The cumulative effects of hypoxia and hyperglycemia on cardiac oxidative stress

A thesis presented to

The Faculty of Graduate Studies

of

Lakehead University

by

Boran Baregzay

In partial fulfillment of requirements
for the degree of
Master of Science in Biology

September 2012

© Boran Baregzay, 2012

Abstract

Hyperglycaemia-induced oxidative stress plays an important role in cardiomyocyte cell death leading to cardiac dysfunction. Autophagy is an intracellular bulk degradation process and can be induced by stressors such as nutrient depletion and acute ischemia, to promote cell survival. Oxidative stress is an important regulator of autophagy in various pathophysiological conditions such as ischemia/reperfusion injury and hypoxia. The role of autophagy in the oxidative stress tolerance of cardiac cells exposed to simultaneous hyperglycemia and hypoxia has not been studied. The aim of the present study is to determine the role of autophagy in cardiac cells in response to combined hyperglycemia and hypoxia. H9c2 rat cardiac cell lines were grown in DMEM supplemented with standard (5.6 mM), moderately high (25 mM) and high (33 mM) glucose concentrations. The cells were then exposed to hypoxia condition (1% oxygen, O_2) for 24h and 48h using the hypoxia chamber. Cell viability and oxidative stress was measured by the 2,5-diphenyl-2H-tetrazolium bromide (MTT) and 2'7'-dichlorofluorescein (DCF) assays respectively. Apoptosis and autophagy was assessed via Caspatag 3/7 In Situ assay and western blotting. Results obtained demonstrated that high glucose andhypoxia additively reduced H9c2 cell viability. Simultaneous high glucose and hypoxia-induced oxidative stress suppresses autophagy and promotes cell death by apoptosis. A biphasic induction of ROS production, caspase 3/7 activity and PAMPK protein expression, in simultaneous high glucose and hypoxia conditions, calls for further research.

Lay Summary

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia and vascular complications that lead to cardiovascular dysfunction such as heart attack. Increased oxidative stress has been proposed as a molecular mechanism for vascular complications in diabetes mellitus. Autophagy, an intracellular bulk degradation process, occurs at basal levels but can be further induced by stressors such as hypoxia or high glucose to promote cell survival or programmed cell death (PCD), respectively. Our research goal aims to investigate and understand the cumulative effects of hyperglycemia and hypoxia on cardiac oxidative stress, by studying autophagic and PCD markers, in an *in vitro* model. Our results suggest that simultaneous high glucose and hypoxia treatments induce reactive oxygen species production, diminish autophagy and promote PCD. Manipulation of these processes may allow for increased cardiac cell survival and contribute to developing better therapeutic approaches for diabetic individuals. The diversity of life is vast but with each study it might just become a little less complicated.

Acknowledgements

I would like to thank my supervisors, Dr. Neelam Khaper and Dr. Zacharias Suntres. Dr. Suntres, thank you for your confidence in my decisions, your guidance and support during this project. Dr. Khaper, I have been very humbled and fortunate to have you as my mentor, not only during this project but throughout my undergraduate studies. I did a lot of growing up during my time with Dr. Khaper and no one could have been more compassionate and supportive. I would like to thank my committee member and favorite undergraduate professor, Dr. Kam Leung, who played a key role in helping shape my experience at Lakehead University. Special thanks to Dr. Simon Lees for his continued assistance, time and energy in the laboratory. I would also like to thank Eveline Fonseca, Stephanie Puukila, Ashley Cerqueira and Nicole Hawdon for their much appreciated assistance in the laboratory. Thank you to all of the members of the Northern Ontario School of Medicine research laboratory for making this wonderful learning experience one that I will never forget.

Abbreviations

AMPK - Adenosine monophosphate-activated protein kinase

ATP – Adenosine triphosphate

Ang II - Angiotensin II

AIF - Apoptosis inducing factor

LC3 - Autophagosomal membrane specific protein light chain 3

Atg - Autophagy related gene protein

Bax - B- -associated X protein

Bcl - B-cell lymphoma

Bad – B-cell lymphoma-associated death promoter

Bak – B- cell lymphoma -2 homologous antagonist/killer

BNIP3 - Bcl-2/adenovirus E1B 19-kDa protein-interacting protein 3

BASMC - Bovine aortic smooth muscle cells

BSA – Bovine serum albumin

CM-H2DCFDA – 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester

CO_2 - Carbon dioxide

CAT - catalase

ddH_2O – Double-distilled water

Endo - Endonuclease

ECL – Enhanced chemiluminescence

ER - Endoplasmic reticulum

eEF2 - Eukaryotic elongation factor-2

FADD - Fas-associated death domain

FasL – Fas ligand

FasR - Fas receptor

FLICA – Fluorochrome inhibitors of caspases

GEC - Glomerular epithelial cells

GLUT - Glucose transporter

HDAC - Histone deacetylases

HRP – Horseradish peroxidase

H_2O_2 - Hydrogen peroxide

OH• - Hydroxyl radical

HIF – Hypoxia-inducible factor

HRE - Hypoxia response element (5’-RCGTG-3’)

IRE - Inositol-requiring enzyme

IGF - Insulin-like growth factor

IGF-1R - Insulin-like growth factor 1 receptor

I/R – Ischemia/reperfusion

JNK – c-Jun N-terminal kinase

Lamp - Lysosome-associated membrane proteins

LDL - Low-density lipoprotein

mTOR - Mammalian target of rapamycin

mTORC1 - mammalian target of rapamycin complex 1

MAPK – Mitogen-activated protein kinase

MCP – Monocyte chemotactic protein-1

MCPIP – Monocyte chemotactic protein-1-induced protein

MEF - Mouse embryonic fibroblast

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N_2 - Nitrogen

NRVM – Neonatal rat ventricular myocytes

NADPH – Nicotinamide adenine dinucleotide phosphate

NO – Nitric oxide

oxLDL - Oxidized LDL

O_2 - Oxygen

PAS - Phagophore assembly site

PBS – Phosphate buffered saline

PE - Phosphatidylethanolamine

PERK - Protein kinase RNA-activated-like ER kinase

PCD - Programmed cell death

PCD-1 - Programmed cell death type 1

PCD-2 - Programmed cell death type 2

Akt - Protein kinase B

PtdIns3K – Phosphatidylinositol 3-kinase

PUMA - p53 up-regulated modulator of apoptosis

Rheb - Ras homolog enriched in brain

ROS – Reactive oxygen species

RPTEC - Renal proximal tubular epithelial cells

SMAC - Second mitochondria-derived activator of caspases

SDS-PAGE – Sodium docecyl sulfate-polyacrylamide gel electrophoresis

STZ - Streptozotocin

SOD – Superoxide dismutase

$O_2\bullet^-$ - Superoxide radical

TBST – Tris-buffered saline containing 0.1% Tween-20

TSC1/2 - Tuberous sclerosis complex

TNF – Tumour necrosis factor

TNFR1 – Tumour necrosis factor receptor 1

ULK1/2 - Unc-51-like kinase 1 or 2

UPR - Unfolded protein response

VEGF - Vascular endothelial growth factor

VSMC – Vascular smooth muscle cell

XO - Xanthine oxidase

Table of Contents

Abstract……………………………………………………………………………......ii

Lay Summary……………………………………………………………….………...iii

Acknowledgements………………………...…………………………….………...iv

Abbreviations…………………………...……………………………….….….….....v

List of Figures……………………...……………………....….................…….....xiii

Introduction……………………………………………………………...……......

1.1 Diabetes...1

1.2 Oxidative stress and its links to cardiovascular disease...……………….............2

1.2.1 *Diabetic Inflammatory response-induced oxidative stress*.....………4

1.3 Apoptosis..5

1.4 Autophagy...8

1.4.1 *Molecular mechanism of autophagy*...9

1.4.2 *Regulation of autophagy*...11

1.4.3 *Autophagy during ischemia and reperfusion*....................………..12

1.4.4 *Oxidative stress can cause autophagy*...……......…………………......14

1.4.4.1 Ischemia and reperfusion..15

1.5 Mammalian cell cross-talk: autophagy and apoptosis Autophagy. A Cooperative and/or negative feedback relationship.......................................16

1.6 High glucose-induced oxidative stress in apoptosis and autophagy...............17

1.6.1 *High glucose-induced oxidative stress and its role in multiple pathways*...17

1.6.2 *High glucose-induced endoplasmic reticulum stress and programmed cell death*..……......... ………………………………………………20

1.7 The effects of high glucose on hypoxia-induced programmed cell death.......23

1.7.1 *Hypoxia-inducible factor (HIF) -1*..23

1.7.2 *The effect of the hypoxia-inducible factor -1 on cardiac cell survival*...24

1.7.3 *Hypoxia-induced oxidative stress and the hypoxia-inducible factor -1*...25

1.8 The effects of high glucose on hypoxia-induced activation of 5'-AMP-activated protein kinase………………………...31

Objectives ....………......……………………………………….......34

Hypothesis.………........………………………………………………….........................34

Methods………………………….………………………………………………….........

. **1.1** H9c2 ventricular cardiomyocyte cell line.……………………....... ………….35

1.2 Treatment conditions……………………………………………..…........35

1.3 Hypoxia chamber…………………………………………….................36

1.4 Cell viability assay…………..……………………………………..……...37

1.5 Measurement of intracellular reactive oxygen species (ROS)............….......37

1.6 Active caspases 3/7-based apoptosis detection assay………………………..38

1.7 Protein extraction..………… ……………………………….…………….39

1.8 Total protein quantification………………………………………………..40

1.9 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)....41

2.0 Electrophoretic transfer……………………………………………....…...41

2.1 Immunoblotting………………………………………..……………..…42

2.2 Enhanced chemiluminesce and densitometry………………...……………..43

2.3 Statistics…………………………………………………….……………..44

Results…………………………………………………………………….......

1.1 Effect of high glucose and hypoxia on cell viability..45

1.2 Effect of high glucose and hypoxia on oxidative stress in H9c2 cardiomyocytes..45

1.3 Effect of high glucose and hypoxia on active caspase 3 and 7 in H9c2 cardiomyocytes..46

1.4 Effect of high glucose and hypoxia on caspase 3, Bax and Bcl-2 protein

expression..46

1.5 Effect of high glucose and hypoxia on LC3B-II protein expression in H9c2 cardiomyocytes..47

1.6 Effect of high glucose and hypoxia on AMPKα and P-AMPKα (Thr172) protein expression..48

Discussion………………………………………………………………………….....

1.1 High glucose and/or hypoxia reduce cell viability and induce oxidative stress in H9c2 cardiomyocytes..59

1.2 High glucose and/or hypoxia-induced apoptotic cell death in H9c2 cardiomyocytes..62

1.3 High glucose and/or hypoxia-induced autophagy in H9c2 cardiomyocytes.....66

1.4 High glucose and/or hypoxia-induced P-AMPK protein expression.................68

1.5 The possible cumulative effects of hypoxia and hyperglycemia on cardiovascular function...73

Summary/conclusion………………...……………………………..........................77

References…………………………………………………………………….........78

List of Figures

Figure 1. Effect of glucose levels on cell viability.

Figure 2. The cumulative effect of high glucose levels and hypoxia on cell viability.

Figure 3. Effect of high glucose and/or hypoxia on oxidative stress in H9c2 cardiomyocytes.

Figure 4. Effect of high glucose and/or hypoxia on apoptosis in H9c2 cardiomyocytes.

Figure 5. Effect of high glucose and/or hypoxia on caspase 3 protein expression in H9c2 cardiomyocytes.

Figure 6. Effect of high glucose and/or hypoxia on Bax protein expression in H9c2 cardiomyocytes.

Figure 7. Effect of high glucose and/or hypoxia on Bcl-2 protein expression in H9c2 cardiomyocytes.

Figure 8. Effect of high glucose and/or hypoxia on LC3B-II protein expression in H9c2 cardiomyocytes.

Figure 9. Effect of high glucose and/or hypoxia on AMPKα protein expression in H9c2 cardiomyocytes.

Figure 10. Effect of high glucose and/or hypoxia on P-AMPKα (Thr172) protein expression in H9c2 cardiomyocytes.

Introduction

1.1 Diabetes

Diabetes mellitus is a metabolic disorder of multiple etiology characterized by chronic hyperglycemia and a set of micro-vascular and macro-vascular complications that substantially increase morbidity and mortality. Diabetes mellitus type 1 involves defective insulin secretion and systemic hyperglycemia (Matschinsky et al. 2002). Diabetes mellitus type 2 is characterized by two main pathogenic disorders: impaired pancreatic β-cell insulin secretion and peripheral insulin resistance in muscle and liver tissue (Alberti et al. 1998, DeFronzo et al. 1992). Diabetes is characterized by endothelial dysfunction (Caballero et al. 1999), which leads to vascular reactivity related to cardiovascular dysfunction such as myocardial infarction and arrhythmia (Halcox et al. 2002).

Mortality from cardiac diseases is approximately two- to four-fold higher in patients with diabetes than in those who have the same magnitude of vascular diseases without diabetes (Johnstone 2000). Diabetes-induced cell death has been observed in multiple organs *in vivo* (Cai et al. 2000; Alici et al. 2000; Srinivasan et al. 2000). In laboratory settings, young adult animals administered with streptozotocin (STZ), a widely used diabetogenic agent, produce metabolic characteristics that resemble the features of type 1 diabetes mellitus (Islam et al. 2009). The apoptotic rate has been shown to increase in the heart of diabetic patients (Frustaci et al. 2000) and STZ-induced diabetic animals (Fiordaliso et al. 2000). Several studies have shown that hyperglycemia, as an independent risk factor, directly causes cardiac damage leading to diabetic

cardiomyopathy (Singh et al. 2000). STZ-induced diabetic animal heart models display cardiomyocyte apoptosis, compensatory myocardial hypertrophy, and reparative interstitial and perivascular fibrosis at the late phase of diabetic cardiomyopathy (Chatham et al. 1996). Because myocytes rarely proliferate in adult cardiac muscles, the loss of cardiac muscle cells, by apoptosis, would eventually lead to compromised cardiac function such as decreased myocardial performance (due to the loss of contractile tissue) (Swynghedauw 1999) and ventricular dilation (Frustaci et al. 2000). These late-phase changes are believed to result from early responses of myocardium to suddenly increased glucose levels (Johnstone 2000; Chatham et al. 1996, Depre et al. 2000). Early responses of myocardial cells to hyperglycemia include metabolic abnormalities and abnormal expression of genes such as decreased expression of glucose transporter (GLUT)-1, GLUT4, sarcoplasmic calcium cation adenosine triphosphatase and carnitine palmitoyltransferase I (Depre et al. 2000). These early responses consequently result in cardiac cell death (Cai and Kang 2001). Inhibition of increased glucose levels, by insulin supplementation, almost completely prevents myocardial morphological abnormalities and partially inhibits myocardial cell death. This indicates that severe hyperglycemia that needs to reach a threshold may be necessary for the development of early morphological abnormalities, whereas apoptotic cell death may be a dose-dependent response to hyperglycemia (Cai et al. 2002).

1.2 Oxidative stress and its links to cardiovascular disease

Diabetes, coronary artery disease, hypertension, smoking and alcoholism are claimed to account for deviations from redox homeostasis (Diaz-Velez et al. 1996).

Redox homeostasis/balance describes repairing unstable, damaging, reduced, reactive oxygen species (ROS) by a process of reduction (gain of electrons) and oxidation (loss of electrons). Ideally there should be a balance between ROS production and the activities of non-enzymatic and enzymatic antioxidant systems, which strive to reduce ROS concentrations (Gutteridge et al. 2000). ROS are very chemically reactive, they can exist as either as a free radical (species with at least one unpaired electron) such as superoxide ($O_2•^{-}$) and hydroxyl radicals (OH•) or non-radical species such as hydrogen peroxide (H_2O_2). Redox stress however; is when there is an imbalance between ROS and antioxidant capacity, this is caused by an overproduction of ROS either via reduction or oxidation or reduced antioxidant reserves. Oxidative stress involves an imbalance due to an excess of ROS produced through oxidation (Hayden et al. 2002). The accumulation of ROS can implement cell death, for example, H_2O_2 has been observed to encourage adult cardiomyocyte death (Kwon et al. 2003) by apoptosis or necrosis. But lower levels of ROS are cytoprotective (Pandya et al. 2002) and may promote cell proliferation, thus the balance between cell growth and cell death can be altered by the amount of oxidative stress within a cell (Finkel et al. 1998).

Increased oxidative stress has been proposed as a molecular mechanism for vascular complications in diabetes mellitus (Creager 2003; Kang et al. 2002). Patients with type 2 diabetes show increased levels of circulating markers of free radical-induced damage and reduced antioxidant defenses (Farahmand et al. 2003; Davi et al. 2005). Factors such as hyperglycemia and glucose autoxidation have been reported to contribute to increased ROS generation in diabetes (Jay et al. 2006). Increased mitochondrial H_2O_2 production and reduced glutathione levels have been observed to occur in diabetic hearts

(Nishio et al. 2004). Endothelial cells exposed to high glucose levels, relevant to clinical diabetes (25 mM), were found to produce increased $O_2\bullet^-$ levels. Hyperglycemia-induced generation of mitochondrial $O_2\bullet^-$ may be the initial trigger of oxidative stress in diabetes (Aronson 2008). Increased $O_2\bullet^-$ production from non-mitochondrial sources include; nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Li et al. 2006). The activity of this enzyme is found to be elevated in vascular tissue obtained from diabetic patients (Guzik et al. 2002).

Interestingly, inactivation of nitric oxide (NO), a signaling molecule, by ROS plays an important role in reducing NO bioavailability and the development of endothelial dysfunction (Creager 2003; Kang et al. 2002). Increased ROS production also contributes to the development of insulin resistance, while reduced ROS levels result in improved insulin sensitivity (Houstis 2006). Markers of oxidative stress are increased with diabetes and insulin resistance (Urakawa 2003).

Pancreatic β-cells are sensitive to oxidative stress because of low antioxidant enzyme expression such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (Tiedge et al. 1997). Increased β-cell death, in diabetes (Mandrup-Poulsen 2001), has been shown to be due to increased oxidative stress and impaired insulin secretion, both of which can be attenuated by antioxidant (N-acetylcysteine) treatment (Robertson et al. 2003). It has also been predicted that the maladaptive decline in antioxidant enzyme expression or activity may contribute to the progression to heart failure (Sam et al. 2005).

1.2.1 *Diabetic Inflammatory response-induced oxidative stress*

An inflammatory response in the diabetic condition may induce oxidative stress and also contribute to the development of diabetic cardiomyopathy (Khansari et al. 2009). In hyperglycemia, both inflammatory cytokine production by monocytes and binding of inflammatory cells to the endothelium is increased (Rolo and Palmeira 2006; Basta et al. 2004). Type 1 and 2 diabetes has been characterized by intra-myocardial inflammation, increased tumor necrosis factor (TNF) α expression, oxidative stress and myocardial fibrosis. TNFα is known to reduce myocyte contractility and increase tissue fibrosis, contributing to cardiac failure. Anti-TNFα antibody treatment has been shown to significantly ameliorate intramyocardial inflammation and myocardial fibrosis associated with diabetic cardiomyopathy (Westermann et al. 2007).

1.3 Apoptosis

Programmed cell death (PCD) is essential for elimination of damaged and diseased cells, malignant cells and tissue remodeling during development. Cellular components undergo a controlled enzymatic digestion, allowing surrounding tissue to escape excessive damage and consequent inflammation due to release of toxic substances from cells that die in an uncontrolled manner, such as through necrosis. Necrotic cell death involves cell swelling, plasma membrane rupture and occurs when the degree of damage is too high and/or PCD is somehow disturbed. Irreversibly damaged, diseased and nonessential cells can initiate a self-killing program in the form of apoptotic or autophagic cell death, followed by phagocytosis of cellular remains by, for example, macrophages (Edinger and Thompson 2004; Bursch 2004; Green and Kroemer 2004).

Apoptotic and autophagic cell death are known as PCD type 1 (PCD-1) and PCD type 2 (PCD-2), respectively (Edinger and Thompson 2004; Bursch, 2004).

Apoptosis (or PCD-1) is a form of caspase-dependent PCD. It is a highly regulated program of cell death hence this process represents a potential target for therapeutic intervention to prevent heart failure. Apoptosis is activated in cardiomyocytes by multiple stressors that are commonly seen in cardiovascular disease such as cytokine production (Kubota et al. 1997; Bryant et al. 1998), increased oxidative stress (Sayen et al. 2003) and DNA damage (Wang et al. 2001). The major pathways involved in apoptotic signaling in the heart involve the extrinsic (death receptor pathway) and intrinsic (mitochondrial) pathways. Lysosomal enzymes contribute to cell death, suggesting an indirect involvement of autophagy.

The extrinsic pathway is activated via a complex signal transduction from the plasma membrane leading to activation of the caspase cascade. Death receptors belong to the TNF/nerve growth factor receptor superfamily. For example, Fas ligand (FasL) -to-Fas receptor (FasR) binding causes receptor activation and the recruiting of the Fas-associated death domain (FADD). This Fas/FADD complex binds and cleaves to pro-caspase 8 (zymogen) to form active caspase 8. The apoptotic signal is spread through the cell via a caspase cascade, in which downstream executioner caspases (3 and 7) are activated, and the release of cytochrome c by mitochondria (Thorburn 2004).

The primary function of mitochondria is to provide energy for the cell in the form of adenosine triphosphate (ATP) through oxidative phosphorylation. However, mitochondria can also contribute to cell death in response to intracellular stress such as increased oxidative stress, serum deprivation and DNA damage. In response to stress,

mitochondria release several pro-apoptotic factors such as cytochrome c, apoptosis inducing factor (AIF), endonuclease (Endo) G and second mitochondria-derived activator of caspases (SMAC), resulting in the initiation of apoptosis. The intrinsic pathway is regulated by pro- and anti-apoptotic B-cell lymphoma (Bcl)-2 proteins. Anti-apoptotic Bcl-2 proteins include Bcl-2 and Bcl-xl, which promote cell survival. The pro-apoptotic Bcl-2 proteins include Bcl-2–associated X protein (Bax), Bcl-2 homologous antagonist/killer (Bak), Bcl-2/adenovirus E1B 19-kDa protein-interacting protein 3 (BNIP3), Bcl-2-associated death promoter (Bad) and p53 up-regulated modulator of apoptosis (PUMA) (Danial and Korsmeyer 2004; Huang and Strasser 2000).

Pro-apoptotic Bcl-2 proteins, Bax/ Bak, activate cell death by permeabilizing the outer mitochondrial membrane, which releases pro-apoptotic proteins such as cytochrome c and AIF from the intermembrane space (Kubli et al. 2008). Anti-apoptotic Bcl-2 has been reported to protect against cell death by inhibiting activation of pro-apoptotic Bax/ Bak (Gustafsson and Gottlieb 2007). Pro-apoptotic Bcl-2 proteins can sense cellular stress and integrate diverse cell death stimuli. For example, BNIP3 is activated in response to increased oxidative stress (Kubli et al. 2008), whereas Bad is activated in response to growth factor deprivation (Zha et al. 1996). In response to a specific stress, activated downstream Bax and/or Bak go on to form of a pore in the outer mitochondrial membrane, which allows for release of proteins from the inter-membrane space. Activated Bax/Bak can also sequester to and inhibit anti-apoptotic Bcl-2/Bcl-xL (Gustafsson and Gottlieb 2007).

Cytochrome c, an essential enzyme complex from the electron transport chain in mitochondria, bind apoptosis-activating factor 1 proteins to form apoptotic bodies

(apoptosomes), which then initiates a cascade of caspase activation (recruits procaspase 9) and the initiation of apoptosis. The SMAC binds and inhibits the activity of inhibitor of apoptisis proteins and is therefore pro-apoptotic. Both the flavoprotein AIF and Endo G are translocated to the nucleus where they cause DNA fragmentation into fragments of about 50kb and degrade single stranded DNA, respectively (Weinberg 2007). In addition, it has been reported that activated caspase 3 translocates to nucleus where it cleaves the DNA repairing enzyme, poly (ADP-ribose) polymerase and activates Endos which also cleave DNA. These events culminate in apoptotic cell death (Li et al. 1997; Pacher and Szabo 2007; de Boer et al. 2000) Moreover, considering the fact that AIF functions as a nicotinamide adenine dinucleotide oxidase involved in the complex I of the electron transport chain (Vahsen et al. 2004), loss of AIF from mitochondria may cause further mitochondrial dysfunction due to reduction in electron transport capacity. Thus, release of AIF not only initiates apoptosis, but also subsequently induces mitochondrial dysfunction, which will ensure cell death. When caspases are inhibited, injured cells are still able to undergo PCD due to mitochondria-to-nucleus translocation of AIF. This caspase independent pathway also causes DNA fragmentation and chromatin condensation (Daugas et al. 2000).

1.4 Autophagy

Autophagy is an intracellular bulk degradation process whereby cytosolic, long-lived proteins and organelles are degraded and recycled (Levine et al. 2004). Autophagy occurs at basal levels but can be further induced by stresses, such as nutrient depletion (Lum et al. 2005) and acute ischemia (Takagi et al. 2007, Matsui et al. 2007). This

cytoplasmic quality control mechanism removes protein aggregates and damaged organelles (Nakai et al. 2007) by autolysosomal degradation of membrane lipids and proteins generates free fatty acids and amino acids, which can be reused to maintain mitochondrial ATP production and protein synthesis, and promote cell survival. The beneficial role of autophagy has been demonstrated in past studies. Inactivation of the autophagy related gene protein (Atg) 5 in adult mice has been shown to result in myocardial dysfunction (Nakai et al. 2007). However, under certain conditions, autophagy also promotes PCD, examples of such conditions include reperfusion (Takagi et al. 2007, Matsui et al. 2007), high glucose exposure (Younce et al. 2010), prolonged endoplasmic reticulum (ER) stress (Takemura et al. 2006, Younce et al. 2010). Together, these results clearly demonstrate that autophagy could be either protective or detrimental depending on the nature of the stimuli and the levels of autophagy induced (Kang and Avery 2008; Matsui et al. 2007).

1.4.1 *Molecular mechanism of autophagy*

In eukaryotes, the process of autophagy consists of four phases, "induction, vesicle nucleation, vesicle expansion and completion and autolysosome formation" (Chen and Klionsky 2011). The "induction phase" can be initiated by activation of the Atg1 complex or beclin-1 (Atg6) (Gustafsson et al. 2009). Followed by "vesicle nucleation," in which Atg's and lipids are recruited to the phagophore assembly site (PAS) and a Class III phosphatidylinositol 3-kinase (PtdIns3K) complex is activated. Higher eukaryotes have three types of PtdIns3K (class I, II and III) (Chang and Neufeld 2010). Mammalian class III PtdIns3K complex is composed of beclin-1, phosphoinositide-3-kinase class 3

and phosphoinositide-3-kinase, regulatory subunit 4 (Yang and Klionsky 2010). A Class III PtdIns3K complex produces PI3-phosphate, a lipid signaling molecule that is crucial in the early stages of autophagosome nucleation. Thirdly, the "vesicle expansion and completion" phase consists of phagophore double membrane expansion and enclosing the sequestered cytosolic cargos by autophagosome formation (Chen and Klionsky 2011). Autophagosomal membrane specific protein light chain 3 (LC3) or Atg8, a marker for autophagosome membrane, is cleaved by the cysteine protease Atg4 to expose a glycine residue that is then covalently conjugated with phosphatidylethanolamine (PE) thereby allowing membrane association. Lipidation of LC3 converts it from its soluble, cytoplasmic form (LC3-I) to the membrane-bound, autophagosome-associated form (LC3-II), which is required for membrane expansion (Ichimura et al. 2000). Atg proteins such as Atg12, Atg5 and Atg16 are covalently conjugated with each other to form the Atg12–Atg5–Atg16 complex, which is recruited to the forming autophagosomal membrane and required for elongation of the isolation membrane. This third phase is a process that requires the two ubiquitin-like conjugation steps, of Atg5–Atg12 and LC3-II–PE, in order to proceed. Conjugation reactions of Atg12 and Atg8 are catalyzed by the E1-like enzyme, Atg7 (for activation), and the E2-like enzymes, Atg10 (for Atg12) and Atg3 (for Atg8) (Geng and Klionsky 2008). The presence, in the preautophagosome, of a protein complex that includes an autophagy-specific class III PtdIns3K complex is necessary to localize the conjugation systems to the forming autophagosome (Kihara et al. 2001). Lastly, the "autolysosome formation" phase, in which the completed autophagosome fuses with a lysosome to form an autolysosome that degrades the cargos by using degradation and the products are released back into cytosol, through permeases,

for reuse (Klionsky 2007).

1.4.2 *Regulation of autophagy*

A study by Kim et al. (2011) provides molecular insights into how 5'- adenosine monophosphate activated protein kinase (AMPK) and mammalian target of rapamycin complex 1 (mTORC1) regulate autophagy through coordinated phosphorylation of Unc-51-like kinase (Ulk) 1/2, examples of fundamental autophagic machinery. AMPK is shown to sense the cellular energy status and activate Ulk1 kinase by a coordinated cascade. Under glucose starvation (4h), the activated AMPK inhibits mTORC1s Ser 757 phosphorylation of Ulk1, leading to Ulk1-AMPK interaction. AMPK then phosphorylates Ulk1 on Ser 317 and Ser 777, activates Ulk1 kinase and eventually leads to autophagy induction. Although AMPK may phosphorylate additional sites that may contribute to Ulk1 activation, phosphorylation of Ser 317/Ser 777 is required for Ulk1 activation and efficient autophagy induction in response to glucose starvation. By overexpression of Ras homolog enriched in brain (Rheb), the mTORC1 activator, Kim et al. (2011) showed mTORC1 to inhibit Ulk1 activation by phosphorylating Ulk1 Ser 757 and disrupting its interaction with AMPK. However, it is worth noting that inhibition of mTORC1 by amino-acid starvation or rapamycin (mTOR inhibitor) treatment can activate Ulk1 in an AMPK-independent manner as these conditions are sufficient to activate Ulk1 and induce autophagy, but do not activate AMPK (Kim et al. 2011).

Overall different protein complexes assemble in the pre-autophagosome when autophagy is activated. Assembly in the pre-autophagosome of conjugation cascades and phosphorylation systems leads to the nucleation/elongation of the isolation membrane.

Progression of this isolation membrane toward the formation of an autophagosome is regulated through the mammalian target of rapamycin (mTOR)-regulated signaling pathway.

Autophagy is also regulated by insulin, various growth factors and conditions that lead to reduced intracellular levels of ATP, because sequestration, fusion and degradation are energy-dependent mechanisms (Ohshita 2000). The effect of some physiological regulators depends on the tissue analyzed. For example, glucagon and beta-adrenergic agonists inhibit rather than stimulate autophagy in cardiac and skeletal muscle, and the inhibitory effect of amino acids is not observed in pancreatic cells (Telbisz and Kovacs 2000).

1.4.3 *Autophagy during ischemia and reperfusion*

In a model of ischemia/reperfusion (I/R), the cellular ATP content decreases with ischemia and rapidly recovers after reperfusion, causing autophagosome formation to be stimulated serially, in two distinct conditions, namely energy-starved and energy-unstarved conditions (Takagi et al. 2007). Matsui et al. (2007) observed an increase in autophagy levels within 20 minutes of ischemia in the mouse heart *in vivo* and a further increase during the reperfusion phase. Decker and Wildenthal (1980) demonstrated that hypoxic conditions for 40 minutes led to an increase in autophagic vacuoles, and that reperfusion after either 20 or 40 minutes of hypoxia induced a dramatic enhancement of autophagy in the perfused rabbit heart (Decker and Wildenthal 1980). Autophagy has also been observed in human and pig hearts under chronic myocardial ischemia (Elsasser et al. 2004; Yan et al. 2006).

Autophagy may serve primarily to maintain energy production during acute ischemia but switch to clearing up damaged organelles during chronic ischemia or reperfusion (Matsui et al. 2007; Takagi et al. 2007). Autophagy has been shown to be protective, in cardiac myocytes, during glucose deprivation or ischemia (Matsui et al. 2007). During myocardial ischemia (an energy starved state) the activation of AMPK leads to the induction of autophagy (Arad et al. 2007).

AMPK is no longer activated during reperfusion, the further increase in autophagy during the reperfusion phase is unlikely to be mediated by AMPK-dependent mechanisms (Matsui et al. 2007). Beclin-1 upregulation, in heart (Matsui et al. 2007), brain (Chien et al. 2007) and kidney cells (Rami et al. 2008), was found to mediate autophagy in the reperfusion phase. It has been proposed that the AMPK-dependent signaling mechanism mediates autophagy during glucose deprivation or ischemia, whereas in the reperfusion phase, beclin-1 mediates autophagy even after the energy supply and the activity of mTOR are restored (Matsui et al. 2007; Takagi et al. 2007).

The inhibition of beclin-1-dependent autophagy was found to be protective during I/R *in vivo* and cell viability in response to H_2O_2 is increased, *in vitro*. It may be that mTOR (inhibition)-independent and beclin-1-dependent autophagy, when cells are not in a starved condition, could be a detrimental process in contrast to the energy-recovering process during starvation which is essential for survival. However the remarkable upregulation of beclin-1 may mean that over-activation of autophagy could just be detrimental to the cell. Thus, specific modulation of autophagy, such as stimulation of AMPK or inhibition of beclin-1, might be a novel strategy to enhance survival under

ischemia and protect against cardiac myocyte death from reperfusion injury *in vivo* (Matsui et al. 2007; Takagi et al. 2007).

Multiple molecular reasons could explain as to why autophagy is detrimental in the reperfusion phase. Firstly, the hyperactivation of autophagy has been shown to cause cell death (Maiuri et al. 2007). Lysosomal enzymes are upregulated in the heart after myocardial infarction (Mayanskaya et al. 2000). Plus dramatic beclin-1 and BNIP3 induction leads to autophagy at supra physiological levels during reperfusion. Secondly, due to the inhibitory effect of Bcl-2 on beclin-1 activity, whether autophagy leads to cell death or survival may depend on balance between Bcl-2 and beclin-1 (Pattingre et al. 2005). Downregulation of Bcl-2 or upregulation of Bcl-2/Bcl-xL binding proteins, such as BNIP3, during the reperfusion phase would stimulate apoptotic and autophagic cell death via beclin-1 activation (Grunenfelder et al. 2001). Thirdly, common mediators of autophagy and apoptosis may play a role during I/R. For example, calpains, which cleave Atg5 and promote apoptosis, are upregulated during I/R (Pyo et al. 2005).

1.4.4 *Oxidative stress can cause autophagy*

At a low level of oxidative stress, autophagy protects the cell against major harm by degrading damaged mitochondria before cytochrome c release occurs. However, prolonged or severe oxidative stress can damage cellular molecules or organelles and promote cell death via apoptotic and autophagic cell death (Kiffin et al. 2006). Damaged lysosomes are unable to fuse with autophagosomes containing damaged components. Instead these lysosomes release potent hydrolases and enhance the degree of cellular damage. Augmented autophagy increases the sensitivity of cells to oxidative stress and

hence contributes to the damaged lysosome-induced generation of ROS. Inhibitors of autophagy, chloroquine or 3-methyladenine prevent ROS formation (Kubota et al. 2010). In addition, Ha et al. (2010) demonstrated oxidative stress-induced autophagic cell death in cardiac H9c2 cells incubated in H_2O_2 (1 mM, 24h) (Ha et al. 2010). This was reversed by treatment with propofol, an antioxidant, (Ha et al. 2010).

Nutrient deprivation (Scherz-Shouval et al. 2007), I/R, hypoxia (Wu et al. 2009) and responses to cell stress (Azad et al. 2009; Moore et al. 2007) all result in increased ROS generation and oxidative stress, which stimulate autophagy. Prolonged nutrient deprivation increases mitochondrial derived H_2O_2 via a PI3K/beclin-1 dependent pathway. The resultant ATG4 oxidation and inhibition promotes ATG8-PE conjugation and hence increased autophagy (Scherz-Shouval et al. 2007).

1.4.4.1 Ischemia/reperfusion

During ischemia, the enzyme xanthine oxidase (XO) forms and XO substrates (xanthine and hypoxanthine) accumulate (Chambers et al. 1985). During reperfusion when oxygen (O_2) is present, xanthine and hypoxanthine are depleted and XO-mediated $O_2\bullet^-$ generation occurs (Misra et al. 2009). A number of reasons could explain the triggering of autophagy in the reperfusion phase. Even though the lack of ATP is alleviated, autophagy is still triggered. This may be explained by ROS production. Mitochondrial ROS production is amplified by ROS-induced ROS release when electron transport resumes during reperfusion (Zorov 2006). Damaged proteins and organelles and lipid peroxidation in mitochondria, due to ROS, promote autophagy (Djavaheri-Mergny et al. 2006). Autophagy can target CAT for degradation, this causes accumulation of

H_2O_2, hence autophagy and oxidative stress can initiate a positive feedback mechanism and cause autophagic cell death (Scherz-Shouval and Elazar 2007). Another explanation may be due to cellular stresses, such as oxidative stress, that impair ER functions. The accumulation of misfolded proteins leads to ER stress and the unfolded protein response (UPR). If prolonged, this can trigger protective autophagy (Ogata et al. 2006; Kouroku et al. 2007). ER stress and the UPR will be introduced in more detail later.

1.5 Mammalian cell cross-talk: autophagy and apoptosis. A Cooperative and/or negative feedback relationship

This section will focus on the cross-talk between the autophagic and apoptotic pathways. Under certain conditions, autophagy and apoptosis are two independent processes (Eisenberg-Lerner et al. 2009), whereas in other situations, the activation of autophagy inhibits apoptosis (Maiuri et al. 2007) or autophagy occurs upstream of apoptosis (Eisenberg-Lerner et al. 2009).

Both apoptosis and autophagy can cooperate to lead to cell death. They may occur simultaneously and even cooperatively, although one may predominate and therefore hide evidence of the other. Alternatively, the second pathway may only be activated upon the impairment/inhibition of the first pathway, it takes over to ensure efficient cell death. The cooperativity and/or negative feedback imply that there is coordination between the pathways (Eisenberg-Lerner et al. 2009). Etoposide, a cytotoxic chemotherapy drug, has been shown to induce both apoptosis and autophagy, in mouse embryonic fibroblasts (MEFs) (Feng et al. 2005). And autophagy is significantly induced when apoptosis is

blocked by the expression of Bcl-2, following ER stress, in cultured MEFs (Gozuacik et al. 2008).

In some cases, autophagy enhances apoptotic cell death and is required for the latter (Eisenberg-Lerner et al. 2009). Autophagy and apoptosis are sequentially observed in light-damaged retinas in the intact mouse or H_2O_2-treated photoreceptor cells, treatments that induce oxidative stress. Autophagy can function both upstream of apoptosis and independently leads to cell death. Inhibition of apoptosis accelerated the induction of autophagy markers and only partially attenuated cell death. Caspase 3 activation and apoptosis were dependent on the release of cathepsin B from an endosomal/ lysosomal compartment, suggesting a possible molecular link between autophagy and apoptosis (Kunchithapautham et al. 2007).

Under other circumstances, autophagy is restrained by the apoptotic pathway and is evident only when the latter is inhibited (Eisenberg-Lerner et al. 2009). For example, autophagic cell death mediated by the receptor-interacting protein and c-Jun N-terminal kinase (JNK), stress kinase, was activated upon inhibition of caspase 8 (Yu et al. 2004). In all of the diverse situations described above, apoptosis, autophagy or both are used to eliminate the cell (Eisenberg-Lerner et al. 2009).

1.6 High glucose-induced oxidative stress in apoptosis and autophagy

1.6.1 *High glucose-induced oxidative stress and its role in multiple pathways*

High glucose toxicity is an important initiator of cardiovascular disease, contributing to the development of cardiomyocyte death and diabetic complications (Yu

et al. 2008). Increased ROS production is observed in type 1 and type 2 diabetes and is considered to be a major contributing factor in the development of diabetic cardiomyopathy (Desco et al. 2002; San Martin et al. 2007). Multiple pathways have been linked to hyperglycemia-induced oxidative stress. In hyperglycemia associated with diabetes mellitus, there is activation of the polyol pathway, hexosamine pathway, protein kinase C and increased advanced glycation end products (Nishikawa et al. 2000; Farahmand et al. 2003; Defraigne 2005). Under normal metabolic circumstances, 2%–5% of glucose entering a cell is processed through the hexosamine pathway (Marshall et al. 1991). During high glucose exposure, excess glucose is directed into the hexosamine pathway in which the end product is UDP-*N*-acetylglucosamine that acts as substrate for glycosylation of vital intracellular factors (McClain and Crook 1996). Active protein kinase B (Akt) phosphorylates and activates endothelial NO synthase (eNOS). The hexosamine pathway attenuates Akt and impairs eNOS activity (Federici et al. 2002). Chemical inhibition of NOS in diabetic and nondiabetic rats increases sorbitol accumulation in the aorta by the polyol pathway, while treatment with L-arginine (an NO precursor) or nitroglycerine prevents this accumulation. This suggests that increased NO production or bioavailability may protect against diabetes-induced complications in the polyol pathway (Ramana et al. 2003). Inappropriate activation of these important regulatory molecules can have deleterious effects on cellular functions and is thought to contribute to the pathogenesis of various diabetic vascular complications (Son 2007).

Atherogenic mechanisms also appear to be involved in pathophysiology of diabetes-associated heart disease. Several atherogenic mechanisms, such as low-density lipoprotein (LDL) oxidation, endothelial dysfunction, and vascular smooth muscle cell

(VSMC) proliferation and migration, could be induced by oxidative stress, leading to cardiomyopathy. There is an upregulation of lectin-like oxidized LDL (oxLDL) receptor in hyperglycemic conditions, which enhances the effect of oxLDL signaling and facilitates foam cell formation from macrophages (Chakravarti et al. 1991). Hyperglycemia-induced ROS have also been shown to increase monocyte adhesion, levels of adhesion molecules, VSMC migration and the secretion of platelet derived growth factor (Devaraj et al. 2004).

Increased oxidative stress may lead to the formation of gene products that cause cellular damage, thereby contributing to the pathogenesis of various diabetic vascular complications (Son 2007; Newsholme et al. 2007). Tyrosine phosphatases, encoded by more than 100 genes in the human genome, play an important role in tissue inflammation, cell development, cellular growth, and control of cell survival involving oxidative stress (Chong and Maiese 2007). Two of these phosphatases, Anti-Src Homology Phosphatases 1 and 2 are important for many growth factors and metabolic pathways implicated in certain diseases, such as diabetes, cancer, and neurodegenerative disorders. In cultured VSMC's exposed to high glucose levels as well as in vascular tissues from animals and patients with diabetes, there were significantly increased levels of oxidants such as glycated compounds, oxLDL, $O_2{\bullet}^-$, nitrotyrosine and markers of oxidative changes in proteins, lipids, and DNA (Son 2007).

High glucose is known to lead to the increased production and activation of angiotensin II (Ang II) signaling pathways in cardiomyoyctes (Singh et al. 2007). Activation of Ang II signaling has been shown to result in monocyte chemotactic protein (MCP) -1 production, a pro-inflammatory cytokine, in cardiac fibroblasts (Omura et al.

2004). Like MCP-1, glucose-induced Ang II can induce NADPH oxidase-mediated ROS production (Kimura et al. 2005).

In addition, Yu et al. (2010), demonstrated the induction of apoptosis, *in vitro*, in H9c2 cells incubated in high glucose (25 mM, after 96h), with time-dependent DNA fragmentation and cell loss. Insulin-like growth factor 1 (IGF-1), a survival factor, exerts its bioactivity via binding to IGF-1 receptor (IGF-1R). Insulin-like growth factor 1 receptor-dependent signaling is crucial for the survival of many cell types, including rat cardiomyocyte H9c2's (Yu et al. 2008). Histone de-acetylation, by histone deacetylases (HDACs), down regulates gene expression (Juan et al. 2000). The tumour suppressor protein, p53, is a critical regulator of apoptosis and transmits signals to genes that control apoptosis when cells are under stress. Several studies indicate that IGF-1R expression is regulated by p53 (Liu et al. 2009). High glucose treatment repressed IGF-1R transcription and protein expression after 24h and 48h respectively. It is proposed that this high glucose-induced IGF-1R repression is mediated by p53 and IGF-1R promoter association and by the subsequent high glucose-dependent p53 enhanced recruitment of chromatin-modifying proteins, such as HDAC1, to the IGF-1R promoter–p53 complex. Transcriptional repression of IGF-1R promoter results due to the de-acetylation and prevention of histone-4 associating with IGF-1R promoter. This suggests that enhancing acetylation of histone 4 and IGF-1R expression may be a useful strategy to prevent diabetic cardiomyopathy (Yu et al. 2010).

1.6.2 *High glucose-induced endoplasmic reticulum stress and programmed cell death*

High glucose exposure (28 mM D-glucose) can induce ER stress in H9c2 cardiomyocytes (Younce et al. 2010; Younce et al. 2010i). The ER is responsible for the synthesis and folding of newly translated protein. ER stress is when the balance between the import of new unfolded proteins and the secretion of folded mature proteins is disturbed. Cells initially cope with ER stress through the UPR, a mechanism which attempts to relieve the ER burden by inhibiting global protein translation and thereby reducing the amount of proteins entering the ER, increasing degradation of misfolded/unfolded proteins and enhancing the ER folding capacity (upregulating protein chaperones). These UPR responses are mediated, respectively, through activation of the ER-resident transmembrane proteins, protein kinase RNA-activated-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6) (Jing et al. 2012).

ER stress-induced cardiomyoycte apoptosis has been reported to occur in the hearts of diabetic animals (Li et al., 2008). Oxidative stress, hyperglycemia, hypoxia and inflammatory factors can disturb ER function and compromise the adaptive UPR, resulting in persistent ER stress and eventually apoptotic cell death (Jing et al. 2012). When the UPR is prolonged, apoptosis is activated via the pro-apoptotic transcription factor CCAAT/enhancer binding protein- homologous protein (CHOP), JNK and/or caspase 4-mediated mechanisms (Szegezdi et al. 2003; Schroder et al. 2005). IRE1α induces stress kinases JNK and p38, which in turn can result in an increase in pro-apoptotic proteins such as p53 and PUMA and eventually cell death (Zhang et al. 2008; Younce et al. 2010i).

Prolonged ER stress is also known to cause the induction of autophagy that is known to be involved in cardiovascular diseases (Takemura et al. 2006). Although autophagy is thought at first to protect the cell from increased stress (Boya et al. 2005) it is known that prolonged autophagy can lead to cell death (Martin et al. 2004). In mammalian cells the accumulation of misfolded proteins leads to the activation of ER stress sensors, such as PERK and IRE1, which stimulates protective autophagy (Ogata et al. 2006; Kouroku et al. 2007).

Diabetes is associated with elevated serum levels of MCP-1, a pro-inflammatory cytokine (Mine et al. 2006). MCP-1 induces a novel zinc-finger protein, MCP-1-induced protein (MCPIP) (Zhou et al. 2006). MCPIP-induced ROS/reactive nitrogen species production induces ER stress, which leads to autophagy and apoptosis through caspase 2 and 12, ER-related caspases and an IRE1α -JNK/p38-p53-PUMA pathway (Younce et al. 2010i).

2-Deoxy-D-glucose (2-DG) is a glucose analog that interferes with glycolysis by blocking the first two reactions in this pathway carried out by hexokinase and phospho-glucose isomerase (Wick et al. 1957). Due to its structural similarity to mannose, 2-DG interferes with oligosaccharide synthesis leading to abnormal N-linked glycosylation (Datema et al. 1978) resulting in ER stress. Xi et al. (2010), showed that in aerobic conditions, autophagy is upregulated in multiple human cancer cell lines in response to 2-DG and is accompanied by ER stress and UPR signaling as well as decreased cellular ATP levels. In contrast they showed that cells grown under anaerobic conditions undergo a more severe drop in ATP levels, 2-DG decreases autophagy even below basal levels, accompanied by a decrease in the ER stress/UPR marker, glucose-regulated protein (Grp)

78, an ER lumen protein. Their study also demonstrated that the autophagy was of a protective nature. The upregulation of autophagy promoted cancer cell survival against 2-DG-induced cytotoxicity by reducing 2-DG-induced ER stress. Inhibition of 2-DG-induced autophagy leads to increased levels of ER stress/UPR markers as well as apoptosis (Xi et al. 2010).

1.7 The effects of high glucose on hypoxia-induced programmed cell death

Under hypoxia and anaerobic conditions, oxidative phosphorylation in mitochondria is inhibited or reduced and hence cells rely mainly on glycolysis for energy production (Maher et al. 2004). Hypoxia results in increased ROS generation and oxidative stress, which stimulate caspase-dependent (apoptosis) (Shimizu et al. 1996) and caspase-independent PCD (autophagy) (Matsui et al. 2008; Wu et al. 2009).

1.7.1 *The hypoxia-inducible factor (HIF)-1*

The hypoxia-induced O_2 sensing transcription factor, HIF-1, can be influenced by cellular ROS generation and highly regulated by cellular O_2 tension. The HIF-1 is found to be consisting of a dimer of subunits HIF-1α and HIF-1β. In normal conditions, HIF-1α is hydroxylated at the proline residues (402 and 564), catalyzed by O_2-dependent prolyl hydroxylase enzymes (Hirota and Semenza 2005; Jaakkola et al. 2001). The tumor suppressor von Hippel-Lindau protein specifically binds with hydroxylated HIF-1α and promotes its ubiquitylation and proteolysis (Jaakkola et al. 2001), whereas HIF-1β is found to be constitutively expressed. In response to hypoxia, HIF-1α is not hydroxylated at the proline residues and therefore is stabilized. The stabilized HIF-1α translocates into

the nucleus and binds to HIF-1β and thus forms the HIF-1 complex, which binds to hypoxia response element (HRE, 5'-RCGTG-3') to increase the transcription of pro-survival-hypoxia-regulated genes by causing their transactivation. Examples of such hypoxia-regulated genes include vascular endothelial growth factor (VEGF) and hence angiogenesis, GLUT1 thereby enhancing glycolysis and erythropoietin for erythropoiesis (Hirota and Semenza 2005). HIF-1α is thought to be a crucial regulator of hypoxia-adaptational responses (Semenza 2000).

1.7.2 *The effect of the hypoxia-inducible factor -1 on cardiac cell survival*

Both hyperglycemia and hypoxia have been suggested to be important causative factors for diabetic retinopathy (Bursell et al. 1996), neuropathy (Newrick et al. 1986), arteriosclerosis (Santilli et al. 1993). High glucose (22 mM) levels suppress serum deprivation-induced apoptosis in VSMC by up-regulating expression of Bcl-2 and Bcl-xL, suggesting that enhanced expression of anti-apoptotic proteins may play an important role in the development of macrovascular complications in diabetes (Li et al. 2005). During hypoxia, cells shift to a mainly glycolytic form of metabolism by up-regulating HIF-1α-dependent glycolytic genes such as GLUT-1 transporters (Semenza 2003), phosphoglycerate kinase 1 (Chen et al. 2001) and hexokinase 1 (Gao et al. 2004), thereby decreasing intracellular pH and increasing lactic acid accumulation that triggers apoptosis (Schmaltz et al. 1998).

In addition, Malhotra et al. (2008) showed that HIF-1α potentiates apoptosis under hypoxic conditions in rat H9c2 cells. Hypoxia-induced apoptosis in H9c2 cells were found to be markedly suppressed by knockdown of HIF-1α. Overexpression of wild

type HIF-1α had little effect in H9c2 cells, suggesting that levels of HIF-1α in these cells during hypoxia were already sufficient to maximally trigger apoptosis. In H9c2 cells overexpression of HIF-1α had little effects on apoptosis, but reduction of HIF-1α inhibited apoptosis. Expression of HIF-1α is also associated with enhanced Bax activation in these systems thereby providing a mechanistic link between hypoxic stress and apoptosis in H9c2 cells (Malhotra et al. 2008).

Hypoxia has also been shown to activate the UPR in cardiac myocytes (Thuerauf et al. 2006) which may potentially protect the myocardium during hypoxic stress, but in the case of prolonged or unresolved ER stress the UPR can also activate apoptotic (Rutkowski et al. 2004) or autophagic cell death (Younce et al. 2010i).

However the opposite effects have also been demonstrated, enhanced glycolytic metabolism and GLUT1 expression have been shown to markedly attenuate hypoxia-induced apoptosis via reduced mitochondrial release of cytochrome c and activation of caspase 9 (Malhotra and Brosius 1999; Lin et al. 2000). Enhanced glycolytic metabolism and GLUT1 expression also suppress levels of HIF-1α in hypoxic cardiomyocytes (H9c2 cells) due to enhanced HIF-1α proteosomal degradation (Malhotra 2002).

1.7.3 *Hypoxia-induced oxidative stress and the hypoxia-inducible factor -1*

It has been reported HIF-1 activity is influenced by cellular ROS generation. Mitochondria produce a burst of ROS in response to hypoxia and that this burst is necessary and sufficient to activate HIF-1 (Guzy et al. 2005; Mansfield et al. 2005). In addition to hypoxic conditions, HIF-1 is also activated in normoxic conditions by physiological stimuli like insulin (Zelzer et al. 1998). Insulin is known to increases

intracellular ROS generation to regulate the post-translational translocation of GLUT4 (Mahadevet al. 2001), it also increases the expression of GLUT1 and phosphoglycerate kinase-1. Insulin increases the transcription of many of its target genes, such as VEGF, Epo and endothelin-1 by activating HIF-1 (Hirota and Semenza 2005; Zelzer et al. 1998). Biswas et al. (2007) demonstrated that insulin-stimulated ROS generation, particularly H_2O_2, by NADPH oxidase is essential for the activation of HIF-1 in insulin-sensitive hepatic cells HepG2 and cardiac myoblasts H9c2. They also found that, in HepG2 cells, the NADPH oxidase-generated ROS regulates phosphorylation of tyrosine residues of insulin receptor tyrosine kinases as well as PtdIns3K activity, essential for HIF-1 activation and subsequent expression of genes like VEGF and GLUT1 (Biswas et al. 2007).

The HIF-1α exerts both anti-apoptotic and pro-apoptotic effects, depending on the cell type (Piret et al. 2002). Activation of HIF-1α by a redox-senstive pathway has also been shown to promote mitochondrial cell death pathway by upregulating BNIP3 (Zhang et al. 2007), Bad and Bax (Bruick 2000; Merighi et al. 2007; chen et al. 2005). BNIP3, a Bcl-2 pro-apoptotic protein, was identified to contain the binding sites for HIF-1α in its promoter region (Sowter et al. 2001). The BNIP3 gene product induces apoptosis by binding and inhibiting the anti-apoptotic proteins Bcl-2 and Bcl-xL (Greijer and Wall 2004). Interestingly, BNIP3 is localized at the mitochondria. In cases of prolonged hypoxia, the HIF-1 complex promotes mitochondrial apoptotic cell death in ventricular cardiac myocytes by significant induction of BNIP3 (Regula and Kirshenbaum 2002) or by stabilizing the tumor suppressor protein p53 (An et al. 1998). In addition to apoptosis, BNIP3 can induce autophagy by inducing mitochondrial dysfunction, activating beclin-1

activity by titrating Bcl-2 and/or Bcl-XL away from beclin-1 (Tracy et al. 2007) or by binding and inhibiting Rheb, the promoter of mTOR activity (Li Y et al. 2007).

Zhou et al. (2010) studied apoptosis in primary neonatal rat ventricular myocytes (NRVM) which were subjected to 24h of hypoxia. Their study also showed hypoxia to increase the expression levels of both HIF-1α and pro-apoptotic protein BNIP3 and hence the rate of apoptosis. The degree of both HIF-1α expression and apoptosis in hypoxia exposed NRVM depends on the degree of hypoxia. When HIF-1α activity is inhibited by 3-(5′-hydroxymethyl-2 - furyl)-1-benzyl indazole (YC-1) treatment, the level of HIF-1α expression decreased. There was also a corresponding decrease in the level of BNIP3 protein expression and the hypoxia-induced rate of apoptosis. This demonstrates that HIF-1α mediated apoptosis in primary NRVM cultured under acute hypoxic conditions, and that the pro-apoptotic protein BNIP3 may be one of the key molecules involved in this effect of HIF-1α (Zhou et al. 2010).

Ornoy et al. (2010) found that 5 and 7 mg glucose/ml (27.8 mM and 38.9 mM glucose, respectively) culture medium produces embryonic damage and enhanced oxidative stress as well as increased HIF-1α in 10.5-day-old rat embryos and their yolk sacs, thus pointing to the possibility that hyperglycemia may also cause hypoxia. In addition, reduced O_2 levels in the culture medium to 15 or 10% (instead of the normoxic 20%) also induces anomalies in the cultured embryos and, similarly to hyperglycemia, also increases HIF-1α levels in the malformed embryos and their yolk sacs (Ornoy et al.2010).

It is clear that hyperglycemia can induce oxidative and nitrosative stress in many cell types (Yoh et al. 2008). Diabetic state is associated with increased oxidative

stress, which plays an important role in the development of diabetic complications. Indeed, several studies have reported that hyperglycemia stimulates the production of advanced glycosylated end products, activates protein kinase C, and enhances the polyol pathway leading to increased $O_2 \bullet^-$ formation (Brownlee 2001; Ceriello 2003). $O_2 \bullet^-$ interacts with NO, forming the potent cytotoxin peroxynitrite, which attacks various biomolecules in the vascular endothelium, VSM and myocardium, leading to cardiovascular dysfunction (Pacher and Szabó 2006).

Moreover, high glucose-induced increase in ROS can attenuate the activity of HIF-1α, under hypoxic conditions. In contrast in normoxia, ROS can stabilize HIF-1 and enhance HIF-1- dependent gene expression (Li et al. 2010). Gao et al. (2007) examined the effects of hyperglycemia (25 mM D-glucose and 5.5 mM control, 5 days) in VSMCs, under normoxic (5% carbon dioxide (CO_2), 95% air) and chronic hypoxic (2% O_2, 5% CO_2, 93% nitrogen (N_2)) conditions. Gao et al. (2007) observed high glucose (25 mM) levels (mimicking hyperglycemia of diabetes) to attenuate hypoxia-induced apoptosis in bovine aortic smooth muscle cells (BASMC). Hypoxia caused a decrease in anti-apoptotic protein, Bcl-xL expression but this was attenuated by simultaneous high glucose (25 mM) and hypoxia. Both pro-apoptotic protein BNIP3L (Bcl-xL suppressor) and HIF-1α expression is increased hypoxia but then attenuated in simultaneous high glucose (25 mM) and hypoxia conditions. This reduction in HIF-1α resulted in a diminished level of HRE-dependent promoter activity concomitant with a decrease in BASMC apoptosis. These data confirm the important link between hyperglycemia and hypoxia since the glucose-mediated recovery from hypoxia-induced apoptosis was significantly attenuated following HIF-1α knockdown (Gao et al., 2007). Mammalian

cells require a constant supply of O_2 to maintain energy balance and sustained hypoxia can result in cell death (Hochachka et al. 1996). In addition, blood glucose has been shown to be in linear relation with fatal outcome after an acute hypoxic challenge, such as acute myocardial infarction (Malmberg et al. 1999). Taken together these studies suggest a potential deleterious influence of hyperglycemia on the tissue's capacity to adapt to low O_2 tensions and that hyperglycemia interferes with the growth response of VSMC and cardiomyocytes to hypoxia, further suggesting a new mechanism for the development of chronic vascular complications of diabetes.

As mentioned earlier VEGF is a hypoxia-regulated gene (Hirota and Semenza 2005). Kim et al. (2002) studied VEGF production by using human renal proximal tubular epithelial cells (RPTEC) and rat glomerular epithelial cells (GEC) exposed to hypoxia (24h) and/or high D-glucose (30 mM, 24h). Their control cells were cultured under normoxic and euglycemic (5 mM) conditions (Kim et al. 2002). Kim et al. (2002) found that VEGF mRNA and protein expression after hypoxia were increased in both RPTEC and GEC and it's suggested that it was this VEGF induction that enhanced capillary formation in culture (Kim et al. 2002). Hypoxia is a well-established potent stimulus for VEGF production in some cells, acting by inducing transcription of VEGF gene and stabilization of VEGF mRNA (Minchenko et al. 1994; Levy et al. 1996). GEC incubated in high glucose showed an increase of VEGF protein expression only, whereas there was an increase of both VEGF mRNA and protein expression in RPTEC. When high D-glucose and hypoxia were combined, VEGF mRNA and protein expression was higher than control in both cell types (except that mRNA was unchanged in GEC) but there were no additive effects of high D-glucose and hypoxia. Overall, Kim et al. (2002)

showed VEGF production is dramatically upregulated by hypoxia and by high glucose alone and that VEGF secreted by proximal tubular epithelial cells support angiogenesis in culture. They also observed that a hyperglycemic, although also stimulatory for VEGF production, fails to augment angiogenesis environment, suppresses hypoxia-induced angiogenesis (Kim et al. 2002).

An interesting question is whether hyperglycemia produces hypoxia through the known changes in glucose metabolism or hypoxia is the result of hyperglycemia-produced oxidative stress. The induction of diabetes has also been shown to produce arterial wall hypoxia preceding the formation of atherosclerotic lesions in an animal model of diabetes (Santilli et al. 1993). This observation suggests a role for local hypoxia in the initiation and/or development of vascular disease in diabetes (Bjornheden et al. 1999). Chandel et al. (1998) noted, while using wild-type Hep3B cells, that mitochondrial enhanced oxidative stress (as observed in diabetes) trigger hypoxia-induced transcription of several genes, thus pointing to the possibility that oxidative stress causes hypoxia. Catrina et al. (2004) showed, using cultured adult human dermal fibroblasts cells and human dermal microvascular cells, that elevated glucose concentrations in the culture medium inhibited, in a dose dependent manner, the upregulation of HIF-1α induced by hypoxia (1% O_2) (Catrina et al. 2004). Moreover, when they studied by immunohistochemistry the level of HIF-1α in skin biopsies from ulcers (thus suffering from hypoxia) in chronic diabetics or following venous thrombosis in non-diabetic patients, they found lower HIF1α levels in the biopsies from the diabetic patients compared to the skin from venous thrombosis ulcers (Catrina et al. 2004). Taken together, these findings imply that diabetic hyperglycemia may cause hypoxia and

excascerbate hyperglycemia-induced damage. High glucose levels, in addition to causing hypoxia may also interfere with the normal response to hypoxia.

1.8 The effects of high glucose on hypoxia-induced activation of 5'-Adenosine monophosphate protein kinase

The AMPK, a serine-threonine kinase, is a heterotrimer protein composed of three subunits, alpha, beta and gamma (Hardie et al. 2003). This kinase, which appears to function as a "sensor" of the energy status of cells, becomes phosphorylated and activated in response to conditions associated with increased intracellular AMP concentration (decreased ATP), changes that are present during hypoxia or inhibition of oxidative phosphorylation (Abbud et al. 2000; Corton et al. 1994).

The α-subunit is the essential part of AMPK with kinase activity. The kinase domain is on the N-terminus of the α-subunit and the β-subunit-binding domain is on the C-terminus. If a Thr172 in the kinase domain is phosphorylated by an upstream kinase (AMPK kinase), kinase activity is increased (Hardie et al. 2005). Dephosphorylation by protein phosphatase-2C inactivates AMPK. In the α-subunit, there is an auto-inhibitory domain that inhibits its kinase activation. The β-subunit is the binding platform between α and γ subunits (Nagata and Hirata 2010; Steinberg et al. 2006). The γ-subunit has four cystathionine β-synthase (CBS) domains having avidity with AMP/ATP. If γ binds to ATP, it enters a locked state. But the binding to AMP unlocks γ and a Thr172 is phosphorylated by an upstream AMPK kinase, this activates the AMPK trimer complex (Hardie et al. 2005; Nagata and Hirata 2010).

Hypoxia and ROS are potent activators of AMPK (Choi et al. 2001). In mammals, ATP depletion inhibits mTOR (Dennis et al. 2001) through activation of AMPK and subsequent phosphorylation of tuberous sclerosis complex (TSC1/2) (Inoki et al. 2003). The class I PtdIns3K/mTOR pathway is a negative regulator of autophagy in mammalian cells (Ravikumar et al. 2004). While Ha et al. (2010) demonstrated oxidative stress-induced autophagic cell death in H9c2 cells was reversed by propofol, an antioxidant, treatment via inhibition of oxidative stress-dependent JNK and AMPK activation (Ha et al. 2010).

Activated AMPK also decreases protein synthesis and inhibits cell growth and hypertrophy (Inoki et al. 2003). In anoxic cardiac myocytes, however, activation of AMPK leads to an inhibition of protein synthesis through phosphorylation of eukaryotic elongation factor-2 (eEF2) rather than by the inhibition of mTOR (Horman et al. 2003). Since eEF2 kinase, which phosphorylates eEF2, regulates autophagy (Wu et al. 2006) ischemia-induced autophagy may be mediated through the AMPK-eEF2 kinase pathway rather than through the AMPK-induced inhibition of mTOR. AMPK may also stabilize p27 (cyclin-dependent kinase) through phosphorylation, which in turn mediates autophagy (Liang et al. 2007). Since the AMPK α2 subunit contains a putative nuclear localization signal (Arad et al. 2007) it is possible that AMPK has direct effects upon the transcription of genes involved in autophagy (Takagi et al. 2007).

Under conditions of stress, such as excessive load or ischemia, AMPK activates the glycolytic pathway by phosphorylating and activating phosphofructokinase-2 (Marsin et al. 2000) and enhancing fatty acid β-oxidation (Kudo et al. 1995). Furthermore, it promotes the translocation of GLUT-4 to the plasma membrane and increases the uptake

of glucose to the skeletal muscle to ameliorate relative ATP deficiencies (Russell et al. 1999). Diabetes remedies, such as metformin or thiazolidine derivates, can activate AMPK (Fryer et al. 2002), suggesting the possibility that AMPK can become a therapeutic target of metabolic abnormalities.

Objectives

There have been no studies to date investigating the cumulative effects of hyperglycemia and hypoxia on cardiac oxidative stress. The role of apoptosis and autophagy in conditions of simultaneous hyperglycemia and hypoxia in cardiac H9c2 cells is still unclear. This study aims to better understand the mechanism(s) of oxidative stress-induced cardiac damage in a hyperglycemia and hypoxia model, multiple markers of oxidative stress, apoptosis and autophagy were investigated.

Hypothesis

In this study we hypothesize that increased oxidative stress in high glucose and/or hypoxia conditions will correlate with increased apoptosis and a decrease in autophagy and cell viability.

Methods

1.1 H9c2 ventricular cardiomyocyte cell line

Rat cardiac ventricular cells (H9c2) were obtained from the American Type Culture Collection (Manassas, VA) and grown in Low Glucose Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone, Logan, Utah), supplemented with 10% fetal bovine serum albumin (Hyclone, Pittsburgh, PA) and 1% antibiotic-antimycotic (Gibco, Aukland, NZ). All H9c2 cells were cultured at 37°C, 5% CO_2 and used for experimentation upon reaching approximately 80 to 90% confluence. This immortalized cell line has been shown to preserve several characteristics of the electrical and hormonal signaling pathways found in adult cardiomyocytes and hence make a useful model for studying various aspects of cell physiology and pathogenic physiology (Hescheler et al. 1991). All experiments were completed with cultures between passage 60 and 72.

1.2 Treatment conditions

High glucose treatments were made by adding the appropriate amounts of D-glucose (Fisher, Fair Lawn, NJ) to serum- and antibiotic-free Low Glucose DMEM (Hyclone, Logan, Utah), followed by filter sterilization. The glucose concentration of the control treatment corresponds to a normal physiologic glucose condition and the glucose concentration of the high glucose treatment mimics the condition of diabetics in highly poor glycemic control (Sainio et al. 2010; Gao et al. 2007).

The following lists the treatment conditions used:

- *Control group;* H9c2 cells incubated in 5.6 mM D-glucose, serum- and antibiotic-free

DMEM, in normoxic (21% O_2) conditions for 24h.

- *High glucose group;* H9c2 cells incubated in 25 mM or 33 mM D-glucose (Fisher, Fair Lawn, NJ), serum- and antibiotic-free DMEM, in normoxic (21% O_2) conditions for 24h.
- *Hypoxia group;* H9c2 cells incubated in 5.6 mM D-glucose, serum- and antibiotic-free DMEM, in hypoxic conditions ($O_2/N_2/CO_2$; 1:94:5) for 24h.
- *High glucose and hypoxia group;* H9c2 cells incubated in 25 mM and 33 mM D-glucose, serum- and antibiotic-free DMEM, in hypoxic conditions ($O_2/N_2/CO_2$; 1:94:5) for 24h.

1.3 Hypoxia treatment

The hypoxia, glove box, chamber was humidified, with built in O_2 and CO_2 sensors and an automatic controller (Coy, Grass Lake, MI, USA). This hypoxia chamber was used for all hypoxia treatments. The controllers were set to 37°C, 5% CO_2 and 1% O_2. A separate, interior, humidified tissue culture chamber acted as the incubator. This inner chamber was covered with aluminum foil to protect cells incubating in light sensitive media/reagents. All interior surfaces, including the shelves in the inner chamber, were routinely wiped and sterilized with ethanol. Cell cultures were carefully taken in/out of the hypoxia chamber via a purge-able airlock chamber. O_2 levels became negligible (very close to 0%) within the airlock chamber after a 115 second purge. This assured that O_2 levels did not fluctuate by more than 1% O_2, during the addition or removal of cell cultures from the chamber.

1.4 Cell viability assay

Cell viability was measured using a quantitative colorimetric assay with thiazolyl blue tetrazolium bromide (MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). MTT reagent is converted to purple formazan crystals in living cells, thereby showing the mitochondrial activity. It is a light sensitive, *in vitro* assay for the measurement of cell proliferation or, when metabolic events lead to apoptosis or necrosis, a reduction in cell viability. H9c2 cells (1 × 10□) were seeded onto a sterile flat-bottomed, 96-well tissue culture plate (Corning) and incubated overnight to achieve the desired confluence. Then plated cells were exposed to normoxic (21% O_2) or hypoxic (1% O_2) conditions at various D-glucose concentrations (5.6, 25, 33 mM) in serum- and antibiotic-free media. With t = 4h of treatment time remaining, MTT reagent (Sigma, St. Louis, MO, USA) was added to each well to achieve a final MTT concentration of 10% (v/v), and cells were incubated at 37°C for an additional 4h. Following this, the media was removed and the reaction was terminated by adding 50 μL of dimethylsulfoxide (DMSO) per well and placed on a Belly Dancer shaker (Stovall, Greensboro, NC, USA) for 10 minutes (highest setting) to solubilize the formazan crystals. Absorbance was measured, using a PowerWave XS Microplate Spectrophotometer (BioTek, Winooski, VT, USA), at wavelengths 570 nm and 650 nm (correction wavelength).

1.5 Measurement of intracellular reactive oxygen species (ROS)

The DCF assay measures the levels of intracellular ROS. Cells are incubated with the CM-H2DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester) which can diffuse through the cell membrane. This molecule remains non-

fluorescent until the acetate groups are removed by intracellular esterases as oxidation occurs within the cell. In addition, esterase cleavage yields a charged form of the dye that is unable to leave the cell. Reaction with ROS, primarily H_2O_2, results in the fluorescent molecule DCF. This DCF fluorescence can be used as a measure for intracellular ROS levels. Cells were seeded onto sterile flat-bottom 25 cm^2 culture flasks (Corning) and grown overnight to achieve the desired confluence. Plated cells were subjected to 24h of normoxia (21% O_2) or hypoxia (1% O_2) at various D-glucose concentrations (5.6, 25, 33 mM) in serum- and antibiotic-free media. Following treatment, cells were washed with phosphate buffered saline (PBS) and stained for 30 minutes with CM-H2DCFDA (Molecular Probes, Eugene) in normoxia. Stained cells were washed with PBS and detached from the plate surface using trypsin (Fisher Scientific) and suspended in PBS for flow cytometric analysis using the FL1-H channel of a BD FACSCalibur Flow Cytometer (BD Biosciences) supported by BD CellQuest Pro Software. A minimum of 1 x 10^6 gated events were acquired per trial. Geometric mean fluorescence is directly proportional to the intracellular ROS levels.

1.6 Active caspases 3/7-based apoptosis detection assay

Fluorochrome inhibitors of Caspase (FLICA), a cell-permeable, carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor, covalently binds to a reactive cysteine residues found on active caspases. This binding thereby inhibits further enzymatic activity of caspase. Bound labeled reagent is retained within the cell, while unbound reagent diffuses out of the cell and is washed away. The amount of active caspase-3 or 7, present in the cell at the time the reagent was added, is measured directly

by a green fluorescent signal. Control and treated cells were stained with FLICA using the CaspaTag Caspase-3/7 In Situ Assay kit (Chemicon International, Temecula, CA). Active caspase-3/7 activity was assessed via flow cytometric analysis (BD Biosciences). Cells were seeded onto sterile flat-bottom 25 cm^2 culture flasks (Corning) and grown overnight to achieve the desired confluence. Plated cells were subjected to 48h of normoxia (21% O_2) or hypoxia (1% O_2) at various D-glucose concentrations (5.6, 25, 33 mM) in serum- and antibiotic-free media. Following treatment, cells were washed with 1X wash buffer (provided), detached from the plate surface using trypsin (Fisher Scientific) and suspended in PBS to achieve 1 x 10□ cells/mL. 10 µL of reconstituted and aliquoted FLICA reagent was added to 300 µL of cell suspension (0.1% bovine serum albumin (BSA; Fisher) /PBS), mixed gently and incubated at 37°C, 21% O_2, 5% CO_2 for 1 hour in the dark (flicking tubes gently every 20 minutes during this incubation). Following two washes and resuspension in 400 µL of 1X wash buffer, samples were immediately analyzed via flow cytometry with a BD FACSCalibur Flow Cytometer (BD Biosciences) supported by BD CellQuest Pro Software on the FL1-H (FLICA) channel. A minimum of 1×10^6 gated events were acquired per trial. Cells without any FLICA were considered to be living, while cells containing FLICA were considered be to apoptotic.

1.7 Protein Extraction

Cells were seeded onto sterile flat-bottom 75 cm^2 culture flasks (Corning) and grown overnight to achieve the desired confluence. Plated cells were subjected to 24h of normoxia (21% O_2) or hypoxia (1% O_2) at various D-glucose concentrations (5.6, 25, 33

mM) in serum- and antibiotic-free media. Following treatment, cell samples trysinized, collected and centrifuged several times in PBS at 500g for 5 minutes at 4°C. The Nonidet P40 (NP-40) (Roche Diagnostics, Germany) buffer containing 150 mM NaCl, 1% NP-40, 50 mM Tris (pH 8.0) and protease inhibitors phenylmethylsulfonyl fluoride, leupeptin, aprotinin and pepstatin was used to lyse cell samples for immunoblotting. Protease inhibitors were all prepared in advance and stored at -20°C. Inhibitors were added to the NP-40 buffer immediately prior to the buffers application and aliquots were refrozen. Once the ice-cold lysis buffer was applied to pelleted cell samples, the lysate was pipetted up and down repeatedly using a pipette tip. Lysates were gently vortexed, incubated on ice for 10 minutes and then centrifuged for 12 minutes at 1 x 10^4 rpm to remove cellular debris. Supernatant was collected and stored at -80°C and/or kept on ice for immediate quantitation.

Hypoxia treated cells were collected within the hypoxia chamber. Serum-free and antibiotic-free media, PBS and TryLE (trypsin) express (Gibco, Grand Island, NY) were deoxygenated in the hypoxia chamber prior to use. Lids of sample vials were tightened and covered with parafilm when samples were going to and from ambient O_2 during the extraction process. Samples were quickly placed on ice and stabilized with protease and phosphatase inhibitors in the NP-40 lysis buffer.

1.8 Total protein quantification

A colorimetric, Lowry-based protein assay was used to quantitate protein lysates. Using a sterile flat-bottomed, 96-well tissue culture plate (Corning), the DC Protein Assay (Bio-Rad) was performed. This assay requires 5 µL of protein lysate, full-strength

samples to be measured alongside a range of BSA (Fisher) protein standard concentrations from 0.2-1.5 mg/mL. Following a 15 minute incubation period in room temperature, standard and sample absorbance was measured, using a PowerWave XS Microplate Spectrophotometer (BioTek), at a wavelength of 750 nm. Excel for Macintosh was used to create a standard curve from the BSA standards, and the equation of the line of best fit was used to calculate the sample protein concentrations. To minimize degradation of stock sample lysates, they were kept on ice the whole time.

1.9 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Once sample lysates were quantified, 6x Laemmli sample loading buffer containing ß- mercaptoethanol (Sigma-Aldrich) was added to the sample lysates, which were then placed in a 97°C hot plate for 5 minutes. Boiled lysates were then cooled on ice, spun down for 30 seconds at 1×10^4 rpm and loaded into 8%, 10% or 15% SDS-PAGs at 25 or 30 µg per well (Bio-Rad Mini PROTEAN 3 Cell System apparatus). Approximately 10 µL (equivalent to 10 µg) of Spectra Multicolor High Range Protein Ladder (Thermo Scientific) or Precision Plus Kaleidoscope (Bio-Rad) protein standard was loaded alongside the sample wells. The electrophoresis apparatus was filled with 1x Running buffer (standard Laemmli 10x Running buffer diluted by 1:10 in double distilled water (ddH$_2$O)). Gels were run at 80V until the sample front cleared the stacking gel (for about 30 minutes), followed by 100V until the appropriate kDa range had migrated through the resolving gel.

2.0 Electrophoretic transfer

To prevent protein band dissociation, gels were removed from the electrophoresis assembly immediately upon completion of SDS-PAGE. Gels were trimmed at the edges, orientation marks added, then soaked in 1x Transfer buffer for 10 minutes for equilibration (standard Laemmli 10x Transfer buffer diluted by 1:2:7 in 70% methanol (Fisher) and ddH_2O). Polyvinylidene fluoride transfer membrane (PVDF, Pierce, Rockford, IL, USA) was soaked in methanol for 15 minutes. The transfer cassette was assembled as per the manufacturer's instructions and filled with 1x Transfer buffer. The transfer was performed at 100V for 2h, with ddH_2O ice block, while in a box of crushed ice.

2.1 Immunoblotting

Once electrophoretic transfer was completed, gels were soaked in Coomassie Blue dye (Sigma) on the Belly Dancer Shaker (Stovall, Greensboro, NC, USA) for at least 1h and then soaked in de-stain (70% ethanol and ddH_2O) solution to verify successful protein gel migration and transfer (bands observed are of proteins not transferred). Membranes were stained with Ponceau S solution (Bio-Rad) for 10 minutes to further verify transfer efficiency. To remove Ponceau S stain, the membrane was immersed in 0.1M sodium hydroxide for 30 seconds followed by successive washes in ddH_2O. The membranes were then soaked in 1x Tris-buffered saline containing 0.1% Tween-20 detergent (TBST) (Sigma) three times, 5 minutes each time (protein side up) to wash and equilibrate them before being blocked with 5% dehydrated milk powder in TBST solution overnight (~16h) at 4°C.

Blocked membranes were rinsed in TBST three times, 10 minutes each time, before

incubation with primary antibodies. Antibodies were diluted by 1:100-1:1000 in 5% BSA and TBST and stored at 4°C. The primary antibodies used were: β-actin (loading control), Bax, Bcl-2 (Santa Cruz Biothechnology, Santa Cruz, CA), caspase 3, AMPKα, Phospho-AMPKα (Thr172, P-AMPKα) and LC3B-II (Cell Signaling Technology, New England). Membranes were then washed in TBST three times for 10 minutes. Following this step, 1:100-1:1000 horseradish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-mouse (Pierce and R&D Systems) IgG secondary antibodies in 5% BSA solution were used to probe the membrane on a shaker for 2h at room temperature or overnight (~16h) at 4°C. Following this step, membranes were again washed with TBST three times for 10 minutes. Following enhanced chemiluminescent (ECL) imaging, a 'medium' strength stripping buffer, containing SDS, was used to sufficiently remove antibodies from the membranes for immediate re-blotting.

2.2 Enhanced chemiluminescence and densitometry

The method of ECL utilizes HRP enzyme tethered to a molecule of interest through a labelled immunoglobulin that specifically recognizes that molecule. The ECL substrate is converted into a sensitized compound, H_2O_2 oxidizes this to emit light upon decay. The HRP enzyme complex catalyzes this reaction. Standard ECL was performed to detect protein bands on the immunoblots via 10 minute High-Sensitivity Chemiluminescent exposures using a Chemidoc XRS imager (Bio-Rad) supported by Quantity One software for Windows (Bio-Rad). Epi-White exposures were also collected and membranes were scanned in order to decipher banding patterns and protein ladders. Blots were later analyzed via densitometry using the Quanity One software, with target protein densities

normalized to the β-actin loading control.

2.3 Statistics

The data is presented as mean ± standard error of the mean (SEM). An $n \geq 3$ independent experiments was obtained for all read outs. GraphPad Prism software was used for statistical analyses. One-way ANOVA with Tukey's multiple comparison test were utilized, with $p < 0.05$ considered as significant. Asterisks are used herein to denote significance according to the following scheme: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Results

1.1 Effect of high glucose and hypoxia on cell viability

Cell viability was assessed via the colourimetric MTT assay. H9c2 cells were subjected to various concentrations of D-glucose treatment (5.6, 25 and 33 mM D-glucose) for 12h, 24h, 48h (data not shown) and 96h at 37°C, 5% CO_2. Cell viability was significantly reduced by 33 mM D-glucose in normoxic after 96h (25 ± 5.8%, $p < 0.01$) when compared to the control (**Figure 1**). Cells treated for 24h with 25 mM (14.49 ± 2.7%, $p < 0.01$) and 33 mM D-glucose (25.8 ± 2.9%, $p < 0.001$) under hypoxic conditions showed significantly decreased cell viabilities when compared to control (**Figure 2**).

1.2 Effect of high glucose and hypoxia on oxidative stress in H9c2 cardiomyocytes

High glucose and hypoxia-induced oxidative stress was investigated in cultured H9c2 cells. Intracellular ROS levels were assessed using the CM-H_2DCFDA assay and measured via flow cytometry. A 48h treatment with increasing D-glucose concentrations, in normoxia, did not cause significant increases in ROS generation (**Figure 3**). However cells exposed to 25 mM D-glucose for 48h in hypoxia caused a significant increase (44 ± 6.6, $p < 0.001$) in ROS generation when compared to all the other treatments, including the control (in normoxia: control, 10.6 ± 2.1, $p < 0.001$; 25 mM, 18.2 ± 4.3, $p < 0.01$; 33 mM, 19 ± 2.9, $p < 0.01$ and in hypoxia: 5.6 mM, 23.2 ± 2.3, $p < 0.05$; 33 mM, 23.1 ± 2.9, $p < 0.05$). There is a slight but non-significant increase in ROS generation in the hypoxia treatments versus the normoxia treatments. These data indicate a pronounced elaboration

of ROS resulting from the simultaneous treatment of 25 mM D-glucose and hypoxia in H9c2 cells. The non-significant differences in ROS generation between the 5.6 mM and 33 mM D-glucose treatments in hypoxia indicate a biphasic trend (**Figure 3**).

1.3 Effect of high glucose and hypoxia on active caspase 3 and 7 activity in H9c2 cardiomyocytes

To determine whether high glucose and hypoxia-induced ROS generation resulted in cell death via apoptotic mechanisms, the activity of apoptotic enzymes, caspases 3 and 7, via the CaspaTag caspase 3/7 In Situ Assay kit and flow cytometry were examined. High glucose, in normoxia for 24h, caused a non-significant decrease in caspase 3 and 7 activity. In hypoxia, high glucose induced increased levels of caspase 3 and 7 activity, of which the 25 mM D-glucose treatment was significant. Cells exposed to 24h of simultaneous 25 mM D-glucose and hypoxia resulted in significantly increased caspase 3 and 7 activity (29.4 ± 4.9, $p < 0.001$) when compared to all other treatments including the control (in normoxia: control, 12.7 ± 2.2, $p < 0.001$; 25 mM, 11.8 ± 1.7, $p < 0.001$; 33 mM, 10.4 ± 1.5, $p < 0.001$ and in hypoxia: 5.6 mM, 18.7 ± 1.0, $p < 0.05$; 33 mM, 16.7 ± 1.2, $p < 0.01$). These findings suggest that simultaneous treatment of high glucose and hypoxia induce activation of apoptotic effector caspases. Treatment with simultaneous 33 mM D-glucose and hypoxia resulted in a decrease in apoptosis (**Figure 4**).

1.4 Effect of high glucose and hypoxia on caspase 3, Bax and Bcl-2 protein expression

Given the increased ROS generation (**Figure 3**) and increased caspase 3 and 7

activity (**Figure 4**) due to simultaneous treatment with high glucose and hypoxia, we decided to further investigate high glucose and hypoxia-induced apoptosis by assessing caspase 3, Bax, and Bcl-2 protein expression via immunoblotting. Caspase 3 protein expression, displayed as a ratio of cleaved (active)/non-cleaved (inactive) caspase 3, did not change significantly after 24h of high glucose and/or hypoxia treatment when compared to control, but a trend, of increasing protein expression with high glucose and/or hypoxia, was observed (**Figure 5**). Bax protein expression was significantly increased after 24h of 25 mM D-glucose in hypoxia (1.5 ± 0.1 fold, $p < 0.05$) when compared to control (**Figure 6**). Bcl-2 protein expression did not change significantly after 24h of high glucose and/or hypoxia, when compared to control. However, Bcl-2 protein expression was observed to gradually decrease with increased D-glucose concentrations and/or hypoxia (**Figure 7**). These findings further indicate simultaneous high glucose and hypoxia-induced apoptotic cell death.

1.5 Effect of high glucose and hypoxia on LC3B-II protein expression in H9c2 cardiomyocytes

Protein expression of the autophagy marker, LC3B-II was assessed via immunoblotting. LC3B-II protein expression, shown as a ratio of LC3B-II/LC3B-I protein expression, did not change significantly after 24h of high glucose and/or hypoxia, when compared to control. However, LC3B-II/LC3B-I protein expression was observed to gradually decrease with increased D-glucose concentrations and/or hypoxia (**Figure 8**). These results suggest that high glucose treatment diminishes autophagy levels and this effect is exacerbated by hypoxia treatment.

1.6 Effect of high glucose and hypoxia on AMPKα and P-AMPKα (Thr172) protein expression

Hypoxia and ROS have been known as potent activators of AMPK, a central modulator in metabolic regulation and autophagy (Choi et al. 2001). Phosphorylation of the Thr172 residue, in the kinase domain of AMPKα, activates kinase activity (Hardie et al., 2005). AMPKα and P-AMPKα protein expression was assessed via immunoblotting and protein expression of both were not significantly altered after 24h of high glucose and/or hypoxia treatment, when compared to control levels (**Figure 9 and 10**). P-AMPKα protein expression showed a gradual decrease with increased D-glucose concentrations, followed by a biphasic response to glucose and hypoxia. P-AMPKα protein expression increased in response to 24h of 5 mM and 33 mM D-glucose and hypoxia but was decreased in response to 25 mM D-glucose and hypoxia (**Figure 9 and 10**). This finding suggests that AMPKα protein regulation could be post-translational under high glucose and/or hypoxia conditions. These results also suggest that high glucose can lead to a decrease in P-AMPKα and cell survival, and this decrease is reversed after exposure to simultaneous glucose and hypoxia.

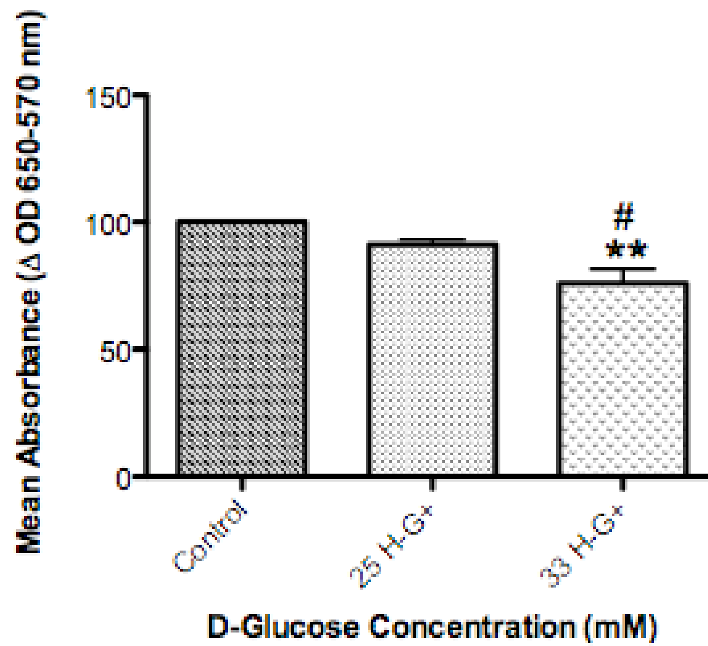

Figure 1. Effect of glucose levels on cell viability. H9c2 cells were treated with serum-free medium containing 5.6 mM (control, 5.6 H-G+), 25 mM and 33 mM D-glucose, in normoxia (21% O_2), 96h (H-G+ indicates normoxia and D-glucose). Cell viability was assessed by the MTT assay. Bars represent mean ± SEM of 3 independent experiments. Data is expressed as mean absorbance (** = $p < 0.01$ versus control; # = $p < 0.05$ versus 25 mM D-glucose).

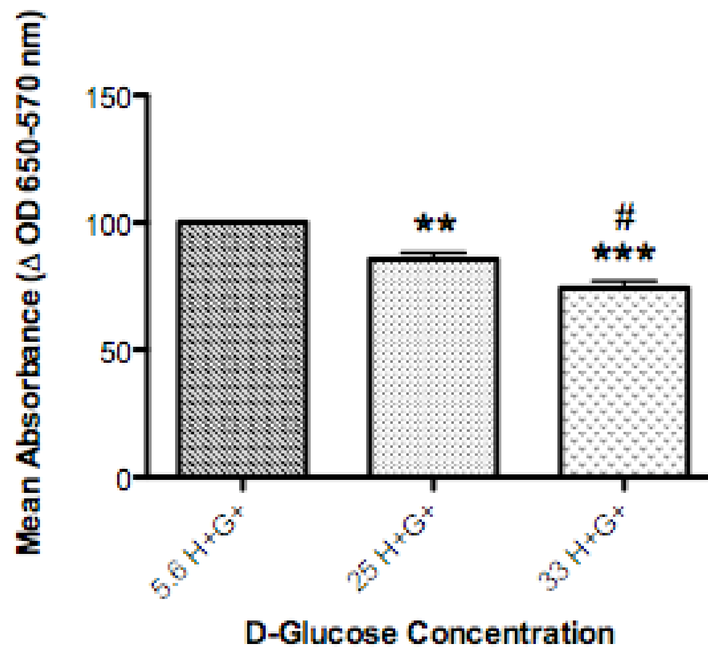

Figure 2. The cumulative effect of high glucose levels and hypoxia on cell viability. Cells were treated with serum-free medium containing 5.6 mM, 25 mM and 33 mM D-glucose, in hypoxia (1% O_2), 96h (H+G+ indicates hypoxia and D-glucose). Bars represent mean ± SEM of 3 independent experiments. Data is expressed as mean absorbance (*** = $p < 0.001$ versus control; ** = $p < 0.01$ versus control; # = $p < 0.05$ versus 25 mM D-glucose and hypoxia).

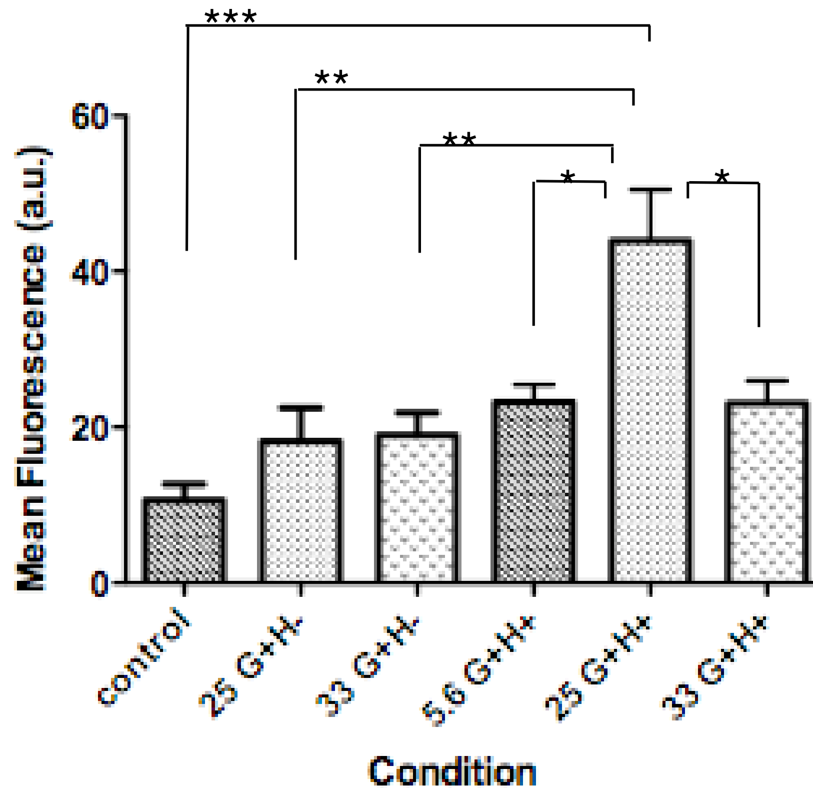

Figure 3. Effect of high glucose and/or hypoxia on oxidative stress in H9c2 cardiomyocytes. The effect of high glucose and/or hypoxia (1% O_2) on intracellular ROS levels in H9c2 cells was assessed via fluorescent detection of oxidation-induced esterase activity (CM-H2DCFDA assay), and analyzed via flow cytometry. Cells were treated in medium containing 5.6 (control, H-G+), 25 and 33 mM D-glucose and/or hypoxia (1% O_2) (H-G+ indicates normoxia and D-glucose; H+G+ indicates hypoxia and D-glucose). Bars represent mean ± SEM of at least 3 independent experiments. Data is expressed as mean fluorescence arbitrary units (a.u.) (* = $p < 0.05$; ** = $p < 0.01$, ***= $p < 0.001$).

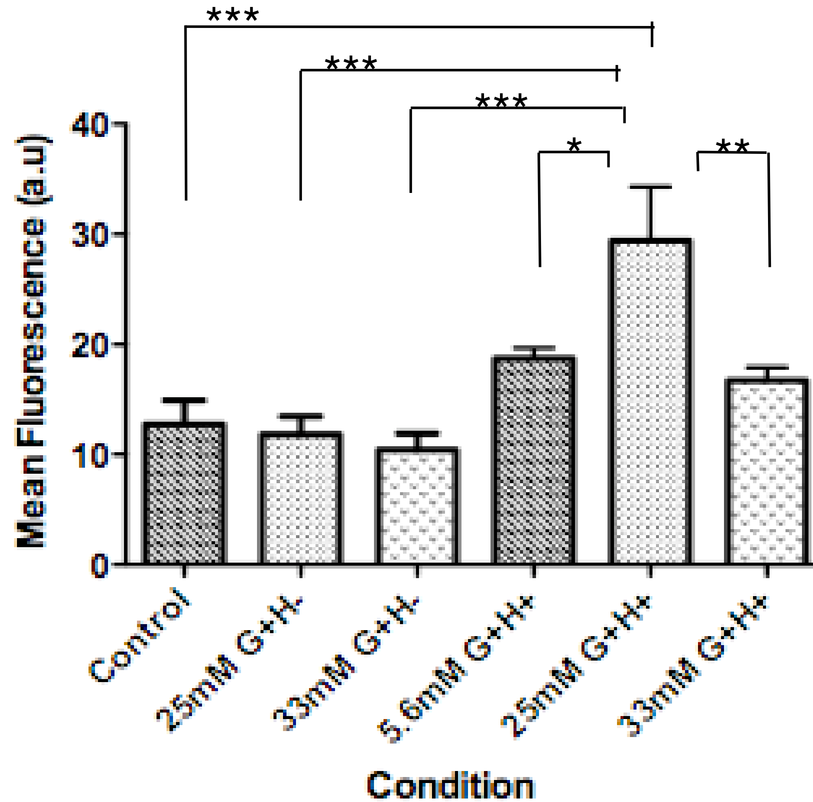

Figure 4. Effect of high glucose and/or hypoxia on apoptosis in H9c2 cardiomyocytes. The effect of high glucose and hypoxia on H9c2 cellular apoptosis (activity of apoptotic enzymes, caspases 3 and 7) was assessed using the CaspaTag caspase 3/7 In Situ Assay kit and measured via flow cytometry. Cells were treated in serum-free media containing 5.6 (control, H-G+), 25 and 33 mM D-glucose and/or hypoxia (1% O_2) (H-G+ indicates normoxia and D-glucose; H+G+ indicates hypoxia and D-glucose). Bars represent mean ± SEM of 3 independent experiments. Data is expressed as mean fluorescence arbitrary units (a.u.) (* = $p < 0.05$; ** = $p < 0.01$, ***= $p < 0.001$).

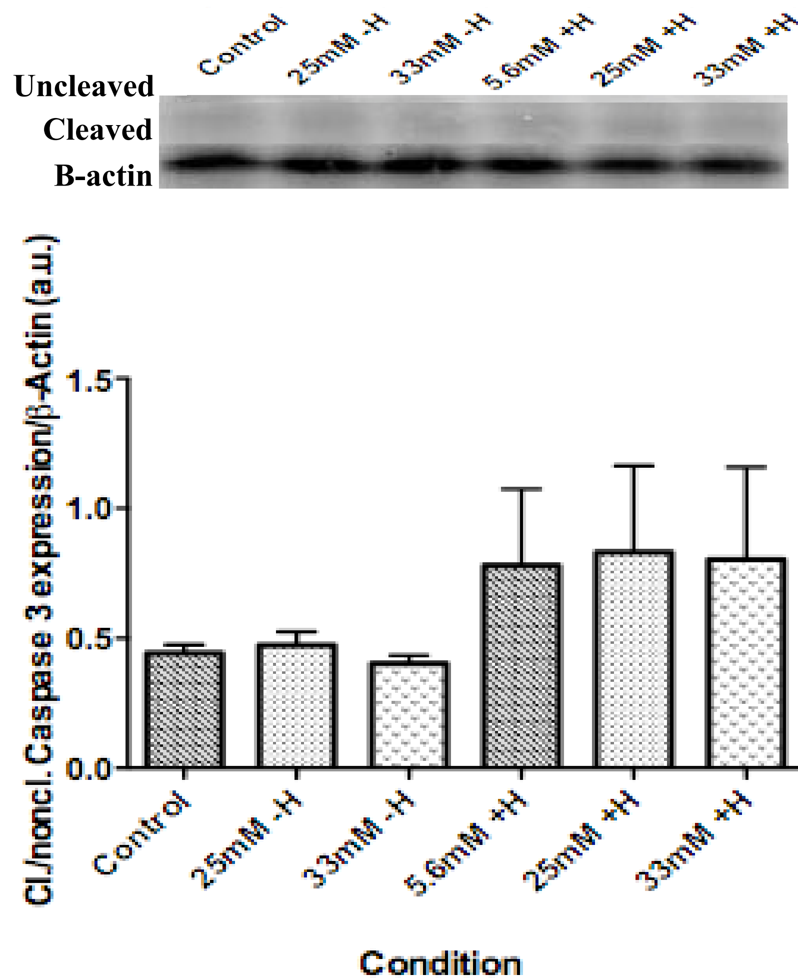

Figure 5. Effect of high glucose and/or hypoxia on Caspase 3 protein expression in H9c2 cardiomyocytes. H9c2 cells were treated in serum-free media containing 5.6 (control, H-G+), 25 and 33 mM D-glucose and/or hypoxia (1% O_2) (H-G+ indicates normoxia and D-glucose; H+G+ indicates hypoxia and D-glucose) and cleaved/non-cleaved (cl./noncl.) Caspase 3 protein expression was assessed via immunoblotting by lysing with NP-40 buffer and quantifying proteins via DC Protein assay (Bio-Rad). Coomassie Blue stain verified total protein separated via SDS-PAGE and Ponceau S stain verified gel-to-PVDF membrane transfer of proteins. Bars represent mean ± SEM of 4 independent experiments. Data is expressed as cl./noncl. Caspase 3 expression, normalized to β-actin (loading control), arbitrary units (a.u.).

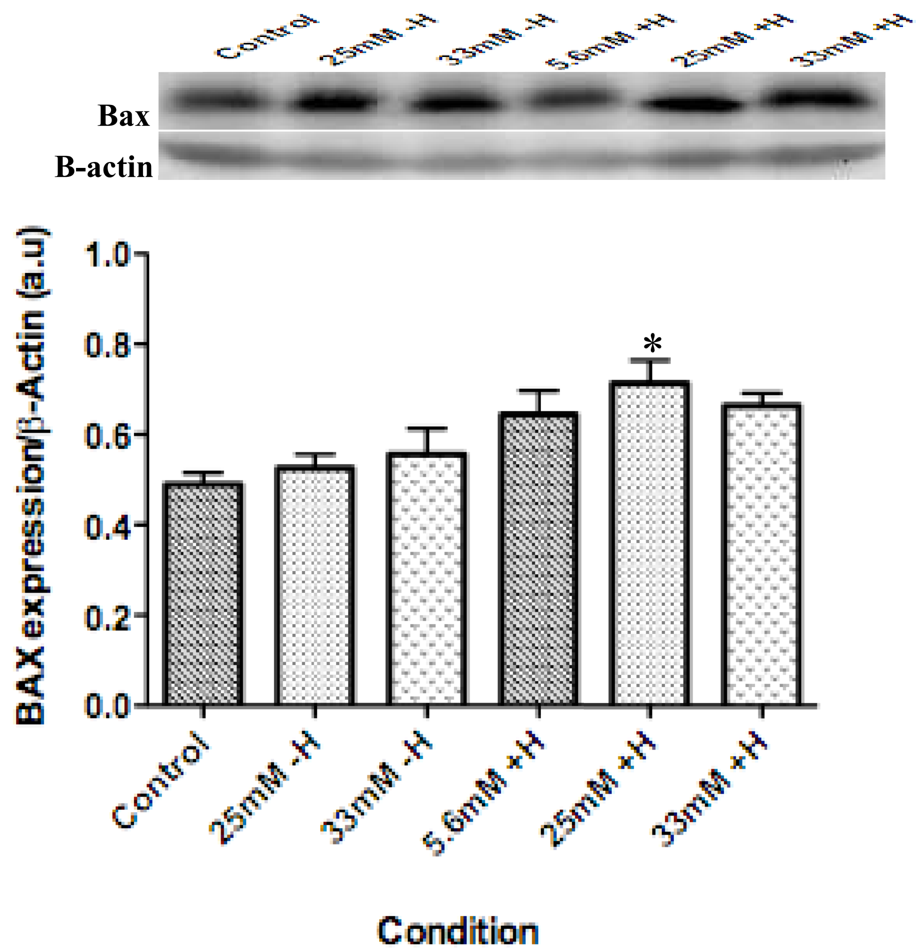

Figure 6. Effect of high glucose and/or hypoxia on Bax protein expression in H9c2 cardiomyocytes. H9c2 cells were treated in serum-free media containing 5.6 (control, H-G+), 25 and 33 mM D-glucose and/or hypoxia (1% O_2) (H-G+ indicates normoxia and D-glucose; H+G+ indicates hypoxia and D-glucose) and pro-apoptotic Bax protein expression was assessed via immunoblotting by lysing with NP-40 buffer and quantifying proteins via DC Protein assay (Bio-Rad). Coomassie Blue stain verified total protein separated via SDS-PAGE and Ponceau S stain verified gel-to-PVDF membrane transfer of proteins. Bars represent mean ± SEM of 4 independent experiments. Data is expressed as Bax expression, normalized to β-actin (loading control), arbitrary units (a.u.). (* = $p < 0.05$).

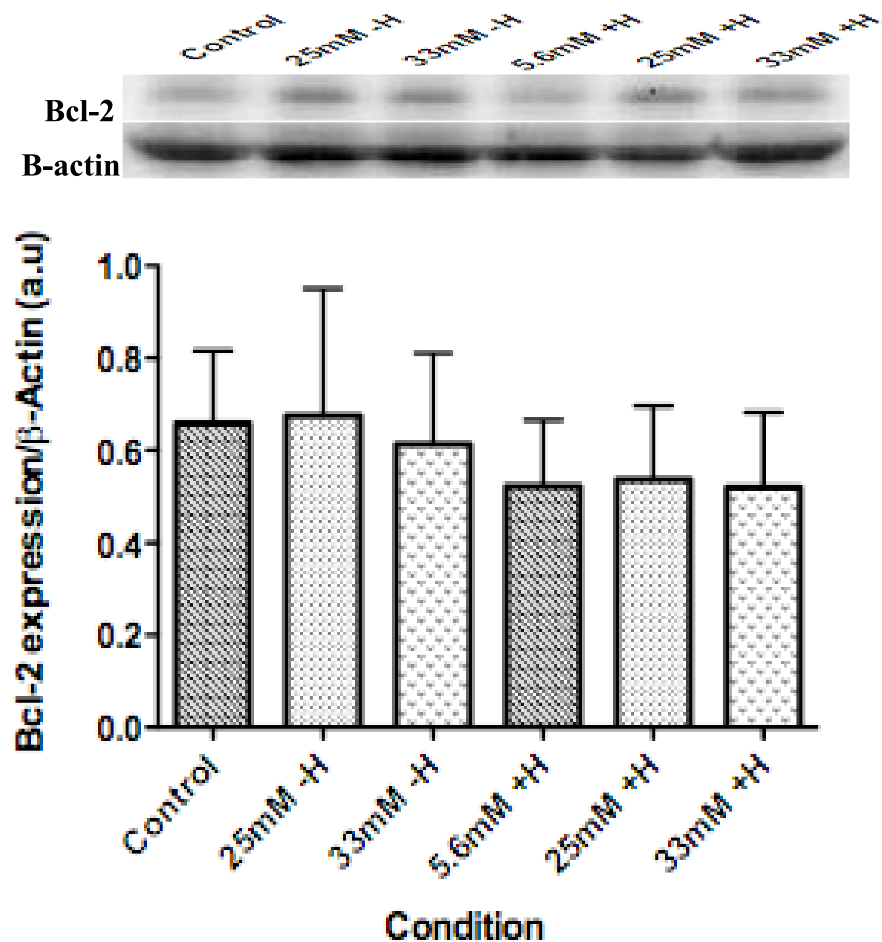

Figure 7. Effect of high glucose and/or hypoxia on Bcl-2 protein expression in H9c2 cardiomyocytes. H9c2 cells were treated in serum-free media containing 5.6 (control, H-G+), 25 and 33 mM D-glucose and/or hypoxia (1% O_2) (H-G+ indicates normoxia and D-glucose; H+G+ indicates hypoxia and D-glucose) and anti-apoptotic Bcl-2 protein expression was assessed via immunoblotting by lysing with NP-40 buffer and quantifying proteins via DC Protein assay (Bio-Rad). Coomassie Blue stain verified total protein separated via SDS-PAGE and Ponceau S stain verified gel-to-PVDF membrane transfer of proteins. Bars represent mean ± SEM of 4 independent experiments. Data is expressed as Bcl-2 expression, normalized to β-actin (loading control), arbitrary units (a.u.).

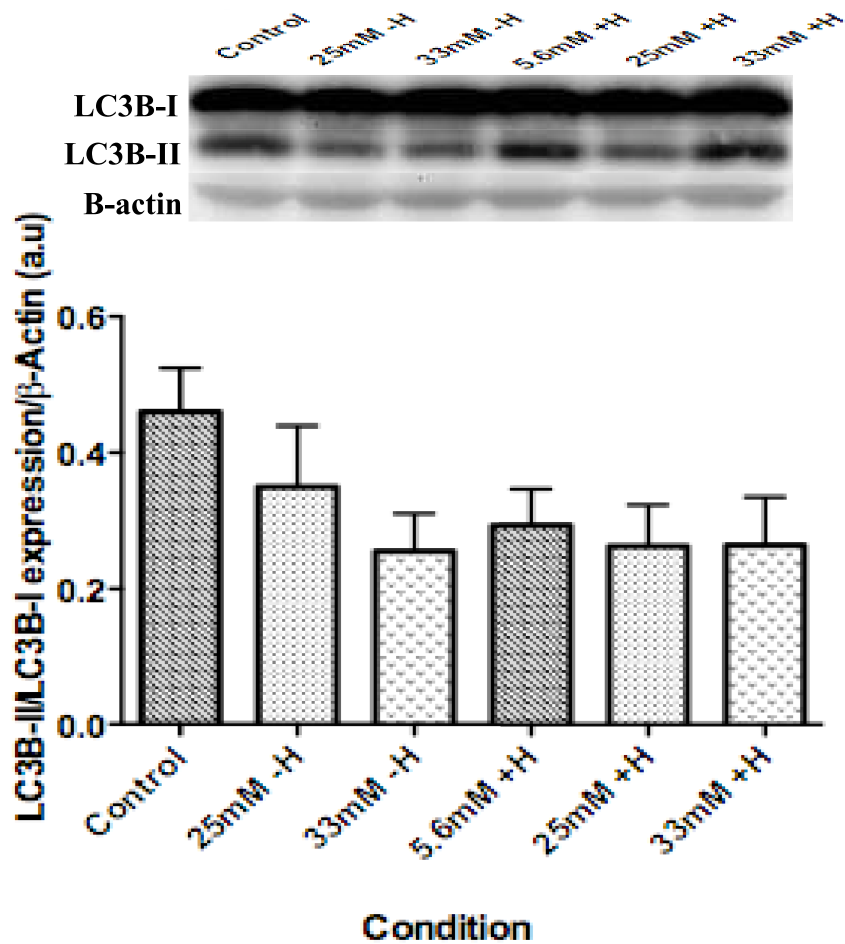

Figure 8. Effect of high glucose and/or hypoxia on LC3B-II protein expression in H9c2 cardiomyocytes. H9c2 cells were treated in serum-free media containing 5.6 (control, H-G+), 25 and 33 mM D-glucose and/or hypoxia (1% O_2) (H-G+ indicates normoxia and D-glucose; H+G+ indicates hypoxia and D-glucose) and LC3B-I/LC3B-II, the autophagy marker, protein expression was assessed via immunoblotting by lysing with NP-40 buffer and quantifying proteins via DC Protein assay (Bio-Rad). Coomassie Blue stain verified total protein separated via SDS-PAGE and Ponceau S stain verified gel-to-PVDF membrane transfer of proteins. Bars represent mean ± SEM of 4 independent experiments. Data is expressed as LC3B-I/LC3B-II expression, normalized to β-actin expression (loading control), arbitrary units (a.u.).

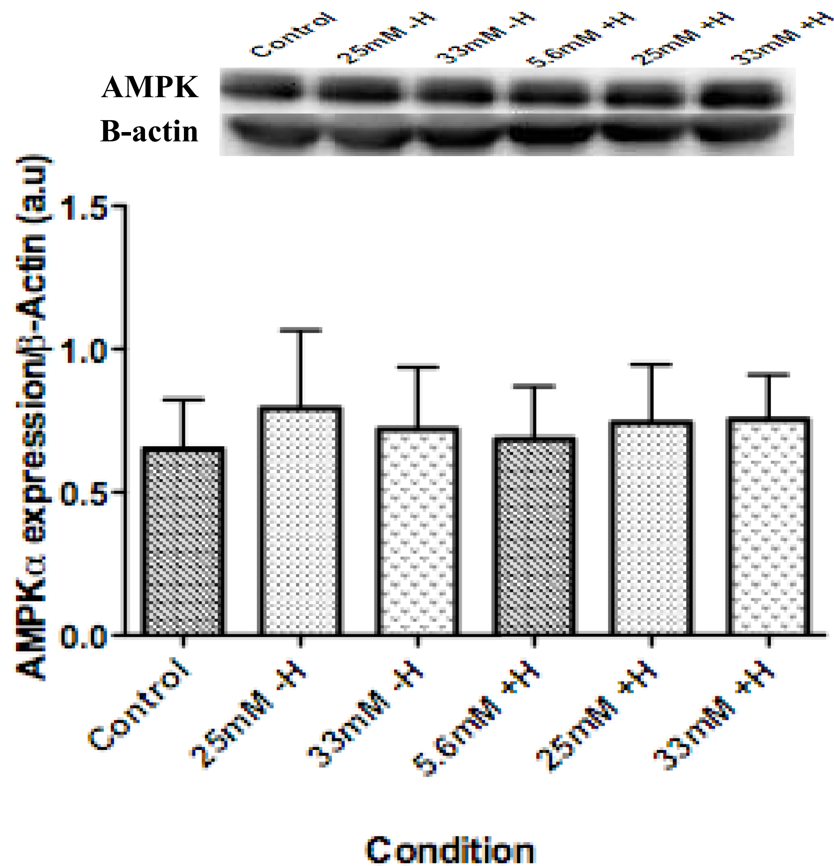

Figure 9. Effect of high glucose and/or hypoxia on AMPKα protein expression in H9c2 cardiomyocytes. H9c2 cells were treated in serum-free media containing 5.6 (control, H-G+), 25 and 33 mM D-glucose and/or hypoxia (1% O_2) (H-G+ indicates normoxia and D-glucose; H+G+ indicates hypoxia and D-glucose) and AMPKα protein expression was assessed via immunoblotting by lysing with NP-40 buffer and quantifying proteins via DC Protein assay (Bio-Rad). Coomassie Blue stain verified total protein separated via SDS-PAGE and Ponceau S stain verified gel-to-PVDF membrane transfer of proteins. Bars represent mean ± SEM of 4 independent experiments. Data is expressed as AMPKα expression, normalized to β-actin expression (loading control), arbitrary units (a.u.).

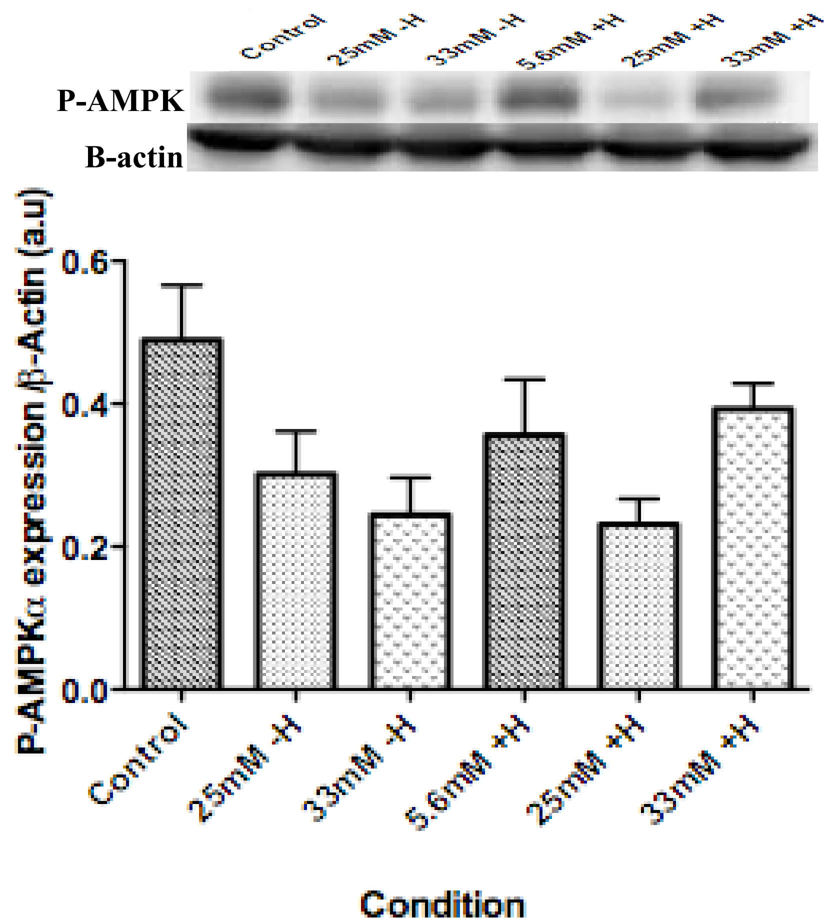

Figure 10. Effect of high glucose and/or hypoxia on P-AMPKα (Thr172) protein expression in H9c2 cardiomyocytes. H9c2 cells were treated in serum-free media containing 5.6 (control, H-G+), 25 and 33 mM D-glucose and/or hypoxia (1% O_2) (H-G+ indicates normoxia and D-glucose; H+G+ indicates hypoxia and D-glucose) and P-AMPKα (Thr172) protein expression was assessed via immunoblotting by lysing with NP-40 buffer and quantifying proteins via DC Protein assay (Bio-Rad). Coomassie Blue stain verified total protein separated via SDS-PAGE and Ponceau S stain verified gel-to-PVDF membrane transfer of proteins. Bars represent mean ± SEM of 4 independent experiments. Data is expressed as P-AMPKα expression, normalized to β-actin expression (loading control), arbitrary units (a.u.).

Discussion

1.1 High glucose and/or hypoxia reduce cell viability and induce oxidative stress in H9c2 cardiomyocytes

Exposure of cardiomyocytes to high glucose decreased cell viability (**Figure 1**), a treatment effect exacerbated in the presence of hypoxia (**Figure 2**). In support of our results in high glucose and normoxia, Younce et al. (2010) exposed H9c2 cardiomyoblasts to high glucose (28 mM D-glucose, 24h) and found cell death was induced in a concentration- and time-dependent manner (Younce et al. 2010). Yu et al. (2010) used intracellular calcein fluorescence to demonstrate a significant decrease in cell viability of H9c2 cells incubated in high glucose (25 mM D-glucose, control; 5 mM), after 96h. H9c2 cells were shown to exhibit significant concentration- and time-dependent DNA fragmentation and cell loss (Yu et al. 2010). In another experiment, Cai et al. (2002) showed that incubation on H9c2 cells with high D-glucose (22, 33 and 60 mM, control; 5.5 mM) concentrations resulted in significant decreases in cell viability. In their study, significant decreases in cell viability with 33 mM D-glucose were not observed until 96hof exposure (Cai et al. 2002). Cultured VSMCs grown in high glucose media concentrations were found to increase their proliferative rate when compared to their normoglycemic control (Ruiz et al. 2006). Enhanced VSMC proliferation has also been demonstrated in both human and experimental models of diabetes (Reddy et al. 2006; Rizzoni and Rosei 2006). The lack of proliferation and the decreased cell viability in H9c2 cells exposed to high glucose treatments suggests that cell survival and growth may be suppressed by the promotion of PCD.

The results of our study showed that high glucose treatment promotes cell death and this is exacerbated in conditions of simultaneous high glucose and hypoxia (**Figure 2**). Gao et al. (2007) used bovine aortic smooth muscle cells (BASMC) to examine the effects of high glucose (25 mM D-glucose and 5.5 mM control, 5 days) on BASMC growth (proliferation and apoptosis) under normoxic (5% CO_2, 95% air) and chronic hypoxic (2% O_2, 5% CO_2, 93% N_2) conditions and reported that simultaneous high glucose and hypoxia treatment significantly increased the rate of BASMC proliferation. Hypoxia alone decreased cell proliferation when compared to normoxia, in which the proliferative state of these cells did not change (Gao et al. 2007).

Increased ROS production is observed in type 1 and type 2 diabetes and is considered to be a major contributing factor in the development of diabetic cardiomyopathy (Desco et al. 2002; San Martin et al. 2007). A 75 gram glucose challenge *in vivo* has been shown to induce increased $O_2•^-$ generation by leukocytes and subunits of NADPH oxidase, the enzyme that converts molecular O_2 to $O_2•^-$ radical (Mohanty et al. 2000). Cai et al. (2002) also showed significant high glucose (33 mM)-induced ROS formation in cardiac H9c2 myocytes (Cai et al., 2002). In addition, Younce et al. (2010i) demonstrated high glucose (28 mM D-glucose, 24h)-induced MCP-1 production, in H9c2 cardiomyoblasts and NRVM. MCP-1 was observed to induce MCPIP, which mediates high glucose-induced cell death via its induction of ROS. It was revealed that MCPIP mediates glucose-induced NADPH oxidase activation, a major source of ROS (Younce et al. 2010i). However, our results showed that exposure of cells to high glucose concentrations did not stimulate the production of ROS (**Figure 3**). We observed a slight, but non significant, increase in ROS generation at high glucose levels.

Nonetheless, in our study, the generation of ROS was significantly increased in conditions of simultaneous 25 mM D-glucose and hypoxia treatment (**Figure 3**). This is supported by Mrianda et al. (2011), who also investigated, *in vitro*, high glucose (25 mM D-glucose and 5 mM D-glucose control, t=18 days, at 37°C, 5% CO_2) and/or hypoxia (at (t-6h) for control and (t-24h) for high glucose group, 1% O_2) using ARPE-19 cells, a spontaneously immortalized human retinal pigment epithelium cell line. In addition to increased ROS generation, their study observed that simultaneous high glucose and hypoxia treatment also upregulates CHOP expression, triggers activation of JNK and p38 mitogen-activated protein kinase (MAPK), reflective of ER stress, and provokes the disruption of tight junctions in ARPE-19 cells (Miranda et al. 2011). Ornoy et al. (2010) found that 5 and 7 mg glucose/ml (27.8 mM and 38.9 mM glucose, respectively) culture medium produces embryonic damage and enhanced oxidative stress as well as increased HIF 1a in 10.5-day-old rat embryos and their yolk sacs, thus pointing to the possibility that hyperglycemia may also cause hypoxia (Ornoy et al. 2010). Ornoy et al. (2011) demonstrates that both hypoxia (10% O_2, 24h, followed by 20% O_2 , 4h) and high glucose (556mg/dl or 30.89 mM D-glucose, 28h) and their combination, produce embryonic damage in 10.5-day-old cultured rat embryos probably by increasing embryonic oxidative stress (Ornoy et al. 2011). In pregnant mice hyperglycemia was found to induce oxidative stress, reduce pax-3 gene expression, a gene necessary for the closure of the neural tube, thus causing an increased rate of neural tube defects (Chang et al. 2003). Li et al. (2005) then showed that hypoxia intensifies the effects of hyperglycemia by further decreasing the activity of Pax 3 gene in mouse embryos, whereas hyperoxia reduced these damaging effects (Li et al. 2005). In these studies the antioxidant vitamin E succinate was found to

block both the effects of hypoxia and of hyperglycemia (Li et al. 2005). Thus, a clear connection between hyperglycemia and hypoxia, both inducing oxidative stress, appears. However, our results show a biphasic trend, in which simultaneous 33 mM D-glucose and hypoxia decreased ROS production, down to control levels (**Figure 3**). In light of the studies mentioned, our results suggest that H9c2 cardiomyocytes are prone to oxidative stress due to high glucose (25 mM D-glucose) and hypoxia-induced increases in ROS production. We need to further investigate why H9c2 cardiomyocytes respond in a biphasic fashion when exposed to an even higher D-glucose (33 mM) concentration, in hypoxic conditions.

1.2 High glucose and/or hypoxia-induced apoptotic cell death in H9c2 cardiomyocytes

Apoptosis is a form of caspase-dependent PCD. Caspases, cytosolic cysteine endopeptidases, are activated either following stimulation of death receptors on the plasma membrane (extrinsic pathway), such as tumour necrosis factor receptor 1 (TNFR1), or the intrinsic mitochondrial pathway. Both pathways involve the cleavage and activation of executioner caspases 3 and 7. The morphological characteristics of PCD-1 include chromatin condensation and fragmentation of nucleus and cytoplasm into apoptotic bodies (Edinger and Thompson 2004; Green and Kroemer 2004). Apoptosis is increased in STZ-induced diabetic mice myocardium and H9c2 cardiomyocytes exposed to high levels of glucose. High glucose exposure has been demonstrated to directly cause apoptosis in H9c2 cells (Cai et al. 2002). Mitochondrial cytochrome c release is a key event in the activation of caspase-3 and 7 (Roy 2000). Mitochondrial cytochrome c

release and caspase-3 activation have been found to be associated with hyperglycemia-induced myocardial apoptosis, *in vivo* and *in vitro*. There is a positive correlation between ROS production and mitochondrial cytochrome c release–mediated caspase-3 activation, in H9c2 cells. Stress-induced ROS production has been suggested to play, at least in part, a critical triggering role in the mitochondrial cytochrome c–mediated caspase-3 activation pathway of apoptotic cell death (Cai et al. 2002).

Hypoxia induces caspase-dependent (apoptosis) and caspase-independent PCD (necrosis) (Shimizu et al. 1996). During cell death by hypoxia, death effectors are released from the intermembrane spaces of mitochondria and into the cytoplasm where they proceed to initiate apoptotic and necrotic pathways (Wang 2001i; Gustafsson et al. 2008). Oxidative stress, hypoxia and hyperglycemia can disturb ER function and compromise the adaptive UPR, resulting in persistent ER stress. This leads to sustained activation of CHOP (proapoptotic transcription factor) and JNK (stress kinase). Both JNK and CHOP attenuate the function of the pro-survival factor Bcl-2, but enhances the activity of pro-apoptotic Bcl-2 proteins such as Bim, Bax, and PUMA, resulting in mitochondrial dysfunction and cytochrome *c* release. In addition, caspase-12 is activated during ER stress, which sequentially activates caspase-7 and/or caspase-3, leading to mitochondria-independent apoptosis (Jing et al. 2012). Both mitochondrial-dependent and independent apoptotic pathways may be triggered in the 25 mM D-glucose and hypoxia treatment. This may explain the significant increase in activated caspase 3/7 in high glucose (25 mM D-glucose) and hypoxia treated cells compared to treatment with 33 mM D-glucose and hypoxia, which did not alter the levels of activated caspase 3/7 levels.

Aki et al. (2010) have demonstrated that glucose uptake and glycolysis rescues H9c2 cells from ATP depletion and subsequent caspase-independent (necrosis) death, during hypoxia. However, their results suggested these protective effects of glucose are limited to concentrations at which cells can assimilate them. In partial support of our results, Aki et al. (2010) discovered a bifurcate property of glucose and proposed that different types of cell death are induced by hypoxia depending on extracellular glucose concentrations. According to their study, glucose concentrations of 2.78–16.7 mM were found to protect H9c2 cells from hypoxic death in a dose-dependent manner, in contrast to high concentrations of glucose, 19.7–25 mM, which promote hypoxia-induced apoptotic cell death (Aki et al. 2010). We observed increased apoptosis in H9c2 cells exposed to a 24h co-treatment of 25 mM D-glucose and hypoxia. However, Malliopoulou et al. (2006) observed acute high glucose pretreatment (22 mM, 15 minutes) to reduce cell death and protect H9c2 cells from hypoxic injury (serum-, glucose- and O_2-free). High glucose exposed-H9c2 cells displayed increased intracellular glucose concentrations and glucose is shown to exert its early protective effect against hypoxic insult, as an energy providing substrate in glucose metabolism (Malliopoulou et al. 2006). Pastukh et al. (2005) also described a similar phenomenon of protection using NRVM exposed for 3 days in high glucose (25 mM) media. Their high glucose treatment reduced apoptosis and necrosis induced by chemical hypoxia (Pastukh et al. 2005). Our results suggest that a combination of 25 mM D-glucose and hypoxia promotes apoptotic cell death, whereas 33 mM D-glucose and hypoxia conditions attenuate this pro-apoptotic signal. Considering the concentration dependent decreases in cell viability, in glucose and hypoxia conditions (**Figure 2**), it is worth investigating other cell death-inducing pathways such as

autophagy and necrosis.

To further investigate this biphasic trend and simultaneous high glucose and hypoxia-induced apoptosis, caspase 3, Bax, and Bcl-2 protein expression was assessed via immunoblotting. Both caspase 3 and Bax protein expression show a trend of increasing protein expression with simultaneous high glucose and hypoxia compared to normoxic conditions (**Figure 5 and 6**). However, only Bax showed a significant increase in protein expression when cells were exposed to simultaneous high glucose and hypoxia, compared to the control. Bcl-2 protein expression did not change significantly but its protein expression was observed to gradually decrease with increased D-glucose concentrations and/or hypoxia (**Figure 7**).

In contrast to our findings, hyperglycemia, alongside hypoxia, has been demonstrated to inhibit HIF-1α expression, inhibit hypoxia-induced decreased proliferation of VSMCs and a concomitant decrease in HRE promoter transactivation (Gao et al. 2007). In a rat model of cerebral ischemia both mRNA and protein expression of HIF-1α and procaspase 3 increased after 12h and 24h of ischemic insult, there was also an increase in specific HIF-1 binding to the caspase 3 gene promoter (Van Hoecke et al. 2007). Since hypoxia leads to increased mitochondrial ROS generation (Mansfield et al. 2005), it is enough of an increase to activate HIF-1α (Chandel et al. 2000). It is also suggested that HIF-1α promotes apoptosis by activating the pro-apoptotic Bcl-2 family protein Bax. The HIF-1 complex is suggested to be necessary for hypoxia-induced Bax expression (Ozawa et al. 2005). The complex also stabilizes p53 (An et al. 1998), which can then induce Bax translocation and thus initiate the mitochondrial cell death pathway (Ding et al. 2007).

The pro-apoptotic Bcl-2 proteins have been widely implicated in cardiovascular disease. In the myocardial cells, Bax is activated in response to oxidative stress (Gustafsson et al. 2004) and simulated I/R (Kubli et al. 2007; Capano and Crompton, 2006; Hamacher-Brady et al. 2006). Moreover, hearts of Bax deficient mice have reduced mitochondrial damage and infarcts size compared to wild type mice, suggesting that Bax is an important contributor to I/R injury (Hochhauser et al. 2003). The relative level of the pro- and anti- apoptotic Bcl-2 proteins determines whether a cell will survive or die following an apoptotic stimulus (Latif et al. 2000). Given the increase in caspase 3 and 7 activity, the increased protein of caspase 3 and pro-apoptotic Bax and decreased protein expression of anti-apoptotic Bcl-2 protein expression, these data indicate simultaneous high glucose and hypoxia-induced oxidative results in increased apoptosis.

1.3 High glucose and/or hypoxia-induced autophagy in H9c2 cardiomyocytes.

Nutrient depletion (Lum et al. 2005) and acute ischemia (Takagi et al. 2007) both induce autophagy to maintain mitochondria and ER function and promote cell survival. However, certain conditions, such as reperfusion (Takagi et al. 2007), high glucose exposure (Younce et al. 2010) and prolonged ER stress (Takemura et al. 2006, Younce et al. 2010), stimulate detrimental autophagy that promotes PCD. Autophagy depends on several key proteins including beclin-1 and LC3B (Takemura et al. 2006). Our results of the autophagy marker, LC3B-II, show a trend of gradually decreasing protein expression, with increased D-glucose concentrations and/or hypoxia (**Figure 8**). High glucose treatment is observed to diminish autophagy levels (**Figure 8**) while stimulating pro-apoptotic protein (caspase 3, 7 and Bax) expression (**Figure 5 and 6**) and diminishing

anti-apoptotic Bcl-2 protein expression (**Figure 7**) in high glucose and/or hypoxia conditions.

It has been reported that high glucose (28 mM D-glucose) exposure of H9c2 cardiomyoblasts and cardiomyocytes has been observed to induce oxidative stress and ER stress which eventually leads to increased autophagy (increased beclin-1 and LC3-II (LC3-I-PE) expression) and cell death (Younce et al. 2010). ER stress induces autophagy that is associated with cardiomyocyte death involved in heart failure (Takemura et al. 2006). Younce et al. (2010i) provide a molecular explanation into how hyperglycemia can result in cardiomyocyte cell death that contributes to the pathophysiological progression of diabetic cardiomyopathy (Younce et al. 2010i). Intriguingly, ROS-induced autophagic cell death occurs via a beclin-1 dependent pathway (De Meyer and Martinet 2009). Increased ROS levels activate the ubiquitin-proteosome system (Breitschopf et al. 2000), which degrade anti-apoptotic, beclin-1 binding and inhibiting (Pattingre et al. 2005), Bcl-2. This allows for beclin-1 activation and autophagic cell death (De Meyer and Martinet 2009).

Mitochondrial damage and/or failure of mitochondria to make enough ATP levels (such as during cell starvation) also leads to increased autophagic cell death (Gozuacik and Kimchi 2004; Levine and Yuan 2005). However we observed autophagy levels to decrease with increased D-glucose exposure, in normoxia (**Figure 8**). In normoxic and hypoxic conditions, we observed the decreases in autophagy to coincide with the decreases in H9c2 cell viability. This finding suggests that the autophagy may be beneficial/protective and that high glucose levels in hypoxia prevented the beneficial effects of autophagy in H9c2 cells.

In response to high glucose and hypoxia, Miranda et al. (2011), observed the induction of ER stress and UPR (Miranda et al. 2011), using ARPE19 cells. Autophagy is deregulated in chronic obesity-related ER stress (Yang et al. 2010). Interestingly, their study demonstrated that at 24h of high glucose and hypoxia, a decrease in autophagy was evident due to a decrease in LC3B-II content, indicating defective autophagosome formation. This coincided with an up-regulation of caspase 3 and a down-regulation of anti-apoptotic Bcl-xL. Miranda et al. (2011) concluded that apoptosis might be a delayed response triggered when autophagy-mediated protection is impaired (Miranda et al. 2011). We also observed a gradual decrease in the protein expressions of anti-apoptotic Bcl-2 and the autophagy marker (**Figure 7 and 8**). This also coincided with the increased protein expression of caspase 3 and pro-apoptotic Bax (**Figure 5 and 6**). Our results hence suggest the possible impairment of protective autophagy and the induction of apoptotic cell death in H9c2 cardiomyocyte exposed to high glucose and/or hypoxia.

1.4 High glucose and/or hypoxia-induced P-AMPK protein expression

The AMPK protein is the inactive form of the protein, no particular trend in the protein expression of inactive AMPK was observed in our study (**Figure 9**), suggesting that modification of AMPK activity is post-translational. Once it is in an activated/phosphorylated form (P-AMPK), it has the ability to function as an "energy sensor" to respond to increased intracellular AMP concentrations (and decreased ATP) during hypoxia (Abbud et al. 2000). This accounts for the increased P-AMPK protein expression in simultaneous 5.6 mM and 33 mM D-glucose and hypoxia. Despite the presence of 33 mM D-glucose containing media during hypoxia, P-AMPK protein

expression was still induced but still less than the control (5.6 mM normoxia) (**Figure 10**). Under hypoxic conditions, Jing et al. (2008) found that AMPK phosphorylation and activation is followed by the selective stimulation of the ERK1/2 pathway in H9c2 cells. The stimulation of this pathway is essential in the GLUT1 glucose transport response (Jing et al. 2008) of P-AMPK, in which it stimulates glucose transport into the mammalian cell to maintain ATP production (Fryer et al. 2002; Abbud et al. 2000). This serine-threonine kinase, AMPK, is inactive in ATP sufficient environments (Abbud et al.2000), hence the D-glucose concentration dependent decrease in P-AMPK protein expression (**Figure 8**) observed in normoxia.

AMPK plays an essential role in mediating glucose deprivation- or ischemia-induced autophagy in primary NRVM, this is accompanied by the inhibition of mTOR and the subsequent stimulation of autophagy in the mouse heart in vivo (Matsui et al. 2007, Takagi et al. 2007). The HIF-1 is upregulated in the heart under ischemic conditions (Kido et al. 2005) and HIF-1 is activated by AMPK (Lee et al. 2003). During myocardial ischemia (an energy starved state) a rapid drop in intracellular ATP leads to the activation of AMPK (Arad et al. 2007). The induction of autophagy by P-AMPK may contribute to the preservation of ATP content as well as promotion of cell survival (inhibits apoptotic cell death) in the ischemic heart. The activation of AMPK enhances ATP production through multiple mechanisms, including increases in glucose uptake, glycolysis, and fatty acid oxidation (Arad et al. 2007). The induction of autophagy allows for myocardial recovery from prolonged ischemia without significant myocardial injury (Decker and Wildenthal 1980). During ischemia, the ubiquitin proteasome pathway is generally inhibited in heart, (Powell 2006) so oxidized, misfolded and polyubiquitinated

protein aggregates accumulate. Autophagy is an alternative measure to scavenge and eliminate these aggregates in the heart during ischemia (Mizushima 2007). BNIP3-induced autophagosomes often contain fragmented mitochondria in cardiac myocytes, (Hamacher-Brady et al. 2007) suggesting that autophagy removes damaged mitochondria. Taken together, our findings suggest that the model we have used in our study voids the ATP depletion caused by hypoxia. The lack or attenuation of ATP depletion by glucose may result in H9c2 treated cells to deviate away from this ischemia/AMPK/mTOR/cardio-protective autophagy pathway. Mitigation of cardio-protective autophagy, needed to remove damaged mitochondria, triggers mitochondrial ROS-induced ROS release and generalized permeability transition pore opening, leading to the intrinsic pathway of apoptosis.

There is constitutive autophagic turnover of intracellular organelles such as mitochondria in highly oxidative tissues, including cardiac myocytes. Opening of the mitochondrial permeability transition and loss of mitochondrial membrane potential triggers their autophagic scavenging (Kim et al. 2007). Autophagic sequestration is also elicited by fission and subsequent depolarization of mitochondria (Twig et al. 2008). Thus, autophagy can protect from mitochondria that might otherwise trigger apoptotic cell death.

In mammals, P-AMPK is observed to inhibit mTOR (autophagy inhibitor) in ATP depleting conditions (Dennis et al. 2001) by phosphorylating and activating TSC1/2. P-AMPK is able to decrease protein synthesis and inhibit cell growth and hypertrophy. Despite the observed increases in P-AMPK, in 5.6 mM and 33 mM D-glucose and hypoxia (**Figure 10**), autophagy declined (**Figure 8**) during simultaneous glucose and

hypoxia treatment. Kanamori et al. (2011) demonstrated the induction of cardio-protective autophagy in an *in vivo* model of myocardial infarction. The inhibition of autophagy, using Bafilomycin A1 (an autophagy inhibitor), resulted in elevated P-AMPK protein levels, while enhancing autophagy, with Rapamycin (an autophagy enhancer) treatment, reduced P-AMPK protein levels. This suggests Bafilomycin A1-mediated inhibition of autophagy interferes with the supply of ATP to the ischemic myocardium, leading to activation of AMPK, while Rapamycin-mediated activation of autophagy does the reverse (Kanamori et al. 2011). In light of this study, our results may indicate a defect or malfunction of the autophagy mechanism in 5 mM and 33 mM D-glucose and hypoxia treatments. Defective autophagosome or autophagolysosome formation could inhibit autophagy, reduce autophagic flux and the maintenance of ATP production in hypoxic conditions leading to elevated activation of AMPK proteins.

This finding is further supported by electron microscopy evidence from pancreases retrieved from diabetic human cadavers that present with a massive increase in overloaded autophagosomes. Decreased lysosome formation can lead to abnormal autophagosome morphology. Diabetic pancreatic β -cells have been shown to have significantly reduced levels of various lysosomal genes, including cathepsins B and D, and of lysosome-associated membrane proteins (Lamp)-2, which are respectively involved in protein degradation and in fusion with the autophagosomes. Pancreatic islets exposed to high fatty acid concentrations for 24h present with impaired autophagosome morphology and decreased Lamp-2 gene expression (Bernales et al. 2007). Autophagic turnover is partially blocked by fatty acids in hepatocytes (Rubinsztein 2006; Arrasate et al. 2004). Decreased autophagic flux associated with decreased lysosomal activity

suggests that lysosomal function is a rate-limiting step halting autophagosome maturation. This reduces autophagic degradation and leads to more accumulation of damaged mitochondria and ER stress-damaged protein.

Another reason, as to why we observe increased P-AMPK protein levels coinciding with diminished autophagy levels in simultaneous glucose and hypoxia treatments, may be the activity of Akt. Interestingly Akt shows just the reverse action from that of P-AMPK because it inhibits mTOR inhibition by phosphorylating another domain of TSC (Inoki et al. 2003).

Interestingly, in simultaneous 25 mM D-glucose and hypoxia treatments we observed a decline in P-AMPK protein expression (**Figure 10**), which coincides with significant increases in ROS production, caspase 3 and 7 activity and Bax protein expression (**Figure 3, 4 and 6**). The relationships mentioned above and the biphasic P-AMPK protein expression may be explained by P-AMPK- p27kip interactions. The cell cycle regulator, p27kip, is involved in G1 arrest. In human mammary cancer-derived cells (MCF-7s), apoptosis occurring in serum depletion is inhibited when p27kip Thr198 is phosphorylated by P-AMPK; p27kip break down is inhibited and autophagy occurs. When p27kip expression is reduced, autophagy is inhibited and apoptosis occurs efficiently. That is, the amount of p27kip determines whether apoptosis or autophagy is initiated, and apoptosis is avoided to some extent. It seems that P-AMPK can induce autophagy and prolong the period leading to cell death (Liang et al. 2007). The findings of this previous study may explain the increased apoptotic cell death observed in simultaneous 25 mM D-glucose and hypoxia treatments. Thus our results suggest that the decrease in PAMPK, in hypoxia, not only fails to activate protective

autophagy but also allows for efficient apoptosis to occur.

Overall PAMPK protein levels, in normoxia, decreased with increasing D-glucose levels due the sufficient ATP states. Our treatments of simultaneous glucose and hypoxia voids the ATP depletion caused by hypoxia and the ischemia/AMPK/mTOR/cardio-protective autophagy pathway is not followed. Mitigation of cardio-protective autophagy leads to the accumulation of damaged mitochondria and promotes the intrinsic pathway of apoptosis. Despite elevated P-AMPK protein levels, autophagy is still diminished in simultaneous glucose and hypoxia conditions. This may be due to a defective step in the autophagy mechanism or the opposing activity of Akt. Diminished autophagy levels and elevated ROS, caspase 3 and 7 activity and pro-apoptotic Bax protein expression, in simultaneous 25 mM D-glucose and hypoxia treatments, may be due to declined P-AMPK protein levels not only failing to activate protective autophagy but also allowing for efficient apoptosis to occur.

1.5 The possible cumulative effects of hypoxia and hyperglycemia on cardiovascular function

Increased ROS production was shown to be associated with increased apoptosis in hearts of diabetic mice (Barouch et al. 2003). It has been proposed that ROS- mediated cell death could promote abnormal cardiac remodeling, which may contribute to the characteristic morphological and functional abnormalities that are associated with diabetic cardiomyopathy (Boudina and Abel 2007). Increased ROS production in diabetic hearts can trigger the accumulation of ceramide, which is involved in triggering

cardiomyocyte apoptosis, cardiac pump failure, and cardiac arrhythmias (Hayat et al. 2004).

It is evident that apoptosis plays a key role in the pathogenesis of a variety of cardiovascular diseases including myocardial infarction, dilated cardiomyopathy and end-stage heart failure (Olivetti et al. 1996; Saraste et al. 1997; Narula 1996; Aharinejad et al. 2008) as well as in animal models of ischemia– reperfusion injury (Cheng et al. 1996; Gottlieb et al. 1994; Gao et al. 2008).

Anti-apoptotic proteins Bcl-2 and Bcl-xl protect cardiomyocytes against various stressors. Over-expression of Bcl-xl in H9c2 cardiomyocytes protects against hypoxia-mediated apoptosis by preserving mitochondrial integrity (Reeve et al. 2007), and Bcl-2 can prevent p53-mediated apoptosis in cardiomyocytes (Kirshenbaum and de Moissac 1997). Transgenic mice overexpressing Bcl-2 in the heart have reduced I/R injury and show fewer apoptotic cells compared to wild type mice, suggesting that the cardio-protective effect of Bcl-2 is via inhibition of the mitochondrial death pathway (Imahashi et al. 2004; Brocheriou et al. 2000; Chen et al. 2001i).

Moreover, in failing hearts with adverse remodeling, increased wall stress represents a significant barrier against which cardiomyocytes must contract. These cells are expected to require a greater energy supply than those in a healthy heart, which could lead to relative hyponutrition, trigger AMPK activation, and induce autophagy. On the other hand, it was recently reported that there is latent hypoxia in the failing heart (Sano et al. 2007) which could interfere with ATP synthesis and reduce both the contraction and relaxation of cardiomyocytes. Baldi et al. (2002) reported that apoptotic loss of surviving cardiomyocytes contribute to the progression of post-infarction cardiac remodeling and

dysfunction (Baldi et al. 2002).

The inhibition of autophagy has been observed to trigger apoptosis (Boya et al. 2005). ATG5 knockout, in vitro, in NRVM decreases autophagic activity, induces a classic hypertrophic response, increased cell size, caspase 12 cleavage and decreased cell viability. Loss of autophagy, in the heart, triggers hypertrophic growth, cardiac dysfunction, and ultimately heart failure (Nakai et al. 2007). Lamp-2 protein deficiency blocks autophagosomes-lysosomes fusion, the resultant impairment of autophagic degradation and excessive accumulation of autophagasomes and long-lived proteins progresses into in cardiomyopathy (Maron et al. 2009). Together, these results point to the crucial housekeeping role of autophagy in cardiac cells in conditions of high glucose and hypoxia.

It is quite clear that apoptosis and autophagy, in the cases mentioned, seems to be in an antagonistic relationship with each other. Each process consists of opposing goals. Kanamori et al. (2011) induced myocardial infarction in mice by ligating the left coronary artery to investigate autophagy levels post-infarction. Autophagy was activated in surviving cardiomyocytes and chemical inhibition of autophagy significantly aggravated post-infarction cardiac dysfunction, remodeling and cardiac hypertrophy. Chemically enhancing autophagy reduced cardiac dysfunction and adverse remodeling. This suggests that activation of autophagic machinery is a compensatory response aimed at increasing the energy supply to meet the cellular demand in the face of excessive wall stress and hypoxia, which can cause a low-energy state in cardiomyocytes (Kanamori et al. 2011). Buss et al. (2009), further supports these findings by demonstrating that mTOR

inhibition increased autophagy and attenuated adverse remodeling following myocardial infarction (Buss et al. 2009).

Summary/Conclusion

Accordingly to our results the combined effects of high glucose and hypoxia reduce H9c2 cell viability. Simultaneous high glucose and hypoxia-induced oxidative stress suppresses cardio-protective autophagy and promotes cell death by apoptosis. The biphasic induction of ROS production, caspase 3/7 activity and PAMPK protein expression, in simultaneous high glucose and hypoxia conditions, calls for further research. Simultaneous high glucose and hypoxia likely voids the ATP depletion caused by hypoxia alone and the ischemia/AMPK/mTOR/cardio-protective autophagy pathway may not be followed. Further investigation into targets upstream and downstream of AMPK and of cellular ATP levels is advised to confirm. Mitigation of cardio-protective autophagy, needed to remove damaged mitochondria, may trigger mitochondrial ROS-induced ROS release and induction of the intrinsic pathway of apoptosis. PAMPK protein expression patterns, during high glucose and hypoxia, suggest the impairment of protective autophagy and the consequent induction of apoptotic cell death in H9c2 cardiomyocytes. It may be possible that defective step(s) in the autophagy process inhibit autophagy, reduce autophagic flux and the maintenance of ATP production in simultaneous high glucose and hypoxia conditions, leading to elevated activation of AMPK proteins.

References

Abbud W, Habinowski S, Zhang JZ, Kendrew J, Elkairi FS, Kemp BE, Witters LA, Ismail-Beigi F. Stimulation of amp-activated protein kinase (ampk) is associated with enhancement of glut1-mediated glucose transport. *Archives of biochemistry and biophysics*. 2000;380:347-352

Aharinejad S, Andrukhova O, Lucas T, Zuckermann A, Wieselthaler G, Wolner E, Grimm M. Programmed cell death in idiopathic dilated cardiomyopathy is mediated by suppression of the apoptosis inhibitor apollon. *The Annals of thoracic surgery*. 2008;86:109-114; discussion 114

Aki T, Nara A, Funakoshi T, Uemura K. Bifurcate effects of glucose on caspase-independent cell death during hypoxia. *Biochemical and biophysical research communications*. 2010;396:614-618

Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: Diagnosis and classification of diabetes mellitus provisional report of a who consultation. *Diabetic medicine : a journal of the British Diabetic Association*. 1998;15:539-553

Alici B, Gumustas MK, Ozkara H, Akkus E, Demirel G, Yencilek F, Hattat H. Apoptosis in the erectile tissues of diabetic and healthy rats. *BJU international*. 2000;85:326-329

An WG, Kanekal M, Simon MC, Maltepe E, Blagosklonny MV, Neckers LM. Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. *Nature*. 1998;392:405-408

Arad M, Seidman CE, Seidman JG. Amp-activated protein kinase in the heart: Role during health and disease. *Circulation research*. 2007;100:474-488

Aronson D. Hyperglycemia and the pathobiology of diabetic complications. *Advances in cardiology*. 2008;45:1-16

Arrasate M, Mitra S, Schweitzer ES, Segal MR, Finkbeiner S. Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death. *Nature*. 2004;431:805-810

Azad MB, Chen Y, Gibson SB. Regulation of autophagy by reactive oxygen species (ros): Implications for cancer progression and treatment. *Antioxidants & redox signaling*. 2009;11:777-790

Baldi A, Abbate A, Bussani R, Patti G, Melfi R, Angelini A, Dobrina A, Rossiello R, Silvestri F, Baldi F, Di Sciascio G. Apoptosis and post-infarction left ventricular remodeling. *Journal of molecular and cellular cardiology*. 2002;34:165-174

Barouch LA, Berkowitz DE, Harrison RW, O'Donnell CP, Hare JM. Disruption of leptin signaling contributes to cardiac hypertrophy independently of body weight in mice. *Circulation*. 2003;108:754-759

Basta G, Schmidt AM, De Caterina R. Advanced glycation end products and vascular inflammation: Implications for accelerated atherosclerosis in diabetes. *Cardiovascular research*. 2004;63:582-592

Bernales S, Schuck S, Walter P. Er-phagy: Selective autophagy of the endoplasmic reticulum. *Autophagy*. 2007;3:285-287

Biswas S, Gupta MK, Chattopadhyay D, Mukhopadhyay CK. Insulin-induced activation of hypoxia-inducible factor-1 requires generation of reactive oxygen species by nadph oxidase. *American journal of physiology. Heart and circulatory physiology*. 2007;292:H758-766

Bjornheden T, Levin M, Evaldsson M, Wiklund O. Evidence of hypoxic areas within the arterial wall in vivo. *Arteriosclerosis, thrombosis, and vascular biology*. 1999;19:870-876

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

Boya P, Gonzalez-Polo RA, Casares N, Perfettini JL, Dessen P, Larochette N, Metivier D, Meley D, Souquere S, Yoshimori T, Pierron G, Codogno P, Kroemer G. Inhibition of macroautophagy triggers apoptosis. *Molecular and cellular biology*. 2005;25:1025-1040

Breitschopf K, Haendeler J, Malchow P, Zeiher AM, Dimmeler S. Posttranslational modification of bcl-2 facilitates its proteasome-dependent degradation: Molecular characterization of the involved signaling pathway. *Molecular and cellular biology*. 2000;20:1886-1896

Brocheriou V, Hagege AA, Oubenaissa A, Lambert M, Mallet VO, Duriez M, Wassef M, Kahn A, Menasche P, Gilgenkrantz H. Cardiac functional improvement by a human bcl-2 transgene in a mouse model of ischemia/reperfusion injury. *The journal of*

gene medicine. 2000;2:326-333

Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature*. 2001;414:813-820

Bruick RK. Expression of the gene encoding the proapoptotic nip3 protein is induced by hypoxia. *Proceedings of the National Academy of Sciences of the United States of America*. 2000;97:9082-9087

Bryant D, Becker L, Richardson J, Shelton J, Franco F, Peshock R, Thompson M, Giroir B. Cardiac failure in transgenic mice with myocardial expression of tumor necrosis factor-alpha. *Circulation*. 1998;97:1375-1381

Bursch W. Multiple cell death programs: Charon's lifts to hades. *FEMS yeast research*. 2004;5:101-110

Bursell SE, Clermont AC, Kinsley BT, Simonson DC, Aiello LM, Wolpert HA. Retinal blood flow changes in patients with insulin-dependent diabetes mellitus and no diabetic retinopathy. *Investigative ophthalmology & visual science*. 1996;37:886-897

Buss SJ, Muenz S, Riffel JH, Malekar P, Hagenmueller M, Weiss CS, Bea F, Bekeredjian R, Schinke-Braun M, Izumo S, Katus HA, Hardt SE. Beneficial effects of mammalian target of rapamycin inhibition on left ventricular remodeling after myocardial infarction. *Journal of the American College of Cardiology*. 2009;54:2435-2446

Caballero AE, Arora S, Saouaf R, Lim SC, Smakowski P, Park JY, King GL, LoGerfo FW, Horton ES, Veves A. Microvascular and macrovascular reactivity is reduced in subjects at risk for type 2 diabetes. *Diabetes*. 1999;48:1856-1862

Cai L, Chen S, Evans T, Deng DX, Mukherjee K, Chakrabarti S. Apoptotic germ-cell death and testicular damage in experimental diabetes: Prevention by endothelin antagonism. *Urological research*. 2000;28:342-347

Cai L, Kang YJ. Oxidative stress and diabetic cardiomyopathy: A brief review. *Cardiovascular toxicology*. 2001;1:181-193

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

Capano M, Crompton M. Bax translocates to mitochondria of heart cells during simulated ischaemia: Involvement of amp-activated and p38 mitogen-activated protein kinases. *The Biochemical journal*. 2006;395:57-64

Catrina SB, Okamoto K, Pereira T, Brismar K, Poellinger L. Hyperglycemia regulates hypoxia-inducible factor-1alpha protein stability and function. *Diabetes*. 2004;53:3226-3232

Ceriello A. New insights on oxidative stress and diabetic complications may lead to a "causal" antioxidant therapy. *Diabetes care*. 2003;26:1589-1596

Chakravarti RN, Kirshenbaum LA, Singal PK. Atherosclerosis: its pathophysiology with special references to lipid peroxidation. *Journal of applied cardiology*. 1991;6:91–112

Chambers DE, Parks DA, Patterson G, Roy R, McCord JM, Yoshida S, Parmley LF, Downey JM. Xanthine oxidase as a source of free radical damage in myocardial ischemia. *Journal of molecular and cellular cardiology*. 1985;17:145-152

Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC, Schumacker PT. Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. *Proceedings of the National Academy of Sciences of the United States of America*. 1998;95:11715-11720

Chandel NS, McClintock DS, Feliciano CE, Wood TM, Melendez JA, Rodriguez AM, Schumacker PT. Reactive oxygen species generated at mitochondrial complex iii stabilize hypoxia-inducible factor-1alpha during hypoxia: A mechanism of o2 sensing. *The Journal of biological chemistry*. 2000;275:25130-25138

Chang TI, Horal M, Jain SK, Wang F, Patel R, Loeken MR. Oxidant regulation of gene expression and neural tube development: Insights gained from diabetic pregnancy on molecular causes of neural tube defects. *Diabetologia*. 2003;46:538-545

Chang YY, Neufeld TP. Autophagy takes flight in drosophila. *FEBS letters*. 2010;584:1342-1349

Chatham JC, Forder JR, McNeill JH. The Heart in Diabetes. Norwell, MA: Kluwer Academic Publishers, 1996

Chen C, Pore N, Behrooz A, Ismail-Beigi F, Maity A. Regulation of glut1 mrna by hypoxia-inducible factor-1. Interaction between h-ras and hypoxia. *The Journal of biological chemistry*. 2001;276:9519-9525

Chen Z, Chua CC, Ho YS, Hamdy RC, Chua BH. Overexpression of bcl-2 attenuates apoptosis and protects against myocardial i/r injury in transgenic mice. *American journal of physiology. Heart and circulatory physiology*. 2001i;280:H2313-2320

Chen JK, Hu LJ, Wang J, Lamborn KR, Kong EL, Deen DF. Hypoxia-induced bax overexpression and radiation killing of hypoxic glioblastoma cells. *Radiation research*. 2005;163:644-653

Chen Y, Klionsky DJ. The regulation of autophagy - unanswered questions. *Journal of cell science*. 2011;124:161-170

Cheng W, Kajstura J, Nitahara JA, Li B, Reiss K, Liu Y, Clark WA, Krajewski S, Reed JC, Olivetti G, Anversa P. Programmed myocyte cell death affects the viable myocardium after infarction in rats. *Experimental cell research*. 1996;226:316-327

Chien CT, Shyue SK, Lai MK. Bcl-xl augmentation potentially reduces ischemia/reperfusion induced proximal and distal tubular apoptosis and autophagy. *Transplantation*. 2007;84:1183-1190

Choi SL, Kim SJ, Lee KT, Kim J, Mu J, Birnbaum MJ, Soo Kim S, Ha J. The regulation of amp-activated protein kinase by h(2)o(2). *Biochemical and biophysical research communications*. 2001;287:92-97

Chong ZZ, Maiese K. The src homology 2 domain tyrosine phosphatases shp-1 and shp-2: Diversified control of cell growth, inflammation, and injury. *Histology and histopathology*. 2007;22:1251-1267

Corton JM, Gillespie JG, Hardie DG. Role of the amp-activated protein kinase in the cellular stress response. *Current biology : CB*. 1994;4:315-324

Creager MA, Luscher TF, Cosentino F, Beckman JA. Diabetes and vascular disease: Pathophysiology, clinical consequences, and medical therapy: Part i. *Circulation*. 2003;108:1527-1532

Danial NN, Korsmeyer SJ. Cell death: Critical control points. *Cell*. 2004;116:205-219

Datema R, Schwarz RT. Formation of 2-deoxyglucose-containing lipid-linked oligosaccharides. Interference with glycosylation of glycoproteins. *European journal of biochemistry / FEBS*. 1978;90:505-516

Daugas E, Susin SA, Zamzami N, Ferri KF, Irinopoulou T, Larochette N, Prevost MC, Leber B, Andrews D, Penninger J, Kroemer G. Mitochondrio-nuclear translocation of aif in apoptosis and necrosis. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2000;14:729-739

Davi G, Falco A, Patrono C. Lipid peroxidation in diabetes mellitus. *Antioxidants & redox signaling*. 2005;7:256-268

de Boer RA, van Veldhuisen DJ, van der Wijk J, Brouwer RM, de Jonge N, Cole GM, Suurmeijer AJ. Additional use of immunostaining for active caspase 3 and cleaved actin and parp fragments to detect apoptosis in patients with chronic heart failure. *Journal of cardiac failure*. 2000;6:330-337

De Meyer GR, Martinet W. Autophagy in the cardiovascular system. *Biochimica et biophysica acta*. 2009;1793:1485-1495

Decker RS, Wildenthal K. Lysosomal alterations in hypoxic and reoxygenated hearts. I. Ultrastructural and cytochemical changes. *The American journal of pathology*. 1980;98:425-444

Dennis PB, Jaeschke A, Saitoh M, Fowler B, Kozma SC, Thomas G. Mammalian tor: A homeostatic atp sensor. *Science*. 2001;294:1102-1105

Desco MC, Asensi M, Marquez R, Martinez-Valls J, Vento M, Pallardo FV, Sastre J, Vina J. Xanthine oxidase is involved in free radical production in type 1 diabetes: Protection by allopurinol. *Diabetes*. 2002;51:1118-1124

Defraigne JO. [a central pathological mechanism explaining diabetic complications?]. *Revue medicale de Liege*. 2005;60:472-478

DeFronzo RA, Bonadonna RC, Ferrannini E. Pathogenesis of niddm. A balanced overview. *Diabetes care*. 1992;15:318-368

Depre C, Young ME, Ying J, Ahuja HS, Han Q, Garza N, Davies PJ, Taegtmeyer H. Streptozotocin-induced changes in cardiac gene expression in the absence of severe contractile dysfunction. *Journal of molecular and cellular cardiology*. 2000;32:985-996

Devaraj S, Vega-Lopez S, Jialal I. Antioxidants, oxidative stress, and inflammation in diabetes. In: Marso SP, Stern DM, eds. Diabetes and cardiovascular disease. Philadelphia, PA: Lippincott Williams and Wilkins, 2004: 19-35.

Diaz-Velez CR, Garcia-Castineiras S, Mendoza-Ramos E, Hernandez-Lopez E. Increased malondialdehyde in peripheral blood of patients with congestive heart failure. *American heart journal*. 1996;131:146-152

Ding WX, Ni HM, Chen X, Yu J, Zhang L, Yin XM. A coordinated action of bax, puma, and p53 promotes mg132-induced mitochondria activation and apoptosis in colon cancer cells. *Molecular cancer therapeutics*. 2007;6:1062-1069

Djavaheri-Mergny M, Amelotti M, Mathieu J, Besancon F, Bauvy C, Souquere S, Pierron G, Codogno P. Nf-kappab activation represses tumor necrosis factor-alpha-induced autophagy. *The Journal of biological chemistry*. 2006;281:30373-30382

Edinger AL, Thompson CB. Death by design: Apoptosis, necrosis and autophagy. *Current opinion in cell biology*. 2004;16:663-669

Eisenberg-Lerner A, Bialik S, Simon HU, Kimchi A. Life and death partners: Apoptosis, autophagy and the cross-talk between them. *Cell death and differentiation*. 2009;16:966-975

Elsasser A, Vogt AM, Nef H, Kostin S, Mollmann H, Skwara W, Bode C, Hamm C, Schaper J. Human hibernating myocardium is jeopardized by apoptotic and autophagic cell death. *Journal of the American College of Cardiology*. 2004;43:2191-2199

Farahmand F, Lou H, Singal PK. Oxidative stress in cardiovascular complications of diabetes. *In* Atherosclerosis, hypertension, and diabetes eds. Pierce GN, Nagano M, Zahradka P, Dhalla NS. Kluwer Academic Publisher, Boston. 2003;427–435

Federici M, Menghini R, Mauriello A, Hribal ML, Ferrelli F, Lauro D, Sbraccia P, Spagnoli LG, Sesti G, Lauro R. Insulin-dependent activation of endothelial nitric oxide synthase is impaired by o-linked glycosylation modification of signaling proteins in human coronary endothelial cells. *Circulation*. 2002;106:466-472

Feng Z, Zhang H, Levine AJ, Jin S. The coordinate regulation of the p53 and mtor pathways in cells. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102:8204-8209

Finkel T. Oxygen radicals and signaling. *Current opinion in cell biology*. 1998;10:248-253

Fiordaliso F, Li B, Latini R, Sonnenblick EH, Anversa P, Leri A, Kajstura J. Myocyte death in streptozotocin-induced diabetes in rats in angiotensin ii- dependent. *Laboratory investigation; a journal of technical methods and pathology*. 2000;80:513-527

Frustaci A, Kajstura J, Chimenti C, Jakoniuk I, Leri A, Maseri A, Nadal-Ginard B, Anversa P. Myocardial cell death in human diabetes. *Circulation research*. 2000;87:1123-1132

Fryer LG, Foufelle F, Barnes K, Baldwin SA, Woods A, Carling D. Characterization of the role of the amp-activated protein kinase in the stimulation of glucose transport in skeletal muscle cells. *The Biochemical journal*. 2002;363:167-174

Gao L, Mejias R, Echevarria M, Lopez-Barneo J. Induction of the glucose-6-phosphate dehydrogenase gene expression by chronic hypoxia in pc12 cells. *FEBS letters*. 2004;569:256-260

Gao W, Ferguson G, Connell P, Walshe T, Murphy R, Birney YA, O'Brien C, Cahill PA. High glucose concentrations alter hypoxia-induced control of vascular smooth

muscle cell growth via a hif-1alpha-dependent pathway. *Journal of molecular and cellular cardiology*. 2007;42:609-619

Gao HK, Yin Z, Zhou N, Feng XY, Gao F, Wang HC. Glycogen synthase kinase 3 inhibition protects the heart from acute ischemia-reperfusion injury via inhibition of inflammation and apoptosis. *Journal of cardiovascular pharmacology*. 2008;52:286-292

Geng J, Klionsky DJ. The atg8 and atg12 ubiquitin-like conjugation systems in macroautophagy. 'Protein modifications: Beyond the usual suspects' review series. *EMBO reports*. 2008;9:859-864

Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *The Journal of clinical investigation*. 1994;94:1621-1628

Gozuacik D, Bialik S, Raveh T, Mitou G, Shohat G, Sabanay H, Mizushima N, Yoshimori T, Kimchi A. Dap-kinase is a mediator of endoplasmic reticulum stress-induced caspase activation and autophagic cell death. *Cell death and differentiation*. 2008;15:1875-1886

Gozuacik D, Kimchi A. Autophagy as a cell death and tumor suppressor mechanism. *Oncogene*. 2004;23:2891-2906

Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. *Science*. 2004;305:626-629

Greijer AE, van der Wall E. The role of hypoxia inducible factor 1 (hif-1) in hypoxia induced apoptosis. *Journal of clinical pathology*. 2004;57:1009-1014

Grunenfelder J, Miniati DN, Murata S, Falk V, Hoyt EG, Kown M, Koransky ML, Robbins RC. Upregulation of bcl-2 through caspase-3 inhibition ameliorates ischemia/reperfusion injury in rat cardiac allografts. *Circulation*. 2001;104:I202-206

Gustafsson AB, Gottlieb RA. Bcl-2 family members and apoptosis, taken to heart. *American journal of physiology. Cell physiology*. 2007;292:C45-51

Gustafsson AB, Gottlieb RA. Heart mitochondria: Gates of life and death. *Cardiovascular research*. 2008;77:334-343

Gustafsson AB, Gottlieb RA. Autophagy in ischemic heart disease. *Circulation research*. 2009;104:150-158

Gustafsson AB, Tsai JG, Logue SE, Crow MT, Gottlieb RA. Apoptosis repressor with caspase recruitment domain protects against cell death by interfering with bax activation. *The Journal of biological chemistry*. 2004;279:21233-21238

Gutteridge JM, Halliwell B. Free radicals and antioxidants in the year 2000. A historical look to the future. *Annals of the New York Academy of Sciences*. 2000;899:136-147

Guzik TJ, Mussa S, Gastaldi D, Sadowski J, Ratnatunga C, Pillai R, Channon KM. Mechanisms of increased vascular superoxide production in human diabetes mellitus: Role of nad(p)h oxidase and endothelial nitric oxide synthase. *Circulation*. 2002;105:1656-1662

Guzy RD, Hoyos B, Robin E, Chen H, Liu L, Mansfield KD, Simon MC, Hammerling U, Schumacker PT. Mitochondrial complex iii is required for hypoxia-induced ros production and cellular oxygen sensing. *Cell metabolism*. 2005;1:401-408

Ha JH, Noh HS, Shin IW, Hahm JR, Kim DR. Mitigation of H2O2-induced autophagic cell death by propofol in H9c2 cardiomyocytes. *Cell Biology and Toxicology*. 2012;28:1:19-29

Halcox JP, Schenke WH, Zalos G, Mincemoyer R, Prasad A, Waclawiw MA, Nour KR, Quyyumi AA. Prognostic value of coronary vascular endothelial dysfunction. *Circulation*. 2002;106:653-658

Hamacher-Brady A, Brady NR, Gottlieb RA. Enhancing macroautophagy protects against ischemia/reperfusion injury in cardiac myocytes. *The Journal of biological chemistry*. 2006;281:29776-29787

Hardie DG. Minireview: The amp-activated protein kinase cascade: The key sensor of cellular energy status. *Endocrinology*. 2003;144:5179-5183

Hardie DG. New roles for the lkb1-->ampk pathway. *Current opinion in cell biology*. 2005;17:167-173

Hayat SA, Patel B, Khattar RS, Malik RA. Diabetic cardiomyopathy: Mechanisms, diagnosis and treatment. *Clin Sci (Lond)*. 2004;107:539-557

Hayden MR, Tyagi SC. Intimal redox stress: Accelerated atherosclerosis in metabolic syndrome and type 2 diabetes mellitus. Atheroscleropathy. *Cardiovascular diabetology*. 2002;1:1-27

Hescheler J, Meyer R, Plant S, Krautwurst D, Rosenthal W, Schultz G. Morphological, biochemical, and electrophysiological characterization of a clonal cell (h9c2) line from rat heart. *Circulation research*. 1991;69:1476-1486

Hirota K, Semenza GL. Regulation of hypoxia-inducible factor 1 by prolyl and asparaginyl hydroxylases. *Biochemical and biophysical research communications*. 2005;338:610-616

Hochachka PW, Buck LT, Doll CJ, Land SC. Unifying theory of hypoxia tolerance: Molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. *Proceedings of the National Academy of Sciences of the United States of America*. 1996;93:9493-9498

Hochhauser E, Kivity S, Offen D, Maulik N, Otani H, Barhum Y, Pannet H, Shneyvays V, Shainberg A, Goldshtaub V, Tobar A, Vidne BA. Bax ablation protects against myocardial ischemia-reperfusion injury in transgenic mice. *American journal of physiology. Heart and circulatory physiology*. 2003;284:H2351-2359

Horman S, Beauloye C, Vertommen D, Vanoverschelde JL, Hue L, Rider MH. Myocardial ischemia and increased heart work modulate the phosphorylation state of eukaryotic elongation factor-2. *The Journal of biological chemistry*. 2003;278:41970-41976

Houstis N, Rosen ED, Lander ES. Reactive oxygen species have a causal role in multiple forms of insulin resistance. *Nature*. 2006;440:944-948

Huang DC, Strasser A. Bh3-only proteins-essential initiators of apoptotic cell death. *Cell*. 2000;103:839-842

Ichimura Y, Kirisako T, Takao T, Satomi Y, Shimonishi Y, Ishihara N, Mizushima N, Tanida I, Kominami E, Ohsumi M. A ubiquitin-like system mediates protein lipidation. *Nature*. 2000;408:488-492.

Imahashi K, Schneider MD, Steenbergen C, Murphy E. Transgenic expression of bcl-2 modulates energy metabolism, prevents cytosolic acidification during ischemia, and reduces ischemia/reperfusion injury. *Circulation research*. 2004;95:734-741

Inoki K, Zhu T, Guan KL. Tsc2 mediates cellular energy response to control cell growth and survival. *Cell*. 2003;115:577-590

Islam MS, Loots du T. Experimental rodent models of type 2 diabetes: A review. *Methods and findings in experimental and clinical pharmacology*. 2009;31:249-261

Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ. Targeting of hif-alpha to the von hippel-lindau ubiquitylation complex by o2-regulated prolyl hydroxylation. *Science*. 2001;292:468-472

Jay D, Hitomi H, Griendling KK. Oxidative stress and diabetic cardiovascular complications. *Free radical biology & medicine*. 2006;40:183-192

Jing M, Cheruvu VK, Ismail-Beigi F. Stimulation of glucose transport in response to activation of distinct ampk signaling pathways. *American journal of physiology. Cell physiology*. 2008;295:C1071-1082

Jing G, Wang JJ, Zhang SX. Er stress and apoptosis: A new mechanism for retinal cell death. *Experimental diabetes research*. 2012;2012:589589

Johnstone M: Diabetes and Cardiovascular Disease. Totowa, NJ, Humana, 2000.

Juan LJ, Shia WJ, Chen MH, Yang WM, Seto E, Lin YS, Wu CW. Histone deacetylases specifically down-regulate p53-dependent gene activation. *The Journal of biological chemistry*. 2000;275:20436-20443

Kanamori H, Takemura G, Goto K, Maruyama R, Tsujimoto A, Ogino A, Takeyama T, Kawaguchi T, Watanabe T, Fujiwara T, Fujiwara H, Seishima M, Minatoguchi S. The role of autophagy emerging in postinfarction cardiac remodelling. *Cardiovascular research*. 2011;91:330-339

Kang C, Avery L. To be or not to be, the level of autophagy is the question: Dual roles of autophagy in the survival response to starvation. *Autophagy*. 2008;4:82-84

Kang SM, Chung N, Kim JY, Koo BK, Choi D, Jang Y, Cho SY. Relation of vasodilator response of the brachial artery to inflammatory markers in patients with coronary artery disease. *Echocardiography*. 2002;19:661-667

Khansari N, Shakiba Y, Mahmoudi M. Chronic inflammation and oxidative stress as a major cause of age-related diseases and cancer. *Recent patents on inflammation & allergy drug discovery*. 2009;3:73-80

Kido M, Du L, Sullivan CC, Li X, Deutsch R, Jamieson SW, Thistlethwaite PA. Hypoxia-inducible factor 1-alpha reduces infarction and attenuates progression of cardiac

dysfunction after myocardial infarction in the mouse. *Journal of the American College of Cardiology*. 2005;46:2116-2124

Kiffin R, Bandyopadhyay U, Cuervo AM. Oxidative stress and autophagy. *Antioxidants & redox signaling*. 2006;8:152-162

Kihara A, Noda T, Ishihara N, Ohsumi Y. Two distinct vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase y sorting in saccharomyces cerevisiae. *The Journal of cell biology*. 2001;152:519-530

Kim BS, Chen J, Weinstein T, Noiri E, Goligorsky MS. Vegf expression in hypoxia and hyperglycemia: Reciprocal effect on branching angiogenesis in epithelial-endothelial co-cultures. *Journal of the American Society of Nephrology : JASN*. 2002;13:2027-2036

Kim J, Kundu M, Viollet B, Guan KL. Ampk and mtor regulate autophagy through direct phosphorylation of ulk1. *Nature cell biology*. 2011;13:132-141

Kim I, Rodriguez-Enriquez S, Lemasters JJ. Selective degradation of mitochondria by mitophagy. *Archives of biochemistry and biophysics*. 2007;462:245-253

Kimura S, Zhang GX, Nishiyama A, Shokoji T, Yao L, Fan YY, Rahman M, Suzuki T, Maeta H, Abe Y. Role of nad(p)h oxidase- and mitochondria-derived reactive oxygen species in cardioprotection of ischemic reperfusion injury by angiotensin ii. *Hypertension*. 2005;45:860-866

Kirshenbaum LA, de Moissac D. The bcl-2 gene product prevents programmed cell death of ventricular myocytes. *Circulation*. 1997;96:1580-1585

Klionsky DJ. Autophagy: From phenomenology to molecular understanding in less than a decade. *Nature reviews. Molecular cell biology*. 2007;8:931-937

Kouroku Y, Fujita E, Tanida I, Ueno T, Isoai A, Kumagai H, Ogawa S, Kaufman RJ, Kominami E, Momoi T. Er stress (perk/eif2alpha phosphorylation) mediates the polyglutamine-induced lc3 conversion, an essential step for autophagy formation. *Cell death and differentiation*. 2007;14:230-239

Kubli DA, Quinsay MN, Huang C, Lee Y, Gustafsson AB. Bnip3 functions as a mitochondrial sensor of oxidative stress during myocardial ischemia and reperfusion. *American journal of physiology. Heart and circulatory physiology*. 2008;295:H2025-2031

Kubli DA, Ycaza JE, Gustafsson AB. Bnip3 mediates mitochondrial dysfunction and cell death through bax and bak. *The Biochemical journal*. 2007;405:407-415

Kubota T, McTiernan CF, Frye CS, Slawson SE, Lemster BH, Koretsky AP, Demetris AJ, Feldman AM. Dilated cardiomyopathy in transgenic mice with cardiac-specific overexpression of tumor necrosis factor-alpha. *Circulation research*. 1997;81:627-635

Kubota C, Torii S, Hou, N, Saito N, Yoshimoto Y, Imai H, Takeuchi T. Constitutive reactive oxygen species generation from autophagosome/lysosome in neuronal oxidative toxicity. *J. Biol. Chem.* 2010; 285: 667-674

Kudo N, Barr AJ, Barr RL, Desai S, Lopaschuk GD. High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in

malonyl-coa levels due to an increase in 5'-amp-activated protein kinase inhibition of acetyl-coa carboxylase. *The Journal of biological chemistry*. 1995;270:17513-17520

Kunchithapautham K, Rohrer B. Apoptosis and autophagy in photoreceptors exposed to oxidative stress. *Autophagy*. 2007;3:433-441

Kwon SH, Pimentel DR, Remondino A, Sawyer DB, Colucci WS. H(2)o(2) regulates cardiac myocyte phenotype via concentration-dependent activation of distinct kinase pathways. *Journal of molecular and cellular cardiology*. 2003;35:615-621

Latif N, Khan MA, Birks E, O'Farrell A, Westbrook J, Dunn MJ, Yacoub MH. Upregulation of the bcl-2 family of proteins in end stage heart failure. *Journal of the American College of Cardiology*. 2000;35:1769-1777

Lee P, Sata M, Lefer DJ, Factor SM, Walsh K, Kitsis RN. Fas pathway is a critical mediator of cardiac myocyte death and mi during ischemia-reperfusion in vivo. *American journal of physiology. Heart and circulatory physiology*. 2003;284:H456-463

Levine B, Klionsky DJ. Development by self-digestion: Molecular mechanisms and biological functions of autophagy. *Developmental cell*. 2004;6:463-477

Levine B, Yuan J. Autophagy in cell death: An innocent convict? *The Journal of clinical investigation*. 2005;115:2679-2688

Levy AP, Levy NS, Goldberg MA. Post-transcriptional regulation of vascular endothelial growth factor by hypoxia. *The Journal of biological chemistry*. 1996;271:2746-2753

Liang J, Shao SH, Xu ZX, Hennessy B, Ding Z, Larrea M, Kondo S, Dumont DJ, Gutterman JU, Walker CL, Slingerland JM, Mills GB. The energy sensing lkb1-ampk pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. *Nature cell biology*. 2007;9:218-224

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cytochrome c and datp-dependent formation of apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell*. 1997;91:479-489

Li H, Telemaque S, Miller RE, Marsh JD. High glucose inhibits apoptosis induced by serum deprivation in vascular smooth muscle cells via upregulation of bcl-2 and bcl-xl. *Diabetes*. 2005;54:540-545

Li R, Chase M, Jung SK, Smith PJ, Loeken MR. Hypoxic stress in diabetic pregnancy contributes to impaired embryo gene expression and defective development by inducing oxidative stress. *American journal of physiology. Endocrinology and metabolism*. 2005i;289:E591-599

Li J, Wang JJ, Yu Q, Chen K, Mahadev K, Zhang SX. Inhibition of reactive oxygen species by lovastatin downregulates vascular endothelial growth factor expression and ameliorates blood-retinal barrier breakdown in db/db mice: Role of nadph oxidase 4. *Diabetes*. 2010;59:1528-1538

Li SY, Yang X, Ceylan-Isik AF, Du M, Sreejayan N, Ren J. Cardiac contractile dysfunction in lep/lep obesity is accompanied by nadph oxidase activation, oxidative modification of sarco(endo)plasmic reticulum ca2+-atpase and myosin heavy chain isozyme switch. *Diabetologia*. 2006;49:1434-1446

Li Z, Zhang T, Dai H, Liu G, Wang H, Sun Y, Zhang Y, Ge Z. Endoplasmic reticulum stress is involved in myocardial apoptosis of streptozocin-induced diabetic rats. *The Journal of endocrinology*. 2008;196:565-572

Lin Z, Weinberg JM, Malhotra R, Merritt SE, Holzman LB, Brosius FC, 3rd. Glut-1 reduces hypoxia-induced apoptosis and jnk pathway activation. *American journal of physiology. Endocrinology and metabolism*. 2000;278:E958-966

Liu Q, Wang G, Zhou G, Tan Y, Wang X, Wei W, Liu L, Xue W, Feng W, Cai L. Angiotensin ii-induced p53-dependent cardiac apoptotic cell death: Its prevention by metallothionein. *Toxicology letters*. 2009;191:314-320

Lum JJ, DeBerardinis RJ, Thompson CB. Autophagy in metazoans: Cell survival in the land of plenty. *Nature reviews. Molecular cell biology*. 2005;6:439-448

Mahadev K, Zilbering A, Zhu L, Goldstein BJ. Insulin-stimulated hydrogen peroxide reversibly inhibits protein-tyrosine phosphatase 1b in vivo and enhances the early insulin action cascade. *The Journal of biological chemistry*. 2001;276:21938-21942

Maher JC, Krishan A, Lampidis TJ. Greater cell cycle inhibition and cytotoxicity induced by 2-deoxy-d-glucose in tumor cells treated under hypoxic vs aerobic conditions. *Cancer chemotherapy and pharmacology*. 2004;53:116-122

Maiuri M C, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self- killing: crosstalk between autophagy and apoptosis. *Nature Reviews Molecular Cell Biology*. 2007; 8:741- 752

Malhotra R, Brosius FC, 3rd. Glucose uptake and glycolysis reduce hypoxia-induced apoptosis in cultured neonatal rat cardiac myocytes. *The Journal of biological chemistry*. 1999;274:12567-12575

Malhotra R, Tyson DG, Sone H, Aoki K, Kumagai AK, Brosius FC, 3rd. Glucose uptake and adenoviral mediated glut1 infection decrease hypoxia-induced hif-1alpha levels in cardiac myocytes. *Journal of molecular and cellular cardiology*. 2002;34:1063-1073

Malhotra R, Tyson DW, Rosevear HM, Brosius FC, 3rd. Hypoxia-inducible factor-1alpha is a critical mediator of hypoxia induced apoptosis in cardiac h9c2 and kidney epithelial hk-2 cells. *BMC cardiovascular disorders*. 2008;8:9

Malliopoulou V, Xinaris C, Mourouzis I, Cokkinos AD, Katsilambros N, Pantos C, Kardami E, Cokkinos DV. High glucose protects embryonic cardiac cells against simulated ischemia. *Molecular and cellular biochemistry*. 2006;284:87-93

Malmberg K, Norhammar A, Wedel H, Ryden L. Glycometabolic state at admission: Important risk marker of mortality in conventionally treated patients with diabetes mellitus and acute myocardial infarction: Long-term results from the diabetes and insulin-glucose infusion in acute myocardial infarction (digami) study. *Circulation*. 1999;99:2626-2632

Mandrup-Poulsen T. Beta-cell apoptosis: Stimuli and signaling. *Diabetes*. 2001;50 Suppl 1:S58-63

Mansfield KD, Guzy RD, Pan Y, Young RM, Cash TP, Schumacker PT, Simon MC. Mitochondrial dysfunction resulting from loss of cytochrome c impairs cellular oxygen sensing and hypoxic hif-alpha activation. *Cell metabolism*. 2005;1:393-399

Maron BJ, Roberts WC, Arad M, Haas TS, Spirito P, Wright GB, Almquist AK, Baffa JM, Saul JP, Ho CY, Seidman J, Seidman CE. Clinical outcome and phenotypic expression in lamp2 cardiomyopathy. *JAMA : the journal of the American Medical Association*. 2009;301:1253-1259

Marshall S, Bacote V, Traxinger RR. Discovery of a metabolic pathway mediating glucose-induced desensitization of the glucose transport system. Role of hexosamine biosynthesis in the induction of insulin resistance. *The Journal of biological chemistry*. 1991;266:4706-4712

Marsin AS, Bertrand L, Rider MH, Deprez J, Beauloye C, Vincent MF, Van den Berghe G, Carling D, Hue L. Phosphorylation and activation of heart pfk-2 by ampk has a role in the stimulation of glycolysis during ischaemia. *Current biology : CB*. 2000;10:1247-1255

Martin DN, Baehrecke EH. Caspases function in autophagic programmed cell death in drosophila. *Development*. 2004;131:275-284

Matschinsky FM. Regulation of pancreatic beta-cell glucokinase: From basics to therapeutics. *Diabetes*. 2002;51 Suppl 3:S394-404

Matsui Y, Kyoi S, Takagi H, Hsu CP, Hariharan N, Ago T, Vatner SF, Sadoshima J. Molecular mechanisms and physiological significance of autophagy during myocardial ischemia and reperfusion. *Autophagy*. 2008;4:409-415

Matsui Y, Takagi H, Qu X, Abdellatif M, Sakoda H, Asano T, Levine B, Sadoshima J. Distinct roles of autophagy in the heart during ischemia and reperfusion: Roles of amp-activated protein kinase and beclin 1 in mediating autophagy. *Circulation research*. 2007;100:914-922

Mayanskaya SD, Mayanskaya NN, Efremov AV, Yakobson GS. Activity of lysosomal apparatus in rat myocardium during experimental coronary and noncoronary myocardial damage. *Bulletin of Experimental Biology and Medicine*. 2000;129:530–532

McClain DA, Crook ED. Hexosamines and insulin resistance. *Diabetes*. 1996;45:1003-1009

Merighi S, Benini A, Mirandola P, Gessi S, Varani K, Leung E, Maclennan S, Baraldi PG, Borea PA. Hypoxia inhibits paclitaxel-induced apoptosis through adenosine-mediated phosphorylation of bad in glioblastoma cells. *Molecular pharmacology*. 2007;72:162-172

Minchenko A, Bauer T, Salceda S, Caro J. Hypoxic stimulation of vascular endothelial growth factor expression in vitro and in vivo. *Laboratory investigation; a journal of technical methods and pathology*. 1994;71:374-379

Mine S, Okada Y, Tanikawa T, Kawahara C, Tabata T, Tanaka Y. Increased expression levels of monocyte ccr2 and monocyte chemoattractant protein-1 in patients

with diabetes mellitus. *Biochemical and biophysical research communications*. 2006;344:780-785

Miranda S, Gonzalez-Rodriguez A, Garcia-Ramirez M, Revuelta-Cervantes J, Hernandez C, Simo R, Valverde AM. Beneficial effects of fenofibrate in retinal pigment epithelium by the modulation of stress and survival signaling under diabetic conditions. *Journal of cellular physiology*. 2012;227:2352-2362

Misra MK, Sarwat M, Bhakuni P, Tuteja R, Tuteja N. Oxidative stress and ischemic myocardial syndromes. *Medical science monitor : international medical journal of experimental and clinical research*. 2009;15:RA209-219

Mizushima N. Autophagy: process and function. *Genes and Development*. 2007;21:2861–2873

Mohanty P, Hamouda W, Garg R, Aljada A, Ghanim H, Dandona P. Glucose challenge stimulates reactive oxygen species (ros) generation by leucocytes. *The Journal of clinical endocrinology and metabolism*. 2000;85:2970-2973

Moore MN, Viarengo A, Donkin P, Hawkins AJ. Autophagic and lysosomal reactions to stress in the hepatopancreas of blue mussels. *Aquat Toxicol*. 2007;84:80-91

Nakai A, Yamaguchi O, Takeda T, Higuchi Y, Hikoso S, Taniike M, Omiya S, Mizote I, Matsumura Y, Asahi M, Nishida K, Hori M, Mizushima N, Otsu K. The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. *Nature medicine*. 2007;13:619-624

Nagata D, Hirata Y. The role of amp-activated protein kinase in the cardiovascular system. *Hypertension research: official journal of the Japanese Society of Hypertension*. 2010;33:22-28

Narula J, Haider N, Virmani R, DiSalvo TG, Kolodgie FD, Hajjar RJ, Schmidt U, Semigran MJ, Dec GW, Khaw BA. Apoptosis in myocytes in end-stage heart failure. *The New England journal of medicine*. 1996;335:1182-1189

Newrick PG, Wilson AJ, Jakubowski J, Boulton AJ, Ward JD. Sural nerve oxygen tension in diabetes. *Br Med J (Clin Res Ed)*. 1986;293:1053-1054

Newsholme P, Haber EP, Hirabara SM, Rebelato EL, Procopio J, Morgan D, Oliveira-Emilio HC, Carpinelli AR, Curi R. Diabetes associated cell stress and dysfunction: Role of mitochondrial and non-mitochondrial ros production and activity. *The Journal of physiology*. 2007;583:9-24

Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature*. 2000;404:787-790

Nishio Y, Kanazawa A, Nagai Y, Inagaki H, Kashiwagi A. Regulation and role of the mitochondrial transcription factor in the diabetic rat heart. *Annals of the New York Academy of Sciences*. 2004;1011:78-85

Ogata M, Hino S, Saito A, Morikawa K, Kondo S, Kanemoto S, Murakami T, Taniguchi M, Tanii I, Yoshinaga K, Shiosaka S, Hammarback JA, Urano F, Imaizumi K.

Autophagy is activated for cell survival after endoplasmic reticulum stress. *Molecular and cellular biology*. 2006;26:9220-9231

Ohshita T. Suppression of autophagy by ethionine administration in male rat liver in vivo. *Toxicology*. 2000;147: 51–57

Olivetti G, Quaini F, Sala R, Lagrasta C, Corradi D, Bonacina E, Gambert SR, Cigola E, Anversa P. Acute myocardial infarction in humans is associated with activation of programmed myocyte cell death in the surviving portion of the heart. *Journal of molecular and cellular cardiology*. 1996;28:2005-2016

Omura T, Yoshiyama M, Kim S, Matsumoto R, Nakamura Y, Izumi Y, Ichijo H, Sudo T, Akioka K, Iwao H, Takeuchi K, Yoshikawa J. Involvement of apoptosis signal-regulating kinase-1 on angiotensin ii-induced monocyte chemoattractant protein-1 expression. *Arteriosclerosis, thrombosis, and vascular biology*. 2004;24:270-275

Ornoy A, Rand SB, Bischitz N. Hyperglycemia and hypoxia are interrelated in their teratogenic mechanism: Studies on cultured rat embryos. *Birth defects research. Part B, Developmental and reproductive toxicology*. 2010;89:106-115

Ornoy A, Livshitz A, Ergaz Z, Stodgell CJ, Miller RK. Hyperglycemia, hypoxia and their combination exert oxidative stress and changes in antioxidant gene expression: studies on cultured rat embryos. *Birth Defects Research: Part B, Developmental and Reproductive Toxicology*. 2011;92(3):231-9

Ozawa T, Hu JL, Hu LJ, Kong EL, Bollen AW, Lamborn KR, Deen DF. Functionality of hypoxia-induced bax expression in a human glioblastoma xenograft model. *Cancer gene therapy*. 2005;12:449-455

Pacher P, Szabo C. Role of poly(adp-ribose) polymerase 1 (parp-1) in cardiovascular diseases: The therapeutic potential of parp inhibitors. *Cardiovascular drug reviews*. 2007;25:235-260

Pacher P, Szabo C. Role of peroxynitrite in the pathogenesis of cardiovascular complications of diabetes. *Current opinion in pharmacology*. 2006;6:136-141

Pandya HC, Snetkov VA, Twort CHC, Ward JPT, Hirst SJ. Oxygen regulates mitogen-stimulated proliferation of fetal human airway smooth muscle cells. *Lung Cellula and Molecular Physiology.* 2002:283: 6: L1220-L1230

Pastukh V, Wu S, Ricci C, Mozaffari M, Schaffer S. Reversal of hyperglycemic preconditioning by angiotensin ii: Role of calcium transport. *American journal of physiology. Heart and circulatory physiology*. 2005;288:H1965-1975

Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, Packer M, Schneider MD, Levine B. Bcl-2 antiapoptotic proteins inhibit beclin 1-dependent autophagy. *Cell*. 2005;122:927-939

Piret JP, Mottet D, Raes M, Michiels C. Is hif-1alpha a pro- or an anti-apoptotic protein? *Biochemical pharmacology*. 2002;64:889-892

Powell SR. The ubiquitin-proteasome system in cardiac physiology and pathology. *American Journal of Physiology - Heart and Circulatory Physiology*. 2006;291:H1–H19

Pyo JO, Jang MH, Kwon YK, Lee HJ, Jun JI, Woo HN, Cho DH, Choi B, Lee H, Kim JH, Mizushima N, Oshumi Y, Jung YK. Essential roles of Atg5 and FADD in

autophagic cell death: dissection of autophagic cell death into vacuole formation and cell death. *Journal of Biological Chemistry*. 2005;280:20722–20729

Ramana KV, Chandra D, Srivastava S, Bhatnagar A, Srivastava SK. Nitric oxide regulates the polyol pathway of glucose metabolism in vascular smooth muscle cells. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*. 2003;17:417-425

Rami A, Langhagen A, Steiger S. Focal cerebral ischemia induces upregulation of beclin 1 and autophagy-like cell death. *Neurobiology of disease*. 2008;29:132-141

Ravikumar B, Vacher C, Berger Z, Davies JE, Luo S, Oroz LG, Scaravilli F, Easton DF, Duden R, O'Kane CJ, Rubinsztein DC. Inhibition of mtor induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of huntington disease. *Nature genetics*. 2004;36:585-595

Reddy HK, Koshy SK, Wasson S, Quan EE, Pagni S, Roberts AM, Joshua IG, Tyagi SC. Adaptive-outward and maladaptive-inward arterial remodeling measured by intravascular ultrasound in hyperhomocysteinemia and diabetes. *Journal of cardiovascular pharmacology and therapeutics*. 2006;11:65-76

Reeve JL, Szegezdi E, Logue SE, Chonghaile TN, O'Brien T, Ritter T, Samali A. Distinct mechanisms of cardiomyocyte apoptosis induced by doxorubicin and hypoxia converge on mitochondria and are inhibited by bcl-xl. *Journal of cellular and molecular medicine*. 2007;11:509-520

Regula KM, Ens K, Kirshenbaum LA. Inducible expression of BNIP3 provokes mitochondrial defects and hypoxia-mediated cell death of ventricular myocytes. *Circulation Research*. 2002;91:226–231

Rizzoni D, Agabiti Rosei E. Small artery remodeling in hypertension and diabetes. *Current hypertension reports*. 2006;8:90-95

Robertson RP, Harmon J, Tran PO, Tanaka Y, Takahashi H. Glucose toxicity in beta-cells: Type 2 diabetes, good radicals gone bad, and the glutathione connection. *Diabetes*. 2003;52:581-587

Rolo AP, Palmeira CM. Diabetes and mitochondrial function: Role of hyperglycemia and oxidative stress. *Toxicology and applied pharmacology*. 2006;212:167-178

Roy S. Caspase at the heart of the apoptotic cell death pathway. *Chemical research in Toxicology*. 2000;13:961–962

Rubinsztein DC. The roles of intracellular protein-degradation pathways in neurodegeneration. *Nature*. 2006;443:780-786

Ruiz E, Gordillo-Moscoso A, Padilla E, Redondo S, Rodriguez E, Reguillo F, Briones AM, van Breemen C, Okon E, Tejerina T. Human vascular smooth muscle cells from diabetic patients are resistant to induced apoptosis due to high bcl-2 expression. *Diabetes*. 2006;55:1243-1251

Russell RR, 3rd, Bergeron R, Shulman GI, Young LH. Translocation of myocardial glut-4 and increased glucose uptake through activation of ampk by aicar. *The American journal of physiology*. 1999;277:H643-649

Rutkowski DT, Kaufman RJ. A trip to the er: Coping with stress. *Trends in cell biology*. 2004;14:20-28

Sainio A, Jokela T, Tammi MI, Jarvelainen H. Hyperglycemic conditions modulate connective tissue reorganization by human vascular smooth muscle cells through stimulation of hyaluronan synthesis. *Glycobiology*. 2010;20:1117-1126

Sam F, Kerstetter DL, Pimental DR, Mulukutla S, Tabaee A, Bristow MR, Colucci WS, Sawyer DB. Increased reactive oxygen species production and functional alterations in antioxidant enzymes in human failing myocardium. *Journal of cardiac failure*. 2005;11:473-480

San Martin A, Du P, Dikalova A, Lassegue B, Aleman M, Gongora MC, Brown K, Joseph G, Harrison DG, Taylor WR, Jo H, Griendling KK. Reactive oxygen species-selective regulation of aortic inflammatory gene expression in type 2 diabetes. *American journal of physiology. Heart and circulatory physiology*. 2007;292:H2073-2082

Sano M, Minamino T, Toko H, Miyauchi H, Orimo M, Qin Y, Akazawa H, Tateno K, Kayama Y, Harada M, Shimizu I, Asahara T, Hamada H, Tomita S, Molkentin JD, Zou Y, Komuro I. P53-induced inhibition of hif-1 causes cardiac dysfunction during pressure overload. *Nature*. 2007;446:444-448

Santilli SM, Fiegel VD, Knighton DR. Alloxan diabetes alters the rabbit transarterial wall oxygen gradient. *Journal of vascular surgery : official publication, the Society for Vascular Surgery [and] International Society for Cardiovascular Surgery, North American Chapter*. 1993;18:227-233

Schmaltz C, Hardenbergh PH, Wells A, Fisher DE. Regulation of proliferation-survival decisions during tumor cell hypoxia. *Molecular and cellular biology*. 1998;18:2845-2854

Saraste A, Pulkki K, Kallajoki M, Henriksen K, Parvinen M, Voipio-Pulkki LM. Apoptosis in human acute myocardial infarction. *Circulation*. 1997;95:320-323

Sayen MR, Gustafsson AB, Sussman MA, Molkentin JD, Gottlieb RA. Calcineurin transgenic mice have mitochondrial dysfunction and elevated superoxide production. *American journal of physiology. Cell physiology*. 2003;284:C562-570

Scherz-Shouval R, Shvets E, Fass E, Shorer H, Gil L, Elazar Z. Reactive oxygen species are essential for autophagy and specifically regulate the activity of atg4. *The EMBO journal*. 2007;26:1749-1760

Schroder M, Kaufman RJ. Er stress and the unfolded protein response. *Mutation research*. 2005;569:29-63

Semenza GL. Surviving ischemia: Adaptive responses mediated by hypoxia-inducible factor 1. *The Journal of clinical investigation*. 2000;106:809-812

Semenza GL. Targeting hif-1 for cancer therapy. *Nature reviews. Cancer*. 2003;3:721-732

Shimizu S, Eguchi Y, Kamiike W, Itoh Y, Hasegawa J, Yamabe K, Otsuki Y, Matsuda H, Tsujimoto Y. Induction of apoptosis as well as necrosis by hypoxia and predominant prevention of apoptosis by bcl-2 and bcl-xl. *Cancer research*. 1996;56:2161-2166

Singh JP, Larson MG, O'Donnell CJ, Wilson PF, Tsuji H, Lloyd-Jones DM, Levy D. Association of hyperglycemia with reduced heart rate variability (the framingham heart study). *The American journal of cardiology*. 2000;86:309-312

Singh VP, Le B, Bhat VB, Baker KM, Kumar R. High-glucose-induced regulation of intracellular ang ii synthesis and nuclear redistribution in cardiac myocytes. *American journal of physiology. Heart and circulatory physiology*. 2007;293:H939-948

Son SM. Role of vascular reactive oxygen species in development of vascular abnormalities in diabetes. *Diabetes research and clinical practice*. 2007;77 Suppl 1:S65-70

Sowter HM, Ratcliffe PJ, Watson P, Greenberg AH, Harris AL. Hif-1-dependent regulation of hypoxic induction of the cell death factors bnip3 and nix in human tumors. *Cancer research*. 2001;61:6669-6673

Srinivasan S, Stevens M, Wiley JW. Diabetic peripheral neuropathy: Evidence for apoptosis and associated mitochondrial dysfunction. *Diabetes*. 2000;49:1932-1938

Steinberg GR, Michell BJ, van Denderen BJ, Watt MJ, Carey AL, Fam BC, Andrikopoulos S, Proietto J, Gorgun CZ, Carling D, Hotamisligil GS, Febbraio MA, Kay

TW, Kemp BE. Tumor necrosis factor alpha-induced skeletal muscle insulin resistance involves suppression of amp-kinase signaling. *Cell metabolism*. 2006;4:465-474

Swynghedauw B. Molecular mechanisms of myocardial remodeling. *Physiological reviews*. 1999;79:215-262

Szegezdi E, Fitzgerald U, Samali A. Caspase-12 and er-stress-mediated apoptosis: The story so far. *Annals of the New York Academy of Sciences*. 2003;1010:186-194

Takagi H, Matsui Y, Hirotani S, Sakoda H, Asano T, Sadoshima J. Ampk mediates autophagy during myocardial ischemia in vivo. *Autophagy*. 2007;3:405-407

Takemura G, Miyata S, Kawase Y, Okada H, Maruyama R, Fujiwara H. Autophagic degeneration and death of cardiomyocytes in heart failure. *Autophagy*. 2006;2:212-214

Tanaka M, Ito H, Adachi S, Akimoto H, Nishikawa T, Kasajima T, Marumo F, Hiroe M. Hypoxia induces apoptosis with enhanced expression of fas antigen messenger rna in cultured neonatal rat cardiomyocytes. *Circulation research*. 1994;75:426-433

Tiedge M, Lortz S, Drinkgern J, Lenzen S. Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. *Diabetes*. 1997;46:1733-1742

Tracy K, Dibling BC, Spike BT, Knabb JR, Schumacker P, Macleod KF. Bnip3 is an rb/e2f target gene required for hypoxia-induced autophagy. *Molecular and cellular biology*. 2007;27:6229-6242

Thorburn A. Death receptor-induced cell killing. *Cellular signalling*. 2004;16:139-144

Twig G, Elorza A, Molina AJ, Mohamed H, Wikstrom JD, Walzer G, Stiles L, Haigh SE, Katz S, Las G, Alroy J, Wu M, Py BF, Yuan J, Deeney JT, Corkey BE, Shirihai OS. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. *The EMBO journal*. 2008;27:433-446

Urakawa H, Katsuki A, Sumida Y, Gabazza EC, Murashima S, Morioka K, Maruyama N, Kitagawa N, Tanaka T, Hori Y, Nakatani K, Yano Y, Adachi Y. Oxidative stress is associated with adiposity and insulin resistance in men. *The Journal of clinical endocrinology and metabolism*. 2003;88:4673-4676

Vahsen N, Cande C, Briere JJ, Benit P, Joza N, Larochette N, Mastroberardino PG, Pequignot MO, Casares N, Lazar V, Feraud O, Debili N, Wissing S, Engelhardt S, Madeo F, Piacentini M, Penninger JM, Schagger H, Rustin P, Kroemer G. Aif deficiency compromises oxidative phosphorylation. *The EMBO journal*. 2004;23:4679-4689

Van Hoecke M, Prigent-Tessier AS, Garnier PE, Bertrand NM, Filomenko R, Bettaieb A, Marie C, Beley AG. Evidence of hif-1 functional binding activity to caspase-3 promoter after photothrombotic cerebral ischemia. *Molecular and cellular neurosciences*. 2007;34:40-47

Wang J, Silva JP, Gustafsson CM, Rustin P, Larsson NG. Increased in vivo apoptosis in cells lacking mitochondrial DNA gene expression. *Proceedings of the National Academy of Sciences of the United States of America*. 2001;98:4038-4043

Wang X. The expanding role of mitochondria in apoptosis. *Genes & development*. 2001i;15:2922-2933

Westermann D, Van Linthout S, Dhayat S, Dhayat N, Schmidt A, Noutsias M, Song XY, Spillmann F, Riad A, Schultheiss HP, Tschope C. Tumor necrosis factor-alpha antagonism protects from myocardial inflammation and fibrosis in experimental diabetic cardiomyopathy. *Basic research in cardiology*. 2007;102:500-507

Wick AN, Drury DR, Nakada HI, Wolfe JB. Localization of the primary metabolic block produced by 2-deoxyglucose. *The Journal of biological chemistry*. 1957;224:963-969

Weinberg R. The Biology of Cancer. Garland Science, New York; 2007

Wu HH, Hsiao TY, Chien CT, Lai MK. Ischemic conditioning by short periods of reperfusion attenuates renal ischemia/reperfusion induced apoptosis and autophagy in the rat. *Journal of biomedical science*. 2009;16:19

Wu H, Yang JM, Jin S, Zhang H, Hait WN. Elongation factor-2 kinase regulates autophagy in human glioblastoma cells. *Cancer research*. 2006;66:3015-3023

Xi H, Kurtoglu M, Liu H, Wangpaichitr M, You M, Liu X, Savaraj N, Lampidis TJ. 2-deoxy-d-glucose activates autophagy via endoplasmic reticulum stress rather than atp depletion. *Cancer chemotherapy and pharmacology*. 2011;67:899-910

Yan L, Sadoshima J, Vatner DE, Vatner SF. Autophagy: A novel protective mechanism in chronic ischemia. *Cell Cycle*. 2006;5:1175-1177

Yang Z, Klionsky DJ. Mammalian autophagy: Core molecular machinery and signaling regulation. *Current opinion in cell biology*. 2010;22:124-131

Yang L, Li P, Fu S, Calay ES, Hotamisligil GS. Defective hepatic autophagy in obesity promotes er stress and causes insulin resistance. *Cell metabolism*. 2010;11:467-478

Yoh K, Hirayama A, Ishizaki K, Yamada A, Takeuchi M, Yamagishi S, Morito N, Nakano T, Ojima M, Shimohata H, Itoh K, Takahashi S, Yamamoto M. Hyperglycemia induces oxidative and nitrosative stress and increases renal functional impairment in nrf2-deficient mice. *Genes to cells: devoted to molecular & cellular mechanisms*. 2008;13:1159-1170

Younce CW, Kolattukudy PE. Mcp-1 causes cardiomyoblast death via autophagy resulting from er stress caused by oxidative stress generated by inducing a novel zinc-finger protein, mcpip. *The Biochemical journal*. 2010;426:43-53

Younce CW, Wang K, Kolattukudy PE. Hyperglycaemia-induced cardiomyocyte death is mediated via mcp-1 production and induction of a novel zinc-finger protein mcpip. *Cardiovascular research*. 2010i;87:665-674

Yu L, Alva A, Su H, Dutt P, Freundt E, Welsh S, Baehrecke EH, Lenardo MJ. Regulation of an atg7-beclin 1 program of autophagic cell death by caspase-8. *Science*. 2004;304:1500-1502

Yu XY, Geng YJ, Liang JL, Lin QX, Lin SG, Zhang S, Li Y. High levels of glucose induce apoptosis in cardiomyocyte via epigenetic regulation of the insulin-like growth factor receptor. *Experimental cell research*. 2010;316:2903-2909

Yu XY, Song YH, Geng YJ, Lin QX, Shan ZX, Lin SG, Li Y. Glucose induces apoptosis of cardiomyocytes via microrna-1 and igf-1. *Biochemical and biophysical research communications*. 2008;376:548-552

Zelzer E, Levy Y, Kahana C, Shilo BZ, Rubinstein M, Cohen B. Insulin induces transcription of target genes through the hypoxia-inducible factor hif-1alpha/arnt. *The EMBO journal*. 1998;17:5085-5094

Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. Serine phosphorylation of death agonist bad in response to survival factor results in binding to 14-3-3 not bcl-x(l). *Cell*. 1996;87:619-628

Zhang L, Li L, Liu H, Prabhakaran K, Zhang X, Borowitz JL, Isom GE. Hif-1alpha activation by a redox-sensitive pathway mediates cyanide-induced bnip3 upregulation and mitochondrial-dependent cell death. *Free radical biology & medicine*. 2007;43:117-127

Zhang K, Kaufman RJ. From endoplasmic-reticulum stress to the inflammatory response. *Nature*. 2008;454:455-462

Zhou L, Azfer A, Niu J, Graham S, Choudhury M, Adamski FM, Younce C, Binkley PF, Kolattukudy PE. Monocyte chemoattractant protein-1 induces a novel transcription factor that causes cardiac myocyte apoptosis and ventricular dysfunction. *Circulation research*. 2006;98:1177-1185

Zhou YF, Zheng XW, Zhang GH, Zong ZH, Qi GX. The effect of hypoxia-inducible factor 1-alpha on hypoxia-induced apoptosis in primary neonatal rat ventricular myocytes. *Cardiovascular journal of Africa*. 2010;21:37-41

Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial ROS-induced ROS release: an update and review. *Biochimica et Biophysica Acta*. 2006;1757: 509–517.