THE ROLE AND MECHANISMS OF HEXOKINASE-2-MEDIATED PROTECTION AGAINST CARDIAC CELL DEATH AND DISEASE

A Dissertation presented to

the Faculty of the Graduate School

At the University of Missouri

In Partial Fulfillment

Of the Requirement for the Degree

Doctor of Philosophy

---

by

KYLE STEPHAN MCCOMMIS

Dr. Christopher P. Baines, Thesis Supervisor

JULY 2013

The undersigned, appointed by the dean of the Graduate School have examined the dissertation entitled

THE ROLE AND MECHANISMS OF HEXOKINASE-2-MEDIATED PROTECTION AGAINST CARDIAC CELL DEATH AND DISEASE

Presented by Kyle Stephan McCommis

A candidate for the degree of doctor of philosophy

And hereby certify that, in their opinion, it is worthy of acceptance

_____

Christopher P. Baines, PhD

_____

Maike Krenz, MD

_____

Douglas K. Bowles, PhD

_____

M. Harold Laughlin, PhD

DEDICATION

I would like to dedicate this dissertation to my son, James McCommis. You are the happiest, healthiest little boy. It has truly been a pleasure to witness you grow up thus far.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor, Dr. Christopher Baines, for his continued support and guidance through the years. I am incredibly grateful for his leadership and direction during this project. He also insisted upon extending my research focus to all available models and projects, from which I have gained immeasurable experience. This accomplishment would not be possible, were it not for his dedication and mentorship.

I would also like to thank my doctoral advisory committee, Drs. Maike Krenz, Douglas Bowles, and M. Harold Laughlin for their thoughtful instruction and constructive criticism.

I would like to extend my gratitude to everyone I have had the pleasure to work with during my time in the Baines / Krenz labs: Sarah Clay, Kathryn Crombie, Diana Douglas, Michelle Edwards, Manuel Gutierrez-Aguilar, Kurt Marshall, John Matera, Allison McGee, Ashley Reeb, and Christine Schramm. You have made my time in the lab most enjoyable.

Lastly, I would also like to thank my wife, Brenda McCommis, and my parents, Jim and Laura McCommis, for their unending support and desire for me to succeed in all endeavors.

TABLE OF CONTENTS

LIST OF FIGURES

FIGURE Page

ABBREVIATIONS

$\Delta\Psi_M$	Mitochondrial Membrane Potential
ADP	Adenosine Diphosphate
ANGII	Angiotensin II
ANP	Atrial Natriuretic Peptide
ANT	Adenine Nucleotide Transporter
ATP	Adenosine Triphosphate
ATP-S	ATP Synthase
Bcl2	B-cell Lymphoma 2
BH3	Bcl2 Homology Domain 3
βGal	β-Galactosidase
BNP	B-type Natriuretic Peptide
CypD	Cyclophilin D
DHEA	Dehydroxyepiandrosterone
DISC	Death-Inducing Signaling Complex
DKO	Double Knockout
DRP1	Dynamin Related Protein 1
ETC	Electron Transport Chain
FA	Fatty Acid
FADD	FAS-Associated Death Domain
FAO	Fatty Acid Oxidation
FFA	Free Fatty Acid

G6PDH	Glucose-6-Phosphate Dehydrogenase
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GLUT	Glucose transporter
H_2O_2	Hydrogen Peroxide
HIF	Hypoxia-inducible Factor
HK	Hexokinase
I/R	Ischemia / Reperfusion
IMM	Inner Mitochondrial Membrane
KO	Knockout
LDH	Lactate Dehydrogenase
LV	Left Ventricle
MEF	Murine Embryonic Fibroblast
MHC	Myosin Heavy Chain
MLKL	Mixed Lineage Kinase Domain-Like
MOI	Multiplicity of Infection
MPT	Mitochondrial Permeability Transition
MPTP	Mitochondrial Permeability Transition Pore
NRCM	Neonatal Rat Cardiomyocyte
NTG	Non-transgenic
O-GlcNAc	*O*-linked *N*-acetylglucosamine
OMM	Outer Mitochondrial Membrane
PARP	Poly(Adenosine Diphosphate-Ribose) Polymerase
PE	Phenylephrine

PGAM5	Phosphoglycerate Mutase 5
PGC-1	Peroxisome Proliferator-Activated Receptor Gamma-Coactivator-1
PiC	Mitochondrial Phosphate Carrier
PPARα	Peroxisome Proliferator-Activated Receptor Alpha
PPP	Pentose Phosphate Pathway
RIP	Receptor Interacting Protein
ROS	Reactive Oxygen Species
RV	Right Ventricle
siRNA	Small Interfering RNA
TCA	Tricarboxylic Acid
TG	Transgenic
TMRE	Tetramethylrhodamine, Ethyl Ester, Perchlorate
TNF	Tumor Necrosis Factor
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
UDP-GlcNAc	Uridine diphospho-*N*-acetylglucosamine
VDAC	Voltage-Dependent Anion Channel
WT	Wild-type

THE ROLE AND MECHANISMS OF HEXOKINASE-2-MEDIATED PROTECTION AGAINST CARDIAC CELL DEATH AND DISEASE

Kyle S. McCommis

Dr. Christopher P. Baines, Thesis Supervisor

ABSTRACT

Numerous cancer studies demonstrate that transformed cells exhibit both increased glucose metabolism and an increased propensity to survive in the face of stressful stimuli. These studies overwhelmingly correlate with overexpression of the rate-limiting glycolysis enzyme hexokinase-2 (HK2). However, the mechanisms linking increased glucose metabolism and survival are unknown. Additionally, there is limited evidence of increased glucose utilization being beneficial in normal cell cultures, as well as *in vivo*. Therefore, the current studies were designed to determine the mechanisms by which increased HK2 expression promotes cell survival, as well as investigate whether HK2 overexpression could attenuate a model of cardiac disease *in vivo*. Findings in **AIM1** demonstrate that HK2 binding to the voltage-dependent anion channel 3 (VDAC3) on the mitochondria is important for protection against reactive oxygen species (ROS)-induced cell death. In **AIM2**, we show that cardiac HK2 overexpression limits cardiomyocyte hypertrophy and cell death in response to chronic isoproterenol administration *in vivo*. Results from these studies demonstrate that HK2 limits ROS accumulation during cardiac hypertrophy, and that this attenuation of ROS is mediated via the pentose-phosphate pathway. Collectively, our data establish several mechanisms by which HK2 overexpression and increased glucose utilization protect against cardiac cell death and disease. Additionally, these findings raise the possibility that activation of HK2 may be a therapeutic target in cardiac pathologies.

CHAPTER 1: Introduction

1. GLUCOSE UTILIZATION PATHWAYS

Hexokinase-2 (HK2) – the focus of this dissertation, is the first enzyme utilized in essentially all glucose metabolism pathways, as described below. Therefore, it is important to first depict the importance of glucose metabolism and all of the glucose utilization pathways to understand how altering HK2 expression may affect these various pathways.

1.1 *Maintenance of whole body glucose*

Glucose and other 6-carbon sugars are important energy substrates utilized by nearly all organisms through a largely conserved set of metabolic pathways. While it is somewhat of an accessory metabolite for most tissues, it is the primary metabolite for the brain (Scheinberg, 1949). Therefore, it is critically important for blood glucose levels to remain relatively steady. Blood glucose is increased either by dietary carbohydrates, or hepatic output. Blood glucose is decreased through cessation of hepatic output, and glucose uptake into the major glucose sinks: muscle and adipose tissue. Influx of dietary carbohydrates stimulates insulin production from pancreatic β-cells, which promotes storage of carbohydrates and fat and prevents hepatic glucose production. On the other hand, there are a host of defenses to combat hypoglycemia (Cryer, 1993). Hypoglycemia causes a decrease in insulin secretion, and stimulates glucagon secretion from the α-cells of the pancreas. Glucagon raises blood glucose levels by raising hepatic glucose output through a combination of glycogenolysis and gluconeogenesis. Exercise also induces

glucagon production to increase blood glucose levels by hepatic output to support the increased muscle glucose uptake (Holloszy, 1996).

1.2 *Cellular glucose entry*

The first step of glucose metabolism involves glucose entry into the cell. For most mammalian cells, this occurs through glucose transporter (GLUT) proteins in the plasma membrane (Zierler, 1999). Epithelial cells transport glucose by combination of GLUTs and the Na^+/glucose cotransporters (Wright, 1997). There are six GLUT isoforms, all with considerable homology but varying in their tissue expression, abundance, and affinity for glucose. GLUT1 is widely distributed to most tissues, while GLUT4 is primarily in insulin sensitive tissues. Under basal conditions, GLUT4 is localized almost entirely to intracellular vesicles. Insulin (Rea, 1997) or exercise (Ren, 1994) cause rapid fusion of these vesicles with the plasma membrane, thereby facilitating glucose transport through GLUT4. Thus, under basal conditions, glucose delivery and entry into cells are rate-limiting steps for glucose utilization. During insulin stimulation or exercise however, the phosphorylation of glucose by hexokinase (HK) becomes the limiting barrier to glucose uptake (Fueger, 2004; Halseth, 1998).

1.3 *Glycolysis*

After entry into cells, glucose can be directed towards any one of the multiple glucose utilization pathways (**Figure 1**). Glycolysis, the breakdown of 6-carbon glucose into two 3-carbon pyruvate molecules for further metabolism, is considered the preferred route for glucose. Louis Pasteur discovered the process of glycolysis in 1860 while studying beer

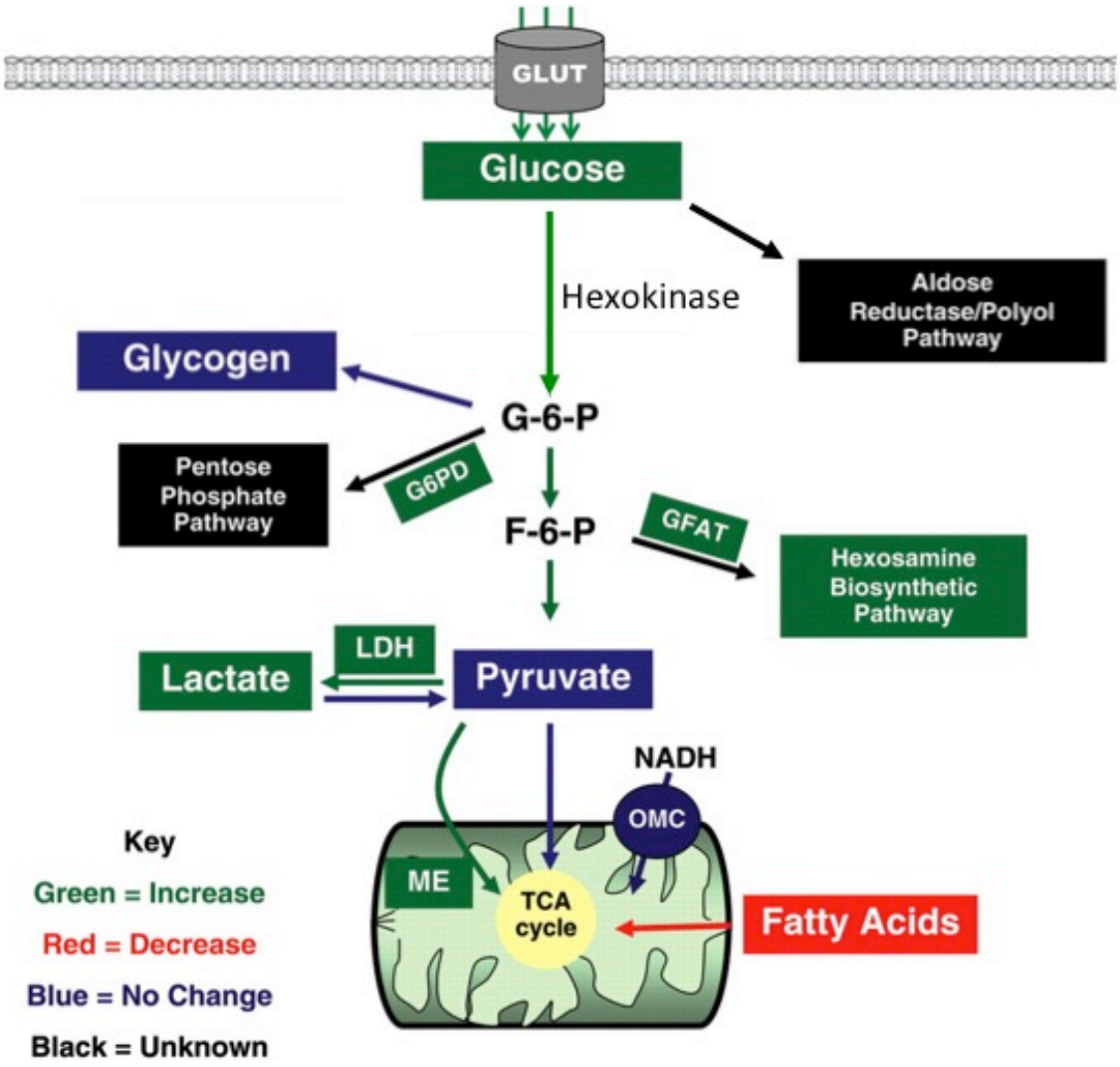

Figure 1. Glucose utilization pathways. Schematic illustrating the various glucose utilization pathways. Glycolysis is the "preferred" pathway for glucose catabolism; however, the accessory pathways are important for the creation of other metabolites. Importantly, hexokinase-mediated phosphorylation of glucose is the initial step for virtually all glucose utilization pathways. The color-coding shown in the key indicates changes observed during cardiac hypertrophy (See section 3.3).

Adapted from: Kolwicz SC. et al.; Cardiovasc Res. 90, 194-201, 2011.

and wine fermentation (Pasteur, 1861). It wasn't until 1940 that the combined work of Gustav Embden, Otto Warburg, and Otto Fritz Meyerhof unearthed the complete glycolytic pathway (Kresge, 2005), which is shown in **Figure 2**. Overall, glycolysis can be divided into two parts: a "preparatory" phase, which requires energy expenditure and cleavage of a 6-carbon molecule into two 3-carbon molecules, and then the "payoff" phase where energy is released. The first step in glycolysis involves the phosphorylation of glucose into glucose-6-phosphate by HK. This is an energy consuming reaction involving the hydrolysis of ATP. Importantly, the formation of glucose-6-phosphate is the initiating step for essentially all other glucose utilization pathways as well. Investigating the effects of increased HK expression and activity in settings of increased oxidative stress will make up the focus of this dissertation.

The rest of the "preparatory" phase of glycolysis consists of four reactions. First, glucose-6-phosphate is rearranged to fructose-6-phosphate by the enzyme phosphoglucose isomerase. Next, there is a second energy consuming reaction which hydrolyzes another ATP to phosphorylate fructose-6-phosphate into fructose-1,6-bisphosphate. This step is catalyzed by the enzyme phosphofructokinase. Fructose-1,6-bisphosphate is then cleaved by fructose-bisphosphate aldolase into two 3-carbon molecules: glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Glyceraldehyde-3-phosphate is the molecule that proceeds down the "payoff" phase of glycolysis; therefore the enzyme triosephosphate isomerase converts the dihydroxyacetone phosphate into a second glyceraldehyde-3-phosphate molecule. Thus, the "preparatory" phase of glycolysis has burned two ATPs, and two 3-carbon sugars remain for the "payoff" phase.

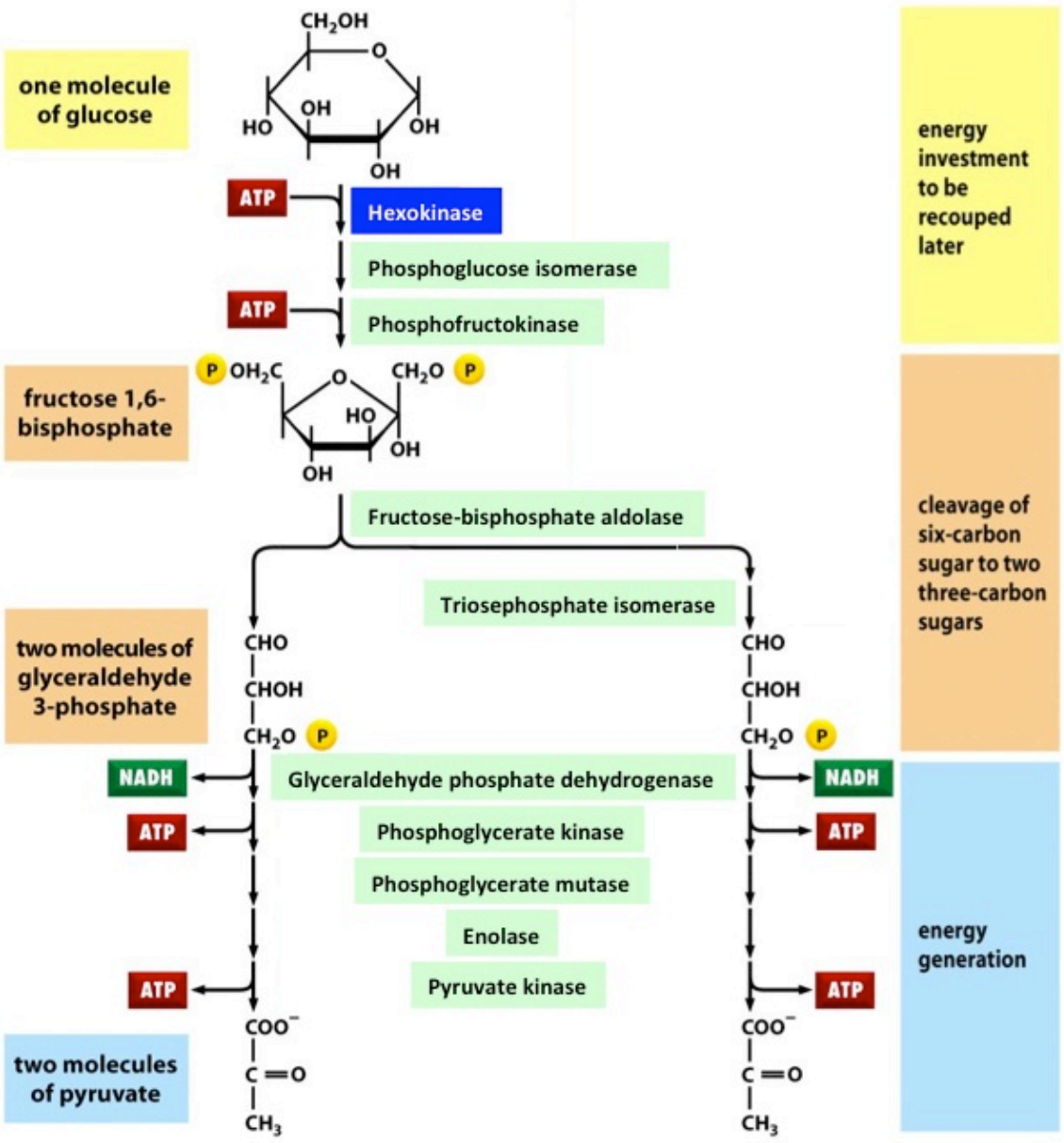

Figure 2. Glycolysis cascade. Schematic illustrating the glycolytic pathway in which 6-carbon glucose is broken down into two 3-carbon pyruvate molecules. The enzymes for each step are highlighted in green, with the exception of Hexokinase – the focus of this dissertation – which is highlighted in blue. Overall, glycolysis first requires energy input in the "preparatory" phase, which also involves the splitting of the 6-carbon fructose-1,6-bisphosphate. Net energy is then released in the "payoff" phase in the form of 2 NADH and 2 ATP per glucose molecule.

Adapted from: Alberts B. et al.; Molecular Biology of the Cell, 5th Ed., Garland Science; 2008.

The first reaction of the "payoff" phase involves the dehydration and phosphorylation of glyceraldehyde-3-phosphate into 1,3-bisphosphoglycerate by the enzyme glyceraldehyde phosphate dehydrogenase (GAPDH). This reaction reduces an NAD^+ molecule into NADH, an energetic compound utilized to reduce oxidized compounds as well as in oxidative phosphorylation (**See section 1.4**). In the next step, phosphoglycerate kinase catalyzes the dephosphorylation of 1,3-bisphosphoglycerate into 3-phosphoglycerate, and forms one ATP. This is the first of two phosphorylation steps requiring ADP; therefore, when a cell has an abundance of ATP and little ADP, this reaction does not occur. Phosphoglycerate mutase converts the 3-phosphoglycerate into 2-phosphoglycerate, which is then dehydrated by enolase into phosphoenolpyruvate. Lastly, pyruvate kinase removes the phosphate from phosphoenolpyruvate and forms pyruvate and ATP. Similar to the phosphoglycerate kinase reaction, ADP is required; therefore when there is an abundance of ATP this reaction is prevented. Since the "preparatory" phase of glycolysis created two glyceraldehyde-3-phosphate molecules, all of the "payoff" phase reactions are performed twice per molecule of glucose. Therefore, per molecule of glucose, two pyruvates, two NADHs, and two ATPs are formed.

1.4 *Post-glycolysis metabolic processes*

Pyruvate is further metabolized for two reasons: 1) most cells have a higher energy demand than the two ATP created by glycolysis, and 2) glycolysis utilizes two NAD^+ molecules, which need to be replenished so that glycolysis can keep moving in the forward direction. By far the simplest way of replenishing NAD^+ is for the enzyme lactate dehydrogenase to oxidize pyruvate into lactate, with concomitant reduction of

NADH back to NAD^+. This lactic acid fermentation is so simple, that it occurs mainly in unicellular organisms or during hypoxic conditions in multicellular organisms.

To produce much more energy per glucose molecule, aerobic organisms have developed processes known as the tricarboxylic acid (TCA) cycle and oxidative phosphorylation. Both of these processes occur in the mitochondria, with the TCA cycle taking place in the matrix and oxidative phosphorylation within the inner mitochondrial membrane (IMM). The ultimate purpose of the TCA cycle is to metabolize pyruvate and form more NADH. Oxidative phosphorylation then both produces large amounts of energy, and replenishes the NAD^+ pool so that glycolysis and the TCA cycle can proceed. To begin the TCA cycle, pyruvate is decarboxylated and reduced to yield acetyl-CoA, CO_2, and NADH. Citrate synthase then combines acetyl-CoA and oxaloacetate to form citrate. Seven other steps are then performed in the cycle to regenerate oxaloacetate and form in total 3 NADH, 3 H^+, 1 $FADH_2$, 1 GTP (which can be converted to ATP), and 2 CO_2. Importantly, per glucose molecule, these figures can be doubled since glucose produced two pyruvate molecules.

The process of oxidative phosphorylation involves the coupling of the flow of electrons through the electron transport chain and the synthesis of ATP. The electron donors are the NADHs (and $FADH_2$) formed in the TCA cycle. The electrons are shuttled through four IMM complexes, aptly named complexes I-IV, releasing energy, which is then used to transport H^+ ions across the IMM into the intermembrane space. This creates both an electrical potential and a pH gradient across the IMM, which is then used by the ATP synthase (aka complex V), which allows H^+ ions to flow down their electrochemical gradient thereby generating the energy for the synthase to form ATP from ADP + P_i. The

number of protons required to flow through the ATP synthase to create one ATP has been estimated to be either three or four (Yoshida, 2001). Therefore, through the combined processes of glycolysis, TCA cycle, and oxidative phosphorylation, the total amount of ATP formed per glucose molecule could theoretically be 38, although is likely closer to 34 (Porter, 1995) or 30 (Rich, 2003) due to proton leak across the IMM.

1.5 ***Accessory glucose utilization pathways***

1.5.1 Glycogenesis and glycogenolysis

Glycogenesis, or glycogen formation, is an important process that occurs when glucose is in excess. Glycogenesis is extremely important in the liver where glycogen is stored until it is broken down and released during times of hypoglycemia. Glycogen is also made and stored in muscles, and used as a rapidly available glucose reserve during times of high energy demand. Glycogen is a large, branched polymer of glucose molecules, stored as granules in the cytoplasm. The glucose monomers are linked together linearly by α(1-4) glycosidic bonds, whereas new branches of glycogen are added by α(1-6) glycosidic bonds. After glucose is phosphorylated by HK into glucose-6-phosphate, phosphoglucomutase converts it to glucose-1-phosphate. This molecule is then converted to UDP-glucose by uridyl transferase, and monomers of UDP-glucose are then linked by glycogen synthase. Branches are created by the "branching enzyme", cutting the end section off of a long branch, and forming a α(1-6) glycosidic bond onto an earlier section of the glycogen chain.

Glycogenolysis, or the breakdown of glycogen is particularly important in the liver in response to hypoglycemia, or in muscles during strenuous exercise. Glycogen phosphorylase removes glucose monomers by cutting the α(1-4) glycosidic bond, and phosphorylating the monomer into glucose-1-phosphate. Glycogen debranching enzyme is responsible for removing the last four glucose monomers at a branch site, and adding them to the end of a branch, where they can be removed by glycogen phosphorylase. The removed glucose-1-phosphates can then be converted to glucose-6-phosphate by phosphoglucomutase, and at this point be shuttled through the glycolysis cascade or other glucose utilization pathways.

1.5.2 Pentose Phosphate Pathway

It is estimated that for most cells, 80-90% of glucose oxidation is performed by glycolysis, with the remaining 10-20% of glucose oxidation occurring through the pentose phosphate pathway (PPP), also known as the hexose monophosphate shunt (Wamelink, 2008). A schematic of the PPP is shown in **Figure 3**. The pathway is present within the cytosol of all cells, and conveniently does not require oxygen, but does not form ATP. Overall, the pathway can be divided into two branches with two distinct roles: 1) an oxidative, non-reversible branch to produce NADPH to be used as a reducing agent important for biosynthetic or metabolic pathways, and also for protection against oxidative damage, and 2) a non-oxidative, completely reversible branch for synthesis of ribose-5-phosphate, which is required for nucleotide and nucleic acid synthesis. Additionally, the non-oxidative pathway generates both fructose-6-phosphate and glyceraldehyde-3-phosphate, which can be shuttled back into the glycolytic cascade.

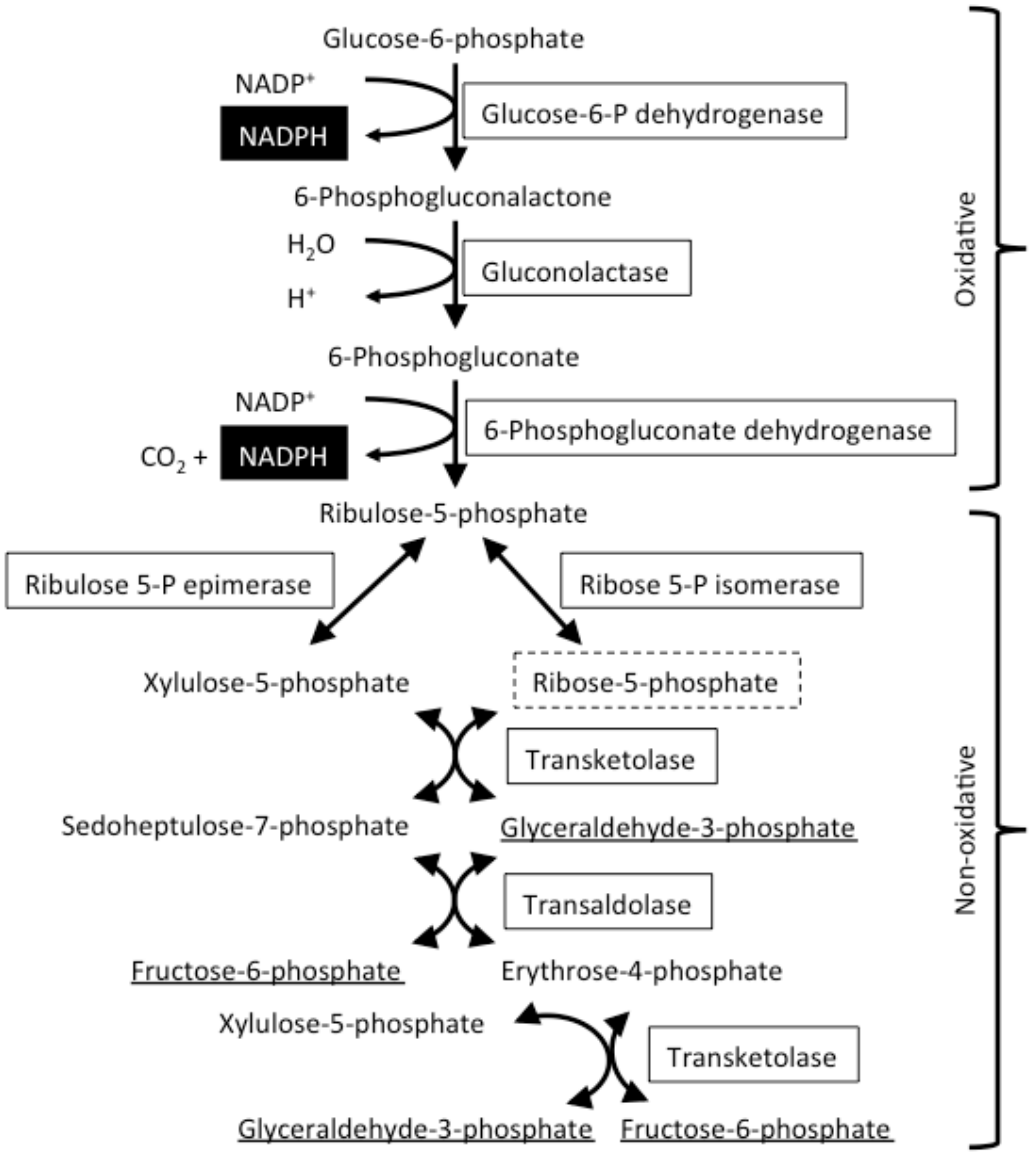

Figure 3. The pentose phosphate pathway. Schematic illustrating the pentose phosphate pathway (PPP). The enzymes for each step are noted with a solid border. Formation of NADPH reducing units (highlighted in black) during the oxidative stage is the first purpose of the PPP. Formation of Ribose-5-phosphate (noted with dashed border), necessary for nucleic acid synthesis, is the second purpose for the pathway. Additionally, the non-oxidative stage of the PPP is able to regenerate glycolytic intermediates, noted with underlines.

Glucose-6-phosphate dehydrogenase (G6PDH) is both the first, and the rate-limiting enzyme of the PPP. Interestingly, the gene for G6PDH is the most commonly mutated gene in the world, with an estimated 400 million people exhibiting at least slight G6PDH deficiency (Nkhoma, 2009). G6PDH is regulated by the NADPH/$NADP^+$ ratio (Holten, 1976), as well as various hormones and oxidants (Kletzien, 1994). Due to the role of the PPP in formation of ribose-5-phosphate, and thus nucleic acids, it is no surprise that G6PDH activity is increased during both normal cell and cancer cell growth (Tian, 1998). Additionally, as G6PDH is the most important enzyme for the maintenance of NADPH, G6PDH has been proven to be essential for preventing ROS-mediated cell death (Tian, 1999). Essentially the entire antioxidant system (glutathione reductase, catalase, and superoxide dismutase) depends on NADPH (Stanton, 2012). Other major enzyme systems that are dependent on NADPH are nitric oxide synthase, NADPH oxidase, dihydrofolate reductase, cytochrome p450 oxidoreductase, as well as several lipid synthesis enzymes (particularly HMG CoA reductase) (Stanton, 2012). Therefore, the PPP is truly at the center of many essential metabolic and stress defense pathways, and could potentially have a large role in protection against cell death and disease.

1.5.3 Aldose Reductase/Polyol Pathway

The aldose reductase or polyol pathway is a simple two-step process of converting glucose to sorbitol, and then sorbitol to fructose. The first reaction is catalyzed by aldose reductase and requires NADPH. The second reaction is catalyzed by sorbitol dehydrogenase and involves the formation of one NADH. Glucose can also be converted to fructose via the first steps of glycolysis and then hydrolysis of fructose-6-phosphate,

but the aldose reductase pathway is more advantageous for this purpose as it does not require ATP. Under normal glycemic conditions, very little glucose enters the aldose reductase pathway, however, during conditions of chronic hyperglycemia such as diabetes, the amount of glucose converted to sorbitol or fructose is dramatically increased (Gabbay, 1966). Buildup of sorbitol or fructose is therefore believed to be responsible for diabetic complications in a number of organs, i.e. diabetic retinopathy where sorbitol accumulation in retinal microvasculature ultimately leads to blindness.

1.5.4 Hexosamine Biosynthetic Pathway

One last possible fate for glucose is passage into the hexosamine biosynthetic pathway, ultimately leading to the formation of uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc). It is estimated that less than 5% of intracellular glucose is shuttled into the hexosamine pathway (Marshall, 1991). The final result of this pathway is the *O*-linked GlcNAcylation (*O*-GlcNAc) of proteins, a posttranslational modification of many nuclear, cytosolic, and mitochondrial proteins (Ngoh, 2010). For the hexosamine pathway, glucose is phosphorylated by HK into glucose-6-phosphate, converted to fructose-6-phosphate, but then exits the glycolysis cascade and an amine is added by glucosamine-fructose-6-phosphate aminotransferase. The glucosamine-6-phosphate is then acetylated, and lastly undergoes the addition of UDP. *O*-GlcNAc is then added to proteins by *O*-GlcNAc transferase.

O-GlcNAc is different from protein glycosylation in many ways, and is most similar to protein phosphorylation (Ngoh, 2010). Like phosphorylation, single *O*-GlcNAc residues can be added to serine and threonine residues of virtually any cellular protein.

Glycosylation, on the other hand, involves addition of complex polysaccharides to predominantly cell surface proteins. An interesting difference between *O*-GlcNAcylation and phosphorylation is that there is only one enzyme for adding *O*-GlcNAc to proteins, and one enzyme for removing *O*-GlcNAc from proteins, compared to the hundreds of kinases and phosphatases responsible for controlling phosphorylation. Overall, although *O*-GlcNAc is one of the most common posttranslational modifications of proteins (Hart, 2007), very little is known of the significance of this modification on specific proteins. In general, since the precursors of *O*-GlcNAc are nutrient derived, it is suspected that *O*-GlcNAcylation of proteins acts as a metabolic sensor, potentially altering the cell cycle and stress signaling (Ngoh, 2010).

2. MECHANISMS OF CELL DEATH

2.1 *Overview*

Mammalian cell death can be categorized into two distinct mechanisms: apoptosis and necrosis (Kroemer, 2009). Traditionally, apoptosis has been described as genetically controlled or "programmed", while necrosis is depicted as accidental or "non-programmed". However, more recent evidence indicates that necrosis in many cases is also initiated and modulated by a cell death "program"; therefore, this dogma is gradually being laid to rest. There are several factors which determine how a cell dies: 1) type and intensity of stressor, 2) cellular ATP concentration, and 3) cell type (Galluzzi, 2007). However, it is becoming understood that a great deal of cross-talk exists between these pathways, and blockade of one pathway will likely divert a cell down the alternate pathway if the noxious signal persists (Hotchkiss, 2009). Since certain steps of apoptosis

are ATP-dependent (Eguchi, 1999), depletion of ATP will cause an apoptotic stimulus to instead induce ATP-independent necrosis (Leist, 1997).

Autophagy is the controlled breakdown and digestion of macromolecules and cellular organelles, which can allow a starving cell to survive (Klionsky, 2007). In excess, autophagy can lead to cell death, and is therefore considered by some to be a third cell death process. However, there is no direct evidence to show that cessation of excessive autophagy is enough to inhibit cell death (Kroemer, 2009). Additionally, genetic deletion of autophagic genes has actually been shown to accelerate apoptotic death, further emphasizing the predominant survival role of autophagy (Galluzzi, 2007). Therefore, this dissertation will focus on apoptotic and necrotic cell death.

2.2 *Apoptosis*

The hallmarks of apoptotic death are: cell shrinkage, maintenance of an intact plasma membrane, and nuclear condensation with DNA fragmentation. Example electron micrographs of apoptotic and necrotic cells are shown in **Figure 4**. In general, apoptosis is thought of as a clean, non-immunogenic process since the plasma membrane remains intact; however this is not entirely the case (Apetoh, 2007; Obeid, 2007). In general, the plasma membrane makes small blebs, engulfing cytoplasmic contents and organelles, which can then be phagocytized by macrophages.

There are two distinct, but convergent pathways leading to apoptosis: an extrinsic death-receptor-mediated pathway, and an intrinsic mitochondrial-mediated pathway (**Figure 5**). The death-receptor pathway is triggered by a death signal of the tumor necrosis factor (TNF) superfamily binding a cell surface death receptor. The ligation of

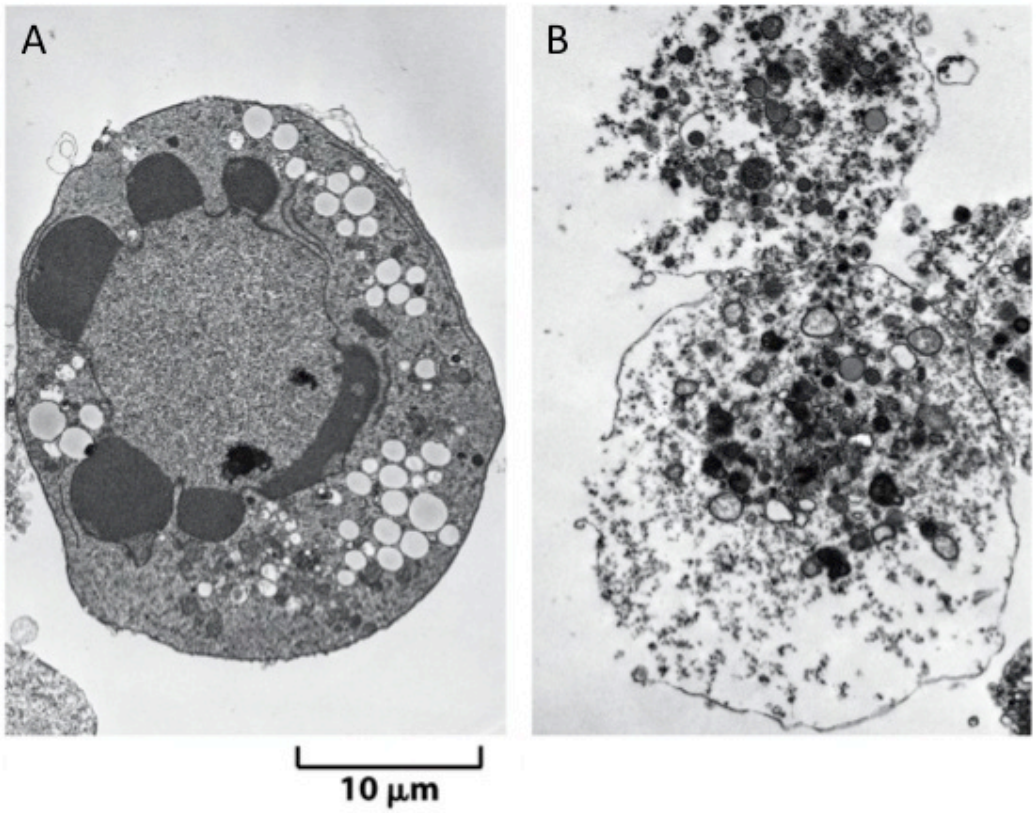

Figure 4. Apoptotic versus necrotic cell death. Electron micrographs depicting an apoptotic cell (A) and a necrotic cell (B). During apoptosis, the nuclear envelope disassembles, and chromatin is condensed, as seen in the dark, electron-dense blobs and crescent shape. In this particular cell, there is also cytosolic vacuolization, which is a variable apoptotic response. The plasma membrane remains intact in apoptotic cells, and eventually blebs off to be consumed by phagocytic cells. The necrotic cell on the other hand shows plasma membrane failure and release of intracellular components.

Adapted from: Alberts B. et al.; Molecular Biology of the Cell, 5th Ed., Garland Science; 2008.

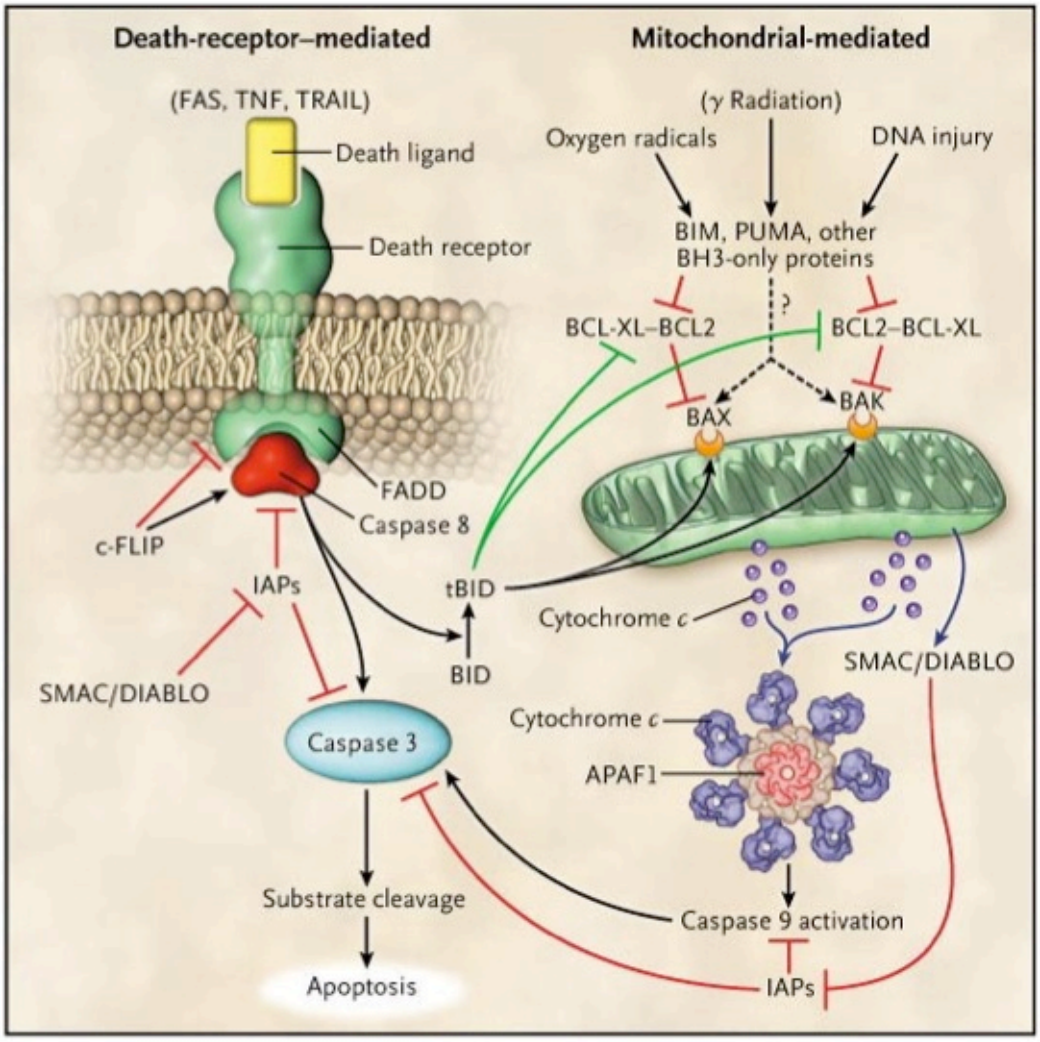

Figure 5. Death receptor-mediated and mitochondrial-mediated apoptosis pathways. Schematic illustrating the two major pathways of apoptosis. In the extrinsic, or death receptor-mediated pathway, a death ligand binds to the death receptor, and recruits the FAS-associated death domain (FADD). FADD recruits caspase 8, which ultimately activates caspase 3, which is a key executioner caspase responsible for cleavage of proteins as well as DNA fragmentation. The intrinsic or mitochondrial-mediated pathway involves a noxious stimulus activating proapoptotic BH3 proteins, which inhibit anti-apoptotic Bcl2 proteins, thus, allowing the proapoptotic Bcl2 proteins, Bax and Bak to induce outer mitochondrial membrane permeabilization. Cytochrome c is then allowed to escape the mitochondrion, assist in the formation of the apoptosome, which then activates caspase 9, and ultimately the executioner caspase 3.

Adapted from: Hotchkiss RS. et al.; N Engl J Med. 361, 1570-1583, 2009.

this death receptor recruits the FAS-associated death domain (FADD) and caspase 8, together comprising the death-inducing signaling complex (DISC) (Lemasters, 2005). Formation of the DISC activates caspase 8, which subsequently activates executioner caspases such as caspase 3, leading to protein and nucleic acid breakdown.

The mitochondrial pathway begins as a competition between proapoptotic and antiapoptotic B-cell lymphoma 2 (Bcl2) proteins. The mitochondrial pathway can be initiated by a variety of intracellular signals such as: reactive oxygen species (ROS), DNA damage, the unfolded protein response, and deprivation of growth factors. These signals activate proapoptotic Bcl2 homology domain 3 (BH3) proteins, which inhibit the antiapoptotic Bcl2 proteins. Inhibition of the antiapoptotic Bcl2 proteins allows for activation of the proapoptotic Bcl2 family members Bax and Bak, which induce outer mitochondrial membrane (OMM) permeabilization (Kuwana, 2005). Subsequent release of cytochrome c leads to formation of the "apoptosome", caspase 9 activation, and subsequently caspase 3 activation to induce protein breakdown and DNase activation to cleave DNA (Li, 1997). Release of other "apoptogens" from the mitochondria inhibits antiapoptotic proteins that prevent these processes, thus further allowing apoptosis to proceed. Therefore, regardless of which pathway is utilized, the endpoint of apoptosis is the activation of executioner caspases, which allows for cleavage of proteins and DNA fragmentation.

2.3 *Necrosis*

The hallmarks of necrosis are nearly the exact opposite of apoptosis – that is: plasma membrane disruption causing cell and organelle swelling. Additionally, necrosis

is typically considered immunogenic, since there is membrane failure and spillage of intracellular contents into the extracellular milieu (**Figure 4**). The compromise of organellar membranes ultimately leads to necrotic death. Disruption of lysosome membranes allows release of proteolytic enzymes into the cytosol (Conus, 2008). Additionally, disruption of mitochondrial membranes results in rapid metabolic failure and depletion of ATP (Leist, 1997).

2.3.1 The Necrotic program

While blunt trauma to cell membranes clearly does not involve a genetically encoded cell death program, recent data has shown that in other cases, necrosis may in fact be programmed. Specifically, it is becoming understood that death receptor activation or other cell stressors can activate members of the receptor interacting protein (RIP) kinase family and induce necrosis (Vandenabeele, 2010). Particularly, RIP1 and RIP3 appear to coordinately mediate necrosis (Cho, 2009; He, 2009; Zhang, 2009). However, the signaling downstream of RIPs is not well understood. Early evidence suggested that TNF stimulation signaled to the mitochondria, and increased ROS production by excessive electron flux through the electron complex chain complex I (Goossens, 1995; Goossens, 1999). RIP3 has been shown to be complexed with several metabolic enzymes: glycogen phosphorylase, glutamate ammonia ligase, and glutamate dehydrogenase, and RIP3 kinase activity enhances the activity of these enzymes (Zhang, 2009). The activity of these metabolic enzymes ultimately leads to excessive ROS production (**Figure 6**).

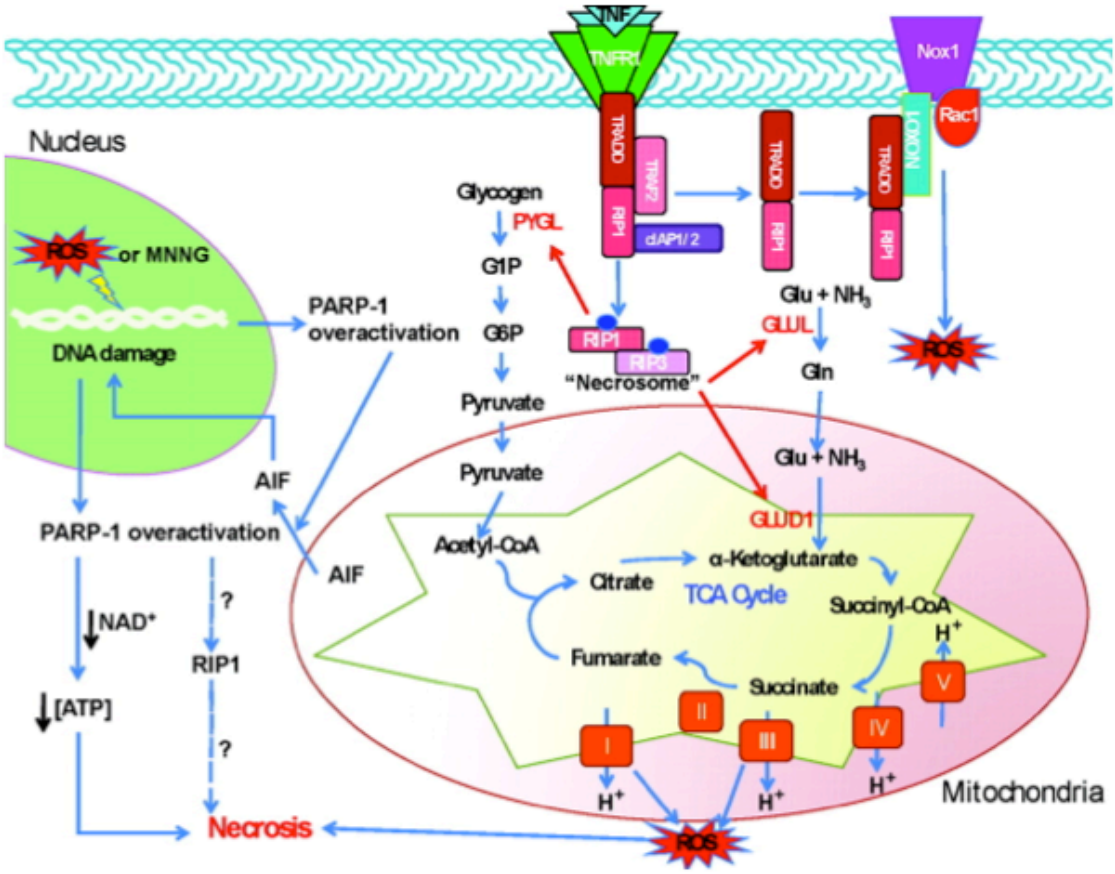

Figure 6. Programmed necrosis. Schematic illustrating the molecular events during necrotic cell death. Stimulation of a death receptor induces formation of the RIP1-RIP3 "necrosome" complex, which stimulates several glucose metabolism enzymes to ultimately increase mitochondrial oxidative phosphorylation and exacerbate mitochondrial ROS production. ROS then causes DNA damage, and the energy consuming process of repairing DNA performed by PARP1 is not inhibited, therefore energy stores are depleted.

Adapted from: Kung G. et al.; Circ Res. 108, 1017-1036, 2011.

Additionally, during the necrotic program, ATP consuming processes such as translation (Saelens, 2005) and proteasomal degradation (Sun, 2004) are not repressed as they are during apoptosis. Another major ATP-depleting process is DNA repair via poly(adenosine diphosphate-ribose) polymerase-1 (PARP1), which is inhibited during apoptosis, but not necrosis (Los, 2002). PARP1 activity is greatly increased with TNF activation, likely due to ROS-induced DNA damage (Los, 2002), and this also is a major contributor to ATP depletion (Alano, 2004; Xu, 2006). Lastly, a direct link has also been proposed for RIP1 reduction of ATP levels as RIP1 was shown to inhibit flux through the mitochondrial adenine nucleotide translocase (ANT) upon TNF stimulation (Temkin, 2006). Thus, the apparent goal of the necrotic program is to deplete cellular energy stores by a vast combination of direct and indirect pathways, many of which culminate in excessive metabolic flux leading to ROS production.

Lastly, recent evidence has suggested a few more players downstream of RIP activation. Mixed lineage kinase domain-like (MLKL) was shown to interact with RIP3, and knockdown of MLKL blocked TNF-induced necrosis (Sun, 2012; Zhao, 2012). RIP1-RIP3 binding and phosphorylation was not affected by MLKL knockdown (Zhao, 2012), and RIP3 was shown to phosphorylate MLKL (Sun, 2012). MLKL also was necessary for the generation of ROS during necrosis (Zhao, 2012); however, the mechanism linking MLKL to ROS remains elusive. One other recent study suggested that the RIP1-RIP3-MLKL complex translocates to the mitochondria, binds to and phosphorylates a mitochondrial phosphatase named phosphoglycerate mutase 5 (PGAM5) (Wang, 2012). Knockdown of PGAM5 attenuated both TNF- and ROS-induced necrosis (Wang, 2012). Additionally, PGAM5 was shown to recruit and activate

dynamin-related protein 1 (DRP1), an enzyme important for mitochondrial fission, resulting in mitochondrial breakdown (Wang, 2012). This depletion of mitochondria could be yet another mechanism of necrosis exhausting cellular ATP, but the role that ROS plays in this scheme remains unclear.

2.3.2 The mitochondrial permeability transition pore

One potential mechanism for rapid mitochondrial ROS production and cessation of ATP production is a phenomenon known as mitochondrial permeability transition (MPT). Opening of the redox, Ca^{2+}, voltage, and pH sensitive MPT pore (MPTP) allows water and solutes up to 1.5 kDa to permeate the inner mitochondrial membrane (IMM) and enter the mitochondrial matrix (Hunter, 1976). This permeabilization of the IMM dissipates the proton electrochemical gradient ($\Delta\Psi_M$), disrupting the electron transport chain, leading to rapid ATP depletion. Dysfunctional electron transport results in ROS production, and ultimately the mitochondria will swell and rupture (Baines, 2010, Kroemer, 2007). Ca^{2+} and ROS are potent openers of the MPTP, while adenine nucleotides inhibit pore opening (Baines, 2010).

The molecular component(s) of the MPTP have yet to be fully elucidated. Originally, the pore was proposed to be a complex of the voltage-dependent anion channel (VDAC) in the OMM, ANT in the IMM, and cyclophilin D (CypD) in the mitochondrial matrix (Baines, 2010). However, mice lacking ANT (Kokoszka, 2004), and cells lacking all three isoforms of VDAC (Baines, 2007), maintain a classical MPT response. In fact, it is now currently believed that there may be no OMM component of the pore, since the OMM is tremendously permeable under normal conditions (Baines,

2010). On the other hand, CypD null mice are resistant to both Ca^{2+} and ROS-induced MPT (Baines, 2005; Nakagawa, 2005; Schinzel, 2005). Thus, while the matrix protein CypD is clearly not the integral membrane pore, it functions in an enzymatic capacity to induce pore opening by binding and regulating the unknown pore proteins.

It was initially thought that the MPTP contributed to both apoptotic and necrotic cell death, however, the CypD null mouse studies suggest that the MPTP is heavily pro-necrotic. The proapoptotic Bcl2 proteins Bax, Bak, and Bid, and the effects of apoptotic stimuli such as staurosporine and etoposide were unaltered by CypD deletion (Baines, 2005; Nakagawa, 2005; Schinzel, 2005). In contrast, necrotic cell death induced by oxidative stress is significantly attenuated in CypD null cells and mice (Baines, 2005; Nakagawa, 2005; Schinzel, 2005). There is some evidence that the RIP-mediated necrotic program ultimately results in MPTP opening (Lim, 2007), but the signaling between these two complexes, as well as the requirement for MPT in necrosis remains controversial.

2.4 *Evidence for a role of hexokinase in cell death*

In the 1920s, Otto Warburg made the discovery that solid tumors overwhelmingly utilized glucose, even when well oxygenated (Warburg, 1927), a discovery that has since been coined the "Warburg effect". Fifty years later, an ingenious set of experiments determined that HK2, which is massively upregulated and localized to the OMM in most tumors, is the primary enzyme responsible for the increased glucose utilization in tumors (Bustamante, 1977; Bustamante, 1981). Since another characteristic of cancer cells is a propensity to survive, it was easy to surmise that perhaps this increased glucose

utilization also played a role in protection against cell death. Since HK2 was shown to be necessary for the increased glucose utilization, and was localized to the mitochondria – the organelle most important for mediating cell death, it was hypothesized that HK2 could be playing a role in this protection against cell death. Indeed, many studies in various cell types have shown that increased mitochondrial HK2 expression and/or activity is protective against cell death (Majewski, 2004; Mergenthaler, 2012; Miyamoto, 2008; Pastorino, 2002; Sun, 2008; Wu 2011).

Despite the wealth of evidence connecting HK2 to cell survival, the mechanism of how HK2 is protective is still largely unknown. Mitochondrial localization of HK2 and hexokinase 1 (HK1) is governed by a 21-amino-acid sequence in the N-termini of these HKs, which allows for interaction with VDACs on the OMM (Fiek, 1982; Linden, 1982). One study suggested that HK1 binding induced VDAC closure and prevented MPTP opening (Azoulay-Zohar, 2004). However, this finding has been largely discredited for several reasons. First of all, HK causing VDAC closure does not make sense biochemically, as VDAC closure would inhibit nucleotide exchange and hinder the ATP supply by which HK depends on for its enzymatic activity. Secondly, VDAC is no longer considered a molecular component of the MPTP (Baines, 2007; Chiara, 2008). The current hypothesis is that HK maintains VDAC in the open conformation, therefore allowing ATP/ADP flux and maintenance of $\Delta\Psi_M$ (McCommis, 2012). Clearly, the role HK plays in mediating MPTP opening remains uncertain.

The bulk of the current evidence points toward HK protecting against necrosis, but not apoptosis. Nearly all the studies thus far to show HK-mediated protection involve ROS, or hypoxic/ischemic stimuli, which are largely inducers of necrotic cell death. In

fact, one study showed that HK2 overexpression was protective against combined oxygen-glucose deprivation, but could not protect against the apoptosis inducers actinomycin D or etoposide (Mergenthaler, 2012). Additionally, removal of HK2 from the mitochondria has been shown to cause rapid necrosis, not apoptosis (Smeele, 2011). That being said, some studies have shown that HK2 competes with Bax for a common mitochondrial binding site (Gall, 2011; Pastorino, 2002), and therefore may have some role in regulating apoptosis.

2.5 ***Mitochondrial voltage-dependent anion channels (VDACs)***

The mitochondrial VDAC channels, also known as mitochondrial porins, are the most abundant proteins in the outer mitochondrial membrane. The mammalian VDAC family of proteins consists of three isoforms from three separate genes (VDAC1, VDAC2, and VDAC3). While it has been established that VDACs regulate the ion and metabolite flux between mitochondria and the cytosol (Bathori, 1993; Lee, 1996; Rostovtseva, 2002), most other properties and even the structure of the channels are still highly debated (De Pinto, 2008; Lemasters, 2006; Rostovtseva, 2005). The three isoforms are highly conserved (Sampson, 1997); however determining different functions for separate isoforms has been a challenge that has only recently started to be confronted (Raghavan, 2012).

2.5.1 VDACs involvement in metabolism

VDACs are critically important in regulating cellular metabolism for two reasons: 1) they control metabolite flux of essentially all metabolites between the mitochondria

and cytosol, and 2) they bind to HK1 or HK2 and regulate HK activity and overall glucose utilization. While in their open state, the VDAC channels display slight anion selectivity and solutes can be up to 5 kDa in size (Colombini, 1983). In their "closed" state, VDACs selectivity allow permeability of cations and solutes up to 1.5 kDa across the OMM (McCommis, 2012). Thus, VDAC closure effectively blocks movement of organic anions, including the respiratory substrates, ATP, ADP, and P_i (Lemasters, 2006). Therefore, VDAC closure could substantially alter cellular energy supply and mitochondrial respiration.

VDACs are also the binding site for the mitochondrially localized HKs (both HK1 and HK2) (Linden, 1982; Sui, 1997). This mitochondrial localization is believed to provide HK preferential access to its substrate, mitochondrial-derived ATP (Arora, 1988). This mitochondrial binding also prevents product inhibition by glucose-6-phosphate and decreases HK's K_m for ATP, therefore increasing HK activity (Bustamante, 1977). This alteration in HK activity by the HK-VDAC interaction has also been shown to affect glucose utilization in oxidative muscles. In the muscles of VDAC1$^{-/-}$ mice, whole-cell HK activity was unaltered, but significantly reduced mitochondrial HK activity was observed; and the mice displayed significantly decreased glucose tolerance (Anflous-Pharayra, 2007). Interestingly, VDAC3$^{-/-}$ muscles displayed slight decreases in both mitochondrial and whole-cell HK activity, yet glucose tolerance was not affected (Anflous-Pharayra, 2007). Thus, HK binding to VDACs, namely VDAC1, is vitally important for glucose metabolism.

2.5.2 VDACs involvement in cell death

VDACs have also been proposed to play a role in cell death by several mechanisms (McCommis, 2012; **see Appendix**). One early theory was that VDACs comprised the outer membrane component of the MPTP. This theory was supported by the fact that the electrical conductance properties of VDAC were similar to those of the MPTP (Szabó, 1993). The MPTP was also determined to be redox, Ca^{2+}, voltage, adenine nucleotide, and pH sensitive (Weiss, 2003) – all attributes that applied to VDAC as well. Genetic models however have debunked this theory, as isolated mouse mitochondria and cells essentially devoid of all three VDAC isoforms maintain an intact MPT response (Baines, 2007). Therefore, VDACs appear to be dispensable for MPT, and are not an essential component of the MPTP.

VDAC2 has been shown to bind to the proapoptotic Bcl-2 protein Bak, and it is believed that VDAC2 sequesters and inactivates Bak in the outer mitochondrial membrane (Cheng, 2003; Lazarou, 2010). VDAC2 also appears to be required for tBid- (Roy, 2009) and Bax- (Yamagata, 2009) induced outer membrane permeabilization and apoptosis. Thus, VDAC2 appears to be directly involved in the mitochondrial apoptotic pathway.

The current most widely accepted model of VDAC controlling cell death relies on the observation that VDAC closure reduces adenine nucleotide exchange between mitochondria and cytosol and results in mitochondrial dysfunction (McCommis, 2012). Evidence supporting this model involves the finding that anti-apoptotic Bcl-xL binds VDAC and promotes its open configuration and maintains ATP/ADP exchange (Malia, 2007; Vander Heiden, 2001). VDAC closure has also been suggested to result in

oxidative stress, decrease the mitochondrial membrane potential, and induce death (Maldonado, 2010; Tikunov, 2010). Therefore, while the mechanisms are unclear, a substantial amount of evidence exists that VDACs play some role in cell death.

3. CARDIAC GLUCOSE METABOLISM AND CARDIAC DISEASE

3.1 *Overview of cardiac metabolism*

The heart is ideal for studying metabolism as it requires by far the most energy of any organ in the body, and is capable of utilizing a diverse array of substrates (**Figure 7**). The rate of ATP hydrolysis is quite high even in the normal resting heart (~0.5 μmol / g wet weight / s), and ATP content is maintained at a relatively low level (5 μmol / g wet weight), therefore complete turnover of ATP occurs nearly every 10 seconds in the heart under baseline conditions (Stanley, 2005). The normal fasting adult heart relies heavily on fatty acid (FA) oxidation (FAO) for its energy metabolism (60-80%), while glucose, lactate, and minor amounts of ketone metabolism covers the remaining 20-40% (Neely, 1972). Approximately equal amounts of glycolysis and lactate oxidation create the pyruvate for this 20-40% of non-FAO metabolism (Gertz, 1988; Stanley, 1997). Of note, there are substantial species differences in myocardial substrate utilization. Mice are more reliant on glucose and lactate metabolism (60-70%), and less on FAO (30-40%) (Stowe, 2006). In general, 95% of the ATP formed in the heart comes from mitochondrial oxidative phosphorylation, while glycolytic ATP and TCA cycle GTP make up only 5% of the organ's derived energy (Stanley, 2005). However, this relatively small amount of ATP from glycolysis has been shown to be important for ion channels and ion

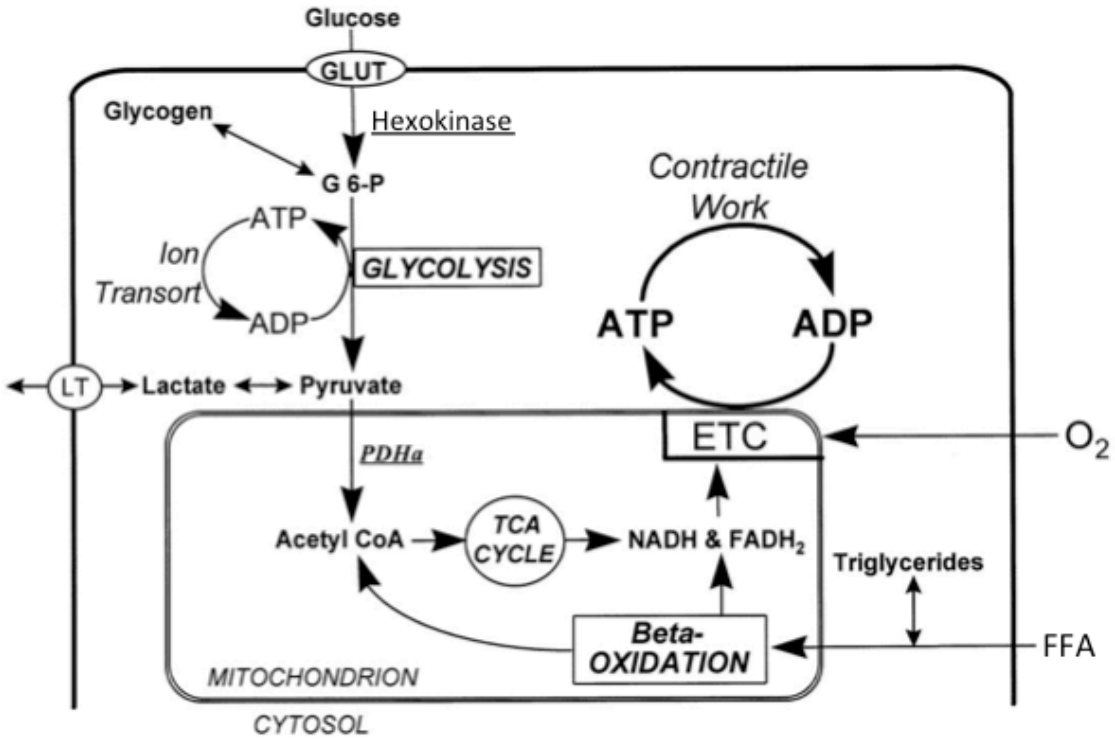

Figure 7. Cardiac substrate metabolism. Schematic depicting the major pathways of cardiac substrate metabolism. In normal hearts, the majority of energy comes from fatty acid oxidation of either free fatty acids (FFA) or intracellular triglycerides. Glycolysis and lactate oxidation produce roughly equal amounts of pyruvate, which enters the TCA cycle to make up the remainder of the heart's energy metabolism. While glycolysis produces only a small percentage of the cells ATP, it has been shown to be important for ion channels to maintain ion homeostasis including sarcoplasmic reticulum Ca^{2+} uptake, and therefore is vital to maintain optimal cardiac relaxation. The large amounts of energy derived from oxidative phosphorylation through the electron transport chain (ETC) fuel the contractile units to perform cardiac work.

Adapted from: Stanley WC. et al.; Cardiovasc Res. 33, 243-257, 1997.

homeostasis, including sarcoplasmic reticulum Ca^{2+} uptake (Entman, 1977), and therefore is essential for optimal diastolic relaxation (Jeremy, 1992; Kusuoka, 1994).

Under normal conditions, glycogen is thought to make a relatively small contribution to glucose utilization in the heart (Stanley, 2005). The heart's glycogen pool is relatively small compared to skeletal muscle (~30 versus 150 μmol / g wet weight, respectively) (Botker, 1994). While this concentration is relatively stable, there is fairly rapid turnover (Henning, 1996). Myocardial glycogen content can be increased by hyperglycemia and hyperinsulinemia (Kruszynska, 1991).

It is important to note that metabolic rates through each pathway are controlled by both the enzyme/transporter expression, as well as a large degree of allosteric regulation of these proteins. In general, the expression of metabolic enzymes, particularly glycolysis enzymes, does not greatly vary based on cardiac work requirements (Stanley, 2005). At rest, the heart operates at only 15-20% of its maximum oxidative capacity; therefore allosteric modification of enzymes in these pathways can significantly and rapidly increase the metabolic flux of the pathway (Fell, 1998).

Another interesting aspect of the heart is that it is quite metabolically adaptable. While it typically relies on FAO, the heart can switch to alternate substrates based on tissue perfusion, nutritional status, and hormonal status (Stanley, 2005). The fetal heart depends more upon glucose and lactate metabolism due to the low oxygen pressure in utero (Goldberg, 2012). In general, older animals and humans also tend to rely more heavily on glucose and less on FAO (Goldberg, 2012). Lactate is a major source of pyruvate for the heart under normal conditions, and lactate utilization can even exceed glucose utilization during exercise (Gertz, 1988). While cardiomyocytes are capable of

both lactate uptake and efflux, the heart is typically a net lactate consumer, not producer, even during high cardiac work (Kaijser, 1992; Stanley, 1991). Under pathologic conditions however, the heart can become a net lactate producer (**See section 3.2 and 3.3**).

3.2 *Cardiac metabolism during ischemia*

The severity of ischemia regulates the degree of alteration in myocardial substrate metabolism (Stanley, 1997). Of course, complete blood flow cessation rapidly depletes ATP, resulting in contractile dysfunction and eventually cardiomyocyte necrosis. Moderate ischemia impairs oxidation of pyruvate and mitochondrial oxidative phosphorylation, therefore resulting in decreased ATP concentrations and net production and output of lactate (Fedele, 1988; Pantely, 1990). Over 30-90 minutes, lactate output decreases (Fedele, 1988) and ATP levels are partially restored (Pantely, 1990); however, contractile function remains modestly decreased. The mechanisms behind this maintained decreased contractile function remain unclear, and do not appear to be due to increased anaerobic glycolysis as pyruvate dehydrogenase activation by dichloroacetate to reduce lactate production does not improve contractile function (Mazer, 1995). This is likely due to the fact that under moderate ischemia, the primary oxidative fuel remains free fatty acids (FFAs), despite reduced myocardial oxygen consumption (Liedtke, 1978; Liedtke, 1984).

More severe ischemia correlates to greater lactate accumulation due to further decreased pyruvate dehydrogenase activity. Decreases in oxidative metabolism results in increased dependence on anaerobic glycolysis, and decreased blood flow causes glycogen

to be the major source of substrate (Guth, 1990). Since the decreased tissue perfusion inhibits lactate washout, intracellular pH decreases and the rate of glycolysis is reduced due to H^+-induced inhibition of phosphofructokinase (Stanley, 1997). Additionally, glyceraldehyde 3-phosphate dehydrogenase activity can be limited due to reduced cytosolic NADH (Rovetto, 1975), further reducing the glycolytic rate. Thus, severe ischemia results in drastically reduced oxidative metabolism, initially leading to excessive anaerobic metabolism. However, the non-oxidative metabolism cannot be maintained, and therefore results in cardiomyocyte death if blood flow is not corrected.

3.2.1 Hexokinase during ischemia

There have been several studies investigating a role for hexokinase during myocardial ischemia. Ischemia causes HK2 detachment from the mitochondria and therefore an increase in cytosolic HK2 expression and activity in Langendorff-perfused rat hearts (Gürel, 2009). Myocardial infarction also causes increased cytosolic HK2 activity (Yeih, 2011). Interestingly, ischemic preconditioning, a phenomenon involving several short periods of no-flow ischemia that has been shown to decrease injury during long-term ischemia and reperfusion, increases mitochondrial HK2 expression and activity and partially maintains this mitochondrial HK2 localization during ischemia and reperfusion (Gürel, 2009; Zuurbier, 2005). Ischemic preconditioning also increases total hexokinase activity in pig hearts, which increases glucose uptake and glycogen levels (McFalls, 2002). The increase in hexokinase expression is likely driven by the hypoxia-inducible factor 1 (HIF1) element found within the HK2 promoter (Mathupala, 2001). The enhanced mitochondrial localization is likely due to ischemic preconditioning

activating Akt (Kunuthur, 2012), which phosphorylates HK2, causing mitochondrial translocation (Miyamoto, 2008). Lastly, the amount of mitochondrial HK2 negatively correlates with infarct size after ischemia-reperfusion injury (Pasdois, 2012; Wu, 2011). Thus, the increased expression and mitochondrial localization of HK2 induced by ischemic preconditioning likely play a role in the cardioprotection from ischemia-reperfusion; however, the metabolic effects of these HK2 alterations during ischemia largely remain unresolved.

3.3 *Cardiac metabolism during hypertrophy*

As discussed earlier, the normal adult heart relies predominantly on FAO for the majority of ATP synthesis. The fetal heart relies more on glucose and lactate, and switches to predominantly lipid oxidation shortly after birth (Lopaschuk, 1991). Animal models of cardiac hypertrophy recapitulate this "fetal metabolic program", showing both increased glucose utilization and the reappearance of other fetal genes (Barger, 1999; Razeghi, 2001). Importantly, human patients with idiopathic cardiomyopathy also show this transition to increased glucose utilization (Dávila-Román, 2002). With the concordant appearance of other fetal genes during this metabolic transition, it was long thought that this switch to increased glucose metabolism was integral to the pathological remodeling of the heart.

The metabolic changes known to occur during cardiac hypertrophy are depicted in **Figure 1**. Cardiomyocyte glucose uptake is significantly increased (Nascimben, 2004; Zhang, 1995), leading to increased glycolytic rates (Allard, 1994; El Alaoui-Talibi, 1997). Despite the increased glycolysis, most studies observe no change, or even a

decrease in pyruvate oxidation, suggesting an uncoupling of glucose oxidation in hypertrophy (Allard, 1994; El Alaoui-Talibi, 1997). The activity of lactate dehydrogenase is increased, thus production and efflux of lactate is increased in hypertrophied cardiomyocytes (Taegtmeyer, 1988). Although lactate production is increased, lactate oxidation appears to be unaltered during hypertrophy (Allard, 1994).

While the increased glucose utilization was initially regarded as an integral pathologic process of hypertrophy, it is now generally believed to be compensatory to the significant downregulation of mitochondrial oxidative metabolism genes (Kolwicz, 2011). Peroxisome proliferator-activated receptor alpha (PPARα) and peroxisome proliferator-activated receptor gamma-coactivator-1 (PGC-1), master regulators of genes involved in fatty acid oxidation and mitochondrial biogenesis, respectively, are both downregulated in hypertrophy (Barger, 2000; Sack, 1997). Additionally, carnitine-palmitoyl transferase 1 (CPT1) and carnitine, which together facilitate fatty acid transport into the mitochondria are reduced in hypertrophy (Barger, 2000; El Alaoui-Talibi, 1992). Decreases in plasma membrane fatty acid transporters are also seen in hypertrophy (Aitman, 1999; Vork, 1992). Therefore, the decrease in cellular and mitochondrial transport of fatty acids is largely responsible for the reduced fatty acid metabolism observed in cardiac hypertrophy. Genetic modulation strongly suggests that alteration of these genes ultimately regulates cardiac hypertrophy. For example, PPARα overexpression increases FAO, decreases glucose utilization, and results in hypertrophy at baseline in 9 month old mice (Finck, 2002). Likewise, deletion of the GLUT4 transporter decreases glucose utilization and results in cardiac hypertrophy even under normal conditions (Abel, 1999). Conversely, GLUT1 overexpression decreases

hypertrophy and improves survival after pressure overload (Liao, 2002). Therefore, alterations in metabolism drive cardiac hypertrophy, and it appears that increased glucose utilization is a beneficial, compensatory mechanism for limiting hypertrophy.

3.3.1 Accessory glucose utilization pathways during hypertrophy

Overall, fairly little is known regarding the accessory glucose utilization pathways during cardiac hypertrophy. The changes in glucose utilization known to occur during hypertrophy are depicted in **Figure 1**. Glucose uptake is increased in hypertrophied cardiomyocytes (Nascimben, 2004); therefore, one would assume that potentially all the glucose utilization pathways show increased flux; and this is most likely true.

Interestingly, hypertrophied hearts appear to preferentially oxidize glucose from glycogen vs. exogenous glucose (Allard, 1997). However, glycogen content remains similar in normal compared to pressure-overloaded hearts (Allard, 1997), and therefore hypertrophy may enhance glycogen turnover, but not necessarily prompt a higher dependence on glycogen metabolism. Glycogen synthase kinase 3α (GSK-3α) is a negative regulator of glycogen synthase, and very recently, GSK-3α-null mice were shown to exhibit hypertrophied hearts compared to WT littermates (Zhou, 2013). Unfortunately, glycogen was not assessed in this study, but one would surmise that GSK-3α-null mice could have constitutively active glycogen synthase and therefore increased glycogen levels. If this were the case, it would argue against the evidence for increased glycogen and glucose utilization being beneficial during cardiac hypertrophy. However, glycogen synthase is also negatively regulated by AMPK and protein kinase A (Soderling, 1977), which could fill in for GSK-3α to inactivate glycogen synthase.

Additionally, GSK-3α is known to phosphorylate over forty substrates (Jope, 2004), thus it is difficult to draw conclusions regarding the role of glycogen in hypertrophy from this specific study.

Early metabolic studies of hypertrophied hearts observed increased pentose phosphate pathway flux (Meerson, 1967; Zimmer, 1980), due to increased activity of the rate-limiting glucose-6-phosphate dehydrogenase (G6PDH) enzyme (Chess, 2009; Gupte, 2006). Mice deficient in G6PDH spontaneously develop hypertrophy at ~9 months of age (Jain, 2003), and display increased hypertrophy after myocardial infarction or pressure overload (Hecker, 2013). Pentose phosphate pathway flux is also increased in the salt-sensitive rat model of hypertrophy and heart failure (Kato, 2010). In this study, pentose phosphate pathway flux was increased during hypertrophy, and further elevated after the transition to heart failure. Treatment with dichloroacetate increased glucose utilization including pentose phosphate pathway flux, decreased reactive oxygen species, and prevented the transition from hypertrophy to heart failure (Kato, 2010). In fact, the majority of these studies associate pentose phosphate pathway activity with decreased oxidative stress (Hecker, 2013; Jain, 2003; Kato, 2010); however, the link between hypertrophy, the pentose phosphate pathway, and ROS is unclear.

Flux through the hexosamine biosynthetic pathway also appears to be increased during hypertrophy. UDP-GlcNAc levels are increased in hypertrophied hearts (Watson, 2010; Young, 2007). Two enzymes important for UDP-GlcNAc synthesis and protein linkage, *O*-linked β-N-acetylglucosamine transferase (Watson, 2010), and glutamine fructose-6-phosphate aminotransferase (Young, 2007) are increased by hypertrophy. This increase in GlcNAc levels during hypertrophy appears to be beneficial as deletion of *O*-

linked β-N-acetylglucosamine transferase decreases GlcNAc levels, increases hypertrophy, increases cardiomyocyte apoptosis, and exacerbates cardiac dysfunction in response to myocardial infarction (Watson, 2010). Thus, increased hexosamine biosynthetic pathway flux may be a survival mechanism for hypertrophied cardiomyocytes.

Extremely little is known about role of the aldose reductase pathway in cardiac hypertrophy. The aldose reductase gene shows a nearly two-fold increase in human heart failure patients (Yang, 2000). Also, aldose reductase inhibition is known to increase cardiac function in diabetic patients with heart failure (Johnson, 2004). Thus, flux through the aldose reductase pathway may potentially be a negative side effect of increased glucose utilization in hypertrophy and heart failure.

3.3.2 Hexokinase during hypertrophy

Several early studies of cardiac metabolism during hypertension hypothesized a potential role for hexokinase during hypertension-induced hypertrophy. Hypertension in rats induced cardiac hypertrophy and significantly increased hexokinase activity (Koehler, 1985). Moderate hypertension in rabbits induced slight cardiac hypertrophy and increased hexokinase activity in these hearts as well (Taegtmeyer, 1988).

Several recent studies also provide evidence suggesting a potential role for hexokinase during cardiac hypertrophy. In one study, mice were treated with streptozotocin to induce type-I diabetes, and the hearts showed decreased HK2 levels as well as increased cardiomyocyte size (Xue, 2010). Cardiac-specific overexpression of HIF-1α was then shown to prevent the diabetes-induced decrease in HK2 protein, and

cardiac hypertrophy was also avoided (Xue, 2010). Since HK2 is just one of hundreds of HIF1α target genes, only limited conclusions can be drawn from these results. However, a recent study did draw a direct link between altered HK2 expression and cardiac hypertrophy. Mice with ~50% decreased HK2 expression and activity were shown to have exaggerated cardiac hypertrophy after aortic constriction (Wu, 2012). This study proposed that decreased mitochondrial HK2 expression increased ROS, which was responsible for the exaggerated hypertrophy. However, the role of mitochondrial HK2 versus cytosolic HK2, or total HK2 activity, is still debatable. Regardless, these studies clearly indicate that HK2 expression is altered in hypertrophy, and genetic manipulation of HK2 can mediate the extent of hypertrophy.

4. STUDY HYPOTHESES AND AIMS

The overall hypotheses for the studies detailed in this dissertation are that increased HK2 expression and activity is beneficial for the heart by both protecting against cell death and attenuating hypertrophy. The mechanisms of both these actions may be due to the ability of HK2 to reduce ROS levels. This ability to modulate ROS levels may or may not be due to mitochondrial binding of HK2. In **AIM 1**, we will investigate the interaction of HK2 with the three VDAC channel isoforms to more thoroughly determine how HK2 binds mitochondria. Additionally, we will assess the ability of HK2 to protect cells devoid of each of the three VDAC isoforms to determine which, if any, play a role in the HK2-mediated protection against cell death. In **AIM 2**, we will determine whether increased HK2 expression and activity has an affect on the development of cardiac hypertrophy.

This study has two major aims:

AIM 1: Define which VDAC isoform(s) binds HK2, and determine if any of the VDAC isoforms are necessary for HK2-mediated protection against cell death.

Hypothesis: *Since mitochondria are important for mediating cell death and HK2 binds to VDACs on the mitochondrial outer membrane, VDAC1, the primary isoform, will be required for HK2-mediated protection against cell death.*

Rationale: As described previously, mitochondria are the primary organelle responsible for mediating both apoptotic and necrotic cell death. The VDAC channels on the outer mitochondrial membrane have been implicated in cell death due to their importance for metabolite shuttling between mitochondria and the cytosol. It is well accepted that HK2 binds to these VDAC channels, however, it is less clear which of the three VDAC isoforms is important for HK2 binding. Additionally, numerous studies have observed a protective effect of HK2 overexpression and/or mitochondrial binding; however, the role VDACs play in this protection is unclear. Therefore, in this aim, we will study HK2 expression and localization in cells devoid of each of the three VDAC isoforms. Additionally, we will overexpress HK2 in these cells and challenge them with H_2O_2 to determine if any of the VDAC isoforms are necessary for HK2-mediated protection against cell death.

AIM 2: Determine whether cardiac-specific HK2 overexpression affects cardiac hypertrophy.

Hypothesis: *Mice overexpressing HK2 will demonstrate increased glucose utilization and attenuated cardiac hypertrophy.*

Rationale: Evidence depicts a switch in cardiac substrate metabolism during hypertrophy in that glucose utilization is markedly increased. Recent studies suggest that a decrease in fatty acid metabolism is an integral process contributing to hypertrophy, and that increased glucose utilization is a beneficial compensatory effect. While the roles for each glucose utilization pathway during hypertrophy are relatively uncertain, it is important to again note that HK2-mediated phosphorylation of glucose is the starting point for all pathways with the exception of the aldose reductase pathway. Thus, we believe that a heart overexpressing HK2 will display increased glucose utilization, potentially through increased flux through all pathways, and will exhibit an attenuated hypertrophic response.

CHAPTER 2: Mitochondrial Voltage-Dependent Anion Channel 3 Is Required For Hexokinase-2 Mediated Protection Against ROS-Induced Necrotic Cell Death

1. ABSTRACT

Hexokinases (HK)-1 and -2 are capable of binding the outer mitochondrial membrane due to a hydrophobic N-terminal amino acid sequence allowing for interaction with Voltage-Dependent Anion Channels (VDACs). However, very little evidence exists regarding which of the three VDAC isoforms HK2 binds. Additionally, it is unknown whether any of the VDAC isoforms are required for HK2 to protect against cell death. Therefore we studied HK2 expression and activity in murine embryonic fibroblasts (MEFs) deficient in each of the three VDAC isoforms. Both VDAC1 and VDAC3 were shown to be important for HK2-mitochondrial localization. Interestingly, *Vdac3*$^{-/-}$ MEFs were shown to have significantly reduced HK2 expression and activity. HK2 overexpression resulted in increases in both cytosolic and mitochondrial expression, maintained the mitochondrial membrane potential in response to H_2O_2 treatment, and significantly protected WT MEFs against H_2O_2-induced cell death. Surprisingly, HK2 overexpression remained protective in *Vdac1*$^{-/-}$ MEFs, but protection was lost in both VDAC2-deficient and *Vdac3*$^{-/-}$ MEFs. However, VDAC2 is known to sequester Bak in the outer mitochondrial membrane, therefore knockdown of VDAC2 considerably increases cell death. HK2 overexpression regained protection against H_2O_2 in *Bax/Bak*$^{-/-}$ cells depleted of VDAC2. These results implicate an important role for mitochondrial VDAC3 in protection against cell death.

2. INTRODUCTION

Cellular glucose utilization begins with glucose phosphorylation to glucose-6-phosphate by the enzyme Hexokinase (HK). There are 4 mammalian HK isoforms: HK1, HK2, HK3, and HK4, also known as glucokinase. HK1 is expressed in most tissues, while HK2 is expressed mainly in insulin-sensitive tissues (Wilson, 2003).

It has become well understood that many cancer cells have increased glucose metabolism even in the presence of oxygen, a trait known as the "Warburg effect" (Pedersen, 2007). Upregulation and mitochondrial localization of HK2 have been shown to be responsible for the "Warburg effect" in cancer cells (Bustamante, 1977; Bustamante, 1981; Parry, 1983). Both HK1 and HK2 contain an N-terminal 21 amino-acid sequence that allows for a hydrophobic interaction with voltage-dependent anion channels (VDACs) on the outer mitochondrial membrane (Linden, 1982; Nakashima, 1986; Sui, 1997). Interestingly, this mitochondrial localization of HK2 has also been shown to be important in defense against cell death (Pastorino, 2002; Sun, 2008), another trait of cancer cells.

The mammalian VDAC protein family consists of 3 gene products termed VDAC1, VDAC2, and VDAC3 (Sampson, 1997). The three isoforms are highly conserved (Sampson, 1997), and determining distinct functions for separate isoforms has been a challenge that is just now starting to be confronted (Raghavan, 2012). To our knowledge, only one study has been performed investigating which VDAC isoform HK binds, as well as the metabolic effects resulting from alterations of this mitochondrial localization. In this study, VDAC1 was observed to bind the majority of HK on both

heart and soleus muscle mitochondria; however, VDAC3 did also slightly bind HK (Anflous-Pharayra, 2007). Interestingly, VDAC1 ablation reduced mitochondrial HK activity, had no effect on whole cell HK activity, yet the mice displayed significantly decreased glucose tolerance (Anflous-Pharayra, 2007). Conversely, VDAC3 deletion resulted in only slight, non-significant decreases of both mitochondrial and whole cell HK activity, and had no effect on glucose tolerance (Anflous-Pharayra, 2007). These data suggested that HK is bound mostly to VDAC1 in oxidative muscles, and disruption of the HK-VDAC1 binding results in a significant decrease in glucose utilization.

Very few studies have investigated a direct role for HK-VDAC binding in cell death; however, a large amount of evidence implicates general HK mitochondrial binding as important for HK-mediated protection. Akt activation phosphorylates HK2, resulting in increased mitochondrial HK2 localization and protection against H_2O_2 and myocardial ischemia-reperfusion injury (I/R) (Miyamoto, 2008; Miyamoto, 2010). Conversely, removal of HK2 from the mitochondria by a TAT-HK2 peptide decreases the mitochondrial membrane potential and reduces cell viability (Chiara, 2008; Wu, 2011). These findings have led to speculation that HK induced VDAC closure and inhibited opening of the mitochondrial permeability transition (MPT) pore (MPTP) (Azoulay-Zohar, 2004). However, another study suggested that HK binding prevents VDAC closure (Majewski, 2004), thus maintaining HK's access to mitochondrial-generated ATP (Bustamante, 1981). Additionally, HK removal from the mitochondria was later determined to induce MPT even in the absence of VDAC1 and VDAC3 (Chiara, 2008), and a portion of HK1 has been reported to bind to the mitochondria via unknown non-VDAC sites (Neumann, 2010). Thus, while it is clear mitochondrial binding of HK2 is

important for protection against cell death, it remains uncertain whether or not any specific VDAC isoform(s) are required for this HK2-mediated protection against death.

In the current study, we thus sought to determine which VDAC isoform(s) bound HK2 in murine embryonic fibroblasts (MEFs), as well as investigate which VDAC isoform, if any, plays a role in HK2-mediated cytoprotection. We hypothesized that HK2 would bind mostly to VDAC1 in MEFs, and loss of VDAC1 would significantly decrease the protection afforded by HK2 overexpression. We show here that HK2 binds mostly to VDAC1, but also to VDAC3, while there was no detectable effect with knockdown of VDAC2. Adenoviral overexpression of HK2 resulted in increases in both cytosolic and mitochondrial HK2 expression. Increased HK2 expression maintained the mitochondrial membrane potential ($\Delta\Psi_M$) during H_2O_2 treatment and resulted in less H_2O_2-induced cell death. Lastly, cells lacking VDAC1 or VDAC2 could still be protected from H_2O_2-induced death by HK2; however, cells lacking VDAC3 were no longer protected by HK2 overexpression.

3. METHODS AND MATERIALS

3.1 *Reagents*

Lipofectamine RNAiMAX, Mitotracker-Red, TMRE, and Sytox Green were from Invitrogen; DMEM medium, Hanks's buffered saline solution (HBSS), penicillin/streptomycin, sodium pyruvate, non-essential amino acids, and fetal bovine serum (FBS) were from ThermoFisher; the In Situ Cell Death Detection (TUNEL) kit

was from Roche; all other chemicals/reagents were from either ThermoFisher or Sigma-Aldrich.

3.2 *MEF Cell Cultures*

The gene-targeting strategies for all three mammalian *Vdac* genes, *Bax*, and *Bak* have been previously reported (Cheng, 2003; Wu, 1999). Primary cultured wildtype (WT), *Vdac1*$^{-/-}$, and *Vdac3*$^{-/-}$ MEFs were harvested from E13.5-15.5 embryos by trypsin digestion as described previously (Cheng, 2003). Immortalized Bax/Bak double null MEFs (Cheng, 2003) were a kind gift from the late Stanley Korsmeyer. All MEFs were then maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mg/ml sodium pyruvate, and 1X non-essential amino acids. The harvesting of mouse embryos was approved by the University of Missouri-Columbia Animal Care and Use Committee and conformed to the NIH guidelines for the use and care of animals.

3.3 *siRNA Transfection*

Vdac2 deletion results in embryonic lethality and enhanced activation of the intrinsic apoptotic pathway due to increased activation of the pro-death Bcl-2 family member Bak on the mitochondria (Cheng, 2003). Therefore, VDAC2 was targeted in WT MEFs by use of a siRNA. MEFs were transfected with 100nM of either a non-targeting control siRNA or a VDAC2-targeting siRNA (both from Invitrogen) using Lipofectamine RNAiMax. The *Vdac2* siRNA sequence was 5'-

GGCUCAUCUAAUACAGACACUGGUA-3'. Cells were transfected for 48 hours before experimentation.

3.4 *HK2 Adenovirus Construction and Infection*

Replication-deficient adenoviruses for β-Galactosidase and mouse HK2 were generated using the AdEasy adenoviral system (Stratagene). MEFs were infected with adenovirus at a MOI of 100 plaque-forming units, and cultured for 24 hours before experimentation.

3.5 *Western Blot Analysis*

Cells grown on 10-cm plates were harvested by scraping and solubilized in lysis buffer containing 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, and 1% Triton-X100. Samples were sonicated and then centrifuged at 13,000 rpm for 10 minutes to remove insoluble debris. For subcellular fractionation experiments, MEFs were grown on 15-cm plates, harvested by scraping, and subfractionated by differential centrifugation as previously described (Baines, 2007). Proteins were resolved on 10% SDS/PAGE gels, transferred onto PVDF membranes, and blocked with 10% milk in TBS-T. The membranes were then immunoblotted using the following commercially available antibodies: anti-ATP Synthase, anti-HK1, anti-HK2, and anti-VDAC/Porin (Abcam); anti-VDAC-2 (Thermo Scientific); anti-Bak, anti-Bax, and anti-LDH (Santa Cruz Biotechnology); and anti-GAPDH (Millipore). The polyclonal mitochondrial phosphate carrier (PiC) antibody was custom made by Yenzyme. Membranes were then incubated

with the appropriate alkaline phosphatase-linked secondary antibody (Santa Cruz Biotechnology) and visualized by enhanced chemifluorescence (Amersham Biosciences).

3.6 *HK Activity Assay*

HK enzymatic activity was assessed as described previously (Wilson, 1989). Total HK, HK1, and HK2 activity were measured simultaneously, by means of HK2 denaturation at 45°C as formerly described (Wilson, 1998). Briefly, cell lysates were divided into two aliquots, one kept on ice, and one put into a 45°C water bath for 1 hour to denature HK2. At the end of the denaturing step, hexokinase activity was determined in both aliquots by measuring glucose-6-phosphate formation coupled to NADPH formation spectrophotometrically at 340 nm. The assay buffer consisted of 100 mM Tris-HCl pH 8.0, 10 mM glucose, 0.4 mM $NADP^+$, 10 mM $MgCl_2$, 5 mM ATP, and 0.15 U of glucose-6-phosphate dehydrogenase.

3.7 *Immunocytochemistry*

Cells were seeded onto glass chamber slides at 30,000 cells/ml. After a 24-hour incubation, cells were then incubated with 100 nM Mitotracker-Red (Invitrogen) in HBSS for 30 minutes. Cells were then fixed in 4% paraformaldehyde for 30 minutes. The slides were then blocked in a solution consisting of 1% BSA, 0.1% cold water fish skin gelatin, 0.1% Tween-20, and 0.05% NaN_3 in PBS for 1 hour. Anti-HK2 primary antibody (Santa Cruz Biotechnology) was then incubated on the slides overnight at 4°C. Slides were then washed thoroughly with PBS and incubated with the secondary antibody (Alexa, Invitrogen) for 2 hours. The slides were then washed thoroughly with PBS,

mounted with a glass cover slip, and imaged on a fluorescence microscope (Olympus IX51) connected to a digital camera.

3.8 *Measurement of Mitochondrial Membrane Potential ($\Delta\Psi_m$)*

Cells were seeded onto glass chamber slides at 30,000 cells/ml, and transfected/infected where applicable. After the 24-48 hour incubation, cells were then treated with 100 nM TMRE in HBSS for 30 min. Cells were then washed with HBSS and imaged on a fluorescence microscope. Average TMRE fluorescence per cell was determined with NIH ImageJ.

3.9 *Cell Death Assays*

Cells were seeded onto 12-well dishes at 30,000 cells/ml, and transfected and/or infected where applicable. After the 24-48 hour incubation, cells were treated with vehicle or varying concentrations of H_2O_2 for 3 hours. Cells were then co-stained with Sytox Green to label dead cells and bis-benzimide to label all cell nuclei. For apoptosis experiments, cells were treated with vehicle or 100 nM staurosporine for 18 hours. The cells were then TUNEL stained using a commercial kit to label apoptotic cells, and stained with Bis-Benzimide to label all cell nuclei. After Sytox Green or TUNEL staining, cells were imaged on an epifluorescence microscope, and the percentage of Sytox or TUNEL stained cells was determined by image analysis with NIH ImageJ.

3.10 *Statistical Analysis*

Data are presented as mean ± S.E. Statistical significance was assessed by two-tailed Student's *t*-test for comparisons between two groups. One-way ANOVA followed by Scheffe's post hoc test was used to determine the difference between three groups. A p value of ≤ 0.05 was used to consider statistical significance.

4. RESULTS

4.1 *VDAC3 Deletion Results in Altered HK2 Expression and Activity*

The previous report by Anflous-Pharayra et al. (Anflous-Pharayra, 2007), demonstrated that in heart and soleus muscle, there are some modest decreases in total HK activity when VDAC1 or VDAC3 are deleted. We therefore wished to characterize HK expression and activity in MEFs deficient in the three VDAC isoforms. Deletion of VDAC1 had no significant effect on HK2 expression (**Figure 8a,c**). Total HK activity, as well as HK1- and HK2-specific activities, was similarly unaffected (**Figure 8d**). In contrast, *Vdac3*$^{-/-}$ MEFs displayed a 30% reduction in HK2 expression (**Figure 8b,c**) with a concomitant reduction in HK2-specific activity (**Figure 8d**), although no significant changes in total HK activity were observed. Transfection of WT MEFs with a VDAC2 siRNA reduced total Vdac2 levels by ~70% (**Figure 9a,b**). However, like with VDAC1, depletion of VDAC2 had no significant effect on HK2 expression (**Figure 9c**) or activity (**Figure 9d**).

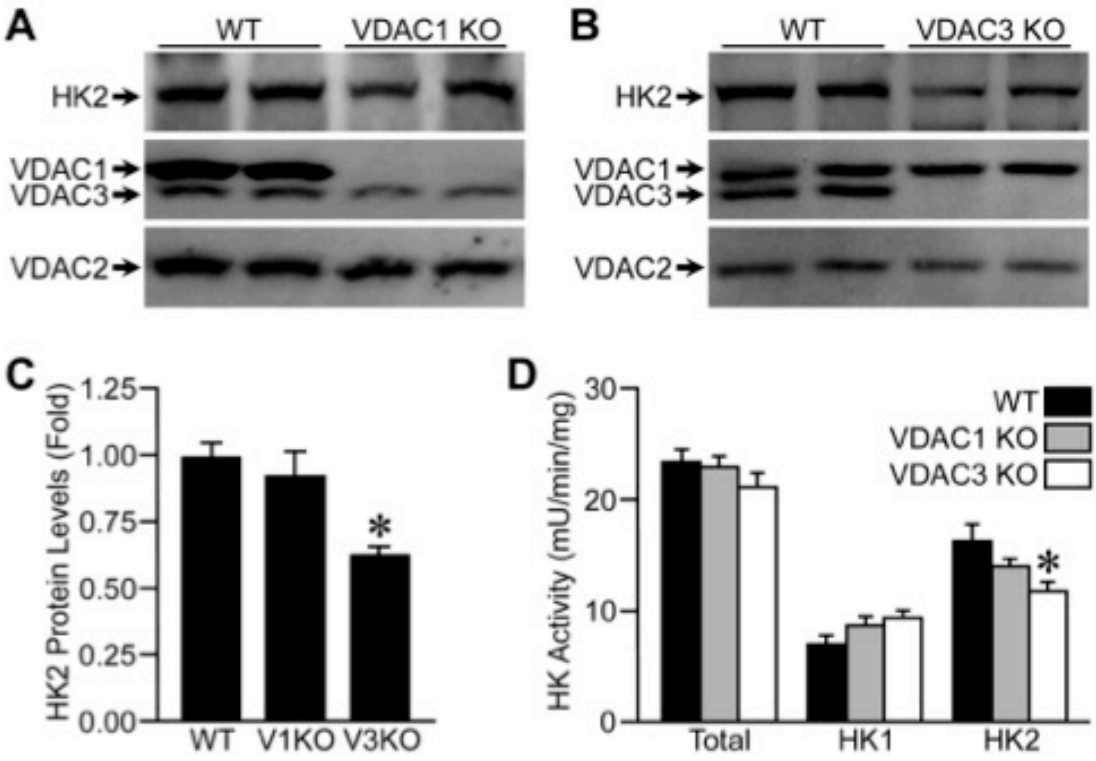

Figure 8. VDAC3 deletion decreases total HK2 expression and activity. *A,* Western blots for HK2, VDAC1/3 and VDAC2 in wildtype (WT) and VDAC1 knockout (KO) MEFs. *B,* Western blots for HK2, VDAC1/3 and VDAC2 in WT and VDAC3 KO MEFs. *C,* Quantification of HK2 expression in WT, VDAC1 KO, and VDAC3 KO MEFs. *D,* Total HK, HK1, and HK2 activities in WT, VDAC1 KO, and VDAC3 KO MEFs. Results shown are representative of 3-4 independent experiments performed in duplicate. Error bars indicate s.e.m. with **P*<0.05 versus WT.

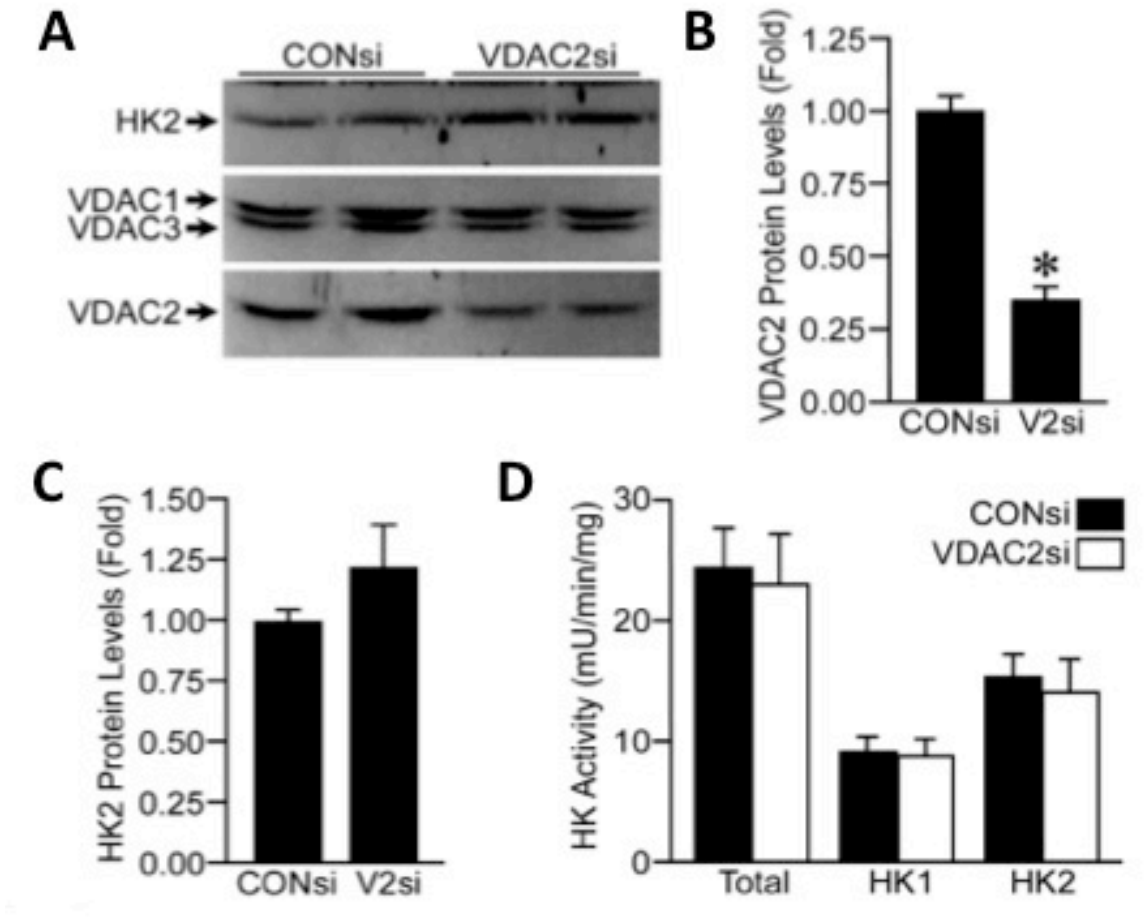

Figure 9. VDAC2 knockdown does not affect total HK2 expression or activity. *A*, Western blots for HK2, VDAC1/3 and VDAC2 in MEFs transfected with either control (CONsi) or VDAC2-specific siRNAs. *B,* Quantification of VDAC2 knockdown. *C,,* Quantification of HK2 expression in control and VDAC2 siRNA-transfected MEFs. *D,* Total HK, HK1, and HK2 activities in control and VDAC2 siRNA-transfected MEFs. Results shown are representative of 3-4 independent experiments performed in duplicate. Error bars indicate s.e.m. with **P*<0.05 versus CONsi.

4.2 *VDAC1 and VDAC3 Are Important For HK2 Mitochondrial Localization*

We next wanted to investigate which VDAC isoform was the binding site for HK2 in MEFs. Thus, we grew up WT and VDAC-deficient/depleted MEFs and subfractionated the cells into cytosolic and mitochondrial lysates. As expected, in WT MEFs HK2 expression and activity was localized primarily in the mitochondrial fraction (**Figure 10a,b**). In *Vdac1*$^{-/-}$ MEFs, there was marked reduction in mitochondrial HK2 expression and activity, with concomitant increases in the cytosolic fraction (**Figure 10a,b**). siRNA-mediated depletion of VDAC2 appeared to have no effect on HK2 localization as both HK2 expression and activity remained predominantly mitochondrial, similar to wild-type MEFs (**Figure 10a,c**). Lastly, deletion of VDAC3 resulted in decreased mitochondrial HK2 expression and activity, and increased cytosolic expression compared to WT (**Figure 10a,b**). However, the redistribution of HK2 in *Vdac3*$^{-/-}$ MEFs was less dramatic than that observed in the *Vdac1*$^{-/-}$ MEFs, indicating that HK2 binds both VDAC1 and VDAC3, but favors VDAC1 binding.

These results were then confirmed by immunocytochemistry. In addition to cytosolic HK2 labeling, a high degree of colocalization was observed between anti-HK2 antibody staining and Mitotracker-Red in WT MEFs (**Figure 11**). Consistent with the Western blotting data, this colocalization was largely lost in *Vdac1*$^{-/-}$ MEFs, and was partially lost in *Vdac3*$^{-/-}$ MEFs (**Figure 11**). VDAC2 depletion had no effect on the HK2 mitochondrial localization (**Figure 11**). These findings again suggest that HK2 binds predominantly to VDAC1, by a lesser extent to VDAC3, and does not bind VDAC2.

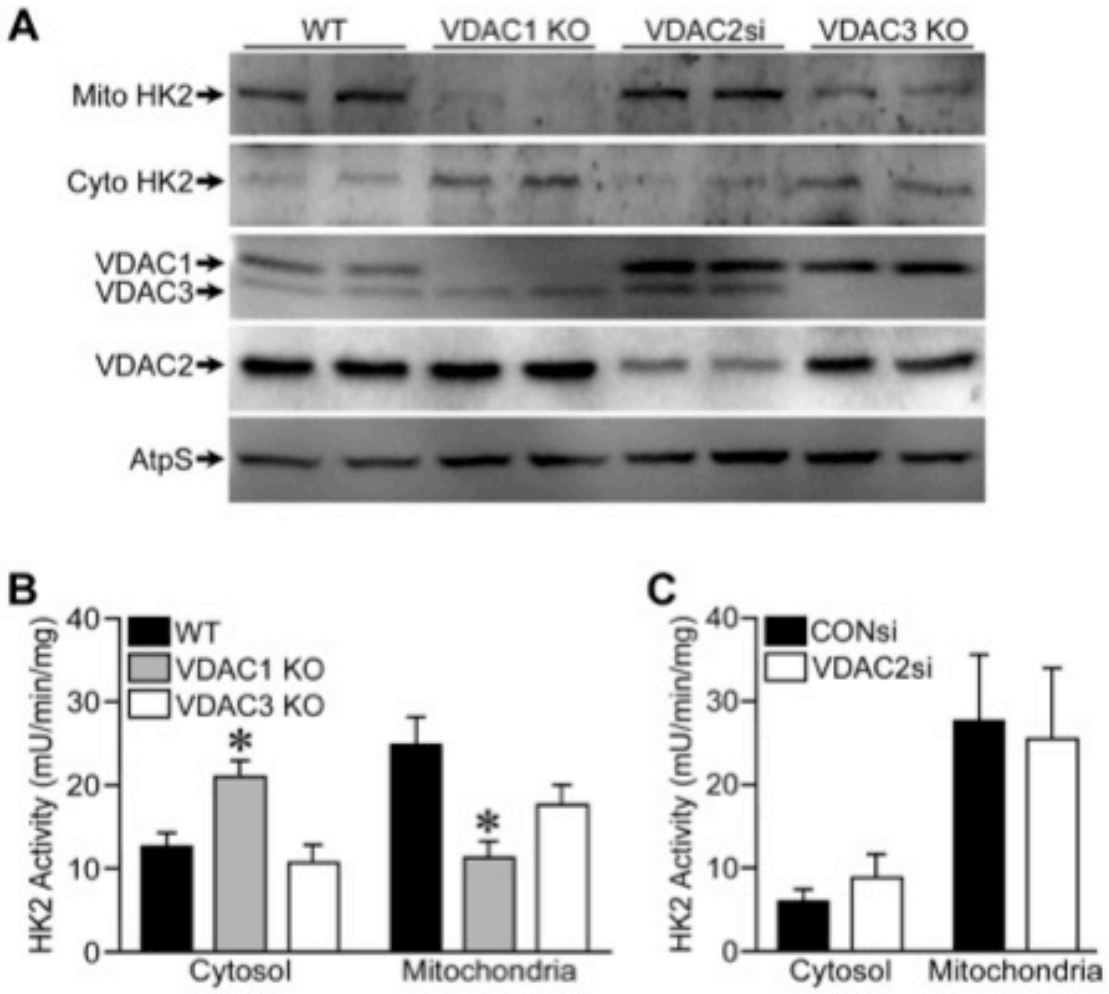

Figure 10. HK2 binds to VDAC1 and VDAC3. *A*, Subcellular mitochondrial and cytosolic fractions were prepared from wildtype (WT) and VDAC-deficient MEFs by differential centrifugation, and then blotted for HK2. Blots for mitochondrial VDAC1/3, VDAC2 and ATP synthase (AtpS) are also shown. *B,* HK2 activity in the cytosolic and mitochondrial fractions of WT, VDAC1 knockout (KO), and VDAC3 KO MEFs. *C,* HK2 activity in the cytosolic and mitochondrial fractions of control and VDAC2 siRNA-transfected MEFs. Results shown are representative of 3-4 independent experiments performed in duplicate. Error bars indicate s.e.m. with **P*<0.05 versus WT.

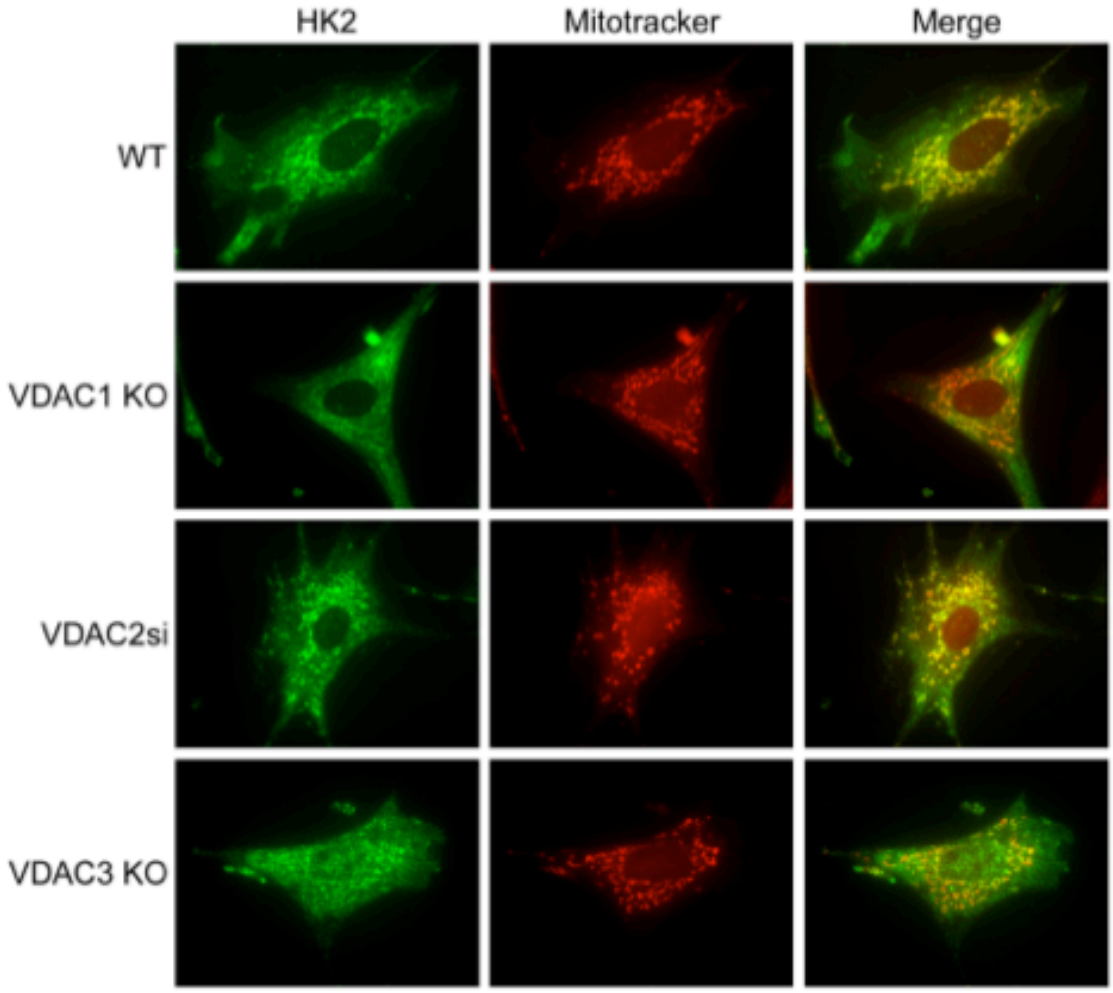

Figure 11. Immunohistochemistry confirms that HK2 binds to VDAC1 and VDAC3. Representative immunocytochemistry images for HK2 in VDAC-deficient MEFs. HK2 was immunolabeled with a goat Anti-HK2 primary antibody and green fluorescent Anti-Goat secondary antibody. Mitochondria were labeled with Mitotracker-Red.

4.3 *HK2 Overexpression Protects Against H_2O_2-Induced Loss of $\Delta\Psi_m$ and Cell Death*

To investigate HK2-mediated protection against cell death we created an adenovirus encoding murine HK2. Infection of WT MEFs with the virus resulted in an ~4-fold increase in total HK2 protein levels compared to control β-galactosidase (βGal)-infected cells (**Figure 12a,c**). HK2 activity was similarly increased in the HK2-infected cells (**Figure 12d**). Importantly, neither HK1 expression nor activity were affected by HK2 overexpression (**Figure 12b,d**). Somewhat surprisingly, subfractionation of infected WT MEFs showed that the majority of the HK2 overexpression occurred in the cytosolic fraction (**Figure 13a,b**). However, there was also a small but significant increase in mitochondrial HK2 levels (**Figure 13a,b**). When treated with increasing concentrations of H_2O_2, βGal-infected WT MEFs showed a concentration-dependent decrease in $\Delta\Psi_m$, which was markedly attenuated by HK2 overexpression (**Figure 14a,b**). Consistent with this, H_2O_2 treatment resulted in concentration-dependent increases in cell death, as measured by Sytox Green (**Figure 14c**). Again, the level of cell mortality was significantly reduced (~50%) by HK2 overexpression (**Figure 14c**). These results indicate HK2 overexpression protects the cells from oxidative stress-induced mitochondrial dysfunction and cell death. As H_2O_2-induced death is primarily necrotic in nature, we next wanted to test whether HK2 overexpression could protect against apoptosis. We therefore incubated βGal- and HK2-infected MEFs with staurosporine, which induces apoptosis in a Bax/Bak-dependent manner (Wei, 2001). However, unlike with H_2O_2, HK2 overexpression was unable to reduce staurosporine-induced TUNEL positivity (**Figure 14d**). These results indicate that HK2 protects against ROS-induced mitochondrial dysfunction and necrosis, but not apoptosis.

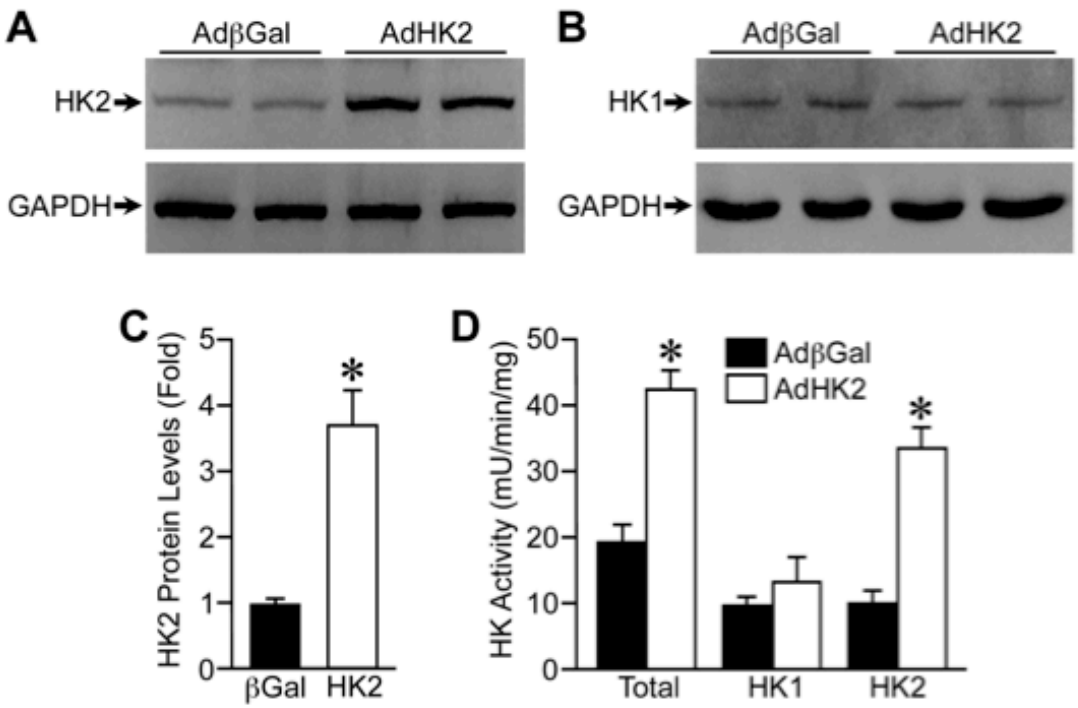

Figure 12. HK2 overexpression with adenovirus. *A,* Western blotting for HK2 and GAPDH in MEFs infected with β-galactosidase (βGal, control) or HK2 adenoviruses. *B,* Western blotting for HK1 and GAPDH in βGal- and HK2-infected MEFs. *C,* Quantification of HK2 expression in βGal- and HK2-infected MEFs. *D,* Total HK, HK1 and HK2 activities in βGal- and HK2-infected MEFs. Results shown are representative of 3 independent experiments performed in duplicate. Error bars indicate s.e.m. with **P*<0.05 versus βGal.

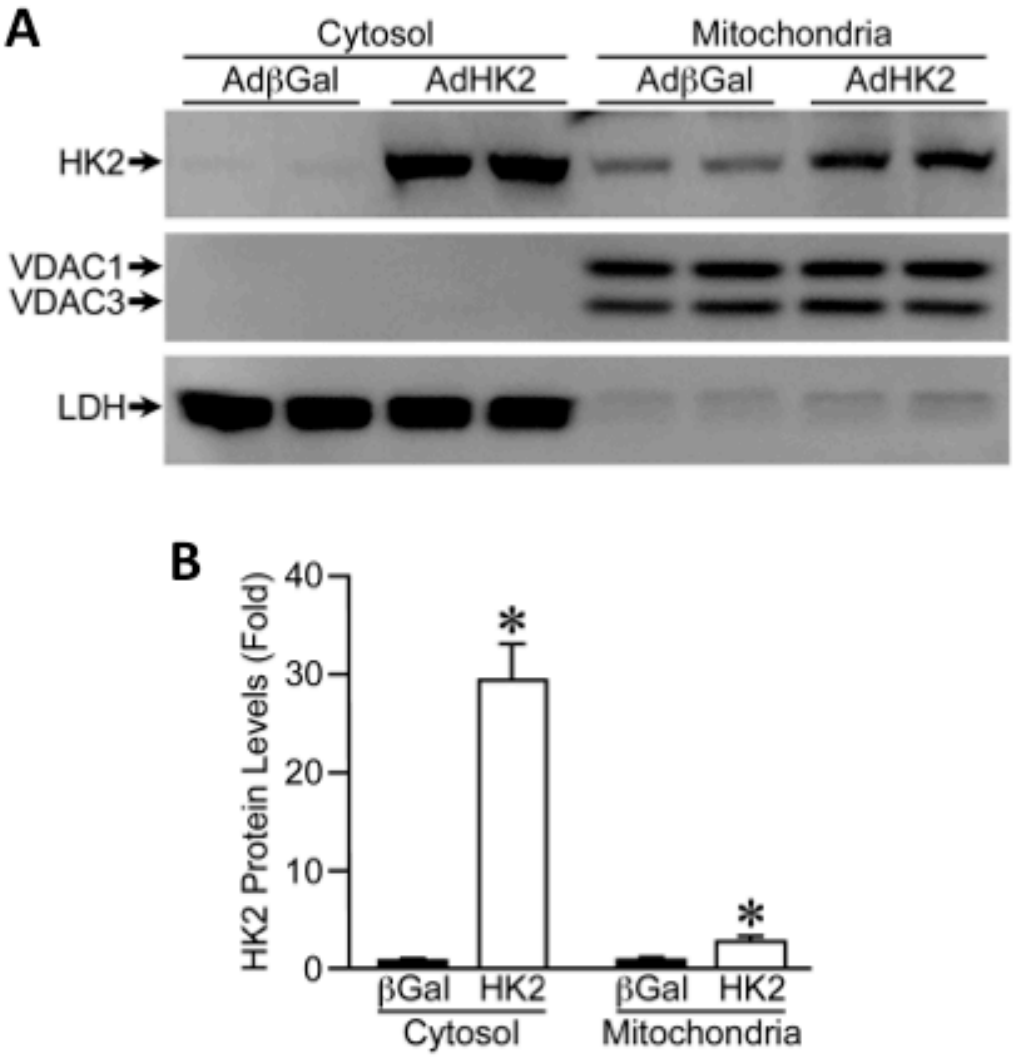

Figure 13. HK2 overexpression is cytosolic and mitochondrial. *A,* Western blotting for HK2, VDAC1/3, and LDH in cytosolic and mitochondrial fractions from βGal- and HK2-infected MEFs. *B,* Quantification of cytosolic and mitochondrial HK2 expression in βGal- and HK2-infected MEFs. Results shown are representative of 3 independent experiments performed in duplicate. Error bars indicate s.e.m. with **P*<0.05 versus βGal.

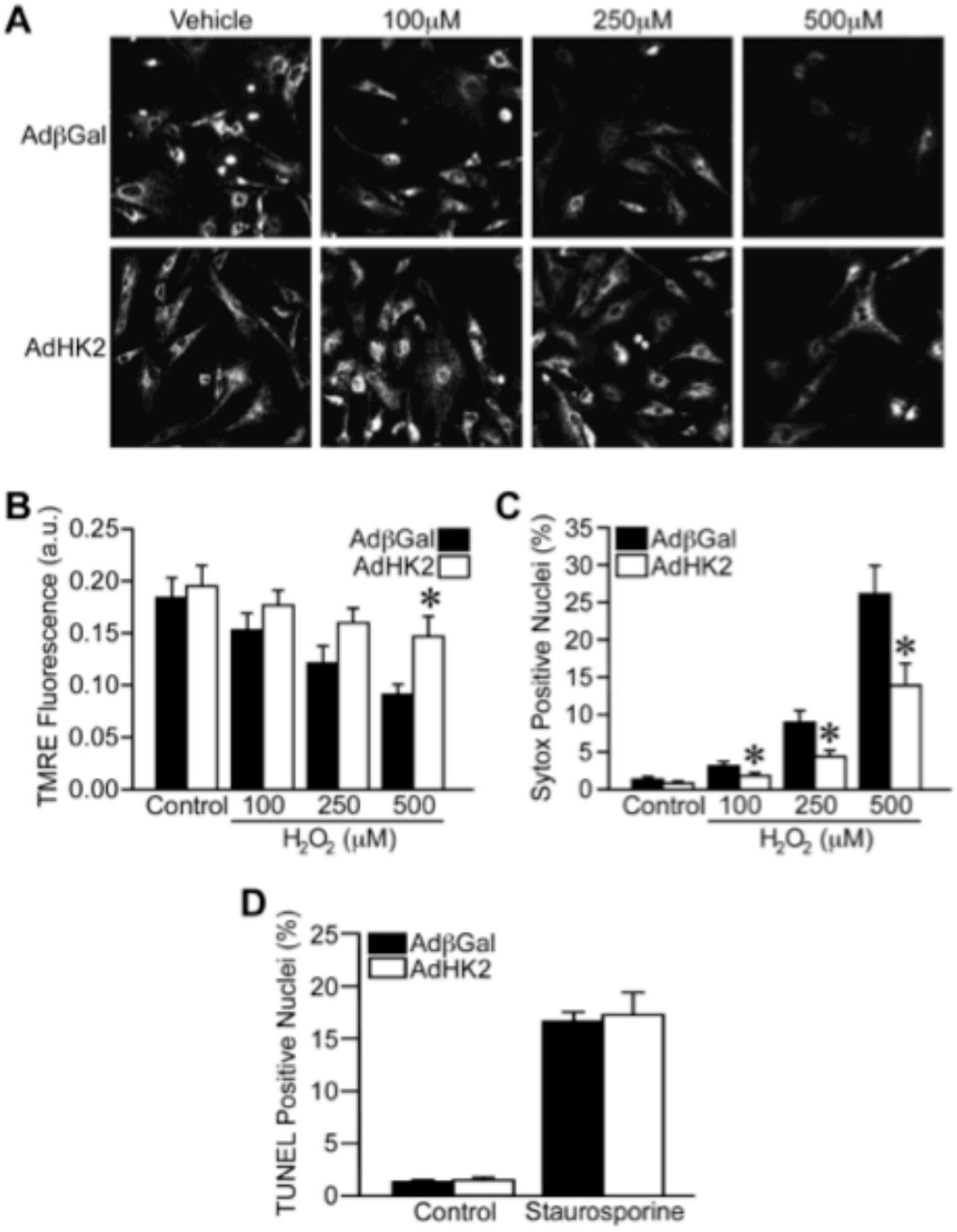

Figure 14. HK2 overexpression protects against H_2O_2-induced loss of $\Delta\Psi_m$ and cell death. *A,* $\Delta\Psi_m$ was determined using TMRE fluorescence in βGal- and HK2-infected MEFs treated with increasing concentrations of H_2O_2 for 2 hrs. *B*, Quantification of the TMRE fluorescence intensity for each condition. *C,* Sytox Green staining in βGal- and HK2-infected MEFs treated with increasing concentrations of H_2O_2 for 3 hrs. *D,* TUNEL staining in βGal- and HK2-infected MEFs treated with vehicle or 100nM staurosporine for 18 hrs. Results shown are representative of 3 independent experiments performed in duplicate. Error bars indicate s.e.m. with **P*<0.05 versus βGal.

4.4 *HK2 Overexpression Protects VDAC1- but not VDAC3-deficient MEFs Against H_2O_2-induced Death*

After observing that HK2 binds to both VDAC1 and VDAC3, we next wanted to investigate the effects of VDAC isoform deletion on HK2-mediated protection. Treatment of *Vdac1*$^{-/-}$ MEFs with 500 μM H_2O_2 resulted in a slightly higher level of death than WT cells, but was not significantly different. However, even though HK2 primarily binds to VDAC1, HK2 overexpression was still able to protect *Vdac1*$^{-/-}$ MEFs against H_2O_2-induced death to a similar extent as that seen in WT cells (~50%, **Figure 15a,b**). VDAC3 deletion did not result in increased cell death at baseline or with H_2O_2 treatment compared to WT cells. However, in contrast to the *Vdac1*$^{-/-}$ MEFs, HK2 overexpression in *Vdac3*$^{-/-}$ MEFs (**Figure 15c**) resulted in complete loss of HK2-mediated protection against cell death (**Figure 15d**). These results suggest that VDAC3 is necessary for HK2-mediated protection against H_2O_2-induced death.

4.5 *HK2 Overexpression Protects VDAC2-depleted MEFs Against H_2O_2-induced Death in the absence of Bak and Bax*

Although we were unable to detect any interaction between HK2 and VDAC2, we still wanted to test the effect of depleting VDAC2 on HK2-mediated protection. To our surprise, HK2 overexpression in WT MEFs transfected with a VDAC2 siRNA (**Figure 16a**) resulted in no protection against cell death (**Figure 16b**). However, depletion of VDAC2 caused a striking increase in H_2O_2-induced cell death, which we suspect masked the beneficial effect of HK2 overexpression. The large increase in death with VDAC2

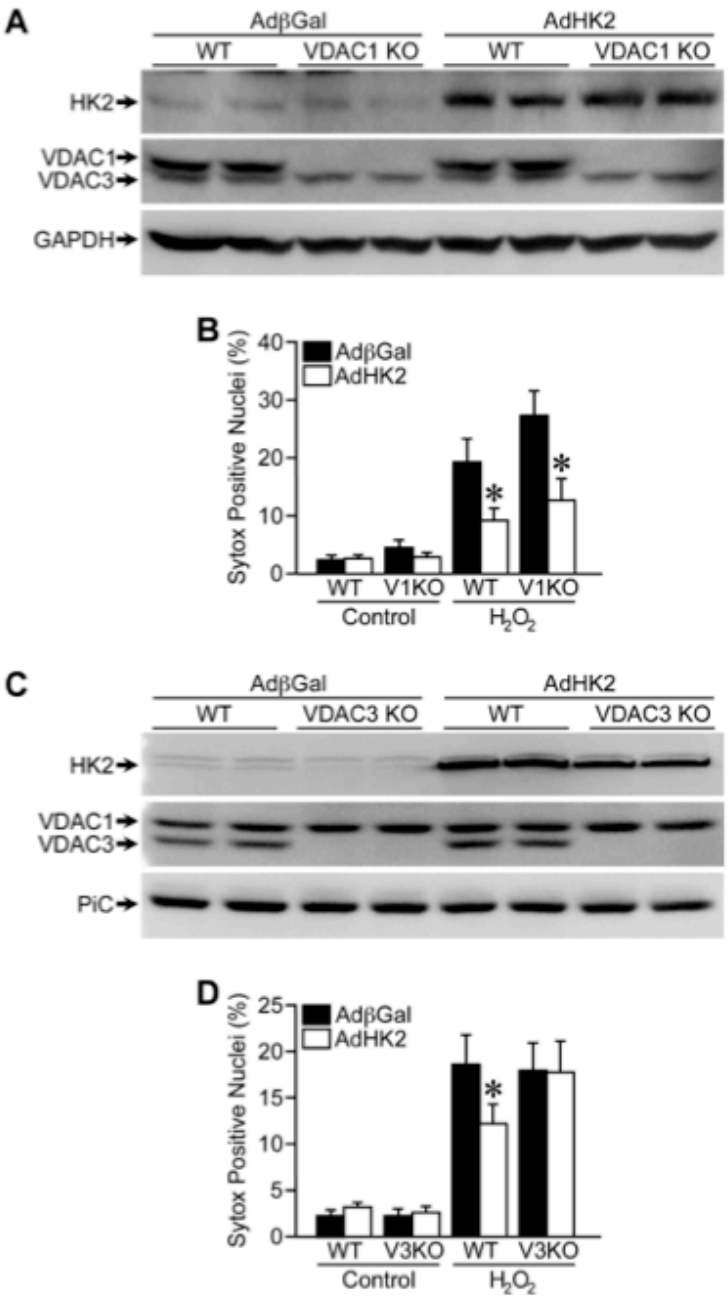

Figure 15. HK2 overexpression protects VDAC1 but not VDAC3-deficient MEFs. *A,* Western blotting for HK2, VDAC1/3, and GAPDH in wildtype (WT) and VDAC1 knockout (KO) MEFs infected with β-galactosidase (βGal, control) or HK2 adenoviruses. *B,* Sytox Green staining in βGal- and HK2-infected WT and VDAC1 KO MEFs treated with 500 μM H_2O_2 for 3 hrs. *C,* Western blotting for HK2, VDAC1/3 and the mitochondrial phosphate carrier (PiC) in WT and VDAC3 KO MEFs infected with βGal or HK2 adenoviruses. *D,* Sytox Green staining in βGal- and HK2-infected WT and VDAC3 KO MEFs treated with 500 μM H_2O_2 for 3 hrs. Results shown are representative of 3-4 independent experiments performed in duplicate. Error bars indicate s.e.m. with **P*<0.05 versus βGal.

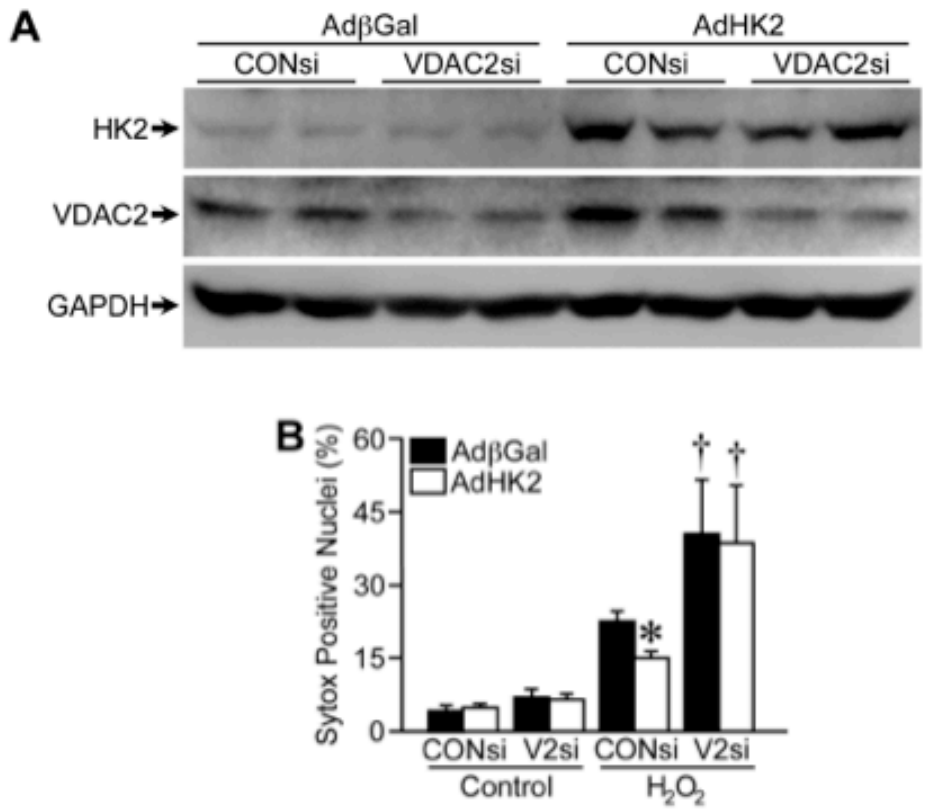

Figure 16. HK2 overexpression does not protect WT MEFs deficient in VDAC2. *A,* Western blotting for HK2, VDAC2, and GAPDH in control and VDAC2 siRNA-transfected MEFs infected with β-galactosidase (βGal, control) or HK2 adenoviruses. *B,* Sytox Green staining in βGal- and HK2-infected control and VDAC2 siRNA-transfected MEFs treated with 500 μM H_2O_2 for 3 hrs. Results shown are representative of 3-4 independent experiments performed in duplicate. Error bars indicate s.e.m. with **P*<0.05 versus βGal and †*P*<0.05 versus CONsi.

knockdown was possibly due to activation of the proapoptotic Bcl2 protein Bak, as VDAC2 has been shown to sequester and inhibit Bak (Cheng, 2003). Likewise, VDAC2 has been suggested to be required for Bax-mediated apoptosis as well (Yamagata, 2009). Therefore, we tested the effect of VDAC2 knockdown in both WT and *Bax/Bak* double-null immortalized MEFs (**Figure 17a**). The WT immortalized cells nicely recapitulated the findings from our primary WT MEF cell culture in that VDAC2 knockdown resulted in a large increase in cell death and a loss of HK2-mediated protection (**Figure 17b**). Conversely, in *Bax/Bak* double-null cells, VDAC2 knockdown did not result in significantly increased death, and HK2 regained the ability to protect against H_2O_2-induced cell death (**Figure 17c**). Thus, depletion of VDAC2 results in activation of proapoptotic Bax and/or Bak, which HK2 overexpression cannot protect against. This is consistent with HK2's inability to protect against staurosporine-induced death (**Figure 14d**). Taken together, these results suggest that VDAC3 is required for HK2-mediated protection against oxidant-induced cell death while VDAC1 and VDAC2 are not.

5. DISCUSSION

In this study, we found that HK2 is bound mostly to VDAC1, but also significantly to VDAC3 in MEF cell cultures. We then determined that HK2 overexpression is protective against H_2O_2-induced cell death, and that VDAC3 deletion results in abolishment of this HK2-mediated protection against death.

These HK2-VDAC interaction findings are largely in agreement with a previous study investigating total HK (both HK1 and HK2) activity in heart and soleus muscles of

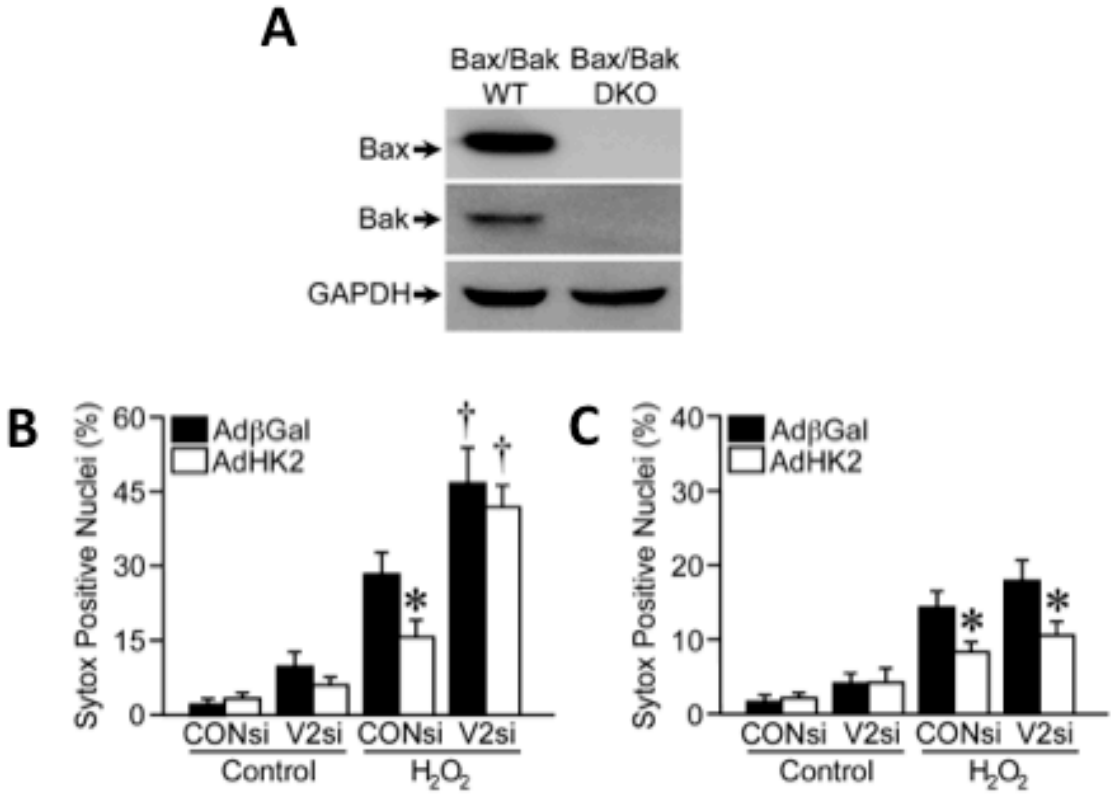

Figure 17. HK2 overexpression protects VDAC2-deficient cells in the absence of Bax and Bak. *A,* Western blotting for Bax, Bak, and GAPDH in Bax/Bak wildtype (WT) and double knockout (DKO) immortalized MEFs. *B,* Sytox Green staining in βGal- and HK2-infected control and VDAC2 siRNA-transfected Bax/Bak WT MEFs treated with 500 μM H_2O_2 for 3 hrs. *C,* Sytox Green staining in βGal- and HK2-infected control and VDAC2 siRNA-transfected Bax/Bak DKO MEFs treated with 500 μM H_2O_2 for 3 hrs. Results shown are representative of 3-4 independent experiments performed in duplicate. Error bars indicate s.e.m. with **P*<0.05 versus βGal and †*P*<0.05 versus CONsi.

Vdac1$^{-/-}$ and *Vdac3*$^{-/-}$ mice (Anflous-Pharayra, 2007). In this previous study, VDAC1 deletion resulted in significantly decreased mitochondrial HK activity in both heart and soleus muscle. VDAC3$^{-/-}$ mice also demonstrated decreased mitochondrial HK activity, but not to the extent of *Vdac1*$^{-/-}$ muscles. Interestingly, in this previous study VDAC1 deletion did not have any effect on whole cell HK activity, while *Vdac3*$^{-/-}$ muscles exhibited a ~20% decrease in whole cell HK activity. We therefore investigated whole cell HK2 expression and activity in the VDAC-deficient MEFs. We similarly observed no significant difference in whole cell HK2 expression/activity in *Vdac1*$^{-/-}$ cells, but observed significantly decreased HK2 expression and activity in *Vdac3*$^{-/-}$ MEFs.

Although the results of the current study and the previous *in vivo* study align almost perfectly, there is one methodological difference that should be noted. In the current study, HK1 and HK2 activity were measured separately. In the previous study, total hexokinase activity was measured, which for muscles would be a combination of HK1 and HK2 activity. This is particularly true for the heart where HK1 and HK2 are both abundantly expressed (Katzen, 1965). In the skeletal muscle, HK2 is the predominant isoform, with far less HK1 expression (Katzen, 1965). Interestingly, in the previous *in vivo* study, both heart and soleus muscles showed the same effects of VDAC deletion on HK activity; however, the effects in the soleus muscle appeared to be slightly more pronounced than in the heart (Anflous-Pharayra, 2007). This is likely due to the heart expressing a combination of HK1 and HK2, while the soleus is expressing predominantly HK2.

The next step in determining which VDAC isoforms played a role in HK2-mediated protection against cell death involved creating a HK2 adenovirus. Infection of

WT MEFs with the HK2 adenovirus resulted in significantly increased HK2 expression and activity, which was observed to be both cytosolic and mitochondrial. This HK2 overexpression was then shown to maintain $\Delta\Psi_M$ during H_2O_2 treatment as well as protect WT MEFs against H_2O_2-induced death. While the effects of cytosolic vs. mitochondrial HK2 cannot be separated in our study, these results agree with several other studies depicting an importance of mitochondrial binding for HK2 to be protective. For example, one study found that HK constructs transfected into 293 cells needed to be both catalytically active and bound to the mitochondria to be fully protective against H_2O_2 (Sun, 2008). A recent report also showed that in primary cultured neurons, overexpression of full-length HK2 was protective against oxygen-glucose deprivation; however, expression of a truncated HK2 construct unable to bind the mitochondria did not afford any protection (Mergenthaler, 2012). Conversely, HK2 heterozygote mice with a ~50% decrease in total and mitochondrial HK2 expression are more susceptible to myocardial ischemia-reperfusion injury (Wu, 2011). Additionally, removal of HK2 from the mitochondria by treatment with a TAT-HK2 peptide in neonatal rat cardiomyocytes was shown to decrease $\Delta\Psi_M$ and reduce cell viability (Miyamoto, 2008; Wu, 2011). Thus, the current study and others suggest that mitochondrial-binding of HK2 is important for maintenance of $\Delta\Psi_M$ and protection against cell death.

Once determining that HK2 bound to both VDAC1 and VDAC3, and that HK2-mitochondrial binding was likely important for protecting against cell death, we decided to investigate whether any of the VDAC isoforms played a role in the HK2-mediated protection. Since the interaction data demonstrated that HK2 bound mostly to VDAC1, we hypothesized that loss of VDAC1 would abolish or attenuate the HK2-mediated

protection. To our surprise, HK2 overexpression was still able to protect *Vdac1*$^{-/-}$ cells to a similar extent as WT cells. VDAC3, on the other hand, only moderately bound HK2, yet deletion of VDAC3 completely abolished the HK2-mediated protection against H_2O_2.

Perhaps the biggest surprise of these experiments was that knockdown of VDAC2 in WT MEFs, which we determined had no effect on HK2 localization, also completely abolished the HK2-mediated protection against cell death. Importantly, VDAC2 knockdown drastically increased cell death with H_2O_2 treatment compared to control cells, whereas deletion of VDAC3 did not result in increased cell death at baseline or with H_2O_2 treatment. VDAC2 has been shown to sequester and "deactivate" Bak on the outer mitochondrial membrane (Cheng, 2003). VDAC2 has also been described to be necessary for Bax-mediated apoptosis (Yamagata, 2009). Indeed knockdown of VDAC2 has been previously shown to increase H_2O_2-induced cell death in MEFs (Baines, 2007). We therefore hypothesized that VDAC2 knockdown was activating proapoptotic Bax and Bak, resulting in increased cell death with H_2O_2 treatment, and preventing HK2 overexpression from being protective. Indeed, Bax/Bak double-null cells no longer exhibited the marked increases in cell death observed with VDAC2 knockdown, and HK2 overexpression was now able to protect against H_2O_2 in these Bax/Bak$^{-/-}$ cells devoid of VDAC2. Therefore, VDAC2 is not required for HK2-mediated protection against death.

The VDAC2 knockdown studies do present some interesting data in regards to the protection afforded by HK2 overexpression. The data suggest that while HK2 is protective against ROS-induced death, which is primarily a necrotic stimulus, HK2 overexpression may not afford protection against apoptosis. To test this hypothesis, we

treated cells with staurosporine to induce apoptosis, and indeed, HK2 overexpression was not protective. These findings are in agreement with a recent study showing that HK2 overexpression protected cultured primary neurons from oxygen-glucose deprivation, but was unable to protect against the apoptosis inducers actinomycin D or etoposide (Mergenthaler, 2012). However, there are also several studies showing that detachment of HK2 from the mitochondria results in increased mitochondrial Bax (Gall, 2011; Pastorino, 2002), therefore, HK2 and Bax may be competing for the same mitochondrial binding site. Thus, the ability of HK2 to protect against apoptotic vs. necrotic stimuli requires much further study.

There is limited evidence to speculate as to why the VDAC3 interaction and not VDAC1 would lead to protection against cell death. Interestingly, VDAC3 was recently determined to be the most important isoform for maintaining $\Delta\Psi_m$ in hepatic cancer cells even though it was the least expressed isoform (Maldonado, 2013). The current study and others show that HK2 helps maintain $\Delta\Psi_m$ (Chiara, 2008; Wu, 2011), and perhaps this is mediated through binding to VDAC3. We also cannot rule out the possibility that perhaps it is not a direct HK2-VDAC3 interaction that is protective, but that some other binding partner is removed when VDAC3 is deleted. Indeed, there have been several recent findings of "accessory proteins" binding to HK2 on the mitochondria and mediating protection against cell death (Cheung, 2012; Mailloux, 2011; Mergenthaler, 2012; Tchakarska, 2011). HK2 interaction with these proteins is not well studied, and it is unknown how VDAC3 deletion affects the interaction with these other proposed binding partners. Obviously, much more work is needed to thoroughly investigate the HK2-VDAC3 interaction.

In conclusion, HK2 binds to both VDAC1 and VDAC3, and the VDAC3 interaction appears to be necessary for HK2-mediated protection against cell death. These findings describe a unique relationship between HK2 and VDAC3 important for regulating cell death, while the HK2-VDAC1 interaction appears to be more important for cellular glucose utilization (Anflous-Pharayra, 2007). Since HK2 overexpression and resistance to cell death are characteristics of cancer cells, it would be interesting to determine if HK2-VDAC3 binding is increased in cancer cells. Additionally, investigation of the susceptibility of *Vdac3*$^{-/-}$ cells to transform into cancer cells would also be interesting in determining the importance of the HK2-VDAC3 interaction.

CHAPTER 3: Cardiac-Specific Hexokinase 2 Overexpression Attenuates Hypertrophy by Increasing Pentose Phosphate Pathway Flux

1. ABSTRACT

Background The enzyme hexokinase-2 (HK2) phosphorylates glucose, which is the initiating step in virtually all glucose utilization pathways. Cardiac hypertrophy involves a switch towards increased glucose metabolism and decreased fatty acid metabolism. Recent evidence suggests increased glucose utilization is compensatory to the down-regulated fatty acid metabolism in pathologic hypertrophy. Therefore, we hypothesized that HK2 overexpression would decrease cardiac hypertrophy.

Methods and Results Mice with cardiac-specific HK2 overexpression displayed decreased hypertrophy in response to isoproterenol. Neonatal rat ventricular myocytes (NRVMs) infected with a HK2 adenovirus similarly displayed decreased hypertrophy in response to phenylephrine. Hypertrophy increased ROS levels and HK2 overexpression attenuated ROS accumulation, thereby decreasing NRVM hypertrophy and death. HK2 appears to modulate ROS via the pentose phosphate pathway, as inhibition of glucose-6-phosphate dehydrogenase with dehydroepiandrosterone abolished HK2's ability to diminish ROS and hypertrophy.

Conclusions These results suggest that HK2 attenuates cardiac hypertrophy by decreasing ROS accumulation via increased pentose phosphate pathway flux.

2. INTRODUCTION

Under normal situations, the heart relies heavily on fatty acid oxidation for its energy metabolism (Neely, 1972). However, during cardiac hypertrophy there is a switch to increased glucose utilization and decreased fatty acid metabolism (Barger, 1999; Dávila-Román, 2002; Taegtmeyer, 1988). Glucose metabolism is initiated by cellular glucose uptake mediated by glucose transporters (GLUT), and glucose phosphorylation by a hexokinase (HK) to form glucose-6-phosphate (Katzen, 1967). Four mammalian HK isozymes exist, with both HK1 and HK2 expressed in the heart (Wilson, 2003). As HK2 expression is dynamically regulated by factors such as insulin and hypoxia (Osawa, 1995; Printz, 1997), HK2 receives most of the attention as the predominant muscle HK. In addition to HK1 or HK2's kinase function, both enzymes contain a hydrophobic region of 21-amino acids in their N-termini, which allows for association with the voltage-dependent anion channel (VDAC) on the outer mitochondrial membrane (Fiek, 1982; Linden, 1982). This mitochondrial localization protects cells against death (Majewski, 2004; Mergenthaler, 2012; Miyamoto, 2008; Pastorino, 2002; Sun, 2008), and this protection has mainly been attributed to a reduction in reactive oxygen species (ROS) (Da-Silva, 2004; Mergenthaler, 2012; Miyamoto, 2008; Sun, 2008).

It is still debated whether the increase in glucose utilization during hypertrophy is an integral pathologic process. However, genetic models mostly argue that altered fatty acid metabolism is the driver of hypertrophy, and that increased glucose utilization is compensatory. For example, mice with cardiac-specific overexpression of peroxisome proliferator-activated receptor α (PPARα) demonstrate increased fatty acid metabolism, decreased glucose metabolism, and hypertrophied hearts compared to WT littermates

(Finck, 2002). Deletion of PPAR-gamma coactivator 1α (PGC1α) increases hypertrophy and accelerates heart failure after pressure overload (Arany, 2006). Cardiac-specific overexpression of the GLUT1 transporter attenuated cardiac hypertrophy and improved survival after pressure overload (Liao, 2002). Antithetically, cardiac-specific deletion of the GLUT4 transporter spontaneously results in hypertrophied hearts (Abel, 1999). Lastly, hearts from HK2 heterozygous mice were recently shown to have exacerbated cardiac hypertrophy after pressure overload (Wu, 2012). It was suggested that decreased HK2 expression resulted in decreased mitochondrial HK2, and increased ROS production due to mitochondrial permeability transition (Wu, 2012). Thus, these studies suggest that increasing glucose metabolism is likely a compensatory mechanism during hypertrophy.

Little is known about how increased glucose metabolism decreases cardiac hypertrophy. While glycolysis is considered the preferred route for glucose, other glucose utilization pathways consist of glycogen formation, the pentose phosphate pathway, and the hexosamine biosynthetic pathway (Kolwicz, 2011). Importantly, phosphorylation of glucose by HK is the initiating step in all of these pathways. While glycogen does not appear to be altered during hypertrophy (Allard, 1997), flux through the pentose phosphate pathway and the hexosamine biosynthetic pathway increase during hypertrophy (Chess, 2009; Gupte, 2006; Watson, 2010). We therefore hypothesized that mice with cardiac-specific overexpression of HK2 would demonstrate decreased hypertrophy in response to chronic isoproterenol infusion. Indeed, HK2 overexpression did result in attenuated cardiac and cardiomyocyte hypertrophy in response to isoproterenol. HK2 overexpression also protected against isoproterenol-induced cardiomyocyte death. Overexpression of HK2 also decreased hypertrophy in cultured

neonatal rat ventricular myocytes (NRVMs) treated with phenylephrine. Hypertrophy was associated with an increase in ROS accumulation, which was attenuated by HK2. HK2 overexpression increased glucose-6-phosphate dehydrogenase (G6PDH) activity within the pentose phosphate pathway, and inhibition of G6PDH blocked the ability of HK2 to attenuate ROS and hypertrophy. Taken together, these results suggest that HK2 overexpression is anti-hypertrophic due to increased glucose shuttling to the pentose phosphate pathway and reduction of ROS accumulation.

3. METHODS AND MATERIALS

Animals were handled as approved by the University of Missouri Animal Care and Usage Committee in accordance with the *Guidelines for the Care and Use of Laboratory Animals* published by the National Institutes of Health.

3.1 *HK2 Transgenic Mice and Isoproterenol Infusion*

The cDNA for mouse HK2 was inserted into the α-myosin heavy chain (αMHC) promoter cassette and injected into fertilized FVB/N oocytes. Mice were maintained in a pure FVB/N background. Transgenic (TG) mice were identified by PCR. Nontransgenic (NTG) littermates were used as controls. Chronic isoproterenol infusion (60 mg/kg/day for 14 days) was administered by implantation of mini-osmotic pump (Alzet) into 2-3 month-old mice. Vehicle treated mice were implanted with pumps to perfuse 0.9% saline.

3.2 *Echocardiography*

Echocardiograms were performed under isofluorane anesthesia (1.2-1.8%, 0.6 L

flow of O_2) using a GE Vivid 7 ultrasound system (GE Healthcare) with a 12-mHz transducer. The echocardiographer was blinded to the treatment group. M-mode echocardiography was performed using the parasternal short-axis view of the left-ventricle. Images were captured digitally and six consecutive cardiac cycles were measures and averaged for each animal.

3.3 *Histological Assessments*

Mice underwent deep inhalation anesthesia (2-3% isofluorane) to arrest hearts in end diastole. Hearts were perfused through the apex with 4% paraformaldehyde in PBS containing 25 mM KCl and 5% dextrose. Hearts were then frozen in OTC and sectioned by microtome. Sections were stained with fluorescently labeled wheat germ agglutinin (WGA) or Gomori's Trichrome. For the WGA labeling, 20 cells per field for a total of 10 fields were planimetered using NIH ImageJ. Sections were also stained for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) utilizing a cell death detection kit (Roche).

3.4 *qRT-PCR Gene Expression Analysis*

RNA was extracted from mouse ventricles with TRIzol (Invitrogen) for first strand DNA synthesis (Superscript III First-Strand Synthesis System; Invitrogen). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using a Biorad cycler with Sybr green intercalating dye (Takara SYBR Premix Ex Taq). Primer sequences were obtained from Roche's Universal ProbeLibrary.

3.5 *Mitochondrial Isolation and Measurement of Mitochondrial Respiration*

For subcellular fractionation experiments, mouse hearts were subfractionated by differential centrifugation as previously described (Baines, 2007). Mitochondrial respiration was assessed by a Clark type electrode (Qubit) using Vernier LoggerPro software. Isolated mitochondria (125 μg) were suspended in buffer consisting of 150 mM KCl, 5 mM KH_2PO_4, 10 mM Tris pH 7.4, 2.5 mM $MgCl_2$, and 5 mM glutamate/malate or 5 mM succinate to obtain State 2 respiration. 200 μM ADP was then added to initiate State 3 respiration.

3.6 *Western Blotting Analysis*

Mouse tissue or cell culture lysates were solubilized in lysis buffer containing 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, and 1% Triton-X100. Samples were sonicated and then centrifuged at 13,000 rpm for 10 minutes to remove insoluble debris. Protein concentration was measured by the Bradford method. Proteins were resolved on 10% SDS/PAGE gels, transferred onto PVDF membranes, and blocked with 10% milk in TBS-T. The membranes were then immunoblotted using the following commercially available antibodies: anti-HK2, anti-HK1, and anti-VDAC (Abcam); anti-LDH (Santa Cruz); and anti-GAPDH (Millipore). Membranes were then incubated with the appropriate alkaline phosphatase-linked secondary antibody (Santa Cruz Biotechnology) and visualized by enhanced chemifluorescence (Amersham Biosciences).

3.7 *HK2 Activity Assay*

HK enzymatic activity was assessed as described previously (Wilson, 1989).

Total HK, HK1, and HK2 activity were measured simultaneously, by means of HK2 denaturation at 45°C as formerly described (Wilson, 1998). Briefly, total, cytosolic and mitochondrial lysates were divided into two aliquots, one kept on ice, and one put into a 45°C water bath for 1 hour to denature HK2. At the end of the denaturing step, hexokinase activity was determined in both aliquots by measuring glucose-6-phosphate formation coupled to NADPH formation spectrophotometrically at 340 nm. The assay buffer consisted of 100 mM Tris-HCl pH 8.0, 10 mM glucose, 0.4 mM $NADP^+$, 10 mM $MgCl_2$, 5 mM ATP, and 0.15 U of G6PDH. Activity was normalized to protein concentration as measured by the Bradford method.

3.8 *Mitochondrial Swelling*

Mitochondria were isolated as described above and resuspended in swelling buffer (150 mM KCl, 5 mM KH_2PO_4, 10 mM Tris pH 7.4) to a final concentration of 0.25 mg/ml. De-energized mitochondria received no substrate, and 5 mM glutamate/malate was added to the buffer to energize mitochondria. Mitochondrial swelling, an index of permeability transition, was induced by the addition of 250 μM $CaCl_2$ or 100 μM $CaCl_2$ for de-energized and energized mitochondria, respectively, and absorbance was measured spectrophotometrically at 520 nm (Baines, 2005).

3.9 *Isolation and Culture of Rat Ventricular Myocytes*

Neonatal rat ventricular myocytes (NRVMs) were isolated as previously described (Schramm, 2012). Briefly, 1-3 day-old Sprague-Dawley rat pup heart ventricles were digested with collagenase overnight according to the manufacturer's instructions

(Worthington). Isolated cells were pre-plated on uncoated polystyrene dishes to remove cardiac fibroblasts, and then plated onto gelatin-coated dishes. Cells were maintained in M199 medium supplemented with 10% BGS for 24 hours, then serum-free M199 for the duration of experimentation. Hypertrophy was induced by treatment with 25 μM phenylephrine (PE) or 2 μM Angiotensin-II for a period of 48 hours. Glucose-6-phosphate dehydrogenase (G6PDH) activity was inhibited by treatment with 25 μM dehydroepiandrosterone (DHEA) for 48 hours, concurrent with the hypertrophic drug treatment.

3.10 *Creation and Infection of HK2 Adenovirus*

Replication-deficient adenoviruses for β-galactosidase and mouse HK2 were generated using the AdEasy adenoviral system (Stratagene). NRVMs were infected with adenovirus at a MOI of 100 plaque-forming units, and cultured for 24 hours before experimentation.

3.11 *siRNA Transfection*

NRVMs were transfected with 100 nM of either a non-targeting control siRNA or an HK2-targeting siRNA pool (Dharmacon siGENOME Cat: M-051128-01) using Lipofectamine RNAiMax. Cells were transfected for 48 hours before experimentation.

3.12 *Measurement of NRVM Cell Area*

For cell surface area determination, NRVMs were immunostained for tropomyosin (Sigma) and all cardiomyocytes in each random field were planimetered

using NIH ImageJ to obtain one average per field. Data from ≥ 4 independent isolates with ≥ 4 random fields per group were summarized.

3.13 *Measurement of ROS Accumulation*

ROS was measured in NRVMs by staining with 1μM 2',7'-dichlorofluorescein (DCF) (Invitrogen) in Hank's buffered saline solution for 30 min. Cells were then washed with HBSS and imaged on a fluorescence microscope (Olympus IX51). Average DCF fluorescence per cell was determined with NIH ImageJ.

3.14 *Measurement of NRVM Cell Death*

NRVMs were treated with 50 μM H_2O_2 for 1 hour, and then costained with Sytox Green to label dead cell nuclei and bis-benzimide to label all cell nuclei. Cells were then imaged on a fluorescence microscope and the percentage of dead cells was determined with NIH ImageJ.

3.15 *G6PDH Activity Assay*

The enzymatic activity of G6PDH was measured as performed previously (Tian, 1994). Briefly, 100 μl of whole cell lysates were added to a cuvette with 900 μl of buffer (50 mM Tris, 1 mM $MgCl_2$, and 100 μM $NADP^+$, pH-8.1). Enzyme activity was measured by increase in absorbance at 340 nm, due to the conversion of $NADP^+$ to NADPH, which is formed by both G6PDH and 6-phosphogluconate dehydrogenase (6PGDH) in the pentose phosphate pathway. Thus, two reactions were measured for each sample, one containing substrates for both G6PDH and 6PGDH (total dehydrogenase

activity) and one reaction with substrate only for 6PGDH. G6PDH activity was then calculated by subtracting the 6PGDH activity from the total dehydrogenase activity. Substrate concentrations were 200 μM glucose-6-phosphate and 200 μM 6-phosphogluconate. Activity was normalized to protein concentration as measured by the Bradford method.

3.16 *Statistical Analysis*

All data are expressed as mean ± SEM. Statistical significance was determined between 2 groups by an unpaired Student's t-test or between multiple groups with ANOVA followed by Scheffe's post-hoc test. A *P* value of less than 0.05 was considered significant.

4. RESULTS

4.1 *Characterization of αMHC-HK2 Mice*

Cardiac-specific HK2 overexpression was performed by cloning the mouse HK2 cDNA into the αMHC promoter cassette. TG mouse hearts displayed ~3 fold increased HK2 expression compared to NTG littermates (**Figure 18a,b**). Total HK activity and HK2-specific activity were accordingly increased in HK2 TG mice (**Figure 18c**). Importantly, neither HK1 expression nor HK1 activity was altered by HK2 overexpression (**Figure 18a,c**). TG hearts were normal in appearance and displayed HW/BW ratios similar to NTG littermates (**Figure 19a,b**). Moreover, fractional shortening and chamber size were not different in the TG hearts when compared to the NTG controls (**Figure 19c-e**).

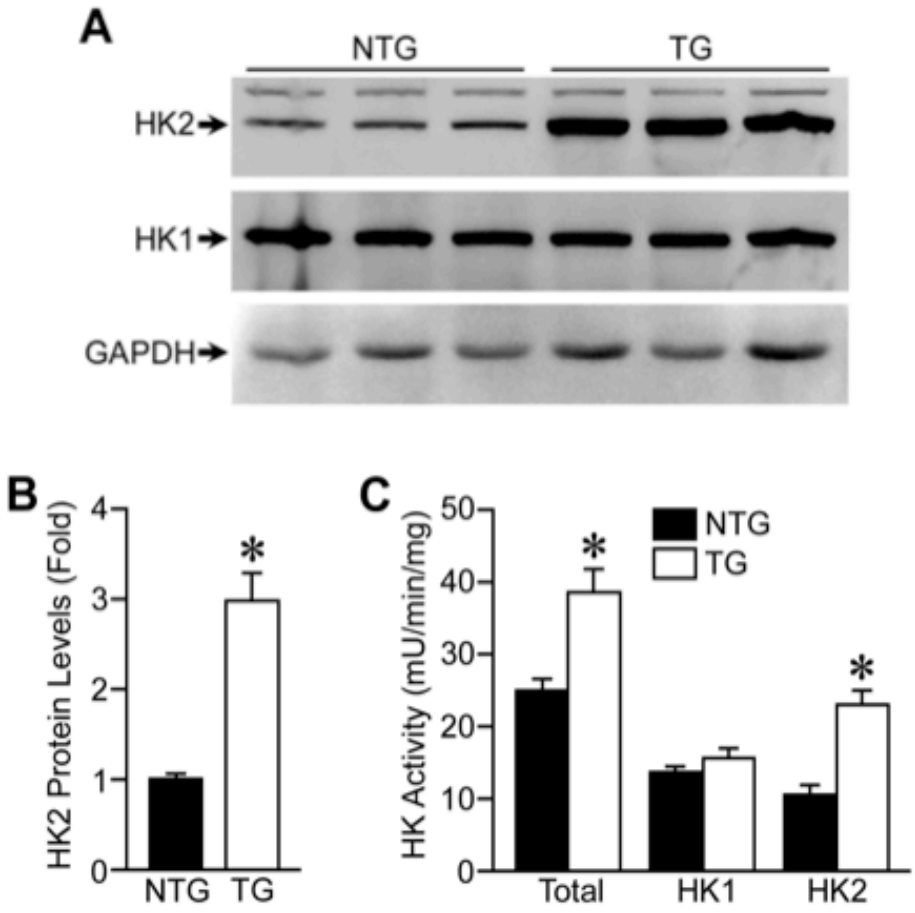

Figure 18. Creation of cardiac-specific HK2 transgenic mouse. *A,* HK2 and HK1 protein levels in αMHC-HK2 transgenic (TG) and non-transgenic (NTG) littermate whole-heart lysates. *B*, Quantified HK2 protein expression from western blots as shown in A. *C*, Total (HK1 + HK2), HK1, and HK2 enzymatic activities from whole heart lysates of NTG and TG mice (n= 4). Error bars indicate s.e.m. with **P*<0.05 versus NTG.

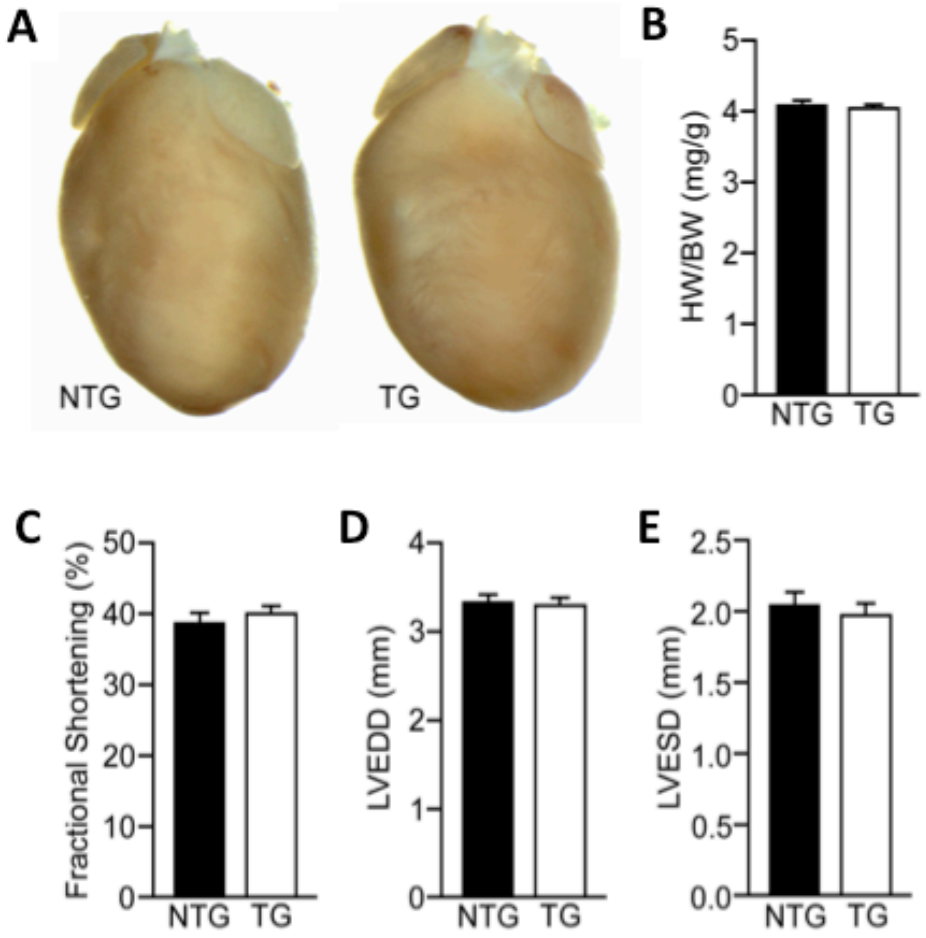

Figure 19. Characterization of αMHC-HK2 transgenic mice. *A,* Representative heart images from NTG and TG littermates. *B*, Gravimetric analysis of heart weight / body weight ratio (HW/BW) for NTG and TG mice. *C-E*, m-mode echocardiography measures of fractional shortening, left ventricular end diastolic dimension (LVEDD), and left ventricular end systolic dimension (LVESD) in NTG and TG mice (n= 10). Error bars indicate s.e.m. with **P*<0.05 versus NTG.

As HK2 can be localized to both the cytosol and mitochondria, we subfractionated NTG and TG hearts into cytosolic and mitochondrial fractions to assess the localization of the overexpressed HK2. Surprisingly, the majority of the overexpressed HK2 was found in the cytosolic fraction (**Figure 20a,b**). However, there was a small but significant increase in mitochondrial HK2 expression as well (**Figure 20a,b**). Total HK and HK2 activity were correspondingly increased mainly in the cytosolic fraction (**Figure 20c**), but also in the mitochondrial fraction (**Figure 20d**). Oxygen consumption was then measured in isolated cardiac mitochondria to assess functional differences. TG heart mitochondria exhibited a significant reduction in state3 respiration when stimulated with complex I substrates (glutamate/malate), but not with complex II substrate (succinate) (**Figure 21a**). The moderate uncoupling observed in TG heart mitochondria likely reflects an increased dependence on glucose metabolism and decreased mitochondrial metabolism in these mice.

Mitochondrial HK2 has been reported to inhibit the mitochondrial permeability transition pore. To determine whether mitochondrial permeability transition was inhibited in the TG mice, heart mitochondria were isolated and subjected to mitochondrial swelling induced by calcium. Despite a small yet significant HK2 overexpression on the mitochondria (**Figure 20a,b**), αMHC-HK2 hearts showed no protection against mitochondrial swelling under either de-energized (**Figure 21b,d**) or energized (**Figure 21c,d**) conditions.

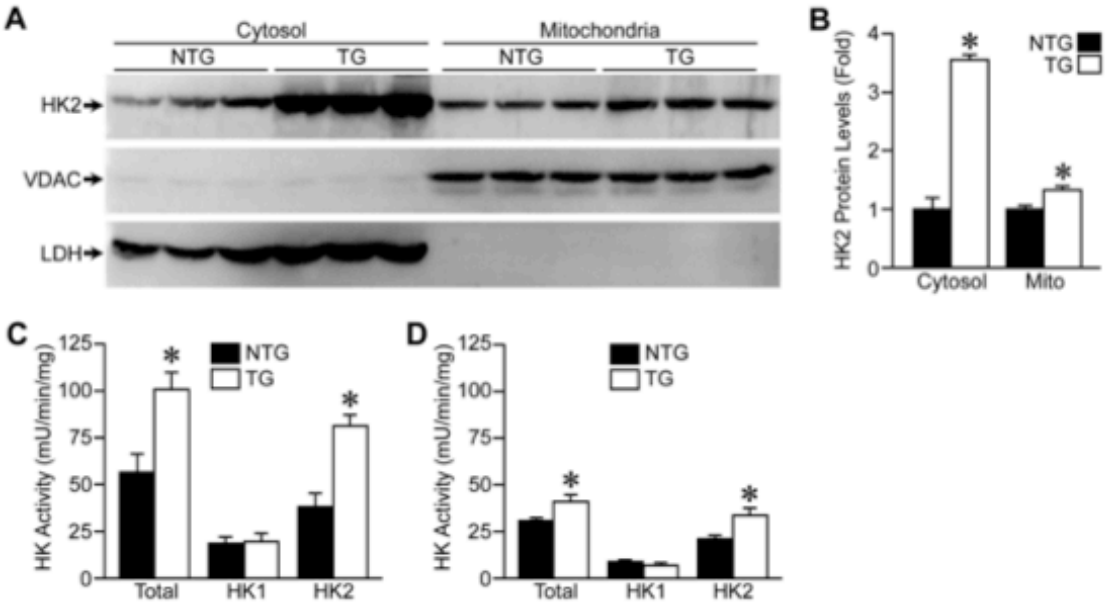

Figure 20. HK2 overexpression is mostly cytosolic. *A,* Cytosolic and mitochondrial HK2 protein expression in subfractionated hearts of NTG and TG mice. Voltage-dependent anion channel (VDAC) demarcates mitochondrial lysates and lactate dehydrogenase (LDH) labels cytosolic lysates. *B*, Quantified HK2 expression from western blots as shown in A. C, Total (HK1 + HK2), HK1, and HK2 enzymatic activities from cytosolic lysates of NTG and TG mouse hearts. *D*, Total, HK1, and HK2 enzymatic activities from mitochondrial lysates of NTG and TG mouse hearts (n= 4). Error bars indicate s.e.m. with **P*<0.05 versus NTG.

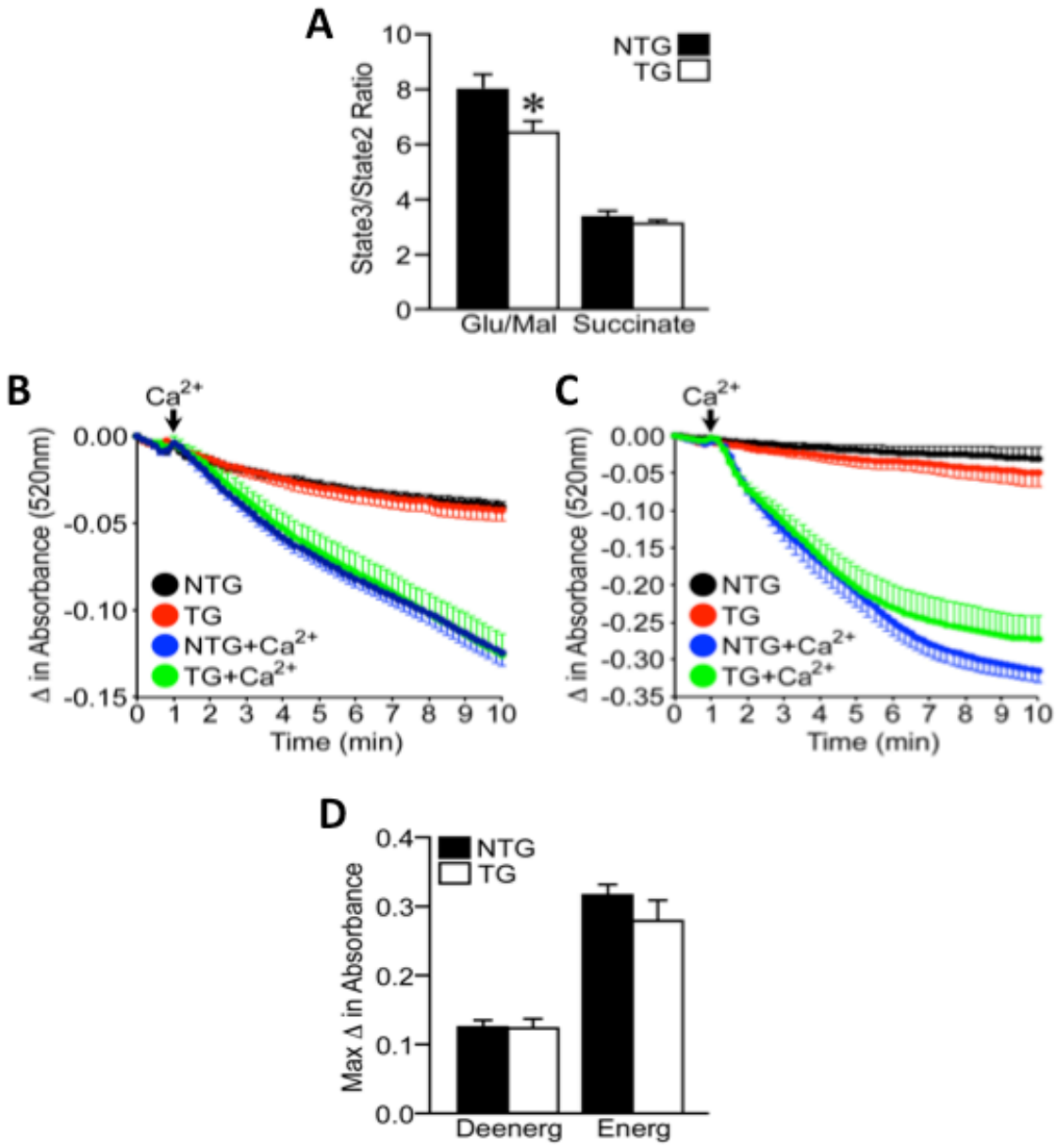

Figure 21. Mitochondrial function in αMHC-HK2 mouse hearts. *A,* Ratio of ADP-stimulated (State 3) to non-stimulated (State 2) respiration measured in isolated mitochondria from NTG and TG hearts. *B*, Mitochondrial swelling average traces for deenergized NTG and TG heart isolated mitochondrial treated with 250 μM Ca^{2+}. *C*, Mitochondrial swelling average traces for Glutamate/Malate energized NTG and TG heart isolated mitochondrial treated with 100 μM Ca^{2+}. *D*, Max change in absorbance from mitochondrial swelling experiments shown in B and C (n= 11-12). Error bars indicate s.e.m. with **P*<0.05 versus NTG.

4.2 *αMHC-HK2 Mice Display Decreased Cardiac Hypertrophy in Response to Isoproterenol*

We next investigated the effects of HK2 transgenesis on the hypertrophic response. Isoproterenol infusion resulted in increased heart size (**Figure 22a**), which significantly increased the HW/BW ratio (**Figure 22b**). HK2 overexpression attenuated the hypertrophic response to isoproterenol (**Figure 22a,b**). Wheat-germ agglutinin staining of LV heart sections indicated increased cardiomyocyte cross-sectional area with isoproterenol, and HK2 overexpression decreased this response (**Figure 22c,d**). Cardiomyocyte cross-sectional area trends were similar for septal wall cardiomyocytes, however, RV cardiomyocytes showed only ~15% increase in cell size with isoproterenol and HK2 overexpression had no effect (data not shown).

We then assessed the effects of HK2 overexpression on cardiomyocyte survival and cardiac function after isoproterenol infusion. In general, there were no signs of heart failure induced by the isoproterenol infusion. LV ejection fraction and fractional shortening remained normal with isoproterenol, and was unaltered by HK2 overexpression (data not shown). No overt fibrosis was seen in either group as well (data not shown). Isoproterenol infusion did result in a small, yet significant increase in cardiomyocyte apoptosis, as measured by TUNEL staining of heart sections, and HK2 overexpression attenuated this increase in cardiomyocyte death (**Figure 22e**). Altogether, these results suggest that isoproterenol infusion induced cardiac hypertrophy, but not heart failure, and that HK2 overexpression reduces the cardiomyocyte hypertrophy.

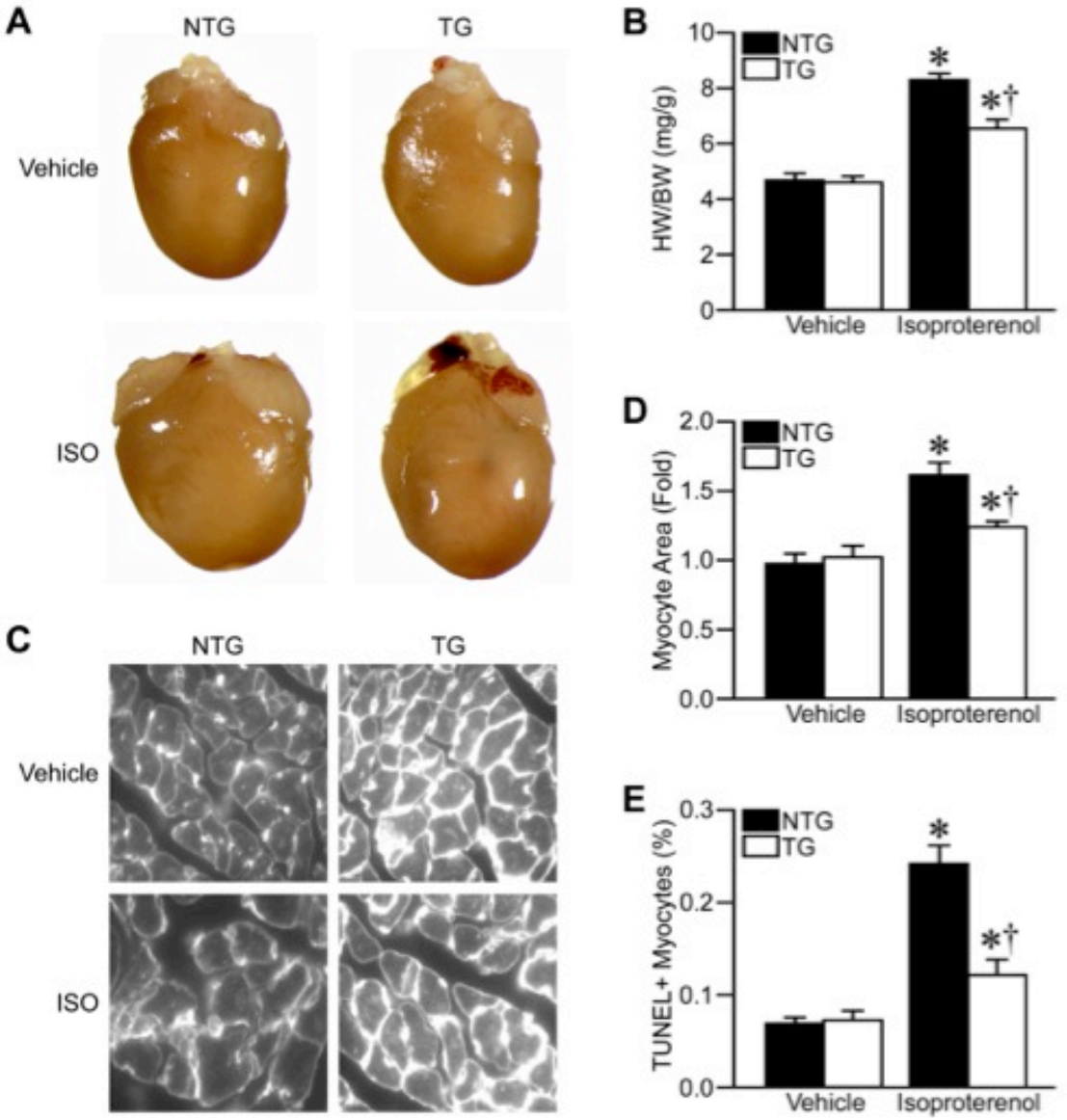

Figure 22. HK2 overexpression attenuates cardiac hypertrophy. *A,* Representative heart images from NTG and TG littermates treated with 0.9% saline (Vehicle) or Isoproterenol (ISO). *B*, Gravimetric analysis of heart weight / body weight ratio (HW/BW) for NTG and TG mice treated with Vehicle or ISO. *C*, Representative wheat-germ agglutinin (WGA) images from LV sections of NTG and TG mice treated with Vehicle or ISO. *D*, LV cardiomyocyte cross-sectional area measured from WGA images as in C. *E*, Apoptotic rate in NTG and TG mouse hearts treated with Vehicle or ISO (n= 10-12). Error bars indicate s.e.m. with **P*<0.05 versus Vehicle and † *P*<0.05 versus NTG.

Isoproterenol infusion also increased transcripts for ANP, BNP, and βMHC, common markers of the "fetal gene program" (**Figure 23**). As HK2 overexpression limited hypertrophy, it was expected that the αMHC-HK2 hearts would exhibit decreased transcripts for these markers. In fact, HK2 overexpression resulted in non-significantly increased ANP, BNP, and βMHC transcript expression (**Figure 23a-c**). However, these transcripts were also slightly elevated in vehicle treated TG mice compared to NTG littermates. Increased levels of these "hypertrophy" markers have also been shown in other models of increased glucose utilization (Young, 2007).

4.3 *Overexpression of HK2 in vitro Attenuates Cardiomyocyte Hypertrophy*

To better study the mechanisms of HK2's anti-hypertrophic effect, we wanted to recapitulate our *in vivo* findings in a cell culture system. NRVMs were infected with either control β-galactosidase adenovirus or a mouse HK2 adenovirus (**Figure 24a,b**), and treated with phenylephrine (PE) to induce hypertrophy. 25 μM PE resulted in a 37% increase in NRVM area, and HK2 overexpression reduced this hypertrophic response to only 16% (**Figure 24c,d**). To confirm that this was not specific to PE we also tested angiotensin-II (AngII). 2 μM AngII increased NRVM area by 27%, while HK2 overexpression limited the AngII-induced hypertrophy to only 10% (**Figure 24e**). These results confirm our *in vivo* findings in that HK2 overexpression attenuates cardiomyocyte hypertrophy. We also investigated the effect of reduced HK2 expression by transfecting NRVMs with a HK2 siRNA, which reduced HK2 protein expression 60%, but did not affect HK1 (**Figure 25a,b**). In agreement with our HK2 overexpression results, decreased

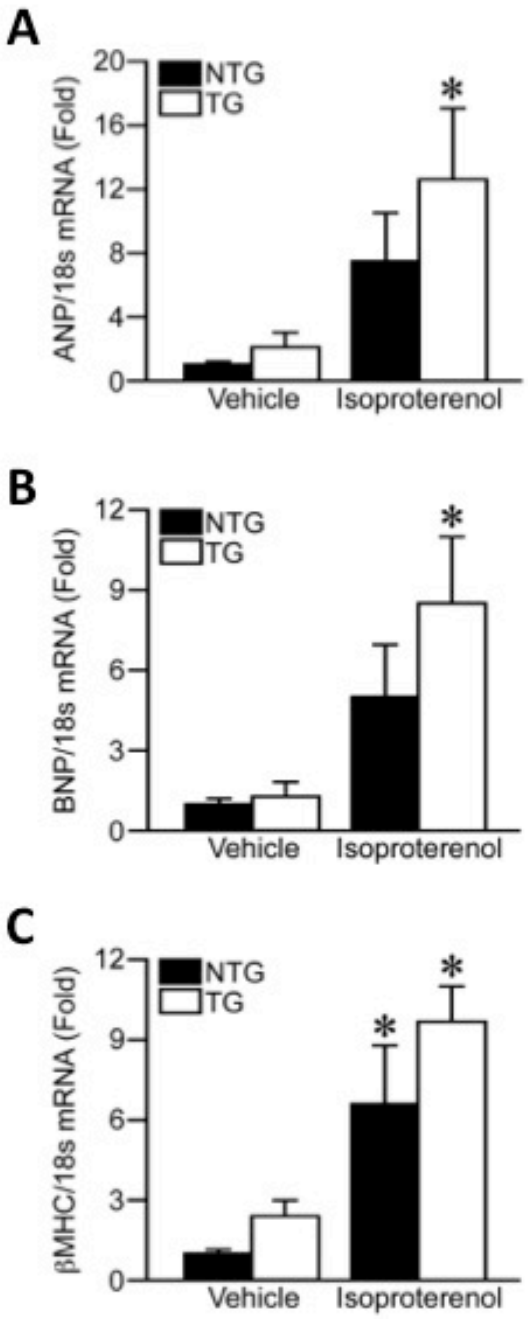

Figure 23. HK2 overexpression increases markers of the fetal metabolic program. *A-C,* mRNA expression measured by qRT-PCR for atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and b-Myosin heavy chain (bMHC) with the 18s ribosomal subunit used as loading control (n= 6). Error bars indicate s.e.m. with **P*<0.05 versus Vehicle.

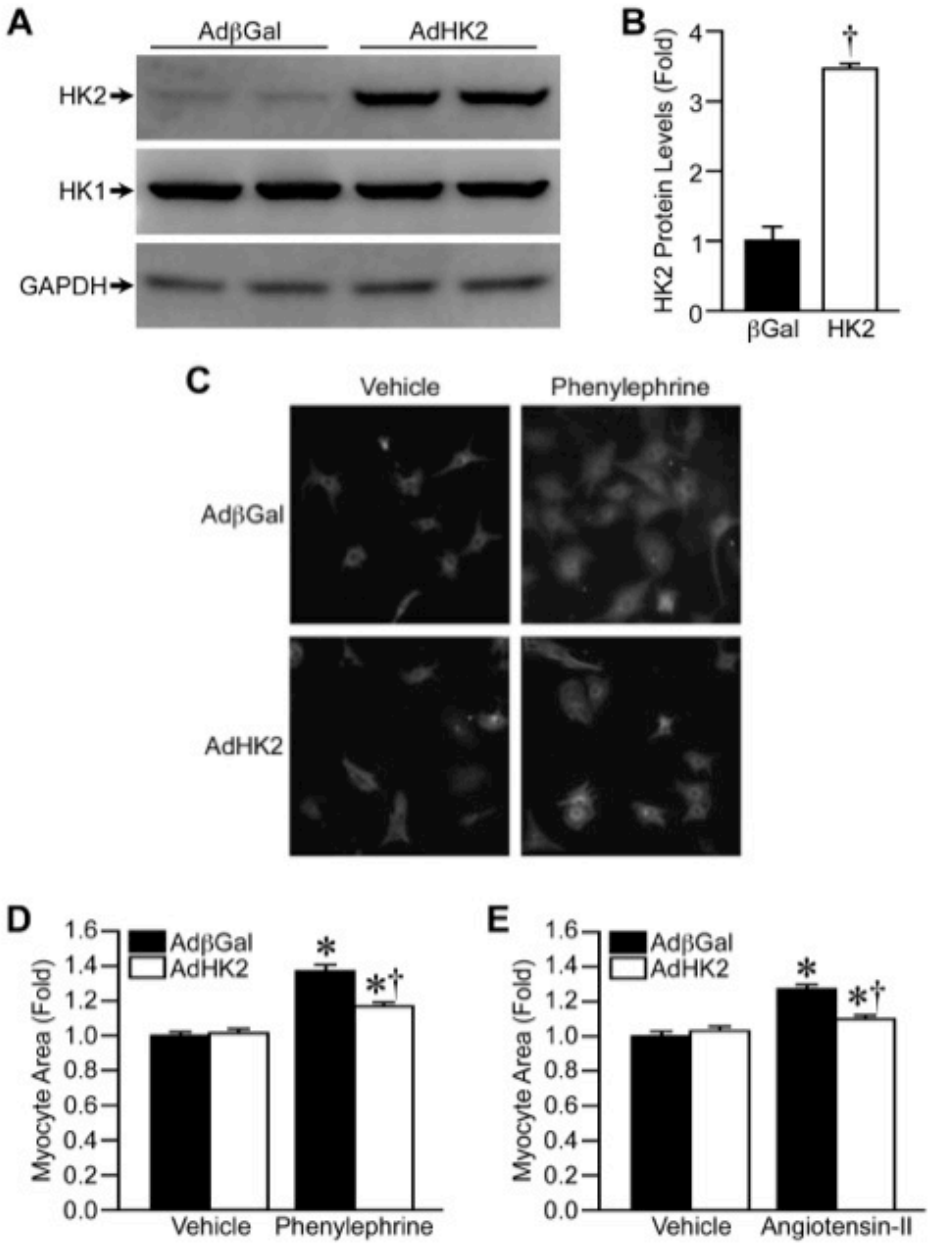

Figure 24. HK2 overexpression decreases cardiomyocyte hypertrophy *in vitro*. *A,* HK2 and HK1 protein levels in isolated neonatal rat ventricular cardiomyocytes (NRVMs) infected with adenovirus expressing either β-Galactosidase (AdβGal) or HK2 (AdHK2). *B*, Quantified HK2 protein expression from western blots as shown in A. *C*, Representative images of NRVMs infected with AdβGal or AdHK2, treated with Phenylephrine, and immunostained for tropomyosin. *D*, Mean cell area for NRVMs infected with AdβGal or AdHK2 and treated with Phenylephrine as in C. *E*, Mean cell area for NRVMs infected with AdβGal or AdHK2 and treated with Angiotensin-II (n= 4). Error bars indicate s.e.m. with **P*<0.05 versus Vehicle and † *P*<0.05 versus AdβGal.

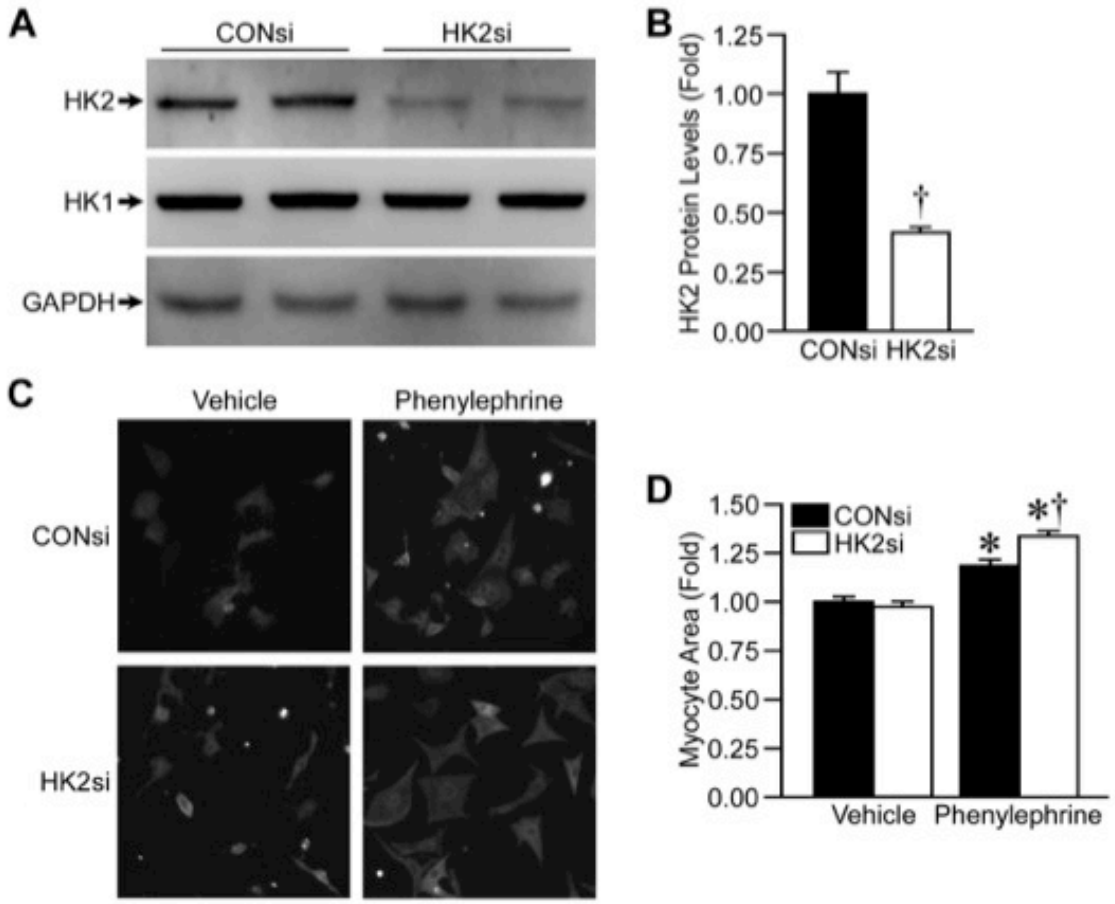

Figure 25. HK2 knockdown increases hypertrophy *in vitro*. *A,* HK2 and HK1 protein levels in NRVMs transfected with a control (CONsi) or Anti-HK2 siRNA (HK2si). *B*, Quantified HK2 expression from western blots as shown in A. *C*, Representative images of NRVMs transfected with CONsi or HK2si, treated with Phenylephrine, and immunostained for tropomyosin. *D*, Mean cell area for NRVMs transfected with CONsi or HK2si and treated with Phenylephrine as in C (n= 4). Error bars indicate s.e.m. with **P*<0.05 versus Vehicle and † *P*<0.05 versus CONsi.

HK2 expression led to significantly increased NRVM hypertrophy in response to 25 μM PE (**Figure 25c,d**). These results further indicate that HK2 expression modulates cardiomyocyte hypertrophy.

4.4 *HK2 Modulates ROS During Cardiomyocyte Hypertrophy*

As ROS levels have been shown to be elevated during hypertrophy (Sabri, 2003; Seddon, 2007; Takimoto, 2007; Wu, 2012), and HK2 is known to be protective against ROS-induced death (Da-Silva, 2004; Majewski, 2004; Mergenthaler, 2012; Miyamoto, 2008; Sun, 2008), we decided to investigate whether HK2 expression affected ROS levels during cardiomyocyte hypertrophy. PE treatment increased NRVM ROS levels by 65% as measured by DCF fluorescence (**Figure 26a**). HK2 overexpression reduced the ROS accumulation during hypertrophy, to an increase of only 24% (**Figure 26a**). HK2 depletion by siRNA exacerbated the accumulation of ROS during PE-induced hypertrophy (**Figure 26b**). This modulation of ROS levels during hypertrophy led us to question whether HK2 could decrease generalized oxidative stress; therefore we measured ROS levels in NRVMs overexpressing HK2 after treatment with 50 μM H_2O_2. ROS levels were increased nearly 3-fold with H_2O_2 treatment, and HK2 overexpression significantly reduced this ROS accumulation (**Figure 26c**). HK2 overexpression also significantly reduced H_2O_2-induced NRVM death (**Figure 26d**). These results suggest that HK2 expression mediates ROS levels during cardiomyocyte hypertrophy and can protect against ROS-induced cardiomyocyte death.

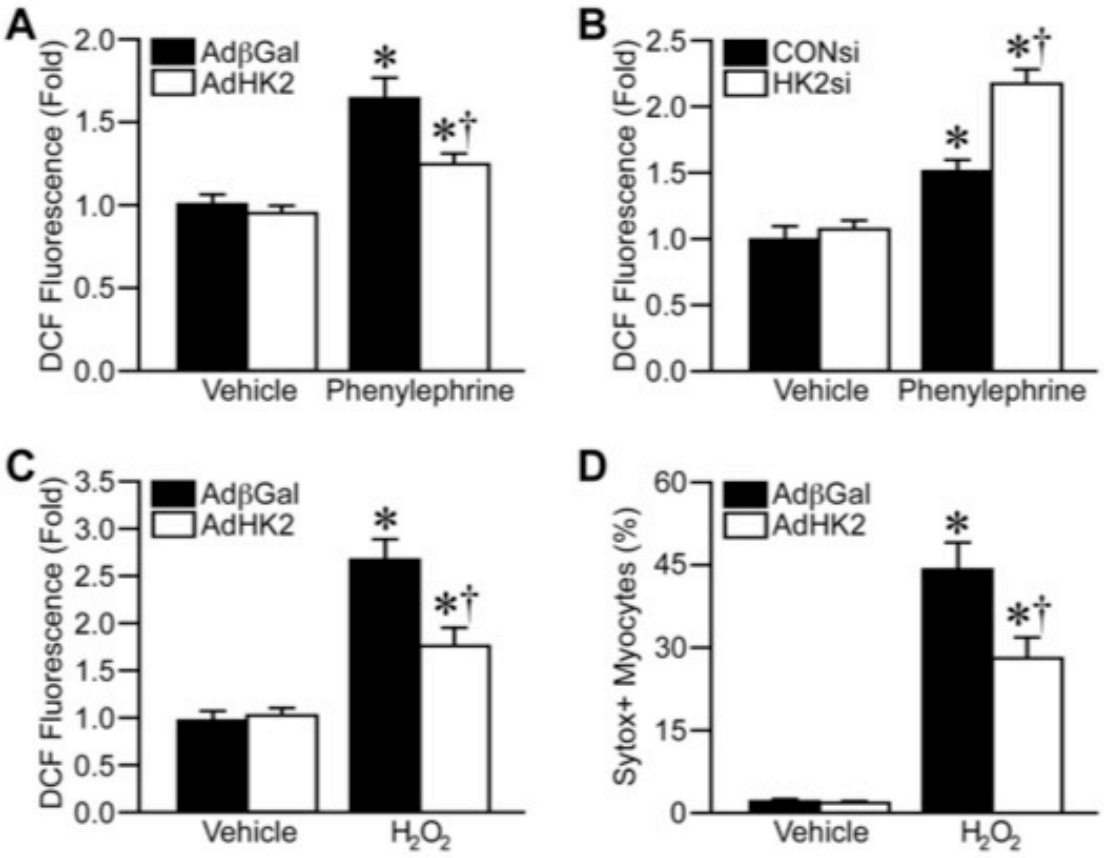

Figure 26. HK2 modulates reactive oxygen species (ROS) levels during hypertrophy. *A,* Mean ROS levels as measured by 2',7'-di-chlorofluorescein (DCF) fluorescence in NRVMs infected with AdβGal or AdHK2 and treated with Phenylephrine. *B*, Mean ROS levels as measured by DCF fluorescence in NRVMs transfected with CONsi or HK2si and treated with Phenylephrine. *C*, Mean ROS levels as measured by DCF fluorescence in NRVMs infected with AdβGal or AdHK2 and treated with 50 μM H_2O_2. *D*, Mean NRVM cell death measured by Sytox Green in NRVMs infected with AdβGal or AdHK2 and treated with 50 μM H_2O_2 (n= 5). Error bars indicate s.e.m. with *P<0.05 versus Vehicle and † P<0.05 versus AdβGal or CONsi.

4.5 *HK2 Modulates ROS via the Pentose Phosphate Pathway*

We lastly wanted to determine how HK2 controlled ROS levels during cardiomyocyte hypertrophy. One possible connection between glucose utilization and ROS is the pentose phosphate pathway. The initial step in this pathway is the conversion of glucose-6-phosphate into 6-phosphogluconolactone by G6PDH, which involves the reduction of $NADP^+$ to NADPH. NADPH can then be utilized to reduce glutathione and control ROS levels (Robey, 2006). We therefore decided to test whether HK2 overexpression was controlling ROS levels by increasing flux through the pentose phosphate pathway. We treated NRVMs with dehydroepiandrosterone (DHEA), an inhibitor of G6PDH (Tian, 1998) to decrease pentose phosphate pathway flux. 25 μM DHEA treatment significantly decreased G6PDH activity by 55% (**Figure 27a**). HK2 overexpression resulted in significantly increased G6PDH activity (27% increase); however, DHEA maintained the ability to inhibit G6PDH activity despite HK2 overexpression (**Figure 27a**). Similar as to shown before, HK2 overexpression decreased ROS accumulation during PE-induced hypertrophy in vehicle treated NRVMs; however, DHEA treatment abolished HK2's ability to decrease ROS accumulation (**Figure 27b**). DHEA treatment also blocked HK2's ability to attenuate cardiomyocyte hypertrophy (**Figure 27c**). Together, these results suggest that HK2 overexpression attenuates ROS levels and hypertrophy by increasing glucose flux through the pentose phosphate pathway.

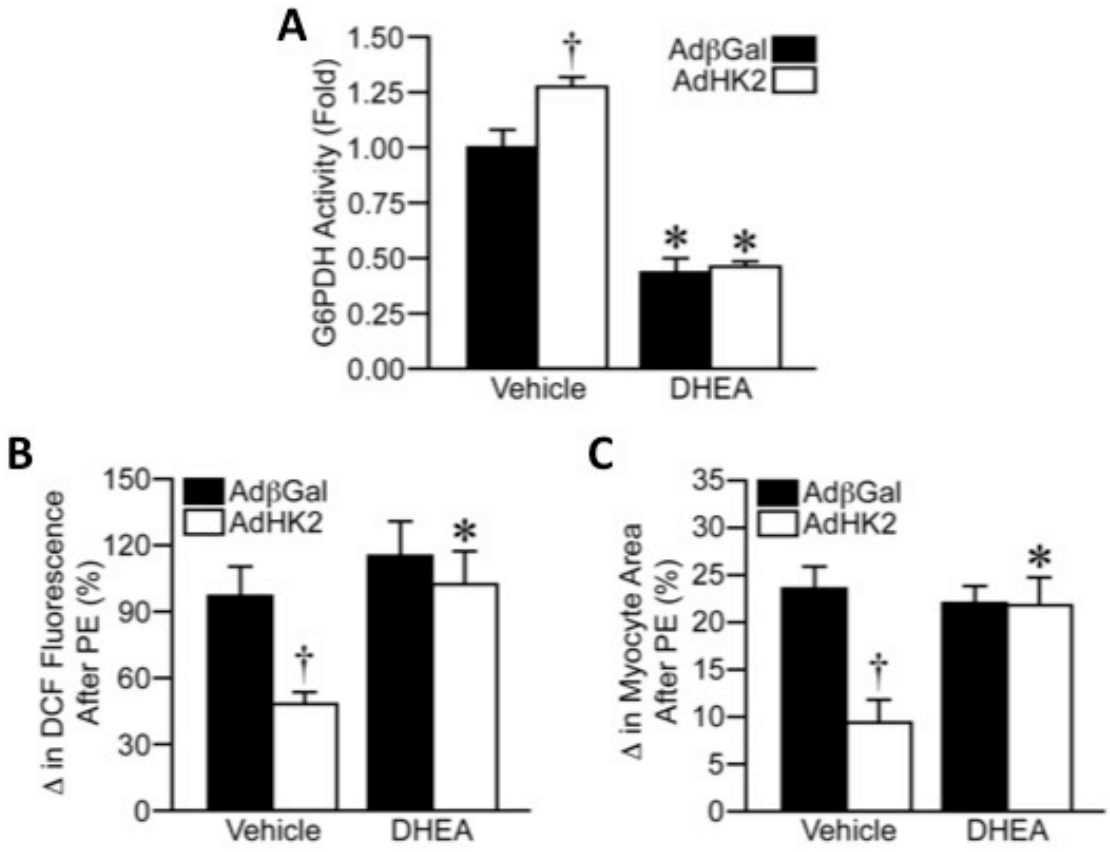

Figure 27. HK2 modulates ROS levels during hypertrophy by increasing pentose phosphate pathway flux. *A,* Glucose-6-phosphate dehydrogenase (G6PDH) enzymatic activity in NRVMs infected with AdβGal or AdHK2 and treated with either Vehicle or the G6PDH inhibitor dehydroepiandrosterone (DHEA). *B*, Mean phenylephrine (PE)-induced increase in ROS levels in NRVMs infected with AdβGal or AdHK2 and treated with Vehicle or DHEA. *C*, Mean PE-induced increase in NRVM area in NRVMs infected with AdβGal or AdHK2 and treated with Vehicle or DHEA (n= 4). Error bars indicate s.e.m. with *P<0.05 versus Vehicle and † P<0.05 versus AdβGal.

5. DISCUSSION

Cardiac hypertrophy is characterized by a transition into the "fetal metabolic program", involving decreased fatty acid metabolism and increased glucose utilization. Recent evidence from genetic models suggests that altered fatty acid metabolism is the driving force of pathologic hypertrophy, and increased glucose utilization may be a beneficial compensation. We therefore hypothesized that a mouse model of increased cardiac HK2 expression would result in a decrease in hypertrophy in response to chronic β-adrenergic agonism. Indeed, αMHC-HK2 mice displayed decreased cardiac and cardiomyocyte hypertrophy in response to isoproterenol. HK2 overexpression also attenuated the hypertrophic response *in vitro*, in NRVMs treated with PE or AngII. Oppositely, HK2 depletion by treatment of NRVMs with a HK2 siRNA exacerbated PE-induced hypertrophy. These results are fully in agreement with most other studies investigating cardiac hypertrophy in models of altered glucose utilization. Cardiac-specific deletion of the GLUT4 transporter was shown to induce cardiac hypertrophy at basal conditions (Abel, 1999). Antithetically, cardiac-specific GLUT1 overexpression was shown to decrease hypertrophy, attenuate the transition to heart failure, and increase survival in response to pressure overload (Liao, 2002). Cardiac-specific PPARα overexpression has also been shown to increase fatty acid metabolism, decrease glucose utilization, and results in cardiac hypertrophy at basal conditions (Finck, 2002). Most recently, HK2 heterozygote mice were shown to have increased hypertrophy and exacerbated heart failure in response to pressure overload (Wu, 2012).

ROS levels have been shown to be elevated during hypertrophy (Sabri, 2003; Seddon, 2007; Takimoto, 2007; Wu, 2012), and HK2 is known to be protective against

ROS-induced death (Da-Silva, 2004; Mergenthaler, 2012; Miyamoto, 2008; Sun, 2008). Moreover, the increased hypertrophy observed with decreased HK2 expression was suggested to be due to increased ROS accumulation due to increased mitochondrial permeability transition (Wu, 2012). Consistent with these data we found that treatment of NRVMs with PE resulted in increased ROS accumulation, and HK2 overexpression attenuated this increase in ROS levels. Conversely, HK2 knockdown further increased the ROS accumulation during PE-induced hypertrophy. However, isolated mitochondria from αMHC-HK2 hearts showed no difference in mitochondrial swelling experiments, suggesting that inhibition of permeability transition may not play a role in HK2's anti-oxidant and anti-hypertrophic effects. We cannot fully rule out a mitochondrial mechanism, as there is a small but significant increase in HK2 mitochondrial expression in our overexpression model. Nevertheless, the much greater increase in HK2 expression in the cytosolic fraction in our model leads one to surmise that any phenotype is likely due to a more cytosolic or glucose utilization mechanism, rather than a mitochondrial mechanism. Regardless, these other studies and the present one strongly suggest that increased glucose utilization can attenuate cardiac hypertrophy.

We investigated how HK2 reduced ROS levels, and tested the theory that increased HK2 expression could increase pentose phosphate pathway flux, leading to reduced ROS. The pentose phosphate pathway is important for the formation of NADPH reducing equivalents that are necessary for glutathione peroxidase activity and production of reduced glutathione (Robey, 2006). Thus, the pentose phosphate pathway is vitally important in controlling oxidative stress. Glucose-6-phosphate, the product of the reaction catalyzed by HK2, is the substrate for the initial reaction of the pentose

phosphate pathway performed by G6PDH. Increased G6PDH activity (Chess, 2009; Gupte, 2006), and increased pentose phosphate pathway flux (Kato, 2010; Meerson, 1967; Zimmer, 1980) have been observed during cardiac hypertrophy. Genetic models of altered pentose phosphate pathway activity also suggest a prominent connection between the pathway and hypertrophy. Mice that are deficient in G6PDH develop hypertrophy ~9 months of age (Jain, 2003), and display increased hypertrophy after myocardial infarction or pressure overload (Hecker, 2013). In the present study, DHEA treatment was utilized to decrease G6PDH activity by ~55%. This level of inhibition did not result in significantly increased ROS accumulation or cardiomyocyte hypertrophy. However, inhibition of G6PDH did abolish the beneficial effects of HK2 overexpression against α-adrenergic stimulation-induced ROS production and cardiomyocyte hypertrophy. Interestingly, our ~3-fold HK2 overexpression increased G6PDH activity ~30%, which fits nicely with the estimation that 10-20% of glucose oxidation occurs through the pentose phosphate pathway (Wamelink, 2008). These results thus indicate that increased pentose phosphate pathway flux is an important component of HK2's anti-hypertrophic effect.

Another possible fate of glucose-6-phosphate is the formation of *O*-linked β-N-acetylglucosamine residues, also known as protein GlcNAcylation, by the hexosamine biosynthetic pathway. Flux through this pathway also appears to be increased during cardiac hypertrophy as GlcNAc levels are increased in hypertrophied hearts (Watson, 2010; Young, 2007). Hypertrophy also increases the expression of *O*-linked β-N-acetylglucosamine transferase, the enzyme responsible for adding GlcNAc residues to proteins; and deletion of this enzyme increases cardiomyocyte hypertrophy, apoptosis,

and dysfunction (Watson, 2010). We did not thoroughly investigate the hexosamine biosynthetic pathway in this study; however, the wheat germ agglutinin used in this study is a marker of N-acetylglucosamine residues on cell plasma membranes. Analysis of the fluorescence intensity of these images does suggest that both HK2 overexpression and hypertrophy increase N-acetylglucosamine levels (data not shown). Therefore, increased protein *O*-GlcNAcylation could potentially be another mechanism decreasing both cardiomyocyte hypertrophy and apoptosis in our HK2 overexpression model. Further study is warranted to thoroughly investigate the hexosamine biosynthetic pathway with altered HK2 expression.

To conclude, our results show that HK2 overexpression attenuates cardiomyocyte hypertrophy and death in response to adrenergic stimulation. Our *in vitro* studies suggest that HK2 modulates hypertrophy by controlling cellular ROS levels by increasing pentose phosphate pathway flux. Thus, therapeutic techniques to increase HK2 expression or pentose phosphate pathway flux may assist in the treatment of pathologic cardiac hypertrophy.

CHAPTER 4: Summary And Future Directions

Hexokinases, including Hexokinase-2 (HK2), are well recognized for their importance as the first enzymatic step in the glycolysis cascade. The findings that HK1 and HK2 can be localized to the outer mitochondrial membrane (Linden, 1982; Nakashima, 1986; Sui, 1997), and that HK2 overexpression is integral to cancer cell growth and survival (Bustamante, 1977; Bustamante, 1981; Parry, 1983) opened up a whole new set of doors for studying the role of HK2 in cell survival. Perhaps most convincing thus far is that HK2 somehow has the capacity to act as an antioxidant, and increases cell survival in the face of oxidative stress (Da-Silva, 2004; Sun, 2008). In this regard, the findings of the present studies support a role for HK2 in attenuating oxidative stress during several cardiac disease models. We show that 1) HK2 overexpression protects against reactive oxygen species (ROS)-induced cell death by binding to VDAC3 on the outer mitochondrial membrane, 2) HK2 overexpression attenuates cardiac hypertrophy by reducing cellular ROS levels, and 3) HK2 modulates ROS during hypertrophy by increasing flux through the pentose phosphate pathway.

In **AIM 1**, we addressed the role of mitochondrial binding of HK2 during protection against ROS-induced cell death. While many studies have demonstrated that HK2 binds to VDACs on the mitochondria (Anflous-Pharayra, 2007; Linden, 1982; Nakashima, 1986), and that mitochondrial HK2 localization is important or even necessary for protection against cell death (Miyamoto, 2008; Sun, 2008), we provide the first evidence that a VDAC isoform, specifically VDAC3, is required for this HK2-mediated protection against cell death. While the true mechanism is still unknown, this

study helps to clarify how mitochondrial localization of HK2 can protect cells from ROS-induced death.

After demonstrating the importance of HK2 overexpression *in vitro*, we began to translate our research to an *in vivo* model of cardiac disease. In **AIM 2**, we uncovered a role for HK2 in the modulation of ROS levels during cardiac hypertrophy. Interestingly, under these circumstances, the effects from HK2 overexpression appeared to be unrelated to mitochondrial localization. Instead, HK2 overexpression in the heart emerged as a mechanism to increase glucose utilization, including increasing flux through the pentose phosphate pathway, which was responsible for modulating ROS levels during hypertrophy.

Collectively, our data demonstrate that overexpression of HK2 is a potent mechanism capable of reducing cellular ROS levels to both alter cell size, as well as increase cell viability. In addition, these findings suggest that HK2 decreases oxidative stress by at least two different mechanisms: 1) binding to VDAC3 on the mitochondrial outer membrane, and 2) increasing glucose flux through the pentose phosphate pathway and potentially other glucose utilization pathways.

1. Discussion of initial hypotheses

1.1 AIM 1 hypotheses

Due to a previous report in muscles of VDAC null mice (Anflous-Pharayra, 2007), in **AIM 1** we hypothesized that HK2 would bind mostly to VDAC1 in our murine embryonic fibroblast (MEF) cell cultures, and therefore, deletion of VDAC1 would abolish HK2's ability to protect against cell death. The first half of our hypothesis was

confirmed as western blotting and immunohistochemistry determined that HK2 was bound primarily to VDAC1, only slightly to VDAC3, and apparently not at all to VDAC2. Contrary to the second half of our hypothesis however, deletion of VDAC1 had no effect on HK2's ability to protect against H_2O_2-induced death. Instead, the VDAC3 isoform that HK2 bound much less significantly was found to be necessary for HK2-mediated protection against death. In this study we were also initially surprised by the fact that depleting VDAC2 expression also abolished HK2-mediated protection in our primary MEF cell cultures even though HK2 appeared to not bind VDAC2. However, we were able to deduce that depleting VDAC2 levels did not have a direct effect on HK2, but rather upregulated the proapoptotic Bcl2 proteins Bax and Bak, which masked the pro-survival affects of HK2 overexpression. Depletion of VDAC2 in Bax/Bak double-null cells then was shown to not affect HK2-mediated protection.

Explaining our incorrect hypothesis and how HK2-VDAC3 binding protects against cell death is challenging, as distinct roles for individual VDAC isoforms are largely unknown. From these results, our current working hypothesis is that HK2 binds mostly to VDAC1 to regulate glucose metabolism (Anflous-Pharayra, 2007), but also binds to VDAC3 to protect against cell death.

1.2 AIM 2 hypotheses

In preparation for **AIM 2**, we poured over a substantial amount of evidence and concluded that pathologic hypertrophy appears to be driven by aberrant fatty acid metabolism, and altering glucose metabolism could be a beneficial adaptation during cardiac hypertrophy. We thus hypothesized that increased glucose phosphorylation due to

HK2 overexpression would attenuate cardiac hypertrophy. Our hypothesis was confirmed as cardiac-specific HK2 overexpression significantly decreased the hypertrophic response to isoproterenol infusion. HK2 overexpression in NRVM cell cultures also attenuated hypertrophy in response to phenylephrine and angiotensin-II treatment. A recent report investigating hypertrophy in HK2 heterozygote mice found the exact opposite results of our findings (Wu, 2012), as would be expected for a model opposite of our own (decreased HK2 expression). Their results suggested that decreased HK2 expression caused "low levels" of mitochondrial permeability transition, which increased cellular ROS levels and exacerbated hypertrophy. Interestingly, our results also determined altered ROS accumulation during hypertrophy, but in our model, HK2 expression was more significantly altered in the cytosol than on the mitochondria. Additionally, we found permeability transition to be unaltered in isolated mitochondria from our αMHC-HK2 hearts. This led us to hypothesize that the HK2-associated phenotype was due to altered glucose metabolism and not to mitochondrial binding. Indeed, HK2 overexpression was determined to increase glucose-6-phosphate dehydrogenase activity, and therefore, increase flux through the pentose phosphate pathway. As this "accessory" glucose utilization pathway is important for maintaining cellular ROS levels (Stanton, 2012), we hypothesized that inhibition of the pathway would abrogate the beneficial effects of HK2 overexpression. Indeed, inhibition of G6PDH with dehydroepiandrosterone (DHEA) fully removed HK2's ability to decrease ROS as well as maintain cardiomyocyte size during hypertrophy. Therefore, our main hypothesis for AIM 2 was confirmed in that increased HK2 expression attenuated cardiac hypertrophy. The mechanism of how HK2 overexpression decreased hypertrophy however was both novel and initially unforeseen.

2. ***Why HK2 overexpression?***

One potential observation of these studies is that focus is given largely to HK2 overexpression and not to decreased expression of HK2. We opted to study the effects of HK2 overexpression, as this would potentially model the mechanisms of how tumor cells metabolize and defend against cell death (Pedersen, 2007). This was chosen because in many cases, what works for a tumor, works for the heart; and many anti-tumor therapies having cardiotoxic side effects provide further evidence for this paradigm (Lal, 2013). Additionally, for the hypertrophy studies, the bulk of existing evidence indicates that increasing glucose utilization attenuates hypertrophy (Abel, 1999; Liao, 2002), and we therefore hypothesized that increasing glucose phosphorylation would decrease hypertrophy.

There may be several diseases that could be modeled by decreased HK2 expression. In particular, diabetes mellitus has been shown to decrease cardiac HK2 expression (Da Silva, 2012; Xue, 2010; Xue, 2012), just one of many mechanisms to decrease glucose metabolism during diabetes. However, models of decreased HK2 expression have significant limitations (**see section 2.2 below**). Conversely, determining whether HK2 overexpression can protect against diabetic cardiomyopathy also remains to be thoroughly investigated.

2.1 Limitations of HK2 overexpression as a model

Likely the largest limitation of a HK2 overexpression model is that HK2 can be overexpressed in the cytosol, the mitochondria, or both. Overexpression of the full-length

cDNA of HK2 as in these studies resulted in significantly greater cytosolic HK2 overexpression with only slightly increased mitochondrial HK2 expression. This is believed to be due to a limiting number of mitochondrial HK2 binding sites (presumably mostly, if not all VDACs). Regardless, this makes data interpretation more difficult as results cannot be fully attributed to only a cytosolic or only a mitochondrial mechanism. Overexpression of truncated HK2 proteins expressing only the catalytic portions of the enzyme and not the mitochondrial binding regions or vice versa would be one way to remove this limitation.

2.2 Limitations of HK2 knockdown as a model

Reducing HK2 expression would have the same limitation as HK2 overexpression in that both cytosolic and mitochondrial HK2 expression would be reduced. Similar studies have been performed in HK2 heterozygote mice, and indeed, both HK2 pools show decreased expression (Wu, 2011). Therefore, results are hard to interpret as due to solely a cytosolic versus mitochondrial mechanism. Another major limitation of decreasing HK2 expression is that decreasing mitochondrial HK2 leaves VDAC channels unbound. This could have major consequences, as HK2 binding promotes the open conformation of VDACs, and supports ATP and ADP flux into and out of the mitochondria (**see Appendix**). Thus, decreasing HK2 expression may result in closed VDAC channels and inhibition of mitochondrial metabolism, which would play a large role in the negative phenotypes associated with HK2 knockdown. Therefore, it would be difficult to determine effects of HK2 knockdown that were not simply due to closed VDAC channels and altered mitochondrial function.

3. ***Use of H_2O_2 as a cell death stimulus in vitro***

While not extremely physiological, the use of acute H_2O_2 treatment to induce death in cell viability studies has been adopted by many investigators in the field (Kroemer, 2009). Treatment with H_2O_2 mimics the underlying mechanism of the necrotic program, which is to induce oxidative stress. It is therefore no surprise that treatment with H_2O_2 induces mainly necrotic cell death, and not apoptosis. The breakdown of cell membranes during necrosis, but not apoptosis, allows for the use of exclusion dyes such as the Sytox green used in these studies to label the dead cells (**see Figure 4**). Some investigators have transitioned to more physiological death stimuli in cell culture studies, such as hypoxia-reoxygenation. While this more closely mimics the pathology of cardiac ischemia-reperfusion injury, results should be scrutinized as hypoxia inducible factor (HIF) is known to have hundreds of target genes, and would be upregulated during hypoxia. We therefore chose H_2O_2 treatment as our death stimulus to avoid confounding our data interpretation by inducing a HIF response.

4. ***Use of isoproterenol infusion to induce cardiac hypertrophy***

Chronic β-adrenergic stimulation with isoproterenol is widely used as a model of cardiac hypertrophy (Galindo, 2009). We adopted this model mostly for its ease of implementation. Unfortunately, this model did not induce a transition from hypertrophy to heart failure, and therefore we were unable to investigate the affects of HK2 overexpression on cardiac function or survival from heart failure. Other models, such as aortic constriction may have allowed us to perform these studies.

5. ***Translation of findings to human disease***

Of utmost importance for all physiological research is that the results should be translatable to human disease. In general, our findings do offer insight into potential mechanisms occurring during human pathologies. In **AIM 1**, we focused on HK2 mitochondrial binding and protection against cell death. The ideas for this study derive their roots strait from human disease, as it has long been established that cancer cells metabolize a large amount of glucose, and are not easily killed (Pedersen, 2007; Warburg, 1927). The finding that overexpression and mitochondrial localization is ultimately responsible for this increased glucose metabolism in cancer cells (Bustamante, 1977; Bustamante, 1981; Parry, 1983) was the motivating factor of this study. Additionally, targeting HK2 in cancers could potentially affect both major cancer phenotypes: increased glucose utilization and resistance to death. Therefore, it is critically important to better understand how HK2 binds to mitochondria, increases glucose utilization, and protects against cell death.

Additionally, mitochondrial expression and activity of HK2 is so vitally important to disease that it is the basis for positron emission tomography (PET) imaging utilizing ^{18}F-deoxyglucose (FDG). This technique relies on hexokinase to phosphorylate FDG into FDG-6-phosphate, which is subsequently trapped in the cell and can be detected by PET imaging. Just five years after the initial finding that mitochondrial HK2 is critical for the increased glucose utilization in cancers (Bustamante, 1977), FDG-PET was utilized to detect brain tumors (Di Chiro, 1982). This technique remains the gold standard for detection and treatment monitoring of tumors. Similar PET tracers are used in more

advanced imaging centers to investigate glucose metabolism in other pathologies such as cardiomyopathies (Dávila-Román, 2002).

In **AIM 2**, we investigated the effects of HK2 overexpression on cardiac hypertrophy in a murine model. In the normal human heart, oxidation of fatty acids contributes to the majority of ATP generation (Neely, 1972). Mouse cardiac metabolism does not perfectly resemble human cardiac metabolism, as mice are more reliant on glucose and lactate metabolism and less on fatty acids (Stowe, 2006). However, increased glucose utilization during hypertrophy is characteristic of both animal models (Allard, 1994; Nascimben, 2004) and human pathology (Dávila-Román, 2002). Therefore, results from this study should be readily applicable to human cardiac pathology.

6. *Summary*

Our present studies have demonstrated that HK2 overexpression is profoundly protective against both cell death and cardiac hypertrophy. Given that HK2 is bound to the mitochondria, mostly to VDAC1, we hypothesized that VDAC1 would be necessary for HK2 to protect against cell death. To our surprise, VDAC1 was not necessary for HK2-mediated protection, while VDAC3 binding was required. These findings demonstrate that HK2 mitochondrial localization is necessary for HK2-mediated protection against ROS-induced cell death.

In addition, we also investigated the affects of HK2 overexpression in cardiac hypertrophy. HK2 overexpression attenuated cardiac hypertrophy, and this affect also appeared to be due to HK2's ability to modulate oxidative stress. Interestingly, in the setting of hypertrophy, HK2's affects did not appear to be mediated by mitochondrial

binding. Instead, it was determined that HK2 overexpression modulated ROS levels through the pentose phosphate pathway, an accessory glucose utilization pathway, during cardiac hypertrophy. Therefore, HK2 overexpression can potentially decrease oxidative stress in several different ways depending on the underlying stress. These findings therefore identify HK2 as an important candidate for the modulation of ROS, and also raise the possibility that HK2 and/or increased glucose metabolism may be a therapeutic target for cardiac pathologies involving hypertrophy and cardiomyocyte death.

7. *Potential future experiments*

These studies have clearly demonstrated an important role for HK2 in attenuating cardiomyocyte death and hypertrophy, and as such, have paved the way for additional questions to be addressed. For example, in **AIM 1**, HK2-VDAC3 binding was shown to be necessary for HK2 to protect against H_2O_2-induced death. But how this HK2-VDAC3 interaction attenuates oxidative stress remains a mystery. To address this question, one logical step would be to pull-down or immunoprecipitate HK2-VDAC3 complexes and determine if any other binding partners exist. Indeed, several other proteins have recently been shown to bind HK2 at the mitochondria and assist HK2 in protecting against ROS (Cheung, 2012; Mailloux, 2011; Mergenthaler, 2012; Tchakarska, 2011). However, it is unknown if these proteins are also complexed with VDAC3. If they were found to be complexed with VDAC3 as well, loss and/or gain of function experiments for these proteins would likely provide fruitful information on how the HK2-VDAC3 complex protects against H_2O_2-induced death. Analyzing the expression and function of these proteins in the VDAC3 null cells and animals would also be warranted.

Furthermore, given that both HK1 and HK2 bind to mitochondria, and are both well expressed in the heart, one major question that arises is whether or not HK1 can perform all the functions of HK2. We have constructed an HK1 adenovirus, and it was shown to be protective against H_2O_2-induced death in MEFs and NRVMs, as well as decrease cardiomyocyte hypertrophy *in vitro* (data not shown). However, the binding characteristics between the three VDAC isoforms and HK1 were not investigated. Also, the ability of HK1 to decrease cardiomyocyte hypertrophy or death *in vivo* remains to be explored.

In **AIM 2**, HK2 overexpression was shown to decrease cardiomyocyte hypertrophy induced by adrenergic stimulation. Unfortunately, this technique did not test whether HK2 overexpression could prevent the transition to heart failure. A more severe hypertrophic stimulus such as pressure overload by aortic constriction would be necessary to address this question. This would also allow for the investigation of HK2's effects on cardiac function during heart failure.

Additionally, while HK2 overexpression was shown to protect against H_2O_2-induced death in cell cultures and low levels of Isoproterenol-induced death *in vivo*, further studies are required to determine whether HK2 can protect cardiomyocytes against more substantial death stimuli *in vivo*. Causing a transition to heart failure with aortic constriction would likely induce more significant cardiomyocyte death then our model of isoproterenol infusion. Investigating whether HK2 overexpression protects cardiomyocytes during ischemia-reperfusion (I/R) would be the most definitive answer to this question. If HK2 was found to be protective against I/R injury, repeating the study in HK2 overexpressing / VDAC3$^{-/-}$ animals would be interesting in attempt to translate the

in vitro findings from **AIM 1** to an *in vivo* animal model. Before the pursuit of more severe surgical interventions such as aortic banding or I/R injury, it is highly suggested that our FVBN αMHC-HK2 line be backcrossed to a C57/black-6 strain, as FVBN lines have been shown to be inherently cardioprotected (Guo, 2012).

In addition, since HK2 overexpression was shown to alter glucose utilization during hypertrophy in **AIM 2**, it would be interesting to investigate HK2 overexpression in diabetic cardiomyopathy, a directly metabolic disease. Studies could be performed to investigate whether HK2 overexpression increases cardiac glucose uptake and utilization during diabetes. Moreover, diabetic cardiomyopathy is associated with increased cardiomyocyte death, and it could be investigated whether HK2 overexpression normalizes the death response of cardiomyocytes in diabetic hearts towards normal levels. As HK2 overexpression can protect normal cardiomyocytes, we would expect it could also protect cardiomyocytes from diabetic hearts, unless any required metabolic pathway(s) downstream of HK2 are too badly compromised in the diabetic state.

Lastly, the effects that HK2 overexpression has on all of the "accessory" glucose utilization pathways should be more thoroughly investigated. We provide evidence in **AIM 2** that HK2 overexpression increases pentose phosphate pathway flux during hypertrophy. While whole body HK2 transgenic mice were not shown to have increased muscle glycogen levels during sedentary or exercised conditions (Fueger, 2004), different disease states could allow for HK2 overexpression to affect glycogen formation. Additionally, HK2 could be increasing flux through the hexosamine biosynthetic pathway. This is particularly interesting, as both HK2 overexpression and the hexosamine pathway have been shown to decrease cardiomyocyte hypertrophy and death (Watson,

2010). And lastly, HK2's affect on the aldose-reductase pathway should also be investigated. Since the aldose-reductase pathway uses glucose (not HK-formed glucose-6-phosphate like all other glucose utilization pathways), HK2 overexpression could potentially steal glucose from the aldose-reductase pathway. If true, this would likely be beneficial as the aldose-reductase pathway and sorbitol formation is generally associated with disease complications such as diabetic retinopathy (Gabbay, 1966). Combining a model of HK2 overexpression with either pharmacological or genetic inhibition of integral parts of these glucose utilization pathways would help address these questions.

REFERENCES

1. **Abel ED, Kaulbach HC, Tian R, Hopkins JC, Duffy J, Doetschman T, Minnemann T, Boers ME, Hadro E, Oberste-Berghaus C, Quist W, Lowell BB, Ingwall JS, Kahn BB.** Cardiac hypertrophy with preserved contractile function after selective deletion of GLUT4 from the heart. *J Clin Invest* 104:1703-1714, 1999.

2. **Aitman TJ, Glazier AM, Wallace CA, Cooper LD, Norsworthy PJ, Wahid FN, Al-Majali KM, Trembling PM, Mann CJ, Shoulders CC, Graf D, St Lezin E, Kurtz TW, Kren V, Pravenec M, Ibrahimi A, Abumrad NA, Stanton LW, Scott J.** Identification of Cd36 (Fat) as an insulin-resistance gene causing defective fatty acid and glucose metabolism in hypertensive rats. *Nat Genet* 21:76-83, 1999.

3. **Alano CC, Ying W, Swanson RA.** Poly(ADP-ribose) polymerase-1-mediated cell death in astrocytes requires NAD+ depletion and mitochondrial permeability transition. *J Biol Chem* 279:18895-18902, 2004.

4. **Allard MF, Schonekess BO, Henning SL, English DR, Lopaschuk GD.** Contribution of oxidative metabolism and glycolysis to ATP production in hypertrophied hearts. *Am J Physiol Heart Circ Physiol* 267:H742-H750, 1994.

5. **Allard MF, Henning SL, Walbolt RB, Granleese SR, English DR, Lopaschuk GD.** Glycogen metabolism in the aerobic hypertrophied rat heart. *Circulation* 96:676-682, 1997.

6. **Anflous-Pharayra K, Zong-Jin C, Craigen WJ.** VDAC1 serves as a mitochondrial binding site for hexokinase in oxidative muscles. *Biochim Biophys Acta* 1767:136-142, 2007.

7. **Apetoh L, Ghiringelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, Mignot G, Maiuri MC, Ullrich E, Saulnier P, Yang H, Amigorena S, Ryffel B, Barrat FJ, Saftig P, Levi F, Lidereau R, Nogues C, Mira JP, Chompret A, Joulin V, Clavel-Chapelon F, Bourhis J, André F, Delaloge S, Tursz T, Kroemer G, Zitvogel L.** Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat Med* 13:1050-1059, 2007.

8. **Arany Z, Novikov M, Chin S, Ma Y, Rosenzweig A, Spiegelman BM.** Transverse aortic constriction leads to accelerated heart failure in mice lacking PPAR-gamma coactivator 1alpha. *Proc Natl Acad Sci USA* 103:10086-10091, 2006.

9. **Arora KK, Pedersen PL.** Functional significance of mitochondrial bound hexokinase in tumor cell metabolism. Evidence for preferential phosphorylation of glucose by intramitochondrially generated ATP. *J Biol Chem* 263:17422-17428, 1988.

10. **Azoulay-Zohar H, Israelson A, Abu-Hamad S, Shoshan-Barmatz V.** In self-defense: hexokinase promotes voltage-dependent anion channel closure and prevents mitochondria-mediated apoptotic cell death. *Biochem J* 377:347-355, 2004.

11. **Baines CP, Kaiser RA, Purcell NH, Blair NS, Osinska H, Hambleton MA, Brunskill EW, Sayen MR, Gottlieb RA, Dorn GW, Robbins J, Molkentin JD.** Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature* 434:658-662, 2005.

12. **Baines CP, Kaiser RA, Sheiko T, Craigen WJ, Molkentin JD.** Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. *Nat Cell Biol* 9:550-555, 2007.

13. **Baines CP.** The cardiac mitochondrion: nexus of stress. *Annu Rev Physiol* 72:61-80, 2010.

14. **Barger PM, Kelly DP.** Fatty acid utilization in the hypertrophied and failing heart: molecular regulatory mechanisms. *Am J Med Sci* 318:36-42, 1999.

15. **Barger PM, Brandt JM, Leone TC, Weinheimer CJ, Kelly DP.** Deactivation of peroxisome proliferator-activated receptor-alpha during cardiac hypertrophic growth. *J Clin Invest* 105:1723-1730, 2000.

16. **Bathori G, Sahin-Toth M, Fonyo A, Ligeti E.** Transport properties and inhibitor sensitivity of isolated and reconstituted porin differ from those of intact mitochondria. *Biochim Biophys Acta* 1145:168-176, 1993.

17. **Botker HE, Helligso P, Kimose HH, Thomassen AR, Nielsen TT.** Determination of high energy phosphates and glycogen in cardiac and skeletal muscle biopsies, with special reference to influence of biopsy technique and delayed freezing. *Cardiovasc Res* 28:524-527, 1994.

18. **Bustamante E, Pedersen PL.** High aerobic glycolysis of rat hepatoma cells in culture: role of mitochondrial hexokinase. *Proc Natl Acad Sci USA* 74:3735-3739, 1977.

19. **Bustamante E, Morris HP, Pedersen PL.** Energy metabolism of tumor cells. Requirement for a form of hexokinase with a propensity for mitochondrial binding. *J Biol Chem* 256:8699-8704, 1981.

20. **Cheng EHY, Sheiko TV, Fisher JK, Craigen WJ, Korsmeyer SJ.** VDAC2 inhibits Bak activation and mitochondrial apoptosis. *Science* 301:513-517, 2003.

21. **Chess DJ, Khairallah RJ, O'Shea KM, Xu W, Stanley WC.** A high-fat diet increases adiposity but maintains mitochondrial oxidative enzymes without affecting development of heart failure with pressure overload. *Am J Physiol Heart Circ Physiol* 297:H1585-H1593, 2009.

22. **Cheung EC, Ludwig RL, Vousden KH.** Mitochondrial localization of TIGAR under hypoxia stimulates HK2 and lowers ROS and cell death. *Proc Natl Acad Sci USA* 109:20491-20496, 2012.

23. **Chiara F, Castellaro D, Marin O, Petronilli V, Brusilow WS, Juhaszova M, Sollott SJ, Forte M, Bernardi P, Rasola A.** Hexokinase II detachment from mitochondria triggers apoptosis through the permeability transition pore independent of voltage-dependent anion channels. *PLoS One* 3:e1851, 2008.

24. **Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, Chan FK.** Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell* 137:1112-1123, 2009.

25. **Colombini M.** Purification of VDAC (voltage-dependent anion-selective channel) from rat liver mitochondria. *J Membr Biol* 74:115-121, 1983.

26. **Conus S, Simon HU.** Cathepsins: key modulators of cell death and inflammatory responses. *Biochem Pharmacol* 76:1374-1382, 2008.

27. **Cryer PE.** Glucose counterregulation: prevention and correction of hypoglycemia in humans. *Am J Physiol Endocrinol Metab* 264:E149-E155, 1993.

28. **Da Silva D, Ausina P, Alencar EM, Coelho WS, Zancan P, Sola-Penna M.** Metformin reverses hexokinase and phosphofructokinase downregulation and intracellular distribution in the heart of diabetic mice. *IUBMB Life* 64:766-774, 2012.

29. **Da-Silva WS, Gomez-Puyou A, de Gomez-Puyou MT, Moreno-Sanchez R, De Felice FG, de Meis L, Oliviera MF, Galina A.** Mitochondrial bound hexokinase activity as a preventive antioxidant defense: steady-state ADP formation as a regulatory mechanism of membrane potential and reactive oxygen species generation in mitochondria. *J Biol Chem* 279:39846-39855, 2004.

30. **Dávila-Román V, Vedala G, Herrero P, de las Fuentes L, Rogers JG, Kelly DP, Gropler RJ.** Altered myocardial fatty acid and glucose metabolism in idiopathic dilated cardiomyopathy. *J Am Coll Cardiol* 40:271-277, 2002.

31. **De Pinto V, Reina S, Guarino F, Messina A.** Structure of the voltage dependent anion channel: state of the art. *J Bioenerg Biomembr* 40:139-147, 2008.

32. **Di Chiro G, DeLaPaz RL, Brooks RA, Sokoloff L, Kornblith PL, Smith BH, Patronas NJ, Kufta CV, Kessler RM, Johnston GS, Manning RG, Wolf AP.** Glucose utilization of cerebral gliomas measured by [18F] fluorodeoxyglucose and positron emission tomography. *Neurology* 32:1323-1329, 1982.

33. **Eguchi Y, Srinivasan A, Tomaselli KJ, Shimizu S, Tsujimoto Y.** ATP-dependent steps in apoptotic signal transduction. *Cancer Res* 59:2174-2181, 1999.

34. **El Alaoui-Talibi Z, Landormy S, Loireau A, Moravec J.** Fatty acid oxidation and mechanical performance of volume-overloaded rat hearts. *Am J Physiol Heart Circ Physiol* 262:H1068-H1074, 1992.

35. **El Alaoui-Talibi Z, Guendouz A, Moravec M, Moravec J.** Control of oxidative metabolism in volume-overloaded rat hearts: effect of propionyl-L-carnitine. *Am J Physiol Heart Circ Physiol* 272:H1615-H1624, 1997.

36. **Entman ML, Bornet EP, van Winkle WB, Goldstein MA, Schwartz A.** Association of glycogenolysis with cardiac sarcoplasmic reticulum. II. Effect of glycogen depletion, deoxycholate solubilization and cardiac ischemia: evidence for a phorphorylase kinase membrane complex. *J Mol Cell Cardiol* 9:515-528, 1977.

37. **Fedele FA, Gewirtz H, Cappone RJ, Sharaf B, Most AS.** Metabolic response to prolonged reduction of myocardial blood flow distal to a severe coronary artery stenosis. *Circulation* 78:729-735, 1988.

38. **Fell DA.** Increasing the flux in metabolic pathways: a metabolic control analysis perspective. *Biotechnol Bioeng* 58:121-124, 1998.

39. **Fiek C, Benz R, Roos N, Brdiczka D.** Evidence for identity between the hexokinase-binding protein and the mitochondrial porin in the outer membrane of rat liver mitochondria. *Biochim Biophys Acta* 688:429-440, 1982.

40. **Finck BN, Lehman JJ, Leone TC, Welch MJ, Bennett MJ, Kovacs A, Han X, Gross RW, Kozak R, Lopaschuk GD, Kelly DP.** The cardiac phenotype induced by PPARα overexpression mimics that caused by diabetes mellitus. *J Clin Invest* 109:121-130, 2002.

41. **Fueger PT, Hess HS, Posey KA, Bracy DP, Pencek RR, Charron MJ, Wasserman DH.** Control of exercise-stimulated muscle glucose uptake by GLUT4 is dependent on glucose phosphorylation capacity in the conscious mouse. *J Biol Chem* 279:50956-50961, 2004.

42. **Gabbay KH, Merola LO, Field RA.** Sorbitol pathway: presence in nerve and cord with substrate accumulation in diabetes. *Science* 151:209-210, 1966.

43. **Galindo CL, Skinner MA, Errami M, Olson LD, Watson DA, Li J, McCormick JF, McIver LJ, Kumar NM, Pham TQ, Garner HR.** Transcriptional profile of isoproterenol-induced cardiomyopathy and comparison to exercise-induced cardiac hypertrophy and human cardiac failure. *BMC Physiology* 9:23, 2009.

44. **Gall JM, Wong V, Pimental D, Havasi A, Wang Z, Pastorino JG, Bonegio R, Schwartz JH, Borkan SC.** Hexokinase regulates susceptibility to Bax-mediated mitochondrial membrane injury after ischemic stress. *Kidney Int* 79:1207-1216, 2011.

45. **Galluzzi L, Maiuri MC, Vitale I, Zischka H, Castedo M, Zitvogel L, Kroemer G.** Cell death modalities: classification and pathophysiological implications. *Cell Death Differ* 14:1237-1243, 2007.

46. **Gertz EW, Wisneski JA, Stanley WC, Neese RA.** Myocardial substrate utilization during exercise in humans. Dual carbon-labeled carbohydrate isotope experiments. *J Clin Invest* 82:2017-2025, 1988.

47. **Goldberg IJ, Trent CM, Schulze C.** Lipid metabolism and toxicity in the heart. *Cell Metab* 15:805-812, 2012.

48. **Goossens V, Grooten J, De Vos K, Fiers W.** Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity. *Proc Natl Acad Sci USA* 92:8115-8119, 1995.

49. **Goossens V, Stangé G, Moens K, Pipeleers D, Grooten J.** Regulation of tumor necrosis factor-induced, mitochondria- and reactive oxygen species-dependent cell death by the electron flux through electron transport chain complex I. *Antioxid Redox Signal* 1:285-295, 1999.

50. **Guo Y, Flaherty MP, Wu WJ, Tan W, Zhu X, Li Q, Bolli R.** Genetic background, gender, age, body temperature, and arterial blood pH have a major impact on myocardial infarct size in the mouse and need to be carefully measured and/or taken into account: results of a comprehensive analysis of determinants of infarct size in 1,074 mice. *Basic Res Cardiol* 107:288, 2012.

51. **Gupte SA, Levine RJ, Gupte RS, Young ME, Lionetti V, Labinskyy V, Floyd BC, Ojaimi C, Bellomo M, Wolin MS, Recchia FA.** Glucose-6-phosphate dehydrogenase-derived NADPH fuels superoxide production in the failing heart. *J Mol Cell Cardiol* 41:340-349, 2006.

52. **Gürel E, Smeele KM, Eerbeek O, Koeman A, Demirci C, Hollman MW, Zuurbier CJ.** Ischemic preconditioning affects hexokinase activity and HKII in different subcellular compartments throughout cardiac ischemia-reperfusion. *J Appl Physiol* 106:1909-1916, 2009.

53. **Guth BD, Wisneski JA, Neese RA, White FC, Heusch G, Mazer CD.** Myocardial lactate release during ischemia in swine. Relation to regional blood flow. *Circulation* 81:1948-1958, 1990.

54. **Halseth AE, Bracy DP, Wasserman DH.** Limitations to exercise- and maximal insulin-stimulated muscle glucose uptake. *J Appl Physiol* 85:2305-2313, 1998.

55. **Hart GW, Housley MP, Slawson C.** Cycling of O-linked β-N-acetylglucosamine on nucleocytoplasmic proteins. *Nature* 446:1017-1022, 2007.

56. **He S, Wang L, Miao L, Wang T, Du F, Zhao L, Wang X.** Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. *Cell* 137:1100-1111, 2009.

57. **Hecker PA, Lionetti V, Ribeiro RF, Rastogi S, Brown BH, O'Connell KA, Cox JW, Shekar KC, Gamble DM, Sabbah HN, Leopold JA, Gupte SA, Recchia FA, Stanley WC.** Glucose-6-phosphate dehydrogenase deficiency increases redox stress and moderately accelerates the development of heart failure. *Circ Heart Fail* 6:118-126, 2013.

58. **Henning SL, Wambolt RB, Schonekess BO, Lopaschuk GD, Allard MF.** Contribution of glycogen to aerobic myocardial glucose utilization. *Circulation* 93:1549-1555, 1996.

59. **Holloszy JO, Kohrt WM.** Regulation of carbohydrate and fat metabolism during and after exercise. *Annu Rev Nutr* 16:121-138, 1996.

60. **Holten D, Procsal D, Chang HL.** Regulation of pentose phosphate pathway dehydrogenases by NADP+/NADPH ratios. *Biochem Biophys Res Commun* 68:436-441, 1976.

61. **Hotchkiss RS, Strasser A, McDunn JE, Swanson PE.** Mechanisms of disease: Cell Death. *New Engl J Med* 361:1570-1583, 2009.

62. **Hunter DR, Haworth RA, Southard JH.** Relationship between configuration, function, and permeability in calcium-treated mitochondria. *J Biol Chem* 251:5069-5077, 1976.

63. **Jain M, Brenner DA, Cui L, Lim CC, Wang B, Pimentel DR, Koh S, Sawyer DB, Leopold JA, Handy DE, Loscalzo J, Apstein CS, Liao R.** Glucose-6-phosphate dehydrogenase modulates cytosolic redox status and contractile phenotype in adult cardiomyocytes. *Circ Res* 93:e9-16, 2003.

64. **Jeremy RW, Koretsune Y, Marban E, Becker LC.** Relation between glycolysis and ion homeostasis in postischemic myocardium. *Circ Res* 70:1180-1190, 1992.

65. **Johnson BF, Nesto RW, Pfeifer MA, Slater WR, Vinik AI, Chyun DA, Law G, Wackers FJ, Young LH.** Cardiac abnormalities in diabetic patients with neuropathy: effects of aldose reductase inhibitor administration. *Diabetes Care* 27:448-454, 2004.

66. **Jope RS, Johnson GV.** The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem Sci* 29:95-102, 2004.

67. **Kaijser L, Berglund B.** Myocardial lactate extraction and release at rest and during heavy exercise in healthy men. *Acta Physiol Scand* 144:39-45, 1992.

68. **Kato T, Niizuma S, Inuzuka Y, Kawashima T, Okuda J, Tamaki Y, Iwanaga Y, Narazaki M, Matsuda T, Soga T, Kita T, Kimura T, Shioi T.** Analysis of metabolic remodeling in compensated left ventricular hypertrophy and heart failure. *Circ Heart Fail* 3:420-430, 2010.

69. **Katzen HM.** The multiple forms of mammalian hexokinase and their significance to the action of insulin. *Adv Enzyme Regul* 5:335-336, 1967.

70. **Katzen HM, Schimke RT.** Multiple isoforms of hexokinase in the rat: tissue distribution, age dependency, and properties. *Proc Natl Acad Sci USA* 54:1218-1225, 1965.

71. **Kletzien RF, Harris PK, Foellmi LA.** Glucose-6-phosphate dehydrogenase: a "housekeeping" enzyme subject to tissue-specific regulation by hormones, nutrients, and oxidant stress. *FASEB J* 8:174-181, 1994.

72. **Klionsky DJ.** Autophagy: from phenomenology to molecular understanding in less than a decade. *Nat Rev Mol Cell Biol* 8:931-937, 2007.

73. **Koehler U, Medugorac I.** Left ventricular enzyme activities of the energy-supplying metabolism in Goldblatt-II rats. *Res Exp Med (Berl)* 185:299-307, 1985.

74. **Kokoszka JE, Waymire KG, Levy SE, Sligh JE, Cai J, Jones DP, MacGregor GR, Wallace DC.** The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore. *Nature* 427:461-465, 2004.

75. **Kolwicz SC, Tian R.** Glucose metabolism and cardiac hypertrophy. *Cardiovasc Res* 90:194-201, 2011.

76. **Kresge N, Simoni RD, Hill RL.** Otto Fritz Meyerhof and the elucidation of the glycolytic pathway. *J Biol Chem* 280:e3, 2005.

77. **Kroemer G, Galluzzi L, Brenner C.** Mitochondrial membrane permeabilization in cell death. *Physiol Rev* 87:99-163, 2007.

78. **Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR, Hengartner M, Knight RA, Kumar S, Lipton SA, Malorni W, Nuñez G, Peter ME, Tschopp J, Yuan J, Piacentini M, Zhivotovsky B, Melino G; Nomenclature Committee on Cell Death 2009.** Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ* 16:3-11, 2009.

79. **Kruszynska YT, McCormack JG, McIntyre N.** Effects of glycogen stores and non-esterified fatty acid availability on insulin-stimulated glucose metabolism and tissue pyruvate dehydrogenase activity in the rat. *Diabetologia* 34:205-211, 1991.

80. **Kunuthur SP, Mocanu MM, Hemmings BA, Hausenloy DJ, Yellon DM.** The Akt1 isoform is an essential mediator of ischemic preconditioning. *J Cell Mol Med* 16:1739-1749, 2012.

81. **Kusuoka H, Marban E.** Mechanism of the diastolic dysfunction induced by glycolytic inhibition. Does adenosine triphosphate derived from glycolysis play a favored role in cellular Ca2+ homeostasis in ferret myocardium? *J Clin Invest* 93:1216-1223, 1994.

82. **Kuwana T, Bouchier-Hayes L, Chipuk JE, Bonzon C, Sullivan BA, Green DR, Newmeyer DD.** BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell* 17:525-535, 2005.

83. **Lal H, Kolaja KL, Force T.** Cancer genetics and the cardiotoxicity of the therapeutics. *J Am Coll Cardiol* 61:267-274, 2013.

84. **Lazarou M, Stojanovski D, Frazier AE, Kotevski A, Dewson G, Craigen WJ, Kluck RM, Vaux DL, Ryan MT.** Inhibition of Bak activation by VDAC2 is dependent on the Bak transmembrane anchor. *J Biol Chem* 285:36876-36883, 2010.

85. **Lee AC, Xu X, Colombini M.** The role of pyridine dinucleotides in regulating the permeability of the mitochondrial outer membranes. *J Biol Chem* 271:26724-26731, 1996.

86. **Leist M, Single B, Castoldi AF, Kühnle S, Nicotera P.** Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J Exp Med* 185:1481-1486, 1997.

87. **Lemasters JJ.** Dying a thousand deaths: redundant pathways from different organelles to apoptosis and necrosis. *Gastroenterology* 129:351-360, 2005.

88. **Lemasters JJ, Holmuhamedov E.** Voltage-dependent anion channel (VDAC) as mitochondrial governator- thinking outside the box. *Biochim Biophys Acta* 1762:181-190, 2006.

89. **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* 91:479-489, 1997.

90. **Liao R, Jain M, Cui L, D'Agostino J, Aiello F, Luptak I, Ngoy S, Mortensen RM, Tian R.** Cardiac-specific overexpression of GLUT1 prevents the development of heart failure attributable to pressure overload in mice. *Circulation* 106:2125-2131, 2002.

91. **Liedtke AJ, Nellis SH, Neely JR.** Effects of excess free fatty acids on mechanical and metabolic function in normal and ischemia myocardium in swine. *Circ Res* 43:652-661, 1978.

92. **Liedtke AJ, Nellis SH, Mjos OD.** Effects of reducing fatty acid metabolism on mechanical function in regionally ischemia hearts. *Am J Physiol Heart Circ Physiol* 247:H387-H394, 1984.

93. **Lim SY, Davidson SM, Mocanu MM, Yellon DM, Smith CCT.** The cardioprotective effect of necrostatin requires the cyclophilin-D component of the mitochondrial permeability transition pore. *Cardiovasc Drug Ther* 21:467-469, 2007.

94. **Linden M, Gellerfors P, Nelson BD.** Pore protein and the hexokinase binding protein from the outer membrane of rat liver mitochondria are identical. *FEBS Lett* 141:189-192, 1982.

95. **Lopaschuk GD, Spafford MA, Marsh DR.** Glycolysis is predominant source of myocardial ATP production immediately after birth. *Am J Physiol Heart Circ Physiol* 261:H1698-H1705, 1991.

96. **Los M, Mozoluk M, Ferrari D, Stepczynska A, Stroh C, Renz A, Herceg Z, Wang ZQ, Schulze-Osthoff K.** Activation and caspase-mediated inhibition of PARP: a molecular switch between fibroblasts necrosis and apoptosis in death receptor signaling. *Mol Biol Cell* 13:978-988, 2002.

97. **Mailloux RJ, Dumouchel T, Aguer C, deKemp R, Beanlands R, Harper ME.** Hexokinase II acts through UCP3 to suppress mitochondrial reactive oxygen species production and maintain aerobic respiration. *Biochem J* 437:301-311, 2011.

98. **Majewski N, Nogueira V, Bhasker P, Coy PE, Skeen JE, Gottlob K, Chandel NS, Thompson CB, Robey RB, Hay N.** Hexokinase-mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak. *Mol Cell* 16:819-830, 2004.

99. **Maldonaldo EN, Patnaik J, Mullins MR, Lemasters JJ.** Free tubulin modulates mitochondrial membrane potential in cancer cells. *Cancer Res* 70:10192-10201, 2010.

100. **Maldonado EN, Sheldon KL, DeHart DN, Patnaik J, Manevich Y, Townsend DM, Bezrukov SM, Rostovtseva TK, Lemasters JJ.** Voltage-dependent anion channels modulate mitochondrial metabolism in cancer cells. *J Biol Chem* 288:11920-11929, 2013.

101. **Malia TJ, Wagner G.** NMR structural investigation of the mitochondrial outer membrane protein VDAC and its interaction with antiapoptotic Bcl-xL. *Biochemistry* 46:514-525, 2007.

102. **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. *J Biol Chem* 266:4706-4712, 1991.

103. **Mathupala SP, Rempel A, Pedersen PL.** Glucose catabolism in cancer cells. Identification and characterization of a marked activation response of the type II hexokinase gene to hypoxic conditions. *J Biol Chem* 276:43407-43412, 2001.

104. **Mazer CD, Cason BA, Stanley WC, Wisneski JA, Shnier CB, Hickey RF.** Dichloroacetate reduces lactate production but does not improve function in ischemic swine myocardium. *Am J Physiol Heart Circ Physiol* 268:H879-H885, 1995.

105. **McCommis KS, Baines CP.** The role of VDAC in cell death: friend or foe? *Biochim Biophys Acta* 1818:1444-1450, 2012.

106. **McFalls EO, Murad B, Liow JS, Gannon MC, Haspel HC, Lange A, Marx D, Sikora J, Ward HB.** Glucose uptake and glycogen levels are increased in pig heart after repetitive ischemia. *Am J Physiol Heart Circ Physiol* 282:H205-H211, 2002.

107. **Meerson FZ, Spiritchev VB, Pshennikova MG, Djachkova LV.** The role of the pentose-phosphate pathway in adjustment of the heart to a high load and the development of myocardial hypertrophy. *Experientia* 23:530-532, 1967.

108. **Mergenthaler P, Kahl A, Kamitz A, van Laak V, Stohlmann K, Thomsen S, Klawitter H, Przesdzing I, Neeb L, Freyer D, Priller J, Collins TJ, Megow D, Dirnagl U, Andrews DW, Meisel A.** Mitochondrial hexokinase II (HKII) and phosphoprotein enriched in astrocytes (PEA15) form a molecular switch governing cellular fate depending on the metabolic state. *Proc Natl Acad Sci USA* 109:1518-1523, 2012.

109. **Miyamoto S, Murphy AN, Brown JH.** Akt mediates mitochondrial protection in cardiomyocytes through phosphorylation of mitochondrial hexokinase-II. *Cell Death Differ* 15:521-529, 2008.

110. **Miyamoto S, Purcell NH, Smith JM, Gao T, Whittaker R, Huang K, Castillo R, Glembotski CC, Sussman MA, Newton AC, Brown JH.** PHLPP-1 negatively regulates Akt activity and survival in the heart. *Circ Res* 107:476-484, 2010.

111. **Nakagawa T, Shimizu S, Watanabe T, Yamaguchi O, Otsu K, Yamagata H, Inohara H, Kubo T, Tsujimoto Y.** Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. *Nature* 434:652-658, 2005.

112. **Nakashima RA, Mangan PS, Colombini M, Pedersen PL.** Hexokinase receptor complex in hepatoma mitochondria: evidence from N,N'-dicyclohexylcarbodiimide-labeling studies for the involvement of the pore forming protein VDAC. *Biochemistry* 25:1015-1021, 1986.

113. **Nascimben L, Ingwall JS, Lorell BH, Pinz I, Schultz V, Tornheim K, Tian R.** Mechanisms for increased glycolysis in the hypertrophied rat heart. *Hypertension* 44:662-667, 2004.

114. **Neely JR, Rovetto MJ, Oram JF.** Myocardial utilization of carbohydrate and lipids. *Prog Cardiovasc Dis* 15:289-329, 1972.

115. **Neumann D, Bückers J, Kastrup L, Hell SW, Jakobs S.** Two-color STED microscopy reveals different degrees of colocalization between hexokinase-1 and the three human VDAC isoforms. *PMC Biophys* 3:4, 2010.

116. **Ngoh GA, Facundo HT, Zafir A, Jones SP.** *O*-GlcNAc signaling in the cardiovascular system. *Circ Res* 107:171-185, 2010.

117. **Nkhoma ET, Poole C, Vannappagari V, Hall SA, Beutler E.** The global prevalence of glucose-6-phosphate dehydrogenase deficiency: a systematic review and meta-analysis. *Blood Cells Mol Dis* 42:267-278, 2009.

118. **Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL, Castedo M, Mignot G, Panaretakis T, Casares N, Métivier D, Larochette N, van Endert P, Ciccosanti F, Piacentini M, Zitvogel L, Kroemer G.** Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat Med* 13:54-61, 2007.

119. **Osawa H, Printz RL, Whitesell RR, Granner DK.** Regulation of hexokinase II gene transcription and glucose phosphorylation by catecholamines, cyclic AMP, and insulin. *Diabetes* 44:1426-1432, 1995.

120. **Pantely GA, Malone SA, Rhen WS, Anselone CG, Arai A, Bristow J, Bristow JD.** Regeneration of myocardial phosphocreatine in pigs despite continued moderate ischemia. *Circ Res* 67:1481-1493, 1990.

121. **Parry DM, Pedersen PL.** Intracellular localization and properties of particulate hexokinase in the Novikoff ascites tumor. Evidence for an outer mitochondrial membrane location. *J Biol Chem* 258:10904-10912, 1983.

122. **Pasdois P, Parker JE, Halestrap AP.** Extent of mitochondrial hexokinase II dissociation during ischemia correlates with mitochondrial cytochrome c release, reactive oxygen species production, and infarct size on reperfusion. *J Am Heart Assoc* 2:e005645, 2012.

123. **Pasteur L.** Expériences et vues nouvelles sur la nature des fermentations. *Comp Rend Acad Sci* 52:1260-1264, 1861.

124. **Pastorino JG, Shulga N, Hoek JB.** Mitochondrial binding of hexokinase II inhibits Bax-induced cytochrome c release and apoptosis. *J Biol Chem* 277:7610-7618, 2002.

125. **Pedersen PL.** Warburg, me and hexokinase 2: multiple discoveries of key molecular events underlying one of cancers' most common phenotypes, the "Warburg effect", i.e., elevated glycolysis in the presence of oxygen. *J Bioenerg Biomembr* 39:211-222, 2007.

126. **Porter RK, Brand MD.** Mitochondrial proton conductance and H+/O ratio are independent of electron transport rate in isolated hepatocytes. *Biochem J* 310:379-382, 1995.

127. **Printz RL, Osawa H, Ardehali H, Koch S, Granner DK.** Hexokinase II gene: structure, regulation and promoter organization. *Biochem Soc Trans* 25:107-112, 1997.

128. **Raghavan A, Sheiko T, Graham BH, Craigen WJ.** Voltage-dependent anion channels: novel insights into isoform function through genetic models. *Biochim Biophys Acta* 1818:1477-1485, 2012.

129. **Razeghi P, Young ME, Alcorn JL, Moravec CS, Frazier OH, Taegtmeyer H.** Metabolic gene expression in fetal and failing human heart. *Circulation* 104:2923-2931, 2001.

130. **Rea S, James DE.** Moving of GLUT4: the biogenesis and trafficking of GLUT4 storage vesicles. *Diabetes* 46:1667-1677, 1997.

131. **Ren JM, Semenkovish CF, Gulve EA, Gao J, Holloszy JO.** Exercise induces rapid increases in GLUT4 expression, glucose transport capacity, and insulin stimulated glycogen storage in muscle. *J Biol Chem* 269:14396-14401, 1994.

132. **Rich PR.** The molecular machinery of Kellin's respiratory chain. *Biochem Soc Trans* 31:1095-1105, 2003.

133. **Robey RB, Hay N.** Mitochondrial hexokinases, novel mediators of the antiapoptotic effects of growth factors and Akt. *Oncogene* 25:4683-4696, 2006.

134. **Rostovtseva TK, Komarov A, Bezukov SM, Colombini M.** Dynamics of nucleotides in VDAC channels: structure-specific noise generation. *Biophys J* 82:193-205, 2002.

135. **Rostovtseva TK, Tan W, Colombini M.** On the role of VDAC in apoptosis: fact and fiction. *J Bioenerg Biomembr* 37:129-142, 2005.

136. **Rovetto MJ, Lamberton WF, Neely JR.** Mechanism of glycolytic inhibition in ischemic rat hearts. *Circ Res* 37:742-751, 1975.

137. **Roy SS, Ehrlich AM, Craigen WJ, Hajnoczky G.** VDAC2 is required for truncated BID-induced mitochondrial apoptosis by recruiting Bak to the mitochondria. *EMBO Rep* 10:1341-1347, 2009.

138. **Sabri A, Hughie HH, Lucchesi PA.** Regulation of hypertrophic and apoptotic signaling pathways by reactive oxygen species in cardiac myocytes. *Antioxid Redox Signal* 5:731-740, 2003.

139. **Sack MN, Disch DL, Rockman HA, Kelly DP.** A role for Sp and nuclear receptor transcription factors in a cardiac hypertrophic growth program. *Proc Natl Acad Sci USA* 94:6438-6443, 1997.

140. **Saelens X, Festjens N, Parthoens E, Vanoverberghe I, Kalai M, van Kuppeveld F, Vandenabeele P.** Protein synthesis persists during necrotic cell death. *J Cell Biol* 168:545-551, 2005.

141. **Sampson MJ, Lovell RS, Craigen WJ.** The murine voltage dependent anion channel gene family. Conserved structure and function. *J Biol Chem* 272:18966-18973, 1997.

142. **Scheinberg P, Stead EA.** The cerebral blood flow in male subjects as measured by the nitrous oxide technique. Normal values for blood flow, oxygen utilization, glucose utilization, and peripheral resistance, with observations on the effects of tilting and anxiety. *J Clin Invest* 28:1163-1171, 1949.

143. **Schinzel AC, Takeuchi O, Huang Z, Fisher JK, Zhou Z, Rubens J, Hetz C, Danial NN, Moskowitz MA, Korsmeyer SJ.** Cyclophilin D is a component of mitochondrial permeability transition and mediates neuronal cell death after focal cerebral ischemia. *Proc Natl Acad Sci USA* 102:12005-12010, 2005.

144. **Schramm C, Fine DM, Edwards MA, Reeb AN, Krenz M.** The PTPN11 loss-of-function mutation Q510E-Shp2 causes hypertrophic cardiomyopathy by dysregulating mTOR signaling. *Am J Physiol Heart Circ Physiol* 302:H231-243, 2012.

145. **Seddon M, Looi YH, Shah AM.** Oxidative stress and redox signaling in cardiac hypertrophy and heart failure. *Heart* 93:903-907, 2007.

146. **Smeele KMA, Southworth R, Wu R, Xie C, Nederlof R, Warley A, Nelson JK, van Horssen P, van den Wijngaard JP, Heikkinen S, Laakso M, Koeman A, Siebes M, Eerbeek O, Akar FG, Ardehali H, Hollmann MW, Zuurbier CJ.** Disruption of hexokinase II-mitochondrial binding blocks ischemic preconditioning and causes rapid cardiac necrosis. *Circ Res* 108:1165-1169, 2011.

147. **Soderling TR, Jett MF, Hutson NJ, Khatra BS.** Regulation of glycogen synthase. Phosphorylation specificities of cAMP-dependent and cAMP-independent kinases for skeletal muscle synthase. *J Biol Chem* 252:7517-7524, 1977.

148. **Stanley WC**. Myocardial lactate metabolism during exercise. *Med Sci Sports Exercise* 23:920-924, 1991.

149. **Stanley WC, Lopaschuk GD, Hall JL, McCormack JG.** Regulation of myocardial carbohydrate metabolism under normal and ischaemic conditions. Potential for pharmacological interventions. *Cardiovasc Res* 33:243-257, 1997.

150. **Stanley WC, Recchia FA, Lopaschuk GD.** Myocardial substrate metabolism in the normal and failing heart. *Physiol Rev* 85:1093-1129, 2005.

151. **Stanton RC.** Glucose-6-phosphate dehydrogenase, NADPH, and cell survival. *IUBMB Life* 64:362-369, 2012.

152. **Stowe KA, Burgess SC, Merritt M, Sherry AD, Malloy CR.** Storage and oxidation of long-chain fatty acids in the C57/BL6 mouse heart as measured by NMR spectroscopy. *FEBS Lett* 580:4282-4287, 2006.

153. **Sui D, Wilson JE.** Structural determinants for the intracellular localization of the isozymes of mammalian hexokinase: intracellular localization of fusion constructs incorporating structural elements from the hexokinase isozymes and the green fluorescent protein. *Arch Biochem Biophys* 345:111-125, 1997.

154. **Sun L, Shukair S, Naik TJ, Moazed F, Ardehali H.** Glucose phosphorylation and mitochondrial binding are required for the protective effects of hexokinases I and II. *Mol Cell Biol* 28:1007-1017, 2008.

155. **Sun L, Wang H, Wang Z, He S, Chen S, Liao D, Wang L, Yan J, Liu W, Lei X, Wang X.** Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. *Cell* 148:213-227, 2012.

156. **Sun XM, Butterworth M, MacFarlane M, Dubiel W, Ciechanover A, Cohen GM.** Caspase activation inhibits proteasome function during apoptosis. *Mol Cell* 14:81-93, 2004.

157. **Szabó I, De Pinto V, Zoratti M.** The mitochondrial permeability transition pore may comprise VDAC molecules. II. The electrophysiological properties of VDAC are compatible with those of the mitochondrial megachannel. *FEBS Lett* 330:206-2210, 1993.

158. **Taegtmeyer H, Overturf ML.** Effects of moderate hypertension on cardiac function and metabolism in the rabbit. *Hypertension* 11:416-426, 1988.

159. **Takimoto E, Kass DA.** Role of oxidative stress in cardiac hypertrophy and remodeling. *Hypertension* 49:241-248, 2007.

160. **Tchakarska G, Roussel M, Troussard X, Sola B.** Cyclin D1 inhibits mitochondrial activity in B cells. *Cancer Res* 71:1690-1699, 2011.

161. **Temkin V, Huang Q, Liu H, Osada H, Pope RM.** Inhibition of ADP/ATP exchange in receptor-interacting protein-mediated necrosis. *Mol Cell Biol* 26:2215-2225, 2006.

162. **Tian WN, Pignatare JN, Stanton RC.** Signal transduction proteins that associate with the platelet-derived growth factor (PDGF) receptor mediate the PDGF-induced release of glucose-6-phosphate dehydrogenase from permeabilized cells. *J Biol Chem* 269:14798-14805, 1994.

163. **Tian WN, Braunstein LD, Pang J, Stuhlmeier KM, Xi QC, Tian X, Stanton RC.** Importance of glucose-6-phosphate dehydrogenase activity for cell growth. *J Biol Chem* 273:10609-10617, 1998.

164. **Tian WN, Braunstein LD, Apse K, Pang J, Rose M, Tian X, Stanton RC.** Importance of glucose-6-phosphate dehydrogenase activity in cell death. *Am J Physiol Cell Physiol* 276:C1121-C1131, 1999.

165. **Tikunov A, Johnson CB, Pediaditakis P, Markevich N, Macdonald JM, Lemasters JJ, Holmuhamedov E.** Closure of VDAC causes oxidative stress and accelerates the Ca(2+)-induced mitochondrial permeability transition in rat liver mitochondria. *Arch Biochem Biophys* 495:174-181, 2010.

166. **Vandenabeele P, Decleroq W, Van Herreweghe F, Vanden Berghe T.** The role of the kinases RIP1 and RIP3 in TNF-induced necrosis. *Sci Signal* 3:re4, 2010.

167. **Vander Heiden MG, Li XX, Gottleib E, Hill RB, Thompson CB, Colombini M.** Bcl-xL promotes the open configuration of the voltage-dependent anion channel and metabolite passage through the outer mitochondrial membrane. *J Biol Chem* 276:19414-19419, 2001.

168. **Vork MM, Trigault N, Snoeckx LH, Glatz JF, van der Vusse GJ.** Heterogeneous distribution of fatty acid-binding protein in the hearts of Wistar Kyoto and spontaneously hypertensive rats. *J Mol Cell Cardiol* 24:317-321, 1992.

169. **Wamelink MMC, Struys EA, Jakobs C.** The biochemistry, metabolism and inherited defects of the pentose phosphate pathway: a review. *J Inherit Metab Dis* 31:703-717, 2008.

170. **Wang Z, Jiang H, Chen S, Du F, Wang X.** The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways. *Cell* 148:228-243, 2012.

171. **Warburg O, Wind F, Negelein E.** The metabolism of tumors in the body. *J Gen Physiol* 8:519-530, 1927.

172. **Watson LJ, Facundo HT, Ngoh GA, Ameen M, Brainard RE, Lemma KM, Long BW, Prabhu SD, Xuan YT, Jones SP.** O-linked β-N-acetylglucosamine transferase is indispensable in the failing heart. *Proc Natl Acad Sci USA* 107:17797-17802, 2010.

173. **Wei MC, Zong WX, Cheng EHY, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB, Korsmeyer SJ.** Proapoptotic BAX and BAK: A requisite gateway to mitochondrial dysfunction and death. *Science* 292:727-730, 2001.

174. **Weiss JN, Korge P, Honda HM, Ping P.** Role of the mitochondrial permeability transition in myocardial disease. *Circ Res* 93:292-301, 2003.

175. **Wilson JE.** Rapid purification of mitochondrial hexokinase from rat brain by a single affinity chromatography step on AffiGel Blue. *Prep Biochem* 19:13-21, 1989.

176. **Wilson JE.** Distinguishing the type I and type II isozymes of hexokinase: the need for a reexamination of past practice. *Diabetes* 47:1544-1548, 1998.

177. **Wilson JE.** Isozymes of mammalian hexokinase: structure, subcellular localization and metabolic function. *J Exp Biol* 206:2049-2057, 2003.

178. **Wright EM, Hirsch JR, Loo DD, Zampighi GA.** Regulation of Na+/glucose cotransporters. *J Exp Biol* 200:287-293, 1997.

179. **Wu R, Smeele KM, Wyatt E, Ichikawa Y, Eerbeek O, Sun L, Chawla K, Hollmann MW, Nagpal V, Heikkinen S, Laakso M, Jujo K, Wasserstrom JA, Zuurbier CJ, Ardehali H.** Reduction in hexokinase II levels results in decreased cardiac function and altered remodeling after ischemia/reperfusion injury. *Circ Res* 108:60-69, 2011.

180. **Wu R, Wyatt E, Chawla K, Tran M, Ghanefar M, Laakso M, Epting CL, Ardehali H.** Hexokinase II knockdown results in exaggerated cardiac hypertrophy via increased ROS production. *EMBO Mol Med* 4:633-646, 2012.

181. **Wu S, Sampson MJ, Decker WK, Craigen WJ.** Each mammalian mitochondrial outer membrane porin protein is dispensable: effects on cellular respiration. *Biochim Biophys Acta* 1452:68-78, 1999.

182. **Xu Y, Huang S, Liu ZG, Han J.** Poly(ADP-ribose) polymerase-1 signaling to mitochondria in necrotic cell death requires RIP1/TRAF2-mediated JNK1 activation. *J Biol Chem* 281:8788-8795, 2006.

183. **Xue W, Cai L, Tan Y, Thistlethwaite P, Kang YJ, Li X, Feng W.** Cardiac-specific overexpression of HIF-1α prevents deterioration of glycolytic pathway and cardiac remodeling in streptozotocin-induced diabetic mice. *Am J Pathol* 177:97-105, 2010.

184. **Xue W, Liu Y, Zhao J, Cai L, Li X, Feng W.** Activation of HIF-1 by metallothionein contributes to cardiac protection in the diabetic heart. *Am J Physiol Heart Circ Physiol* 302:H2528-H2535, 2012.

185. **Yamagata H, Shimizu S, Nishida Y, Watanabe Y, Craigen WJ, Tsujimoto Y.** Requirement of voltage-dependent anion channel 2 for pro-apoptotic activity of Bax. *Oncogene* 28:3563-3572, 2009.

186. **Yang J, Moravec CS, Sussman MA, DiPaola NR, Fu D, Hawthorn L, Mitchell CA, Young JB, Francis GS, McCarthy PM, Bond M.** Decreased SLIM1 expression and increased gelsolin expression in failing human hearts measured by high-density oligonucleotide arrays. *Circulation* 102:3046-3052, 2000.

187. **Yeih DF, Yeh HI, Lin LY, Tsay YG, Chiang FT, Tseng CD, Tseng YZ.** Enhanced activity and subcellular redistribution of myocardial hexokinase after acute myocardial infarction. *Int J Cardiol* 149:74-79, 2011.

188. **Yoshida M, Muneyuki E, Hisabori T.** ATP synthase- a marvellous rotary engine of the cell. *Nat Rev Mol Cell Biol* 2:669-677, 2001.

189. **Young ME, Yan J, Razeghi P, Cooksey RC, Guthrie PH, Stepkowski SM, McClain DA, Tian R, Taegtmeyer H.** Proposed regulation of gene expression by glucose in the rodent heart. *Gene Regul Syst Biol* 5:251-262, 2007.

190. **Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC, Dong MQ, Han J.** RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. *Science* 325:332-336, 2009.

191. **Zhang J, Duncker DJ, Ya X, Zhang Y, Pavek T, Wei H, Merkle H, Uğurbil K, From AH, Bache RJ.** Effect of left ventricular hypertrophy secondary to chronic pressure overload on Transmural myocardial 2-deoxyglucose uptake. A 31P NMR spectroscopic study. *Circulation* 92:1274-1283, 1995.

192. **Zhao J, Jitkaew S, Cai Z, Choksi S, Li Q, Luo J, Liu ZG.** Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. *Proc Natl Acad Sci USA* 109:5322-5327, 2012.

193. **Zhou J, Freeman TA, Ahmad F, Shang X, Mangano E, Gao E, Farber J, Wang Y, Ma XL, Woodgett J, Vagnozzi RJ, Lal H, Force T.** GSK-3α is a central regulator of age-related pathologies in mice. *J Clin Invest* 123:1821-1832, 2013.

194. **Zierler K.** Whole body glucose metabolism. *Am J Physiol Endocrinol Metab* 276:E409-E426, 1999.

195. **Zimmer HG, Ibel H, Steinkopff G.** Studies on the hexose monophosphate shunt in the myocardium during development of hypertrophy. *Adv Myocardiol* 1:487-492, 1980.

196. **Zuurbier CJ, Eerbeek O, Meijer AJ.** Ischemic preconditioning, insulin, and morphine all cause hexokinase redistribution. *Am J Physiol Heart Circ Physiol* 289:H496-H499, 2005.

APPENDIX: The Role of VDAC in Cell Death: Friend or Foe?

Kyle S. McCommis and Christopher P. Baines

1. ABSTRACT

As the voltage-dependent anion channel (VDAC) forms the interface between mitochondria and the cytosol, its importance in metabolism is well understood. However, research on VDAC's role in cell death is a rapidly growing field, unfortunately with much confusing and contradictory results. The fact that VDAC plays a role in outer mitochondrial membrane permeabilization is undeniable, however, the mechanisms behind this remain very poorly understood. In this review, we will summarize the studies that show evidence of VDAC playing a role in cell death. To begin, we will discuss the evidence for and against VDAC's involvement in mitochondrial permeability transition (MPT) and attempt to clarify that VDAC is not an essential component of the MPT pore (MPTP). Next, we will evaluate the remaining literature on VDAC in cell death which can be divided into three models: proapoptotic agents escaping through VDAC, VDAC homo- or hetero-oligomerization, or VDAC closure resulting in outer mitochondrial membrane permeabilization through an unknown pathway. We will then discuss the growing list of modulators of VDAC activity that have been associated with induction/protection against cell death.

2. Introduction

The mitochondrial voltage-dependent anion channel (VDAC), also known as mitochondrial porin, is the most abundant protein in the outer mitochondrial membrane.

The VDAC family of proteins consists of three isoforms from three separate genes (VDAC1, VDAC2, and VDAC3). As most studies on VDAC using defined isoforms have been performed with VDAC1, the main focus of this review will be on VDAC1. However, significant findings from the other two isoforms will be noted. For this review, 'VDAC' will be used to reference studies of undefined isoforms.

While it has been established that VDACs regulate the ion and metabolite flux between mitochondria and cytosol [1,2,3], most other properties and even the structure of the channels are still highly debated and have been the focus of several past reviews [4,5,6,7,8]. Additionally, up-to-date reviews on VDAC structure, function, and regulation of metabolism can be found within this special issue. The purpose of this review is to highlight the role of VDAC and its regulators in mitochondrial membrane permeabilization and cell death.

3. VDAC and the Mitochondrial Permeability Transition Pore (MPTP)

Mitochondrial permeability transition (MPT) is the sudden permeabilization of the inner mitochondrial membrane in response to a noxious stimulus such as oxidative stress, Ca^{2+} overload, hypoxia, and cytotoxic drugs [9,10,11,12,13]. The degree of permeabilization is fairly substantial; although not large enough to allow the passage of proteins, solutes and metabolites up to 1.5 kD in size can now freely pass across the normally impermeable inner membrane. This includes protons such that the $\Delta\Psi m$ is dissipated upon MPT, thereby inhibiting ATP synthesis. Moreover, water can now move into the mitochondrial matrix, down its osmotic gradient, causing the mitochondrion to swell and, if left unchecked, rupture completely. MPT appears to play a critical role in

cell death, especially necrosis [14], and has been implicated in the development and progression of many diseases including ischemia/reperfusion injury, muscular dystrophy, Alzheimer's disease, and cardiotoxicity. MPT is believed to be mediated by opening of the MPT pore (MPTP), a highly debated, and undefined protein complex thought to span both the inner and outer mitochondrial membranes at membrane contact sites. Needless to say, due to the important role of MPT in disease pathogenesis there has been a concerted effort to try and elucidate the molecular makeup for the MPTP.

As VDAC is the most abundant protein in the outer mitochondrial membrane, it has long been considered a candidate for the outer membrane component of the MPTP. It was first proposed as an MPTP constituent nearly 20 years ago by Mario Zoratti's group who put forward that the electrical conductance properties of VDAC were similar to those described for the MPTP [15,16]. Moreover, it was well established that the MPTP was redox, Ca^{2+}, voltage, adenine nucleotide, and pH sensitive [10,11,12,13] – all attributes that applied to VDAC as well. However, what was strange was how this concept of VDAC as the outer membrane component of the MPTP slowly morphed from hypothesis to dogma without much in the way of substantiation in between.

As far as experimental data, initial support for a role for VDAC in MPT came from Crompton's laboratory, which demonstrated that a GST-CypD fusion protein was able to pull down VDAC along with the adenine nucleotide translocase (ANT), another putative MPTP component from mitochondrial lysates [17]. Reconstitution of this VDAC-ANT-CypD complex then resulted in a Ca^{2+}-dependent, cyclosporine-sensitive channel that was reminiscent of the MPTP [17]. Additional supporting evidence for VDAC's involvement in MPT came from experiments using putative VDAC inhibitors

and anti-VDAC antibodies. Monoclonal antibodies purported to block VDAC's channel activity were reported to prevent MPT in isolated mitochondria. [18,19]. Yet the specificity of these antibodies for VDAC has been cast into doubt [6]. A study by Cesura et al., also suggested that VDAC1 is the outer membrane component of the MPTP as radioactively labeled MPT inhibitor Ro 68-3400 was shown to bind a protein of ~32 kDa, which was identified as VDAC1 by mass spectrometry [20]. However, the same group has since demonstrated that Ro 68-3400 does not in fact bind to any VDAC isoform [21].

Certainly from a biochemical perspective VDAC would not fit very well into the MPTP paradigm. The "opened" configuration of VDAC is the most conductive, and shows a significant preference for anions, particularly metabolic anions (ATP/ADP) [6]. The "closed" state greatly diminishes, but does not abolish flux of metabolic anions, and now favors conductance of cations. If VDAC were part of the MPTP, it would make sense for VDAC closure to equal MPTP closure. However, Marco Colombini has demonstrated that closure of VDAC actually increases Ca^{2+} flux, which if anything should actually promote opening of the MPTP [80]. Indeed, Tikunov et al [23] utilized G3139, an 18-mer phosphorothioate blocker of VDAC to cause VDAC closure, and MPT was accelerated. Moreover, even its closed state, the VDAC channel is still large enough to pass solutes up to 1.5 kD in size, i.e., the closed state of VDAC is the same as the open state of the MPTP [6]. Using a similar approach to Crompton's initial reconstitution of a VDAC-containing MPTP, Halestrap's group was only able to pulldown ANT (and the mitochondrial phosphate carrier as it later turned out), but not VDAC with GST-CypD [24]. Yet this VDAC-less precipitate was still capable of generating an MPTP-like channel in liposomes [24].

Genetic studies have most recently been employed to decipher the role, if any, of VDAC in MPT. Kroemer's group reported that VDAC-deficient yeast were more resistant to HIV Vpr-induced MPT [25]. A similar loss of MPT response was observed in response to ethanol in ΔVDAC yeast mitochondria [26]. However, the nature of MPT, both in terms of properties and regulation, appears to be inherently different in yeast [27], thus making extrapolations to the mammalian system difficult. In this regard, we have observed an intact MPT response in isolated mouse mitochondria and cells essentially deficient for all three VDAC isoforms, suggesting that VDACs are dispensable for MPT and are not an essential component of the MPTP [28]. Paolo Bernardi's group has also reported a maintained MPT response in VDAC1$^{-/-}$ [21] and VDAC1/3$^{-/-}$ mitochondria [29]. Thus, in light of both the nonspecific VDAC "blocking" agents used in previous studies and the more recent findings of maintained MPT responses in VDAC-deficient null mitochondria, we must conclude that VDAC is not an essential component of the MPTP. We found that mitochondria and cells lacking VDAC have an exacerbated MPT and death response [28], suggesting that if anything VDAC appears to protect against, rather than contribute to, MPTP. Consistent with this concept, closure of VDAC using Koenig's polyanion, phosphorothioates, or the anion channel blocker DIDS, actually enhances the MPT response in isolated mitochondria [23,30,31].

4. VDAC and apoptosis

While evidence for a role of VDAC in outer mitochondrial membrane permeabilization and apoptosis persistently accumulates, the mechanisms behind VDAC's role remain very poorly understood. In fact, it is still debated whether VDAC

opening, or closure results in apoptosis [6]. The existing evidence of how VDAC is involved in outer membrane permeability and release of cytochrome c can be grouped into roughly three models: model #1) VDAC is part of the MPTP complex and pro-apoptotic agents therefore induce cytochrome c release indirectly through activation of the MPTP, model #2) VDAC homo- or hetero-oligomerization creating a larger pore capable of releasing cytochrome c, and model #3) VDAC closure resulting in a build up of mitochondrial metabolites, mitochondrial swelling, and either outer membrane permeabilization through an specific undefined mechanism or generalized rupture (**Figure 1**). In the "VDAC and MPTP" section above, we have already discussed the evidence that largely discredits model #1. We will next discuss the evidence both for and against models #2 and #3 of VDAC-mediated outer membrane permeabilization.

4.1 Model #2 – VDAC homo- or hetero-oligomerization leading to outer mitochondrial membrane permeabilization

Studies in several cell types overexpressing human, murine, yeast, *Paralichthys olivaceus*, or rice VDAC have observed increased apoptosis [32,33,34,35,36]. Two reports suggest elevated reactive oxygen species (ROS) production leads to the increase in cell death observed with VDAC overexpression [37,38], however, the mechanisms have not been thoroughly investigated. A recent report tested the viability of yeast cells transfected with the three human VDAC isoforms individually [39]. Interestingly, cells that expressed VDAC1 and VDAC2 showed similar levels of ROS and similar viability to wildtype yeast. However, yeast transformed with VDAC3 had higher levels of ROS and decreased viability (increased death) [39]. In contrast, we have shown that murine

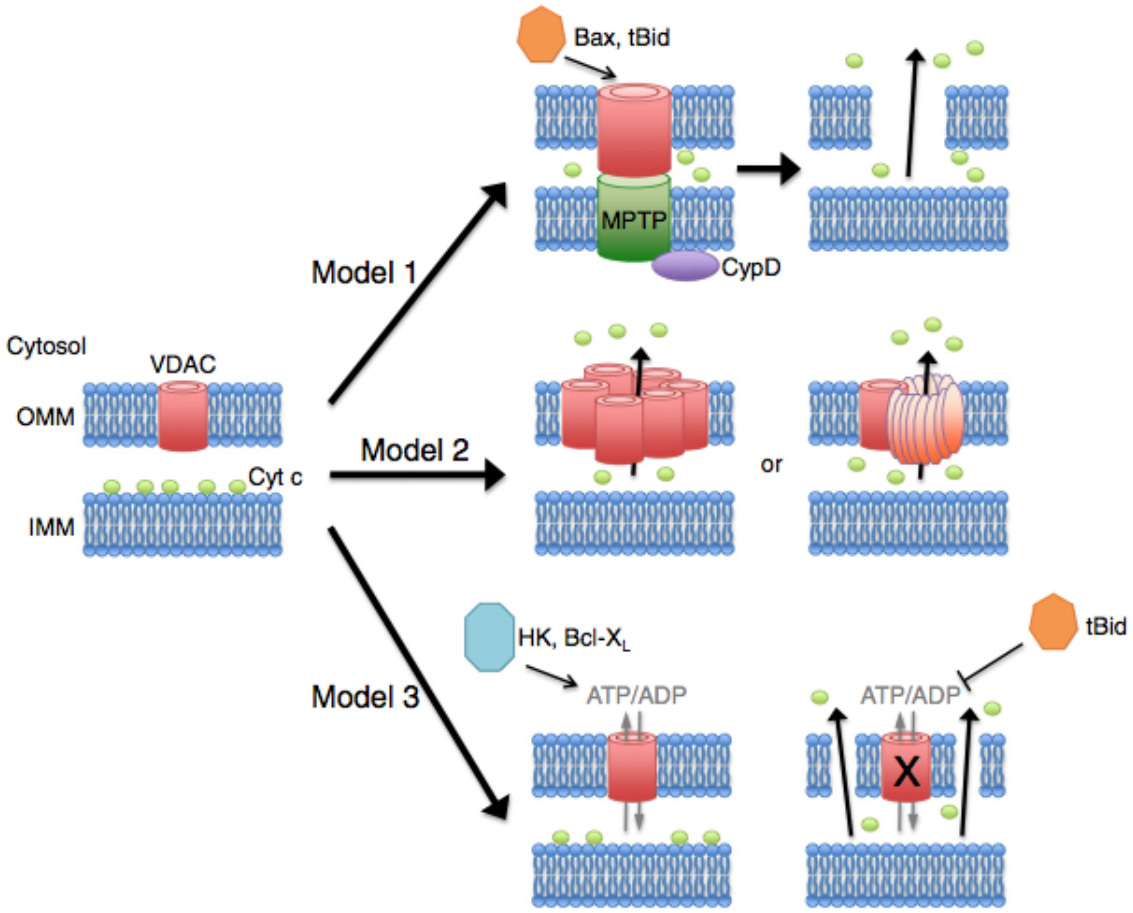

Figure 1. Proposed models for VDAC's role in outer mitochondrial membrane permeabilization and induction of apoptosis. In model #1, the VDAC is a component of the MPTP and therefore causes cytochrome c release indirectly through the swelling and rupture of mitochondria. This model has largely been discredited, as mitochondria lacking VDACs still possess a normal MPT response. Model #2 involves either homo-oligomerization of VDAC channels or hetero-oligomerization with VDAC and Bax/Bak to produce a large channel capable of releasing cytochrome c. However, this model is also highly questionable as cells devoid of all VDACs actually exhibit enhanced cell death in response to apoptotic stimuli, thus suggesting that VDAC plays a protective role against cell death. Evidence of VDAC's interaction with Bax or Bak is limited and has also been questioned. Model #3 details how anti-apoptotic agents modulate VDAC to keep it in the open state, maintaining adenine nucleotide flux, thus maintaining outer membrane permeability. On the other hand, a growing list of pro-apoptotic modulators of VDAC have been shown to close VDAC, inhibiting adenine nucleotide flux, causing mitochondrial permeability and cell death. This model #3 thus fits with concept of a functional VDAC channel being cytoprotective.

fibroblasts essentially devoid of all three VDAC isoforms are actually more sensitive to staurosporine-, and TNFα-induced death [28], suggesting that VDAC may actually play an anti-apoptotic, rather than pro-apoptotic, role.

One explanation for VDAC overexpression leading to increased cell death is that higher VDAC expression favors VDAC oligomerization (**Figure 1**, model #2). Indeed, arrays of VDAC in plant outer mitochondrial membranes were very early observations [40]. It has been suggested that oligomeric VDAC1 mediates the release of cytochrome c [41], since the internal diameter of a single VDAC pore is 2.5-3.0 nm, which is insufficient to pass a folded protein. VDAC1 has been shown to assemble into dimers, trimers, tetramers, and multimers [41,42,43,44,45,46]. Interestingly, overexpression of the anti-apoptotic protein Bcl-xL has been shown to prevent VDAC homo-dimerization [19].

VDAC has also been suggested to assemble into hetero-oligomers with pro- and anti-apoptotic Bcl-2 proteins. There are two models of VDAC associating with Bcl-2 family proteins (**Figure 1**). The first model involving Bcl-2 proteins (model #2) entails pro-apoptotic Bax binding to VDAC, creating a large VDAC-Bax channel, and causing cytochrome c release [18,26,47,48,49]. Model #2 concludes that, although the channels formed by Bax or VDAC alone are unable to translocate cytochrome c, the new larger Bax-VDAC channel is permeable to cytochrome c [47]. In complete disagreement with this model, mammalian VDAC reconstituted into planar phospholipid membranes has shown Bax to not have any effect on the properties of VDAC channels [50]. While monomeric Bax is found in the cytosol of healthy cells [51,52], tBid triggers Bax oligomerization [53,54] causing Bax to form channels in the outer membrane that allow

for cytochrome c release [55,56]. Neither the monomeric nor oligomeric form of Bax showed any interaction with VDAC channels [50]. Additionally, immunoprecipitations have failed to detect Bax-VDAC interaction [57], and the levels of Bax required for killing in VDAC1$^{-/-}$ yeast cells was similar to wild-type cells [58].

The VDAC2 isoform has been shown to bind the pro-apoptotic Bak, however, its role is still controversial. The first report by Cheng et al. [59] suggested that VDAC2, but not VDAC1, binds inactive Bak in normal healthy cells. Treatment with tBid released Bak from VDAC2, causing activation of Bak and apoptosis. In this study, VDAC2$^{-/-}$ cells had greater activation (oligomerization) of Bak, and increased sensitivity to apoptotic stimuli [59]. Bax was not shown to make complexes with VDAC2 even in apoptotic situations, which argues against model #2. Interestingly, in Bax-deficient cells, the VDAC2-Bak interaction is no longer observed with or without apoptotic stimulation [60]. A more recent study showed that VDAC2 is required for the formation of the inactive Bak complex, and that the Bak transmembrane anchor is required for interacting with VDAC2 [61]. Furthermore, another study suggests that VDAC2 is required for tBid-induced Bak activation and apoptosis [62]. This study showed that although VDAC1$^{-/-}$, VDAC3$^{-/-}$, and VDAC1/3$^{-/-}$ fibroblasts respond normally to tBid, VDAC2$^{-/-}$ fibroblasts are virtually insensitive to tBid-induced outer membrane permeabilization and apoptosis [62]. Additionally, VDAC2 may be required for Bax-induced outer membrane permeability and apoptosis in Bak$^{-/-}$ cells [63]. Thus, these studies suggest VDAC2 interacts with Bak and mediates Bak-activated apoptosis in a Bax- and tBid-dependent manner [60,62,64].

4.2 Model #3 – Proteins and metabolites alter VDAC conductance, resulting in outer membrane permeabilization through unknown mechanisms

In a new model (**Figure 1**, model #3), tBid was shown to induce VDAC closure, reducing adenine nucleotide exchange between mitochondria and cytosol, thus creating mitochondrial dysfunction [50]. Conversely, but also along the lines of model #3, the anti-apoptotic Bcl-xL has been shown to bind VDAC [44] and promote its open configuration and maintain ATP/ADP exchange [65]. Thus, model#3 describes how proteins and metabolites modulate VDAC conductance. VDAC closure prevents the exchange of ATP/ADP and all other larger respiratory metabolites, which then leads to outer membrane permeabilization and release of proteins from the intermembrane space [65,66]. It is unknown how the permeabilization occurs, either through undefined pathways or via swelling and rupture of the outer membrane. It is also not known how or if tBid and VDAC physically interact.

Although the mechanism(s) are unclear, it is apparent that VDACs modulate outer membrane permeability and therefore the release of intermembrane proteins and apoptosis. The next section of this review will detail several metabolites and non-Bcl-2 family proteins that have been shown to modulate VDAC permeability.

5. Regulators of VDAC permeability and their role in cell death

To date, a number of metabolites and proteins have been shown to bind and modulate VDAC conductance. However, the effects of altered VDAC permeability on cell death are still unclear. While ATP/ADP [67,68], glutamate [69], ROS [70], the 18 kDa translocator protein [71], and hexokinase [72] have all been suggested to modulate

MPT, it is easy to propose that these agents are working through VDAC to affect MPT (Model #1). However, as VDACs are dispensable for MPT [28,29], these agents must be working through another mechanism, perhaps by altering VDAC structure and preventing Bcl-2 protein interaction (Model #2) or by altering VDAC conductance and creating outer mitochondrial membrane permeability through an unknown mechanism or outer membrane rupture (Model #3) as discussed above.

5.1 Adenine nucleotides

As the main purpose of VDAC is to shuttle adenine nucleotides between the mitochondria and cytosol, it only makes sense that adenine nucleotides can modulate VDAC behavior. The discovery of at least two nucleotide-binding sites on VDAC [73,74,75] explains how NAD(P)H, ATP, and ADP can decrease VDAC channel conductance [3,76]. Indeed, ATP bound to the nucleotide-binding site on VDAC is suggested to sterically block VDAC [3,76]. Mechanistically, this interaction of VDAC with ATP/ADP and NAD(P)H explains how energetic pathway intermediates can modulate outer mitochondrial membrane permeability and respiration rates to adjust to cellular energy requirements.

The effect of adenine nucleotides on mitochondrial permeability (and subsequent cell death) has been thoroughly studied. Yehezkel et al [75] showed that T-Rex-293 cells expressing VDAC1 with a mutation in the nucleotide-binding site had severely diminished ATP synthesis and ATP levels. NAD(P)H, ADP, and particularly ATP inhibit the MPTP [67]. During ischemia, ATP and ADP are degraded to nucleosides and bases, and thus the inhibition of the MPTP is lost. Therefore, ischemia or other noxious stimuli

that decrease the exchange of adenine nucleotides through VDAC can cause MPT and subsequently, cell death. Thus, excess adenine nucleotides may decrease VDAC conductance, cause outer membrane permeabilization, resulting in cell death, which fits model #3 (**Figure 1**).

5.2 Glutamate

Much of a cells glutamate is formed in the mitochondria through the action of glutamate dehydrogenase on the TCA cycle intermediate α-ketoglutarate. The glutamate formed in the mitochondria can then be exported to the cytosol, crossing the outer membrane through VDAC. Studies suggest that VDAC contains a glutamate-binding site, as L-glutamate has been shown to cause VDAC to oscillate between the stable (more closed) and open conformations decreasing Ca^{2+} transport [69,77,78]. The decreased Ca^{2+} import caused by L-glutamate has been shown to inhibit MPTP opening, decrease mitochondrial swelling, as well as decrease cytochrome c release from mitochondria [69]. Thus, it appears that glutamate is protective against cell death by inhibiting mitochondrial Ca^{2+} import through VDAC. The decreased VDAC conductance caused by glutamate clearly fits with model #3 in that decreased VDAC conductance can result in outer membrane permeabilization.

5.3 Tubulin

Tubulin, the heterodimeric subunit of microtubules has been shown to bind mitochondria via VDAC [79,80]. Dimeric tubulin closes VDAC channels reconstituted into planar phospholipid membranes [81]. Tubulin decreased outer mitochondrial

membrane permeability to adenine nucleotides in isolated brain mitochondria and permeabilized cardiomyocytes [81,82]. Recently, the microtubule destabilizing compounds rotenone, colchicine, and nocodazole were shown to increase free tubulin and decrease the mitochondrial membrane potential; conversely, the microtubule stabilizer paclitaxel decreased free tubulin and hyperpolarized mitochondria of HepG2 cells (cancerous hepatoma cells) and primary rat hepatocytes [83]. Thus, free tubulin can decrease VDAC conductance and ultimately lead to outer mitochondrial membrane permeabilization and cell death, which fits model #3. This inhibition of VDAC by free tubulin limits mitochondrial respiration, and may explain the Warburg effect in cancer cells, which by nature require excess tubulin to support rapid division. Only time will tell if this interaction can be utilized to control cancer cell death *in vivo*.

5.4 Reactive oxygen species

Mitochondrially produced ROS has been shown to be involved in cell death, and Ca^{2+} accumulation by mitochondria is associated with mitochondrial ROS generation by mechanism(s) that are poorly understood [84,85]. Several studies suggest that VDAC plays a role in this mechanism. Mitochondrial-generated ROS has been shown to induce cytochrome c release, which is inhibited by VDAC blockers or anti-VDAC antibody [70,86]. Interestingly, the same effect is not seen with the MPTP inhibitor cyclosporine A [86]. ROS caused cardiolipin peroxidation, which was interpreted as the means by which cytochrome c was released from its cardiolipin-mediated attachment to the inner mitochondrial membrane [70]. However, how the cytochrome c is released and the role of VDAC in its release remains unknown. As discussed earlier, cytochrome c is too large

to be released through monomeric VDAC. Possible mechanisms include oligomeric VDAC forming a larger channel [41] (model #2), or that mitochondrial membrane lipid peroxidation impairs membrane function and alters VDAC properties, resulting in outer mitochondrial membrane permeabilization (model #3). Indeed, the gating properties of VDAC channels reconstituted into lipid bilayers were significantly affected by lipid composition [87].

5.5 Peripheral Benzodiazepine Receptor (PBR) or 18kDa translocator protein (TSPO)

The peripheral benzodiazepine receptor (PBR), now known as the 18kDa translocator protein (TSPO), is expressed on the outer mitochondrial membrane and mediates cholesterol import into the mitochondria for steroidogenesis [88]. TSPO interacts with VDAC on the outer membrane, and may alter its properties [89]. Importantly, the TSPO has been implicated in ROS production in models of neurodegeneration and cancer [90,91]. The proapoptotic agent erucylphosphohomcholine (ErPC3) was shown to increase ROS generation and cause oxidation of cardiolipin on the inner mitochondrial membrane, while the TSPO ligand PK11195 prevented this ROS generation and induction of apoptosis [71]. Interestingly, electron microscopy studies have found large groupings of the TSPO around VDAC channels [92], potentially increasing ROS concentrations in the proximity of VDAC. Therefore, the 18kDa TSPO likely affects VDAC conductance by production of ROS, causing outer membrane permeabilization (model #3).

5.6 Hexokinases

Perhaps the most studied regulator of VDAC function is the glycolytic enzyme hexokinase. A 21-amino-acid sequence in the N-termini of hexokinase isoforms 1 (HK1) and 2 (HK2) are predicted to form a hydrophobic α-helix, which is essential and sufficient for binding to mitochondria [93,94]. Interaction with VDAC is believed to cause the specificity of hexokinases binding to the outer mitochondrial membrane [95,96]. Binding of HK2 to isolated mitochondria or overexpression of HK2 in HeLa cells has been shown to inhibit the mitochondrial translocation of Bax and the release of cytochrome c [97]. Majewski et al [98] confirmed that mitochondrial HK inhibits cytochrome c release and apoptosis; however, this was also true in Bax- and Bak-deficient cells. This study suggested that mitochondrial hexokinase prevented VDAC closure. Thus, this study is in agreement with Vander Heiden et al [65,66] in that VDAC closure (due to HK detachment) leads to mitochondrial swelling and consequently to cell death via an undefined pathway. HK binding and inhibiting closure of VDAC therefore fits into model #3 and explains how mitochondrial-bound HK protects against outer mitochondrial membrane permeabilization and cell death.

Conversely, a direct inhibitory effect of HK1 has been reported on VDAC channels reconstituted into planar membranes [72]. This study showed that HK1 induced VDAC closure, and that addition of hexokinase's reaction product glucose-6-phosphate released HK from VDAC and allowed VDAC reopening [72]. These authors suggest that HK1-induced closure of VDAC inhibits MPTP opening, which is not in agreement with VDACs being inessential for MPT [28,29]. Biochemically, HK1-mediated closure of VDAC does not make sense, as VDAC closure would inhibit nucleotide exchange and

hinder the ATP supply by which HK1 depends on for its enzymatic activity. In support of this notion, hexokinases preferentially utilize intramitochondrially generated ATP from oxidative phosphorylation [99]. In fact, in most cells, mitochondrial respiration increases dramatically after addition of glucose, a phenomenon termed the Pasteur effect [100]. Therefore, HK1-mediated closure of VDAC causing decreased MPT argues against not only the biochemical activity of HK, but also does not fit with model #3. While this finding should be highly scrutinized, it does imply that the mechanism of how HK protects against cell death and what role, if any, VDAC plays in this mechanism still remains to be definitely described.

6. Questions raised by genetic models of VDAC deficiency/overexpression

One conundrum raised by the genetic ablation of VDAC isoforms, is how do mitochondria devoid of VDAC still function metabolically, and perhaps more importantly, what has changed? The same argument can be made for overexpression of VDAC isoforms. Certainly cells lacking essentially all 3 VDAC isoforms still seem to exist in culture relatively easily, although, as mentioned above and later on, they are more susceptible to death stimuli than their wildtype counterparts [6]. It is conceivable that other outer membrane anion channels such as the peripheral benzodiazepine receptor could compensate electrically and/or metabolically for the loss of VDACs, indicating that adaptations occur that could also influence cell death end-points. However, VDAC1$^{-/-}$ cells have enlarged mitochondria [6,101], and VDAC1$^{-/-}$ and VDAC1/VDAC3$^{-/-}$ muscle mitochondria have substantially less HK activity [102], suggesting that substantial mitochondrial alterations can occur. Thus, the question that arises is this: are the effects

(or lack thereof in the case of the MPTP) of VDAC depletion indicative of a primary role for VDAC in that process, or are they due to secondary changes in mitochondrial structure and/or function caused by the general impairment of metabolite transport across the outer membrane? Can these even be separated? For example, model #3 suggests that the role of VDAC in modulating cell death is entirely due to its ability to greatly influence metabolite flux across the outer membrane.

Another question that is raised is the concept of redundant versus non-redundant functions of each VDAC isoform. For the most part, one mammalian VDAC seems to be able to compensate for the lack of another [103]. This of course brings up the issue of a negative phenotype simply being due to redundancy, and that is certainly why we, and others, attempt to ablate all 3 VDAC isoforms in a cell at a given time. However, it is becoming more apparent that there are some functional differences between isoforms especially with regards to tissue-specificity. For instance, VDAC1 deletion affects mitochondrial respiration similarly in both heart and skeletal muscles, whereas VDAC3 ablation primarily only affects the mixed glycolytic/oxidative skeletal muscle [101,104]. The relative expression of each VDAC isoform also differs between tissues [105], which in turn could also influence mitochondrial function and, ultimately, cell death progression in a tissue-specific manner.

These are all issues that need to be considered when using any genetic model, but especially when there are several isoforms involved, both with regards to the designing and interpretation of experiments. The refinement of genetic approaches (tissue-specificity, temporal induction, reconstitution of selective isofoms etc.) should hopefully help address these issues.

7. Conclusions

Although VDAC undoubtedly plays a role in outer mitochondrial membrane permeabilization and induction of cell death, the mechanisms remain poorly understood. As we have discussed, VDAC on its own cannot induce apoptosis. The diameter of the VDAC pore cannot be drastically increased to allow passage of proapoptotic proteins. Likewise, while VDAC overexpression may increase VDAC homo-oligomerization, we have shown that mitochondria devoid of all VDACs have an increased MPT response, portraying not a pro-death role, but rather a pro-survival role for VDAC. Lastly, modulation of VDAC by cytosolic mediators is the most definitive mechanism for causing outer membrane permeabilization. Future research is needed to further characterize the mechanisms of these VDAC-modulating agents.

8. References

[1] G. Bathori, M. Sahin-Toth, A. Fonyo, E. Ligeti, Transport properties and inhibitor sensitivity of isolated and reconstituted porin differ from those of intact mitochondria, Biochim. Biophys. Acta. 1145 (1993) 168-176.

[2] A.C. Lee, X. Xu, M. Colombini, The role of pyridine dinucleotides in regulating the permeability of the mitochondrial outer membranes, J. Biol. Chem. 271 (1996) 26724-26731.

[3] T.K. Rostovtseva, A. Komarov, S.M. Bezukov, M. Colombini, Dynamics of nucleotides in VDAC channels: structure-specific noise generation, Biophys. J. 82 (2002) 193-205.

[4] V. De Pinto, S. Reina, F. Guarino, A. Messina, Structure of the voltage dependent anion channel: state of the art, J. Bioenerg. Biomembr. 40 (2008) 139-147.

[5] J.J. Lemasters, E. Holmuhamedov, Voltage-dependent anion channel (VDAC) as mitochondrial governator- thinking outside the box, Biochemica et Biophysica Acta. 1762 (2006) 181-190

[6] T.K. Rostovtseva, W. Tan, M. Colombini, On the role of VDAC in apoptosis: fact and fiction, J. Bioenerg. Biomembr. 37 (2005) 129-142.

[7] V. Shoshan-Barmatz, N. Keinan, H. Zaid. Uncovering the role of VDAC in the regulation of cell life and death, J. Bioenerg. Biomembr. 40 (2008) 183-191.

[8] K. Zeth, M. Thein, Porins in prokaryotes and eukaryotes: common themes and variations, Biochem. J. 431 (2010) 13-22.

[9] C.P. Baines, The cardiac mitochondrion: nexus of stress. Annu. Rev. Physiol. 72 (2010) 61-80.

[10] F. Di Lisa, M. Canton, R. Menabo, N. Kaludercic, P. Bernardi, Mitochondria and cardioprotection, Heart Fail. Rev. 12 (2007) 249-260.

[11] A.P. Halestrap, S.J. Clarke, S.A. Javadov, Mitochondrial permeability transition pore opening during myocardial reperfusion-a target for cardioprotection, Cardiovasc. Res. 61 (2004) 372-385.

[12] E. Murphy, C. Steenbergen, Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury, Physiol. Rev. 88 (2008) 581-609.

[13] J.N. Weiss, P. Korge, H.M. Honda, P. Ping, Role of the mitochondrial permeability transition in myocardial disease, Circ. Res. 93 (2003) 292-301.

[14] C.P. Baines, The mitochondrial permeability transition pore and the cardiac necrotic program, Pediatr. Cardiol. 32 (2011) 258-262.

[15] I. Szabó, M. Zoratti. The mitochondrial permeability transition pore may comprise VDAC molecules. I. Binary structure and voltage dependence of the pore. FEBS Lett. 330 (1993) 201-205.

[16] I. Szabó, V. De Pinto, M. Zoratti. The mitochondrial permeability transition pore may comprise VDAC molecules. II. The electrophysiological properties of VDAC are compatible with those of the mitochondrial megachannel. FEBS Lett. 330 (1993) 206-210.

[17] M. Crompton, S. Virji, J.M. Ward, Cyclophilin-D binds strongly to complexes of the voltage-dependent anion channel and the adenine nucleotide translocase to form the permeability transition pore, Eur. J. Biochem. 258 (1998) 729-735.

[18] S. Shimizu, Y. Matsuoka, Y. Shinohara, Y. Yoneda, Y. Tsujimoto, Essential role of voltage-dependent anion channel in various forms of apoptosis in mammalian cells, J. Cell Biol. 152 (2001) 237-250.

[19] Y. Zheng, Y. Shi, C. Tian, C. Jiang, H. Jin, J. Chen, A. Almasan, H. Tang, Q. Chen, Essential role of the voltage-dependent anion channel (VDAC) in mitochondrial permeability transition pore opening and cytochrome c release by arsenic trioxide, Oncogene 23 (2004) 1239-1247.

[20] A.M. Cesura, E. Pinard, R. Schubenel, V. Goetschy, A. Friedlein, H. Langen, P. Polcic, M.A. Forte, P. Bernardi, J.A. Kemp, The voltage-dependent anion channel in the target for a new class of inhibitors of the mitochondrial permeability transition pore, J. Biol. Chem. 278 (2003) 49812-49818.

[21] A. Krauskopf, O. Eriksson, W.J. Craigen, M.A. Forte, P. Bernardi, Properties of the permeability transition in VDAC1(-/-) mitochondria, Biochim. Biophys. Acta. 1757 (2006) 590-595.

[22] W. Tan, M. Colombini. VDAC closure increases calcium ion flux. Biochim. Biophys. Acta. 1768 (2007) 2510-2515.

[23] A. Tikunov, C.B. Johnson, P. Pediaditakis, N. Markevich, J.M. Macdonald, J.J. Lemasters, E. Holmuhamedov. Closure of VDAC causes oxidative stress and accelerates the Ca^{2+}-induced mitochondrial permeability transition in rat liver mitochondria. Arch. Biochem. Biophys. 495 (2010) 174-181.

[24] K. Woodfield, A. Ruck, D. Brdiczka, A.P. Halestrap, Direct demonstration of a specific interaction between cyclophilin-D and the adenine nucleotide translocase confirms their role in the mitochondrial permeability transition, Biochem. J. 336 (1998) 287-290.

[25] E. Jacotot, L. Ravagnan, M. Loeffler, K.F. Ferri, H.L. Vieira, N. Zamzami, P. Costantini, S. Druillennec, J. Hoebeke, J.P. Briand, T. Irinopoulou, E. Daugas, S.A. Susin, D. Cointe, Z.H. Xie, J.C. Reed, B.P. Roques, G. Kroemer. The HIV-1 viral protein R induces apoptosis via a direct effect on the mitochondrial permeability transition pore. J. Exp. Med. 191 (2000):33-46.

[26] S. Shimizu, Y. Shinohara, Y. Tsujimoto, Bax and Bcl-xL independently regulate apoptotic changes of yeast mitochondria that require VDAC but not the adenine nucleotide translocator, Oncogene 19 (2000) 4309-4318.

[27] S. Uribe-Carvajal, L.A. Luévano-Martínez, S. Guerrero-Castillo, A. Cabrera-Orefice, N.A. Corona-de-la-Peña, M. Gutiérrez-Aguilar. Mitochondrial Unselective Channels throughout the eukaryotic domain. Mitochondrion 11 (2011) 382-390.

[28] C.P. Baines, R.A. Kaiser, T. Sheiko, W.J. Craigen, J.D. Molkentin, Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death, Nat. Cell Biol. 9 (2007) 550-555.

[29] F. Chiara, D. Castellaro, O. Marin, V. Petronilli, W.S. Brusilow, M. Juhaszova, S.J. Sollott, M. Forte, P. Bernardi, A. Rasola, Hexokinase II detachment from mitochondria triggers apoptosis through the permeability transition pore independent of voltage-dependent anion channels, PLoS ONE. 3 (2008) e1852.

[30] C.F. Bernardes, J.R. Meyer-Fernandes, D.S. Basseres, R.F. Castilho, A.E. Vercesi. Ca^{2+}-dependent permeabilization of the inner mitochondrial membrane by 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), Biochim. Biophys. Acta. 1188 (1994) 93-100.

[31] N. Brustovetsky, T. Brustovetsky, R. Jemmerson, J.M. Dubinsky. Calcium-induced cytochrome c release from CNS mitochondria is associated with the permeability transition and rupture of the outer membrane. J. Neurochem. 80 (2002) 207-218.

[32] S. Abu-Hamad, H. Zaid, A. Israelson, E. Nahon, V. Shoshan-Barmatz, Hexokinase-I protection against apoptotic cell death is mediated via interaction with the voltage-dependent anion channel-1: mapping the site of binding, J. Biol. Chem. 283 (2008) 13482-13490.

[33] T. Ghosh, N. Pandey, A. Maitra, S.K. Brahmachari, B. Pillai, Role for voltage-dependent anion channel VDAC1 in polyglutamine-mediated neuronal cell death, PLoS ONE. 2 (2007) e1170.

[34] A. Godbole, J. Varghese, A. Sarin, M.K. Mathew, VDAC is a conserved element of death pathways in plant and animal systems, Biochim. Biophys. Acta. 1642 (2003) 87-96.

[35] A.J. Lu, C.W. Dong, C.S. Du, Q.Y. Zhang, Characterization and expression analysis of Paralichthys olivaceus voltage-dependent anion channel (VDAC) gene in response to virus infection, Fish Shellfish Immunol. 23 (2007) 601-613.

[36] H. Zaid, S. Abu-Hamad, A. Israelson, I. Nathan, V. Shoshan Barmatz, The voltage-dependent anion channel-1 modulates apoptotic cell death, Cell Death Differ. 12 (2005) 751-760.

[37] E. Simamura, K. Hirai, H. Shimada, J. Koyama, Y. Niwa, S. Shimizu, Furanonaphthoquinones cause apoptosis of cancer cells by inducing the production of reactive oxygen species by the mitochondrial voltage-dependent anion channel, Cancer Biol. Ther. 5 (2006) 1523-1529.

[38] S. Yuan, Y. Fu, X. Wang, H. Shi, Y. Huang, X. Song, L. Li, N. Song, Y. Luo, Voltage-dependent anion channel 1 is involved in endostatin-induced endothelial cell apoptosis, FASEB J. 22 (2008) 2809-2820.

[39] V. De Pinto, F. Guarino, A. Guarnera, A. Messina, S. Reina, F.M. Tomasello, V. Palermo, C. Mazzoni, Characterization of human VDAC isoforms: A peculiar function for VDAC3?, Biochim. Biophys. Acta. 1797 (2010) 1268-1275.

[40] D.F. Parsons, W.D. Bonner, J.G. Verboon, Electron microscopy of isolated plant mitochondria and plastids using both the thin-section and negative-staining techniques, Can. J. Bot. 43 (1965) 647-655.

[41] R. Zalk, A. Israelson, E.S. Garty, H. Azoulay-Zohar, V. Shoshan-Barmatz, Oligomeric states of the voltage-dependent anion channel and cytochrome c release from mitochondria, Biochem. J. 386 (2005) 73-83.

[42] R.P. Gonclaves, N. Buzhynskyy, V. Prima, J.N. Sturgis, S. Scheuring, Supramolecular assembly of VDAC in native mitochondrial outer membranes, J. Mol. Biol. 369 (2007) 413-418.

[43] B.W. Hoogenboom, K. Suda, A. Engel, D. Fotadis, The supramolecular assemblies of voltage-dependent anion channels in the native membrane, J. Mol. Biol. 370 (2007) 246-255.

[44] T.J. Malia, G. Wagner, NMR structural investigation of the mitochondrial outer membrane protein VDAC and its interaction with antiapoptotic Bcl-xL, Biochemistry 46 (2007) 514-525.

[45] Y. Shi, C. Jiang, Q. Chen, H. Tang, One-step on-column affinity refolding purification and functional analysis of recombinant human VDAC1, Biochem. Biophys. Res. Commun. 303 (2003) 475-482.

[46] V. Shoshan-Barmatz, R. Zalk, D. Gincel, N. Vardi, Subcellular localization of VDAC in mitochondria and ER in the cerebellum, Biochim. Biophys. Acta. 1657 (2004) 105-114.

[47] S. Shimizu, T. Ide, T. Yanagida, Y. Tsujimoto, Electrophysiological study of a novel large pore formed by Bax and the voltage-dependent anion channel that is permeable to cytochrome c, J. Biol. Chem. 275 (2000) 12321-12325.

[48] S. Shimizu, A. Konishi, T. Kodama, Y. Tsujimoto, BH4 domain of antiapoptotic Bcl-2 family members closes voltage-dependent anion channel and inhibits apoptotic mitochondrial changes and cell death, Proc. Natl. Acad. Sci. USA. 97 (2000) 3100-3105.

[49] S. Shimizu, M. Narita, Y. Tsujimoto, Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC, Nature 399 (1999) 483-487.

[50] T.K. Rostovtseva, B. Antonsson, M. Suzuki, R.J. Youle, M. Colombini, S.M. Bezrukov, Bid, but not Bax, regulates VDAC channels, J. Biol. Chem. 279 (2004) 13575-13583.

[51] B. Antonsson, S. Montessuit, S. Lauper, R. Eskes, J.C. Martinou, Bax oligomerization is required for channel-forming activity in liposomes and to trigger cytochrome c release from mitochondria. Biochem. J. 345 (2000) 271-278.

[52] Y.T. Hsu, R.J. Youle, Bax in murine thymus is a soluble monomeric protein that displays differential detergent-induced conformations, J. Biol. Chem. 273 (1998) 10777-10783.

[53] R. Eskes, S. Desagher, B. Antonsson, J.C. Martinou, Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane, Mol. Cell Biol. 20 (2000) 929-935.

[54] M.C. Wei, T. Lindsten, V.K. Mootha, S. Weiler, A. Gross, M. Ashiya, C.B. Thompson, S.J. Korsmeyer, tBid, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c, Genes Dev. 14 (2000) 2060-2071.

[55] B. Antonsson, F. Conti, A. Ciavatta, S. Montessuit, S. Lewis, I. Martinou, L. Bernasconi, A. Bernard, J.J. Mermod, G. Mazzei, K. Maundrell, F. Gambale, R. Sadoul, J.C. Martinou, Inhibition of Bax channel-forming activity by Bcl-2, Science 277 (1997) 370-372.

[56] P.H. Schlesinger, A. Gross, X.M. Yin, K. Yamamoto, M. Saito, G. Waksman, S.J. Korsmeyer, Comparison of the ion channel characteristics of proapoptotic BAX and antiapoptotic BCL-2, Proc. Natl. Acad. Sci. USA. 94 (1997) 11357-11362.

[57] V. Mikhailov, M. Mikhailova, D.J. Pulkrabek, Z. Dong, M.A. Venkatachalam, P. Saikumar, Bcl-2 prevents Bax oligomerization in the mitochondrial outer membrane, J. Biol. Chem. 276 (2001) 18361-18374.

[58] P. Polcic, M. Forte, Response of yeast to the regulated expression of proteins in the Bcl-2 family, Biochem. J. 374 (2003) 393-402.

[59] E.H.Y. Cheng, T.V. Sheiko, J.K. Fisher, W.J. Craigen, S.J. Korsmeyer, VDAC2 inhibits Bak activation and mitochondrial apoptosis, Science 301 (2003) 513-517.

[60] D. Chandra, G. Choy, P.T. Daniel, D.G. Tang, Bax-dependent regulation of Bak by voltage-dependent anion channel 2, J. Biol. Chem. 280 (2005) 19051-19061.

[61] M. Lazarou, D. Stojanovski, A.E. Frazier, A. Kotevski, G. Dewson, W.J. Craigen, R.M. Kluck, D.L. Vaux, M.T. Ryan, Inhibition of Bak activation by VDAC2 is dependent on the Bak transmembrane anchor, J. Biol. Chem. 285 (2010) 36876-36883.

[62] S.S. Roy, A.M. Ehrlich, W.J. Craigen, G. Hajnoczky, VDAC2 is required for truncated BID-induced mitochondrial apoptosis by recruiting Bak to the mitochondria, EMBO Rep. 10 (2009) 1341-1347.

[63] H. Yamagata, S. Shimizu, Y. Nishida, Y. Watanabe, W.J. Craigen, Y. Tsujimoto, Requirement of voltage-dependent anion channel 2 for pro-apoptotic activity of Bax, Oncogene 28 (2009) 3563-3572.

[64] K. Setoguchi, H. Otera, K. Mihara, Cytosolic factor- and TOM-independent import of C-tail-anchored mitochondrial outer membrane proteins, EMBO J. 25 (2006) 5635-5647.

[65] M.G. Vander Heiden, X.X. Li, E. Gottleib, R.B. Hill, C.B. Thompson, M. Colombini, Bcl-xL promotes the open configuration of the voltage-dependent anion channel and metabolite passage through the outer mitochondrial membrane, J. Biol. Chem. 276 (2001) 19414-19419.

[66] M.G. Vander Heiden, N.S. Chandel, X.X. Li, P.T. Schumacher, M. Colombini, C.B. Thompson, Outer mitochondrial membrane permeability can regulate coupled respiration and cell survival, Proc. Natl. Acad. Sci. USA. 97 (2000) 4666-4671.

[67] M. Crompton, The mitochondrial permeability transition pore and its role in cell death, Biochem J. 341 (1999) 233-249.

[68] M. Zoratti, I. Szabo, The mitochondrial permeability transition, Biochim. Biophys. Acta. 1241 (1995) 139-176.

[69] D. Gincel, V. Shoshan-Barmatz, Glutamate interacts with VDAC and modulates opening of the mitochondrial permeability transition pore, J. Bioenerg. Biomembr. 36 (2004) 179-186.

[70] G. Petrosillo, F.M. Ruggiero, M. Pistolese, G. Paradies, Ca2+-induced reactive oxygen species production promotes cytochrome c release from rat liver mitochondria via mitochondrial permeability transition (MPT)-dependent and MPT-independent mechanisms: role of cardiolipin, J. Biol. Chem. 279 (2004) 53103-53108.

[71] L. Veenman, Y. Shandalov, M. Gavish, VDAC activation by the 18 kDa translocator protein (TSPO), implications for apoptosis, J. Bioenerg. Biomembr. 40 (2008) 199-205.

[72] H. Azoulay-Zohar, A. Israelson, S. Abu-Hamad, V. Shoshan-Barmatz, In self-defense: hexokinase promotes voltage-dependent anion channel closure and prevents mitochondria-mediated apoptotic cell death, Biochem. J. 377 (2004) 347-355.

[73] H. Florke, F.P. Thinnes, H. Winkelbach, U. Stadtmuller, G. Paetzold, C. Morys-Wortmann, D. Hesse, H. Sternbach, B. Zimmermann, P. Kaufmann-Kolle, Channel active mammalian porin, purified from crude membrane fractions of human B lymphocytes and bovine skeletal muscle, reversibly binds adenosine triphosphate (ATP), Biol. Chem. Hoppe Seyler 375 (1994) 513-520.

[74] G. Yehezkel, N. Hadad, H. Zaid, S. Sivan, V. Shoshan-Barmatz, Nucleotide-binding sites in the voltage-dependent anion channel: characterization and localization, J. Biol. Chem. 281 (2006) 5938-5946.

[75] G. Yehezkel, S. Abu-Hamad, V. Shoshan-Barmatz, An N-terminal nucleotide-binding site in VDAC1: involvement in regulating mitochondrial function, J. Cell Physiol. 212 (2007) 551-561.

[76] T.K. Rostovtseva, S.M. Bezrukoz, Atp transport through a single mitochondrial channel, VDAC, studied by current fluctuation analysis, Biophys. J. 74 (1998) 2365-2373.

[77] A. Deniaud, C. Rossi, A. Berquand, J. Homand, S. Campagna, W. Knoll, C. Brenner, J. Chopineau, Voltage-dependent anion channel transports calcium ions through biomimetic membranes, Langmuir 23 (2007) 3898-3905.

[78] D. Gincel, S.D. Silberberg, V. Shoshan-Barmatz, Modulation of the voltage-dependent anion channel (VDAC) by glutamate, J. Bioenerg. Biomembr. 32 (2000) 571-583.

[79] F. Bernier-Valentin, B. Rousset, Interaction of tubulin with rat liver mitochondria, J. Biol. Chem. 257 (1982) 7092-7099.

[80] M. Carre, N. Andre, G. Carles, H. Borghi, L. Brichese, C. Briand, D. Braguer, Tubulin is an inherent component of mitochondrial membranes that interacts with the voltage-dependent anion channel, J. Biol. Chem. 277 (2002) 33664-33669.

[81] T.K. Rostovtseva, K.L Sheldon, E. Hassanzadeh, C. Monge, V. Saks, S.M. Bezrukov, D.L. Sackett, Tubulin binding blocks mitochondrial voltage-dependent anion channel and regulates respiration, Proc. Natl. Acad. Sci. USA. 105 (2008) 18746-18751.

[82] N. Timohhina, R. Guzman, K. Tepp, C. Monge, M. Varikmaa, H. Vija, P. Sikk, T. Kaambre, D. Sackett, V. Saks, Direct measurement of energy fluxes from mitochondria into cytoplasm in permeabilized cardiac cells in situ: some evidence for mitochondrial interactosome, J. Bioenerg. Biomembr. 41 (2009) 259-75.

[83] E.N. Maldonaldo, J. Patnaik, M.R. Mullins, J.J. Lemasters, Free tubulin modulates mitochondrial membrane potential in cancer cells, Cancer Res. 70 (2010) 10192-10201.

[84] A.J. Kowaltowski, R.F. Castilho, A.E. Vercesi, Ca(2+)-induced mitochondrial membrane permeabilization: role of coenzyme Q redox state, Am. J. Physiol. Cell Physiol. 269 (1995) C141-147.

[85] A.A. Starkov, B.M. Polster, G. Fiskum, Regulation of hydrogen peroxide production by brain mitochondria by calcium and Bax, J. Neurochem. 83 (2002) 220-228.

[86] M. Madesh, G. Hajnoczky, VDAC-dependent permeabilization of the outer mitochondrial membrane by superoxide induces rapid and massive cytochrome c release, J. Cell Biol. 155 (2001) 1003-1015.

[87] T.K. Rostovtseva, N. Kazemi, M. Weinrich, S.M. Bezrukov, Voltage gating of VDAC is regulated by nonlamellar lipids of mitochondrial membranes, J. Biol. Chem. 281 (2005) 37496-37506.

[88] H. Li, V. Papadopoulos, Peripheral-type benzodiazepine receptor function in cholesterol transport. Identification of a putative cholesterol recognition/interaction amino acid sequence and consensus pattern, Endocrinology 139 (1998) 4991-4997.

[89] M.W. McEnery, A.M. Snowman, R.R. Trifiletti, S.H. Snyder, Isolation of the mitochondrial benzodiazepine receptor: association with the voltage-dependent anion channel and the adenine nucleotide carrier, Proc. Natl. Acad. Sci. USA. 89 (1992) 3170-3174.

[90] V. Papadopoulos, M. Baraldi, T.R. Guilarte, T.B. Knudsen, J.J. Lacapere, P. Lindemann, M.D. Norenberg, D. Nutt, A. Weizman, M.R. Zhang, M. Gavish,

Translocator protein (18kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function, Trends Pharmacol. Sci. 27 (2006) 402-409.

[91] L. Veenman, M. Gavish, The peripheral-type benzodiazepine receptor and the cardiovascular system. Implications for drug development, Pharmacol. Ther. 110 (2006) 503-524.

[92] V. Papadopoulos, N. Boujrad, M.D. Ikonomovic, P. Ferrara, B. Vidic, Topography of the Leydig cell mitochondrial peripheral-type benzodiazepine receptor, Mol. Cell Endocrinol. 104 (1994) R5-9.

[93] J.C. Xie, J.E. Wilson, Rat brain hexokinase: the hydrophobic N-terminus of the mitochondrially bound enzyme is inserted in the lipid bilayer, Arch. Biochem. Biophys. 267 (1988) 803-810.

[94] D. Sui, J.E. Wilson, Structural determinants for the intracellular localization of the isozymes of mammalian hexokinase: intracellular localization of fusion constructs incorporating structural elements from the hexokinase isozymes and the green fluorescent protein, Arch. Biochem. Biophys. 345 (1997) 111-125.

[95] C. Fiek, R. Benz, N. Roos, D. Brdiczka, Evidence for identity between the hexokinase-binding protein and the mitochondrial porin in the outer membrane of rat liver mitochondria, Biochem. Biophys. Acta. 688 (1982) 429-440.

[96] M. Linden, P. Gellerfors, B.D. Nelson, Pore protein and the hexokinase binding protein from the outer membrane of rat liver mitochondria are identical, FEBS Lett. 141 (1982) 189-192.

[97] J.G. Pastorino, N. Shulga, J.B. Hoek, Mitochondrial binding of hexokinase II inhibits Bax-induced cytochrome c release and apoptosos, J. Biol. Chem. 277 (2002) 7610-7618.

[98] N. Majewski, V. Nogueira, P. Bhasker, P.E. Coy, J.E. Skeen, K. Gottlob, N.S. Chandel, C.B. Thompson, R.B. Robey, N. Hay, Hexokinase-mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak, Mol. Cell 16 (2004) 819-830.

[99] K.K. Arora, P.L. Pederson, Functional significance of mitochondrial bound hexokinase in tumor cell metabolism. Evidence for preferential phosphorylation of glucose by intramitochondrially generated ATP, J. Biol. Chem. 263 (1988) 17422-17428.

[100] E. Racker, History of the Pasteur effect and its pathobiology, Mol. Cell Biochem. 5 (1974) 17-23.

[101] K. Anflous, D.D. Armstrong, W.J. Craigen, Altered mitochondrial sensitivity for ADP and maintenance of creatine-stimulated respiration in oxidative striated muscles from VDAC1-deficient mice. J. Biol. Chem. 276 (2001) 1954-1960.

[102] K. Anflous-Pharayra, Z.J. Cai, W.J. Craigen, VDAC1 serves as a mitochondrial binding site for hexokinase in oxidative muscles. Biochim. Biophys. Acta. 1767 (2007) 136-142.

[103] S. Wu, M.J. Sampson, W.K. Decker, W.J. Craigen, Each mammalian mitochondrial outer membrane porin protein is dispensable: effects on cellular respiration. Biochim. Biophys. Acta. 1452 (1999) 68-78.

[104] K. Anflous-Pharayra, N. Lee, D.L. Armstrong, W.J. Craigen, VDAC3 has differing mitochondrial functions in two types of striated muscles, Biochim. Biophys. Acta. 1807 (2011) 150-156.

[105] T. Yamamoto, A. Yamada, N. Watanabe, Y. Yoshimura, N. Yamazaki, Y. Yoshimura, T. Yamauchi, M Kataoka, T. Nagata, H. Terada, Y. Shinohara, VDAC1, having a shorter N-terminus than VDAC2 but showing the same migration in an SDS-polyacrylamide gel, is the predominant form expressed in mitochondria of various tissues. J. Proteome Res. 5 (2006) 3336-3344.

VITA

Kyle Stephan McCommis, son of Jim and Laura McCommis, was born in St. Louis, Missouri on November 24, 1984. He received a Bachelor of Science degree in Cell Biology from the University of Kansas in 2006. He then worked as a research technician in the Cardiovascular Imaging Laboratory at the Washington University School of Medicine until July 2009. Kyle then entered into the Biomedical Sciences PhD program in August 2009, where he joined the laboratory of Dr. Christopher Baines. In May 2011, Kyle was awarded an American Heart Association predoctoral fellowship, which assisted in funding this research. Kyle has accepted a postdoctoral research fellowship in the Nutritional Sciences division of the Department of Internal Medicine at Washington University in St. Louis, under the guidance of Dr. Brian Finck.