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MITOCHONDRIAL THERAPEUTICS DURING ISCHEMIA-REPERFUSION; MODULATION OF COMPLEX I: EFFECT OF METFORMIN.

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MITOCHONDRIAL THERAPEUTICS DURING ISCHEMIA-REPERFUSION;

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Physiology & Biophysics at Virginia Commonwealth University.

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List of Abbreviations

AA – Antimycin A

ADP – Adenosine diphosphate

ANOVA – Analysis of Variance

ATP – Adenosine triphosphate

BSA – Bovine serum albumin

CAC – Citric acid cycle

CP1 – Chapell-Perry 1

CP2 – Chapell-Perry 2

CRC – Calcium retention capacity

DNP – Dinitrophenol

EDTA – Ethylenediaminetetraacetic acid

EGTA – Ethylene glycol-bis (2-aminoethylether-N,N,N’,N’-tetraacetic acid)

ETC – Electron transport chain

H_2O_2 – Hydrogen peroxide

HRP – Horse Radish Peroxidase

IMM – Inner mitochondrial membrane

IMS – Intermembrane space

KME – 100 mM KCl, 50 mM MOPS, and 0.5 mM EGTA

$MgSO_4$ – Magnesium Sulfate

MOPS – 3-(N-morpholino)propanesulfonic acid

MPTP – Mitochondrial permeability transition pore

NADH – Nicotinamide adenine dinucleotide

NO – Nitric oxide

$\cdot O_2^-$ - Superoxide

OMM – Outer mitochondrial membrane

PH – Polytron homogenate

PMF – Protein motive force

P_i – Phosphate

ROS – Reactive Oxygen Species

Rot –Rotenone

SEM – Standard error of the mean

Succ – Succinate

TCA – Tricarboxylic acid cycle

TMRM – Tetramethyl rhodamine methyl ester

ΔpH – Proton gradient

$\Delta\psi$ – Mitochondrial membrane potential

$\Delta\psi_o$ – Mitochondrial membrane potential of a completely uncoupled mitochondria

$\Delta\psi_m$ – Mitochondrial maximum membrane potential

Abstract

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The modulation of the electron transport during ischemia-reperfusion has been shown to be protective. We hypothesized that metformin, a Complex I inhibitor, may exhibit characteristics of a pharmacological agent that could achieve long-term therapeutic intervention against ischemia-reperfusion injury. Mitochondria were harvested from adult male mice and incubated with or without metformin at 30^{o} C for 15 minutes, while being shaken at 300 rpm. Metformin decreased Complex I oxidative phosphorylation and Complex I activity. However, metformin also increased injury and decreased the maximum membrane potential. Even though there was a decrease in maximum membrane potential, the proton motive force (PMF) was still intact as the ADP/O ratio was not affected. In conclusion, metformin does exhibit some

characteristics of a drug that could achieve long-term therapeutic benefit against ischemia-reperfusion.

CHAPTER 1: INTRODUCTION

1.1. Mitochondria Background

Mitochondria are required for the production of cellular energy through a process known as cellular respiration. The last step in cellular respiration is oxidative phosphorylation. Oxidative phosphorylation begins with the transfer of electrons from an electron donor to an electron acceptor in the respiratory chain. The last electron acceptor in the respiratory chain is molecular oxygen. Upon acceptance of an electron, oxygen becomes reduced to water. As these electrons are passed down the respiratory chain, the energy released from electron transfer is used to drive protons from the matrix into the intermembrane space (IMS). The buildup of protons in the IMS creates both a proton gradient and a potential difference across this membrane. These protons move down this electrochemical gradient traversing through ATP (Adenosine Triphosphate) Synthase molecules and back into the matrix. The energy released from the movement of protons down their gradient is used to drive ATP Synthase to phosphorylate an ADP (Adenosine Diphosphate) forming an ATP molecule. Newly synthesized ATP molecules are then transported out of the matrix to be used as fuel by the cell[1].

But what happens when this process is disturbed either by injury or disease? For instance, in a circumstance of anoxia or severe hypoxia, there would be a low supply of oxygen, and without oxygen as an electron accepter, ATP synthesis would be inhibited. Consequently, the main source of energy would not be provided by cellular respiration, but by

anaerobic respiration. At first glance, this may seem like a good thing as a loss in energy is being compensated for, but there is a large differential in how much ATP is produced by both processes. The production of ATP in cellular respiration is ~ 30-32 ATP/Glucose, while the production of ATP in anaerobic respiration is ~2 ATP/Glucose. Therefore, it is very important for mitochondria to maintain their ability to carry out oxidative phosphorylation in order to provide sufficient energy for cellular processes. Our goal was to find a way to enable mitochondria to stay intact after ischemia-reperfusion in order for oxidative phosphorylation to be able to still occur even after injury.

1.2. Mitochondria in Ischemia-Reperfusion (I-R)

Mitochondria are both the main source of injury and the main target of injury in ischemia-reperfusion. It is a vicious cycle in where mitochondrial damage brings greater damage upon itself. Ischemia-Reperfusion damages the mitochondria first by decreasing the activity of Complex I, Complex III, and Complex IV[2, 3, 4, 5]. In addition, there is a decrease in cardiolipin content[6]. Cardiolipin is a key phospholipid necessary for Complex IV to work. As a result, a decrease in cardiolipin leads to a decrease in Complex IV activity[6]. The final result is a decrease in respiration and an increase in reactive oxygen species (ROS) produced by the ETC of the mitochondria. These ROS then cause oxidative damage to the membranes of the same mitochondria. The result is greater permeation of the outer mitochondrial membrane (OMM)[7]. Eventually, cytochrome c is able to exit from the mitochondria and activate caspase 3. Caspase 3 is responsible for triggering apoptosis in a cell[7]. Therefore, our hope is to save mitochondria from itself in order to preserve both the mitochondria and the cell.

1.3. Electron Transport Chain (ETC) under Damage

Under pathological conditions such as ischemia, oxygen consumption is stalled. As a result, electrons can no longer be passed from electron carrier to electron carrier down the respiratory chain. More importantly, without the transfer of electrons, protons are not transferred either. Consequently, there is no energy to drive the phosphorylation of ADP to synthesize ATP. The result is a decrease in cellular energy. However, this is not the only problem involved when it comes to a pathological circumstance. A key factor in injury to mitochondria with the occurrence of a disease are the electrons that are prevented from being transferred to distal sites. These electrons react with other molecules to produce a number of ROS from the respiratory chain. These ROS molecules cause oxidative damage to the membranes of the mitochondria causing degradation of the electrochemical gradient of protons. In addition, the increase in membrane permeability allows for a massive influx in Ca^{+2}. This overload of Ca^{+2} activates the opening of mitochondrial permeability transition pores (MPTP). Cytochrome c is then released through these pores as a signal to Caspase-3 activating apoptosis (programmed cell death)[8].

However, normally, there are compensatory mechanisms in place to deal with ROS molecules such as manganese superoxide dismutases (MnSOD), glutathione peroxidases, and the process of autodismutation. Sometimes even a moderate production of ROS can actually even be beneficial as this could trigger a cytoprotective mechanism[9, 10]. However, in ischemia-reperfusion, there is an excess in production of ROS that far exceeds either the compensatory mechanisms in place or the beneficial effects from having ROS. This is because, in ischemia, the lack of oxygen to the heart would result in the slowing down of oxidative phosphorylation and consequently the transfer of electrons down the electron transport chain. This would allow time for remaining unused oxygen molecules to become substrates for further ROS generation in the

mitochondria. The fraction of ROS that is not scavenged by mitochondria are what is responsible for the damage caused by Ischemia-Reperfusion in the mitochondria and the cell.

From previous, studies, the sites of ROS production have been localized to Complex I and Complex III[11, 12]. These sites were discovered by the addition of a complex inhibitor before ischemia. Inhibition of a complex upstream from damage would prevent an increase in ROS production, while inhibition of a complex downstream from damage would not. Therefore, our hope was to find a pharmacological agent that could inhibit a complex in the beginning of the respiratory chain.

1.4. Blockade of Electron Transport Chain (ETC) in protection

During myocardial ischemia, the mitochondria become a major source of cellular injury as it generates ROS products and releases cytochrome c. Therefore, a novel approach was to modulate the ETC during ischemia in order to protect against damage by the mitochondria. Previous studies showed this approach to be protective [11, 13, 14, 15]. One such study used rotenone, an irreversible inhibitor of Complex I, to prevent further electron transport into Complex III during ischemia. The result was the blockade of electron transport during ischemia resulting in the preservation of cardiolipin, cytochrome c, and respiration through Complex IV [11, 16]. Similarly, the application of amobarbital, a reversible Complex I inhibitor, immediately before ischemia also resulted in the preservation of the electron transport during ischemia and mitochondrial protection during reperfusion [11, 17]. However, the modulation of the ETC under aerobic conditions actually leads to cellular injury which leads to cell death[11, 18]. Thus, modulation of the ETC only under pathologic conditions such as ischemia, when mitochondrial respiration is responsible for cellular injury, is beneficial. Therefore, our hope was to find an

agent that could partially inhibit Complex I so as to not be toxic under aerobic conditions, but still maintain protective characteristics upon the occurrence of ischemia.

1.5. Approaches to block the Electron Transport Chain (ETC)

One approach to block the ETC is direct inhibition of a complex. However, the location of inhibition is very important. As seen in the previous section, the blockade of Complex I during ischemia protects the ETC from mitochondrial damage. However, blockade of Complex IV does not exhibit the same protective effects. The reason being that the blockade of Complex IV is a blockade of a distal electron transport. Therefore, electrons would still be able to accumulate at upstream complexes and generate ROS. Thus, the focus was to study inhibitors that could block electron flow proximal to Complex IV.

Another approach was to use non-specific inhibitors that bind to several complexes. For instance, nitric oxide (NO) actually inhibits both Complex I and Complex IV. Complex I inhibition does decrease myocardial injury, but Complex IV inhibition actually increases myocardial injury [11, 19]. Thankfully, the effects of Complex I inhibition by NO far outweighs the effects of Complex IV inhibition [11, 19]. However, our hope was to find an inhibitor that would be specific in order to prevent the possibility of both helping and harming the mitochondria at the same time.

Site-specific posttranslational modifications such as acetylation/deacetylation, glutathionylation, and nitrosation can also inhibit ETC complexes[11, 20, 21, 22]. Previous studies have shown that the use of an S-nitrosating agent before ischemia or during reperfusion rescued Complex I activity through inhibition of activity [11, 22]. Inhibition was also seen to be reversed two

hours after reperfusion [11, 22, 23]. However, it remains to be questioned whether this method would have off-target effects on multiple non-Complex I proteins as well.

The last approach that will be discussed in this section is a genetic approach to modulate electron transport. Complete knock out of a protein complex could cause inhibition, but it would be severe and permanent. Thus, a complete knock out would be detrimental to the mitochondria under aerobic conditions. On the other hand, heterozygous knock outs of a complex would most likely exhibit partial inhibition. However, a genetic approach in vivo is not as feasible as changes are irreversible. This would mean that the electron transport chain would be inhibited not only during ischemia-reperfusion, but under aerobic conditions as well. From previous sections, we know that the blockade of the ETC during aerobic conditions is actually detrimental to cells. Therefore, our hope was to find a pharmacological drug that was not only mild in inhibition, but reversible as well.

1.6. MLS-STAT3E mouse protects Acutely & Chronically

STAT3 is a transcription factor induced by IL-6 and activates acute phase genes. The expression of STAT3 plays a key role in the control of cell growth and host response to inflammation and cellular stress through an increase in expression of antiapoptotic and antioxidant genes [11, 24, 25]. Another study showed the deletion of STAT3 in mouse cardiomyocytes actually enhances inflammation and oxidative stress [11, 26]. Thus, it is perceived that STAT3 plays a key role in the protection against a variety of stresses chronically.

Besides the transgenic approach of STAT3, STAT3 also plays a localized role in mitochondria [11, 27, 28, 29]. STAT3 in mitochondria has been shown to result in a partial blockade of Complex I, but was still able to preserve the membrane potential despite the downregulation of

Complex I. These changes observed at baseline in mitochondrial from mice that overexpress a mitochondria-targeted STAT3 in turn protect against ischemic damage to Complex I and the release of cytochrome c [11, 30]. There is also speculation that STAT3 in the mitochondria non-transcriptionally decreases generation of superoxides from Complex I through inhibition. As a result, this would greatly decrease the probability that cardiolipin oxidation would occur with ischemia [11]. Therefore, our hope is to eventually find if metformin works either acutely, chronically, or even through both mechanisms similar to STAT3.

1.7. Metformin as a Complex I Inhibitor

Metformin belongs to a biguanide class and used for the treatment of Type 2 Diabetes (T2D). The reason why metformin is different from other types of antidiabetic drugs is that it exhibits a "superior safety profile"[31]. For instance, the incidence of lactic acidosis with metformin is rarer than non-metformin therapies[31]. In addition, metformin does not induce hypoglycemia or weight gain[31].

In some non-cardiac tissues, metformin decreases cellular respiration by mild and specific inhibition of Complex I [31, 32, 33]. However, it has not been studied whether this mild inhibition occurs in the heart nor if it is sufficient enough for protection during ischemia-reperfusion. Therefore, we decided to explore this aspect of metformin. In addition, the site and mechanism of how metformin inhibits Complex I is still not yet known either. Therefore, our lab decided to test whether metformin directly inhibits Complex I. It is known that the inhibition of Complex I does result in a drop in ATP synthesis. Consequently, there is an increase in both ADP/ATP ratio and the AMP/ATP ratio. The increase in both ratios activates AMP-activated kinase (AMPK)[31, 34]. AMPK is an energy sensor for cellular energy homeostasis that coordinates

protective responses[31, 34]. However, metabolic conditions are not the only factors that can activate AMPK. AMPK can also be activated by an increase in the intracellular levels of ROS regardless of a change in the rate of ATP synthesis[10]. Therefore, we wanted to know whether the amount of ROS produced with the use of metformin could possibly be protective through the activation of AMPK.

1.8. Rationale for the Present Study

The aim of this study was to determine if metformin exhibits characteristics of a pharmacological agent that could cause long-term cardiac or mitochondrial protection. We hypothesized that metformin would exhibit all the characteristics of an agent that could cause long-term protection. These are the partial blockade of electron transport at Complex I, a decrease in the production of ROS, and an unaffected mitochondrial membrane potential[20]. To test this hypothesis, mitochondria were harvested from adult mice and incubated with or without metformin at 30^{o} for 15 minutes with shaking at 300 rpm. The effect of metformin on mitochondria was compared to the incubated control. The rates of oxidative phosphorylation on Complex I and Complex II, the release of H_2O_2, the calcium retention capacity, the maximum membrane potential, and the activity of Complex I were all measured to determine if metformin exhibited characteristics for long-term protection against ischemia-reperfusion.

CHAPTER 2: MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals & Reagents

Chemicals and reagents used for mitochondria isolation and mitochondria-related functional assays were purchased from Sigma-Aldrich, Saint Louis, MO. All other chemicals and reagents were purchased from Fisher Scientific, Pittsburgh, PA unless otherwise stated.

2.1.2. Animals

The mice used in these experiments were C57BL/6 Males purchased and received from Harlan Sprague Dawley, Inc. (Indianapolis, IN) aged eight to twelve weeks through the Division of Animal Resources (DAR) of Virginia Commonwealth University (VCU). These were all housed in a fully AAALAC-accredited Animal Research Facility at VCU. Each mouse used in these experiments was deeply anesthetized with pentobarbital (90 mg/kg; i.p.) and all procedures followed guidelines for the humane use and care of laboratory animals for biomedical research published by the National Institutes of Health. All protocols involving animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of VCU.

2.2. Methods

2.2.1. Isolation of heart tissue homogenates, cytosol, and mitochondria

Male mice were first anesthetized with pentobarbital (90 mg/kg; i.p.) followed by the quick excision of the heart. Afterwards, the heart was placed into a chilled modified Chapell-Perry 1 (CP1) buffer wash [100 mM KCl, 50 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 1mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 5 mM $MgSO_4 \cdot 7H2O$, and 1 mM adenosine 5'-triphosphate disodium (ATP)] (pH 7.4). Next, the heart was dried with Whatman filter paper, weighed, and placed back in an empty beaker on ice. The heart was then minced and transferred to a glass tube for homogenization with 3 ml of CP1 buffer. A polytron tissue blender was then used for 2.5 s at a rheostat setting of 10,000 rpm to further mince the tissue. Once fully homogenated, 50 ul of the polytron homogenate (PH) was saved as a heart tissue extract and the rest was centrifuged at 6,000 x g for 10 min at 4^{o}C. The supernatant was then saved as a crude cytosol for further purification (see below) and the pellet of the homogenate was re-suspended in 3 ml of CP1 buffer. In addition, 5 mg/g (wet weight) of trypsin (#T0303, Sigma-Aldrich, Saint Louis, MO) was placed in with the 3 ml of CP1 and the pellet. The pellet was incubated in trypsin for 15 min at 4^{o}C. After 15 min, 3 ml of CP2 buffer [CP1 and 0.2% of Bovine Serum Albumin (BSA) (#A7030, Sigma Aldrich, Saint Louis, MO)] was added to stop the effect of trypsin. The digested tissue was then transferred into a glass tube where it was homogenized with a tight Teflon pestle twice at a speed of 600 rpm. Afterwards, the homogenate was placed into a 15 ml centrifuge tube and centrifuged at a speed of 500 rpm for 10 min at 4^{o}C. This was done in order to separate the undigested and heavier cell fractions into a pellet and the mitochondria into a supernatant. The supernatant was then poured into a separate 15 ml centrifuge tube and was centrifuged at a speed of 3000 x g for 10 min at 4^o C. The

supernatant was then discarded and the pellet re-suspended in 2 ml of KME buffer [100 mM KCl, 50 mM MOPS, and 0.5 mM EGTA]. The re-suspended mitochondria were centrifuged at 3000 x g for 10 min at 4ºC. The supernatant was then tossed as waste and the mitochondrial pellet re-suspended in 100 ul of KME. Lastly, protein concentration was measured with the use of a Lowry Protein Assay. BSA was used as the standard and sodium deoxycholate (Sigma-Aldrich, Saint Louis, MO) as the detergent.

The crude cytosolic fractions extracted (see above) were then pipetted into two 1 ml Eppendorf's and centrifuged at 22,000 x g for 30 min at 4ºC. Afterwards, a syringe-attached 0.1 um filter was used to extract pure cytosol as the end product. The protein concentration of the cytosol was then determined with the use of the same Lowry Protein Assay mentioned above.

2.2.2. Incubation of mitochondria with and without metformin

Glutamate + malate respiration required 150 ug of isolated mitochondria, while succinate respiration required 100 ug of isolated mitochondria. Two out of the three groups measured required incubation, while the normal non-incubated control ("Control") did not. An incubated control was created with the addition of 20 ul of KME to the appropriate amount of mitochondria (150 ug for G + M respiration & 100 ug for succinate respiration) and was listed as "0 mM Incubated". For a 0.5 mM concentration of metformin, 18 ul of KME and 2 ul of metformin from a stock solution (listed below) were added together with the appropriate amount of mitochondria needed for each run and was listed as "0.5 mM Incubated". For a 5 mM concentration of metformin, 20 ul of metformin from the same stock solution was added together with the appropriate amount of mitochondria needed for each run and was listed as "5 mM Incubated". For the measurement of H_2O_2 production and membrane potential, 200 ug of mitochondria was

used, while 250 ug of mitochondria was used to measure the Calcium Retention Capacity (CRC). For these measurements, only either 20 ul of KME for a 0 mM concentration ("0 mM Incubated") or 20 ul of metformin for a 5 mM concentration ("5 mM Incubated") were added to isolated mitochondria in addition to a normal control without incubation ("Control"). This was done because we only wanted to see the effects of metformin on these assays at a high concentration. Metformin incubated in mitochondria were then spun at 300 rpm for 15 min at 30^{o} C. The stock solution of metformin (127 mM) used above was made with the addition of 21 mg of metformin to 1 ml of KME.

2.2.3. Measurement of oxidative phosphorylation in incubated mitochondria

Oxygen consumption was measured by a 782 Oxygen Meter System Version 3.0 (Strathkelvin Instruments, Glasgow, Scotland) in a glass chamber of a MT200 Mitocell Respirometer (Strathkelvin Instruments, Glasgow, Scotland) with a 1302 Microcathode Oxygen Electrode (Strathkelvin Instruments, Glasgow, Scotland) at a temperature of 30^{o}C. The glass chamber was filled with 500 ul of a respiration buffer [80 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM KH_2PO_4, and 1 mg/ml defatted BSA] (pH 7.4). Complex I respiration was measured with 150 ug of mitochondria and Complex II respiration was measured with 100 ug of mitochondria. After mitochondria was added to the chamber, either 20 mM Glutamate + 5 mM of Malate for Complex I Respiration or 20 mM Succinate + 7.5 uM Rotenone for Complex II Respiration was added to the chamber. 10 ul of 10 mM ADP was then added to the chamber for a final concentration of 0.2 mM ADP in order to measure State 3 and State 4 respiration. This step was repeated once and then followed with the addition of 10 ul of 100 mM of ADP for a final concentration of 2 mM ADP. Recordings taken at this point in time measured the maximum rate

of State 3 respiration (maximum coupled respiration rate). Lastly, 10 ul of 10 mM of the uncoupler DNP (2,4-dinitrophenol) for a final concentration of 0.2 mM was added to measure the maximum rate of State 4 respiration (maximum uncoupled respiration rate).

2.2.4. Incubated mitochondrial H_2O_2 production from complex I

An Amplex Red reagent (10-acetyl-3, 7-dihydroxyphenoxazine, A-12222) (Invitrogen, Carlsbad, CA) was used to measure the hydrogen peroxide production in incubated mitochondria. Specifically, Amplex Red was used to be oxidized in order for hydrogen peroxide to be reduced by horseradish peroxidase (HRP) (Sigma-Aldrich, Saint Louis, MO) which together produces the fluorescent product resorufin. A LS 55 Fluorescence Spectrometer (PerkinElmer Instruments, Waltham, MA) was used to measure the fluorescence of resorufin at a temperature of 30^{o}C in the presence of maximal stirring.

The final volume for this reaction in a cuvette will be 2 ml. The first step to measure hydrogen peroxide production in incubated mitochondria was to add 0.2 mg (4-6 ul based on the sample) of incubated mitochondria with or without metformin into 1 ml of MMC Buffer [Final Concentrations: 150 mM KCl, 5 mM KH_2PO_4, 1 mM EGTA; Chelex Treated Buffer] and 950 ul of H_2O. Afterwards, Amplex Red (Final Concentration: 25 uM) and HRP (Final Concentration: 0.20 units/ml) were added. 10 ul of Glutamate (Final Concentration: 6.67 mM) and 5 ul of Malate (Final Concentration: 3.33 mM) were then added afterwards to measure the H_2O_2 production from Complex I and Complex III, while 2 ul of rotenone (Final Concentration: 5 uM) was added after the Glutamate and Malate addition to measure only Complex I dependent H_2O_2 production. Generated H_2O_2 was then measured and compared to a standard curve with known concentrations.

2.2.5. Determination of Calcium Retention Capacity in incubated mitochondria

Calcium Retention Capacity (CRC) measures the mitochondrial susceptibility to calcium-mediated opening of MPTP. A LS 55 Fluorescence Spectrometer (PerkinElmer Instruments, Watham, MA) was used to calculate the measure of Ca^{+2} uptake with the use of a fluorescent probe, Calcium-Green 5N (Invitrogen, Carlsbad, CA), at excitation and emission wavelengths of 500 and 530 nm respectively. The units of Calcium-Green fluorescence were in mmol of $CaCl_2$/mg of mitochondrial protein and converted with the use of a standard curve to calculate $[Ca^{+2}]$.

The initial step of CRC involves the addition of 10 ul of a 0.5 mM calcium green to 2 ml of a CRC Buffer (150 mM Sucrose, 50 mM KCl, 2 mM KH_2PO_4, and 20 mM Tris/HCl, pH 7.4) at a temperature of 30^{o}C with maximal stirring. Incubated mitochondria (250 ug of protein; 6.15 ul of protein) along with the appropriate dose of metformin was then added to the mixture. After 60 s, 10 ul of 1 mM $CaCl_2$ was added every minute until there were MPTP openings resulting in calcium release. CRC was then recorded as the total concentration of Ca^{+2}-uptake before the release of calcium.

2.2.6. Measurement of mitochondrial membrane potential in incubated mitochondria

Maximum membrane ($\Delta\psi_m$) potential and depolarization potential were measured with a A LS 55 Fluorescence Spectrometer (PerkinElmer Instruments, Waltham, MA) at an emission intensity of 590 nm and with a tetramethylrhodamine methyl ester (TMRM) dye (Invitrogen, Carlsbad, CA). TMRM in the cytosol of an uncoupled mitochondria exhibits a fluorescence at a wavelength of 573 nm, while TMRM that has entered into the mitochondria of coupled or

depolarized mitochondria exhibit a fluorescence at a wavelength of 546. A ratio between these two wavelengths exhibited the membrane potential of a mitochondria.

Two-hundred micrograms of incubated mitochondria and the appropriate amount of metformin were added to 2 ml of a respiration buffer (100 mM KCl, 50 mM MOPS, 5 mM KP_i, 1 mM EGTA) and 10 ul of 0.1 mM TMRM at 30ºC with stirring. The addition of 10 ul of Glutamate (Final Concentration: 5 mM) + 5 ul of Malate (Final Concentration: 1.25 mM) triggered State 2 Respiration and the addition of 10 ul of 10 mM ADP (Final Concentration: 0.2 mM) triggered State3 Respiration (State 3 – protons pass through Complex V to synthesize ATP from ADP). The addition of 2 ul of oligomycin triggers a $State4_{Oligomycin}$ state. A $State4_{Oligomycin}$ state involves the inhibition of Complex V by oligomycin. As a result, protons cannot fall down their concentration gradient and membrane potential is restored. The continuous addition of 10 ul of 10 mM DNP results in the complete uncoupling of the mitochondria ($\Delta\psi_0$). Subtraction of $\Delta\psi_0$ from $State4_{Oligomycin}$ represented physiologic $\Delta\psi_m$ and the difference between State2 and the end of State3 respiration represented the depolarization potential.

2.2.7. Electron transport chain assay of complex I

Spectrophotometric measurements at 30ºC were used to measure the enzyme activities of detergent-solubilized mitochondria. First, 25 ug of frozen mitochondria was thawed and added to 25 ul of 0.5% cholate and the appropriate amount of KME to give a total volume of 250 ul. The end result was a final protein concentration of 1 ug/ul. Calculations of enzyme activity were expressed as nmol/min/mg of mitochondrial protein.

The final volume for this assay will be 1 ml. First, fifteen micrograms of cholate solubilized mitochondria were added to 560 ul of Gimish Buffer (Final Concentrations: 50 mM

KH_2PO_4 pH 7.4, 0.1 mM EDTA, 5% defatted BSA (w/v), 0.015% Sonicated Asolectin), 0.2 mM NADH (final concentration), and 20 ug of Antimycin A (AA). In addition, rotenone was added to one cuvette to calculate Complex I specific activity (rotenone – a specific inhibitor of NADH-ubiquinone activity). After 2 min of incubation, there was an addition of 20 ul of oxidized decylubiquinone (DUQ) (Final Concentration: 0.05 mM). The decrease in the NADH absorbance (340 nm; 6.22 $mM^{-1}cm^{-1}$) was monitored. The oxidation of exogenously added reduced NADH resulted in a decrease of absorbance at 340 nm. This decrease represented Complex I activity. From these readings, an extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ for NADH was used to calculate the enzyme activity of Complex I. Lastly, rotenone sensitivity was measured by subtracting the rate measured with rotenone from the rate without rotenone.

2.2.8. NADH-ferricyanide reductase (NFR) assay

NADH-ferricyanide reductase (NFR) assay measured Complex I activity proximal to the rotenone inhibition binding site. NADH was added as a substrate and reduced added $FeCN_6^-$ in Complex I as an endpoint. The portion proximal to the site of $FeCN_6^-$ reduction was recognized as the rotenone-insensitive proximal portion of Complex I as it would be unaffected by rotenone inhibition. Measurements were recorded as a decrease in absorbance at a wavelength of 340 nm caused by oxidation of added NADH. The final volume of this assay contained 1 ml of solutions (Final Concentrations: 50 mM KH_2PO_4, 0.1 mM EDTA, 0.2% de-fatted BSA (w/v), 0.015% sonicated asolectin, 0.66 mM $K_3Fe(CN)_6$ (potassium ferricyanide – an artificial electron acceptor from FMN), 2 mM NaN_3, and 0.2 mM NADH) and maintained at a pH of 7.4. After a baseline reading, 5 ug of cholate solubilized mitochondria were added, and the NFR activity was measured using an extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ for NADH. However, the actual rates

of NFR activity were calculated by subtracting the baseline rate (all components with no mitochondria) from the NFR readings with mitochondria present.

2.2.9. Data Analysis and Statistics

Data was presented as both mean ± standard error of the mean (SEM) and percentages. Percentages were calculated by dividing the value obtained from an incubated sample to the value obtained from an immediately tested corresponding control. Differences between only two groups were analyzed with a two-tailed Student's t-test. Differences between multiple groups were analyzed with a one-way analysis of variance followed by a Student-Newman-Keuls post-test for a pair-wise comparison of all groups. $p < 0.05$ was considered to be statistically significant.

CHAPTER 3: RESULTS

3.1. Glutamate+Malate-dependent but not succinate-dependent oxidative phosphorylation was decreased by metformin at high concentration

Rates of oxidative phosphorylation were measured in a non-incubated control ("Control"), a no metformin incubated control ("0 mM Incubated), a 0.5 mM metformin final concentration ("0.5 mM Incubated"), and a 5 mM metformin final concentration ("5 mM Incubated"). Complex I respiration was measured by the addition of Glutamate + Malate, while Complex II respiration was measured with the addition of Succinate + Rotenone as substrates. As can be seen in Figure 3.1.1 and Figure 3.1.2., there is a significant decrease between the "Control" and the "0 mM Incubated" group. This can be explained by the fact that some mitochondrial function decreases with incubation at 30^{o} C[34]. Therefore, it was more appropriate to measure the metformin concentrates to the "0 mM Incubated" control group. In Figure 3.1.1., we see that there was no decrease in coupled respiration of Complex I with a low concentration of metformin (0.5 mM), but the addition of a high concentrate (5 mM) caused a 44% decrease in coupled respiration. Figure 3.1.2. shows the same result, but in uncoupled respiration. In the presence of a high concentrate (5 mM), uncoupled respiration decreased by 24%. Figure 3.1.3. shows that there is a greater decrease in Maximal Glutamate + Malate Coupled Respiration than in Maximal Glutamate + Malate Uncoupled Respiration with the addition of a high concentration of metformin. However, Figure 3.1.4. and 3.1.5. shows that even 5 mM of metformin has no effect on either coupled or uncoupled respiration of Complex II. Maximum

coupled respiration was measured with the addition of 2 mM ADP, and uncoupled respiration was measured with the addition of 2,4-dinitrophenol (DNP).

ADP/O ratios were also measured by dividing the rate of ADP consumption over the rate of oxygen consumption within the glass chamber. Neither the addition of a low concentration nor a high concentration of metformin significantly changed the ADP/O ratio as can be seen in Figure 3.1.6.

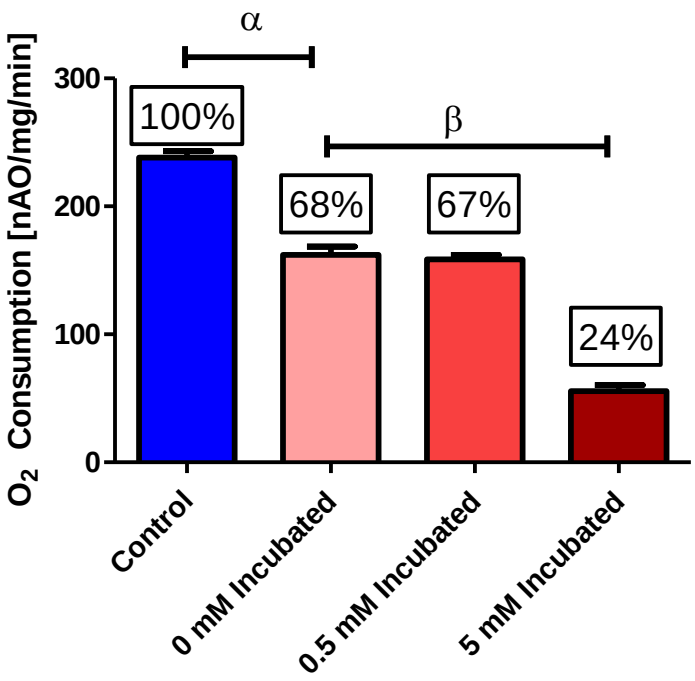

Figure 3.1.1. Metformin decreases Maximal Glutamate + Malate Coupled Respiration of Incubated Mitochondria. Oxygen consumption in incubated mitochondria was measured as described in Materials and Methods. Maximal glutamate coupled respiration was measured with the addition of 10 ul of 100 mM ADP for a final concentration of 2 mM ADP. The unit of measurement was nanoatoms of atomic oxygen/min/mg of mitochondrial protein (nAO/mg/min) and data are presented both as a mean ± SE and a percentage with the normal control as 100%. *A one-way ANOVA test was used to compare all results. n = 3 for all groups, $^{\alpha}P < 0.05$ Control vs. 0 mM Incubated, $^{\beta}P < 0.05$ 0 mM Incubated vs. 5 mM Incubated.

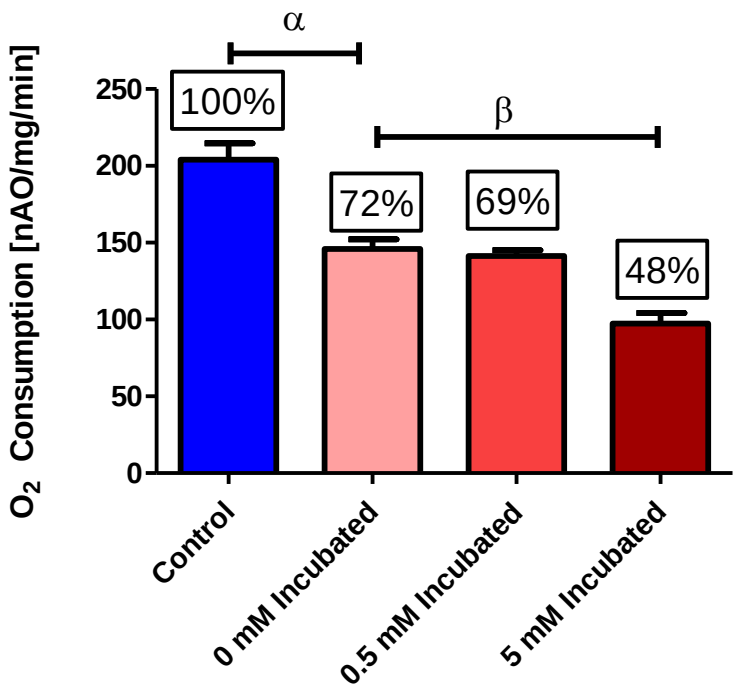

Figure 3.1.2. Metformin decreases Maximal Glutamate + Malate Uncoupled Respiration of Incubated Mitochondria. Oxygen consumption in incubated mitochondria was measured as described in Materials and Methods. Maximal glutamate uncoupled respiration was measured with the addition of 10 ul of 10 mM dinitrophenol (DNP) for a final concentration of 0.4 mM DNP. The unit of measurement was nanoatoms of atomic oxygen/min/mg of mitochondrial protein (nAO/mg/min) and data are presented both as a mean ± SE and a percentage with the normal control as 100%. *A one-way ANOVA test was used to compare all results. n = 3 for all groups, $^{\alpha}P < 0.05$ Control vs. 0 mM Incubated, $^{\beta}P < 0.05$ 0 mM Incubated vs. 5 mM Incubated.

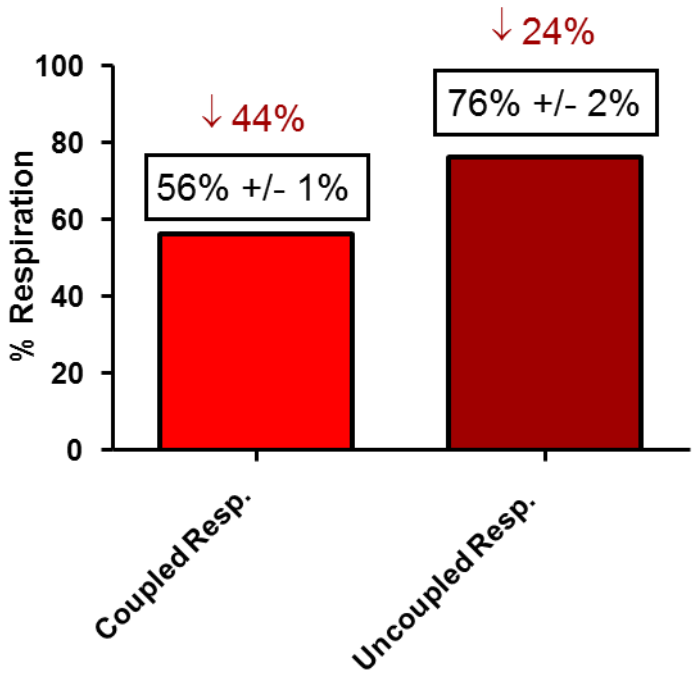

Figure 3.1.3. Metformin decreases Maximal Glutamate + Malate Coupled Respiration more than Maximal Glutamate + Malate Uncoupled Respiration. Maximal Glutamate + Malate Coupled Respiration measurements were taken from Figure 3.1.1., while Maximal Glutamate + Malate Uncoupled Respirations measurements were taken from Figure 3.1.2 n = 3 for all groups.

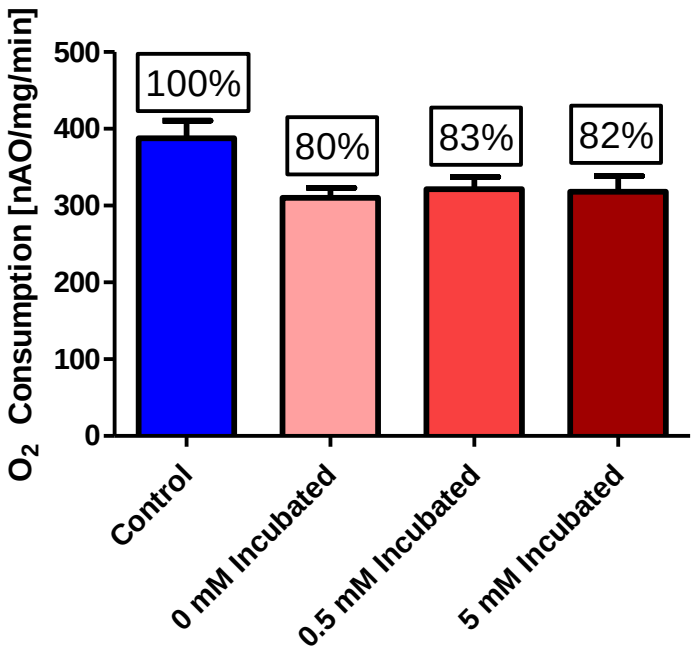

Figure 3.1.4. Metformin does not affect Maximal Succinate Coupled Respiration. Oxygen consumption in incubated mitochondria was measured as described in Materials and Methods. Maximal succinate coupled respiration was measured with the addition of 10 ul of 100 mM ADP for a final concentration of 2 mM ADP. The unit of measurement was nanoatoms of atomic oxygen/min/mg of mitochondrial protein (nAO/mg/min) and data are presented both as a mean ± SE and a percentage with the normal control as 100%. *A one-way ANOVA test was used to compare all results. n = 3 for all groups.

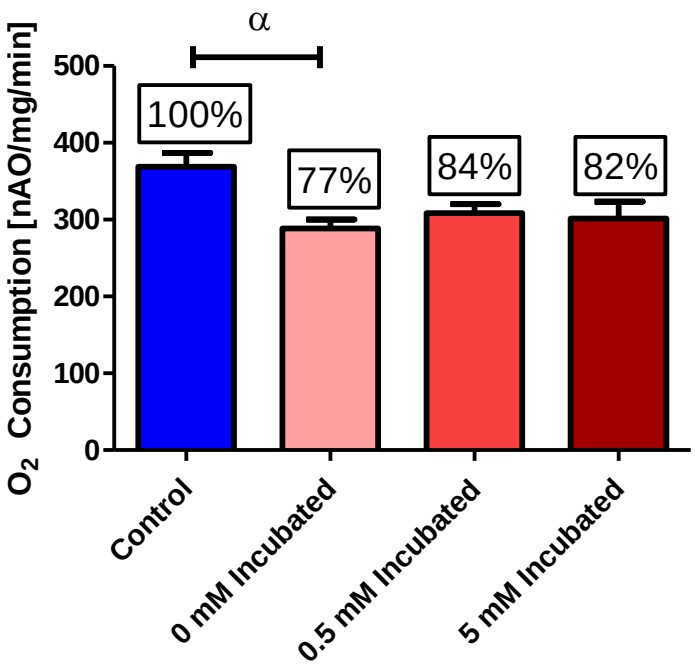

Figure 3.1.5. Metformin does not affect Maximal Succinate Uncoupled Respiration. Oxygen consumption in incubated mitochondria was measured as described in Materials and Methods. Maximal succinate uncoupled respiration was measured with the addition of 10 ul of 10 mM DNP for a final concentration of 0.4 mM DNP. The unit of measurement was nanoatoms of atomic oxygen/min/mg of mitochondrial protein (nAO/mg/min) and data are presented both as a mean ± SE and a percentage with the normal control as 100%. *A one-way ANOVA test was used to compare all results. n = 3 for all groups, $^{\alpha}P < 0.05$ Control vs. 0 mM Incubated.

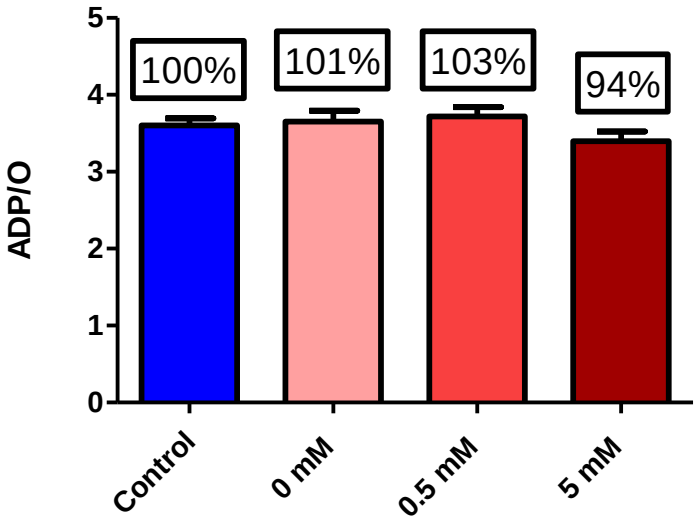

Figure 3.1.6. Metformin does not significantly change the ADP/O ratio. ADP consumption by isolated mitochondria was measured in the oxygen electrode chamber and divided by the amount of oxygen needed to consume added ADP. *A one-way ANOVA test was used to compare all results. n = 3 for all groups.

3.2. The addition of metformin significantly increases the release of ROS from Complex I

Net H_2O_2 release was used as a measure of ROS production in incubated mitochondria. The maximum ability of Complex I to produce H_2O_2 was carried out with the addition of Glutamate + Rotenone. Rotenone blockade maximizes ROS from Complex I[2, 35]. Rotenone is a Complex I inhibitor and would prevent the production of ROS from other complexes in the ETC. As seen in Figure 3.2.1., there is a significant increase in H_2O_2 release from Complex I with the addition of 5 mM metformin.

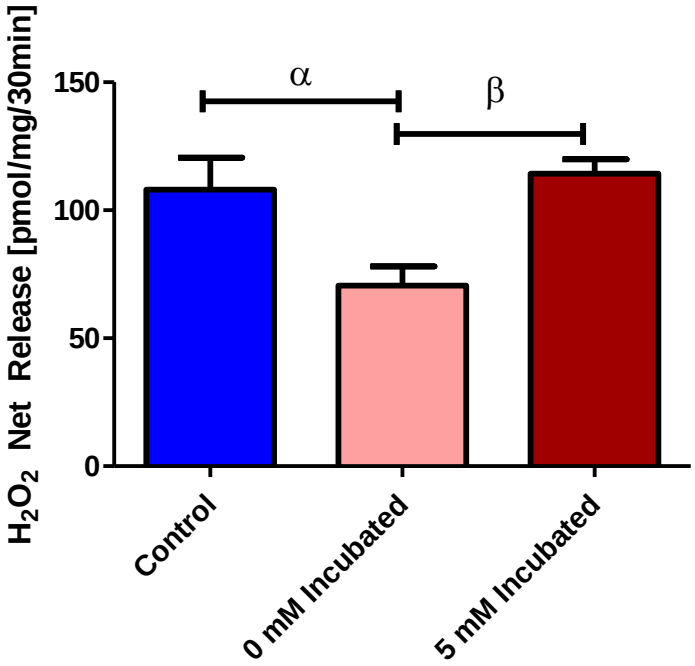

Figure 3.2.1. Metformin significantly increases the release of H_2O_2 from intact mitochondria in the presence of a Complex I substrate. Net production of H_2O_2 from Complex I in incubated mitochondria was measured with glutamate+malate+rotenone, Amplex Red, and HRP. The unit of measurement was pmol/mg/30min and was presented as a mean ± SE. *A one-way ANOVA test was used to compare all results. n = 3 for all groups, $^{\alpha}P < 0.05$ Control vs. 0 mM Incubated, $^{\beta}P < 0.05$ 0 mM Incubated vs. 5 mM Incubated.

3.3. Metformin does not change the susceptibility of mitochondrial permeability transition pore (MPTP) proteins in an incubated mitochondria

At the onset of reperfusion in ischemia-reperfusion, a calcium overload activates mitochondrial permeability transition pore (MPTP) proteins[36]. The result is the opening of the permeability transition pore that bridges the IMM and OMM. Calcium Retention Capacity (CRC) was used as a measure of the susceptibility of incubated mitochondria to the opening of MPTP as described in the methods. Figure 3.3.1. shows metformin does not improve nor worsen mitochondria susceptibility to MPTP openings.

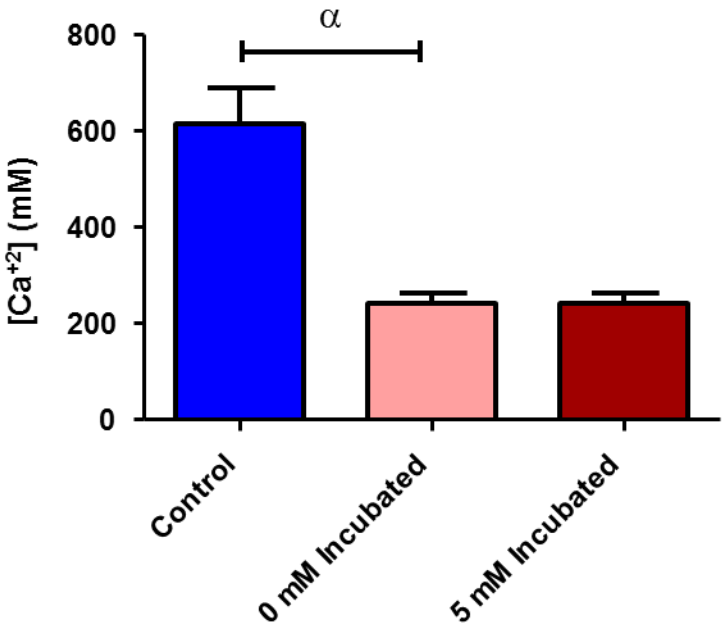

Figure 3.3.1. Metformin does not change MPTP susceptibility in incubated mitochondria. The unit of measurement was presented as a mean ± SE. *A one-way ANOVA test was used to compare all results. n = 3 for all groups, $^{\alpha}P < 0.05$ Control vs. 0 mM Incubated.

3.4. Metformin decreases the maximum mitochondrial potential ($\Delta\psi_m$).

Tetramethyl rhodamine methyl ester (TMRM) dye was used in this assay because it is both positively charged and lipophilic. These characteristics of TMRM allowed it to cross the membrane. As positive charged TMRM enters into the mitochondria, the membrane would become depolarized. Upon polarization, TMRM would have exited the mitochondria and entered into the buffer. The greater the negative charge on the matrix side of the IM, the greater the TMRM uptake and the lower the fluorescence.

Therefore, the addition of a substrate (i.e. Glutamate + Malate) would both result in the exit of the dye and polarization of the mitochondria causing maximum fluorescence. State 2 respiration occurred upon the addition of the Glutamate + Malate substrate. Upon addition of max ADP, positively charged molecules would move into the mitochondria and cause maximum depolarization. When the added ADP no longer aids in ATP synthesis, the graph will flatten out, and at this point, State3 respiration has just finished. The difference between State2 respiration and the end of State3 respiration represented the depolarization potential (depol. ψ) (See Figure 3.4.1.). The depolarization potential represented the ability of mitochondria to meet energy demands, but not maximal energy demands if needed. Figure 3.4.2. shows there was no significant change in the depolarization potential with the addition of metformin.

Maximum mitochondrial membrane potential ($\Delta\psi_m$) represents a way to measure the integrity of the mitochondrial membrane and the ability of protons to be pumped across the inner membrane during electron transport through the respiratory chain. The addition of an oligomycin, an ATP synthase inhibitor, substrate followed by a DNP, an uncoupler, substrate was used to measure the maximum membrane potential of an incubated mitochondria. The addition of an oligomycin would create a maximal inner membrane polarization state as protons would only be able to be moved from the matrix

to the IMS. On the other hand, DNP will allow free flow of protons back into the matrix and fully depolarize the membrane. The difference between the maximal inner membrane polarization state and a fully depolarized membrane represented the maximal mitochondrial potential (See Figure 3.4.1.). Figure 3.4.3. shows there was a significant decrease in the maximum membrane potential not only with incubation, but with the addition of metformin as well.

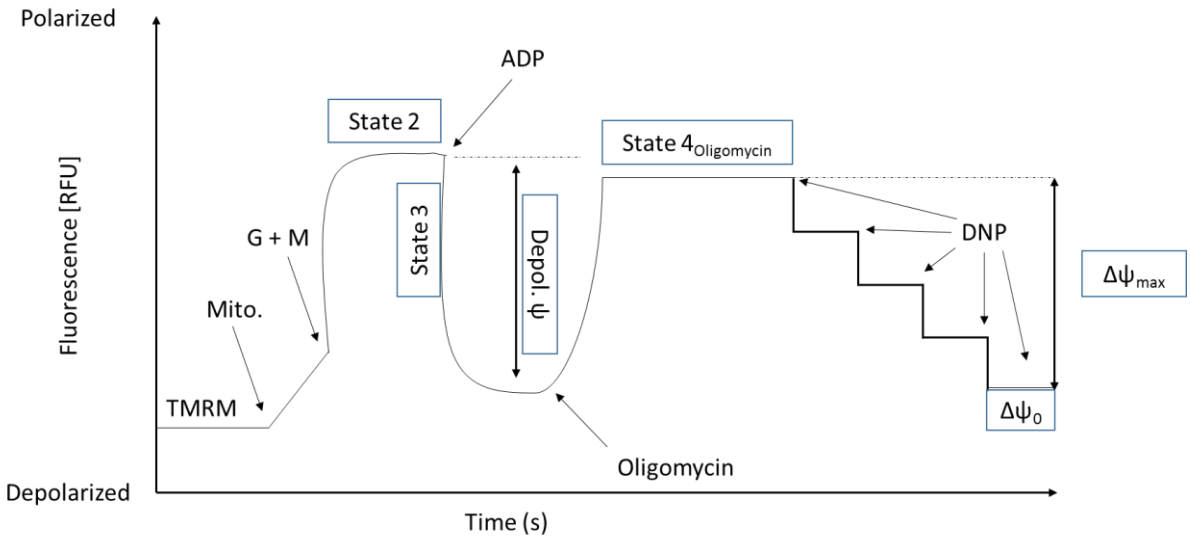

Figure 3.4.1. Mitochondrial Membrane Potential States and Measurements. A 0.1 uM TMRM dye was used to measure the depolarization potential (depol. ψ) and maximum membrane potential ($\Delta\psi_m$) of an incubated mitochondria. Depolarization potential (depol. Ψ) was calculated by taking the difference in membrane potential between State2 and the end of State3 respiration, while maximum membrane potential ($\Delta\psi_m$) was calculated by taking the difference in membrane potential between fully polarized mitochondria ($State4_{Oligomycin}$) and fully uncoupled mitochondria ($\Delta\psi_0$).

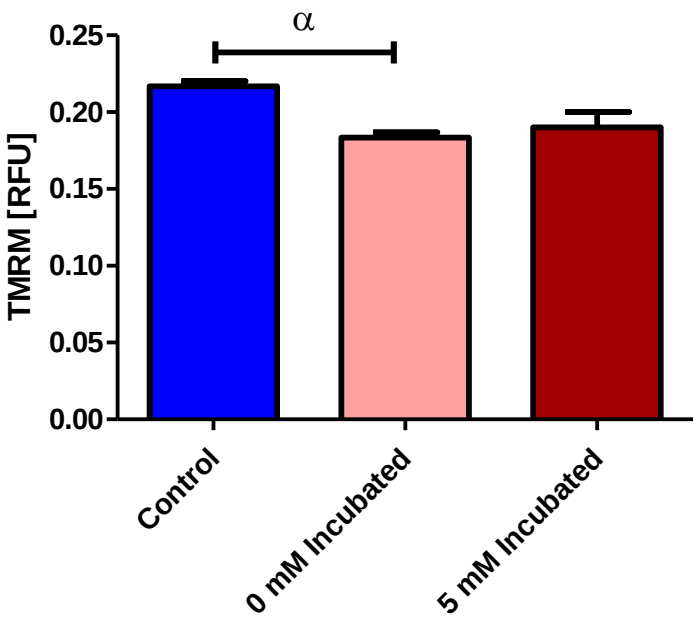

Figure 3.4.2. Metformin does not affect depolarization potential. A 0.1 uM TMRM dye was used to measure the depolarization potential (depol. ψ) of an incubated mitochondria. Depolarization potential (depol. ψ) was calculated by taking the difference in membrane potential between State2 and the end of State3 respiration. The unit of measurement was in RFU (Relative Fluorescence Units) and was presented as a mean ± SE.*A one-way ANOVA test was used to compare all results. n = 3 for all groups, $^{\alpha}P < 0.05$ Control vs. 0 mM Incubated.

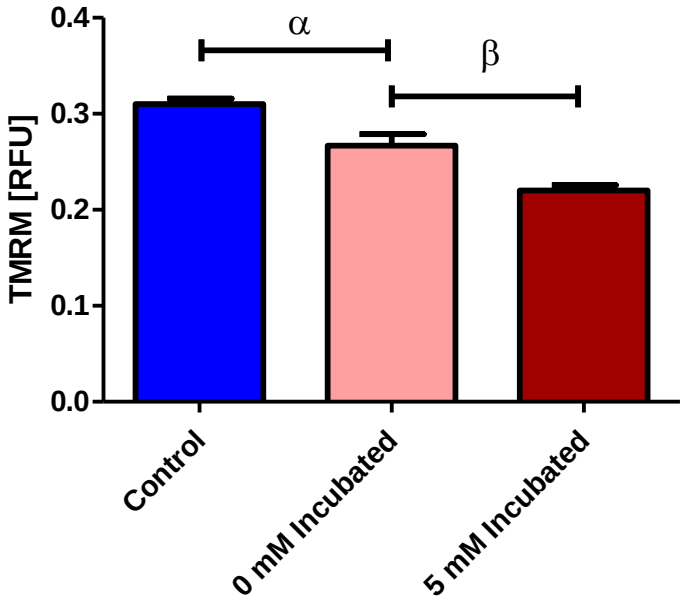

Figure 3.4.3. Metformin decreases the maximum mitochondrial potential ($\Delta\psi_m$). A 0.1 uM TMRM dye was used to measure the maximum membrane potential ($\Delta\psi_m$) of an incubated mitochondria. Maximum membrane potential ($\Delta\psi_m$) was calculated by taking the difference in membrane potential between fully polarized mitochondria and fully uncoupled mitochondria. The unit of measurement was in RFU (Relative Fluorescence Units) and was presented as a mean ± SE. *A one-way ANOVA test was used to compare all results. n = 3 for all groups, $^{\alpha}P < 0.05$ Control vs. 0 mM Incubated, $^{\beta}P < 0.05$ 0 mM Incubated vs. 5 mM Incubated.

3.5. Maximal enzymatic activity of Complex I decreased upon treatment with metformin.

Complex I activity was measured with the consumption of NADH by Complex I in both a normal control and in the presence of 5 mM of metformin. An incubated control was not used here because mitochondria was added into sodium cholate permeabilizing the mitochondrial membrane. Figure 3.5.1. shows Complex I activity decreases by 34% with the addition of 5 mM of metformin.

Figure 3.5.1.a.

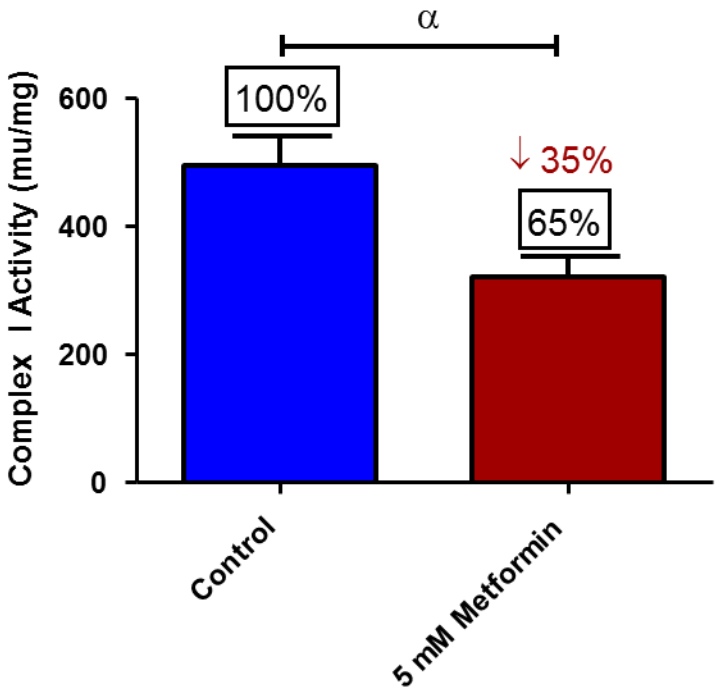

Figure 3.5.1.b.

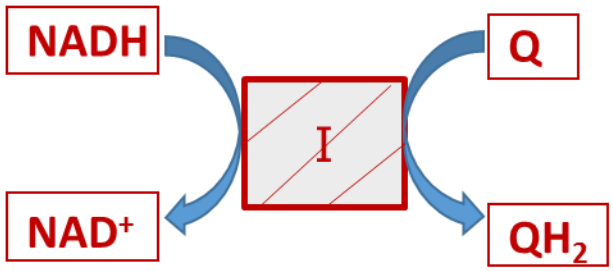

Figure 3.5.1.a. Maximal enzymatic activity of Complex I decreased upon treatment with metformin. Enzymatic activity was spectrophotometrically measured as outlined in the Materials and Methods section. The unit of measurement was in nanomole of substrate decreased per minute per mg of mitochondrial protein [nmol/min/mg] and was presented as a mean ± SE. *A t-test was used to compare results. n = 4 for all groups, $^{\alpha}P < 0.05$ Control vs. 5 mM Incubated.

Figure 3.5.1.b. Site of Complex I Activity Assay. NADH was added as a substrate, and oxidized decylubiquinone was added as a ubiquinone analog in order to act as the final electron acceptor in Complex I. Consequently, the assay represented the entire activity of Complex I and not just a portion.

3.6. Metformin does not react with the rotenone-insensitive proximal portion of Complex I

NFR enzymatic activity was measured with the consumption of NADH by Complex I in both a normal control and in the presence of 5 mM of metformin. An incubated control was not used here because mitochondria was added into sodium cholate permeabilizing the mitochondrial membrane. Figure 3.5.2.a. shows maximal NFR enzymatic activity in Complex I was not significantly changed with the addition of 5 mM of metformin. Figure 3.5.2.b. shows where the site of NFR activity occurs.

Figure 3.5.2.a.

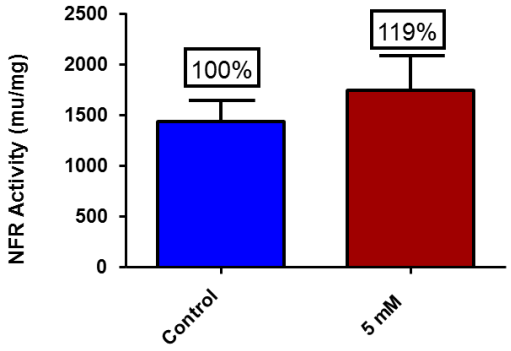

Figure 3.5.2.b.

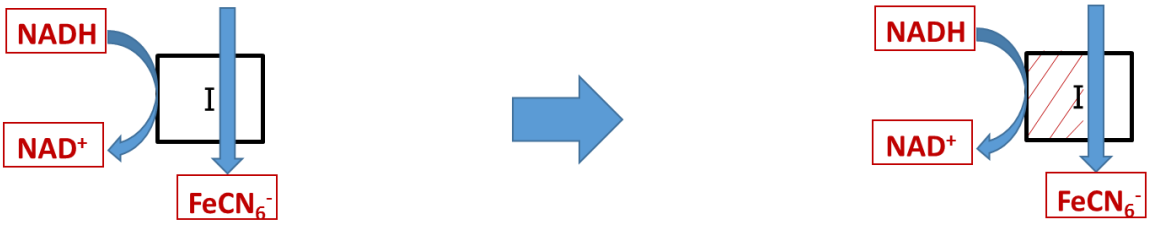

Figure 3.5.2a. Metformin does not affect NFR activity. Enzymatic activity was spectrophotometrically measured as outlined in the Materials and Methods section. The unit of measurement was in nanomole of substrate decreased per minute per mg of mitochondrial protein [nmol/min/mg] and was presented as a mean ± SE. *A t-test was used to compare results. n = 5 for all groups.

Figure 3.5.2.b. Site of NFR Activity. NADH was added as a substrate and the NADH dehydrogenase portion of Complex I will oxidize NADH. The NADH dehydrogenase portion of Complex I is also non-covalently bonded to a flavin-mononucleotide (FMN) molecule. This FMN will molecule will be reduced by the electron donated by NADH and will be oxidized by added $K_3[Fe(CN_6)]$. The portion proximal to the site of electron donation to $K_3[Fe(CN_6)]$ was measured for activity in this assay. Distal portions were not.

CHAPTER 4: DISCUSSION

4.1. Metformin decreases Complex I respiration

In the present study, metformin decreased the rate of Glutamate + Malate oxidative phosphorylation. As this rate represents the oxidative phosphorylation of Complex I, this suggested metformin acts upon Complex I. Furthermore, the addition of metformin did not affect the rate of Succinate oxidative phosphorylation. This suggests that metformin does not affect Complex II, but only Complex I.

However, a decrease between G + M coupled respiration and uncoupled respiration suggests metformin may not only affect Complex I, but Complex V (ATP Synthase) as well. In coupled respiration, oxygen consumption is coupled to ATP synthesis. However, in uncoupled respiration, oxygen consumption is uncoupled with ATP synthesis. Therefore, a greater decrease in coupled respiration rather than uncoupled respiration suggests there might be a decrease in activity of ATP synthase, but further studies are needed.

4.2. Metformin does not significantly affect membrane potential

From the previous section, there is a possibility of a Complex V defect, but the question remains how bad the defect is? A decrease in maximum membrane potential by metformin suggests that the defect may not be due to the ATP synthase molecule, but a reduction in the proton motive force due to a loss in membrane integrity or inhibition of Complex I with decrease in proton pumping from Complex I (See Section 4.3.). However, metformin does not

significantly change the ADP/O ratio. The ADP/O ratio represents the efficiency of phosphorylation of ADP to ATP with the use of the PMF generated with the consumption of oxygen. This suggests that ATP production is not greatly affected and metformin is only a mild inhibitor on Complex V. In addition, the ability to produce ATP suggests the preservation of the PMF, which suggests the membrane potential remains largely intact as well. Confirmed by no change in "depolarization potential" from State3 to State4

4.3. Metformin directly inhibits Complex I

As previously stated, a decrease in both Glutamate + Malate respiration and no change in Succinate respiration suggests metformin inhibits Complex I. We wanted to know if metformin directly inhibited Complex I. Therefore, we studied the enzymatic activity of only Complex I with metformin. The results showed that metformin does indeed directly inhibit Complex I. Further studies showed that metformin directly acts on Complex I downstream of NADH Oxidase.

4.4. Metformin does not work through a MPTP mechanism

Metformin did not change the value of CRC readings. Therefore, the inhibition of Complex I by metformin does not activate MPTP opening as expected. This means that metformin's effect on Complex I inhibition either does not play a factor in the activation of MPTP opening or is protective.

4.5. Metformin increases production of ROS

Metformin does not decrease ROS production from Complex I. The increase in ROS could be deleterious or could in fact be protective. One possible suggestion is that an increase in ROS actually signals for the activation of AMPK as a cytoprotective mechanism[10]. However, more studies must be done in order to confirm this.

4.6. Metformin protects acutely

In this study, metformin at a high concentration exhibited signs of acute protection. This suggests that metformin at a high concentration could be used to directly treat ischemia-reperfusion. In addition, a recent study showed that metformin at low doses could continue its effect[37]. Therefore, there is a possibility that we could use metformin first acutely then chronically, which would be a challenge for most other agents.

In addition, other reversible and transient acute complex I inhibitors have been seen to be protective at the onset of reperfusion. One such compound that has achieved this goal is amobarbital. However, amobarbital will always have translational problems. Therefore, acute administration of metformin might be useful at the onset of reperfusion to compliment the emerging therapy of chronic therapy since it has been seen protective in mice and has the potential to be protective in humans as well.

4.7. Conclusion

Metformin has exhibited several of the characteristics that attribute to long-term protection. The first being a partial blockade of Complex I. In addition, this blockade left the membrane potential largely intact, which satisfies the second requirement for long-term protection. However, the third condition for long-term protection was not met. There was an

increase in ROS production rather than decrease. However, this may in fact be a protective mechanism rather than toxic as the increase in ROS did not increase MPTP openings. An increase in ROS by metformin may in fact activate AMPK in intact heart as an additional "off-target" secondary protective mechanism. Therefore, metformin has the potential to replace amobarbital as the new acute Complex I inhibitor.

LITERATURE CITED

CHAPTER 5: LITERATURE CITED

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CHAPTER 6: VITA

Shawn Youngtae Sunu was born on July 7th, 1989 in Yorba Linda, California. He graduated from Valencia High School in Placentia, CA in 2007. Shawn received a Bachelor of Science in Biochemistry/Cell Biology from the University of California San Diego in December of 2011. After graduating, he matriculated into Virginia Commonwealth University's Premedical Graduate Certificate Program. After completion in 2014, he is now pursuing a Master of Science in Physiology & Biophysics at Virginia Commonwealth University in 2015.