filter paper moistened with 0.1 M NaOH. After 6 hours, the filter paper was removed and the absorbed $^{14}CO_2$ as a product of palmitate-D-[^{14}C (U)] was detected by liquid scintillation. Thereafter, medium was removed and cells washed with 1 mL DPBS. Cells were then lysed with 0.3 M NaOH, and FAU of palmitate-D-[^{14}C (U)] was assessed in the lysate by liquid scintillation (2220 CA, Packard Tri-Carb series, PerkinElmer, Downers Crove, IL, USA. Results were calculated as fmol/mg. Counts per minute (CPM) divided by

specific activity (calculated by Graphpad radioactivity calculator (http://www.graphpad.com/quickcalcs/radcalcform.cfm)), normalized to protein content was used to estimate fmol/mg. Data are expressed in relative to the control in arbitrary units.

2.5.1. 2-Deoxy-[3H]-D-glucose uptake

2-Deoxy-[3H]-D-glucose uptake (GU) was measured using the method described by Muller et al (2012). Briefly, after 6-hours of incubation with the relevant treatments or controls, in 24 well plates, the cells were incubated with a final concentration of 0.5 µCi/mL 2-deoxy-[3H]-D-glucose for 15 minutes at 37 °C in 5% CO_2 and humidified air. 2-Deoxy-[3H]-D-glucose uptake was assessed by liquid scintillation [22]. Results were calculated as fmol/mg as described in subsection 2.5. Data are expressed relative to the control in arbitrary units.

2.5.2. Glucose oxidation

Glucose oxidation (GO) was determined as described by Mazibuko et al (2013) [31]. Briefly, following 6 hour treatment, cells were cultured in DMEM containing 0.5 µCi/mL glucose D-[^{14}C (U)] and CO_2 released from the culture media was trapped with filter paper moistened with 0.1 M NaOH. After 6 hours, the filter paper was removed and placed in scintillation vials for quantification of GO by liquid scintillation. Data are expressed relative to the control in arbitrary units.

2.6. Measurement of pro-oxidant and antioxidant activity

Intracellular production of ROS was detected using 2', 7'-dichlorfluoresceindiacetate (DCFH-DA) fluorescent dye (Cell Biolabs Inc., San Diego, CA, USA). Briefly, 100 µL of a 1 µM DCFH-DA final solution prepared in Hank's balanced salt solution (HBSS) was added to H9c2 cells in a multi well plate and incubated at 37 °C for 30 minutes in humidified air with 5% CO_2. After 30 minutes, the dye aspirated, the cells rinsed in HBSS and DCFH-DA fluorescent intensity measured using a BioTek® FLx800 plate reader. GSH content was determined using 7-amino-4chloromethylcoumarin (CellTracker™ Blue CMAC) (Invitrogen Corporation, Carlsbad, CA, USA). Briefly, 100 µL of 2.5 µM CellTracker solution was added to H9c2 cells and incubated at 37ºC for 30 minutes. Thereafter, media containing CellTracker solution was removed and cells rinsed in DPBS before fluorescence intensity was measured using a BioTek® FLx800 plate reader. SOD activity was quantified using a Biovision kit (Mountain View, CA, USA). Briefly, following treatment, H9c2 cells were lysed by the addition of 100 µL lysis buffer. Ten-microliter cell lysate was then transferred to a new 96 well plate to which 200 µL

tetrazolium working solution was added. SOD activity was measured using a BioTek® ELx800 plate reader.

2.7. Determination of mitochondrial membrane potential

Mitochondrial membrane potential was assessed using the cationic dye 5,5',6,6'-tetrachloro-1,1',3,3-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) (Sigma-Aldrich, St Louis, MO, USA). In healthy cells with normal mitochondrial membrane potential, JC-1 forms complexes known as J-aggregates producing an orange/red fluorescence. In cells with mitochondrial dysfunction, JC-1 remains in the monomeric form and exhibits a green fluorescence. To assess mitochondrial membrane potential, H9c2 cells were washed with DPBS, 2 µM of JC-1 solution was added to the cell mixture and incubated in the dark at 37°C for 30 minutes. JC-1 fluorescent intensity was measured using a BioTek® FLx 800 plate reader (Ex 485 nm, Em 530±25 nm and 590±35). In addition fluorescent photomicrographs were captured at 10x magnification using NIS elements software, with a Nikon Eclipse Ti inverted microscope.

2.8. TUNEL assay

DNA nicks were detected by DeadEnd™ Fluorometric TUNEL assay according to manufacturer's instructions (Promega Corporation, Madison, USA). Briefly, TdT solution was added to the treated cells and incubated at 37°C for 60 minutes. After incubation, cells were rinsed twice in SSC buffer for 15 minutes each. TUNEL-positive cells were detected by direct visualization of fluorescent staining. The apoptotic rate was calculated as the average number of condensed TUNEL-positive cells of non-overlapping fields of 1 mm^2 under x 100 magnification (average of at least 5 fields per well).

2.8. Caspase-3/7 activity

Caspase-3 and caspase-7 activities of H9c2 cells exposed to HG concentration were measured using a Caspase-Glo® 3/7 luminescent kit (Promega Corporation, Madison, USA), following manufacturer's instructions. Briefly, following treatment, cells were lysed and 20 µl of the cell lysate transferred to a white 96-well plate. An equal amount of Caspase-3/7 Glo reagent was added to each well; and incubated for 30 minutes. Luminescence was measured in a BioTek® FLx 800 plate reader. Results were normalized to pre-determined protein content determined using Bradford assay.

2.9. mRNA expression analysis

Total RNA was extracted from H9c2 cells using Trizol reagent. Briefly, H9c2 cells were homogenized using a TissueLyser (Qiagen, Hilden, Germany) at 25 Hz for 2 minutes according to the manufacturer's instructions. RNA was purified using an RNeasy Mini kit (Qiagen, Germantown, MD, USA) and genomic DNA was removed using the Ambion Turbo DNase kit (Applied Biosystems, Austin, Texas, USA) according to the manufacturer's instructions. RNA samples were converted to cDNA using the High Capacity Reverse Transcription kit (Applied Biosystems, Austin, Texas, USA) as recommended by the manufacturers. Quantitative RT-PCR was performed on an ABI 7500 Instrument (Applied Biosystems, Austin, Texas, USA). The reaction mix was prepared by adding 12.5 µL Taqman universal PCR master mix, 1.25 µL Taqman Gene Expression Assay (for *Glut4, ACC and UCP2*), 25 ng of cDNA, and RNAse free water to a final volume of 25 µL. The quantitative RT-PCR protocol was conducted as follows: 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Gene expression data were normalized to hypoxanthine-guanine phosphoribosyltransferase (*HPRT)*.

2.10. Western blot analysis

H9c2 cells were lysed in ice-cold RIPA buffer (Pierce Biotechnologies, Rockford, CA, USA). The lysates were centrifuged and the supernatant collected. Twenty micrograms of cell lysate were denatured and loaded onto a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane. Nonspecific binding on the membranes were blocked, using 5% (w/v) low-fat milk in Tris-buffered saline with Tween-20 (TBS-T; 10 mmol/L Tris-HCl, pH 7.5, 200 mmol/L NaCl, 0.05% Tween-20) at room temperature for 2 hours. Subsequently, the membrane was incubated overnight at 4ºC with the following primary antibodies: anti-Bcl-2 (1:1 000 dilution), anti-Bax (1:1000), pAMPK (Thr^{172}) (1:1000) and CPT1 (1:1000) with the relevant horseradish peroxidase (HRP) conjugated secondary antibodies applied the following day for 90 minutes at room temperature. β-Actin (1:4000) antibody was added as a loading control. Proteins were detected and quantified using a Chemidoc-XRS imager and Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

2.11. Statistical analysis

Data were expressed as the mean ± SEM of three independent biological experiments with each experiment containing at least 3 technical replicates. GraphPad Prism software (GraphPad Software Inc. La Jolla, CA, USA) was used for calculation of one-way multivariate ANOVA, followed by a Tukey

post hoc test or student t-test where appropriate. A p value of ≤ 0.05 was deemed as statistically significant.

3. Results

3.1. Effect of high glucose concentration on cardiac muscle cells

The effect of HG on metabolic activity was determined by exposing H9c2 cells to NG (5.5 mM) and HG (33 mM) over a 24 and 48-hour period, respectively. Culturing cells exposed to 33 mM glucose for 24 hours did not show a significant difference in metabolic activity when compared to the control. However, chronic exposure of cardiomyocytes to HG for 48 hours resulted in a decrease in metabolic activity when compared to the NG control (Figure 1). ASP does not have an effect on H9c2 cells treated with 5.5 mM glucose (unpublished data).

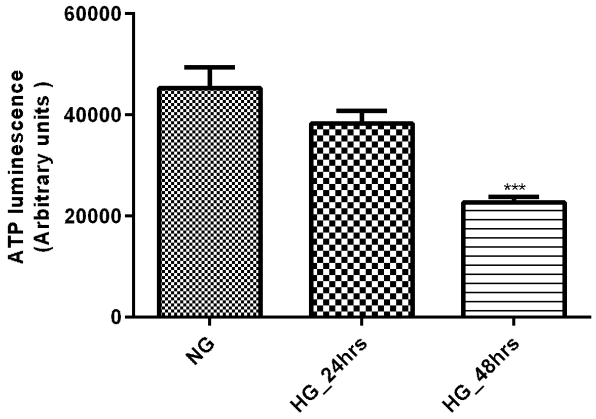


Figure 1. Effect of 33 mM glucose on metabolic activity. H9c2 cells treated with 5.5 mM glucose (normal glucose, NG) and 33 mM glucose (high glucose, HG) for (A) 24 and (B) 48 hours. Results are expressed as the mean ± SEM of three independent experiments. ATP luminescence results are presented in arbitrary units (AU). ***p < 0.0001 versus NG.

3.2. Energy metabolism in the diabetic heart

H9c2 cells exposed to HG showed a significant increase in FAU while GU was significantly decreased (1.5 ± 0.09; p < 0.0001 and 0.6 ± 0.04; p < 0.001). Treatment with MET resulted in a decrease in FAU with a concomitant increase in GU (1.2 ± 0.04; p < 0.01 and 0.9 ± 0.05). Similarly, ASP (1.2 ± 0.07; p < 0.0001 and 0.9 ± 0.07; p < 0.001) and a combination of MET and ASP (1.1 ± 0.04; p < 0.0001 and 0.9 ± 0.09; p < 0.001) were effective in decreasing FAU while increasing GU (Figure 2A and B).

Results obtained also showed that HG elicited a significant increase in FAO with a concomitant decrease in GO (0.84; $p < 0.0001$ and 0.22; $p < 0.001$) (Figure 2C). Treatment with MET decreased FAO by 16% ($p < 0.01$) while GO was increased by 16% ($p < 0.01$). Treatment of H9c2 cells with either ASP or a combination of MET and ASP decreased FAO by 18% ($p < 0.01$) and 23% ($p < 0.001$) and increased GO by 18% and 23% ($p < 0.01$ and $p < 0.0001$), respectively (Figure 2C).

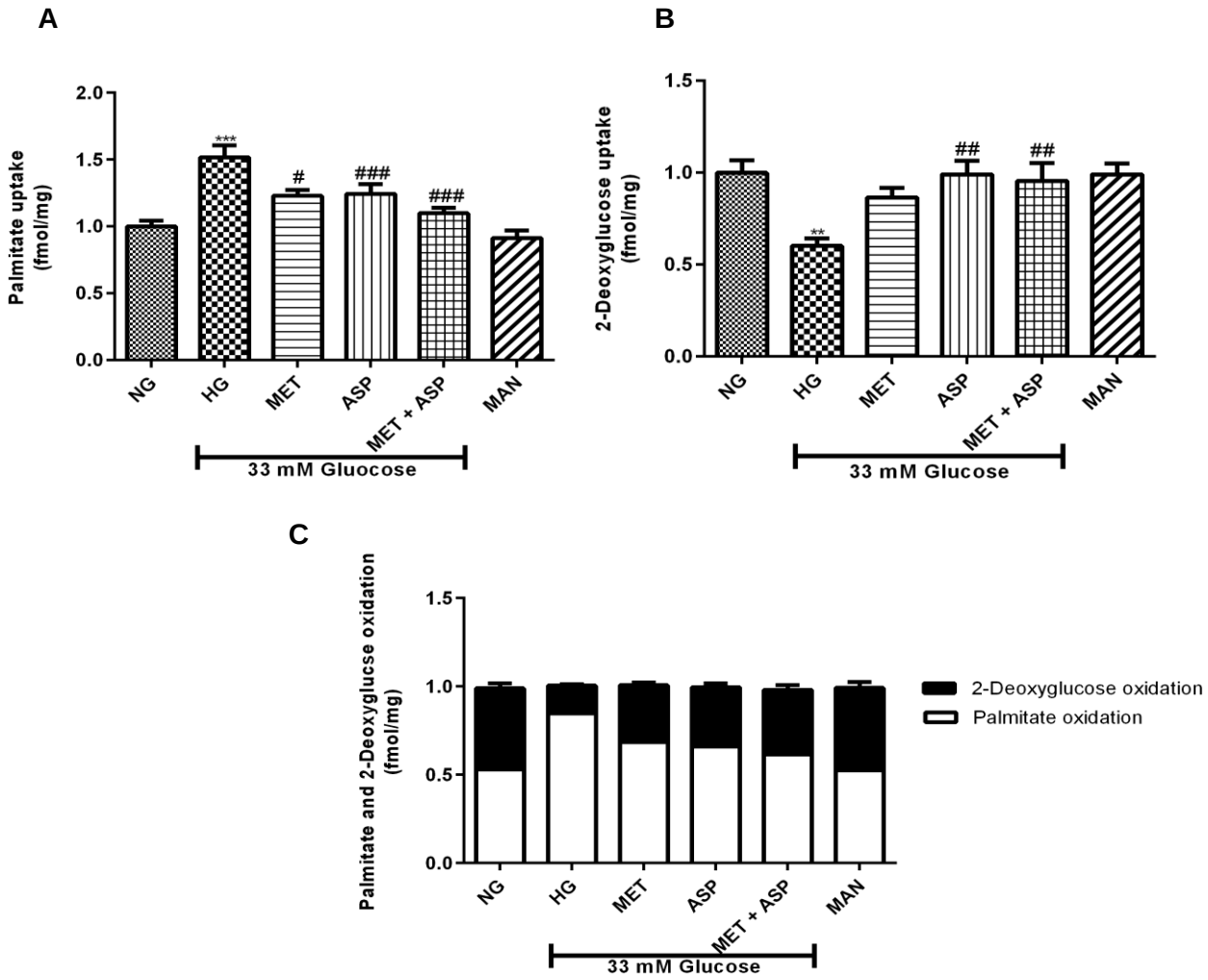


Figure 2. Effect of ASP on shift in substrate preference. Fatty acid uptake (A), glucose uptake (B) and fatty acid and glucose oxidation combination stacked bar graph (C) of H9c2 cells treated with 33 mM glucose (high glucose, HG) for 48 hours and post treated with metformin (MET; 1 μM), aspalathin (ASP; 1 μM) and the combination of MET and ASP (1 μM each) for 6 hours. Mannitol (MAN) at a concentration of 33 mM was used to rule out the effect of osmolarity. Glucose uptake and palmitate uptake are expressed in arbitrary units (AU) for 2-Deoxy-[3H]-D-glucose and palmitate-D-[^{14}C (U)], respectively. Results are expressed as the mean of three independent experiments relative to the normal glucose (NG) control (5.5 mM). **$p < 0.001$ and ***$p < 0.0001$ versus NG control. $^{\#}p < 0.01$, $^{\#\#}p < 0.001$ and $^{\#\#\#}p < 0.0001$ versus HG control.

3.3. Increased ROS production

A 41% increase in ROS production was observed in H9c2 cells exposed to HG (141 ± 2.3%; $p < 0.0001$) (Figure 3). Treatment of cells with MET decreased ROS production by 20% (121 ± 2.2%; $p< 0.0001$), while ASP (121 ± 2.1%; $p < 0.0001$) and the combination of MET and ASP (115 ± 2.6%; $p< 0.0001$) subdued the HG-induced increase of intracellular ROS by 20% and 26% respectively.

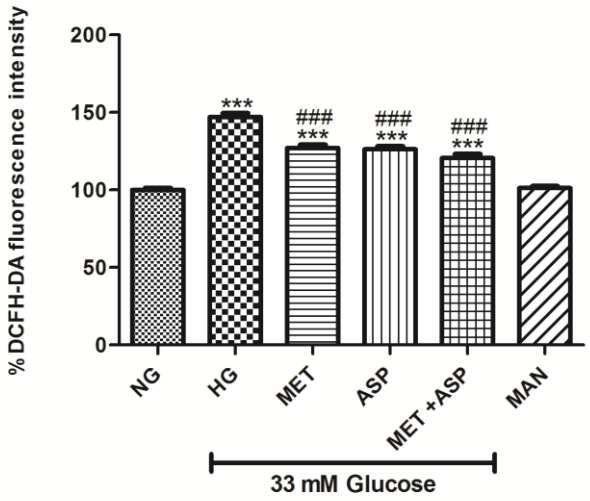
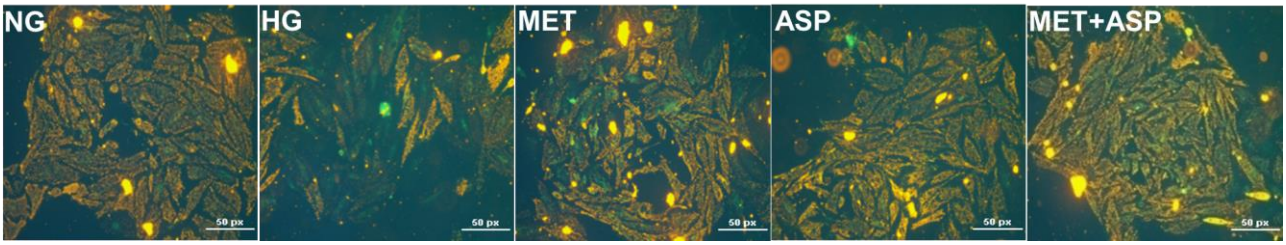


Figure 3. Effect of ASP on intracellular ROS formation. DCFH-DA fluorescence intensity of intracellular ROS formation of H9c2 cells treated with 33 mM glucose (HG) for 48 hours and post treated with metformin (MET; 1 µM), aspalathin (ASP; 1 µM) and the combination of MET and ASP (1 µM each) for 6 hours. Mannitol (MAN) at a concentration of 33 mM was used to rule out the effect of osmolarity. Results are expressed as the mean of three independent experiments relative to the normal glucose (NG) control (5.5 mM) at 100% ± SEM. ***$p < 0.0001$ versus NG control ###$p < 0.0001$ versus HG control.

3.4. Mitochondrial transmembrane potential (ΔΨm)

Pre-treatment of cells with HG resulted in an increase in green fluorescence after H9c2 cells were stained with JC-1 (Figure 4A and B), indicating a reduction in mitochondrial transmembrane potential (ΔΨm). The HG concentration significantly increased mitochondrial depolarization by a ratio of 0.46 (from 1 ± 0.05 to 1.46 ± 0.1; $p < 0.0001$). Both MET (1.16 ± 0.044; $p < 0.01$) and ASP (1.13 ± 0.07; $p < 0.01$) were able to ameliorate this effect. An enhanced effect was observed when H9c2 cells, exposed to glucose, were treated with the MET and ASP combination (1.02 ± 0.07; $p < 0.0001$). The observed effect was similar to that of the normal control and slightly better than either MET or ASP alone.

A



B

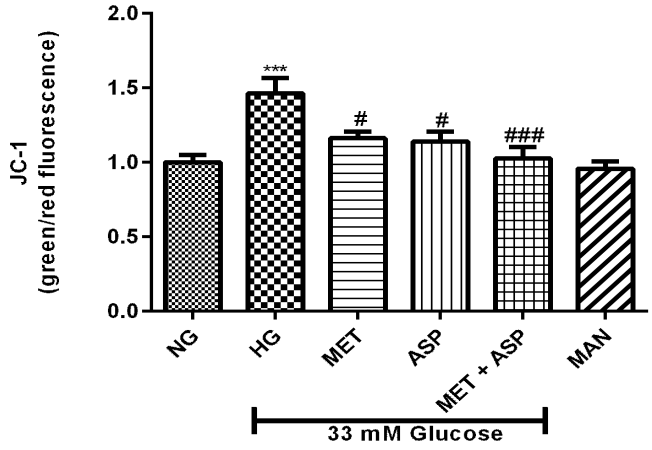


Figure 4. Effect of ASP on mitochondrial membrane potential. Fluorescent photomicrographs of JC-1 fluorescent mitochondrial depolarization of H9c2 cells treated with 33 mM glucose (high glucose, HG) for 48 hours and post treated with metformin (MET; 1 μM), aspalathin (ASP; 1 μM) and the combination of MET and ASP (1 μM each) for 6 hours. Mannitol (MAN) at a concentration of 33 mM was used to rule out the effect of osmolarity. Results are expressed as the mean of three independent experiments relative to the normal glucose (NG) control (5.5 mM). ***$p < 0.0001$ versus NG control. #$p < 0.01$ and ###$p < 0.0001$ versus HG control.

3.5. GSH content and SOD activity

Chronically exposing H9c2 cells to HG decreased SOD activity to 47% (47 ± 9%; $p < 0.0001$). MET increased SOD activity by 57% (104 ± 5.3%; $p < 0.0001$) while both ASP (118 ± 4.9%; $p < 0.0001$) and the combination of MET and ASP (118 ± 5.2%; $p < 0.0001$) increased de-novo SOD synthesis by 87% (Figure 5A). The effect of ASP was marginally better than that of MET alone, although non-significant. HG also reduced the GSH content of H9c2 cells (0.59 ± 0.03; $p < 0.0001$), while treatment with MET (0.82 ± 0.03; $p < 0.0001$), ASP (0.89 ± 0.03; $p < 0.0001$) or the combination of MET and

ASP (0.94 ± 0.02; p < 0.0001) increased GSH content under HG conditions (Figure 5B). The observed effect was comparable to that of the NG control (1 ± 0.02; p = NS).

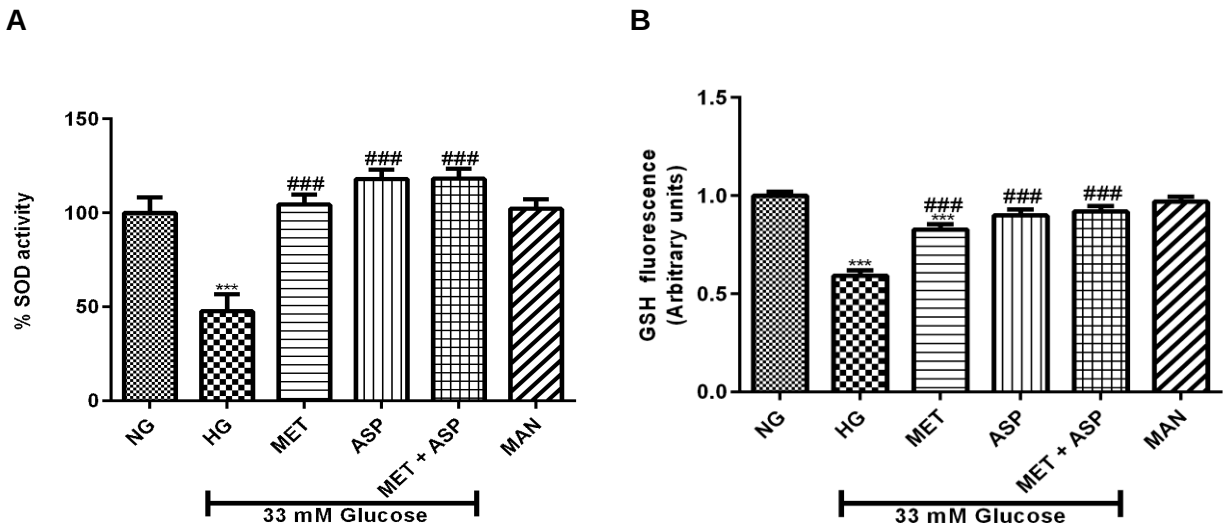


Figure 5. Effect of ASP on SOD activity and GSH fluorescence. Superoxide dismutase (SOD) activity (A) and CellTracker™ Blue CMAC fluorescence as a measure of glutathione (GSH) content (B) was assessed in H9c2 cells treated with 33 mM glucose (high glucose, HG) for 48 hours and post treated with metformin (MET; 1 μM), aspalathin (ASP; 1 μM) and the combination of MET and ASP (1 μM) for 6 hours. Mannitol (MAN) at a concentration of 33 mM was used to rule out the effect of osmolarity. Results are expressed as the mean ± SEM of three independent experiments relative to the normal glucose (NG) control (5.5 mM). ***p < 0.0001 versus NG control. ###p < 0.0001 versus HG control.

3.6. H9c2 cell apoptosis

HG significantly increased Caspase-3/7 activity in H9c2 cell (139 ± 6.9%; p < 0.0001), while treatment with ASP (116 ± 5.3%; p < 0.01) or the combination of MET and ASP (116 ± 3.8%; p < 0.01) decreased HG-induced apoptosis (Figure 6). Increased numbers of TUNEL-positive cells were observed in H9c2 cells exposed to HG compared to that of the NG control (7.7 ± 0.5 compared to 1.2 ± 0.2; p < 0.0001). Treatment with ASP and the combination of MET and ASP significantly decreased the amount of TUNEL positive cells (3.6 ± 0.12; p < 0.001 and 4.4 ± 0.6; p < 0.01). However, no significance was observed with MET treatment (5.2 ± 0.48) (Figure 7).

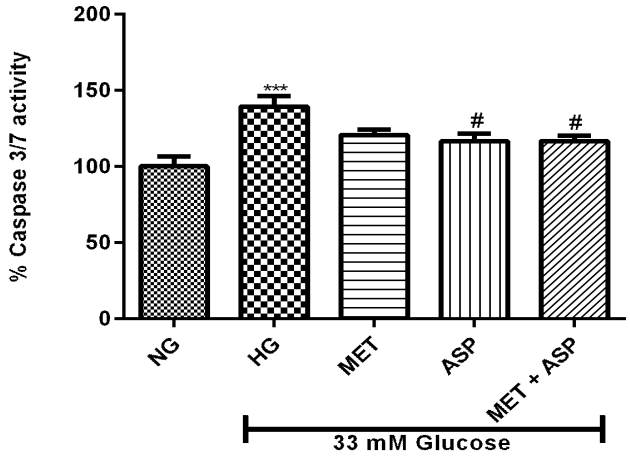


Figure 6. Effect of ASP on cell apoptosis. Caspase-3/7 activity of H9c2 cells treated with 33 mM glucose (high glucose, HG) for 48 hours and post treated with metformin (MET; 1 μM), aspalathin (ASP; 1 μM) and the combination of MET and ASP (1 μM) for 6 hours. Results are expressed as the mean of three independent experiments relative to the normal glucose (NG) control (5.5 mM) at 100% ± SEM. ***$p < 0.001$ versus NG control. #$p < 0.01$ versus HG control.

3.7. Western blot analysis

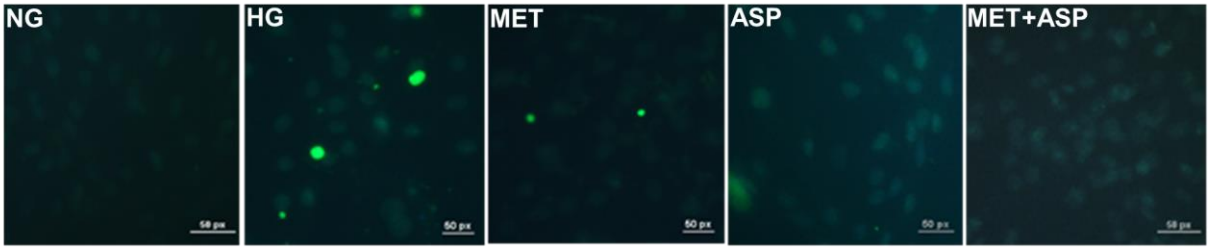
3.7.1. Bcl-2\Bax ratio

A significant decrease in the Bcl-2\Bax ratio was observed when cells were exposed to HG (71 ± 2.2%; $p < 0.01$). Treatment with MET (106 ± 6.1%; $p < 0.001$), ASP (111 ± 6.5%; $p < 0.001$) and a combination of MET and ASP (115 ± 10.7%; $p < 0.0001$) was able to ameliorate this effect significantly (Figure 8).

3.7.2. β-Oxidation

Exposure of H9c2 cells to a HG concentration increased pAMPK (Thr^{172}) and CPT1 expression by 5.9 fold ($p < 0.001$) and 1.4 fold ($p < 0.01$), respectively. Treatment with ASP and the combination of MET and ASP decreased the expression of pAMPK (Thr^{172}) by 1.96 fold ($p < 0.01$) and 1.84 fold ($p < 0.01$), respectively. MET had no effect. Treatment with ASP, MET and combination of MET and ASP decreased CPT1 expression by 1.4 fold ($p < 0.0001$), 2.1 ($p < 0.0001$) and 1.3 fold ($p < 0.0001$), respectively, when compared to the HG control (Figure 9A and B).

A



B

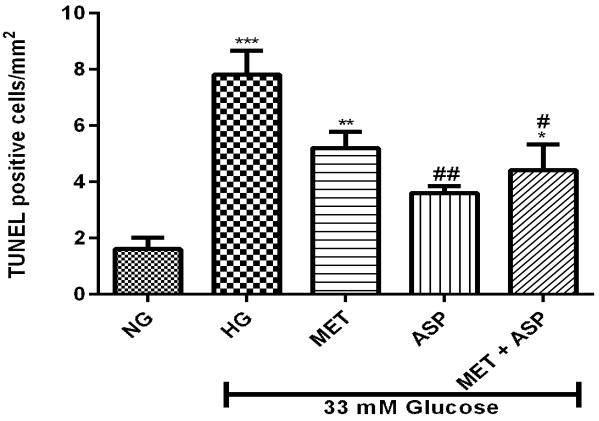


Figure 7. Effect of ASP on diminishing DNA fragmentation of apoptotic cells. Fluorescent photomicrographs of TUNEL positive H9c2 cells and B) treated with 33 mM glucose (high glucose, HG) for 48 hours and post treated with metformin (MET; 1 μM), aspalathin (ASP; 1 μM) and the combination of MET and ASP (1 μM each) for 6 hours. White arrows illustrate apoptotic TUNEL positive cells. Mannitol (MAN) at a concentration of 33 mM was used to rule out the effect of osmolarity. Results are expressed as the mean of three independent experiments relative to the normal glucose (NG) control (5.5 mM) at 100% ± SEM. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$ versus NG control. #$p < 0.01$ and ##$p < 0.001$ versus HG control.

3.8. mRNA expression analysis

Exposure of H9c2 cells to a HG concentration decreased the expression of *GLUT4* by 2.2 fold ($p < 0.01$) and *ACC* by 2.5 fold ($p < 0.001$), respectively when compared to that of the NG control. MET, ASP and the combination of MET and ASP significantly increased mRNA expression of *GLUT4* by 1.1 fold (NS), 2.3 fold ($p< 0.01$) and 2.8 fold ($p < 0.01$) and that of *ACC* by 1.9 fold ($p < 0.0001$), 3.3 fold ($p < 0.0001$) and 4.7 fold ($p < 0.0001$) when compared to that of the HG control (Figure 10A and B). HG also decreased the expression of *UCP2* by 2.2 fold ($p < 0.01$) in H9c2 cells (Figure 10C). Aspalathin

and the combination of MET and ASP reversed this effect by 2.6 fold ($p < 0.001$) and 2.3 fold ($p < 0.001$), respectively. MET monotherapy was less effective.

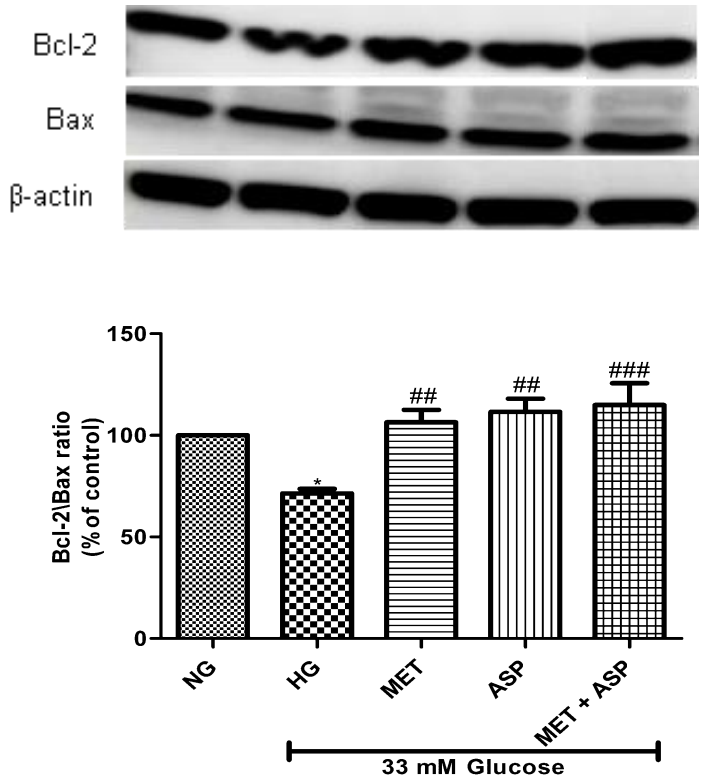
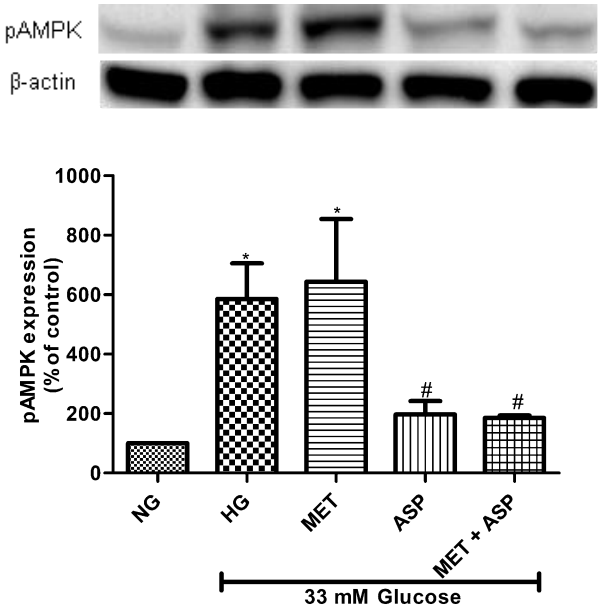


Figure 8. Effect of ASP On Bcl-2 and Bax protein expression. H9c2 cells treated with 33 mM glucose (HG) for 48 hours and post treated with metformin (MET; 1 μM), aspalathin (ASP; 1 μM) and the combination of MET and ASP (1 μM each) for 6 hours. Results are expressed as the mean of three independent experiments relative to the normal glucose (NG) control (5.5 mM) at 100% ± SEM. *$p < 0.01$ versus NG control. ##$p < 0.001$ and ###$p < 0.0001$ versus HG control.

A



B

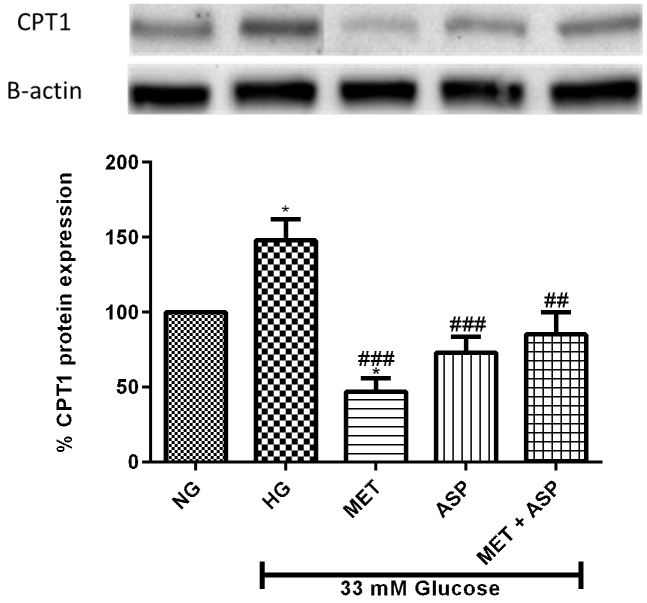


Figure 9. Effect of ASP on pAMPK and CPT1 protein expression. pAMPK(Thr172) (A) and CPT1 (B) protein expression of H9c2 cells treated with 33 mM glucose (high glucose, HG) for 48 hours and post treated with metformin (MET; 1 μM), aspalathin (ASP; 1 μM) and the combination of MET and ASP (1 μM each) for 6 hours. Results are expressed as the mean of three independent experiments relative to the normal glucose (NG) control (5.5 mM) at 100% ± SEM. *$p < 0.01$ versus NG control. $^{\#}p < 0.01$, $^{\#\#}p < 0.001$ and $^{\#\#\#}p < 0.0001$ versus HG control.

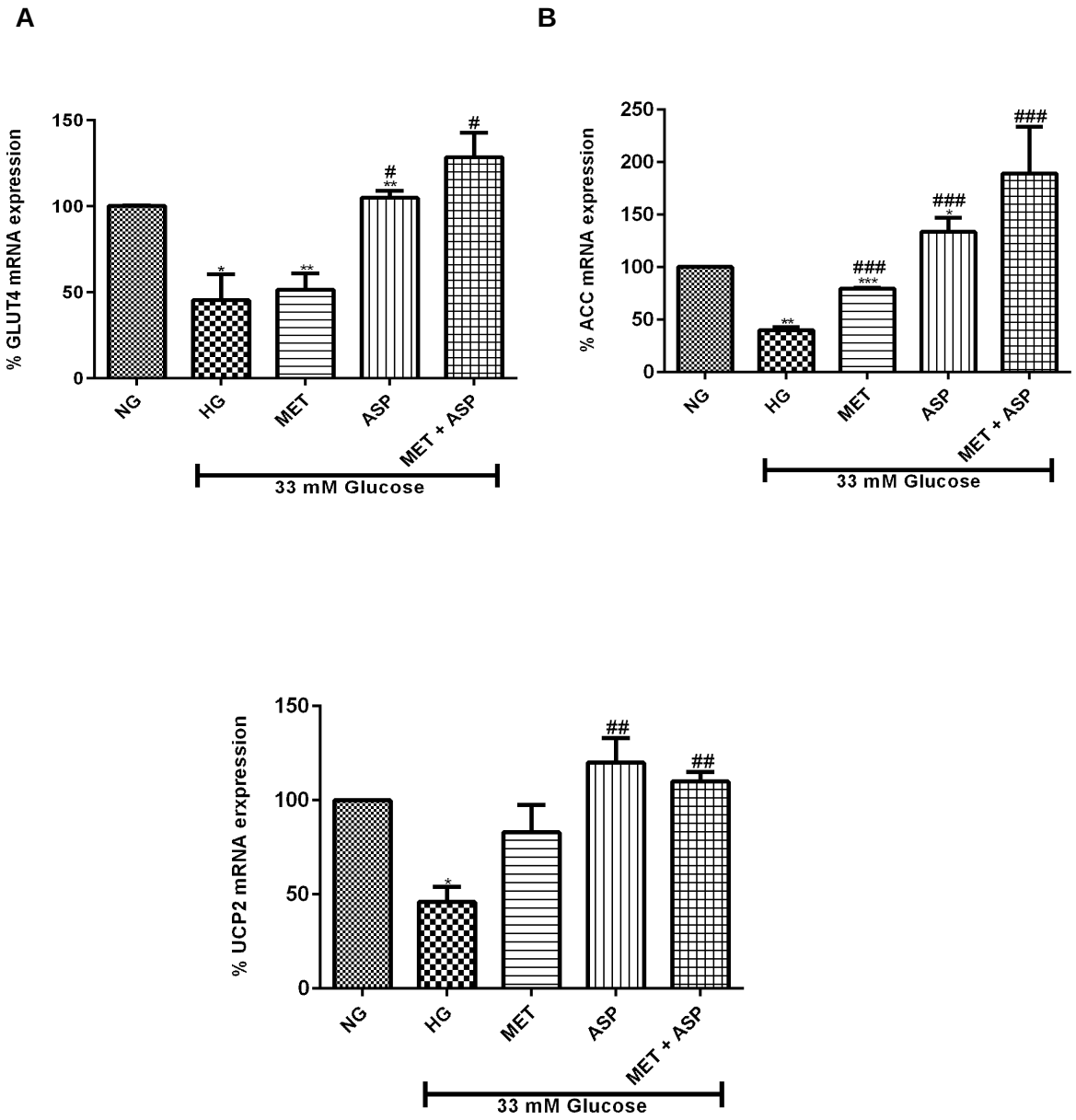


Figure 10. Effect of ASP on *GLUT4, ACC* and *UCP2* mRNA expression. *GLUT4* (A), *ACC* (B) and *UCP2* (C) mRNA expression in H9c2 cells treated with 33 mM glucose (high glucose, HG) for 48 hours and post treated with metformin (MET; 1 μM), aspalathin (ASP; 1 μM) and the combination of MET and ASP (1 μM each) for 6 hours. Results are expressed as the mean of three independent experiments relative to the normal glucose (NG) control (5.5 mM) at 100% ± SEM. *p < 0.01, **p < 0.001 and ***p < 0.0001 versus NG control. #p < 0.01, ##p < 0.001 and ###p < 0.0001 versus HG control.

4. Discussion

In normal cardiomyocytes FAO contributes to approximately 70% and GO to the remaining 30% of energy production. This balance in energy production is crucial for optimal contractile function of the heart. In the diabetic heart, there is a shift in mitochondrial substrate preference from glucose towards an almost complete reliance on FAO (80-90%) as the preferred energy source [10]. This compensatory shift in energy substrate is believed to be involved in impaired cardiac function of the failing heart [32]. Studies done in T2DM rodent models with associated HF, showed a dramatic increase in lipid accumulation and FAO with a concomitant decrease in GO [33, 34]. Similar findings were also reported for studies of the hearts of human patients with T2DM [35]. In this study, a shift in substrate preference with an associated decreased metabolic activity was observed when H9c2 cells were chronically exposed to a HG concentration of 33 mM. ASP, as well as the combination of MET and ASP, was able to reverse this effect. These findings can indirectly improve heart contractile function as energy metabolism is closely linked to cardiac function [36]. Our findings are supported by a study conducted by Kantor et al (2000) demonstrating that trimetazidine, an anti-anginal agent and FA inhibitor, was able to protect the diabetic heart by decreasing FAO and increasing GO in the hearts of Zucker rats [36].

This shift in substrate preference and the ability of cells to survive metabolic dysfunction relies, in part, on the response of intracellular kinases [37]. A change in contractile activity of diabetic cardiomyocytes results in a temporal increase in the adenosine monophosphate/triphosphate (AMP/ATP) ratio, which induces a conformational change within AMPK, making it accessible for phosphorylation (pAMPK). Increased pAMPK suppresses acetyl-CoA carboxylase (*ACC*) activity, decreases malonyl-CoA levels and subsequently the inhibitory effect of malonyl-CoA on CPT1 is lessened with a resultant increase in FAO. Our findings support such a hypothesis whereby exposure of the cells to a HG concentration decreased ATP and increased pAMPK expression. This brought about a decrease in *ACC* and increased CPT1 with a subsequent increase in FAU and FAO. ASP and the combination of MET and ASP was able to reduce FAU and FAO while increasing glucose uptake as observed by decreased fatty acid translocase (FAT/CD36) (supplementary data) and increased *GLUT4* mRNA expression. This infers that ASP can improve myocardial energy balance by switching energy preference from FAO to GO and thus protecting the diabetic heart against contractile dysfunction. This is an important finding, as both FAT/CD36 and GLUT4 are major transporter proteins with fundamental roles in fatty acid and glucose uptake respectively. In T2DM FAT/CD36 remains localized at the sarcolemma whereas *GLUT4* internalizes, resulting in a shift in substrate preference [38]. This shift in substrate utilization results in increased FAU, at the expense of glucose as observed in this study. The effect of ASP on *GLUT4* gene expression with concomitant increased GU results in

an improved glucose to fatty acid utilization ratio. In addition, we showed that ASP has a modulating effect on FAT/CD36 that could culminate in an improved substrate utilization in the diabetic heart.

Chronic hyperglycemia is also known to be responsible for cardiac dysfunction through the generation of increased oxidative stress [4, 6]. Oxidative stress is defined as the imbalance between the production and elimination of ROS, which plays a critical role in left ventricular remodeling and HF [39]. The cardiomyocytes have a very low antioxidant capacity and a shift in mitochondrial substrate preference can result in increased ROS production. Uncoupling protein 2 (UCP2) is a negative regulator of mitochondrial derived ROS and plays an important role in mitochondrial cellular redox homeostasis. Augmentation of *UCP2* expression could result in decreased ROS production and protect the cell against oxidative damage [40, 41]. Data from this study showed that chronic exposure of H9c2 cells to 33 mM glucose triggered increased ROS production. This increase in ROS was associated with decreased *UCP2* mRNA expression. Both ASP alone and the combination of MET and ASP were able to attenuate intracellular ROS through up regulation of *UCP2*. Furthermore, excessive ROS production can induce intracellular mitochondrial damage including opening of the mitochondria permeability transition (MPT), leading to mitochondrial depolarization. This results in the expression and leakage of various proteins including endogenous antioxidant enzymes, GSH and SOD, as observed in this study. Treatment with ASP enhanced the endogenous antioxidant capacity of the cell and potentially could protect the myocardium against HG-induced oxidative stress and subsequent mitochondrial depolarization.

Furthermore, the mitochondrial apoptotic pathway is largely mediated through Bcl-2 family proteins. Bcl-2, a human proto-oncogene, protects against mitochondrial permeability and subsequent programmed cell death [39, 42]. Bax, a pro-apoptotic member of the Bcl-2 family, initiates apoptosis by inserting its C-terminal end into the mitochondrial outer membranes upon induction of cell death. A shift in the Bcl-2\Bax ratio can therefore result in increased mitochondrial depolarization and cytochrome-c release, a crucial molecule in the induction of apoptosis. In this study, the 33 mM glucose (HG) treatment resulted in a significant decrease in the Bcl-2\Bax ratio and a subsequent increase in cell apoptosis. ASP, MET or the combination of ASP and MET were able to reverse HG-induced apoptosis by increasing the Bcl-2\Bax ratio, decreasing mitochondrial membrane depolarization, caspase-3/7 activity and reducing tunnel positive cells. However, in terms of efficacy no differences were found between the treatments, suggesting that there was no additive effect between MET and ASP.

The mechanism by which ASP modulates cellular antioxidant defenses and function of cardiomyocytes remains to be fully elucidated. However, unpublished data from our group suggest that ASP is able to activate the redox-sensitive transcription factor, nuclear factor erythroid 2-related

factor (Nrf2). Nrf2 is a transcriptional regulator that plays a key role in regulating phase II detoxifying and antioxidant enzymes in response to stressful conditions. Niture and Jaiswel (2012) demonstrated that Nrf2 regulates Bcl-2 gene expression and prevents apoptosis in human lung cancer cells [42]. Based on their findings and evidence obtained from the current study, we proposed that ASP also activates Nrf2 during chronic hyperglycemia. Activation of Nrf2 upregulates various antioxidant genes including GSH and SOD [43]. Furthermore, this increase in hyperglycemic-induced oxidative stress activates AMPK, a kinase like Nrf2 that controls the redox-status of the cell. AICAR, an AMPK activator, induces Nrf2 activation by an AMPK-independent mechanism in hepatocarcinoma cells [44]. Thus, we proposed that although both AMPK and Nrf2 maintain cellular homeostasis, they act independently of each other. AMPK increases cell survival by regulating FAT/CD36 expression and β-oxidation while Nrf2 increase cell survival by decreasing oxidative stress and increasing the antioxidant capacity.

In conclusion, our study provides evidence that ASP is able to protect H9c2 cardiomyocytes against HG-induced shifts in substrate preference by decreasing FAU and FAO, inferring that ASP might act as a FAO modulator in the heart of the diabetic individuals. Furthermore, results obtained from this study showed that ASP was as effective as metformin to inhibit glucose-induced increased fatty acid β-oxidation and subsequent increased oxidative stress. Additional evidence produced, demonstrated that ASP enhances endogenous antioxidant defenses and protects against HG-induced membrane depolarization and subsequent apoptotic cell death of vulnerable cardiomyocytes under hyperglycemic conditions. Interestingly, the effect of ASP or the combined effect of ASP and MET was more effective than MET alone.

5. Acknowledgments

This work was funded by the National Research Foundation: Thuthuka Programme Grant 87836 and South African Medical Research Council's Diabetes Discovery Platform baseline funding. The grant holders acknowledge that opinions, findings and conclusions or recommendations expressed in any publication generated by the NRF supported research are those of the authors, and that the NRF accepts no liability whatsoever in this regard.

6. Conflict of Interest

None.

7. References

[1] Alexander, C. M., Landsman, P. B., Teutsch, S. M., Haffner, S. M., NCEP-defined metabolic syndrome, diabetes, and prevalence of coronary heart disease among NHANES III participants age 50 years and older. Diabetes. 2003, 52, 1210-1214.

[2] Lee, T. I., Kao, Y. H., Chen, Y. C., Huang, J. H., et al., Peroxisome proliferator-activated receptors modulate cardiac dysfunction in diabetic cardiomyopathy. Diabetes Res. Clin. Pract. 2013, 100, 330-339.

[3] Mahgoub, M. A., Abd-Elfattah, A. S., Diabetes mellitus and cardiac function. Mol. Cell. Biochem. 1998, 180, 59-64.

[4] Ansley, D. M., Wang, B., Oxidative stress and myocardial injury in the diabetic heart. J. Pathol. 2013, 229, 232-241.

[5] Flores-Mateo, G., Carrillo-Santisteve, P., Elosua, R., Guallar, E., et al., Antioxidant enzyme activity and coronary heart disease: meta-analyses of observational studies. Am. J. Epidemiol. 2009, 170, 135-147.

[6] Khullar, M., Al-Shudiefat, A. A., Ludke, A., Binepal, G., Singal, P. K., Oxidative stress: a key contributor to diabetic cardiomyopathy. Can. J. Physiol. Pharmacol. 2010, 88, 233-240.

[7] Flores-Mateo, G., Elosua, R., Rodriguez-Blanco, T., Basora-Gallisa, J., et al., Oxidative stress is associated with an increased antioxidant defense in elderly subjects: a multilevel approach. PloS One. 2014, 9, e105881.

[8] Mukherjee, S., Banerjee, S. K., Maulik, M., Dinda, A. K., et al., Protection against acute adriamycin-induced cardiotoxicity by garlic: role of endogenous antioxidants and inhibition of TNF-alpha expression. BMC Pharmacol. 2003, 3, 16.

[9] Abou-El-Hassan, M. A., Rabelink, M. J., van der Vijgh, W. J., Bast, A., Hoeben, R. C., A comparative study between catalase gene therapy and the cardioprotector monohydroxyethylrutoside (MonoHER) in protecting against doxorubicin-induced cardiotoxicity in vitro. Br. J. Cancer. 2003, 89, 2140-2146.

[10] Bayeva, M., Sawicki, K. T., Ardehali, H., Taking diabetes to heart--deregulation of myocardial lipid metabolism in diabetic cardiomyopathy. J. Am. Heart Assoc. 2013, 2, e000433.

[11] Lopaschuk, G. D., Ussher, J. R., Folmes, C. D., Jaswal, J. S., Stanley, W. C., Myocardial fatty acid metabolism in health and disease. Physiol. Rev. 2010, 90, 207-258.

[12] Stanley, W. C., Lopaschuk, G. D., McCormack, J. G., Regulation of energy substrate metabolism in the diabetic heart. Cardiovasc. Res. 1997, 34, 25-33.

[13] Fillmore, N., Mori, J., Lopaschuk, G. D., Mitochondrial fatty acid oxidation alterations in heart failure, ischaemic heart disease and diabetic cardiomyopathy. Br. J. Pharmacol. 2014, 171, 2080-2090.

[14] Inzucchi, S. E., Metformin and heart failure: innocent until proven guilty. Diabetes Care. 2005, 28, 2585-2587.

[15] Erejuwa, O. O., Sulaiman, S. A., Wahab, M. S., Salam, S. K., et al., Antioxidant protective effect of glibenclamide and metformin in combination with honey in pancreas of streptozotocin-induced diabetic rats. Int. J. Mol. Sci. 2010, 11, 2056-2066.

[16] Pandey, K. B., Rizvi, S. I., Plant polyphenols as dietary antioxidants in human health and disease. Oxid. Med. Cell. Longev. 2009, 2, 270-278.

[17] Wang, S., Zhang, J., Chen, M., Wang, Y., Delivering flavonoids into solid tumors using nanotechnologies. Expert Opin. Drug Deliv. 2013, 10, 1411-1428.

[18] Bahadoran, Z., Mirmiran, P., Azizi, F., Dietary polyphenols as potential nutraceuticals in management of diabetes: a review. J. Diabes Metab. Disord. 2013, 12, 43.

[19] Esmaeili, M. A., Sonboli, A., Antioxidant, free radical scavenging activities of Salvia brachyantha and its protective effect against oxidative cardiac cell injury. Food Chem. Toxicol. 2010, 48, 846-853.

[20] Jiang, F., Chang, C. W., Dusting, G. J., Cytoprotection by natural and synthetic polyphenols in the heart: novel mechanisms and perspectives. Curr. Pharm. Des. 2010, 16, 4103-4112.

[21] Zhang, P. Y., Xu, X., Li, X. C., Cardiovascular diseases: oxidative damage and antioxidant protection. Eur. Rev. Med. Pharm. Sci. 2014, 18, 3091-3096.

[22] Muller, C. J., Joubert, E., de Beer, D., Sanderson, M., et al., Acute assessment of an aspalathin-enriched green rooibos (Aspalathus linearis) extract with hypoglycemic potential. Phytomedicine. 2012, 20, 32-39.

[23] Son, M. J., Minakawa, M., Miura, Y., Yagasaki, K., Aspalathin improves hyperglycemia and glucose intolerance in obese diabetic ob/ob mice. Eur. J. Nutr. 2013, 52, 1607-1619.

[24] Chen, W., Sudji, I. R., Wang, E., Joubert, E., et al., Ameliorative effect of aspalathin from rooibos (Aspalathus linearis) on acute oxidative stress in Caenorhabditis elegans. Phytomedicine. 2013, 20, 380-386.

[25] Darvesh, A. S., Carroll, R. T., Bishayee, A., Geldenhuys, W. J., Van der Schyf, C. J., Oxidative stress and Alzheimer's disease: dietary polyphenols as potential therapeutic agents. Expert Rev. Neurother. 2010, 10, 729-745.

[26] Baba, H., Ohtsuka, Y., Haruna, H., Lee, T., et al., Studies of anti-inflammatory effects of Rooibos tea in rats. Pediatri. Int. 2009, 51, 700-704.

[27] Dludla, P. V., Muller, C. J., Louw, J., Joubert, E., et al., The cardioprotective effect of an aqueous extract of fermented rooibos (Aspalathus linearis) on cultured cardiomyocytes derived from diabetic rats. Phytomedicine. 2014, 21, 595-601.

[28] Singh, B. K., Singh, A., Kumar, V., Ameliorative Effect of Adjunct Therapy of Metformin with Atorvastatin on Streptozotocin-induced Diabetes Mellitus in Rats. Drug. Res. 2015.

[29] Han, Z., Achilonu, M. C., Kendrekar, P. S., Joubert, E., et al., Concise and scalable synthesis of aspalathin, a powerful plasma sugar-lowering natural product. J. Nat. Prod. 2014, 77, 583-588.

[30] Nugent, C., Prins, J. B., Whitehead, J. P., Wentworth, J. M., et al., Arachidonic acid stimulates glucose uptake in 3T3-L1 adipocytes by increasing GLUT1 and GLUT4 levels at the plasma membrane. Evidence for involvement of lipoxygenase metabolites and peroxisome proliferator-activated receptor gamma. J. Biol. Chem. 2001, 276, 9149-9157.

[31] Mazibuko, S. E., Muller, C. J., Joubert, E., de Beer, D., et al., Amelioration of palmitate-induced insulin resistance in C(2)C(1)(2) muscle cells by rooibos (Aspalathus linearis). Phytomedicine. 2013, 20, 813-819.

[32] Fillmore, N., Lopaschuk, G. D., Targeting mitochondrial oxidative metabolism as an approach to treat heart failure. Biochim. Biophys. Acta. 2013, 1833, 857-865.

[33] Belke, D. D., Larsen, T. S., Gibbs, E. M., Severson, D. L., Altered metabolism causes cardiac dysfunction in perfused hearts from diabetic (db/db) mice. Am. J. of Physiol. Endocrinol Metab. 2000, 279, E1104-1113.

[34] Kenno, K. A., Severson, D. L., Lipolysis in isolated myocardial cells from diabetic rat hearts. Am. J. Physiol 1985, 249, H1024-1030.

[35] Herrero, P., Peterson, L. R., McGill, J. B., Matthew, S., et al., Increased myocardial fatty acid metabolism in patients with type 1 diabetes mellitus. J. Am. Coll. Cardiol. 2006, 47, 598-604.

[36] Kantor, P. F., Lucien, A., Kozak, R., Lopaschuk, G. D., The antianginal drug trimetazidine shifts cardiac energy metabolism from fatty acid oxidation to glucose oxidation by inhibiting mitochondrial long-chain 3-ketoacyl coenzyme A thiolase. Cir. Res. 2000, 86, 580-588.

[37] Nagendran, J., Waller, T. J., Dyck, J. R., AMPK signalling and the control of substrate use in the heart. Mol. Cell. Endocrinol. 2013, 366, 180-193.

[38] Coort, S. L., Bonen, A., van der Vusse, G. J., Glatz, J. F., Luiken, J. J., Cardiac substrate uptake and metabolism in obesity and type-2 diabetes: role of sarcolemmal substrate transporters. Mol. Cell. Biochem. 2007, 299, 5-18.

[39] Sun, X., Chen, R. C., Yang, Z. H., Sun, G. B., et al., Taxifolin prevents diabetic cardiomyopathy in vivo and in vitro by inhibition of oxidative stress and cell apoptosis. Food. and Chem. Toxicol. 2014, 63, 221-232.

[40] Mattiasson, G., Shamloo, M., Gido, G., Mathi, K., et al., Uncoupling protein-2 prevents neuronal death and diminishes brain dysfunction after stroke and brain trauma. Nat. Med. 2003, 9, 1062-1068.

[41] Brand, M. D., Esteves, T. C., Physiological functions of the mitochondrial uncoupling proteins UCP2 and UCP3. Cell. Metab. 2005, 2, 85-93.

[42] Niture, S. K., Jaiswal, A. K., Nrf2 protein up-regulates antiapoptotic protein Bcl-2 and prevents cellular apoptosis. J. Biol. Chem. 2012, 287, 9873-9886.

[43] Zhu, H., Itoh, K., Yamamoto, M., Zweier, J. L., et al., Role of Nrf2 signaling in regulation of antioxidants and phase 2 enzymes in cardiac fibroblasts: protection against reactive oxygen and nitrogen species-induced cell injury. FEBS Lett. 2005, 579, 3029-3036.

[44] Sid, B., Glorieux. C., Valenzuela, M., Rommelaere, G., et al., AICAR induces Nrf2 activation by an AMPK-independent mechanism in hepatocarcinoma cells. Biochem. Pharmacol. 2014, 91,168-180.

Supplementary

mRNA expression analysis

Total RNA was extracted from H9c2 cells using Trizol reagent. Briefly, H9c2 cells were homogenized using a TissueLyser (Qiagen, Hilden, Germany) at 25 Hz for 2 minutes according to the manufacturer's instructions. RNA was purified using an RNeasy Mini kit (Qiagen, Germantown, MD, USA) and genomic DNA was removed using the Ambion Turbo DNase kit (Applied Biosystems, Austin, Texas, USA) according to the manufacturer's instructions.

RT^2-PCR arrays

Quantitative reverse transcription real-time PCR (qRT-PCR) was used to quantify the amounts of mRNA in the H9c2 cells using a Rat Insulin resistant RT^2 Profiler PCR Arrays (PARN -145Z) (Qiagen/SA Biosciences). The arrays included 84 assay genes, 5 housekeeping genes, and reverse transcription efficiency and DNA contamination controls. cDNA was synthesized from 2 µg of RNA using RT^2 PCR Array First Strand Kit (Qiagen/SA Biosciences) and the arrays were performed according to the manufacturer instructions using Qiagen/SA Biosciences Master Mix. Each experiment was performed on RNA pooled samples from 3 biological replicates and repeated only once for each condition.

Results

Fatty acid oxidation is the source of energy for cardiac myocytes and changes in fatty acid metabolism have been implicated in the diabetic-induced cardiac disease state. To give insight into the intracellular transportation of fatty acids, we investigated the expression of fatty acid translocase (FAT/CD36) using an Insulin resistant RT^2-PCR array. The result obtained confirmed an increased in CD36 (3.7 fold) mRNA expression after 33mM glucose treatment, however treatment with ASP and MET + ASP decreased this effect (1.27 and -1.37 fold, respectively).

Table1. Summary of CD36 gene expression in H9c2 cells treated with ASP

Symbol	Description	Name	RT2 Catalog	HG	ASP	MET+ASP
Cd36	CD36	FAT/CD36	PPM03796D	3.75	1.27	-1.37

Chapter 4: Aspalathin protects the heart against hyperglycemia-induced oxidative damage by up-regulating Nrf2 expression

Phiwayinkosi Dludla[a, b], Christo Muller[a], Elizabeth Joubert[c, d], Johan Louw[a], M. Faadiel Essop[e], Kwazi Gabuza[a], Samira Ghoor[a], Barbara Huisamen[a, b] and Rabia Johnson[a, *].

[a]Biomedical Research and Innovation Platform (BRIP), Medical Research Council (MRC), Tygerberg, South Africa.

[b]Division of Medical Physiology, Faculty of Health Sciences, Stellenbosch University, Tygerberg, South Africa.

[c]Post-Harvest and Wine Technology Division, Agricultural Research Council (ARC) Infruitec-Nietvoorbij, Stellenbosch, South Africa.

[d]Department of Food Science, Stellenbosch University, Stellenbosch, South Africa.

[e]Cardio-Metabolic Research Group (CMRG), Department of Physiological Sciences, Stellenbosch University, Stellenbosch, South Africa.

Submitted manuscript: Phytomedicine 2016; PHYMED-D-16-00621

My contribution:

Project conception and design

Performed experiments

Data analysis and interpretation

Wrote the manuscript

Abstract

Background and aim: Aspalathin, a *C*-glucosyl dihydrochalcone, can protect H9c2 cardiomyocytes against high glucose (HG)-induced shifts in myocardial substrate preference, oxidative stress and apoptosis. While the protective mechanism of aspalathin remains unknown, nuclear factor (erythroid-derived 2)-like 2 (*Nrf2*) has emerged as a crucial factor for intracellular responses against oxidative stress. Therefore, we hypothesized that aspalathin protects the myocardium against hyperglycemia-induced oxidative damage by up-regulating *Nrf2* expression in H9c2 cardiomyocytes and diabetic (db/db) mice.

Methods: H9c2 cardiomyocytes exposed to HG for 48 hours were treated with aspalathin (1 μM) for 6 hours. RT^2 Profiler PCR array was used to measure differential expression of genes associated with oxidative damage. *Nrf2* siRNA was employed to further evaluate our hypothesis and to gain novel mechanistic insights. We also evaluated our hypothesis in an *in vivo* context and here db/db mice and their non-diabetic (db/+) littermate controls were treated daily for 6 weeks with either a low (13 mg/kg) or high (130 mg/kg) aspalathin dose versus metformin (150 mg/kg dosage), a known antidiabetic agent. Cardiac fibrosis was assessed by histopathological examination and the degree of oxidative stress determined by qRT-PCR.

Results and discussion: Aspalathin protected H9c2 cardiomyocytes against HG-induced oxidative stress. However, *Nrf2* silencing abolished this protective response and exacerbated cardiomyocyte apoptosis. In parallel, daily aspalathin treatment of db/db mice (130 mg/kg dose for 6 weeks) reversed cardiac fibrosis and oxidative stress versus untreated, control hearts. The higher aspalathin dosage also resulted in the most effective response when compared to the lower aspalathin dosage and metformin. In addition, the higher aspalathin dose elicited enhanced cardiac *Nrf2* expression and its downstream antioxidant genes such as glutathione peroxidase 2, glutathione synthetase and Parkinson protein 7.

Conclusion: These results indicate that aspalathin maintains cellular homeostasis and protects the myocardium against hyperglycemia-induced stress through activation of *Nrf2* and its downstream target genes.

Keywords

Diabetes mellitus; cardiomyopathy; hyperglycemia; oxidative stress; antioxidants, aspalathin.

Introduction

The International Diabetes Federation (IDF) recently reported a rapid rise in cases of diabetes mellitus (DM) worldwide (IDF, 2015). The current number of individuals living with DM is estimated to be 415 million and this figure is expected to reach 642 million by the year 2040 (IDF, 2015). Diabetes is a major risk factor for the development of cardiovascular complications. Hyperglycemia, a hallmark of DM, is associated with rising cardiovascular deaths in the diabetic population (Inzucchi et al., 2015). Coronary artery disease (CAD) remains the causal factor linked to the increase of cardiovascular-related deaths in diabetic persons (Inzucchi et al., 2015). However, diabetic cardiomyopathy (DCM) is a frequent but commonly unrecognized pathology that exists in the absence of CAD or hypertension (Rubler et al., 1972). DCM is characterized by left ventricular (LV) dysfunction leading to decreased cardiac efficiency in diabetic individuals (Fonarow, 2005). Alterations in myocardial substrate preference and mitochondrial dysfunction are some of the metabolic perturbations implicated in the onset of LV dysfunction (Bayeva et al., 2013). Oxidative stress is another factor linked with cardiac structural and functional modifications observed in a diabetic heart (Thandavarayan et al., 2011). The mechanisms related to the development of oxidative injury are often multifactorial and may involve a cascade of events associated with various cell signaling pathways.

Humans are equipped with a defense system that controls free radical species and reduces oxidative stress. The nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is an emerging regulator of cellular resistance to oxidants. Nrf2 plays a crucial role in controlling basal and induced expression of an array of cytoprotective and antioxidant defense genes important for the regulation of physiological and pathophysiological outcomes in response to oxidative exposure (He et al., 2009). In cultured cardiomyocytes and endothelial cells, suppression of *Nrf2* expression results in oxidative stress-induced insulin resistance (Tan et al., 2011), while heart tissues isolated from *Nrf2* knockout mice display a marked increase in the production of reactive oxygen species (ROS) compared to wild type mice (He et al., 2009). Nrf2 preserves intracellular redox homeostasis by increasing the expression of antioxidant and detoxifying genes such as glutathione peroxidase 2 (*Gpx2*), superoxide dismutase (*SOD*) and NAD(P)H dehydrogenase [quinone] 1 (*Nqo1*) (Chen et al., 2013). Under conditions of oxidative stress, Nrf2 is activated by disassociating from its negative regulator Kelch-like ECH-associated protein 1 (*KEAP1*) and translocating to the nucleus, where it binds to the antioxidant response element (ARE), activating detoxifying enzymes and genes to inactivate the stressors and restore homeostasis (Bai et al., 2013).

Various phytochemicals from food substances, such as sulforaphane, derived from a major glucoslinolate of broccoli and quercetin, an aglycone, derived from flavonol glycosides, common to plant foods, can prevent oxidative injury by up-regulating *Nrf2* expression (Bai et al., 2013 and

Granado-Serrano et al., 2012). Several studies carried out in our laboratory demonstrated the beneficial effects of rooibos (*Aspalathus linearis*), a popular South African herbal tea and some of its major polyphenolic compounds in ameliorating metabolic complications associated with DM (Dludla et al., 2014, Mazibuko et al., 2015, Muller et al., 2012 and Sanderson et al., 2014,). Aspalathin (ASP), a *C*-glucosyl dihydrochalcone unique to rooibos, can improve glucose and lipid metabolism by modulating 5' adenosine monophosphate-activated protein kinase (AMPK) expression in 3T3-L1 adipocytes (Mazibuko et al., 2015). Similarly, in a recent study we demonstrated that ASP protects H9c2 cardiomyocytes against high glucose (HG)-induced oxidative damage by improving myocardial substrate metabolism via AMPK regulation (Johnson et al., 2016). While AMPK plays a noticeable role in regulating energy metabolism in cardiomyocytes, the control of hyperglycemia-induced oxidative stress may be additionally enhanced through other redox-related pathways such as Nrf2 (Zimmermann et al., 2015). Hence we tested the hypothesis that ASP can prevent hyperglycemia-induced oxidative injury by up-regulating the transcriptional expression of *Nrf2* by using an *in vitro* H9c2 cardiomyocyte model and a leptin receptor-deficient db/db mouse model.

Materials and methods

Reagents and kits

H9c2 rat derived cardiomyoblasts (ECACC No. 8809294) were purchased from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK), while ASP was synthesized according to Han et al., 2014 and obtained from High Force Research (ca. 98%, batch SZI-356-54) (Durham, UK). Hematoxylin, eosin, xylene, and formalin were obtained from Merck-Millipore (Billerica, MA, USA), halothane was from , Safeline Pharmaceuticals (Johannesburg, RSA), Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's phosphate-buffered saline, penicillin and streptomycin from Lonza (Verviers, Belgium), and fetal bovine serum and horse serum from Biochrom (Berlin, Germany). High Capacity Reverse Transcription Kit, RNAse free water, small interfering RNA (siNrf2) (AM16708), scrambled Nrf2 (scrRNA) (AM4615), Trizol reagent, Turbo DNase Kit, Lipofectamine RNAimax reagent, and all Taqman gene expression assays were supplied by ThermoFisher Scientific, Inc. (Waltham, MA, USA). RT^2 SYBR Green qPCR Master Mix, RT^2 Array First Strand Kit and RNeasy Mini Kit were obtained from Qiagen (Valencia, CA, USA). All other consumables and reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA), unless otherwise specified.

In vitro experiments on H9c2 cardiomyocytes

Cell culture

H9c2 cardiomyoblasts were cultured in supplemented DMEM (10% fetal bovine serum, 100 µg/mL penicillin and 100 µg/mL streptomycin) overnight under standard tissue culture conditions (37°C in humidified air and 5% CO_2). Cells were seeded in a 6-well plate at a seeding density of 2 X 10^4 cells/well. Confluent H9c2 cardiomyoblasts were differentiated into adult cardiomyocytes by substituting growth media with differentiation media consisting of DMEM supplemented with 10 nM all-trans-retinoic acid and 1% horse serum for 6 days (Karagiannis et al., 2010). On day seven, differentiated cells were exposed to 33 mM glucose for 48 h prior to treatment with ASP (1 µM) for an additional 6 h. Cells exposed to either 5.5 mM glucose or 33 mM glucose served as controls for normal glucose (NG) and HG, respectively. Cells exposed to 33 mM mannitol were used to rule out the effect of osmolarity (Johnson et al., 2016).

Preparation of ASP for cell culture treatment

A stock solution of ASP (22.1 mM), prepared in dimethyl sulfoxide (DMSO), was diluted using DMEM media to give a final solution of 1 µM. The toxic effect of DMSO at a concentration of 0.004% was tested and ruled out in all the experiments performed.

RNA isolation and purification

Total RNA was extracted using Trizol reagent, according to a previously described protocol (Johnson et al., 2016). RNA was purified using an RNeasy Mini Kit, while the Turbo DNase Kit was used to remove genomic DNA, as per manufacturer's instructions. RNA integrity was determined using an Agilent 2100 Bioanalyser (Agilent Technologies, Inc., Palo Alto, CA, USA), according to manufacturer's instructions.

RT^2-PCR array analysis

cDNA was synthesized from 2 µg RNA using the RT^2 First Strand Kit, according to manufacturer's instructions. Rat Oxidative Stress and Atherosclerosis RT^2 Profiler PCR Arrays were used (PARN-065ZA and PARN-065ZA) to analyze the differential expression of multiple genes. Briefly, cDNA was added to the RT^2 SYBR Green qPCR Master Mix and aliquotted onto each well of the 96-well RT^2 Profiler PCR Array plate. An ABI 7500 Instrument (ThermoFisher Scientific, Inc., Waltham, MA, USA) was used for mRNA quantification at the following cycling conditions: 50ºC for 1 min, 1 cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Analysis of PCR array data was done using a Microsoft Excel sheet with macros made available by the manufacturer

(http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). As controls, each array with 84 assay genes includes 5 housekeeping genes, as well as a reverse transcription efficiency and DNA contamination control. RNA from 3 biological replicates was pooled and repeated only once for each condition.

Real-time PCR to confirm oxidative stress and apoptosis markers

Quantitative RT-PCR was performed on an ABI 7500 Instrument. cDNA was synthesized from 1 µg of RNA using the High Capacity Reverse Transcription Kit, as per manufacturer's instructions. Thereafter, the PCR reaction mix was prepared by adding 5 µL Taqman universal PCR master mix, 0.5 µL Taqman gene expression assay [*in vitro* study (*Casp3*, *Gpx2*, *Nox4*, *Nrf2*, *Park 7*, *SOD2* and *UCP2*) *and in vivo* study (*Casp3*, *Gpx2*, *Gss*, *Nox4*, *Nrf2* and *Park 7*)], 1 µL of cDNA, and 3.5 µL RNAse free water to a final volume of 10 µL. The quantitative RT-PCR protocol was conducted as follows: 50°C for 1 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. Gene expression data were normalized to hypoxanthine-guanine phosphoribosyltransferase (*Hprt*).

Knockdown of Nrf2 using small interfering RNA

Nrf2-siRNA was done using a Lipofectamine RNAimax reagent, according to the manufacturer's instructions. Briefly, H9c2 cells at approximately 70% confluence were transfected with siNrf2 or scrRNA for 24 h, respectively. Thereafter, transfected cells were exposed to HG for 24 h before treatment with ASP for 6 h. The degree of Nrf2 knockdown after 24 h of transfection was confirmed by RT-PCR. Non-transfected cells, exposed to either 5.5 mM glucose or 33 mM glucose served as controls for NG and HG, respectively.

In vivo experiments using C57BL/KS mice

Animals

Male C57BL/KS leptin receptor-deficient (db/db) mice and nondiabetic heterozygote littermate (db/+) controls were obtained and housed at the Primate Unit and Delft Animal Centre (PUDAC) of the South African Medical Research Council (SAMRC). Animals were kept in a controlled environment with a 12 h light/dark cycle and a temperature range of 23-25ºC (relative humidity: ~50%). The mice received standard laboratory chow pellets (Afresh Vention, Cape Town, South Africa) *ad libitum* and had free

access to water. Ethical clearance for the use of animals in this study was granted by the SAMRC Ethics Committee for Research on Animals (ECRA no. 07/13), and the Stellenbosch University Ethics Committee (SU-ACUM13-00021).

Treatment of mice with ASP

Nine-week old diabetic (db/db) mice and their non-diabetic (db/+) littermate controls were treated daily for 6 weeks through oral gavage with either a low (13 mg/kg) or high (130 mg/kg) ASP dose and compared to metformin (MET) at a dose of 150 mg/kg. Dose selection was based on a previous study by Kawano et al., 2009. Treatment groups (n=6/group) included; i) db/+ untreated controls (db/+_UC), ii) db/db untreated controls (db/db_UC), iii) db/db MET (db/db_MET), iv) db/db ASP low dose (db/db_ASP_LD), v) and db/db ASP high dose (db/db_ASP_HD). ASP and MET were prepared fresh daily for the duration of the study by dissolving in distilled water, while animals belonging to the untreated control groups were given water only.

Heart tissue staining and left ventricular hypertrophic measurements

After the 6-week treatment period, mice were fasted for 4 h before being weighed and anesthetized with halothane. Animals received the anesthetic until no reaction could be recorded by pedal reflex before removal of the heart. The heart tissue was weighed and fixed in 10% formalin for a minimum of 16 h before it was processed using a Leica TP 1020 automated processor (Leica Biosystems, Buffalo Grove, IL, USA) and embedded in paraffin wax. Paraffin embedded tissue was cut into sections and attached to aminopropyltriethoxysilane coated glass slides. Tissues were then stained with H&E as previously described (Page et al., 2004). Stained sections were visualized using a Nikon Eclipse Ti inverted microscope (Tokyo, Japan). Micrographs for heart LV and median wall thickness were taken in non-overlapping fields of 1 mm^2 under 40x magnification and measurements were done using NIS Elements imaging software (Tokyo, Japan).

Measurement of fasting plasma glucose (FPG) concentrations

In mice fasted overnight for 16 h, FPG concentrations were measured by tail prick using a OneTouch® Select® handheld glucometer (LifeScan Inc., Milpitas, CA, USA), according to the manufacturer's instructions.

Oral glucose tolerance tests (OGTTs)

After the 6 weeks of treatment, OGTTs were performed. Briefly, after a 16 h fast, a glucose bolus of 2 g/kg was orally administered through gastric gavage. Plasma glucose levels were determined by tail prick at time intervals of 0, 30, 60, and 120 min.

Statistical analysis

Data were expressed as the mean ± SEM. Results for *in vitro* experiments were expressed as the mean of 3 independent biological experiments with each experiment containing at least 3 technical replicates, unless otherwise stated. For *in vivo* experiments, each treatment group contained 6 mice. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, USA). Comparisons between groups were performed using one-way multivariate ANOVA, followed by unpaired Student t-test, and a p-value of ≤ 0.05 was deemed as statistically significant.

Results

In vitro screening of ASP in H9c2 cardiomyocytes

ASP maintained cellular homeostasis in vitro

High glucose exposure decreased endogenous antioxidant capacity of the cells. RT^2-PCR analysis revealed that ASP post-treatment at a dose of 1 µM was able to enhance mRNA expression of a number of antioxidant genes and phase II detoxification enzymes. Results obtained showed that ASP increased the expression of catalase (*Cat*; 11.8 fold), *Gpx2* (15.8 fold), peroxiredoxin 1, 3, 4, and 6 (2.4, 3.0, 2.1, and 2.8 fold, respectively) *SOD1* and *SOD2* (2.1 and 1.2 fold) (Table 1). The increased expression of antioxidant genes correlated with the up-regulation of glutathione-associated and reducing genes, including glutamate-cysteine ligase catalytic subunit (*Gclc*; 6.9 fold), glutamate-cysteine ligase, modifier subunit (*Gclm*; 5.8 fold) and glutathione reductase (*Gsr*; 3.2 fold) (Table 1). Increased expression of thiol reducing genes and cytoprotective genes by ASP included sulfiredoxin 1 (*Srxn1*; 6.3 fold), thioredoxin 1 (*Txn1*; 2.0 fold), thioredoxin reductase 1 (*Txnrd1*; 13.7 fold), thioredoxin reductase 2 (*Txnrd2*; 1.0 fold), heme oxygenase 1 (*Hmox1*; 3.9 fold), *Nqo1* (11.4 fold), and uncoupling protein 2 (*UCP2*; 2.8 fold) (Table 1). Interestingly, the effect of ASP was not only restricted to activation of antioxidant-associated genes, but the anti-apoptotic properties of this flavonoid were also displayed as it was able to increase the expression of B-cell lymphoma 2 (*Bcl2*; 2.6 fold) and down-regulate that of Caspase 8 (*Casp8*; -1.3) (Table 1).

Table 1. Aspalathin treatment (1 μM) increased the expression of antioxidant genes and phase II cytoprotective enzymes in H9c2 cardiomyocytes pre-exposed to 33 mM glucose for 48 h.

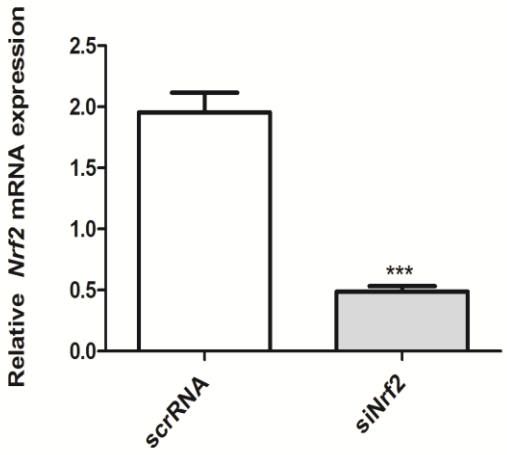
GENE	Fold regulation after high glucose exposure	Fold regulation after post-treatment with aspalathin
Antioxidant genes		
Catalase (*Cat*)	-1.63	11.80
Glutathione peroxidase 2 (*Gpx2*)	-1.20	15.86
Peroxiredoxin 1 (*Prdx1*)	-1.33	2.49
Peroxiredoxin 3 (*Prdx3*)	-1.21	3.09
Peroxiredoxin 4 (*Prdx4*)	-1.72	2.14
Peroxiredoxin 6 (*Prdx6*)	-2.65	2.89
Superoxide dismutase 1 (*SOD1*)	-1.25	2.18
Superoxide dismutase 2 (*SOD2*)	-1.17	1.22
Glutathione synthesis genes		
Glutamate-cysteine ligase catalytic subunit (*Gclc*)	-1.84	6.96
Glutamate-cysteine ligase, modifier subunit (*Gclm*)	-1.40	5.89
Glutathione reductase (*Gsr*)	-1.50	3.29
Reducing agent genes		
Sulfiredoxin 1 (*Srxn1*)	-3.43	6.34
Thioredoxin 1 (*Txn1*)	-1.08	2.05
Thioredoxin Reductase 1 (*Txnrd1*)	-2.88	13.70
Thioredoxin Reductase 2 (*Txnrd2*)	-3.25	1.08
Cytoprotective genes		
Heme oxygenase 1 (*Hmox1*)	-2.77	3.98
NAD(P)H dehydrogenase (quinone 1) (*Nqo1*)	-4.57	11.45
Uncoupling protein 2 (*UCP2*)	-3.95	2.83
Uncoupling protein 3 (*UCP3*)	-2.01	-1.61
Apoptotic genes		
B-cell lymphoma 2 (*Bcl2*)	-1.8	2.6
Caspase 8 (*Casp8*)	3.9	-1.3

ASP regulated expression of Nrf2 and its downstream target genes in vitro

To investigate if ASP activates Nrf2, siRNA was employed. H9c2 cardiomyocytes exposed to siNrf2 for 24 h resulted in a significantly reduced *Nrf2* mRNA expression (0.5 ± 0.05, $p \leq 0.0001$) when compared to the scrRNA control (2.0 ± 0.2) (Fig. 1A). Furthermore, to define the role ASP plays in the activation of Nrf2, HG-exposed H9c2 cells were treated with either siNrf2 or scrRNA. HG exposure decreased the expression of *Nrf2* when compared to the NG control (0.4 ± 0.12 compared to 1.0 ± 0.13, $p \leq 0.001$) (Fig. 1B). ASP treatment ameliorated this effect compared to the HG control (0.8 ±

0.04 compared to 0.4 ± 0.12, p = 0.01) (Fig. 1B). As anticipated, cells treated with siNfr2 + ASP failed to increase *Nrf2* mRNA expression when compared to the scrRNA control (Fig. 1B).

A



B

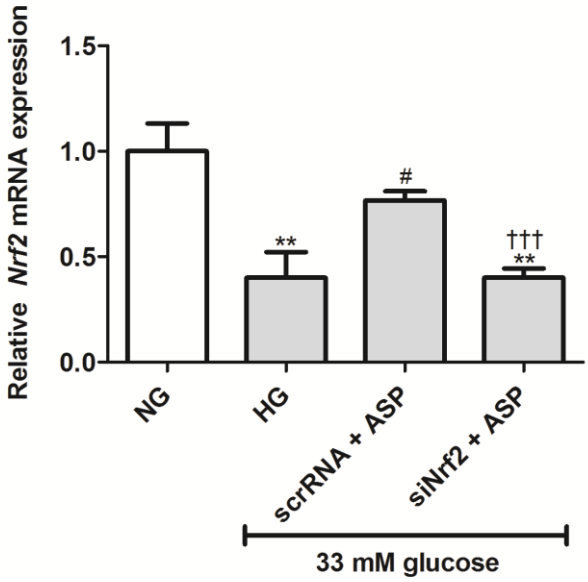
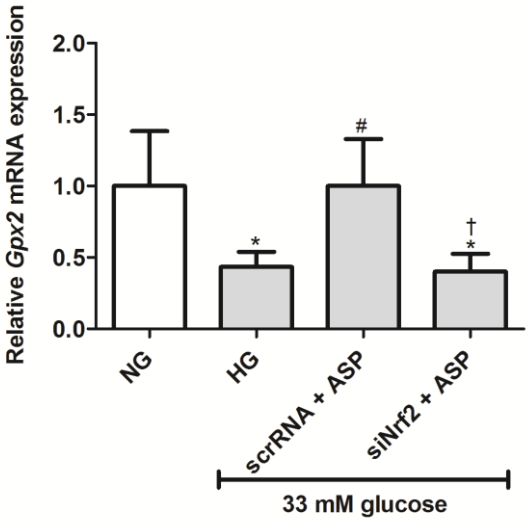
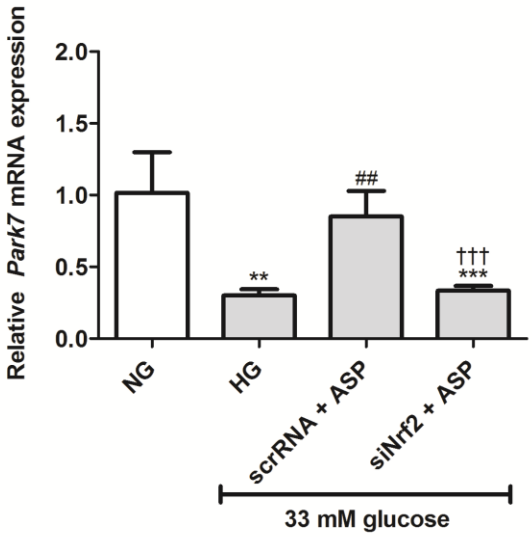


Figure 1. Aspalathin (ASP) increased the expression of *Nrf2* in H9c2 cardiomyocytes. (A) The degree of *Nrf2* knockdown in H9c2 cardiomyocytes after transfection with either small interfering RNA (siNrf2) or scrambled RNA (scrRNA) for 24 h. (B) H9c2 cardiomyocytes transfected with siNrf2 or scrRNA for 24 h were exposed to 33 mM glucose (HG) for 24 h, followed by treatment with 1 μM ASP for 6 h. Results are expressed as the mean ± SEM of 3 independent biological experiments relative to the normal glucose (NG) control, each done in triplicate. **p ≤ 0.001, ***p ≤ 0.0001 vs. NG, #p ≤ 0.05 vs. HG, †††p ≤ 0.0001 vs. scrRNA + ASP.

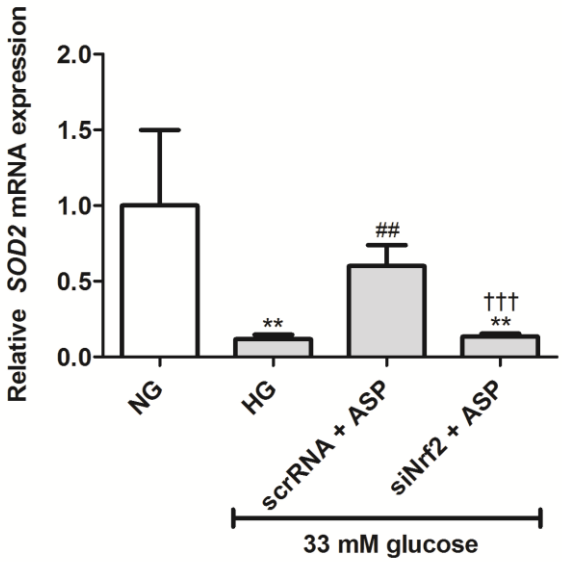
A



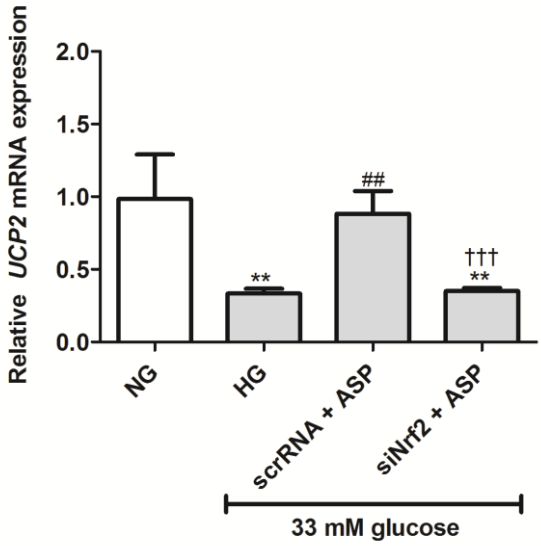
B



C



D



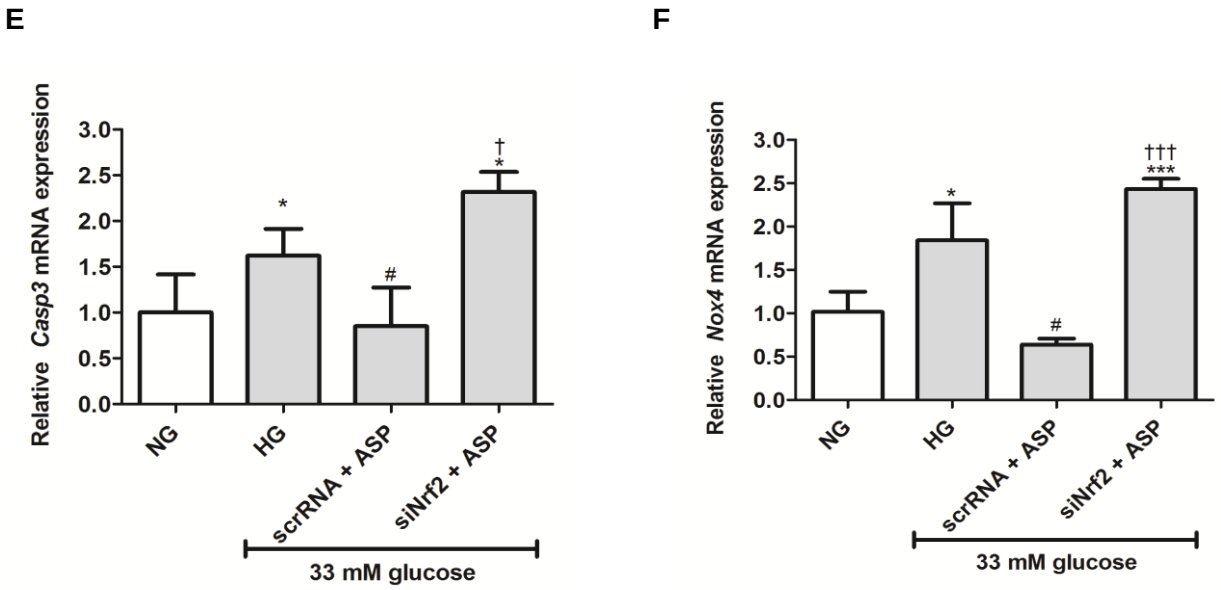


Figure 2. siNrf2 inhibited the effect of aspalathin (ASP) *in vitro*. The relative mRNA expression of antioxidant genes, including (A) *Gpx2*, (B) *Park7*, (C) *SOD2*, and (D) *UCP2*, and oxidative damage associated genes (E) *Casp3* and (F) *Nox4* after transfection of cells with either small interfering RNA (siNrf2) or scrambled RNA (scrRNA) for 24 h followed by treatment with 1 μM ASP for 6 h. Results are expressed as the mean ± SEM of 3 independent biological experiments relative to the normal glucose (NG) control, each done in triplicate. *$p \leq 0.05$, **$p \leq 0.001$, ***$p \leq 0.0001$ vs. NG, $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.001$ vs. HG, $^{\dagger}p \leq 0.05$, $^{\dagger\dagger\dagger}p \leq 0.0001$ vs. scrRNA + ASP.

The effects of siNrf2 on ASP treatment on mRNA expression of antioxidant and oxidative genes. These results show that HG exposure significantly reduced the mRNA expression of oxidative stress protective genes, *Gpx2* (0.4 ± 0.04, $p = 0.01$), *Park7* (0.3 ± 0.04, $p \leq 0.001$), *SOD2* (0.1 ± 0.03, $p \leq 0.001$), and *UCP2* (0.3 ± 0.33, $p \leq 0.001$) when compared to the NG controls (1.0 ± 0.38, 1.0 ± 0.28, 1.0 ± 0.49, and 1.0 ± 0.30, respectively) (Fig. 2A-D). Treatment with ASP at 1 μM for 6 h was able to prevent this by up-regulating the expression of *Gpx2* (1.0 ± 0.32, $p \leq 0.02$), *Park7* (0.85 ± 0.17, $p \leq 0.001$), *SOD2* (0.6 ± 0.13, $p \leq 0.001$), and *UCP2* (0.9 ± 0.15, $p \leq 0.001$) when compared to the HG controls (Fig. 2A-D). Interestingly, the effect on cells exposed to siNrf2 + ASP was down-regulated when compared to scrRNA + ASP and similar to when compared to HG (Fig. 2A-D).

Moreover, the mRNA expression of genes associated with oxidative damage (*Casp3* and *Nox4*) was up-regulated in HG exposed cells (1.6 ± 0.43, $p = 0.04$; and 1.8 ± 0.4, $p = 0.03$) when compared to the NG controls (1.0 ± 0.61, and 1.0 ± 0.20, respectively) (Fig. 2E and F). ASP treatment was able to abolish this effect for both *Casp3* and *Nox4* (0.9 ± 0.42, $p = 0.04$; and 0.6 ± 0.06, $p = 0.01$, respectively) when compared to the HG controls (Fig. 2E and F). However, treatment with siNrf2 +

ASP failed to reduce expression of both these genes when compared to cells subjected to scrRNA + ASP (Fig. 2E and F). The effect was similar to the HG control.

In vivo confirmation studies on db/db mice

Effect of ASP on FPG and OGTT

Db/db mice and their db/+ controls were treated daily with either a low (13 mg/kg) or a high (130 mg/kg) dose of ASP, as well as MET at 150 mg/kg for 6 weeks. The db/db_UC group displayed elevated FPG levels from week 9 to week 14 (21.0 ± 1.73, 15.5 ± 1.29, 23.7 ± 1.15, 24.7 ± 2.91, 19.7 ± 1.46, and 22.3 ± 2.85, respectively) when compared to the untreated db/+_UC control group (6.1 ± 0.30, 5.9 ± 0.50, 6.2 ± 0.38, 5.3 ± 0.44, 6.0 ± 0.20, and 4.3 ± 0.49, respectively) (Fig. 3A). Although both the low and high dose ASP treatments did not reduce the increased FPG levels in db/db mice, MET was able to reduce the raised FPG levels from week 12 to week 14 (17.3 ± 1.92, 17.0 ± 1.67, and 17.4 ± 2.56, respectively), though not significantly (Fig. 3A).

Assessment of OGTTs after administration of a 2 g/kg glucose bolus in mice

Results obtained showed a marked increase in FPG levels 30 min after glucose administration, and this was significantly different when the db/db_UC (33.8 ± 0.40, $p \leq 0.0001$) was compared to the db/+_UC control (9.6 ± 0.50) (Fig. 3B). High dose ASP treatment at 60 and 120 min (30.8 ± 1.53 and 28.6 ± 1.63) was comparable to MET (30.2 ± 1.61 and 28.8 ± 2.22) in reducing the increased FPG concentrations when compared to the untreated diabetic controls, though not significantly (Fig. 3B). Treatment with a low dose of ASP was, however, ineffective.

ASP prevented diabetes-induced LV mass enlargement in vivo

Heart hypertrophy associated measurements, including heart weight (HW)/body weight (BW) ratio, LV wall and interventricular septum thickness, and occurrence of cardiac muscle remodeling were assessed after mice were treated with ASP for 6 weeks. Results showed that the db/db_UC group presented with increased HW/BW ratio (0.0061 ± 0.00005, $p \leq 0.0001$), LV wall and interventricular septum thickness measurements ((1588 ± 42, $p \leq 0.0001$ and 1556 ± 70, $p \leq 0.001$, respectively) occurring concurrent to enhanced cardiac remodeling in comparison to the db/+_UC control group (HW/BW ratio: 0.0048 ± 0.0001, LV wall: 1151 ± 9; and interventricular septum: 1120 ± 29) (Fig. 4A-C). Interestingly, treatment with a high dose ASP was able to ameliorate these dysregulations

associated with cardiac hypertrophy, including prevention of changes in HW/BW ratio (0.0043 ± 0.0003, $p \leq 0.001$), LV wall (1278 ± 96, $p = 0.01$) and interventricular septum thickness (1160 ± 42, $p \leq 0.001$) (Fig. 4A-C). Neither MET or a low dose ASP had any effect on the altered cardiac muscle fibers or ventricular wall thickness.

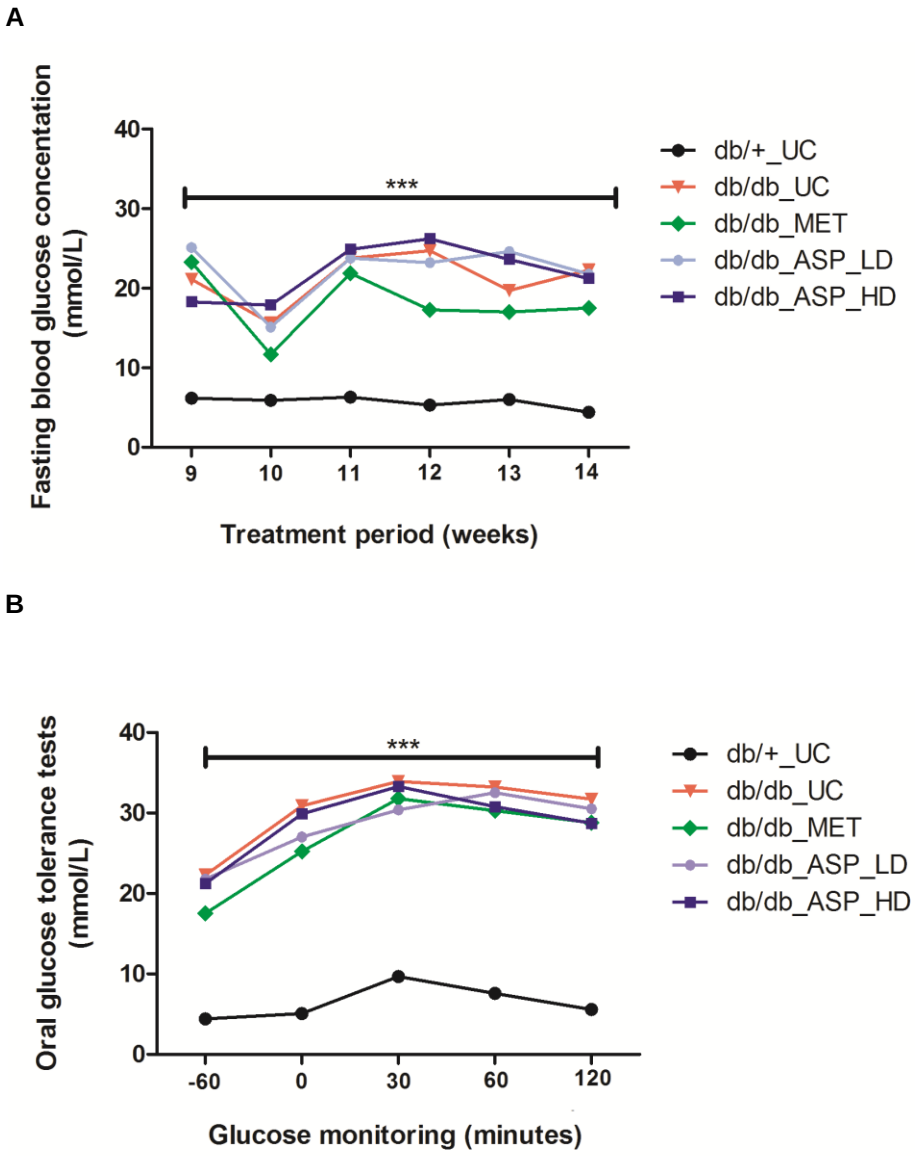


Figure 3. Aspalathin (ASP) treatment did not reduce increasing fasting plasma glucose (FPG) levels, however a high dose of 130 mg/kg of ASP was able to improve oral glucose tolerance in diabetic mice after 60 and 120 minutes from baseline. (A) FPG levels (B) Oral glucose tolerance tests (-60: baseline reading). Results are expressed as the mean ± SEM and each treatment group contained 6 mice. ***$p \leq 0.0001$ diabetic control mice (db/db_UC) vs. untreated nondiabetic mice (db/+_UC). db/db_MET: diabetic mice treated with metformin (150 mg/kg); db/db_ASP_LD: diabetic mice treated with aspalathin low dose (13 mg/kg); db/db_ASP_HD: diabetic mice treated with aspalathin high dose (130 mg/kg).

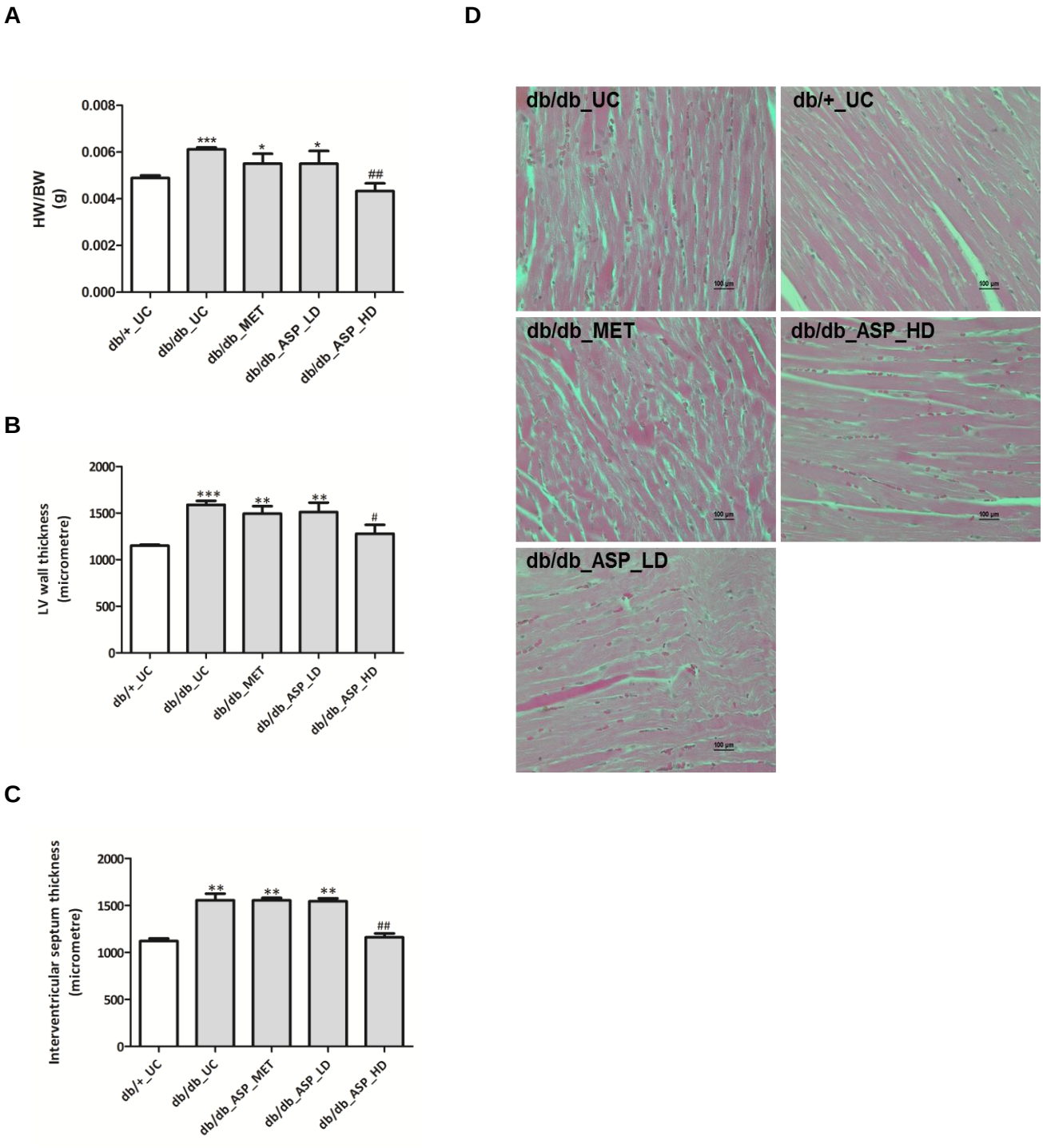


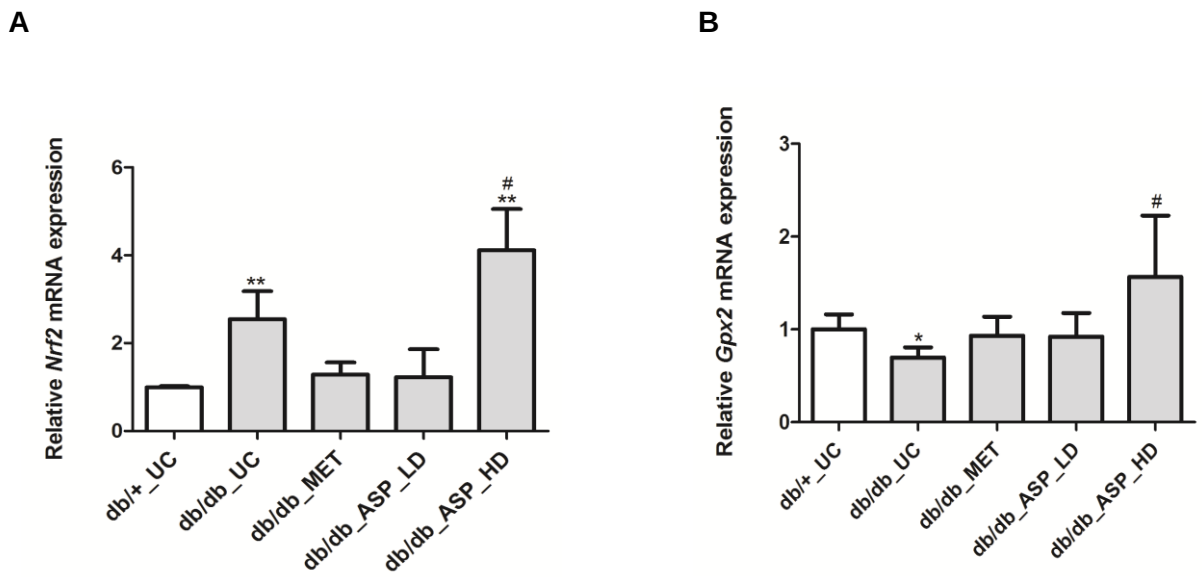
Figure 4. Aspalathin (ASP) prevented diabetes-induced cardiac muscle structure modifications associated with hypertrophy in diabetic mice. (A) Heart weight (HW) to body weight (BW) ratio, (B) left ventricular (LV) wall thickness, (C) interventricular septum thickness, and (D) photomicrographs of cardiac remodeling. Results are expressed as the mean ± SEM and each treatment group contained 6 mice. **p ≤ 0.001, ***p ≤ 0.0001 versus untreated nondiabetic mice (db/+_UC), #p ≤ 0.05, ##p < 0.001 versus untreated diabetic mice (db/db_UC). db/db_MET: diabetic mice treated with metformin (150 mg/kg); db/db_ASP_LD: diabetic mice treated with aspalathin low dose (13 mg/kg); db/db_ASP_HD: diabetic mice treated with aspalathin high dose (130 mg/kg).

ASP regulated the expression of Nrf2 and its target genes in vivo

In support of the *in vitro* data on H9c2 cardiomyocytes, we assessed the expression of *Nrf2* and its associated downstream cytoprotective genes *in vivo*, and results showed a significant enhancement of *Nrf2* mRNA expression in the db/db_UC group (2.54 ± 0.63, p ≤ 0.001) in comparison to the db/+_UC control group (0.99 ± 1.49) (Fig. 5A). Although MET and low dose ASP treatments were not effective, the high dose ASP (4.1 ± 0.93, p = 0.01) significantly increased mRNA expression of *Nrf2* when compared to both db/db_UC and db/+_UC groups (Fig. 5A).

Further analyzing mRNA expression of genes activated by *Nrf2*, it was noted that the expression of *Gpx2* (0.69 ± 0.11, p = 0.04) and *Gss* (0.66 ± 0.05, p = 0.03) was reduced in the db/db_UC group when compared to the db/+_UC group (1.0 ± 0.15, 1.0 ± 0.11, respectively) (Fig. 5B and C). The expression of *Park7* was not significantly affected (0.94 ± 0.06). Treatment with MET and the low dose ASP did not have any effect, while the high dose ASP significantly up-regulated expression of *Gpx2* (1.56 ± 0.66, p = 0.04), *Gss* (1.0 ± 0.15, p = 0.03), and *Park7* (1.35 ± 0.06, p ≤ 0.001) when compared to db/db_UC group (Fig. 5B-D).

Expression of oxidative damage-associated genes (*Casp3* and *Nox4*) was significantly enhanced in the db/db_UC group (1.53 ± 0.24, p = 0.02; and 1.8 ± 0.37, p = 0.01) when compared to the db/+_UC group (1.0 ± 0.06; and 1.0 ± 0.07, respectively) (Fig. 5E and F). No effect was observed for MET or low dose ASP treatments, while the high dose ASP treatment was able to down-regulate the expression of both *Casp3* (0.97 ± 0.03, p = 0.01) and *Nox4* (0.96 ± 0.10, p = 0.01) when compared to db/db_UC group (Fig. 5E and F).



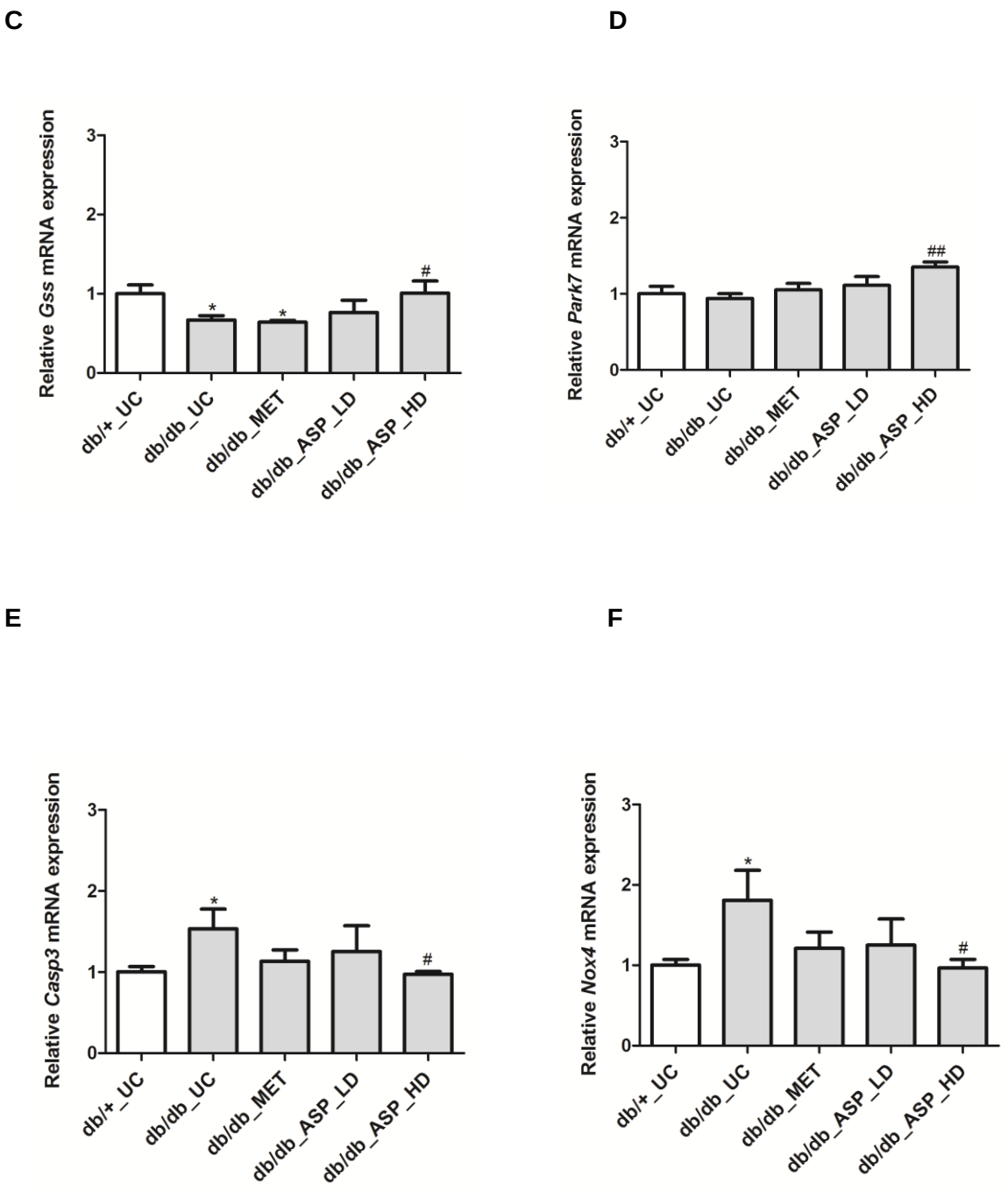


Figure 5. Aspalathin (ASP) prevented oxidative damage by increasing the expression of *Nrf2* and its down-stream target genes in diabetic mice. The relative mRNA expression of (A) *Nrf2* and associated antioxidant genes, including (B) *Gpx2*, (C) *Gss*, and (D) *Park7*, and oxidative damage associated genes (E) *Casp3* and (F) *Nox4* after the 6 week treatment period with metformin, and a low or high dose ASP. Results are expressed as the mean ± SEM and each treatment group contained 6 mice. *p ≤ 0.05, **p ≤ 0.001 versus untreated nondiabetic mice (db/+_UC), #p ≤ 0.05, ##p ≤ 0.001 versus untreated diabetic mice (db/db_UC).

Discussion

Evidence of the antidiabetic properties of ASP, through the use of cell culture and animal models, has been increasingly reported (Kawano et al., 2009, Muller et al., 2012 and Son et al., 2013). In addition to its robust antioxidant properties (Snijman et al., 2009 and von Gadow et al., 1997), this *C*-glucosyl dihydrochalcone protects the myocardium against HG-induced oxidative stress and subsequent metabolic dysregulations (Johnson et al., 2016). Metabolic dysregulations associated with oxidative stress are complex, but two cellular signaling processes, namely AMPK and Nrf2 play crucial roles and are essential in maintaining cellular homeostasis (Bai et al., 2013 and Johnson et al., 2016). Previously, we showed that ASP was able to prevent shifts in substrate preference by decreasing AMPK phosphorylation resulting in reduced oxidative damage (Johnson et al., 2013). In addition, various studies have shown that activation of *Nrf2* decreases oxidative stress by increasing intracellular antioxidant defences (Chen et al., 2013 and He et al., 2009). Hence, this study aimed to investigate if ASP can improve the bioenergetics of the cell through enhancing *Nrf2* expression.

During accelerated free radical damage that occurs mainly through ROS the cell adapts by dissociating *Nrf2* from *KEAP1*. This dissociation results in the translocation of *Nrf2* into the nucleus and binding to the ARE that activate the endogenous antioxidant defense system (Dinkova-Kostova et al., 2015). Accumulative evidence has shown that certain dietary phytochemicals are able to activate *Nrf2* and thereby increase its cytoprotective response (Chen et al., 2013, Cheng et al., 2015 and Granado-Serrano et al., 2012). Presence of a catechol group may be a pivotal structural feature of flavonoids that contributes to *Nrf2* activation as demonstrated for shogaol derivatives (Zhu et al., 2016). Furthermore, a dihydrochalcone glycoside, neohesperidin dihydrochalcone, has recently been shown to prevent carbon tetrachloride-induced hepatic injury by increasing the expression of *Hmox1* and *Nqo1* through activation of *Nrf2* in HepG2 cells (Su et al., 2015). In agreement to others (Bai et al., 2013, Chen et al., 2013 and Su et al., 2015), our study showed that activation of *Nrf2* promoted cell survival by increasing the expression of an array of antioxidant genes and enzymes. We showed that a 6 h treatment with 1 µM ASP protected cardiomyocytes exposed to HG against oxidative damage by up-regulating the expression of antioxidant genes and cytoprotective enzymes such as *Nqo1* and *Hmox1*. In addition, by using RNA interference we showed that activation of *Nrf2* by ASP is crucial for the antioxidant response against hyperglycemia-induced oxidative injury in H9c2 cardiomyocytes. While it was expected that *Nrf2*-knockdown may exacerbate susceptibility of such cells to oxidative damage, ASP treatment showed an even higher capacity to enhance *Nrf2* expression and its downstream target genes and thus protect against HG-induced stress.

Following *in vitro* experiments on H9c2 cardiomyocytes, it was important to confirm such findings in an *in vivo* system. For this we made use of db/db mice, which represent a type 2 diabetic mouse model.

These mice display a whole-body phenotype of DM and are frequently used in the pharmacological study of compounds including ASP (Kawano et al., 2009 and Zheng et al., 2015). Moreover, they have been reported to spontaneously become obese and develop hyperglycemia from the age of eight weeks (Ritchie et al., 2008). At the age of ten to eighteen weeks these mice display signs of cardiac interstitial fibrosis and hypertrophy as a result of increased LV wall thickness, occurring simultaneous to enhanced generation of ROS (Belke et al., 2000 and Ritchie et al., 2008). Accelerated ROS production induces deleterious modifications to DNA, proteins and lipids concomitant to the pathophysiological state observed within the diabetic heart (Mapanga and Essop, 2016). To support this, we showed that, in addition to impaired OGTT, db/db mice presented elevated FPG levels, an increased HW/BW ratio that occurred concomitant to a thickened left ventricle and interstitial fibrosis when compared to the db/+ control group. An enlarged heart LV wall is a conspicuous sign of DCM and is identified as an early defect associated with chronic hyperglycemia in diabetic patients (Fonarow, 2005 and Rubler et al., 1972). The latter state has been associated with elevated oxidative stress and various studies have reported on the effect of oxidative stress on vascular remodeling and how antioxidant therapy prevents progressive remodeling and improves cardiac function (Bai et al., 2013, Dludla et al., 2014 and Johnson et al., 2016). Correspondingly, in this study we observed that morphological derangements within db/db mouse hearts occurred concurrently to accelerated oxidative stress and a modest increase in *Nrf2* expression. It has been previously reported that the expression of *Nrf2* is initially elevated in response to stress, and then diminishes at a later stage leading to adverse complications such as apoptosis (Bai et al., 2013 and Dinkova-Kostova et al., 2015).

Despite inability protective effects displayed by ASP treatment on reducing elevated FPG concentrations, this polyphenol at a dose of 130 mg/kg was able to ameliorate oxidative damage via *Nrf2* activation, resulting in attenuated cardiac hypertrophy and remodeling. However, an unexpected finding was the inability of ASP to attenuate increased FPG concentrations, since it has been previously reported to have blood glucose lowering effects (Kawano et al., 2009 and Muller et al., 2012). A similar finding was observed where treatment of streptozotocin (STZ)-induced diabetic mice with sulforaphane failed to reduce FPG, but was able to protect the heart against oxidative stress associated with heart tissue remodeling (Bai et al., 2013). The inability of ASP to reduce blood glucose can also be due to other factors, such as the duration of treatment, as our group has previously demonstrated that ASP reduces blood glucose concentrations in STZ-induced diabetic rats over a period of 6 h (Muller et al., 2012). In contrast, while current antidiabetic agents including MET effectively reduce FPG concentrations, there is still an increasing number of cardiovascular-related deaths in diabetic patients (Inzucchi et al., 2015). Correspondingly, this study showed that even though MET-treated mice presented with moderately reduced FPG levels, it was unable to prevent

oxidative stress associated myocardial structural modifications. Therefore, the use of potential nutraceutical agents such as ASP, especially in combination with current antidiabetic agents to alleviate oxidative injury and protect diabetic individuals at risk of myocardial infarction are of foremost importance.

In summary, consistent with previous findings, ASP displays strong properties to improve myocardial ultrastructure by preventing HG-associated complications. However, dose selection remains important to induce desired efficacy, since the low dose of 13 mg/kg did not show any effect in protecting the heart when compared to the high dose of 130 mg/kg in our study. In addition to its modulatory effects of AMPK, the ability of ASP to regulate *Nrf2* may be a key factor to protect against DCM. However, further studies to assess the impact of ASP on cardiac functional parameters in diabetic rodents are required to confirm this exciting proposal.

Acknowledgments

This research was funded in part by the National Research Foundation (NRF) Thuthuka Programme Grant 87836 and the South Africa Medical Research Council's Biomedical Research and Innovation Platform. The grantholders acknowledge that opinions, findings and conclusions or recommendations expressed in any publication generated by the NRF supported research are those of the authors, and that the NRF accepts no liability whatsoever in this regard. Funding from Stellenbosch University and Ernst Ethel Erikson trust are also acknowledged. We also like to thank Mr. Desmond Linden for technical support with animal work.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

References

Bai, Y., Cui, W., Xin, Y., Miao, X., Barati, M.T., Zhang, C., Chen, Q., Tan, Y., Cui, T., Zheng, Y., Cai L., 2013. Prevention by sulforaphane of diabetic cardiomyopathy is associated with up-regulation of Nrf2 expression and transcription activation. J. Mol. Cell Cardiol. 57, 82–95.

Bayeva, M., Sawicki, K.T., Ardehali, H., 2013. Taking diabetes to heart-deregulation of myocardial lipid metabolism in diabetic cardiomyopathy. J. Am. Heart Assoc. 2, e000433.

Belke, D.D., Larsen, T.S., Gibbs, E.M., Severson, D.L., 2000. Altered metabolism causes cardiac dysfunction in perfused hearts from diabetic (db/db) mice. Am. J. Physiol. Endocrinol. Metab. 279, E1104–1113.

Chen, X., Liu, J., Chen, S.Y., 2013. Sulforaphane protects against ethanol-induced oxidative stress and apoptosis in neural crest cells by the induction of Nrf2-mediated antioxidant response. Br. J. Pharmacol. 169, 437–448.

Cheng, A.S., Cheng, Y.H., Lee, C.Y., Chung, C.Y., Chang, W.C., 2015. Resveratrol protects against methylglyoxal-induced hyperglycemia and pancreatic damage *in vivo*. Nutrients 7, 2850–2865.

Dinkova-Kostova, A.T., Abramov, A.Y., 2015. The emerging role of Nrf2 in mitochondrial function. Free Radic. Biol .Med. 88, 179–188.

Dludla, P.V., Muller, C.J., Louw, J., Joubert, E., Salie, R., Opoku, A.R., Johnson, R., 2014. The cardioprotective effect of an aqueous extract of fermented rooibos (*Aspalathus linearis*) on cultured cardiomyocytes derived from diabetic rats. Phytomedicine 21, 595–601.

Fonarow, G.C., 2005. An approach to heart failure and diabetes mellitus. Am. J. Cardiol. 96, 47E-52.

Granado-Serrano, A.B., Martín, M.A., Bravo, L., Goya, L., Ramos S., 2012. Quercetin modulates Nrf2 and glutathione-related defenses in HepG2 cells: Involvement of p38. Chem. Biol. Interact. 195, 154–164.

Han, Z., Achilonu, M.C., Kendrekar, P.S., Joubert, E., Ferreira, D., Bonnet, S.L., van der Westhuizen, J.H., 2014. Concise and scalable synthesis of aspalathin, a powerful plasma sugar-lowering natural product. J. Nat. Prod. 77, 583–588.

He, X., Kan, H., Cai, L., Ma, Q., 2009. Nrf2 is critical in defense against high glucose-induced oxidative damage in cardiomyocytes. J. Mol. Cell. Cardiol. 46, 47–58.

International Diabetes Federation (IDF). IDF Diabetes Atlas 7th edition. idf.org [Internet]. Available from: http://www.diabetesatlas.org/. Accessed 12 December 2015.

Inzucchi, S.E., Bergenstal, R.M., Buse, J.B., Diamant, M., Ferrannini, E., Nauck, M., Peters, A.L., Tsapas, A., Wender, R., Matthews, D.R., 2015. Management of hyperglycemia in type 2 diabetes, 2015: A patient-centered approach. Update to a position statement of the American Diabetes Association and the European Association for the Study of Diabetes. Diabetes Care 38, 140–149.

Johnson, R., Dludla, P., Joubert, E., February, F., Mazibuko, S., Ghoor, S., Muller, C., Louw, J., 2016. Aspalathin, a dihydrochalcone C-glucoside, protects H9c2 cardiomyocytes against high glucose-induced shifts in substrate preference and apoptosis. Mol. Nutr. Food Res. 60, 922–934.

Karagiannis, T.C., Lin, A.J., Ververis, K., Chang, L., Tang, M.M., Okabe, J., El-Osta A., 2010. Trichostatin A accentuates doxorubicin-induced hypertrophy in cardiac myocytes. Aging 2, 659–568.

Kawano, A., Nakamura, H., Hata, S., Minakawa, M., Miura, Y., Yagasaki, K., 2009. Hypoglycemic effect of aspalathin, a rooibos tea component from *Aspalathus linearis*, in type 2 diabetic model db/db mice. Phytomedicine 16, 437–443.

Mapanga, R.F., Essop, M.F., 2016. Damaging effects of hyperglycemia on cardiovascular function: spotlight on glucose metabolic pathways. Am. J. Physiol. Heart Circ. Physiol. 310, H153–173.

Mazibuko, S.E., Joubert, E., Johnson, R., Louw, J., Opoku, A.R., Muller, C.J., 2015. Aspalathin improves glucose and lipid metabolism in 3T3-L1 adipocytes exposed to palmitate. Mol. Nutr. Food Res. 59, 2199–2208.

Muller, C.J., Joubert, E., de Beer D., Sanderson, M., Malherbe, C.J., Fey, S.J., Louw J., 2012. Acute assessment of an aspalathin-enriched green rooibos (*Aspalathus linearis*) extract with hypoglycemic potential. Phytomedicine 20, 32–39.

Page, B. J, du Toit, D.F., Muller, C.J., Mattysen, J., Lyners, R., Arends, E., 2004. Autogenous transplantation of a duct ligated pancreas: a functional and histological study. JOP. 5, 71–80.

Ritchie, R., Julius, T., Buxton, K., Xu, Q., Kiriazis, H., McMullen, J., Forbes, J., Du, X., Kaye, D., Tesch, G., 2008. Type 2 diabetic cardiomyopathy in db/db mice is associated with progressive cardiac fibrosis, cardiomyocyte hypertrophy and oxidative stress. Heart Lung Circ. 17, S231.

Rubler, S., Dlugash, J., Yuceoglu, Y.Z., Kumral, T., Branwood, A. W., Grishman, A., 1972. New type of cardiomyopathy associated with diabetic glomerulosclerosis. Am. J. Cardiol. 30, 595–602.

Sanderson, M., Mazibuko, S.E., Joubert, E., de Beer, D., Johnson, R., Pheiffer, C., Louw, J., Muller, C.J., 2014. Effects of fermented rooibos (*Aspalathus linearis*) on adipocyte differentiation. Phytomedicine 21, 109–117.

Snijman, P.W., Joubert, E., Ferreira, D., Li, X.C., Ding, Y., Green, I.R., Gelderblom, W.C., 2009. Antioxidant activity of the dihydrochalcones aspalathin and nothofagin and their corresponding flavones in relation to other rooibos (*Aspalathus linearis*) flavonoids, epigallocatechin gallate, and trolox. J. Agric. Food Chem. 57, 6678–6684.

Son, M.J., Minakawa, M., Miura, Y., Yagasaki, K., 2013. Aspalathin improves hyperglycemia and glucose intolerance in obese diabetic ob/ob mice. Eur. J. Nutr. 52, 1607–1619.

Su, C., Xia, X., Shi, Q., Song, X., Fu, J., Xiao, C., Chen, H., Lu, B., Sun, Z., Wu, S., Yang, S., Li, X., Ye, X., Song, E., Song, Y., 2015. Neohesperidin dihydrochalcone versus CCl_4-induced hepatic injury through different mechanisms: the implication of free radical scavenging and Nrf2 activation. J. Agric. Food Chem. 63, 5468–5475.

Tan, Y., Ichikawa, T., Li, J., Si, Q., Yang, H., Chen, X., Goldblatt, C.S., Meyer, C.J., Li, X., Cai, L., Cui, T., 2011. Diabetic downregulation of Nrf2 activity via ERK contributes to oxidative stress-induced insulin resistance in cardiac cells *in vitro* and *in vivo*. Diabetes 60, 625–633.

Thandavarayan, R.A., Giridharan, V.V., Sari, F.R., Arumugam, S., Veeraveedu, P.T., Pandian, G.N., Palaniyandi, S.S., Ma, M., Suzuki, K., Gurusamy, N., Watanabe, K., 2011. Depletion of 14-3-3 protein exacerbates cardiac oxidative stress, inflammation and remodeling process via modulation of MAPK/NF-κB signaling pathways after streptozotocin-induced diabetes mellitus. Cell Physiol. Biochem. 28, 911–922.

von Gadow, A., Joubert, E., Hansmann, C. F., 1997. Comparison of the antioxidant activity of aspalathin with that of other plant phenols of rooibos tea (*Aspalathus linearis*), α-tocopherol, BHT, and BHA. J. Agric. Food Chem. 45, 632–638.

Zheng, A., Li, H., Xu, J., Cao, K., Li, H., Pu, W., Yang, Z., Peng, Y., Long, J., Liu, J., Feng, Z., 2015. Hydroxytyrosol improves mitochondrial function and reduces oxidative stress in the brain of db/db mice: role of AMP-activated protein kinase activation. Br. J. Nutr. 113, 1667–1676.

Zhu, Y., Wang, P., Zhao, Y., Yang, C., Clark, A., Leung, T., Chen, X., Sang, S., 2016. Synthesis, evaluation, and metabolism of novel [6]-shogaol derivatives as potent Nrf2 activators. Free Radic. Biol. Med. 95, 243–254.

Zimmermann, K., Baldinger, J., Mayerhofer, B., Atanasov, A. G., Dirsch, V. M., Heiss E. H., 2015. Activated AMPK boosts the Nrf2/HO-1 signaling axis-A role for the unfolded protein response. Free Radic. Biol. Med. 88, 417–426.

Chapter 5: Transcription profile unveils the cardioprotective effect of aspalathin against lipid toxicity

Rabia Johnson[1,2], Phiwayinkosi V. Dludla[1,2], Elizabeth Joubert[3,4], Christo J.F. Muller[1], Barbara Huisamen[1,2] M. Faadiel Essop[5] and Johan Louw[1]

[1]Biomedical Research and Innovation Platform, Medical Research Council, Cape Town, South Africa.

[2]Division of Medical Physiology, Faculty of Health Sciences, Stellenbosch University, Tygerberg, South Africa.

[3]Post-Harvest and Wine Technology Division, Agricultural Research Council (ARC) Infruitec-Nietvoorbij, Stellenbosch, South Africa.

[4]Department of Food Science, Stellenbosch University, Stellenbosch, South Africa.

[5]Cardio-Metabolic Research Group (CMRG), Department of Physiological Sciences, Stellenbosch University, Stellenbosch, South Africa.

Submitted manuscript: Plos One, PONE-D-16-34079.

My contribution:

Planning of manuscript outline

Performed experiments

Data analysis and interpretation

Edited the manuscript

Abstract

In the pathological condition of type 2 diabetes mellitus, myocardial fatty acid uptake exceeds the rate of β-oxidation leading to lipid toxicity. Increased lipid storage within the diabetic heart correlates with the development of diabetic cardiomyopathy (DCM). Aspalathin, a *C*-glucosyl dihydrochalcone, can protect cardiomyocytes against hyperglycemia-induced shifts in substrate preference and subsequent apoptosis. However, the precise gene regulatory network associated with the protective effect of aspalathin remains to be fully elucidated. Db/db mice and their non-diabetic (db/+) littermate controls were treated daily for 6 weeks with either a low (13 mg/kg) or high (130 mg/kg) aspalathin dose. Results obtained showed that aspalathin significantly lowered plasma lipid levels. To provide insight into the molecular mechanisms associated with the improved plasma lipid profile, an *in vitro* H9c2 cardiomyocytes model was employed. H9c2 cardiomyocytes were exposed to high glucose for 48 hours before treatment with or without aspalathin (1 μM) for 6 hours. Thereafter, RNA was extracted and RT^2 PCR Profiler Arrays were used to profile the expression of 336 genes involved in cardiac metabolism. Results obtained showed that among the 336 genes assessed, 57 were differentially regulated by more than 2-fold in the high glucose or high glucose + aspalathin treated groups. STRING analysis revealed lipid metabolism and molecular transport to be the top two biological processes that were altered after high glucose treatment, followed by inflammation and apoptosis. Interestingly, aspalathin was able to modulate key regulators associated with lipid metabolism (*Acads*, *Adipoq*, *Apob/e*, *Cd36*, *Cpt1*, *Pparγ*, *Srebf1/2*, *Scd1* and *Vldlr)*, insulin resistance (*Igf1*, *Akt1*, *Pde3* and *Map2k1*), inflammation (*Il3*, *Il6*, *Jak2*, *Lepr*, *Socs3*, and *Tnf13*) and apoptosis (*Bcl2, Bbc3* and *Chuk*). Collectively, our results indicate that aspalathin was able to reverse lipid toxicity that can lead to subsequent inflammation and myocardial death.

Keywords

Diabetes mellitus; cardiomyopathy; hyperglycemia; lipid toxicity; inflammation; apoptosis.

Introduction

In the last decade, there has been much interest in the use of plant-derived polyphenols as nutraceuticals to protect against the development of metabolic diseases [1, 2]. *Aspalathus linearis* (commonly known as rooibos) is a rich source of plant polyphenols with known health promoting properties. In addition to reversing ischemia/reperfusion injury in the isolated perfused rat heart [3], rooibos has been shown to improve lipid profiles as well as oxidative stress in diabetics at risk of developing cardiovascular disease [4, 5]. Furthermore, literature has indicated that polyphenols specific to rooibos may present strong ameliorative properties against diabetes mellitus and its associated complications [6-8]. Of note, aspalathin, a *C*-glucosyl dihydrochalcone found in rooibos has displayed an even greater potency to prevent diabetes-induced cardiovascular complications [9-11]. For example, a fermented rooibos extract containing abundant levels of aspalathin protected cardiomyocytes isolated from diabetic rats against experimentally-induced oxidative stress and ischemia [9]. Moreover, recent data from our laboratory demonstrated that aspalathin protects cultured cardiomyocytes exposed to chronic hyperglycemia from shifts in substrate preference and subsequent cell apoptosis [10].

Enhanced intracellular lipid accumulation that results in impaired cardiac substrate metabolism is consistently reported in diabetic hearts [10, 12]. For example, obese mice display elevated intramyocardial lipid accumulation concomitant to suppressed glucose oxidation that was linked to contractile dysfunction [12, 13]. Increased lipid storage in the hearts of diabetic patients correlates with the development of diabetic cardiomyopathy (DCM) [14]. DCM is a disease of the myocardium that is characterized by a measurable deterioration in the heart function. In animal models of DCM, a significant increase in FFA transporter expression alters intracellular signalling pathways and promotes apoptotic cell death [15, 16]. Therefore, targeting myocardial metabolism as a therapeutic intervention to protect a diabetic heart is a powerful therapeutic strategy. This study made use of RT^2 PCR Profiler Arrays to unravel the transcriptional mechanisms that underlie the cardioprotective effect of aspalathin against the development of high glucose-induced cardiomyopathy.

Materials and methods

Reagents and kits

Pure aspalathin (ca. 98%, batch SZI-356-54) was synthesized according to a previously published protocol [17] and was obtained from High Force Research (Durham, UK) while H9c2 rat derived cardiomyoblasts were purchased from the European Collection of Cell Cultures (ECACC No. 8809294; Wiltshire, UK). Radioimmunoassay Kit was from Linco Research (St. Charles, USA),

halothane from Safeline Pharmaceuticals (Johannesburg, RSA), Dulbecco's Modified Eagle's Medium, penicillin, and streptomycin were obtained from Lonza (Verviers, Belgium), and fetal bovine serum was from Biochrom (Berlin, Germany). RNeasy Mini Kit, RT^2 First Strand Kit, RT^2 Profiler PCR Arrays and RT^2 SYBR Green qPCR Master Mix were from Qiagen (Valencia, USA), while Trizol reagent and Turbo DNase Kit were from ThermoFisher Scientific (Waltham, USA). All other consumables and reagents were purchased from Sigma-Aldrich Corp. (St. Louis, USA), unless otherwise specified.

Animal work

Male C57BL/KS leptin receptor deficient (db/db) mice were obtained from and housed at the Primate Unit and Delft Animal Centre (PUDAC) of the South African Medical Research Council (SAMRC) in a controlled environment with a 12 h light/dark cycle in a temperature range of 23-25ºC (relative humidity: ~50%). The mice received standard laboratory chow pellets (Afresh Vention) *ad libitum* and had free access to drinking water. The study was performed in agreement with the principles and guidelines of the SAMRC (ECRA no. 07/13) as well as Stellenbosch University Ethics Committee (SU-ACUM13-00021).

Animal treatment with aspalathin

After acclimatizing for one week, nine-week old db/db mice together with their nondiabetic lean littermates (db/+) (n=6 per group), caged in individual cages, were randomly divided in five groups. Mice were treated daily for six weeks through oral gavage with either a low (13 mg/kg) or high (130 mg/kg) aspalathin dose and compared to metformin (150 mg/kg). Treatment groups included: i) untreated nondiabetic controls (db/+_UC), ii) untreated diabetic controls (db/db_UC), iii) diabetic treated with metformin (db/db_MET), iv) diabetic treated with low dose aspalathin (db/db_ASP_LD) and v) diabetic treated with high dose aspalathin (db/db_ASP_HD). Aspalathin and metformin were dissolved in water before orally administered at the same time every day for six weeks, while untreated animals were given water in place of treatment.

Homeostasis model assessment: insulin resistance (HOMA-IR) determination

Mice were fasted overnight before plasma glucose levels were measured. This was done on a weekly basis by tail pricks using OneTouch Select handheld glucometer (LifeScan, Milpitas, USA). Fasting plasma insulin was detected using Radioimmunoassay Kit, as per manufacturer's instruction. HOMA-

IR was calculated using fasting plasma glucose levels and insulin values, according to an already described method [18].

Organ weight measurements and lipid profiles

After the 6-week treatment period, mice were fasted for 4 h and body weight (BW) measurements documented before being anesthetized with halothane. Animals received the anesthetic until no reaction could be recorded by pedal reflex before an average of 1 mL of blood was collected from the abdominal vena cava and organs were removed from each mouse. Heart, liver muscle, and fats (gonadal and intraperitoneal) were removed and weighed to determine heart weight (HW), liver weight (LW) and fat weight (FW), respectively, using an Adventurer analytical balance (OHAUS Corp., Parsippany, USA). For lipid profiles, blood was collected and centrifuged at 4000 g at 4ºC for 15 min before serum was removed and stored at -80ºC. Serum was sent to PathCare Medical Diagnostic Laboratories to screen for total cholesterol, triglycerides, low density lipoprotein (LDL), and high-density lipoprotein (HDL).

Cell culture and treatment

Embryonic ventricular rat heart-derived H9c2 cardiomyoblasts were cultured in supplemented Dulbecco's Modified Eagle's Medium (10% foetal bovine serum, 100 U/ml of penicillin, 100 mg/ml of streptomycin) for 48 h under standard tissue culture conditions (37°C in humidified air and 5% CO_2). Confluent cells (60-80%) were seeded at a density of 2 x 10^4 in 6-well multi-plates. Thereafter, H9c2 cardiomyoblasts were differentiated into adult cardiomyocytes for 7 days using retinoic acid according to previously described method [19]. On day 8, H9c2 cells were exposed to 33 mM glucose for 48 h prior to treatment with aspalathin (1 µM) for an additional 6 h. Cells exposed to 5.5 mM glucose were used as a normal glucose control [10].

RT^2-PCR array analysis

Total RNA was extracted using Trizol reagent, according to a previously described protocol [10]. RNA was purified using an RNeasy Mini Kit, while the Turbo DNase Kit was used to remove genomic DNA, as per manufacturer's instructions. RNA integrity was determined using an Agilent 2100 Bioanalyser (Agilent Technologies, Palo Alto, USA) and cDNA was synthesized from two microgram RNA using the RT^2 First Strand Kit, according to relevant manufacturer's instructions. Rat Atherosclerosis (PARN-038ZA-2), Cytokine (PARN-011ZA-2), Fatty Acid Metabolism (PARN-007Z), and Insulin Resistance

(PARN-156ZA-2) RT^2 Profiler PCR Arrays were used for mRNA profiling studies using ABI7500 (ThermoFisher Scientific, Waltham, USA). Analysis of PCR array data was done according to manufacturer's instructions, using a Microsoft Excel sheet with macros (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). Each array contained five house keeping genes (*Actb*, *B2m*, *Gapdh*, *Gusb* and *Hsp90ab1*) against which the sample data was normalized. Using the online software, the transcript level of each candidate gene was quantified by using the ΔΔCT method. Ant Ct value > 35 was not included in the analysis and considered as negative. To determine the gene interaction and network analysis, Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; http://string-db.org/) database was used to represent information of known and anticipated gene interactions [20, 21].

Statistical analysis

Data were expressed as the mean ± SEM of three independent biological experiments with each experiment containing at least three technical replicas, unless stated otherwise. For *in vivo* experiments, each treatment group contained six mice. Statistical analysis was calculated using GraphPad Prism software (La Jolla, USA). Comparisons between groups were performed using one-way multivariate ANOVA followed by using unpaired Student t-test. P-value of ≤ 0.05 was deemed as statistically significant.

Results and discussion

In vivo effect of aspalathin

Hyperglycemia, lipidemia and insulin resistance are the key risk factors associated with the development of cardiovascular complications. Chronic hyperglycemia results in shifts in substrate preference that precede the onset of heart failure. Aspalathin, a *C*-linked dihydrochalcone glucoside, plays an important role in maintaining cellular homeostasis by preventing shifts in substrate preference through reducing excess uptake of free fatty acids that may result in lipid toxicity in H9c2 cardiomyocytes [10].

Effect of aspalathin on biometrical measurements: Results from this study demonstrated that leptin receptor deficient db/db_UC mice displayed significantly increased water and food intake when compare to the db/+_UC littermate control group (Table 1). Concomitantly, an increase in BW, FW, HW and LW was observed. The results obtained were expected as leptin deficient db/db mice have

high circulating leptin levels which are actively associated with increased BW, FW, food intake, HOMA-IR and decreased adiponectin levels in type 2 diabetic individuals [22-24]. Interestingly, chronic treatment (daily for 6 weeks) with a high dose of aspalathin reduced all biometrical measurements except for food intake when compared to the db/db_UC mice. Of interest, aspalathin treatment at a high dose was more effective than either metformin or a low dose of aspalathin treatment (Table 1).

Table 1. The effect of aspalathin on body weight, fat weight, heart weight, liver weights, food intake and water intake.

Weight (g)	db/+_UC	db/db_UC	db/db_MET	db/db_ASP_LD	db/db_ASP_HD
BW	27.1 ± 0.33	37.9 ± 0.82***	38.0 ± 0.61***	37.7 ± 0.74***	35.0 ± 0.99***$^{\#}$
FW	0.93 ± 0.08	2.6 ± 0.12***	2.9 ± 0.17***	2.9 ± 0.19***	1.9 ± 0.15***$^{\#\#}$
HW	0.1 ± 0.01	0.2 ± 0.02**	0.2 ± 0.02	0.2 ± 0.03	0.1 ± 0.01$^{\#}$
LW	1.2 ± 0.08	2.5 ± 0.14***	2.5 ± 0.09***	2.2 ± 0.09***$^{\#}$	1.9 ± 0.15**$^{\#}$
FI	24.9 ± 0.67	45.68 ± 1.87***	41.12 ± 2.20***	44.63 ± 1.50***	40.95 ± 1.59***
WI	57.3 ± 4.73	213.9 ± 6.49***	172.1 ± 7.41***$^{\#\#}$	188.6 ± 3.98***$^{\#}$	181.8 ± 9.86***$^{\#}$

Results are the mean ± SEM, with each treatment group containing six mice. *p < 0.05, **p < 0.001, ***p < 0.0001 versus untreated nondiabetic mice (db/+_UC); $^{\#}$p < 0.05, $^{\#\#}$p < 0.001 versus untreated diabetic mice (db/db_UC). **BW:** body weight, **db/db_ASP_LD:** diabetic mice treated with low dose aspalathin (13 mg/kg), **db/db_ASP_HD:** diabetic mice treated with high dose aspalathin (130 mg/kg), **db/db_MET:** diabetic mice treated with metformin (150 mg/kg), **FI:** food intake, **FW:** fat weight, **HW:** heart weight, **LW:** liver weight, **WI:** water intake.

Effect of aspalathin on lipid profiles: Increased morbidity observed in type 2 diabetes individuals is primarily associated with heart failure with lipid toxicity being the key pathological mechanism underlying the condition [5, 12, 14]. To investigate if aspalathin can improve lipoprotein clearance, a total lipid profile was performed on mice treated with and without aspalathin (Table 2). Interestingly, we observed that the db/db_UC mice had increased HDL levels with associated elevated triglycerides,

total cholesterol, LDL and no change in HOMA-IR when compared to the db/+_UC group (Table 2). Aspalathin treatment at a high dose was able to ablate this response and the effect was better than either the low dose aspalathin or the metformin treated groups. However, similar to metformin, aspalathin treatment was unable to change HDL cholesterol (Table 2). An unexpected finding was the increased HDL levels on untreated db/db mice, while metformin or aspalathin failed to have any effect. However, it had been suggested that obese mice display a marked catabolic defect for the major HDL apolipoprotein A-1 (*Apoa1*) [13].

Table 2. The effect of aspalathin on blood lipid profiles and HOMA-IR.

	db/+_UC	db/db_UC	db/db_MET	db/db_ASP_LD	db/db_ASP_HD
Total cholesterol (mmol/L)	2.4 ± 0.09	3.5 ± 0.18***	2.9 ± 0.18*$^{\#}$	3.2 ± 0.18**	2.7 ± 0.24$^{\#}$
LDL (mmol/L)	0.1 ± 0.06	0.4 ± 0.07*	0.2 ± 0.04$^{\#}$	0.2 ± 0.05	0.1 ± 0.04$^{\#\#}$
HDL (mmol/L)	1.7 ± 0.06	2.5 ± 0.10***	2.2 ± 0.13**	2.3 ± 0.13**	2.3 ± 0.14**
Triglycerides (mmol/L)	0.9 ± 0.04	3.2 ± 0.38***	2.3 ± 0.46*	2.6 ± 0.19***	2.3 ± 0.28**
HOMA-IR	0.1 ± 0.01	0.6 ± 0.28	0.3 ± 0.05	0.4 ± 0.07	0.2 ± 0.03

Results are the mean ± SEM, with each treatment group containing six mice. *p < 0.05, **p < 0.001, ***p < 0.0001 versus untreated nondiabetic mice (db/+_UC); $^{\#}$p < 0.05, $^{\#\#}$p < 0.001 versus untreated diabetic mice (db/db_UC). **db/db_ASP_LD:** diabetic mice treated with low dose aspalathin (13 mg/kg), **db/db_ASP_HD:** diabetic mice treated with high dose aspalathin (130 mg/kg), **db/db_MET:** diabetic mice treated with metformin (150 mg/kg), **HDL:** high density lipoprotein, **HOMA-IR:** homeostasis model assessment: insulin resistance, **LDL:** low density lipoprotein.

In vitro effect of aspalathin

To confirm our *in vivo* findings and to decipher the molecular mechanism used by aspalathin to improve hyperglycemia-associated lipid toxicity in the heart, gene expression profiling was employed using H9c2 cardiomyocytes exposed to high glucose. The expression profiles of 336 genes were assessed, 57 genes (17%) were differentially expressed and of these, 45 and 12 genes (79% and

21%) were hyper-expressed in the high glucose and aspalathin treated groups, respectively (Table 3). STRING data analysis confirmed that aspalathin treatment largely improved the expression of genes involved in metabolic processes, identifying fatty acid and lipid metabolism as the top two regulatory processes.

In vitro effect of aspalathin on fatty acid and lipid metabolism

Data analysis identified 26 of the 57 (46%) differentially expressed genes to be involved in fatty acid and lipid metabolism (Table 3). STRING network analysis identified three clusters encoding interactive nodes representing genes associated with fatty acid/lipid transport, lipid metabolism and fatty acid metabolism (confidence score, 0.7) (Fig. 1). Lipids are the large and diverse group of naturally occurring organic compounds that are essential for energy storage while FFAs are a sub-group of lipids that are commonly derived from triglycerides. FFAs are the preferred substrate for energy production within cardiomyocytes, contributing up to 70% of ATP when compared to other substrates such as glucose and lactate.

Increased β-oxidation: Enhanced FFA uptake and lipid storage are causal factors known to precede the development of diabetic heart failure [14, 25]. In this study, we demonstrated that high glucose increased the expression of fatty acid transporters including; cluster of differentiation 36 (*Cd36*; 3.7-fold), fatty acid-binding proteins 3 (*Fabp3*; 2.7-fold), solute carrier family 25, member 30 (*Slc25a30*; 2.6-fold) and solute carrier family 27, member 1, 3 and 5 (*Slc27a*1, 3 and 5) by 1.0, 6.3-fold and 2.0-fold, respectively (Fig. 1 and Table 3). This increased fatty acid transport was concomitant to raised expression levels of genes associated with β-oxidation; including carnitine palmitoyltransferase 1b (*Cpt1b*; 3.3-fold), acyl-CoA thioesterase 2 (*Acot2*, 2.8-fold), acyl-CoA oxidase 2 (*Acox2*, 2.8-fold) as well as lysophospholipase 1 (*Lypla1*; 3.5-fold) (Fig. 1 and Table 3). Additionally, we observed that high glucose exposure upregulated mRNA expression of stearoyl-CoA desaturase 1 (*Scd1*, 5.5-fold), an enzyme crucial for the synthesis and storage of fatty acids. However, aspalathin treatment supressed this effect, which was associated with reduced FFA uptake and oxidation (Fig. 1 and Table 3). This result was in agreement with our previous findings [10, 26], where we showed that aspalathin and phenyl acid-2-*O*-β-D-glucoside prevented loss of metabolic flexibility by preventing shifts in myocardial substrate preference, from an almost compete reliance on fatty acid oxidation towards increased glucose utilization.

Increased supply of long-chain fatty acids (LCFAs): Chronic hyperglycemia has been associated with increased supply of circulating FFAs in cardiomyocytes [10, 26]. When this enhanced supply of FFAs exceed the rate of β-oxidation, myocardial triglyceride accumulation occurs, leading to lipotoxicity [14, 15]. The latter can subsequently lead to left ventricular dysfunction, a major characteristic of DCM. In a study done by Drosatos et al. [25], they showed that mice with cardiac-specific overexpression of LCFA acyl-CoA synthetase (*Acsl*) genes developed lipid toxicity and diastolic dysfunction, directly implicating LCFAs in the development of cardiac fibrosis and subsequent myocardial remodeling [25]. In our dataset, STRING analysis identified a network of genes with nodes linking long-chain fatty acyl-CoA synthetase enzymes (Fig. 1). These data confirm previous findings [25], where high glucose exposure resulted in increased mRNA expression of acyl-CoA synthetase long-chain family member 4 and 6 (*Acls4*, 5.6-fold and *Acls6*, 2.4-fold) as well as acyl-CoA synthetase medium-chain family member 3 and 4 (*Acsm3*, 14.2-fold and *Acsm4*, 6.1-fold) (Fig. 1 and Table 3). Results obtained showed that aspalathin treatment was able to attenuate this effect.

Altered lipid metabolism and increased cholesterol flux: Scientific evidence has shown that excessive cardiac lipid accumulation may lead to fibrosis and reduced ventricular compliance, contributing to the development of cardiac dysfunction [10-13]. Lipid toxicity in the myocardium has been associated with the transcriptional factor sterol regulatory element-binding protein 1/2 (*Srebf1/2*) and the transcriptional coactivator peroxisome proliferator-activated receptor-gamma (*PPARγ*) [25, 27]. *Srebf1/2* and *PPARγ* are important switches that regulate lipid accumulation and lipotoxicity. In a study done by Marfella et al. [27] on biopsies from diabetic patients with left ventricular dysfunction, they found that increased cardiac lipid deposits were concomitant with enhanced mRNA expression of *Srebf1/2*, *PPARγ* as well as genes associated with accelerated β-oxidation. Notably, in this study, elevated levels of circulating FFAs were observed after high glucose exposure. This was in parallel to enhanced mRNA expression of *Srebf1* and 2 (3.4 and 2.0-fold, respectively) as well as *PPARγ* (8.3-fold) (Fig. 1 and Table 3). Increased expression of *Srebf1* is further linked to the development of an artherogenic apolipoprotein profile, a characteristic of cardiac hypertrophy [29, 30]. The main function of apolipoproteins is to transport triglycerides and cholesterol in the blood [30]. *Apoa1* is the major protein component of HDL. HDL promotes efflux of cholesterol, phospholipids, and other lipophilic molecules from cells by an active process mediated by a cell-membrane transporter, ATP-binding cassette transporter (*Abca1*) [28, 30]. By contrast, apolipoprotein B (*Apob*) is the main apolipoprotein of chylomicrons and LDL and its elevated levels may cause heart failure [28, 30]. In this study, we observed an increased mRNA expression of *Apob* (7.7-fold), apolipoprotein E (*Apoe*; 4.4-fold) and very low density lipoprotein (*Vldlr*; 2.0-fold) that was decreased after aspalathin treatment (Fig.1). Similar to increased HDL cholesterol in db/db mice, *Apoa1* and its transporter *Abca1* were increased

by 4.2-fold and 2.0-fold, respectively, after high glucose exposure. Aspalathin treatment reversed this effect. Of interest, increased *Apob* has been associated with increased expression of *Cd36*, the receptor for oxidized LDL. Furthermore, insulin sensitivity and improved lipotoxicity have also been linked to adiponectin (*Adipoq*) [29]. *Adipoq* encodes the circulating protein adiponectin and is expressed primarily in the adipose tissue [31], but can also be found in vascular tissue [22, 32]. In this study, high glucose exposure resulted in the reduced expression of *Adipoq* (-6.0-fold), while aspalathin was able to reverse this effect.

Our results support the work of others that increased cardiac lipid toxicity as a result of chronic hyperglycemia may lead to heart failure [30, 32]. Aspalathin treatment was able to reverse lipid toxicity by modulating key regulatory genes, such as *Adipoq* and *Sreb1/2,* that are involved in metabolic, hormonal and energy processes. *Adipoq* plays an important role in controlling lipid metabolism by negative regulating *Srebf1/2* [29]. Thus, by upregulating *Adipoq*, aspalathin improved insulin sensitivity and suppressed LDL by reducing *Srebf1/2* (Fig. 1). We predict that aspalathin can prevent lipid accumulation and subsequent death due to cardiac failure.

In vitro effect of aspalathin on the development of insulin resistance

It is well-known that increased LCFAs result in the intramyocardial accumulation of diacylglycerol that activates protein kinase C (theta isoform), leading to the inhibition of insulin receptor substrate 1 (Irs1) and development of insulin resistance [10-12]. The latter can have a profound effect on cardiac performance, as the heart is an insulin-responsive organ and hyperglycemic-induced insulin resistance decreases cardiac performance. Our data analysis revealed that 14 (25%) of the 57 differentially expressed genes were associated with the development of hyperglycemic-induced insulin resistance (Table 3). Network mapping of the differential expressed genes identified two major interconnecting clusters (genes associated with protein kinase activity and development of insulin resistance) with serine/threonine-protein kinase homolog 1 (*Akt1*) and mitogen activated protein kinase (Mapk) being the major nodes of connection (confidence score, 0.7) (Fig. 2).

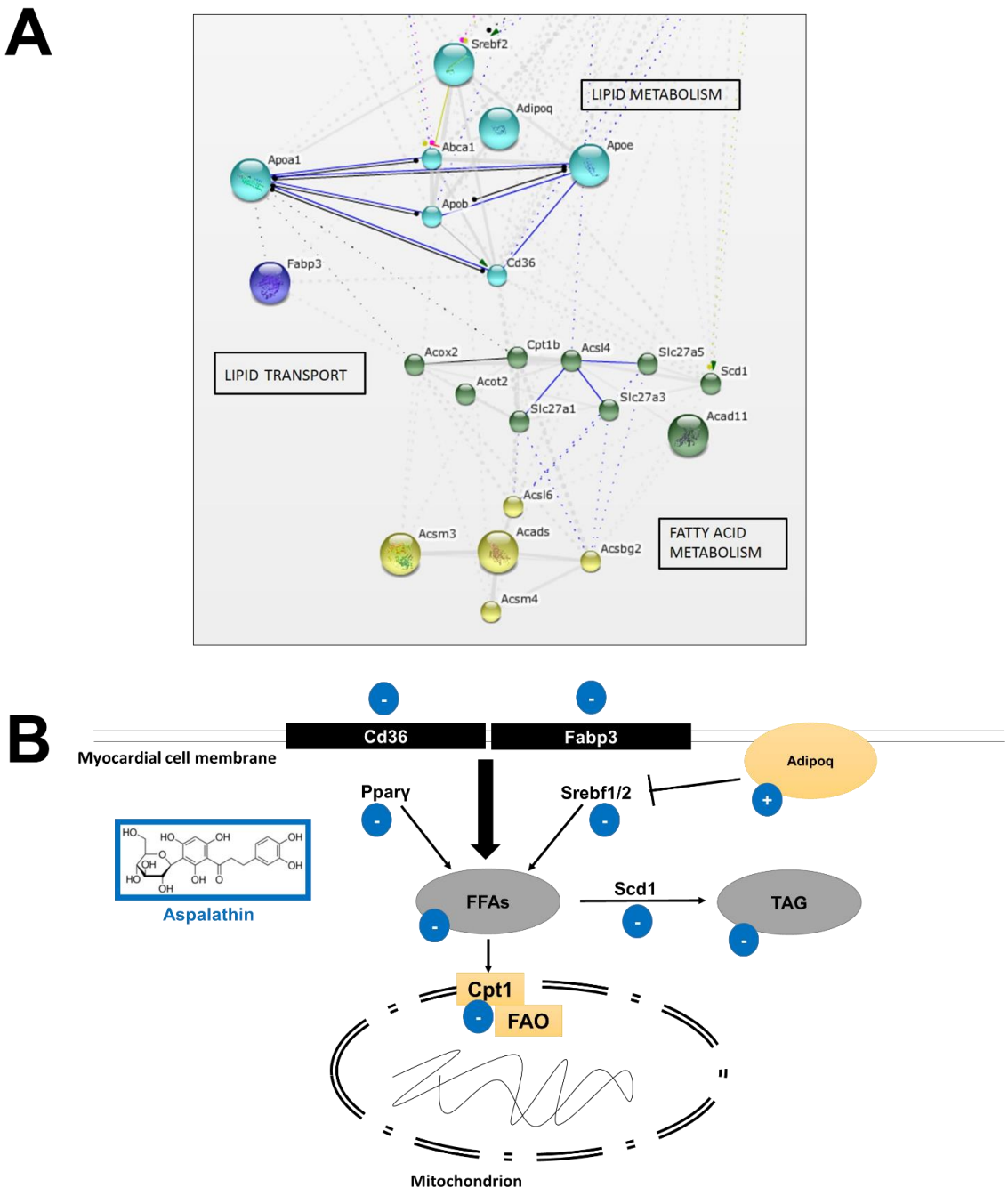


Figure 1. Aspalathin prevented high glucose-induced impaired cardiac substrate metabolism by reducing the uptake and oxidation of free fatty acids. (A) Search Tool for the Retrieval of Interacting Genes (STRING) database confirmed a strong interaction between genes associated with lipid toxicity. (B) Representative diagram of the proposed protective mechanism of aspalathin against increased lipid accumulation and oxidation. **Cd36:** cluster of differentiation 36; **Cpt1:** carnitine palmitoyltransferase 1; **FFAs:** free fatty acids; **Fabp3:** fatty acid binding protein 3; **FAO:** fatty acid oxidation; **Pparɣ:** peroxisome proliferator activated receptor gamma; **Scd1:** stearoyl-Coenzyme A desaturase 1; **Srebf1/2:** Sterol regulatory element binding transcription factor 1/2; **TAG:** triacylglycerides.

Insulin signalling and effect on myocardium: *Akt1* is a pro-survival protein kinase that plays an important role in the regulation of various cellular functions, including metabolism (glucose and lipids), growth, migration, proliferation and cell survival [33]. STRING network analysis identified *Akt1* as the central node associated with 12 of the 14 differentially expressed genes. *Akt1* constitutes an important node with diverse signalling cascades. In this study, a direct link was observed between phosphodiesterase 3B (*Pde3b*) and *Akt1*. Studies have demonstrated that cAMP-dependent protein kinase signaling is impaired in diabetes and is associated with cardiac dysfunction [34, 35]. Cyclic AMP (cAMP) plays a significant role in the thermogenic process as well as potentiating glucose-stimulated insulin release [34, 35]. In contrast, *Pde3b* is a negative regulator of cAMP and increased expression of this gene has been shown to block and diminish the effect of cAMP [28]. *Pde3b* is highly expressed in the myocardium and is known to decrease myocardial smooth muscle contractility [36]. In studies done on isolated rat islets, inhibition of *Pde3* was reported to improve insulin release [37]. In this study, high glucose exposure resulted in the upregulation of *Pde3b* by 2.1-fold (Fig. 1 and Table 3). However, treatment with aspalathin was able to ablate this response, implying that aspalathin might act as a possible inhibitor of *Pde3* and could be used as a potential therapeutic target to protect the diabetic heart. Furthermore, *Akt1* was found to be associated with various antioxidant genes including superoxide dismutase 2 (*Sod2*) and uncoupling protein 1 (*Ucp1*). Although predominantly found in the brown adipose tissue, *Ucp1* is also expressed in cardiac cells where together with *Sod2* inhibit the damaging effects of reactive oxygen species (ROS) [38]. Aspalathin was able to improve *Sod2* (3.1-fold) activity while the effect of high glucose on *Ucp1* (58.6-fold) expression was decreased (-1.7-fold) (Fig. 2). Interestingly, in a study done by Barreto et al [39], where they investigated the mechanism of *Ucp1* action on stress response, they observed that increased *Ucp1* expression induced the upregulation of various antioxidant stress-response genes, such as *Sod1*, glutathione-S-transferase and glutathione peroxidase, concomitantly reducing ROS. Similarly, in a study by Dludla et al. [9], a rooibos extract with abundant aspalathin levels significantly enhanced glutathione levels of cardiomyocytes isolated from diabetic rats exposed to ROS. Based on these findings, we speculate that the observed increased *Ucp1* expression could have been a compensatory mechanism used by the cells to prevent the adverse effects of increased FFA and hyperglycemia-induced ROS.

Impaired myocardial energy metabolism, due to aberrant FFA levels and insulin resistance, activates a myriad of other maladaptive signaling pathways. This includes activation of insulin-like growth factor 1 (*Igf1*), a hormone similar in structure to insulin and known to be a physiological regulator of glucose transport and glycogen synthesis [40]. *Igf1* activates and phosphorylates *Akt1* to attenuate the development of diabetes-induced myocardial apoptosis, through inhibiting tumor protein 53 [41]. In this study, high glucose moderately reduced the mRNA expression of *Igf1* (1.3-fold) and *Akt1* (-2.2-fold)

(Fig. 2). Conversely, the tumor necrosis factor ligand superfamily member 6 (*Faslg*) that interacts with *Akt1* has been shown to induce apoptosis through binding and inhibiting the pro-survival gene Cflar [42]. In this study, high glucose treatment increased mRNA expression of *Faslg* (4.8-fold), while aspalathin ablated this response. Thus, our results infer that aspalathin protected cardiomyocytes exposed to chronic hyperglycemia against *Faslg*-induced apoptosis by activating the Igf-PI3K-Akt pro-survival pathway (Fig. 2). Additionally, STRING data analysis showed that an interactive network was formed between *Igf1*, *Akt1*, serpin peptidase inhibitor, member 1/2 (*Serpin1/2*) and vascular endothelial growth factor A (*Vegfa*) (Fig. 2). High glucose treatment increased the expression of *Vegfa* by 2.0-fold while reducing that of *Serpinb2* and *Serpine1* by -2.7 and -46.1-fold, respectively (Fig. 2 and Table 3). Similarly, Xue et al. [43] as well as Natarajan et al. [44] observed that chronic hyperglycemia upregulates *Vegf* and accelerates diabetic-induced cardiac fibrosis. They further speculate that the observed increase in high glucose-induced *Vegf* expression may be due to elevated inflammation consistent with a hyperglycemic state. The role of *Serpine1* in cardiac fibrosis remains controversial however; *Serpine1* deficient mice display cardiac fibrosis, enhanced inflammation, along with increased microvascular permeability and haemorrhage [45]. Thus, concluding that *Serpine1* is cardio protective, and functions in maintaining normal microvasculature integrity. Thus, based on the latter findings we would speculate that increased expression of *Serpine1* might be cardioprotective. However, this hypothesis requires further investigation.

Protein kinases and mitochondrial function: Damaged structural components of the heart as a result of insulin resistance are known to activate signaling pathways such as p38 mitogen-activated protein kinases (Mapk) [46]. STRING network analysis identified three distinct nodes within this network, including dynamin 1-like (*Dnm1l*), *Map2k1* and cAMP-dependent protein kinase catalytic subunit (*Prkacb*), which is known to be involved in the process of mitophagy. Mitophagy is responsible for the removal of damaged mitochondria proteins as a result of lipotoxicity and defects in this process have been linked to cardiovascular dysfunction [47]. *Dnm1l* is a mitochondrial protein that is involved in the apoptotic process and is crucial for maintaining mitochondrial function through increased *Dnm1l*-dependent mitophagy. Roy et al. [48] showed that mice lacking *Dnm1l* demonstrate impaired heart contraction with an associated reduction in mitochondrial fission, concluding that *Dnm1l* is critical for sustaining mitochondrial morphology and heart function. Interestingly, *Dnm1l* was increased 2.8-fold after aspalathin treatment. Furthermore, mitophagy induction can be regulated/activated through the *Prka-MTOR-ULK1* mediated signaling pathway [48]. STRING analysis showed a strong interaction between protein kinase, AMP-activated, gamma 1 non-catalytic subunit (*Prkag*) and *Mapk*. Activation of *Mapk* has been implicated in FFA-induced cardiac toxicity and subsequent cardiac failure [49]. In this study we observed that high glucose resulted in enhanced expression of *Map2k1* (2.3-fold), with

an associated decrease in the mitophagy activator *Prkag1* (-2.6 fold). However, aspalathin treatment reversed this effect (Fig. 2). Thus, we speculate that aspalathin might possibly protect the myocardium from chronic hyperglycemia through enhanced cAMP-mediated mitophagy, thereby alleviating FFA-induced-MAPK toxicity and subsequent intracellular lipid accumulation *in vitro*.

In vitro effect of aspalathin on inflammation

In a diabetic state, a complex interplay between impaired cardiac substrate metabolism, insulin resistance and inflammation underlies the progression of DCM. Furthermore, accumulating evidence is available on the important role inflammation plays in the development of cardiac hypertrophy and failure. Aberrant and/or prolonged suppressors of cytokine signaling (*Socs*) proteins and JAK-induced signaling is detrimental and can give rise to a number of inflammatory pathologies known to affect cardiac function [50, 51]. Accordingly, 11 of the 57 (19%) differentially expressed genes were associated with cytokine signaling (Table 3). STRING analysis identified a network with five distinct nodes that include interleukins, Cd3e antigen (*Cd3e*), *Socs3*, and leptin receptor precursor (*Lepr*), with Janus kinase 2 (*Jak2*) being the predominant interactive node (Fig. 3).

Leptin action: Leptin is an adipokine that is mainly expressed in adipose tissue, but is also expressed in cardiac heart muscle cells [22]. Accumulative evidence suggests that leptin impairs myocardial energy metabolism by favouring a complete reliance on FFAs as an energy source leading to cardiac hypertrophy [22, 50]. Leptin is known to bind to the leptin receptor to activate the JAK/Signal Transducers and Activators of Transcription (*STAT*) pathway. Activation of this pathway results in the translocation of *STAT* to the nucleus where it can activate *Socs3*, inhibiting leptin action as well as insulin signaling [51]. A meta-analysis of our data showed that the *Lepr* was increased (7.2-fold) after high glucose treatment. Interestingly, this increase was concomitant with an upregulation in the mRNA expression of both *Jak2* (3.9-fold) and *Socs3* (4.5-fold). This result is of interest as increased *Lepr* expression is known to enhance *Socs3* with an associated altered β-oxidation [51], as observed in this study.

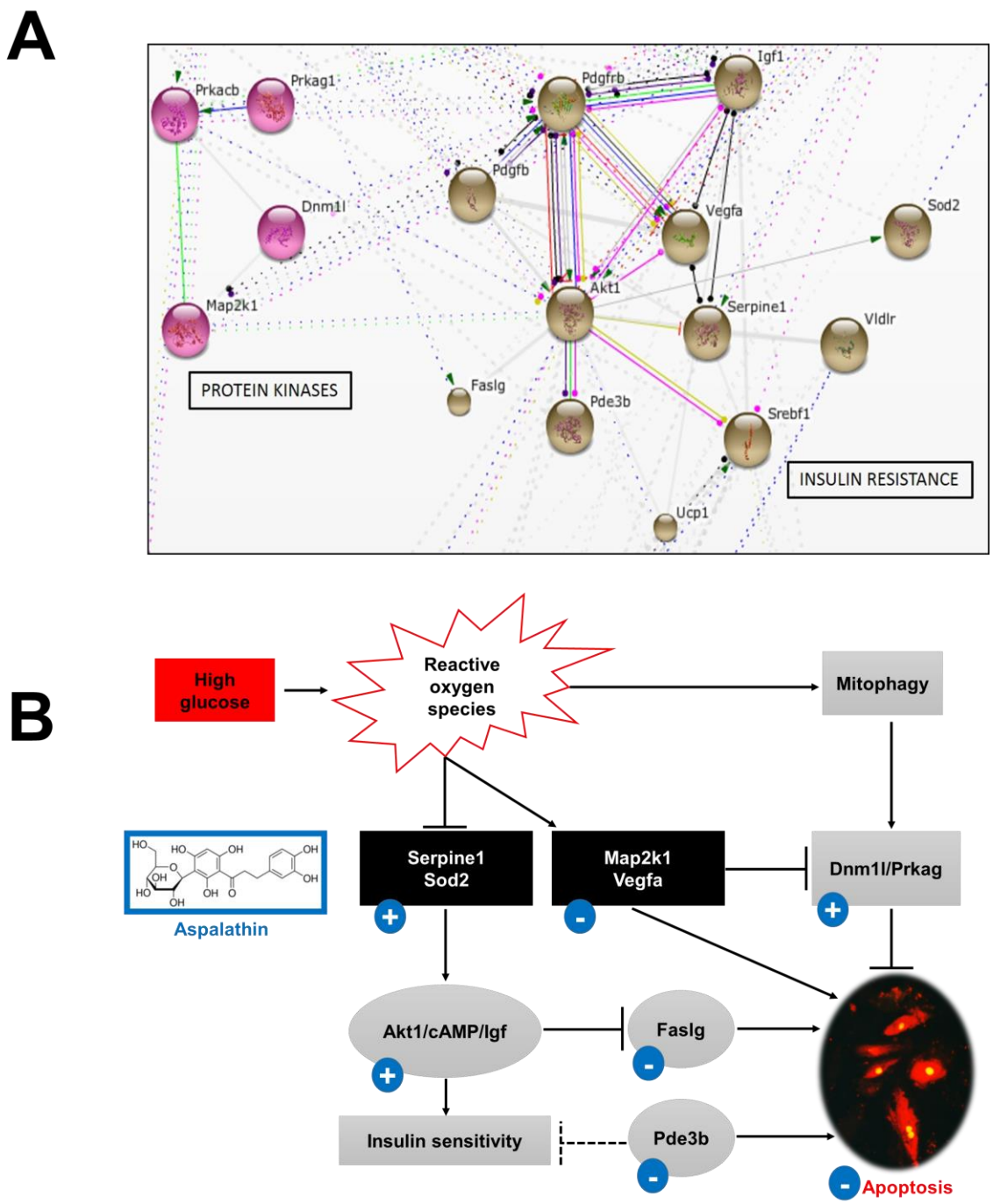


Figure 2. Aspalathin prevented high glucose-induced insulin resistance. (A) Search Tool for the Retrieval of Interacting Genes (STRING) database confirmed a strong interaction between genes associated with insulin resistance. (B) Representative diagram of the proposed protective mechanism of aspalathin against insulin resistance and resultant oxidative stress. **Akt1:** v-akt murine thymoma viral oncogene homolog 1; **cAMP:** cyclic adenosine monophosphate; **Dnm1l:** dynamin 1-like; **Faslg:** fas ligand (TNF superfamily, member 6); **Igf:** insulin-like growth factor 1; **Map2k1:** mitogen-activated protein kinase kinase 1; **Prkag:** protein kinase, AMP-activated, gamma 1 non-catalytic subunit; **Serpine1:** serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1; **Sod2:** superoxide dismutase 2; **Vegfa:** vascular endothelial growth factor A.

Cytokine signaling: In the diabetic heart, increased lipotoxicity and insulin resistance triggers the recruitment of macrophages and leukocytes before the release of pro-inflammatory cytokines such as interleukin 3 and 6 (*Il3* and *Il6*), tumour necrosis factor superfamily (*Tnf*) and cluster of differentiation (Cd). *Il6*, *Tnf* and *Cd* cause the endothelial cells of blood vessels to express cellular adhesion molecules, such as Selectin E (*Sele*) and *Cd44*, resulting in an acute localized cellular inflammation. Both *Cd44* and *Sele* are cell-surface glycoproteins that mediate neutrophil, monocyte, lymphocyte and platelet rolling in the ventricular wall. These glycoproteins have also been found to play an important role in atherosclerotic lesion development and calcification of the lesion [52, 53]. Studies demonstrated that *Cd44* as well as *Sele* null mice display increased atherosclerosis plaque formation [52, 53]. Our results showed that high glucose treatment increased the expression of various pro-inflammatory cytokines including, *Il3* (2.3-fold), *Il6* (2.7-fold), *Tnsf13* (4.7-fold) and *Tnfsf13b* (2.1-fold), *Sele* (13.8-fold) and Cd44 antigen (*Cd44*, 2.3-fold) (Fig. 3 and Table 3). Based on our gene expression data, we propose that during chronic hyperglycemia, pro-inflammatory cytokines (*Il3, Il6* and *Tnf-α*) are activated. This increased inflammatory cytokine response releases *Sele* and *Cd44* from the storage granules in activated platelet and endothelial cells and recruits them to mediate the first step of leukocyte extravasation. Additionally, *Il6* and *Tnf* activate the JAK/STAT and Map kinase pathways respectively, resulting in increased hypertrophy and apoptosis. Increased expression of *Socs3* has been linked to inflammation-induced insulin resistance and enhanced JAK/STAT signaling as observed in this study [54]. However, aspalathin treatment was able to reverse this effect (Fig. 3).

In vitro effect of aspalathin on apoptosis

Chronic hyperglycemia, insulin resistance and inflammation have been shown to exacerbate oxidative stress and cardiomyocyte apoptosis [23, 26]. In this study, 6 of the 57 (11%) differentially expressed genes were associated with apoptosis signaling. STRING data analysis identified one network with five interactive nodes, centralized around B-cell lymphoma 2 (*Bcl2*).

Genes identified in this network included *Bcl2*, Bcl2 binding component 3 (*Bbc3*), mitogen activated protein kinase 3 (*Mapk3*) and optic atrophy 1 homolog (*Opa1*) (Fig. 4 and Table 3). The Bcl2 family of proteins are known to be strong modulators of apoptosis and can induce either pro-apoptosis or cell survival depending on the cell's fate [10, 26]. Two pro-survival genes were identified (*Bcl2* and *Bcl2a1*) and their expression was reduced after high glucose exposure (Fig. 4 and Table 3). Contrary, four pro-apoptotic proteins (*Bbc3*, *Chuk*, *Mapk3* and *Opa1*) were identified. Activation of *Mapk3* together with pro-apoptotic proteins such as *Bbc3* has been associated with myocardial dysfunction [23, 55], while upregulation of *Opa1* is directly linked to the reversal of mitochondrial fragmentation [56]. Furthermore, *in vivo* and *in vitro* data has confirmed that activation of Mapk proteins, including *Mapk3* together with

pro-apoptotic proteins is associated with hyperglycemia induced myocardial dysfunction [23, 55]. Hence, this emphasises the importance of ameliorative strategies that target apoptosis to alleviate myocardial structural remodeling. Increased *Bcl2* expression has an inhibitory effect on *Mapk3*. Thus, we proposed that aspalathin prevents myocardial apoptosis through the inhibition of *Mapk3* while simultaneously increasing *Bcl2*-mediated cardiomyocyte survival.

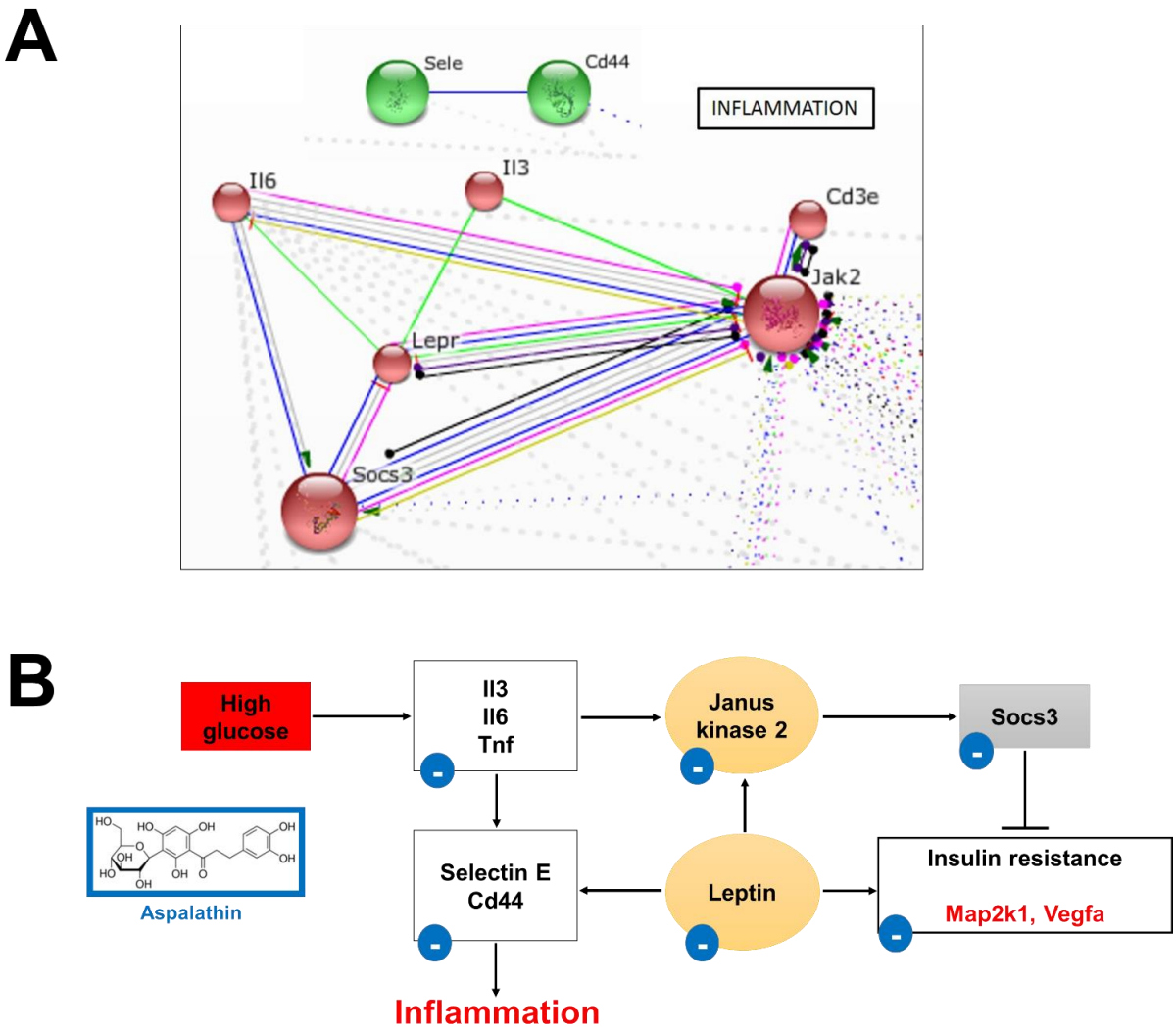


Figure 3. Aspalathin prevented high glucose-induced inflammation. (A) Search Tool for the Retrieval of Interacting Genes (STRING) database confirmed a strong interaction between genes associated with inflammation. (B) Representative diagram of the proposed protective mechanism of aspalathin against high glucose induced inflammation. **Cd44:** cluster of differentiation 44; **Il3:** interleukin 3; **Il6:** interleukin 6; **Map2k1:** mitogen-activated protein kinase kinase 1; **Socs3:** suppressor of cytokine signaling 3; **Tnf:** tumor necrosis factor; **Vegfa:** vascular endothelial growth factor A.

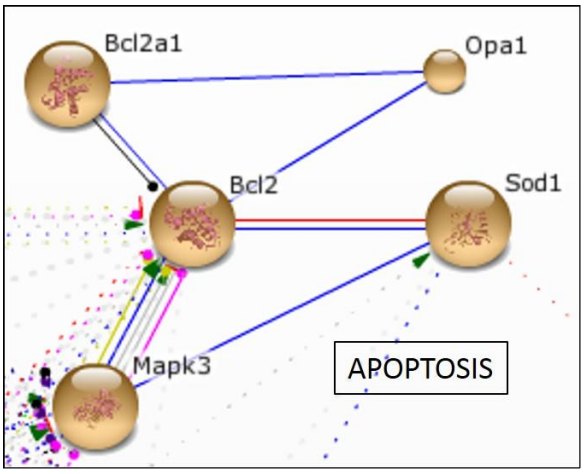


Figure 4. Aspalathin limited high glucose-induced apoptosis. (A) Search Tool for the Retrieval of Interacting Genes (STRING) database confirmed a strong interaction between genes associated with high glucose induced apoptosis.

Conclusion: Results obtained in this study lead us to propose that aspalathin could protect the myocardium against lipid toxicity in two ways. In the first instance, such treatment significantly improves the blood lipid profile to a far less damaging one therefore attenuating fuel substrate availability to the diabetic heart. It is likely that aspalathin mediates such effects by acting on targets in the liver, although this was not further investigated in the current study. Secondly, our data demonstrate that aspalathin also exerts direct, protective effects within the myocardium to protect the heart against excess fuel substrate availability. Here our data indicate that aspalathin may protect the myocardium against lipotoxicity and subsequent cell apoptosis by increasing the expression of *Adipoq* and subsequently decreasing the expression of *Cd36* and Cpt1. Furthermore, aspalathin reduced lipid transport via *Slc27a3/5*, which together with reduced *PPARγ* and *Srebf1* resulted in decreased total cholesterol and subsequent cell apoptosis. Moreover, we proposed that the reversal of lipid toxicity results in a decreased inflammatory response via the *Il6/Jak/Stat* pathway, which together with an observed increase in *Bcl2* prevents myocardium cell apoptosis. Together, these findings propsed a probable mechanism by which aspalathin reverses metabolic abnormalities associated with the failing myocardium (Fig 5). However, this warrants further investigation.

Table 3 Aspalathin improves gene expression derangements associated with hyperglycemia in H9c2 cardiomyocytes.

		Gene fold regulation	
Gene name	**Gene symbol**	**High glucose (33 mM)**	**Aspalathin (1 µM)**
Lipid metabolism			
ATP-binding cassette, subfamily A (ABC1), member 1	*Abca1*	2.0776	-1.1921
Acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain	*Acads*	-1.6611	-2.425
Acyl-CoA thioesterase 2	*Acot2*	2.8239	-1.2178
Acyl-Coenzyme A oxidase 2, branched chain	*Acox2*	2.0959	1.8895
Acyl-CoA synthetase bubblegum family member 2	*Acsbg2*	4.3205	-1.5973
Acyl-CoA synthetase long-chain family member 4	*Acsl4*	5.6547	1.7361
Acyl-CoA synthetase long-chain family member 6	*Acsl6*	2.465	-1.5973
Acyl-CoA synthetase medium-chain family member 3	*Acsm3*	14.2353	5.697
Acyl-CoA synthetase medium-chain family member 4	*Acsm4*	6.1301	3.1806
Adiponectin, C1Q and collagen domain containing	*Adipoq*	-6.0324	3.2588
Apolipoprotein A-I	*Apoa1*	4.2006	2.9178
Apolipoprotein B	*Apob*	7.7651	-3.0228
Apolipoprotein E	*Apoe*	4.4207	-1.0317
CD36 antigen	*Cd36*	3.7512	1.7387
Carnitine palmitoyltransferase 1b, muscle	*Cpt1b*	3.3366	2.0449
Fatty acid binding protein 3, muscle and heart	*Fabp3*	2.7003	-1.5135
Lysophospholipase 1	*Lypla1*	3.5198	1.6047
Peroxisome proliferator activated receptor gamma	*Pparγ*	8.3847	-1.6135
Stearoyl-Coenzyme A desaturase 1	*Scd1*	5.5982	1.0879
Solute carrier family 25, member 30	*Slc25a30*	2.6123	1.6634
Solute carrier family 27 (fatty acid transporter), member 1	*Slc27a1*	1.0514	-2.2919
Solute carrier family 27 (fatty acid transporter), member 3	*Slc27a3*	6.323	2.2207
Solute carrier family 27 (fatty acid transporter), member 5	*Slc27a5*	2.0184	-1.5973
Sterol regulatory element binding transcription factor 1	*Srebf1*	3.4573	-4.544
Sterol regulatory element binding factor 2	*Srebf2*	2.0208	-26.826
Very low density lipoprotein receptor	*Vldlr*	2.0148	1.3077

Insulin resistance			
V-akt murine thymoma viral oncogene homolog 1	*Akt1*	-2.2173	1.2477
Dynamin 1-like	*Dnm1l*	1.4367	2.8522
Fas ligand (TNF superfamily, member 6)	*Faslg*	4.8622	-1.4856
Insulin-like growth factor 1	*Igf1*	1.3447	2.4477
Mitogen-activated protein kinase kinase 1	*Map2k1*	2.3446	-2.1822
Phosphodiesterase 3B, cGMP-inhibited	*Pde3b*	2.1481	1.5951
Protein kinase, cAMP dependent, catalytic, beta	*Prkacb*	1.687	2.4466
Protein kinase, AMP-activated, gamma 1 non-catalytic subunit	*Prkag1*	-2.6381	1.3516
Serpin peptidase inhibitor, clade B (ovalbumin), member 2	*Serpinb2*	-2.7789	1.8746
Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	*Serpine1*	-46.1814	-1.4005
Superoxide dismutase 1, soluble	*Sod1*	2.8805	1.0032
Superoxide dismutase 2, mitochondrial	*Sod2*	-1.5522	3.1195
Uncoupling protein 1 (mitochondrial, proton carrier)	*Ucp1*	58.6622	-1.7821
Vascular endothelial growth factor A	*Vegfa*	2.0015	1.2082
Inflammation			
CD3 antigen, epsilon polypeptide	*Cd3e*	5.4019	1.3557
Cd44 molecule	*Cd44*	2.3883	1.322
Interleukin 3	*Il3*	2.3815	-1.1606
Interleukin 6	*Il6*	2.7362	1.9106
Janus kinase 2	*Jak2*	3.9723	1.721
Leptin receptor	*Lepr*	7.2781	-1.6135
Selectine E	*Sele*	13.8787	-1.5233
Suppressor of cytokine signaling 3	*Socs3*	4.5848	-2.4959
Tumor necrosis factor receptor superfamily, member 1b	*Tnfrsf1b*	1.0099	-3.114
Tumor necrosis factor (ligand) superfamily, member 13	*Tnfsf13*	4.7142	1.2849
Tumor necrosis factor (ligand) superfamily, member 13b	*Tnfsf13b*	2.0522	1.8583
Apoptosis			
Bcl-2 binding component 3	*Bbc3/puma*	-1.3703	-3.1586
B-cell CLL/lymphoma 2	*Bcl2*	-2.6947	1.8312
B-cell leukemia/lymphoma 2 related protein A1d	*Bcl2a1*	-7.13	1.8085
Conserved helix-loop-helix ubiquitous kinase	*Chuk*	4.2959	2.1502

Mitogen-activated protein kinase 3	*Mapk3*	1.2583	-3.0871
Optic atrophy 1 homolog (human)	*Opa1*	1.3949	2.0255

References

1. Gollucke AP, Peres RC, Odair AJ, Ribeiro DA. Polyphenols: a nutraceutical approach against diseases. Recent Pat. Food Nutr. Agric. 2013; 5: 214–219.

2. Bahadoran Z, Mirmiran P, Azizi F. Dietary polyphenols as potential nutraceuticals in management of diabetes: a review. J. Diabetes Metab. Disord. 2013; 12: 43.

3. Pantsi WG, Marnewick JL, Esterhuyse AJ, Rautenbach F, van Rooyen J. Rooibos (*Aspalathus linearis*) offers cardiac protection against ischaemia/reperfusion in the isolated perfused rat heart. Phytomedicine 2011; 18: 1220–1228.

4. Ulicná O, Vancová O, Bozek P, Cársky J, Sebeková K, Boor P, et al. Rooibos tea (*Aspalathus linearis*) partially prevents oxidative stress in streptozotocin-induced diabetic rats. Physiol. Res. 2006; 55: 157–164.

5. Marnewick JL, Rautenbach F, Venter I, Neethling H, Blackhurst DM, Wolmarans P, et al. Effects of rooibos (*Aspalathus linearis*) on oxidative stress and biochemical parameters in adults at risk for cardiovascular disease. J. Ethnopharmacol. 2011; 133: 46–52.

6. Mazibuko SE, Joubert E, Johnson R, Louw J, Opoku AR, Muller CJF. Aspalathin improves glucose and lipid metabolism in 3T3-L1 adipocytes exposed to palmitate. Mol. Nutr. Food Res. 2015; 59: 2199–2208.

7. Muller CJF, Joubert E, De Beer D, Sanderson M, Malherbe CJ, Fey SJ, et al. Acute assessment of an aspalathin-enriched green rooibos (*Aspalathus linearis*) extract with hypoglycemic potential. Phytomedicine 2012; 20: 32–39.

8. Kawano A, Nakamura H, Hata S, Minakawa M, Miura Y, Yagasaki K. Hypoglycemic effect of aspalathin, a rooibos tea component from *Aspalathus linearis*, in type 2 diabetic model db/db mice. Phytomedicine 2009; 16: 437–443.

9. Dludla PV, Muller CJF, Louw J, Joubert E, Salie R, Opoku AR, et al. The cardioprotective effect of an aqueous extract of fermented rooibos (*Aspalathus linearis*) on cultured cardiomyocytes derived from diabetic rats. Phytomedicine 2014; 21: 595–601.

10. Johnson R, Dludla P, Joubert E, February F, Mazibuko S, Ghoor S, et al. Aspalathin, a dihydrochalcone C-glucoside, protects H9c2 cardiomyocytes against high glucose-induced shifts in substrate preference and apoptosis. Mol. Nutr. Food Res. 2016; 60: 922–934.

11. Smit SE. An investigation into the effects of aspalathin on myocardial glucose transport using cardiomyocytes from control and obesity-induced insulin resistant rats, and terminally

differentiated H9c2 cells. M.Sc. Thesis, Stellenbosch University. 2016. Available: http://scholar.sun.ac.za/handle/10019.1/98490

12. Sharma S, Adrogue JV, Golfman L, Uray I, Lemm J, Youker K, et al. Intramyocardial lipid accumulation in the failing human heart resembles the lipotoxic rat heart. FASEB J. 2004; 18: 1692–1700.

13. Silver DL, Jiang XC, Tall AR. Increased high density lipoprotein (HDL), defective hepatic catabolism of ApoA-I and ApoA-II, and decreased ApoA-I mRNA in ob/ob mice. Possible role of leptin in stimulation of HDL turnover. J. Biol. Chem. 1999; 274: 4140–4146.

14. Goldberg IJ, Trent CM, Schulze PC. Lipid metabolism and toxicity in the heart. Cell Metab. 2012; 15: 805–812.

15. Inoue T, Kobayashi K, Inoguchi T, Sonoda N, Maeda Y, Hirata E, et al. Downregulation of adipose triglyceride lipase in the heart aggravates diabetic cardiomyopathy in db/db mice. Biochem. Biophys. Res. Commun. 2013; 438: 224–229.

16. Duncan JG. Mitochondrial dysfunction in diabetic cardiomyopathy. Biochim Biophys Acta. 2011; 1813: 1351–1359.

17. Han Z, Achilonu MC, Kendrekar PS, Joubert E, Ferreira D, Bonnet SL, et al. Concise and scalable synthesis of aspalathin, a powerful plasma sugar-lowering natural product. J. Nat. Prod. 2014; 77: 583–588.

18. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985; 28: 412–419.

19. Karagiannis TC, Lin AJ, Ververis K, Chang L, Tang MM, Okabe J, et al. Trichostatin A accentuates doxorubicin-induced hypertrophy in cardiac myocytes. Aging 2010; 2: 659–568.

20. Jensen LJ, Kuhn M, Stark M, Chaffron S, Creevey C, Muller J, et al. STRING 8-a global view on proteins and their functional interactions in 630 organisms. Nucleic Acids Res. 2009; 37: D412–416.

21. Szklarczyk D, Franceschini A, Wyder S, Forslund K, Heller D, Huerta-Cepas J, et al. STRING v10: protein-protein interaction networks, integrated over the tree of life. Nucleic Acids Res. 2015; 43: D447–452.

22. Rajapurohitam V, Gan XT, Kirshenbaum LA, Karmazyn M. The obesity-associated peptide leptin induces hypertrophy in neonatal rat ventricular myocytes. Circ. Res. 2003; 93: 277–279.

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

24. Paolisso G, Manzella D, Montano N, Gambardella A, Varricchio M. Plasma leptin concentrations and cardiac autonomic nervous system in healthy subjects with different body weights. J. Clin. Endocrinol. Metab. 1999; 85: 1810–1814.

25. Drosatos K, Khan RS, Trent CM, Jiang H, Son N, Blaner WS, et al. PPARγ activation prevents sepsis-related cardiac dysfunction and mortality in mice. Circ. Heart Fail. 2013; 6: 550–562.

26. Dludla PV, Muller CJF, Joubert E, Louw J, Gabuza KB, Huisamen B, et al. Phenylpyruvic acid-2-O-β-D-glucoside attenuates high glucose-induced apoptosis in H9c2 cardiomyocytes. Planta Med. 2016; 82: 1–7.

27. Marfella R, Di Filippo C, Portoghese M, Barbieri M, Ferraraccio F, Siniscalchi M, et al. Myocardial lipid accumulation in patients with pressure-overloaded heart and metabolic syndrome. J. Lipid. Res. 2009; 50: 2314–2323.

28. Tamang HK, Timilsina U, Singh KP, Shrestha S, Raman RK, Panta P, et al. Apo B/Apo A-I ratio is statistically a better predictor of cardiovascular disease (CVD) than conventional lipid profile: a study from Kathmandu valley, Nepal. J. Clin. Diagn. Res. 2014; 8: 34–36.

29. DeFronzo RA. Insulin resistance, lipotoxicity, type 2 diabetes and atherosclerosis: the missing links. The Claude Bernard Lecture 2009. Diabetologia. 2010; 53: 1270–1287.

30. Chan DC, Watts GF. Apolipoproteins as markers and managers of coronary risk. QJM. 2006; 99:277–287.

31. Haim Y, Blüher M, Slutsky N, Goldstein N, Klöting N, Harman-Boehm I, et al. Elevated autophagy gene expression in adipose tissue of obese humans: A potential non-cell-cycle-dependent function of E2F1. Autophagy 2015; 11: 2074–2088.

32. Sundell J, Huupponen R, Raitakari OT, Nuutila P, Knuuti J. High serum leptin is associated with attenuated coronary vasoreactivity. Obes. Res. 2003; 11: 776–782.

33. Zdychová J, Komers R. Emerging role of Akt kinase/protein kinase B signaling in pathophysiology of diabetes and its complications. Physiol. Res. 2005; 54: 1–16.

34. Bockus LB, Humphries KM. cAMP-dependent protein kinase (PKA) signaling is impaired in the diabetic heart. J. Biol. Chem. 2015; 290: 29250–29258.

35. Weber HE, Menahan LA, Chaudhuri SN, Shipp JC. Effect of experimental diabetes and glucagon on cAMP-dependent protein kinase in rat liver. Diabetologia 1977; 13: 153–157.

36. Beca S, Ahmad F, Shen W, Liu J, Makary S, Polidovitch N, et al. PDE3A regulates basal myocardial contractility through Interacting with SERCA2a-signaling complexes in mouse heart. Circ. Res. 2013; 112: 289–297.

37. Hoerter J, Gonzalez-Barroso MD, Couplan E, Mateo P, Gelly C, Cassard-Doulcier AM, et al. Mitochondrial uncoupling protein 1 expressed in the heart of transgenic mice protects against ischemic-reperfusion damage. Circulation 2004; 110: 528–533.

38. Vettor R, Fabris R, Serra R, Lombardi AM, Tonello C, Granzotto M, et al. Changes in FAT/CD36, UCP2, UCP3 and GLUT4 gene expression during lipid infusion in rat skeletal and heart muscle. Int. J. Obes. Relat. Metab. Disord. 2002; 26: 838–847.

39. Barreto P, Okura VK, Neshich IA, Maia Ide G, Arruda P. Overexpression of UCP1 in tobacco induces mitochondrial biogenesis and amplifies a broad stress response. BMC Plant Biol. 2014; 14: 144.

40. Zoidis E, Ghirlanda-Keller C, Schmid C. Stimulation of glucose transport in osteoblastic cells by parathyroid hormone and insulin-like growth factor I. Mol. Cell Biochem. 2011; 348: 33–42.

41. Kajstura J, Fiordaliso F, Andreoli AM, Li B, Chimenti S, Medow MS, et al. IGF-1 overexpression inhibits the development of diabetic cardiomyopathy and angiotensin II-mediated oxidative stress. Diabetes 2001; 50: 1414–1424.

42. Lacana E, D'Adamio L. Regulation of Fas ligand expression and cell death by apoptosis-linked gene 4. Nat. Med. 1999; 5: 542–547.

43. Xue W, Cai L, Tan Y, Thistlethwaite P, Kang YJ, Li X, et al. Cardiac-specific overexpression of HIF-1α prevents deterioration of glycolytic pathway and cardiac remodeling in streptozotocin-induced diabetic mice. Am. J. Pathol. 2010; 177: 97–105.

44. Natarajan R, Bai W, Lanting L, Gonzales N, Nadler J. Effects of high glucose on vascular endothelial growth factor expression in vascular smooth muscle cells. Am. J. Physiol. 1997; 273:H2224–2231.

45. Xu Z, Castellino FJ, Ploplis VA. Plasminogen activator inhibitor-1 (PAI-1) is cardioprotective in mice by maintaining microvascular integrity and cardiac architecture. Blood. 2010; 115: 2038–2047.

46. Arabacilar P, Marber M. The case for inhibiting p38 mitogen-activated protein kinase in heart failure. Front Pharmacol. 2015; 6: 102.

47. Hoshino A, Mita Y, Okawa Y, Ariyoshi M, Iwai-Kanai E, Ueyama T, et al. Cytosolic p53 inhibits Parkin-mediated mitophagy and promotes mitochondrial dysfunction in the mouse heart. Nat Commun. 2013; 4: 2308.

48. Roy M, Kageyama Y, Iijima M, Sesaki H. PARK2/Parkin becomes critical when DNM1L/Drp1 is absent. Autophagy 2015; 11: 573–574.

49. Wende AR, Symons JD, Abel ED. Mechanisms of lipotoxicity in the cardiovascular system. Curr. Hypertens. Rep. 2012; 14: 517–531.

50. Xu FP, Chen MS, Wang YZ, Yi Q, Lin SB, Chen AF, et al. Leptin induces hypertrophy via endothelin-1-reactive oxygen species pathway in cultured neonatal rat cardiomyocytes. Circulation 2004; 110: 1269–1275.

51. Yang R, Barouch LA. Leptin signaling and obesity: cardiovascular consequences. Circ. Res. 2007; 101: 545–559.

52. Cuff CA, Kothapalli D, Azonobi I, Chun S, Zhang Y, Belkin R, et al. The adhesion receptor CD44 promotes atherosclerosis by mediating inflammatory cell recruitment and vascular cell activation. J. Clin. Invest. 2001; 108: 1031–1040.

53. Dong ZM, Chapman SM, Brown AA, Frenette PS, Hynes RO, Wagner DD. The combined role of P- and E-Selectins in atherosclerosis. J. Clin. Invest. 1998; 102: 145–152.

54. Tamiya T, Kashiwagi I, Takahashi R, Yasukawa H, Yoshimura A. Suppressors of cytokine signaling (SOCS) proteins and JAK/STAT pathways: regulation of T-cell inflammation by SOCS1 and SOCS3. Arterioscler. Thromb. Vasc. Biol. 2011; 31: 980–925.

55. Duvezin-Caubet S, Jagasia R, Wagener J, Hofmann S, Trifunovic A, Hansson A, et al. Proteolytic processing of OPA1 links mitochondrial dysfunction to alterations in mitochondrial morphology. J. Biol. Chem. 2006; 281: 37972–37979.

56. Chen SC, Kochan JP, Campfield LA, Burn P, Smeyne RJ. Splice variants of the OB receptor gene are differentially expressed in brain and peripheral tissues of mice. J. Recept. Signal. Transduct. Res. 1999; 19: 245–266.

Chapter 6: General conclusions

Summary of findings

Urbanization and obesity remain important causal factors responsible for the dramatic rise in cases of diabetes mellitus (DM) worldwide (International Diabetes Federation, 2015). The rising incidence of DM has been associated with an increase in cardiovascular mortality. Currently, insulin and metformin remain the leading first line antihyperglycemic drug therapies (Jonassen et al., 2001; Yin et al., 2011). However, these drugs have a limited capacity to protect the diabetic myocardium against the development of cardiovascular complications. Currently, researchers are increasingly exploring the use of plant-derived materials as an adjunct to current drug treatment (Chellan et al., 2014; Mazibuko et al., 2015; Muller et al., 2016). Numerous edible plants are known to contain plant polyphenols, flavonoids, terpenoids and other constituents, which may contribute to the reduction of blood glucose levels as well as counteract oxidative-induced tissue damage through their robust antioxidant effect (Kawano et al., 2009; Bai et al., 2013; Ezuruike et al 2014). Previously, our group and others have demonstrated that *Aspalathus linearis*, a plant indigenous to South Africa and commonly known as rooibos, displayed protective properties against the development of obesity, insulin resistance and cardiovascular disease in various experimental models (Muller et al., 2012; Son et al., 2013; Dludla et al., 2014; Sanderson et al. 2014). In a study done by Dludla et al 2014, we showed that a fermented rooibos extract with abundant levels of phenyl pyruvic acid-2-*O*-β-D-glucoside (PPAG) and aspalathin was able to decrease oxidative stress and subsequent cell apoptosis in primary rat cardiomyocytes from diabetic rats. However, the protective mechanism afforded by PPAG and aspalathin to modulate oxidative-induced myocardial damage remained to be fully elucidated. Therefore, in this study we aimed to unravel the mechanisms by which PPAG and aspalathin protect against the development of hyperglycemia-induced myocardial injury.

PPAG protected against hyperglycemia-induced cardiac apoptosis

The mitochondrial apoptotic pathway is largely mediated through the B-cell lymphoma 2 (*Bcl2*) family of proteins. *Bcl2*, a human proto-oncogene, protects against mitochondrial permeability and subsequent programmed cell death. Following a death signal, pro-apoptotic proteins such as Bax undergo a conformational change that enable it to integrate into the outer mitochondrial membrane and initiate cell death. In contrast, anti-apoptotic proteins such as *Bcl2* are important for protecting the mitochondrial membrane against Bax translocation, making Bcl2/Bax ratio crucial for cell survival. In this study, we observed that PPAG treatment led to an enhanced Bcl2/Bax ratio thus preventing mitochondrial depolarization and subsequent cell apoptosis as observed by a reduced caspase 3/7

activity and inhibition of DNA fragmentation (Fig. 1). We further observed that PPAG treatment resulted in increased glucose uptake with a concomitant decrease in oxidative phosphorylation. It is well-known that tumor suppressor 53 (p53) plays an important role in energy metabolism by activating TP53-inducible glycolysis and apoptosis regulator (TIGAR), resulting in the blockade of glycolysis and accelerated apoptosis. Thus, we speculate that PPAG may protect the myocardium from cell death through an increased Bcl2/Bax ratio as well as improved glucose uptake by inhibiting p53. However, this mechanism will be further explored in a follow up study.

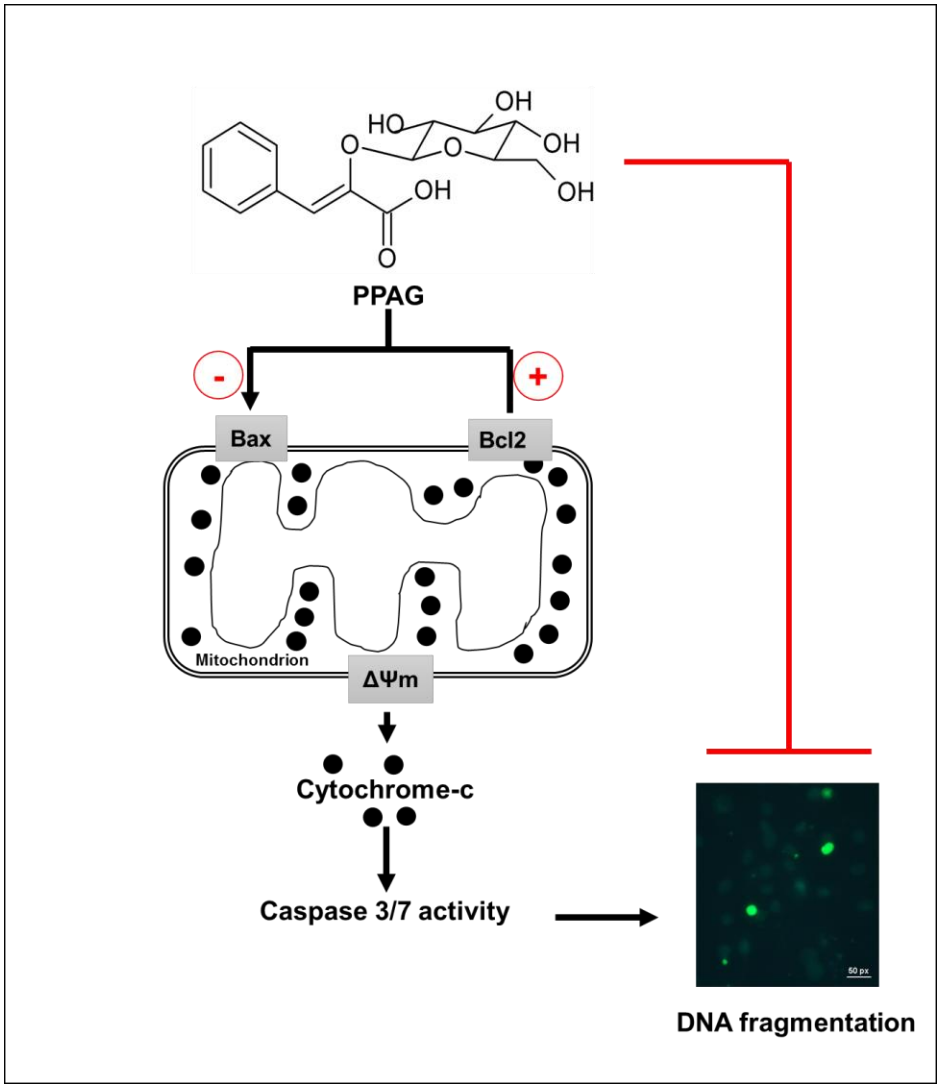


Figure 1. Pyruvic acid-2-*O*-β-D-glucoside (PPAG) protects H9c2 cardiomyocytes against hyperglycemia-induced damage by reducing mitochondrial membrane depolarization and inhibiting apoptosis through increasing Bcl-2/Bax ratio. **ΔΨm:** mitochondrial transmembrane potential, **Bax:** Bcl2-like protein 4, **Bcl2:** B-cell lymphoma 2.

The role of aspalathin in preventing shifts in energy substrate preference, oxidative damage and subsequent lipid toxicity

The role aspalathin plays in modulating fatty acid oxidation (FAO) and glucose oxidation (GO): An important feature of the failing heart is the observed shift in substrate preference that precedes the onset of oxidative damage. The myocardium has a high-energy demand that is primarily achieved through FAO, accounting for 60-80% of ATP production at the expense of glucose. In a diabetic heart, an adaptive response is observed where there is diminished utilization of glucose with an increase in FAO. This results in a shift in substrate preference with an almost complete reliance on FAO as an energy source, with an associated decrease in glucose oxidation. This compensatory shift is believed to be involved in impaired cardiac function of the failing heart. In this study we showed that aspalathin, via 5' AMP-activated protein kinase (AMPK) was able to efficaciously decrease myocardial FAO, causing a reciprocal increase in glucose oxidation. Shifting energy substrate preference away from FAO towards glucose utilization is a promising therapeutic approach that could possibly slow down the progression of heart failure (HF) in a diabetic state. However, there is a fine line in the balance of substrate preference. Decreasing the cell's capacity for FAO may result in the accumulation of long-chain fatty acyl intermediates and triglycerides, which may in turn lead to the generation of toxic lipid build-up and potentially accelerate the progression of HF (Lionetti et al 2011). However, as will be discussed below, we showed that aspalathin was able to decrease lipid toxicity as a result of reduced FAO. Additionally, we showed that aspalathin was able to enhance endogenous antioxidant defense genes and protect against hyperglycemia-induced membrane depolarization and subsequent cell apoptosis (Fig. 2).

Aspalathin decrease oxidative damage by increasing the expression of *Nrf2*: Impaired myocardial energy substrate utilization and increased oxidative stress are linked to the onset of cardiac hypertrophy. In this study, we demonstrated that in addition to impaired substrate preference, cardiomyocytes exposed to a simulated hyperglycemic state displayed increased generation of reactive oxygen species and reduced expression of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Fig. 2). Nrf2 plays a crucial role in the cells ability to response to endogenous oxidative stress, by increasing the expression of an array of cytoprotective and antioxidant defense genes. Correspondingly, in this study, after high glucose exposure, we observed accelerated oxidative stress concurrent to heart tissue morphological derangements and increased apoptosis. However, aspalathin treatment was able to abolish the observed myocardial injury by upregulating the expression of *Nrf2* and its associated down-stream cytoprotective and antioxidant genes (Fig. 2).

Aspalathin role in lipid metabolism: DM has been associated with an increase in FFA availability with a concomitant increase in lipid toxicity. This lipid overload is known to decrease respiratory efficiency of the heart resulting in increased hypertrophy and subsequent HF. In this study, we showed that aspalathin was able to reverse lipid toxicity by increasing the expression of *Adipoq* and subsequently decreased *Cd36* and *Cpt1* mRNA expression (Chapter 5, Fig. 1B). We speculate that *Adipoq* negatively regulates FFA synthesizing genes, such as sterol regulatory element binding transcription factor 1 (*Srebf1*) and stearoyl-Coenzyme A desaturase 1 (*Scd1*), leading to a decrease in lipid accumulation. Moreover, aspalathin reduced intracellular FFA uptake via solute carrier family 27 (fatty acid transporter), member 3/5 (*Slc27a3/5*) which together with reduced *Srebf1* result in the decrease of total cholesterol, hypertrophy and subsequent HF. Moreover, reversal of lipid toxicity was associated with an observed decrease in inflammatory markers, such as interleukin 3 and 6 (*Il6* and *Il3)*, tumor necrosis factor receptor superfamily, member 1b and 13 (*Tnfrsf1b* and *Tnfsf13*), Janus kinase 2 (*Jak2*) and mitogen-activated protein kinase 3 (*Mapk3*) (Fig. 2).

In summary, in this study showed that while PPAG and aspalathin were able to reduce hyperglycemia-induced apoptosis, only aspalathin could ameliorate lipid toxicity and oxidative stress-associated with the insulin resistances state by activating *Nrf2* expression. In conclusion, this study was able to prove the hypothesis that rooibos-specific compounds (PPAG and aspalathin) can protect the myocardium against hyperglycemia-induced oxidative injury by activating *Nrf2* antioxidant response pathway.

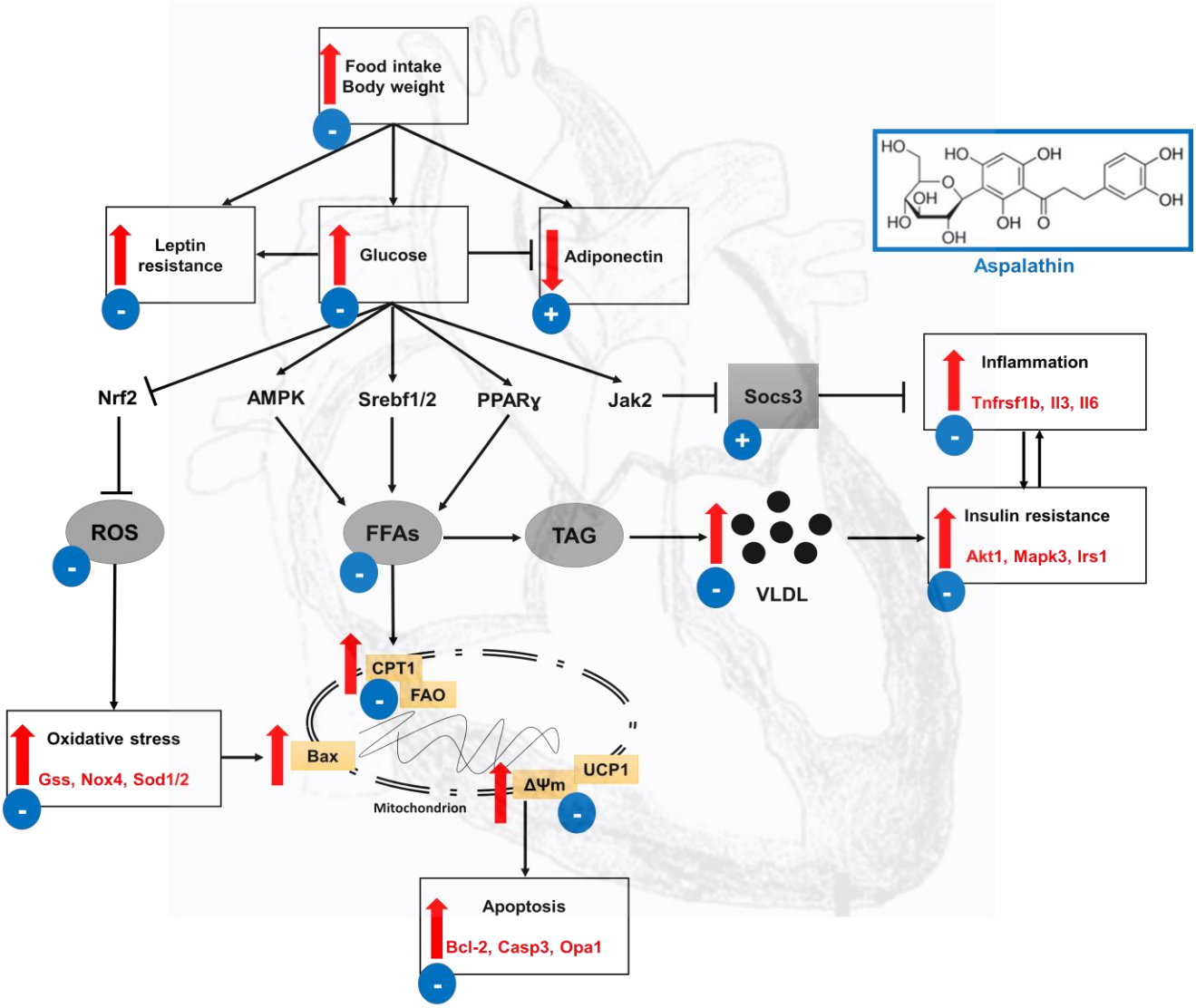


Figure 2. Aspalathin protects against hyperglycemic-induced myocardial injury. A leptin receptor-deficient (db/db) mouse model displayed increased food intake and body weights that was concomitant to elevated lipid profiles. Similarly, cultured cardiomyocytes exposed to a simulated hyperglycemic state demonstrated impaired myocardial energy substrate preference (that was associated with increased inflammation, insulin resistance and subsequent apoptosis. However, aspalathin treatment was able to prevent these complications, with activation of AMPK and Nrf2 the most prominent mechanisms of protection involved. **ΔΨm:** mitochondrial transmembrane potential, **AMPK:** 5' AMP-activated protein kinase, **Bax:** Bcl-2-like protein 4, **Bcl-2:** B-cell lymphoma 2, **Casp3:** caspase 3, **CPT1:** carnitine palmitoyltransferase 1, **FAO:** fatty acid oxidation, **FFAs:** free fatty acids, **Gss:** glutathione synthetase, **Il3:** interleukin 3, **Il6:** interleukin 6, **Jak2:** Janus kinase 2, **Mapk3:** mitogen-activated protein kinase 3, **Nox4:** NADPH oxidase, **Nrf2:** nuclear factor (erythroid-derived 2)-like 2, **Opa1:** Optic atrophy 1 homolog (human) 4, **PPARɣ:** peroxisome proliferator-activated receptor gamma, **ROS:** reactive oxygen species, **Socs3:** suppressor of cytokine signaling 3, **Sod1/2:** superoxide dismutase 1 or 2, **Srebf1/2:** sterol regulatory element-binding protein 1 or 2, **TAG:** triacylglycerides, **Tnfrsf1b:** tumor necrosis factor receptor superfamily, member 1b, **UCP1:** uncoupling protein 1, **VLDL:** very low density lipoprotein.

Study limitations and future work

Limitations of study:

1. Due to financial constraints, we were unable to investigate the precise gene regulatory network used by PPAG to decrease hyperglycemia-induced apoptosis.
2. To establish a successful animal model for DCM, it is crucial to detect the precise age at which animals develop left ventricular dysfunction. A Tissue Doppler Imaging echocardiography (TDI) would have been a more precise and relevant technique to detect such changes. However, this technique could not be used due to the unavailability of a TDI echocardiography sensitive enough to detect left ventricular dysfunction in rodent animals.
3. The combinational use of metformin with either PPAG or aspalathin was effective in enhancing the cardioprotective effect of these polyphenols *in vitro*. However, the *in vivo* potential of metformin in combination with both PPAG and aspalathin was not investigated.

Future work:

PPAG

- Targeting the role of p53, the known modulator of apoptosis within a diabetic heart.
- We will also make use of an *in vivo* model, e.g. type 2 diabetic (db/db) mice to confirm the protective mechanisms of PPAG in relation to apoptosis.
- We will perform additional studies where we treat db/db mice with a combination of metformin and either PPAG or aspalathin before measuring relevant markers associated with hyperglycemia-induced myocardial injury.

Aspalathin

- Although aspalathin was expected to prevent oxidative-induced injury due to its known abundant antioxidants, an unexpected finding was its effect to act as a lipid lowering agent by significantly decreasing cholesterol related markers such as very low density lipoprotein and triglycerides both in high glucose-induced cardiomyocytes and diabetic mice. Thus, it is of interest to further employ more robust *in vitro* techniques such as lentiviral vector gene silencing to unravel the mechanism linked to its ameliorative effect of lipid toxicity and to confirm such findings in an *in vivo* model.

References

Bai, Y., Cui, W., Xin, Y., Miao, X., Barati, M.T., Zhang, C., Chen, Q., Tan, Y., Cui, T., Zheng, Y., Cai, L., 2013. Prevention by sulforaphane of diabetic cardiomyopathy is associated with up-regulation of Nrf2 expression and transcription activation. J. Mol. Cell Cardiol. 57, 82–95.

Chellan, N., Joubert, E., Strijdom, H., Roux, C., Louw, J., Muller, C.J., 2014. Aqueous extract of unfermented honeybush (*Cyclopia maculata*) attenuates STZ-induced diabetes and β-cell cytotoxicity. Planta Med 80, 622–629.

Dludla, P.V, Muller, C.J.F., Louw, J., Joubert, E., Salie, R., Opoku, A.R., Johnson, R., 2014. The cardioprotective effect of an aqueous extract of fermented rooibos (*Aspalathus linearis*) on cultured cardiomyocytes derived from diabetic rats. Phytomedicine. 21, 595–601.

Ezuruike, U.F., Prieto, J.M., 2014. The use of plants in the traditional management of diabetes in Nigeria: pharmacological and toxicological considerations. 155, 857–924.

International Diabetes Federation (IDF). IDF Diabetes Atlas 7th edition. idf.org [Internet]. Available from: http://www.diabetesatlas.org/. Accessed 12 December 2015.

Jonassen, A.K., Sack, M.N., Mjøs, O.D., Yellon, D.M., 2001. Myocardial protection by insulin at reperfusion requires early administration and is mediated via Akt and p70s6 kinase cell-survival signaling. Circ. Res. 89, 1191–1198.

Kawano, A., Nakamura, H., Hata, S., Minakawa, M., Miura, Y., Yagasaki, K., 2009. Hypoglycemic effect of aspalathin, a rooibos tea component from *Aspalathus linearis*, in type 2 diabetic model db/db mice. Phytomedicine. 16, 437–443.

Lionetti, V., Stanley, W.C., Recchia, F.A., 2011. Modulating fatty acid oxidation in heart failure. Cardiovasc. Res. 90, 202–209.

Mazibuko, S.E., Joubert, E., Johnson, R., Louw, J., Opoku, A.R., Muller, C.J., 2015. Aspalathin improves glucose and lipid metabolism in 3T3-L1 adipocytes exposed to palmitate. Mol. Nutr. Food Res. 59, 2199–2208.

Muller, C.J., Malherbe, C.J., Chellan, N., Yagasaki, K., Miura, Y., Joubert, E., 2016. Potential of rooibos, its major *C*-glucosyl flavonoids and z-2-(β-D-glucopyranoloxy)-3-phenylpropenoic acid in prevention of metabolic syndrome. Crit. Rev. Food Sci. Nutr. In press.

Muller, C.J.F., Joubert, E., De Beer, D., Sanderson, M., Malherbe, C.J., Fey, S.J., Louw, J., 2012. Acute assessment of an aspalathin-enriched green rooibos (*Aspalathus linearis*) extract with hypoglycemic potential. Phytomedicine. 20, 32–39.

Sanderson, M., Mazibuko, S.E., Joubert, E., De Beer, D., Johnson, R., Pheiffer, C., Louw, J., Muller, C.J.F., 2014. Effects of fermented rooibos (*Aspalathus linearis*) on adipocyte differentiation. Phytomedicine. 21, 109–117.

Son, M.J., Minakawa, M., Miura, Y., Yagasaki, K., 2013. Aspalathin improves hyperglycemia and glucose intolerance in obese diabetic ob/ob mice. Eur. J. Nutr. 52, 1607–1619.

Yin, M., van der Horst, I.C.C., van Melle, J.P., Qian, C., van Gilst, W.H., Silljé, H.H.W., de Boer, R.A., 2011. Metformin improves cardiac function in a nondiabetic rat model of post-MI heart failure. Am. J. Physiol. Heart Circ. Physiol. 301, H459–H468.

ADDENDUM 1: Animal ethical clearance

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
jou kennisvennoot • your knowledge partner

Protocol Approval

Date: 16-Sep-2013

PI Name: Johnson, Rabia R

Protocol #: SU-ACUM13-00021

Title: Compounds specific to Aspalathus linearis protects the diabetic heart against oxidative stress: A mechanistic study

Dear Rabia Johnson, the Response to Modifications, was reviewed on 23-Jul-2013 by the Research Ethics Committee: Animal Care and Use via committee review procedures and was approved. Please note that this clearance is only valid for a period of twelve months. Ethics 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: 2008 document is available on the Division for Research Developments website www.sun.ac.za/research.

Please remember to use your protocol number, SU-ACUM13-00021 on any documents or correspondence with the REC: ACU concerning your research protocol.

Please note that the REC: ACU has the prerogative and authority to ask further questions, seek additional information, require further modifications or monitor the conduct of your research.

We wish you the best as you conduct your research.

If you have any questions or need further help, please contact the REC: ACU secretariat at WABEUKES@SUN.AC.ZA or .

Sincerely,

Winston Beukes

REC: ACU Secretariat

Research Ethics Committee: Animal Care and Use