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The Role of Mitochondria ATP-sensitive Potassium Channel in Cardioprotection

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THESIS APPROVAL

The abstract and thesis of Ksenia Andrukhiv Everton for the Master of Science in Biology were presented on May 13, 2008, and accepted by the thesis committee and the department.

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ABSTRACT

An abstract of the thesis of Ksenia Andrukhiv Everton for the Master of Science in Biology presented on May 13, 2008.

Title: The role of mitochondrial ATP-sensitive potassium channel in cardioprotection.

Mitochondrial ATP-sensitive potassium channel (mitoK$_{ATP}$) has been suggested to be the mediator of cardiac preconditioning. All of the diverse pharmacological and physiological agents that open mitoK$_{ATP}$ provide protection against ischemia-reperfusion injury. Some investigators have pointed out that some of these mitoK$_{ATP}$ channel openers also inhibit succinate dehydrogenase (SDH), complex II of the electron transport chain. Based on this observation they suggest that it is the inhibition of SDH, and not the opening of mitoK$_{ATP}$ channel, that mediates the observed cardioprotection. In this study, I examined four chemically distinct and unrelated pharmacological agents, diazoxide, 3-Nitropropionic Acid (3-NPA), Protein Kinase G (PKG), and ΨεRACK, all of which have been shown to open mitoK$_{ATP}$, to demonstrate that the said cardioprotective effect mediated by mitoK$_{ATP}$ is entirely independent of SDH. Light scattering technique was utilized to measure the

state of mitoK$_{ATP}$ (open/closed) by measuring mitochondrial volume. An electrode that measures oxygen concentration was utilized to measure mitochondrial respiration. The results of this study confirm that two of the drugs, diazoxide and 3-NPA, inhibit succinate-supported respiration in high doses (IC50 = 140μM and IC50 = 1.05mM, respectively). Both of these drugs, however, do not inhibit succinate-supported respiration at the concentration necessary to open mitoK$_{ATP}$ (30μM and 10nM, respectively). PKG and ΨεRACK, the other two mitoK$_{ATP}$ openers tested in this study, do not inhibit succinate-supported respiration at any concentration tested. These results support the hypothesis that potassium channel openers mediate cardioprotection through the opening of mitoK$_{ATP}$ and not through the inhibition of SDH.

THE ROLE OF MITOCHONDRIA ATP-SENSITIVE POTASSIUM CHANNEL IN CARDIOPROTECTION

by

KSENIA ANDRUKHIV EVERTON

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LIST OF ABBREVIATIONS

3-NPA	3-Nitropropionic Acid
5-HD	5-hydroxydecanoate
ADP	Adenosine diphosphate
AIF	Apoptose-inducing Factor
APD	Action potential duration
ATP	Adenosine triphosphate
cGMP	Cyclic guanylyl monophosphate
CPC	Calcium preconditioning
IMS	Inter-membrane space
IPC	Ischemic preconditioning
KCOs	Potassium channel openers
MitoK_{ATP}	Mitochondrial ATP-sensitive potassium channel
MPT	Mitochondrial Permeability Transition Pore
NHE	Sodium Hydrogen Exchanger
PKC	Protein Kinase C
PKG	Protein Kinase G (cGMP-dependent protein kinase)
PMA	Phorbol 12-myristate 13-acetate
ROS	Reactive Oxygen Species
sarcK_{ATP}	Sarcoplasmic ATP-sensitive potassium channel
SDH	Succinate Dehydrogenase (Complex II)
VDAC	Voltage-dependent ion channel

1. Introduction and Literature Review

1.1 Heart Attacks and Infarct

Heart attacks and their associated complications are the number one cause of death in the United States and worldwide. According to the American Heart Association there are over a million new and recurrent coronary attacks per year, and of these about 38 percent will result in death [1]. In addition, World Health Organization reported that acute myocardial infarction is the leading cause of death throughout the world in both men and women. During coronary thrombosis, commonly associated with atherosclerotic plaque in blood vessels, blood flow through the circulatory system is obstructed resulting in lack of nutrients and oxygen to the heart. Prolonged lack of nutrition may lead to tissue death, also known as infarction. Infarction and other known damage-causing aspects of a heart attack are induced by apoptotic and necrotic cell death mechanisms. This tissue damage is irreversible and begins within approximately 20 minutes of occlusion. Because infarcted tissue cannot regenerate and thus permanently decreases the size of functional cardiac tissue, return of blood flow should be accomplished as soon as possible [2].

1.2 Normoxic Mitochondria

Mitochondria are highly abundant eukaryotic organelles that have been essential to the evolution of complex organisms and are the site of aerobic respiration. They are bound by two specialized membranes (Figure 1) that together create two mitochondrial compartments: intermembrane space (between the two membranes) and the internal matrix (within the inner membrane). The matrix is the site of the citric-acid cycle and the highly convoluted inner membrane is the site of oxidative phosphorylation and the locus of mitochondrial ATP-sensitive potassium channel (mitoK_{ATP}). The outer membrane contains large numbers of integral proteins called porins, which form large aqueous channels through the lipid bilayer making it highly permeable to molecules of 5000 daltons or less. The inner membrane, on the other hand, is highly selective and largely impermeable to ions. Permeability to ADP, ATP, substrate anions and to Na^+, K^+ and Ca^{2+} is mediated largely by specific channels and carriers. A tight proton-electrochemical gradient is maintained across this membrane in order to support the mechanism of ATP synthesis. The electron transport chain and ATP synthase are located within the inner membrane, which has a large surface area due to its cristae (or folds) in order to support these mechanisms.

Proper function of the heart requires a large amount of cellular energy and over 90% of cardiac metabolism is aerobic [3]. In order to maintain this

metabolism, heart cells have a high oxidative capacity. It has been estimated that 25-35% of total cardiomyocyte volume is occupied by mitochondria [4]. About two-thirds of the ATP hydrolyzed by these mitochondria is utilized in the contractile apparatus of the heart, while the remaining is used in active transport in order to maintain ion balance, especially by sarcoplasmic reticulum Ca^{2+} -ATPase and the sarcolemmal Na^{+}/K^{+} -ATPase [3]. In addition to being the site of the electron transport chain and the citric acid cycle, the mitochondria are responsible for mediating necrosis and apoptosis.

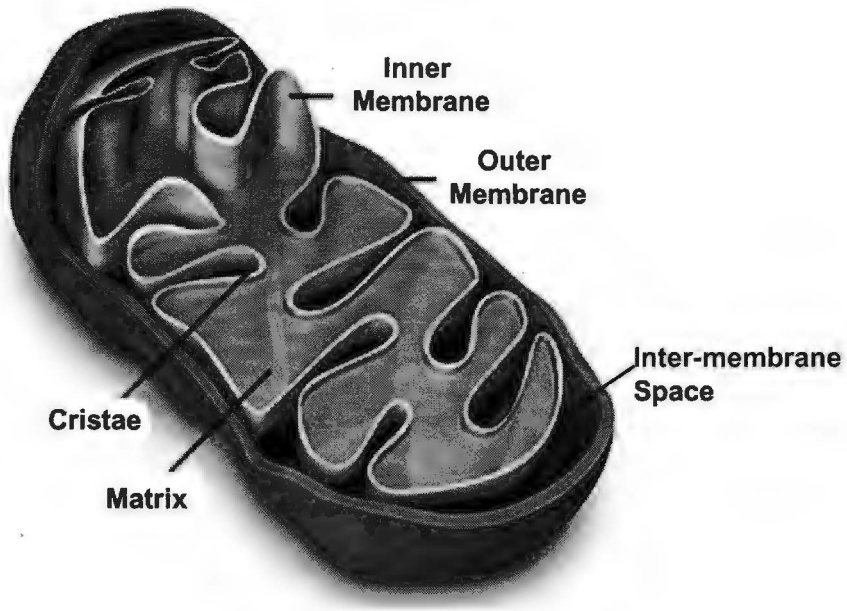

Figure 1. Internal structures of mitochondria (modified from http://www.cartage.org).

1.3 Ischemia-Reperfusion Injury

During cardiac ischemia, blood flow to the heart decreases or stops completely, thus oxygen delivery to the mitochondria by and large ceases. The citric acid cycle is arrested and therefore no energy is available from oxidative phosphorylation. Non-esterified fatty acid levels rise leading to an accumulation of cytoplasmic NADH resulting in a severalfold rise in NADH/NAD ratio [3]. For several minutes after the arrest of oxidative phosphorylation levels of ATP may be maintained by glycolysis. Glycolysis, however, is unable to meet the demand of the beating heart for ATP for more than a few minutes [5]. In addition, after 30 minutes, lactate accumulates in the cell and cytospasmic pH decreases to 5.5-6 resulting in inhibition of glycolysis [3, 6]. ADP levels increase rapidly, while ATP levels fall slowly to about 50% of [ATP] at normoxic conditions after 30 minutes of ischemia [7]. Sodium-hydrogen exchanger (NHE), although relatively quiescent under aerobic conditions, becomes activated by intracellular acidosis, causing the extrusion of H^+ and the influx of the concomitant Na^+ [8]. Due to low ATP levels the sarcolemmal Na^+/K^+ -ATPase and sarcoplasmic reticulum Ca^{2+} -ATPase become non-functional and cytoplasmic [Na^+] and [Ca^{2+}] increase. Na^+ increase causes the Na^+/Ca^{2+} exchanger to work in reverse, which inbibits Ca^{2+} efflux or even enhances the Ca^{2+} influx [9, 10]. It has been reported that with prolonged ischemia NHE is inhibited by extracellular acidosis that eventually exceeds intracellular acidosis [11, 12]. Soon, the

mitochondrial electro-chemical gradient is almost entirely diminished resulting in a great depression of the mitochondrial membrane potential. When in this extreme state of stress mitochondria are likely to trigger necrosis (accidental cell death associated with membrane rupture) and apoptosis (programmed cell death).

Although it is essential to return blood flow to the heart in order to salvage the myocardium and preserve functionality of the organ, the process of reperfusion itself causes additional deleterious effects not observed during ischemia. This phenomenon is collectively referred to as ischemia-reperfusion injury. When blood is reintroduced to an ischemic system, rapid washout of extracellular H^+ reactivates NHE, resulting in further intracellular Na^+ accumulation. Again, Ca^{2+} influx follows causing abnormally high intracellular Ca^{2+} concentration. When oxygen is reintroduced to the respiratory chain, ubiquinone can become partially reduced to ubisemiquinone. This can in turn react with oxygen to produce superoxide that is reduced to hydrogen peroxide by superoxide dismutase [5]. Hydrogen peroxide, in the presence of ferrous ions, will form the highly reactive hydroxyl radical through the Fenton reaction [5, 13]. These reactive oxygen species (ROS), which immediately increase slightly above normal when the heart becomes ischemic and then dramatically upon reperfusion [14] are extremely damaging to mitochondrial proteins. ROS have direct effects on complex 1 and complex 3 of the respiratory chain, as well as other iron sulphur proteins

[5]. In addition, ROS may cause oxidation of glutathione that may then form mixed disulphides with proteins, which in turn inhibit protein pumps [15, 16]. Overall, it is thought that the combined effects of ROS and Ca^{2+} overload are responsible for ischemia-reperfusion injury and determine whether the injury is reversible. In particular, mitochondrial injury by these agents may lead to the opening of mitochondrial permeability transition (MPT).

1.4 Mitochondrial Permeability Transition

The mitochondrial inner membrane barrier to diffusive ion fluxes is essential in order to maintain the chemiosmotic gradient necessary to drive ATP synthesis. During ischemia-reperfusion, several factors including matrix calcium overload, high oxidative stress, high phosphate and low ATP concentrations induce the opening of a non-selective pore in the inner mitochondrial membrane, referred to as mitochondrial permeability transition (MPT) or mitochondrial permeability transition pore. During reperfusion, when oxygen is introduced ROS are produced and, with Ca^{2+} overload that happens during ischemia, MPT opens. This step takes place within 1-2 minutes and this delay enables postconditioning. Once open, MPT allows unselective passage of any molecule up to 1.5kDa across the membrane. This uncouples oxidative phosphorylation, by allowing unrestricted proton movement across the membrane. The consequence of this is not only prevention of ATP synthesis but rapid consumption (hydrolysis) of ATP by

reversing ATP synthase in a futile effort to re-establish the membrane potential [17]. Under these conditions the ionic and metabolic homeostasis of the mitochondria is disrupted and degradative enzymes such as phospholipases, proteases, and nucleases are activated. Irreversible damage will eventually occur and the cell will die via necrosis if MPT remains open [3, 5]. A single opening of MPT is enough to depolarize the organelle as described and will activate further MPT opening within the same mitochondrion. This fully open state will result in a huge influx of small molecules, after which osmotically responsible water will follow resulting in matrix swelling. Swelling ultimately results in the rupture of the outer membrane (inner membrane withstands swelling due to the unfolding of cristae) and release of proapoptotic molecules in the intermembrane space such as cytochrome *c* and apoptosis-inducing factor (AIF) [18, 19]. Both cytochrome *c* and AIF instigate apoptotic processes and lead to cell death [17-21].

1.5 Ischemic Preconditioning

Ischemic preconditioning (IPC) is an experimental physiological phenomenon in which brief episodes of ischemia and reperfusion protect the heart against subsequent lethal ischemic insult [22]. In the process of preconditioning, a series of cell signaling events modify cellular components in a manner that reduces myocardial damage from the sustained ischemia-

reperfusion event. The IPC mechanism results in reduced infarct size, attenuating the incidence and severity of reperfusion-induced arrhythmias and preventing endothelial cell dysfunction [23]. Several endogenously released agents are involved in cardioprotection that results from preconditioning and include: opioids, norepinephrine, free radicals, adenosine, and bradykinin [24, 25].

1.6 Modes of Protection

In addition to ischemic preconditioning, several other modes of cardiac preconditioning have been described. (1) Calcium preconditioning (CPC) mimics IPC when calcium concentration increases briefly within the cardiomyocyte. Exogenous increase in calcium administered just prior to ischemia activates protein kinase C (PKC) and induces myocardial functional protection similar to IPC [26]. (2) Potassium channel openers (KCOs) result in cardiac preconditioning when administered prior to ischemia by increasing K^+ conductivity [27]. (3) Inhibition of sodium/proton exchanger by agents such as cariporide ultimately prevents Ca^{2+} overload and unusual Na^+ gradients when administered prior to ischemia and thus causes decreased cardiomyocyte damage [28, 29]. (4) Ischemic post-conditioning describes a phenomenon in which the heart undergoes ischemia and reperfusion with no pretreatment of any kind. Shortly after reperfusion, however, the heart is subjected to cycles of ischemia and reperfusion, resulting in similar extent of

protection as IPC [30].

1.7 Cardioprotection by Potassium Channel Openers

Grover *et al* discovered that two drugs capable of cardioprotection, cromakalim and pinacidil, were KCOs [27]. Both were known vasodilators. The vasodilator activity of these agents was attributed to their ability to increase ATP-dependant K^+ channel (K_{ATP}) conductance. In addition, there was electrophysiological evidence that indicated that both activated K_{ATP} channels in cardiac myocytes [31]. Activation of K^+ channels in smooth muscle cells leads to hyperpolarization and consequent action potential shortening [32]. Cardioprotective effects of cromakalim and pinacidil was reversed by glyburide (glybenclamide) [27], which has been shown to be a high affinity blocker of ATP-dependent K+ channels [33]. A variety of other KCOs were shown to be cardioprotective when administered prior to ischemia, including nicorandil, cromakalim, pinacidil, bimakalim, aprikalim, and P-1075 [27, 34-36]. These findings were consistent throughout different animal species [37-41]. Cardioprotective effects of all KCOs were reveresed by the blocker glyburide.

1.8 MitoK_{ATP} Mediates Cardioprotection by KCOs

Initially, it was believed that the cardioprotection afforded by KCOs was modulated via the sarcolemmal ATP-sensitive K+ channel (sarcK_{ATP}).

Potassium activation in the cell leads to hyperpolarization of the cell and subsequent action potential shortening. Hyperpolarization due to increased potassium conductance may bring the resting potential further from the threshold that activates calcium channels; inhibiting calcium influx and reducing ischemia. This view was significantly questioned when several experiments showed that cardioprotection is possible without action potential duration (APD) shortening [42-44]. Also, in 1991, Inoue *et al* identified an ATP-sensitive potassium channel in the inner mitochondrial membrane in rat liver through patch clamp experiments, and Paucek reconstituted and partially purified ATP-sensitive potassium channel that was sensitive to glibenclamide (K^+ channel closer) from rat liver and beef heart mitochondria [45, 46]. A few years later, in 1996, work by Garlid *et al* put forth the hypothesis that it is the mitochondrial ATP-sensitive K+ channel ($mitoK_{ATP}$) that is responsible for the cardioprotective effects of KCOs and not $sarcK_{ATP}$ [47]. This finding was based on several independent observations and explained the paradoxical finding that the action potential shortening in cardiac myocytes and cardioprotection through KCOs did not correlate. First, it was demonstrated that various KCOs opened $mitoK_{ATP}$ in isolated mitochondria within their cardioprotective concentration range [47]. Second, $sarcK_{ATP}$ was shown to be almost completely insensitive to diazoxide, a pharmacological agent with known cardioprotective properties, or 5-HD (5-hydroxydecanoate), which blocks cardioprotection, while $mitoK_{ATP}$,

assessed in isolated mitochondria, is sensitive to both in the pharmacological dose range [47-49].

In 1997, Garlid *et al* [48] confirmed the mitoK$_{ATP}$ hypothesis with the following findings: although both diazoxide and cromakalim (non-selective KCO) afforded similar levels of protection against ischemia-reperfusion injury, cardioprotection via cromakalim was accompanied by some action potential duration (APD) shortening, whereas no APD shortening was caused by diazoxide, the selective mitoK$_{ATP}$ opener, demonstrating that diazoxide does not act via sarcK$_{ATP}$. Since this discovery, numerous laboratories have provided evidence to support the paradigm that mitoK$_{ATP}$, and not the sarcK$_{ATP}$, is the mediator of ischemic preconditioning and precondition by KCOs [50-55].

1.9 MitoK$_{ATP}$ During Normal Conditions

It is conceivable that mitoK$_{ATP}$ evolved in order to provide a mode of cardioprotection, via IPC. However, this protein appears to exist in all mammalian species studied, including several that are not susceptible to ischemic heart disease [56]. Thus, it has been proposed that mitoK$_{ATP}$ plays a role in cardiac physiology during normal conditions and protection against ischemia happens to be a beneficial byproduct.

1.9.a *MitoK$_{ATP}$ in normal cell signaling*

There is some evidence that mitoK$_{ATP}$ plays a role in normal cell

signaling process that leads to cell growth. It has been shown that ouabain, a ligand of Na/K-ATPase, stimulates mitochondrial ROS production via a Ras-dependent pathway [57]. Among others, this ROS production is an element that the ouabain and the cardioprotection pathways share in common, thereby linking the two [58]. Furthermore, it has been shown that the ouabain signaling pathway is blocked not only by ROS scavengers but also by 5-HD – increasing support for the hypothesis that the two pathways are linked [58]. The interplays among these pathways in cardiomyocytes lead to changes in the expression of several genes. [56].

1.9.b MitoKATP opening in high work state – maintenance of positive ionotropy

During high work state, when the cardiomyocyte is undergoing high rates of ATP production and consumption, electron transport is high and the membrane potential significantly decreases. As Garlid explains in his review [56], this observation is identical to any other battery system, where drawing high currents ill decrease the output voltage. This decrease in membrane potential will result in the contraction of the mitochondrial matrix (because decrease in membrane potential decreases the influx of ions and the osmotically obligated water that enters with them), and the expansion of the inter-membrane space (IMS). However, mitoK_{ATP}, when open, provides a parallel potassium conductance that counteracts this matrix contraction, resulting in the preservation of matrix and IMS volume. IMS preservation is critical in maintaining the association of VDAC (voltage-dependent ion

channel), which controls outer membrane permeability to ADP and ATP, and creatine kinase. Creatine kinase phosphorylates creatine, using ATP produced by the mitochondrial ATPase, creatine phosphocreatine and ADP. The high-energy phosphate bond of phosphocreatine has more energy than the bond of ATP, 10,300 calories per mole in comparison with 7,300. Phosphocreatine presumably gets shuttled into the cytosol through VDAC and holds enough energy to reconstitute the high-energy bond of ATP. Therefore, this system provides an energy store in the cell and is incredibly important in the heart, where high work states are very frequent [56]. It has been proposed that endogenous signals open mitoK$_{ATP}$ during high work state in order to maintain matrix volume and the association between VDAC and creatine kinase. Garlid and colleagues [56] tested the hypothesis that mitoK$_{ATP}$ opening is required for the positive inotropic response (increased contractility) by testing mitoK$_{ATP}$ inhibitors in this system. Consistent with the hypothesis, these authors found that mitoK$_{ATP}$ blockers (5-HD and TPP^{+}) prevented inotropic response indeed.

1.10 MPT and mitoK$_{ATP}$

Ischemic preconditioning as well as preconditioning by KCOs reduces MPT opening and decreases infarct size [59-61]. In order to describe the mechanism that acts in this situation, Korge *et al* [62] simulated ischemia in isolated mitochondria and caused MPT opening by exposing them to high

Ca^{2+} and phosphate. Diazoxide (mitoK_{ATP} channel opener) and phorbol 12-myristate 13-acetate (PMA, pharmacological agent that activates Protein Kinase C (PKC) by mimicking diacylglycerol – a physiological PKC activator) both blocked MPT opening and the release of cytochrome *c*, while 5-HD (mitoK_{ATP} channel closer) reversed this effect [63]. These results supported the hypothesis that the protective mechanism of blocking MPT operates through PKC [49]. Working with genetically modified mice with cardiac-specific expression of recombinant PKCε, Baines *et al* [64] demonstrated that PKCε activity inhibited MPT opening in heart mitochondria. Other studies have demonstrated that inhibition of PKC by pharmacological agents blocks protection via IPC [65]. Also, hearts from PKCε knock-out mice do not exhibit protection by IPC [66]. In addition to PKCε involvement Kim *et al* [67] suggested that MPT inhibition operated through a guanylyl cyclase-dependent signaling pathway by showing that cyclic GMP (cGMP) prevented MPT opening. Interestingly, it was shown that mitochondria swell in K^+ medium after addition of Protein Kinase G (PKG) and cGMP [68]. This swelling is highly indicative of a potassium channel opening suggesting that PKG + cGMP cause mitoK_{ATP} to open. In addition, KT5823, a specific inhibitor of PKG blocked this effect, while mitoK_{ATP} specific opener, diazoxide, reversed inhibition by KT5823 [68]. In 2006 our laboratory demonstrated the mechanism by which mitoK_{ATP} opening inhibits MPT activation in heart, liver, and brain mitochondria. In agreement with previous experiments, this

mechanism utilizes PKG and PKCε as obligatory intermediates [49]. Upon activation in this process, mitoK$_{ATP}$ increases ROS. Interestingly, ROS (in this experiment H_2O_2), in concentrations at least 50 times smaller than those that induce MPT opening provides protection from MPT opening via PKCε independent of mitoK$_{ATP}$, suggesting that small amounts of ROS may be protective [49, 69]. These data suggest that mitoK$_{ATP}$ opening protects the heart from ischemia-reperfusion by inhibiting MPT and thus cell death by apoptosis and necrosis (Figure 2).

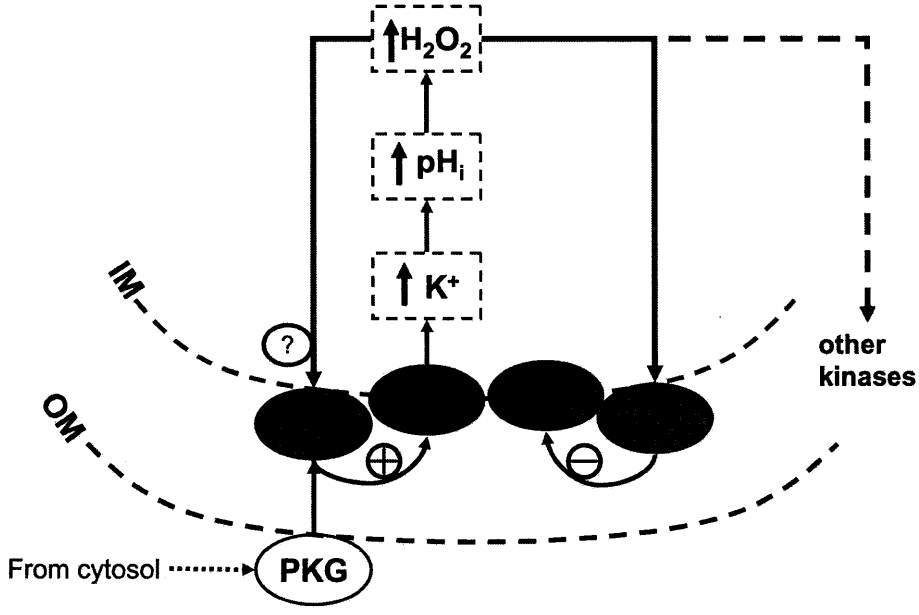

Figure 2. (Modified from [49])
The cardioprotective signaling pathway in mitochondria. Active PKG from the cytosol, which cannot cross the outer membrane of the mitochondria, phosporylates an unknown protein at the surface of the mitochondria. This protein presumably activates PKCε1, that is thought to be bound to the outer surface of the inner mitochondrial membrane. Then, PKCε1 phosphorylates and thus opens mitoK_{ATP}, resulting in an influx of potassium ions. Matrix potassium increase results in matrix alkalinization and eventually a small increase in matrix H_2O_2. This increase in H_2O_2 activates PKCε2, causing the inhibition of MPT. PKCε1 and PKCε2 are identical enzymes localized in different pools within the pathway. It is also known that this modest increase in H_2O_2 activates a number of other kinases.

1.11 Mechanism of Cardioprotection Induced by MitoK_{ATP}

The primary function of mitoK_{ATP} channels is to regulate mitochondrial volume [70, 71]. It is absolutely essential to maintain appropriate mitochondrial matrix volume, and thus vesicular integrity, in the face of the ever changing environment of the inner membrane space, with numerous ions, molecules, and water leaving and entering the inner membrane space. This is largely accomplished by the regulation of K^+ transport, via diffusion and via mitoK_{ATP} [70, 71]. Diffusive uptake of K^+ is exponential with voltage and is thus highly sensitive to the mitochondrial membrane potential. K^+ uptake is followed by uptake of phosphate (Pi) on the Pi/OH^- exchanger, followed by osmotically obligated water [72]. On the other hand, K^+ leaves the inner membrane space via the K^+/H^+ antiporter, again, followed by water. In this way, the balance between the ion (and water) influx is balanced by the activity of K^+/H^+ antiporter. During ischemia, the mitochondrial membrane potential is low, mitochondria depolarize due to anoxia and mitochondrial matrix contraction results. As a result, inner membrane space architecture gets highly distorted, and ATP hydrolysis is accelerated due to disruption of mitochondrial creatine kinase [71]. Increased K^+ conductance through the opening of mitoK_{ATP} may help to maintain inter-membrane space architecture and slow down the hydrolysis of ATP, allowing the use of creatine as substrate upon reperfusion [71].

1.12 MitoK_{ATP} in Other Tissues

MitoK_{ATP} has been shown to provide protection in pancreatic beta-cells by preventing the onset and development of diabetes in rats. The selective mitoK_{ATP} opener, diazoxide, inhibited apoptosis by directly opening mitoK_{ATP} and this effect was completely abolished by 5-HD [73]. As in the heart, ischemia-reperfusion injury may also lead to apoptosis in the brain [74]. Mitochondria in the brain contain seven times more mitoK_{ATP} channels then heart mitochondria [75]. There is support that mitoK_{ATP} protects the brain against ischemia reperfusion injury *in vivo* models: diazoxide preserves neuronal-vascular function after cerebral ischemia in piglets [76] and induces protection induced by hypothermic circulatory arrest in canine models [77], both effects were reversed by 5-HD. A study in cultured cerebellar granule neurons, where H_2O_2 was used to simulate neuronal apoptosis, suggested that mitoK_{ATP} provides neuroprotection by preserving mitochondrial inner membrane potential [78].

1.13 The Argument

Diazoxide and 5-HD have become synonymous with mitoK_{ATP} opening and closing, respectively. The paradigm that mitoK_{ATP} is the mediator of preconditioning via KCOs has been questioned by a number of investigators [79-83]. In particular, it has been proposed that diazoxide and 5-HD mediate cardioprotection independently of mitoK_{ATP} [79, 81, 83]. This controversy has

be intensified by the lack of a molecular identity for mitoK$_{ATP}$. Throughout the last six years or so, several investigators, most recently Minners [79] have concluded that cardioprotection by diazoxide is afforded by respiratory chain inhibition [81, 82, 84]. In fact, it has been known for almost forty years that high doses of diazoxide significantly decrease mitochondrial respiration by inhibiting succinate-dehydrogenase (SDH) [85]. These investigators fail to take into account the differences in the concentrations of this drug in both circumstances. Diazoxide inhibits SDH at high concentrations, while cardioprotection is achieved in much lower doses. In fact, it is very reasonable to suppose that most hydrophobic drugs, such as DZX, when used in very high concentrations will embed themselves into the membrane and cause physiological changes. It is also important to point out that these arguments focus on diazoxide, ignoring the fact that many other mitoK$_{ATP}$ openers provide cardioprotection without inhibiting the respiratory chain.

The same three laboratories proposed that 5-HD, which is a hydroxy derivative of decanoate, antagonizes the effect of diazoxide by being metabolized like other medium-chain fatty acids in the heart. Again, metabolism of 5-HD is already know from previous work [86]. 5-HD has a very short (7 minute) biological half life in dogs [86] and blocks cardioprotection when given 2 minutes, but not 8 minutes, before IPC [87]. These authors suggested that the short half life is due to 5-HD being converted to 5-HD-CoA by mitochondria in the presence of ATP and CoA

[56]. Those that have proposed this explanation for cardioprotection suggest that 5-HD blocks cardioprotection by interfering with normal cardiac metabolism, although do not explain how this may be. Again, these arguments do not explain why glibenclamide [48] and HMR1098 [88] block cardioprotection by diazoxide.

MitoK_{ATP} openers diazoxide, 3-NPA, ΨεRACK, and PKG will be utilized in our experiments. Therefore, they are briefly discussed in sections below.

1.14 3-NPA

3-Nitropropionate (3-NPA) is a widely distributed plant and fungal toxin first identified by Morris et al in 1954 as the primary toxic agent of *Indigofera endecaphylla* [89, 90]. It is produced on moldy crops such as sugarcane and peanuts and causes severe neurological disorders in humans when consumed in sufficient amounts [91-93]. 3-NPA is said to be a suicide inhibitor of succinate dehydrogenase (SDH), associated with the tricarboxylic acid cycle and complex II in the respiratory chain. Alston et al demonstrated that the dianion form of 3-nitropropionate is the inhibitory form [90]. The carbanion form of this compound is isoelectric with succinate (Figure 3), SDH substrate [90], and reacts progressively with N-5 of the covalently bound flavin to form a

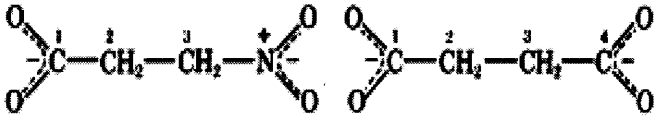

Figure 3. 3-NPA (left), in its carbanion form, is isoelectic with succinate (right).

covalent adduct [94] (Figure 4). This binding is irreversible [90, 94]. Chemical preconditioning with 3-NPA has been shown to have a protective effect against ischemic hypoxic neuronal damage in vitro [95-97]. Interestingly, the symptoms of 3-NPA toxicity closely resemble those of Huntington's disease and thus provide a research model for understanding the mechanisms of Huntington's [98]. Several findings show that the type of neuronal death induced by 3-NPA is dose-dependent [99]. In vivo and in rat hippocampal cell cultures, intermediate doses of the compound result in apoptosis [100], while high doses led to NMDA-receptor dependent excitotoxic cell death. It is speculated that dependent on its concentration 3-NPA can induce three different cellular responses: tolerance (preconditioning) [96], apoptosis, or necrosis. 3-NPA addition caused a pronounced decrease in [creatine phosphate/creatine] and a fall in [ATP/ADP] as well as [GTP/GDP] [98]. It has been well established that reductions in energy metabolism are neuroprotective. However, it is unlikely that preconditioning via low doses of 3-NPA is an example of this phenomenon because 3-NPA preconditioning is evident much later (several days) after normal ATP levels and SDH activity have been reestablished [96]. It may therefore be concluded that inhibition of SDH is not the mechanism by which 3-NPA is protective in the brain.

Riepe *et al* hypothesized that ATP-sensitive potassium channels may be responsible for the effect of administrating sub-lethal doses of 3-NPA after

conducting electrophysiological studies on rat hippocampal slices [101]. Further investigation and *in situ* studies demonstrate that neuroprotection via 3-NPA was abolished by administration of glybenclamide and 5-HD [102]. Additionally, 3-NPA administration resulted in mitochondrial depolarization and these effects were also blocked by 5-HD [102]. These results suggest that mitoK$_{ATP}$ plays a significant role in 3-NPA preconditioning in the brain.

Several studies also suggest that 3-NPA protects the heart from ischemia-reperfusion injury. Experiments on a Langendorff apparatus demonstrated that 3-NPA provides significant cardioprotection [103]. Ockaili and coworkers demonstrated that 3-NPA induces an immediate and delayed cardioprotective effect in the heart as indicated by a significantly decreased infarct size compared with control animals. The mitoK$_{ATP}$ selective opener, 5-HD, abolished both the early and the delayed cardioprotective effects when administered before ischemia-reperfusion [104]. This evidence indicates that mitoK$_{ATP}$ is a mediator in heart preconditioning and cardioprotection via 3-NPA.

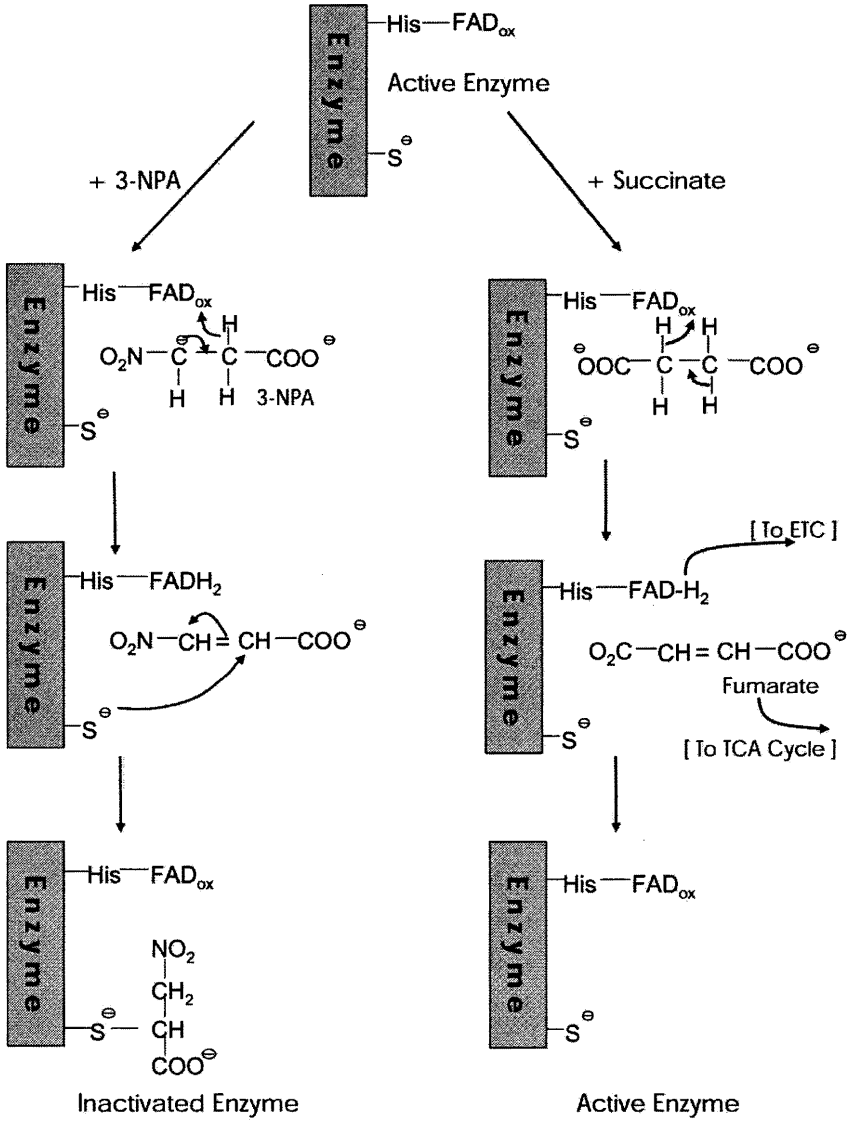

Figure 4. Proposed mechanism by which 3-NPA irreversibly deactivates succinate dehydrogenase (on the left) compared to the cyclic mechanism by which SDH converts succinate into fumarate and supplies electrons into the electron transport chain (ETC) (Modified from [94].

1.15 Diazoxide

Diazoxide (3-methyl-7-chloro-1,2,4-benzo- thiadiazin-1, 1 dioxide) (figure 5) is a synthetic drug that has been used as a diabetogenic agent, by suppressing of insulin secretion in vivo and in vitro [85]. It is the most potent beta-cell K_{ATP} channel opener known and it successfully hyperpolarizes the beta-cell, thereby inhibiting insulin secretion [105] and by preventing apoptosis in pancreatic beta-cells of pre-diabetic and diabetic rats [73]. Diazoxide is also used inter-venously to treat severe hypertension as it relaxes arteriolar smooth muscle [106]. Both of these clinical applications of diazoxide were widely used before the discovery of ATP-sensitive potassium channels. In 1969, Schafer *et al* reported that diazoxide inhibited succinate dehydrogenase (SDH), complex II of the electron transport chain in isolated rat liver mitochondria and beef heart submitochondrial particles [85]. Their findings demonstrated that in high concentrations, diazoxide strongly inhibits succinate-supported respiration but not NADH-linked substrate oxidation. Belyaeva *et al* and Szewczyk *et al* reported that diazoxide (and other KCOs) also act on respiring mitochondria [107, 108]. Their reports suggested that diazoxide effects on mitochondria are pharmacologically irrelevant because its effect was very weak at concentrations much higher than $K_{1/2}$ values observed with sarcK_{ATP} [109]. In

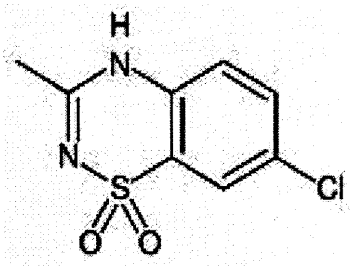

Figure 5. Molecular structure of diazoxide

1996 Garlid *et al* reported that diazoxide was a very potent activator of the ATP-sensitive potassium channel in intact mitochondria and proteoliposomes containing recontituted mitoK_{ATP} [47]. No effect was observed on uninhibited potassium flux, likely explaining the low potencies that Szewczyk and Kelyaeva reported [47]. Due to the observation that diazoxide opens mitoKATP channel at a concentration that has no effect on the sarcK_{ATP} channel, focus shifted from sarcK_{ATP} to mitoK_{ATP} channel as mediator of cardioprotection [47].

1.16 ΨεRACK

ΨεRACK is a selective PKCε (Protein Kinase C epsilon) activator peptide. It is derived from PKCε and is eight amino acids long [HDAPIGYD]. PKC (Ca^{2+}-dependent protein kinase) is a serine/threonine kinase found abundantly in many different cell types. PKCε is one PKC isozyme (one of six) that is constitutively present in heart mitochondria [110]. There are many reports that PKCε activation plays a critical role in transmitting a signal that induces cardioprotection [68, 111-114]. Baines *et al* [64] and Korge *et al* [63] have shown that PKC activation is able to inhibit MPT opening. Costa *et al* [49] demonstrated that ΨεRACK provides cardioprotection through mitoK_{ATP} through the inhibition of MPT. This finding that ΨεRACK provides cardioprotection by mimicking ischemic preconditioning has been demonstrated by a number of laboratories [111, 113, 115, 116].

1.17 PKG

cGMP-dependent protein kinase (PKG) is an abundant serine/threonine kinase activated by cyclic GMP (cGMP), in fact, it is the major intracellular receptor of cGMP [117]. It is present in high amounts in platelets, cerebellum, smooth muscle, cardiac muscle, hippocampus, dorsal root ganglia, neuromuscular end plate, and the kidney vasculature. Low levels have been detected in granulocytes, vascular endothelium, chondrocytes, and osteoclasts. PKG phosphorylates a number of physiologically relevant proteins involved in regulating the contractile activity of smooth muscle cells, regulation of cell differentiation and proliferation, inhibition of platelet aggregation and apoptosis [118, 119]. Han *et al* [120] first suggested that PKG is involved in the signaling pathway that induces cardioprotection via ischemic preconditioning in rabbit ventricular myocytes. In their experiments, PKG was found to be responsible (at least in part) to phosphorylation and activation of K_{ATP} channels. Selective PKG inhibitors abrogated any effects [120]. Oldenburg *et al* [121] studied mitochondrial ROS generation by bradykinin and concluded that PKG activation (in their case by ROS) triggers preconditioning by opening mitoK_{ATP}. In order to uncover the mechanism by which PKG acts on mitoK_{ATP}, Costa *et al* [68] conducted light scattering experiments which demonstrated that active PKG (and cGMP necessary to activate it) is indeed necessary to open mitoK_{ATP} in vitro, and KT5823, a specific PKG inhibitor, blocked this effect. Interestingly, the inhibition of PKG by KT5823 was reversed by the addition of the specific

mitoK_{ATP} opener diazoxide [68]. In addition, PKG-induced effect was reversed by 5-HD (selective mitoK_{ATP} inhibitor) [68]. These investigations suggest that active PKG activates mitoK_{ATP} by indirect phosphorylation, and that this phenomenon is not limited to cardiomyocytes and has been observed in brain and liver mitochondria [68].

1.18 Overall Hypothesis

Several investigators have suggested that cardioprotection afforded by ischemic preconditioning is not mediated through mitoK_{ATP} but results from the inhibition of Complex II of the electron transport chain, succinate dehydrogenase. In this study, I have examined four chemically distinct and unrelated pharmacological agents, all of which have been shown to open mitoK_{ATP}, to demonstrate that the said cardioprotective effect mediated by mitoK_{ATP} is entirely independent of SDH.

2. Methods

2.1 Animals

Male rats of the Sprague-Dawley variety were used in all experiments. The rats were about 2 months old and weighed 225-249g. They were housed two per cage in a 24°C room and were fed and watered *ad libitum*. Diet consisted of standard rodent formula chow from LabDiet. All procedures involving animals are in accordance with the American Physiological Society "Guiding Principles in the Care and Use of Animals" and was approved by Institutional Animal Care and Use Committee at Portland State University.

2.2 Mitochondrial Isolation

One or two rats were anesthetized with CO_2 for 20-30 seconds and immediately decapitated. An incision into the chest cavity was made using surgical scissors and hearts were quickly removed and rinsed in ice-cold Buffer A (250mM sucrose, 10mM HEPES pH 7.2, 5mM K-EGTA, and 1mg/mL fatty-acid free BSA). Hearts were squeezed manually until most of the blood was removed. The hearts were then finely minced in Buffer A containing 1mg/mL protease (Nagarse) for approximately 40 seconds. The suspension was then diluted with Buffer A and homogenized with a motorized Teflon pestle until the homogenate looked uniform (about 45 seconds). The entire homogenate was then divided equally into two 30mL plastic tubes and

diluted with additional Buffer A to bring the volume just shy of the top of the tubes. These were then spun at 1700g for 3 minutes. The resulting supernatant containing the mitochondria was decanted into clean, ice-cold tubes and further centrifuged at 9000g for 5 minutes. Pellet from the first spin containing blood cells and cellular fractions was discarded. The pellet from the second spin was carefully resuspended in Buffer B (Buffer A minus BSA) and centrifuged again for 4 minutes at 2300g. The resulting supernatant was centrifuged one more time to pellet the isolated mitochondria at 9000g for 5 minutes. This final mitochondrial pellet was resuspended in about 200μL of Buffer B and stored on ice.

2.3 Protein Measurement

All protein was measured in Thermo Spectronic Genesys 20 using the Biuret Method first described by Gornall [122].

2.4 Measurement of Mitochondrial Volume

Mitochondrial matrix volume was measured by light scattering technique as first described by Beavis [123]. Mitochondria respond to osmotic pressure of their environment by taking up, or pumping out of water. When osmolarity outside the mitochondria increases and salt begins to enter the mitochondrial membrane – obligated water follows resulting in mitochondrial matrix swelling; shrinking occurs due the reverse. When mitoK_{ATP} is opened, regardless of the cause, potassium ions and water flow

into the matrix and swelling is observed. Matrix contraction is observed when mitoK$_{ATP}$ is closed. Therefore, mitochondrial swelling and shrinking in a potassium rich medium is indicative of the state of mitoK$_{ATP}$. The light scattering technique utilizes a Brinkmann PC 700 colorimeter light probe fitted with a 520 nm filter. A small mirror about 1 cm below the light source reflects the light that has not been absorbed back into the system. Increase in matrix volume is accompanied by a decrease in the intensity of light scattered by the suspension, because as mitochondria swell, they absorb more (and therefore scatter less) light [123, 124]. The probe is normally immersed in 2 mL of assay medium and mitochondria protein (0.1mg/mL) suspension, which is constantly stirred and maintained at 30°C. Absorbance is measured and quantified [123] using a computer program developed in our laboratory. It is based on the principle that reciprocal absorbance (A) of the mitochondrial suspension (proportional to matrix volume, which depends on: medium osmolality, matrix solute content, and the concentration of mitochondria [123], when corrected for the extrapolated absorbance at infinite protein concentration is linearly related to the matrix volume. This absorbance is then converted to beta (•), which corrects for variations in protein concentraion:

$$\beta \equiv \frac{P}{Ps}\left(A^{-1} - \alpha\right)$$

where • is the machine constant (0.25 in the case of our apparatus), A is

absorbance, P is mitochondrial concentration (mg/mL), and Ps (equals 1mg/mL) is introduced to make • a scaled dimensionless quantity [123]. Conversion to • allows comparison of multiple experiments. The light scattering assay medium contains 120mM KCl, 10mM HEPES (pH 7.2), 0.1mM EGTA, 10mM succinate, and 5mM phosphate. Rotenone (5μM), which inhibits complex I of the electron transport chain and the reverse electron transfer from complex II to complex I as well as oligomycin (0.67μM), which inhibits ATP synthesis by the ATPase and consequent decrease in membrane potential are also added to all assay media.

MitoK_{ATP} openers and closers were added to the assay media in appropriate concentrations to determine their effect on mitoK_{ATP} (swelling indicates open channel, shrinkage – closed).

2.5 Measurement of Mitochondrial Respiration

Respiration was measured using a Yellow Springs Instruments oxygen electrode (Clark polarographic sensor), model 53. Electrode is connected to a chamber containing 2mL of respiration solution of constant temperature (25°C). At normal conditions this chamber contains a set amount of oxygen (960ng atoms O in 2mL at 25°C). After the addition of respiring mitochondria, the oxygen amount decreases and this linear rate is plotted on the computer that is connected to the electrode. Chemicals were added to the assay chamber containing the assay medium and mitochondria and their effect on respiration were observed. Respiration assay medium contains: 110mM

sucrose, 10mM HEPES pH 7.2, 2.5mM Pi, 0.1mM EGTA, 0.5mM MgCl2, and 10mM succinate. 5μM rotenone was also added to each run in order to inhibit Complex I of the electron transport chain.

2.6 Solutions

All solutions were made with deionized water from Millipore Synergy 185. For contents of each medium please refer to sections above.

2.7 Chemicals and Reagents

Most chemicals were purchased from Sigma Chemical Co. except for PKG (Promega), cGMP (EZ Biolabs), and ΨεRACK (EZ Biolabs).

2.8 Data Analysis

All data analysis was done using the program ORIGIN by Microcal. Respiration data were analyzed as follows. Each dose was assessed in an independent run. Mitochondria and rotenone were added immediately (at time 0), 100 seconds later CCCP (carbonyl cyanide m-chloro phenyl hydrazone, a mitochondrial uncoupling agent) (35μM) was added, 100 seconds after that the appropriate drug was added. The CCCP alone and CCCP+drug rates were converted into (ng atoms oxygen)*(min^{-1})*(mg $protein^{-1}$). Then a ratio of (CCCP + drug) rate/(CCCP) rate was calculated and multiplied by 100% in order to calculate "% of control". Thus, each individual

run had its own no drug control to account for slight differences in pipetting, stirring, mitochondrial viability, and so on in different runs.

3. Results

3.1 Diazoxide

Diazoxide opens mitoKATP at 30μM in rat heart mitochondria. Figure 6 demonstrates the swelling that is associated with mitoK_{ATP} opening due to the presence of diazoxide. This opening is reversed by 5-HD. Diazoxide concentrations above 1mM inhibit succinate-supported respiration by 75% in rat heart mitochondria, IC50 = 140μM (Figure 7).

3.2 3-NPA

3-NPA opens mitoKATP at 10nM in rat heart mitochondria (Figure 8). This opening was reversed by 5-HD. 3-NPA inhibits succinate supported respiration, IC50 1.05mM.

3.3 PKG

PKG + cGMP do not inhibit succinate-supported respiration below 96% of full function in the dose range tested. Figure 10b demonstrates the raw respiration data for these experiments, while figure 10a shows the dose-response.

3.4 ΨεRACK

ΨεRACK does not inhibit succinate-supported respiration below 92% of full function in the presence of rotenone within the tested dose (Figures 11a-b).

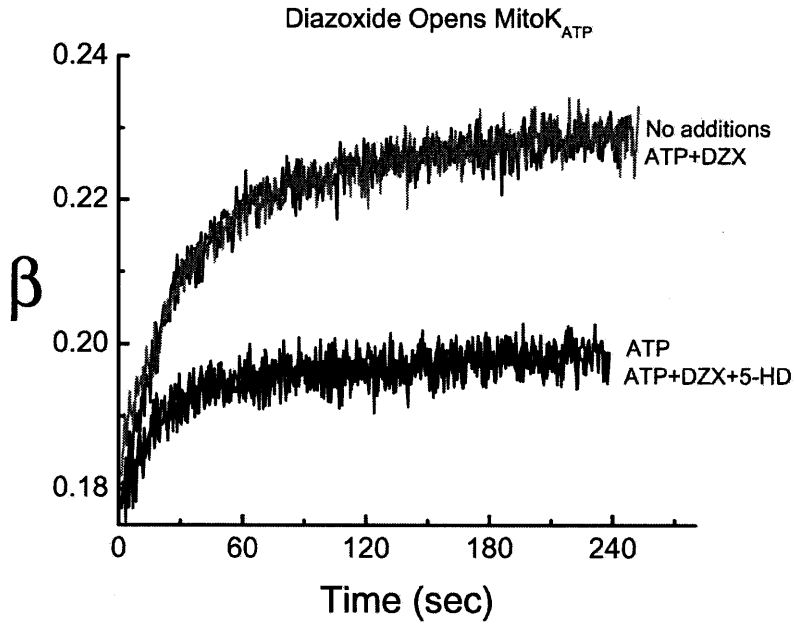

Figure 6. Light scattering assay results demonstrating that diazoxide (30μM) opens mitoK$_{ATP}$ in KCl medium. 5-HD reverses this opening in the presence of ATP (n = 4).

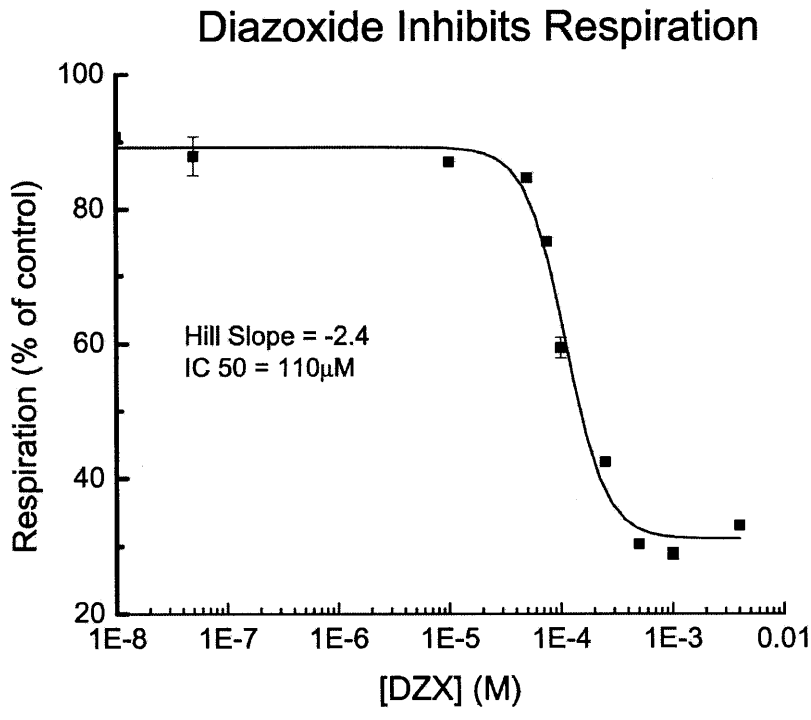

Figure 7. Respiration assay results demonstrating that diazoxide inhibits succinate-supported respiration. Hill slope = -2.4, IC 50=110μM, sucrose medium, in the presence of rotenone (n = 3).

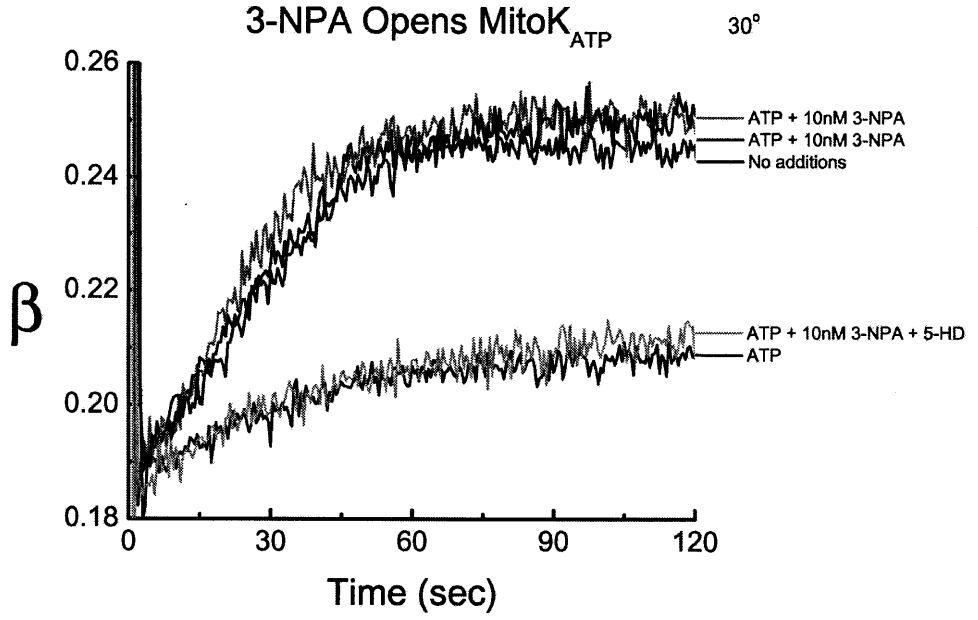

Figure 8. Light scattering assay demonstrating that 3-NPA (10nM) opens mitoK$_{ATP}$ in isotonic KCl medium, in the presence of succinate, rotenone, and oligomycin. This effect is reversed by 5-HD (n = 1).

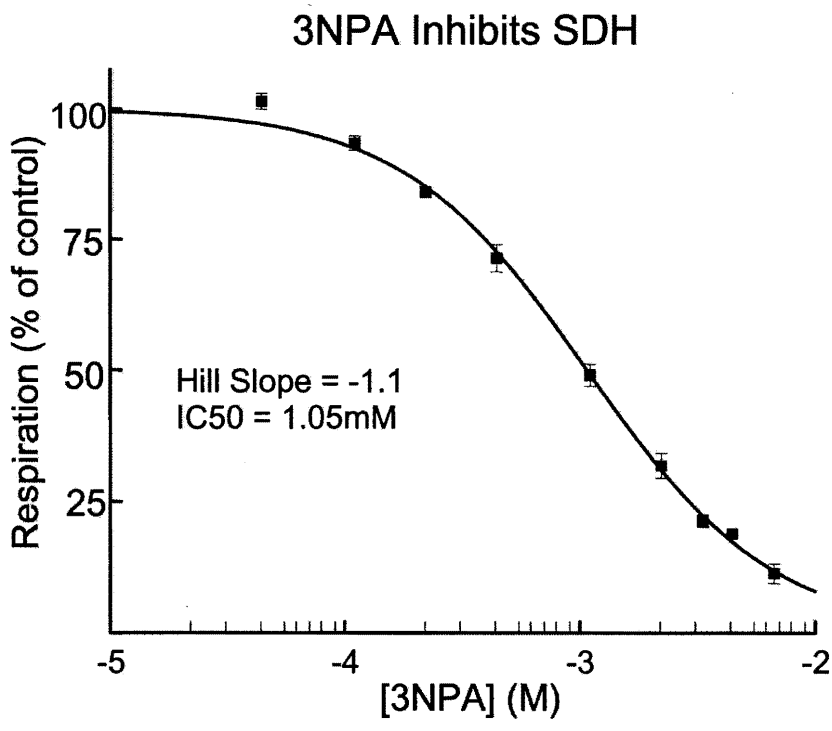

Figure 9. Respiration assay demonstrating that 3-NPA inhibits succinate-supported respiration in sucrose medium, in the presence of rotenone. Hill Slope = -1.1, IC 50 = 1.05mM (n = 4).

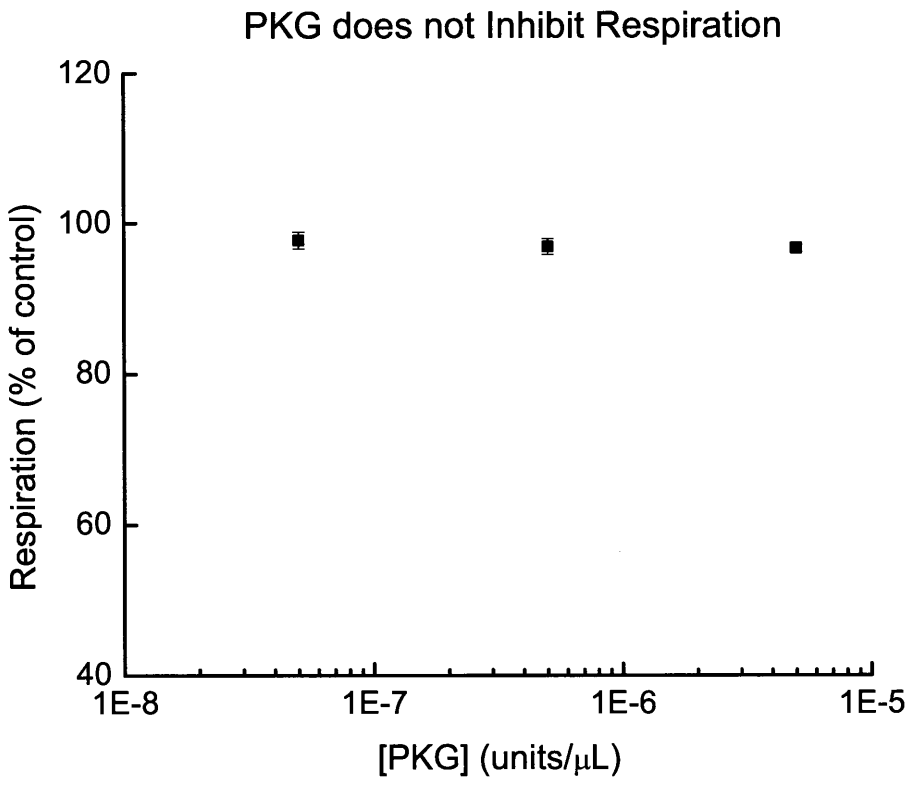

Figure 10a. Respiration assay demonstrating that PKG + cGMP do not inhibit succinate-supported respiration in sucrose medium, in the presence of rotenone (n=3).

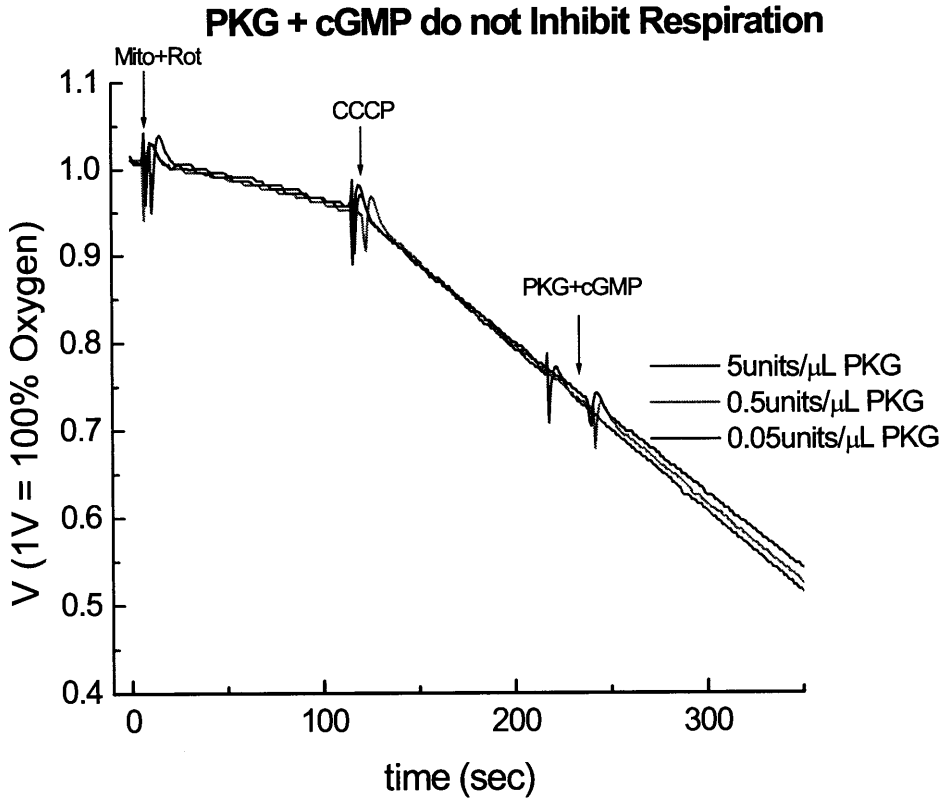

Figure 10b. Raw traces from respiration assay demonstrating that PKG + cGMP do not inhibit succinate-supported respiration in sucrose medium, in the presence of rotenone (n=3). (1 unit = amount of kinase required to incorporate 1pmol of phosphate into the test heptapeptide (RKRSRAE) per minute, at 30•C)

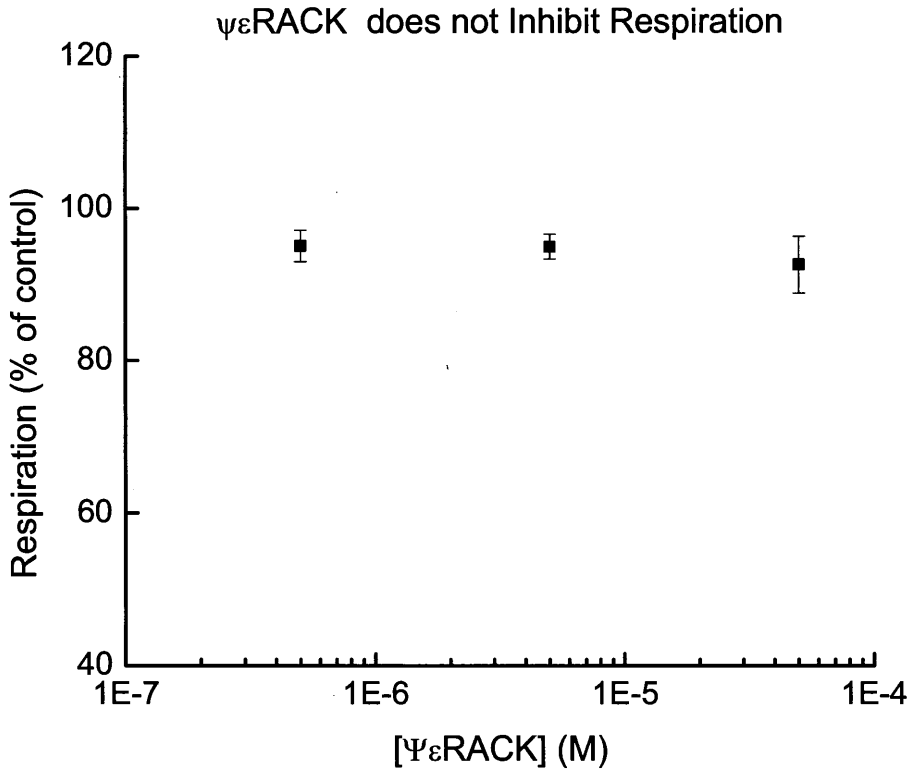

Figure 11a. Respiration assay showing that ΨεRACK does not inhibit succinate-supported respiration in the tested range. Sucrose medium, in the presence of rotenone (n=3).

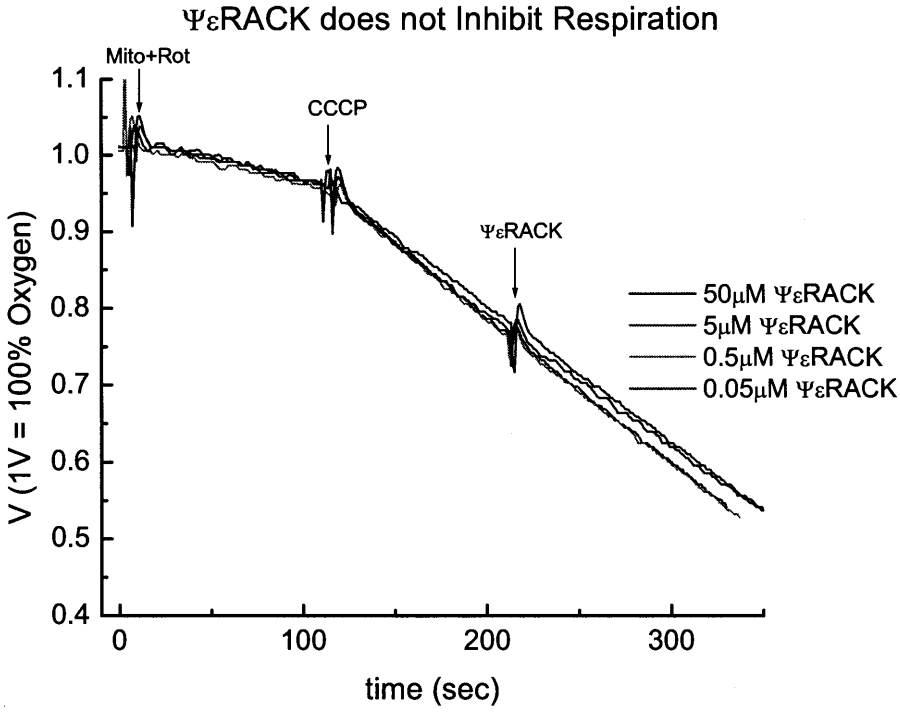

Figure 11b. Raw traces from respiration assay demonstrating that ΨεRACK does not inhibit succinate -respiration in the tested range. Sucrose medium, in the presence of rotenone (n=3).

4. Discussion

In 1986, Murry *et al.* described a paradoxical phenomenon whereby brief ischemic insults reduce damage to the heart caused by a subsequent, prolonged ischemic event [22]. Since 1989, when Grover *et al.* suggested that opening K_{ATP} channels protects the heart from ischemia-reperfusion injury, there has been a growing interest in investigating the details of this mechanism. Although the classical sarcolemal K_{ATP} channel was though to be the mediator of this cardioprotective pathway [125-127], compounding evidence suggested that the mitochondrial K_{ATP} channel, located in the inner mitochondrial membrane, was responsible for this effect [45, 47, 54, 55]. Garlid *et al* demonstrated that it is the mitoK_{ATP} channel that is the site of action of all K_{ATP} channel openers [48]. This shift of paradigm has stirred a lot of debate as the details of this cardioprotective mechanism are investigated.

Some of the difficultly in pinpointing the mechanism by which mitoK_{ATP} induces cardioprotection lies in the fact that molecular identity of this channel remains unclear. Indeed, what is known about mitoK_{ATP} comes largely from pharmacological investigation. SarcK_{ATP} channels have been molecularly defined as an octamer composed of four pore-forming inwardly-rectifying potassium ($K_{ir}6.1$ and $K_{ir}6.2$) subunits and four sulfonylurea receptors (SUR2A), which confer a regulatory role and a sensitivty of the channel to ATP and pharmacological regulators [128]. MitoK_{ATP} channels share some

pharmacological, and thus likely structural, properties with the classical sarcK_{ATP} counterpart. Both channels are activated by pinacidil and inhibited by glibenclamide [46, 48]. In order to study the mechanism of cardioprotection, which has been suggested to operate through mitoK_{ATP}, it is crucial to utilize specific activators and inhibitors of this channel. Obviously, it is also important to ensure that the specific pharmacological agents don't have effects on other, relevant systems.

Diazoxide and 5-hydroxydecanoate have been utilized as a selective mitoK_{ATP} channel opener and closer, respectively. Several investigators, most recently Minners *et al* [79], have rejected the role of mitoK_{ATP} in cardioprotection on the basis that diazoxide (and 5-HD) effects that have been attributed to mitoK_{ATP} are actually due to these drugs' effects on mitochondrial respiration. Therefore, it was a goal of this study to investigate whether the observed effect can be attributed to respiratory inhibition instead of mitoK_{ATP} activity.

It was reported almost forty years ago that at high doses diazoxide inhibits succinate dehydrogenase, complex II of the electron transport chain [85]. Indeed, all hydrophobic drugs including diazoxide (and pinacidil, another K_{ATP} channel opener) exhibit concentration-dependent inhibition of the respiratory chain. This is due to their tendency to embed in the hydrophobic membrane and cause a plethora of effects. This is further supported by the observation that diazoxide is not entirely specific to SDH [129]. The results of the current study strongly suggest that diazoxide inhibits

succinate-supported respiration at high doses (IC50 = 110μM) down to 30% respiratory activity at about 1mM diazoxide (figure 7), as previously described. However, these concentrations are in excess of those necessary to open mitoK$_{ATP}$ (figure 6). In fact, the K$_{1/2}$ for diazoxide mitoK$_{ATP}$ opening is merely 3μM (Garlid 1996). In our studies, 30μM diazoxide is used in light scattering assays in order to ensure complete channel opening. Even at this concentration, no significant respiratory inhibition is observed.

At high concentrations 3-NPA acts as a suicide inhibitor of SDH by binding irreversibly to the enzyme and preventing it from further participating in its metabolic roles. Figure 9 demonstrates that the current study supports these claims; the IC50 = 1.05mM. However, at much lower concentrations (10nM, figure 8) 3-NPA seems to be capable of opening mitoK$_{ATP}$. At this low concentration, 3-NPA appears to have no effect on respiration. It is important to mention here that replicates are necessary to determine the exact dose range at which 3-NPA opens mitoK$_{ATP}$ in our light scattering system.

The influx of K+ ions through mitoK$_{ATP}$, observed as swelling during light scattering experiments, is a respiration driven process. The chemiosmotic gradient that is maintained by mitochondria during normal respiratory conditions is the same gradient that drives K+ into the matrix. If the concentrations of diazoxide and 3-NPA that are used to open mitoK$_{ATP}$ inhibit respiration, then this swelling would not be observed (significantly decreased amount of swelling or even shrinkage would be observed), because inhibition of respiration decreases or abolishes this gradient.

Some investigators [79] imply that the observed cardioprotection results from inhibition of SDH, since diazoxide, a known SDH inhibitor, has been used as a model mitoK$_{ATP}$ opener. However, my results demonstrate (figures 10a-b, 11a-b) that other mitoK$_{ATP}$ openers, such as PKG and ΨεRACK, do not have a significant effect on SDH function. They open mitoK$_{ATP}$ at 0.25units/μL and 0.5μM, respectively [68]. PKG and ΨεRACK do not activate mitoK$_{ATP}$ directly, however, but act upstream of mitoK$_{ATP}$ in the cardioprotection pathway. This fact complicates the current issue and experiments with compounds that activate mitoK$_{ATP}$ directly, but have no effect on any part of the respiratory chain, are necessary; such drugs were unavailable for the current study. Interestingly, glibenclamide, a drug used to inhibit K$_{ATP}$ channel opening, is known to inhibit SDH as well as mitoK$_{ATP}$ opening, again demonstrating that that the mitoK$_{ATP}$ effect is not explained by SDH inhibition.

It has been suggested that ROS (reactive oxygen species) that are produced during respiratory inhibition are responsible for cardioprotection, giving a mechanism for cardioprotection via SDH inhibition. Indeed, ROS is central in all cardioprotection models, including the mitoK$_{ATP}$ hypothesis. A small amount of ROS, regardless of its origin, protects the heart from ischemia [49, 130-133]. This can be clearly demonstrated by including ROS scavengers in an assay that measures cardioprotection or mitoK$_{ATP}$ activity [131]. ROS scavengers also block preconditioning in Langendorff assays. In every case, the resulting effect is identical to that of 5-HD – abolition of

cardioprotection. Small amounts of ROS are known to activate PKCε by oxidizing the zinc fingers the enzyme and changing its conformation [134]. PKCε is a critical element in all modes of cardioprotection as it is thought to phosphorylate and open mitoK$_{ATP}$ [49]. Cardioprotective amounts of ROS, therefore, activate mitoK$_{ATP}$ through PKCε. It has also been shown that inhibition of the respiratory chain produces ROS [79]. It is therefore conceivable that the observed cardioprotective effect includes both slight respiratory inhibition and activation of mitoK$_{ATP}$. If mild inhibition causes some respiration inhibition and therefore causes a production of a small amount of ROS then this ROS could in turn activate and open mitoK$_{ATP}$ resulting in cardioprotection. In short, it is possible that diazoxide and 3-NPA, in small doses, inhibit respiration by an amount that does not cause apoptosis, but causes the production of a small amount of ROS which in turn activates PKCε, which finally activates mitoK$_{ATP}$ and provides cardioprotection, and do not activate mitoK$_{ATP}$ directly.

However, it is highly unlikely that the small amount of respiratory inhibition we observed in the mitoK$_{ATP}$ opening doses has any effect on ROS production. In order to test above hypothesis, it would be necessary to measure ROS production after addition of 3-NPA and to test if, perhaps, marginal inhibition of SDH by an SDH-specific inhibitor (such as malonate) causes cardioprotection *in situ*. At this time there is no known mechanism by which SDH is capable of producing ROS, which is generally produced by

Complex I and Complex III. In addition, recently, Drose *et al* [83] demonstrated that diazoxide does not generate ROS from the respiratory chain, making the above hypothesis even less likely. Controversy on this topic remains however, since Busija *et al* demonstrated that both 3-NPA and diazoxide increase mitochondrial ROS in the brain [135].

Ardehali *et al* have demonstrated that SDH is a part of a protein complex that is capable of transporting potassium ions, implying that SDH is in fact mitoK_{ATP}, or that the two proteins are closely associatedb [136]. This group suggests that the ability of SDH to regulate mitoK_{ATP} activity is entirely independent from its function in the electron transport channel [136]. A very recent study by Wojtovich and Brookes provides additional evidence that SDH and mitoK_{ATP} are closely associated [137]. They demonstrated that malonate, a competative inhibitor of SDH, opens mitoK_{ATP} channel even when the mitochondria are respiring on complex I substrates (glutamate and malate), at concentrations much lower than those necessary to inhibit SDH [137]. These data are in agreement with the dose-dependant effects of SDH inhibitors present in the current study. Interestingly, it has also been demonstrated that IPC decreases SDH activity [138]. From these data it can be speculated that during IPC or preconditioning by pharmacological agents, SDH decreases its function as a Citric Acid Cycle enzyme and electron transport chain enzyme and becomes involved in channeling potassium ions. This would also give an evolutionary reason for why SDH is imbedded in the inner membrane of the mitochondria. It is well established that the

mitochondrial shape is highly dynamic thus quick volume regulation mechanism seems necessary. Although this hypothesis would explain why SDH, which does not pump protons in the electron transport chain, is embedded in the membrane, it does not explain why some mitoK_{ATP} channel openers seem to have no effect on SDH and why some mitoK_{ATP} channel closers also inhibit SDH activity. In order to test this hypothesis, it would be necessary to confirm the speculation that SDH is capable of shuttling K^+ and measure SDH enzymatic activity at the same time as K+ transport (or mitoK_{ATP} activity).

It is well known that many of the agents used in studying cardiac preconditioning have other effects on the heart, seemingly unrelated to their effects on the mitoK_{ATP} channel. It would be ideal to study the effects of these agents in situ, without disruption of cardiac myocytes. It is likely that mitochondrial isolation greatly disrupts the natural shape of each mitochondrion. At this point, unfortunately, there are no available techniques to do this.

The dose-response curves for both 3-NPA and diazoxide yielded a Hill slope that is greater than 1 (figures 7, 9). It is important to point out that the system we tested is not an isolated one. Mitochondria purified in a crude, differential centrifugation manner was utilized in all our assays. Therefore, it is not feasible to create a perfect dose response curve with a Hill slope of 1. For such an experiment, in order to describe the exact binding manner of drug to SDH, it would be necessary to work with isolated SDH. In both cases

the Hill slope indicates some degree of cooperative binding. In fact, it is impossible to determine, in the present study, whether these drugs are acting only on SDH, and not having other unknown effects on the electron transport chain. Conceivably, it is possible that both diazoxide and 3-NPA affect other components of the electron transport chain causing an increase in ROS from complexes I or III. The current design of the study is, however, more relevant to the physiological conditions that we were attempting to assess. Additionally, the purpose of this study was not to obtain exact drug concentrations, but to assess whether or not succinate-supported respiration is significantly inhibited in doses that are used for mitoK_{ATP} opening.

5. Conclusions

Cardiac disease is the most common cause of death in the United States and in the world, therefore, understanding the details of its mechanism is of great importance to public health. Ischemic preconditioning, in particular, is an interesting phenomenon with a possibility of wide implications in the clinical field. Understanding how it works and how it can be manipulated is crucial.

The goal of the research presented in this thesis was to clarify if preconditioning by potassium channel openers is through the inhibition of succinate dehydrogenase, complex II of the electron chain, as has been claimed by some investigators, or if this kind of preconditioning is mediated by mitochondrial ATP-sensitive potassium channel. The results of this study indicate that the doses of potassium channel openers used to open mitoK_{ATP} in light scattering assays are insufficient to cause any significant respiratory inhibition. In addition, PKG and ΨεRACK, which are known to open mitoK_{ATP} do not inhibit respiration at any dose tested.

Therefore, it appears that preconditioning by diazoxide, 3-NPA, PKG, and ΨεRACK is mediated by mitoK_{ATP} as previously suggested and not through the inhibition of succinate dehydrogenase. Many possible directions of further studies exist to clarify the exact mechanism of cardiac preconditioning.

6. References

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