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Modulation of electron transport by Metformin in cardiac protection: role of complex I

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Modulation of electron transport by Metformin in cardiac protection: role of complex I

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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ABBREVIATIONS

ACC - Acetyl-CoA Carboxylase

ADP - Adenosine diphosphate

AMPK - 5' adenosine monophosphate-activated protein kinase

ANOVA - Analysis of variance

Bax - Bcl-2-associated X protein

Bcl-2 - B-cell lymphoma 2

CoQ - Coenzyme Q

Cyt C - Cytochrome c

DMEM - Dulbecco’s modified Eagle’s medium

ETC - Electron transport chain

ETC - Electron transport chain

FITC - Fluorescein isothiocyanate

ISC - Ischemia

KCN - Potassium cyanide

MET - Metformin

MITO - Mitochondria

MnSOD - Mn superoxide dismutase

MPT - Mitochondrial permeability transition pore

NFR - NADH ferricyanide oxidoreductase

NO - Nitric oxide

$O_2^{\bullet-}$ - Superoxide

P-ACC - Phospho-Acetyl-CoA Carboxylase

P-AMPK - Phospho- AMP-activated protein kinase

PI - Propidium iodide

PT - permeability transition

Q – Ubiquinone

REP - Reperfusion

RO - Reoxygenation

ROS - Reactive oxygen species

SI - Simulated ischemia

TTFA - Thenoyltrifluoroacetone

Abstract

MODULATION OF ELECTRON TRANSPORT BY METFORMIN IN CARDIAC PROTECTION: ROLE OF COMPLEX I

By Ahmed Abdul Hussein Mohsin, Ph.D.

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Modulation of mitochondrial complex I during reperfusion reduces cardiac injury. Complex I exists in two structural states: active (A) and deactive (D) with transition from A→D during ischemia. Reperfusion reactivates D→A with an increase in ROS production. Metformin preserves the D-Form. Our aim was to study the contribution of maintenance of deactivation of complex I during early reperfusion by metformin to protect against ischemia reperfusion injury. Our results showed that metformin decreased H9c2 cardiomyoblast apoptosis and total cell death following simulated ischemia for six hours followed by reoxygenation for twenty four hours compared to untreated cells. Reactive oxygen species (ROS) generation was reduced at the onset

of reoxygenation with metformin treatment. Metformin also prevented the acute reactivation of complex I during reoxygenation following 10 minutes of hypoxia accompanied by decreased ROS generation. In addition, the content of C/EBP homologous protein was decreased in metformin treated cells, suggesting that metformin treatment decreased endoplasmic reticulum stress. 5' adenosine monophosphate-activated protein kinase was activated in our model independent of metformin treatment. Intriguingly, metformin protects in 5' adenosine monophosphate-activated protein kinase knock down system. Surprisingly, we found that metformin successfully downregulated p53 compared to untreated simulated ischemia reoxygenation. We sought potential metformin related impact on anti-apoptotic protein B-cell lymphoma 2. Our results showed the expression of the anti-apoptotic protein B-cell lymphoma 2 was markedly decreased in SI6/RO24 and metformin increased expression of B-cell lymphoma 2. Metformin, likely by partial inhibition of complex I with decreased ROS generation, resulted in less sulfhydryl modification and decreased modification of thiol groups by nitrosylation.

We propose that the slowing down of activation of complex I at early stage of reperfusion by acute use of high dose metformin would be protective in cells and hearts against ischemia reperfusion injury. This potential new mechanism of protection is relevant at the onset of reperfusion to directly modulate electron transport to achieve cardiac protection and to decrease cardiac cell injury.

CHAPTER 1: Introduction

Despite recent progress, congestive heart failure remains a major cause of death in western countries (1). Ischemia-reperfusion causes injury to the heart when it's exposed to ischemia when there is cessation of blood flow to the tissue for a period of time followed by reperfusion reflow of the blood. Mitochondria have many important functions in regard to cellular metabolism. They generate ATP in an O_2-dependent manner(2),and serve as a relevant checkpoints in cell death versus survival (3). Mitochondrial oxidative phosphorylation (OXPHOS) defects trigger mitochondrial dysfunction that can stimulate the generation of reactive oxygen species (ROS) and finally abolish ATP generation from mitochondria leading to cell death (4). Ischemia-reperfusion injury induces mitochondrial dysfunction and tissue damage (5). Ischemia causes damage to the electron transport chain that leads to slowing down of oxidative phosphorylation rates (6) with an increase in mitochondrial reactive oxygen species generation (7). Reactive oxygen species generated through the reperfusion is play an important role in complicating tissue injury (8). Reactive oxygen species production is normally localized to complex I and complex III (7,9).

Reversible blockade of complex I during ischemia can protect the heart against ischemic damage (10). Transient inhibition of complex I at the onset of reperfusion by the inhibitor amobarbital reduces cardiac ischemia/reperfusion injury in buffer-perfused hearts. The complex I inhibitor rotenone can cause blockade electron transport in isolated mitochondria and decrease the production of reactive oxygen species (11). However rotenone has a toxic effect (12). In addition, the temporary inhibition of complex I by extracellular acidification at the start of

reperfusion decreases cardiac injury (10). Complex I and complex III represent the main sites of reactive oxygen species production from heart mitochondria exposed to ischemia/reperfusion injury (13).

Metformin (1,1-dimethylbiguanide hydrochloride) is a medication used for treatment of type 2 diabetes mellitus and belongs to the biguanide family (14). Metformin is also 5' adenosine monophosphate-activated protein kinase (AMPK) pathway stimulator and protection has been attributed to AMPK activation and downstream signaling (15). Previous studies in cells and animal models show that metformin protects cells against ischemia/reperfusion (I/R) injury by stimulation of autophagy and mitochondrial biogenesis (16,17). Our work supports the role of metformin in protection derived from the acutely partial inhibition of complex I. The use of higher dose metformin to acutely block complex I, independent of AMPK activation, provides the potential for protective modulation of complex I by a non-toxics compound that has potential to readily translate to clinical treatment. Our hypothesis is metformin can protect against ischemia reperfusion injury in cells and in the intact heart independent of AMPK phosphorylation activation by electron transport chain modulation through mild reversible inhibition of mitochondrial complex I.

Background and Significance

Mitochondrial structure and function

The term “mitochondrion” initiated from the Greeks words “mitos,” thread, and “chondros” grain represents the typical shapes of mitochondria first identified by light microscopy (18). Mitochondria are organelles located in the cytoplasm with a double enclosed membrane. The outer membrane separates the mitochondrion from the cellular cytosol and a

highly folded or convoluted inner membrane is subjacent to the outer membrane which are separated by an intermembrane space. The inner membrane is the site of the electron transport chain for ATP production, infolded into various cristae to amplify the membrane surface area. The inner membrane encloses the mitochondrial matrix also termed “mitoplast” that contains mitochondrial DNA, RNA, citric acid cycle and soluble enzymes that oxidize fatty acids and catalyze both oxidation and decarboxylation of pyruvic acid and further small organic molecules (19-21). Proper cell function depends on the integrity of the outer mitochondrial membrane separating mitochondrial factors from the cytosol. The outer mitochondrial membrane contains channels that enable the transmembrane movement of ions and molecules through a voltage-dependent anion-selective channel (VDAC) (21).

Mitochondria are anchored to the actin cytoskeleton and dispersed in the cytosol as a reticulum (22). Mitochondria have approximately 2000 proteins. Some of these are hydrophobic membrane-based proteins (23). In adult cardiomyocytes, nuclei are surrounded by densely, organized longitudinally grouped mitochondria. Mitochondria produce most of the ATP needed for ion homeostasis and myocardial contraction (21).

Mitochondrial Electron Transport Chain (ETC) is organized into membrane-embedded supramolecular complexes that contain four electron carrier multiprotein enzyme complexes: complex I (Nicotinamide adenine dinucleotide (NADH): ubiquinone oxido-reductase), complex II (succinate: ubiquinone oxido-reductase), complex III (ubiquinone: cytochrome *c* oxido-reductase or cytochrome bc_1 complex), complex IV (cytochrome oxidase). There are two mobile electron carriers, ubiquinone (Q) and cytochrome *c* (cyt *c*) (21). These individual complexes are organized into supercomplexes that contain combinations of the individual electron transport chain complexes.

Complex I consists of at least 45 peptides according to analysis by high - resolution chromatography. In mammals seven of these peptides are highly hydrophobic encoded by the mitochondrial genome and referred as a core subunit (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6) and seven hydrophilic nuclear-encoded subunits (NDUFV1, NDUFV2, NDUFS1, NDUFS2, NDUFS3, NDUFS7, NDUFS8), these fourteen subunits are essential for complex I catalytic function. The other 31 mammalian peptides are encoded by nuclear DNA. Their function is not clearly known and referred as " supernumerary, " " accessory, " or " ancillary " (24). Complex I structure as shown in cryo-electron microscopy is an L – shape, composed of a long and short arm. The long arm is integrated into the inner membrane while the short arm protrudes within the matrix.

The complex I short arm, also be referred to as the peripheral arm contains the FMN cofactor and Fe – S clusters and functions as an NADH dehydrogenase that accepts electrons from NADH into the FMN cofactor located in NDUFV1 subunit (25). This oxidation of NADH is insensitive to the complex I inhibitor rotenone. Then electrons are channeled through the chain of eight iron-sulfur clusters: N3 (NDUFV1), N1b (NDUFS1), N4 (NDUFS1), N5 (NDUFS1), N6a (NDUFS8), N6b (NDUFS8) and N2 (NDUFS7). In the membrane arm, electron transfer from N2 to ubiquinone-binding site to form QH_2 occurs. QH_2 then diffuses into the lipid bilayer (26). The reduction of quinone to quinol is a process of a two-electron reduction, and partially reduced semiquinone intermediates could be formed through the turnover (27,28). The net reaction of complex I activity is summarized below:

$$\text{NADH + ubiquinone (Q) + } 5H^+_{\text{matrix}} \longrightarrow \text{NAD}^+ \text{ + ubiquinol (QH2) + } 4H^+_{\text{IMS}}$$

Complex I is a proton pump because the energy of electron transfer drives the transfer of four protons per pair of electrons from the matrix to the intermembrane space. The movement of

protons from matrix toward intermembrane space makes the matrix become negatively charged while the intermembrane space becomes positively charged. The long, membrane - integrated arm plays a crucial role in proton translocation (21). Complex I couples electron transfer from NADH to ubiquinone with transmembrane proton movement generating the proton motive force used for ATP synthesis (29). There are studies reported that electron transfer and proton translocation are two spatially separated processes in complex I (30). Studies of complex I structure by dissociation of complex I with chaotropes and detergents confirm that its NADH-binding site and redox centers of flavin mononucleotide (FMN) and iron-sulfur clusters are in the peripheral arm although the membrane arm might contain the proton-pumping machinery (31-33).

We can briefly summarize complex I function as : Complex I catalyzes the transfer of a hydride ion from NADH to FMN from which two electrons pass through a chain of Fe-S centers to the N-2 in the matrix arm of the complex I to reach ubiquinone on the membrane arm to form reduced ubiquinone (QH_2). QH_2 reduced ubiquinone moves within the lipid bilayer to reach Complex III. Electron flow from complex I is accompanied by pumping four protons per each pair of electrons. (Figure 1)

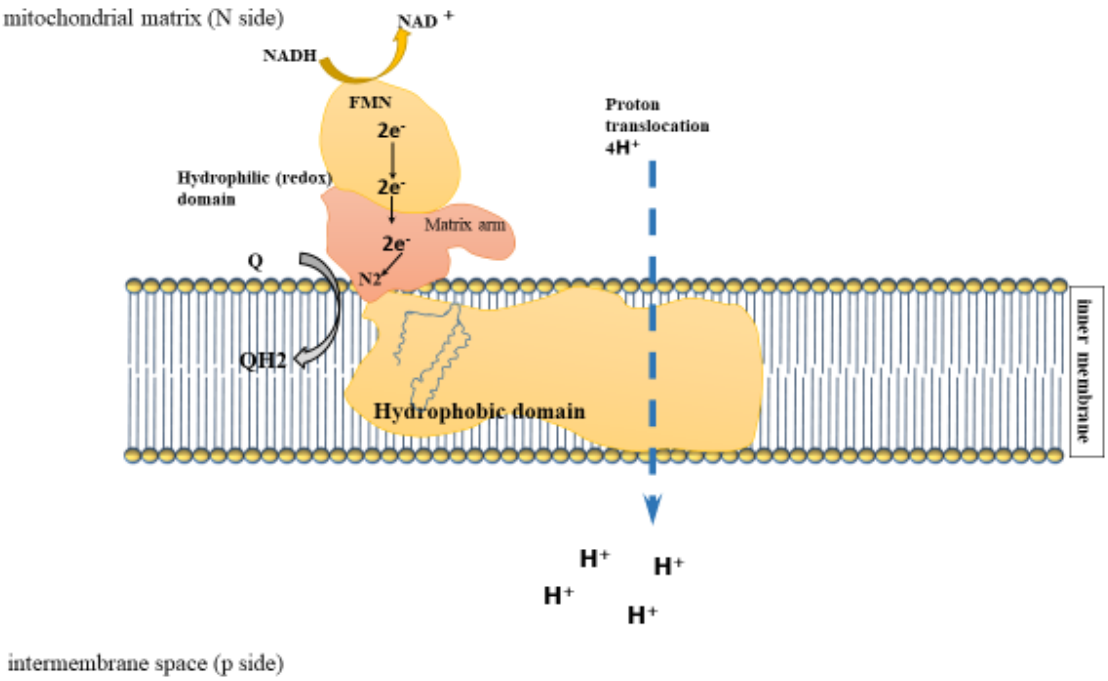


Figure 1. Complex I: NADH: ubiquinone oxidoreductase

Complex I catalyzes oxidation of NADH and transfer of a hydride ion to reduce FMN, two electrons move through a clusters of Fe-S centers to N2 in the complex I matrix arm. Then electrons pass from N2 to reduce ubiquinone to become ubiquinol (QH2) which distributes into the lipid bilayer. This process is accompanied by the pumping of four protons per pair of electrons from matrix toward intermembrane space.

The succinate dehydrogenase component of complex II protein is the only membrane-bound enzyme to contribute in both the citric acid cycle and ETC by catalyzing the oxidation of TCA intermediate succinate to fumarate. Complex II involves of four protein subunits encoded by nuclear-DNA two larger hydrophilic subunits SDHa and SDHb, which project into matrix, and two hydrophobic subunits SDHc and SDHd rooted in inner membrane. The electrons from succinate are accepted at an FAD cofactor.

The electrons from complex I and II transferred to complex III (ubiquinol: cytochrome c oxidoreductase) via QH2 from complex III, the mobile cytochrome *c* carries the electrons to complex IV. The terminal electron acceptor is molecular oxygen which is fully reduced to produce water (21). (Figure 2). Metabolically dynamic organs like heart contain up to several thousand mitochondria per cell (34).

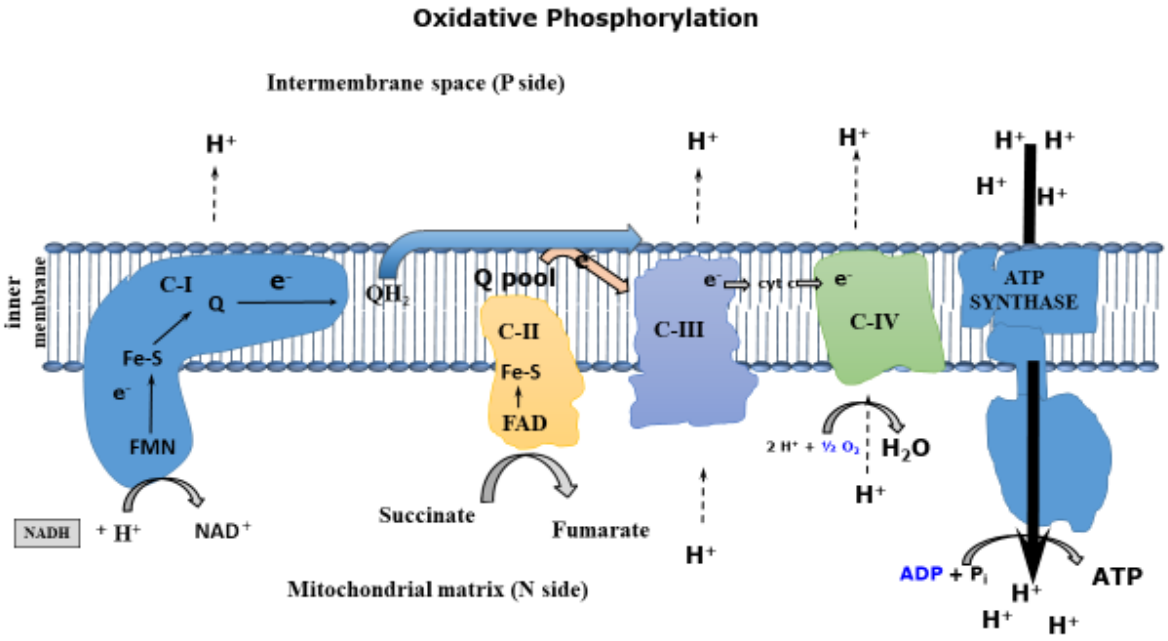


Figure 2. Graphic representation of the mitochondrial electron transport chain (ETC) (Based on Lehninger et al., 2013). Mitochondrial electron transport chain (ETC) involves four multi-subunit enzyme complexes (I, II, III, and IV), coenzyme Q (CoQ), and cytochrome *c* (Cyt C). Electrons (e-) are moved through the ETC from the reducing $NADH/FADH_2$ to O_2 the terminal electron acceptor, eventually, generating H_2O at complex IV.

Mitochondria contribute a crucial role in preserving cellular homeostasis, and its play a critical role in metabolic pathways: - (Figure 2)

1) Oxidative phosphorylation (OXPHOS): OXPHOS results in ATP production. Mitochondria produce 90% of the energy inside the cell in the form of adenosine triphosphate (ATP) by oxidative phosphorylation which represents a very important product for cellular functions.
2) Membrane potential: The mitochondrial membrane potential ($\Delta\Psi m$) results from proton pumps (Complexes I, III and IV) which accompany electron transport chain and it's a critical in the process of ATP synthesis during oxidative phosphorylation. The proton gradient (ΔpH) and mitochondrial membrane potential ($\Delta\Psi m$) forms the transmembrane potential of hydrogen ions which is harnessed by complex V to produce energy. Stability of $\Delta\Psi m$ is required for normal cell function (35-37).
3) Storage of calcium ions: Mitochondria can transiently store calcium, which contribute a critical role in the cell's homeostasis of calcium. Both increased and reduced mitochondrial Ca^{2+} levels have potential impact on the mitochondria function and implicate generation of reactive oxygen species (ROS) (38,39).
4) Synthetic and metabolic function: Gluconeogenesis, citric acid cycle, the urea cycle and the β-oxidation of fatty acids are pathways located in mitochondria. Therefore, any defects in mitochondrial function have extremely serious medical consequences (40). Also there are byproducts from this process, that can result in the production of reactive oxygen species (ROS) (41). Increased ROS generation can affect specific thiol groups that are located in proteins of complexes I, II, and IV that are mainly liable to oxidation and their oxidation leads to decreased mitochondrial complex activity (42).

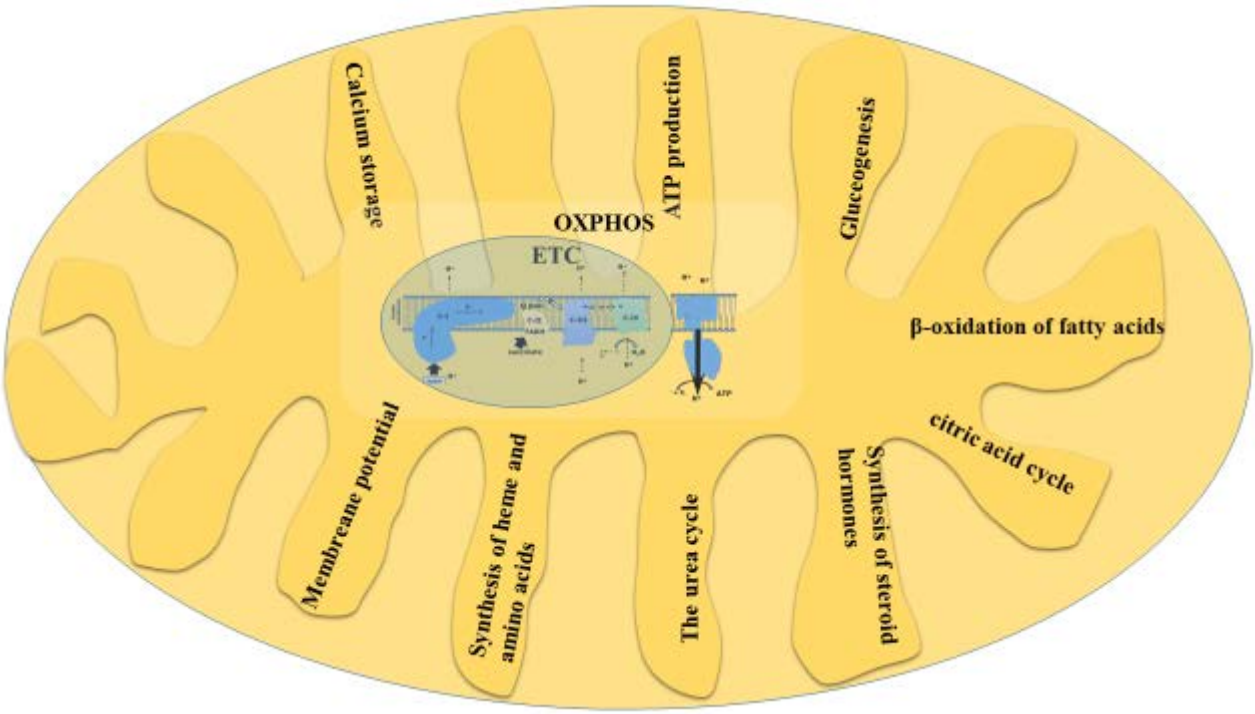


Figure 3. Representative figure of mitochondria function

Important mitochondrial functions were mentioned in this figure in black color: - OXPHOS ATP production, membrane potential, Calcium storage, gluconeogenesis, citric acid cycle, the urea cycle and the β-oxidation of fatty acids.

Role of mitochondria in Ischemia-Reperfusion injury

Ischemia reperfusion causes injury to cardiomyocyte cells when the heart is exposed to an insufficient supply of oxygen and nutrients as a consequence of blockade of blood flow through coronary arteries (ischemia) for a period of time. The restoration of blood supply after ischemia is known as reperfusion (43). Ischemia duration and the severity of ischemia determine the extent of ischemia and reperfusion (IR) injury.

Mitochondria have many important functions in regard to cellular metabolism (2). During ischemia and reperfusion, mitochondria become the sources and targets of cellular damage, through affecting electron transport chain leading to increase production of mitochondria reactive oxygen species (ROS). ROS production by mitochondria in turn stimulates the opening of the mitochondrial permeability transition pore (MPTP) opening and release of cytochrome *c*, finally lead to apoptotic cell death (44-48). Ischemia for longer periods of time causes damage of complex I. Longer times of ischemia causes generation of reactive oxygen species from mitochondria as a result of electron leakage from the electron transport chain leading to reduced activity of complex III, and loss of cytochrome *c* content with diminish complex IV respiration rate (7,49). Throughout ischemia, complex I is reflected to be both the target and a source of damage. Studies have conflicting data regarding the site of complex I damage during ischemia. One study reports that ischemia causes damage to the NADH oxidase FMN – containing component, other studies place site of damage more distal in the Iron sulfur complexes or near the ubiquinone binding site (50,51). Mitochondria have an interesting role in controlling of programmed cell death by release of cytochrome *c* and numerous other proteins (3). The OXPHOS defect due to ischemia-reperfusion triggers mitochondria dysfunction that can stimulate the generation of reactive oxygen species (ROS) and finally abolish ATP generation

from mitochondria leading to cell death (4) and tissue damage (5,6). Increased reactive oxygen species (ROS) production during reperfusion plays an important role in tissue injury (7,8). ROS production is localized to Complex I and Complex III (7,9). Reversible blockade of complex I during ischemia can protect heart against damage (10).

Role of Complex I and ROS generation

Complex I (NADH:ubiquinone oxidoreductase) is important for oxidation of NADH produced by the citric acid cycle and β-oxidation of fatty acids. Also, NADH generated in the cytosol enters mitochondria via the Malate –Aspartate shuttle which is an important process to provide substrate for oxidative phoshorylation in mitochondria (52). Complex I is a source and target of harmful ROS and reactive nitrogen species (RNS) during cell stress (53,54) including ischemia - reperfusion which exposes complex I into oxidative and nitrosative stress (55).

Numerous studies have considered how superoxide is generated by complex I (56,57). There is an argument on the interesting role of the complex I components in superoxide production. Some studies report that FMN is the source for the superoxide (58,59), although others studies proposed that iron-sulfur clusters N1a and N2 are responsible for $O_2^{\cdot -}$ production (37,60,61). Some studies also claim a role for NAD radical and ubisemiquinone were also implicated in the $O_2^{\cdot -}$ generation in complex I (62,63). Oxygen has access to electron carriers most likely at FMN, CoQ sites and terminal FeS centers which can possibly donate an electron reducing O_2 to $O_2^{\cdot -}$ (64). Nevertheless, the superoxide generation from complex I is known to be directed into the matrix of the mitochondria. Ischemia causes damage to the electron transport chain that increase the net ROS generated from complex I and implicates this production in the cardiac injury during reperfusion (7). The blockade of mitochondrial ETC at complex I directly prior of ischemia with the complex I irreversible inhibitor, rotenone, protects against ischemic

damage to the distal ETC (65) Transient, reversible modulation of the ETC has shown the potential to reduce both ischemic mitochondrial damage as well as cell death during reperfusion (66).

Reverse Electron Transport and ROS generation

The initial reactive oxygen species (ROS) formed inside mitochondria is superoxide ($O_2^{\cdot -}$) via a one electron reduction of molecular oxygen. Superoxide is converted to H_2O_2 by the enzyme Mn superoxide dismutase (MnSOD) within the matrix (67).

Reverse electron transport (RET) at complex I generates superoxide ($O_2^{\cdot -}$) that leads to oxidative damage in certain pathological conditions including ischemia reperfusion injury. The formation of $O_2^{\cdot -}$ at complex I can occur by reverse electron transport (RET) in the setting of preserved proton motive force (Δp) and extremely reduced coenzyme Q (CoQ) pool. These conditions drive electrons backwards through complex I and lead to an intense increase in $O_2^{\cdot -}$ production (59). Intriguingly, the accumulation of succinate through ischemia causes a burst reactive oxygen species generation via oxidation of accumulated succinate upon reperfusion through reverse electron transport (68). The site in complex I of the RET-linked superoxide formation is uncertain (69). Notably, one study reports that the superoxide generation site could be the iron-sulfur cluster N2, dependent on its ability to interact with endogenous ubiquinone (7). Other studies proposed that additional RET-related $O_2^{\cdot -}$ generation takes place at a semiquinone radical formed in the Q-binding site of complex I (70). Nevertheless, other studies report that FMN cofactor as the site for ROS generation through reversed electron transfer (71). Thus the site of $O_2^{\cdot -}$ formation during RET remain uncertain.

Mitochondria oxidative stress

Oxidative stress describes as the condition in which the equilibirium between the ROS and endogenous antioxidants in the cell is disturbed (72). Oxygen has the affinity to create reactive oxygen species (ROS) like superoxide radical ($O_2\cdot^-$), hydroxyl radical (·OH) and hydrogen peroxide (H_2O_2) (73). Superoxide is formed due to the electron leakage from the electron transport chain to molecular oxygen (O_2) through the aerobic mitochondria respiration (74). Superoxide anion ($O_2^{\cdot-}$) is the initiator of H_2O_2, throughout the following reaction: $2O_2^{\cdot-} + 2H^+ \rightarrow O_2 + H_2O_2$, that is catalyzed by superoxide dismutase (SOD) or via spontaneous dismutation. Hydrogen peroxide, $O_2^{\cdot-}$ and the reactive $OH^{\cdot}$ radical, produced by reduction of hydrogen peroxide as described in the following reaction ($H_2O_2 + O_2^{\cdot-} + H^+ \rightarrow H_2O + O_2 + OH^{\cdot}$), are known as reactive oxygen species (ROS) (75). SOD enzymes are the main superoxide radical dismutase, and regarded as a major source of H_2O_2 *in vivo* (76). Free oxygen radicals and non-radical ROS, especially hydrogen peroxide, can diffuse and penetrate biological membranes to cause oxidative damage in distant locations compared to the site of its origin (77,78). The normal concentration of H_2O_2 in the cells of 1.0×10^{-8} M (79). H_2O_2 is nonreactive toward DNA. Although H_2O_2 causing DNA damage is due to the production of **.**OH through the Fenton reaction (80). Mitochondrial DNA (mtDNA) is a more sensitive target for oxygen radical attack compared to the nuclear DNA because of its proximity to the sites of oxygen radical generation. Mitochondrial DNA is unguarded by histones, in contrast to nuclear DNA. As a consequence, the level of highly oxidized bases in mtDNA is about 10- to 20-fold greater than that in nuclear DNA (81).

Complex I A-D transition

Mitochondrial complex I oxidizes NADH. Complex I contributes to the formation of inner mitochondrial membrane potential and subsequently ATP synthesis (55). Mitochondrial complex I from mammals has a remarkable feature that called the active/deactive (A/D) transition (82). The catalytically active form (A-form) is predominant during the aerobic respiration while during ischemia and *in vitro* incubation of the complex I at the physiological temperatures complex I A-form will convert into a different conformation which is known as the deactive, dormant form (D-form) (83). A/D equilibrium can be shifted toward formation of the D-form by global ischemia in tissue like the heart. This conversion leads to decrease in the mitochondria respiration rate and low ATP production (55). De-activation of complex I by ischemia may act as a protective mechanism against increased ROS production and could be a physiological mechanism to protect against the oxidative stress by maintaining a low enzymatic activity (D-form) during reperfusion (84).

Mitochondria regulate cell death

Cell death can occur by either of two mechanisms: necrosis or apoptosis. Necrosis, the first type of cell death to be known, is an uncontrolled degenerative condition caused by deleterious stimuli that leads to irreversible failure of membrane function (85). Apoptosis is a programmed cell death, controlled by complex molecular signaling pathways. Since ischemia and reperfusion in different types of tissue activate apoptotic signaling, therapeutic targeting of these apoptosis pathways is considered a potential treatment to protect cellular integrity in important organs such as the heart and brain (86). Bcl-2 contributes in inhibition of apoptosis, it is located on the outer mitochondrial membrane and endoplasmic reticulum. There are other

types of Bcl-2 protein family members that promote apoptosis known as Bcl-2 associated x proteins (Bax) such as Bcl-$x_{S,}$ Bax, Bad and Bak (87,88).

p53 tumor suppressor protein is another critical protein which regulates both autophagy and apoptosis. p53 is an essential factor that regulates repair of cellular DNA and induces apoptosis (89). Under stress, such as hypoxia or γ-irradiation, p53 translocate into mitochondria and induces p53 dependent apoptosis (90). This is a prevalent phenomenon that happens in human and mouse cultured cells (91). Rapid translocation of p53 inside mitochondria affects cytochrome *c* release, mitochondrial membrane potential and procaspase-3 activation (91). Accumulation of p53 in the mitochondrial matrix binds into cyclophilin D leading to MPTP pore opening and cell death (90,92).

Studies report that MPTP opening plays a critical role in ischemia reperfusion injury (93,94). Mitochondrial permeability transition pore (MPTP) opening happens at the onset of and throughout the reperfusion phase as a result of amplified ROS production, calcium overload, reduction of adenine nucleotides, increase in phosphate levels, and mitochondrial depolarization. Inhibition of the MPTP directly by interaction with MPTP or indirectly by reducing calcium loading and decreasing reactive oxygen species generation could protect against reperfusion injury (95). Our lab showed that modulation of pathologic ETC function decreases MPTP opening (96).

Mitochondria and Autophagy

Effective clearance of damaged mitochondria inhibits activation of cell death pathways, decreases reactive oxygen species (ROS) generation, and preserves effective ATP production. The process of mitochondrial clearance is called mitophagy (97). Autophagy could preserve cell

function by eliminating protein aggregates or impaired mitochondria (98). LC3, p62, Beclin-1 are central autophagy-related proteins involved in the autophagy process (99). LC3 is a ubiquitin-like protein, firmly associated with autophagosomal membranes. LC3-I and –II are two forms of LC3, produced post-translationally. LC3-I is cytosolic, while LC3-II is membrane bound (100).

Another important autophagy receptor is p62 that is involved in autophagy-dependent removal of many different ubiqutinated protein aggregates. Since p62 interacts with LC3, it is constantly degraded *via* autophagy (101). Beclin-1 is an autophagy-specific protein that contributes to autophagosome formation (102). Beclin-1 level has an important role as the biomarker for the activation of autophagy. Indeed, a moderate level of Beclin-1 can cause autophagy cytoprotection (103). Ischemia may prompt autophagy via several signaling pathways including endoplasmic reticulum (ER) stress, AMPK activation and Beclin -1 (98).

Modulation of ETC to protect against IR injury

Blockade of electron transport instantly before ischemia strongly protects against ischemia-induced mitochondrial damage (65). Thus the ETC itself is a key mediator of the mitochondrial damage (65,66). Unfortunately, treatment before the onset of ischemia is rarely possible in clinical settings. Therefore, relevant pharmacologic treatment is focused on the modulation of metabolism by damaged mitochondria during early reperfusion in order to limit cardiac injury and cell death.

Study of the blockade of complex I with amobarbital during ischemia in intact hearts showed decreased ROS generation and protection of the heart against IR injury (104). Ischemic damage to the electron transport chain robustly increased ROS production during re-

oxygenation, although prevention of ischemic damage decreases ROS generation throughout reperfusion (7,105).

The mitochondrial permeability transition pore (MPTP) is a non-selective pore spanning both mitochondria inner and outer membranes. MPTP opening is a main contributor to cardiac injury throughout ischemia-reperfusion. MPTP opening is favored at the onset of reperfusion due to increased oxidative stress. Due to the oxidative stress, opening of MPTP happens at the onset of reperfusion (106,107). Oxidative stress leading to ischemia-reperfusion injury by decreased Bcl-2 protein content can also induce MPTP opening (108). The reversible inhibition of ETC could protect against MPTP opening leading to protection of heart tissue throughout reperfusion (109-111).

Interestingly, a reduced cardiac infarct size following ischemia-reperfusion was realized by using extracellular acidification to protect in part by transient and reversible inhibition of complex I leading to decreased ROS generation and reduced susceptibility to MPTP opening throughout reperfusion (10). This study added more support for protection against the heart injury during reperfusion by modulation of complex I activity using physiological or pharmacological approaches (10,110).

The crucial site for protective modulation of the electron transport chain is at mitochondrial complex I, the first enzyme complex in the series of electron transport (112-117). Based upon data from pharmacologic, functional/structural and genetic models, a site distal in the pathway of electrons through complex I is the vital nexus for cardioprotection (7,83,118-120). Modulation of electron flux at this site decreases the production of ROS by blocking forward electron flow from complex I into an ischemia-damaged complex III (121-123). Furthermore, reversible blockade of complex I similarly reduces production of ROS that are

generated from the reverse electron flow that occurs at the onset of reperfusion (83,112,117). Subsequent work in pharmacologic and genetic models has shown that a partial blockade of complex I by STAT3 that does not impair mitochondrial membrane potential but appears to provide optimal protection, resulting in less ROS production with decreased damage to mitochondria during cell stress (9). Therefore, modulation of complex I activity during early reperfusion is a critical approach to decrease cardiac injury during ischemia-reperfusion.

Role of AMPK

AMP-activated protein kinase (AMPK) is a heterotrimeric complex consisting of three subunits, one catalytic α subunit and two regulatory β and γ subunits. Phosphorylation of the Thr^{172} residue found within the activation loop of the catalytic α subunit is regarded as the major mechanism of the control of AMPK activation. The function of the other phosphorylation sites on α and β subunits are not yet clear (124,125).

AMP-activated protein kinase (AMPK) is an important regulatory enzyme and serves as a fuel sensor. AMPK is activated in pathological conditions of hypoxia and ischemia in order to conserve sufficient adenosine triphosphate (ATP) production (126). Physiologically, at the onset of myocardial ischemia, AMPK is phosphorylated within minutes and remains active at least 24 hours after reperfusion (127,128). AMPK is activated by increases in AMP/ATP ratio or by oxidant stress by increased generation of reactive oxygen species (ROS) (129). AMPK adjusts two major metabolic pathways - mitochondrial biogenesis and autophagy.

AMPK inhibits cardiac hypertrophy by inducing the activity of peroxisome proliferator-activated receptor-α (PPAR-α) through activation of the extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway. Subsequently, there is induced expression of carnitine palmitoyl

transferase-1 (CPT-1) and medium-chain acyl-COA dehydrogenase (MCAD), consequently supporting fatty acid oxidation and regularizing myocardial energy metabolism (130-132). Thus, canonical AMPK activation leads to pleotropic activation of pathways that regulates metabolism and cytoprotection.

Intervention by metformin to modulation ETC

Metformin (1,1-dimethylbiguanide hydrochloride) is a medication used for treatment of type 2 diabetes mellitus that belongs to the biguanide family (14). Although well known for its action as an activator of the AMP kinase signaling pathway (133), this action is achieved though chronic dosing that results in a chronic, minimal inhibition of complex I. The complex I inhibition is the mechanism of the subtle energy deficit that that results in activation of the AMPK pathway. Metformin exhibits a superior safety profile and is safe for use in non-diabetic patients (134). Given in standard clinical dosing, metformin is safe and may improve outcomes in patients that sustain a myocardial infarction (135).

Studies suggested that metformin activates AMPK by inhibiting complex I of the mitochondrial respiratory chain leading to subsequent reduction in the intracellular adenosine triphosphate (ATP) concentration and a rise in the adenosine monophosphate (AMP) to ATP ratio (136). Chronic treatment with metformin enhances myocardial resistance to ischemia-reperfusion injury by improving mitochondrial structure through possible effects of activation AMPK and PGC-1α pathway (137). Some previous studies have proposed that metformin might have cardioprotective effect independent of its glucose reducing effect (138). Indeed another study found that metformin protects heart against cardiotoxicity induced by doxorubicin by decrease of oxidative stress levels with contribution of autophagy proteins Beclin-1 and LC3 (139). Other interesting studies showed that metformin protects cells against

ischemia/reperfusion injury by stimulation of autophagy and mitochondrial biogenesis (16,17). Furthermore, extra research verified that metformin effectively improved heart function and attenuated LV hypertrophy through downregulation of p-mTOR (ser2481), p70S6K (Thr389), and S6 phosphorylation in AMPKα2 KO mice, suggesting that metformin protects against systolic overload-induced heart failure by attenuated myocardial mTOR signaling independently of AMPKα2 activation (140).

Metformin binds to mitochondrial complex I and inhibits it with reversible effect (52). Under lack of oxygen in ischemic tissue, complex I is converted into a deactive dormant (D-form) (118). The D-form may prevent reverse electron transport (RET) mediated ROS generation upon tissue reoxygenation (141). Since complex I is the source and target of detrimental ROS and oxidative damage in ischemia-reperfusion injury (142) (143), metformin can inhibit complex I with a high rate of inhibition of NADH oxidation in D-form rather than A-form (52), this binding of metformin to D-form might be important for an intervention strategy (52).We propose that the slowing down of the activation of complex I during early of reperfusion by use of metformin will protect cells and hearts against I-R injury. This potential new mechanism of protection by modulate electron transport to achieve cardiac protection.

Mitochondria defects in aged heart

Aging is linked with pathologic changes leading to a progressive decrease in function of cellular, organ and whole organism. Moreover, aging causes a reduced resistance to superimposed disease - induced stress (144). Aging is associated with increased mitochondrial ROS production and mitochondrial permeability transition (MPTP) pore becomes more sensitive for ischemia-reperfusion injury leading to increased cell death (145). Aging causes a decay in cardiac functional proficiency (146). Mitochondrial function declines during aging simultaneous

with changes in mitochondrial morphology (147). These defective mitochondria predispose to enhance a greater cardiac injury during the stress of ischemia-reperfusion compared to the younger adult heart (148). Aging heart mitochondria are the major target and effector of ischemia injury that enhances cardiac injury by several mechanism included oxidative stress and calcium accumulation leading to cell death (149). In the aged heart, there is decreased mitochondria oxidative phosphorylation (150). Aging induced alteration in mitochondrial function at baseline leads to increased injury in the aged heart following ischemia and reperfusion (151).

CHAPTER 2: Materials and Methods

Cells culture and simulated ischemia-reoxygenation

H9c2(2-1) (ATCC® CRL-1446™) mycoplasma free cardiac myoblasts were grown in complete Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, USA) containing 4.5 mM glucose and supplemented with 10 % fetal bovine serum and 1 % penicillin and streptomycin. Cells were exposed to simulated ischemia by replacing the culture medium with "ischemia buffer" that contained [118 mM NaCl, 24 mM $NaHCO_3$, 1.0 mM NaH_2PO4, 2.5mM $CaCl_2$-$2H_2O$, 1.2mM $MgCl_2$, 20mM sodium lactate, 16 mM KCl, 10 mM 2-deoxyglucose (pH adjusted to 6.2)] (152) and incubating in the Galaxy O_2 controlled incubator at 5% CO_2 and 1% O_2 at 37^oC for 6 hours. Reoxygenation was performed by replacing ischemia buffer with complete DMEM media under aerobic cell culture condition (5% CO_2 and 19% O_2) at 37 °C. Incubation was continued for 24 hours. Chemical hypoxia was performed by using 2 mM KCN incubation in normoxic cell culture media (DMEM, 5% CO_2 and 19% O_2) for 24 hours.

Cell transfection

H9c2 cells were cultured under 5 % CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 mM glucose and supplemented with 10 % fetal bovine serum and 1 % penicillin and streptomycin. Cells were plated at a density of 13000–16000 cells/cm_2. Silencer pre-designed siRNA was used to knock down the AMPK alpha 2 catalytic subunit with the target region GCAACUAUCAAAGACAUAC (Ambion Life Tchnologies, Carisbad, CA, USA). Cells

were grown to 80 % confluence and transfected with 100 nM each siRNA using Lipofectamine RNAiMAX transfection reagent (Invitrogen CA, USA). Twenty four hours later, transfection mixtures were replaced with complete DMEM medium without antibiotic. Fifty four hours after transfection, cells were lysed and proteins analyzed by immunoblotting.

Immunoblot analysis

Cells were trypsinized by using Trypsin-EDTA (0.25%) then resuspended in complete DMEM media and centrifuged at 125 x g. The cell pellets were washed twice with 1x PBS. Pellets were lysed with 1x lysis buffer (Cell Signaling #9803S, MA, USA) supplemented with protease and phosphatase inhibitor cocktails (Roche, Indianapolis, IN) and incubated on ice for 10 minutes after which samples were centrifuged at 125 x g for 10 minutes at 4ºC to remove insoluble cell debris. The supernatant was collected and protein measured using the Lowry method. Samples were combined with 2x Laemmli sample buffer (Bio-Rad #161-0737, California, USA) and boiled for 5 minutes after which proteins were separated using SDS-PAGE on 4%-20% TGX gradient gel (Bio-Rad #567-1093) and transferred to Immobilion-P transfer membrane paper (Millipore #IPVH00010). After blocking non-specific binding sites with 5% milk or 5% bovine albumin fraction V in 1x TBS-T (tris buffered saline with 0.05% tween), membranes were incubated with primary antibody at 4ºC for overnight, washed 3x for 10 minutes each with TBS-T and one time washed with TBS. then membrane were incubated an additional one hour with a secondary antibody. Membranes were then washed 3 times with TBS-T for 10 minutes each and one time with TBS and visualized using ECL Prime Western blotting detection reagent (GE Healthcare, UK#RPN2232) and exposed by ChemiDoc gel imaging system (Bio-Rad, US). Antibodies (CHOP 2895, GAPDH 3683, AMPK alpha 2532S, p-AMPK alpha 2531S, ACC 3661 and p-ACC 3662) were purchased from Cell Signaling Technology

(Danvers, MA). (Bcl-2 Sc-23960, p53 Sc-6243) were purchased from Santa Cruz Biotechnology. Beclin 1 antibody 612112 purchased from BD Biosciences. San Jose, CA.

High resolution respirometry-OROBOROS

Oxygen consumption was measured by the high resolution Oxygraph (OROBOROS Oxygraph-2k, Innsbruck, Austria). Oroboros is a closed-chamber respirometer for high-resolution respirometry (HRR) monitoring of oxygen concentration and consumption in the incubation medium over time.

Measurements were made in 2 ml of MiR05 medium at 37°C. H9c2 cells treated or control were added into MiR05 medium (0.5 mM EGTA, 110 mM sucrose, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM Hepes and 1 g/L BSA, pH 7.1) (153). Multiple substrates and inhibitors were used in the protocol to define the bioenergetic function of mitochondria in H9c2 cells. Briefly, cells were harvested following after the incubation period and resuspended in 2.1 ml MiR-05 pH.7.1. Cell density was determined by counting the cells using a hemacytometer. For measurement of endogenous respiration, glutamate-pyruvate-malate (GPM, 10, 5 and 2mM respectively) were used as the substrate of complex I. State 2 was measured after permeabilization of H9c2 cells by digitonin (dig, 10µg/ml) followed by addition 1mM ADP. Next, inhibition of the complex I by rotenone (0.05 µM) was followed by addition succinate (10mM) as substrate for respiration of complex II. TTFA (40 µM) was then added to inhibit Complex II respiration, Complex IV respiration was measured by addition of TMPD/ascorbate (0.3 and 3 mM). Finally azide (15 mM) was added as a complex IV respiration inhibitor (Table 1). Oroboros DatLab4 software was used to calculate of oxygen consumption.

Hypoxia–reoxygenation experiments were performed in the Oroboros using a similar approach except that after the addition of ADP, the cells were allowed to consume all the oxygen in the chamber. Ten minutes of hypoxia were started at the time of zero oxygen concentration with no evident oxygen consumption. Reoxygenation was performed by opening the chamber and reoxygenation documented by measurement of the oxygen content. Respiration rate was monitored for several minutes, the rate determined, and rotenone added to derive the background rate for complex I. Samples were studies with or without metformin 1 mM added after the initial addition of ADP.

Table 1. Oroboros respiration cell protocol

	Inject	Volume to injected	Substrate	Final Concentration into O2k chamber
1	Inject Cells (x µL)mark with F4 : "Cells"			
2	inject	10 µL	Glutamate (2M)	10 mM
		5 µL	Pyruvate (2M)	5 mM
		5 µL	Malate (0.8M)	2 mM
3	inject	2 µL	Digitonin (10mg/ml)	10 µg/ml
4	inject	4 µL	ADP (0.5M)	1 mM
5	inject	1 µL	Rotenone (0.1mM)	0.05 µM
6	inject	20 µL	Succinate (1M)	10 mM
7	inject	4 µL	TTFA (20mM)	40 µM
8	Inject	25 µL	TMPD/Asc (25-250mM)	0.3-3 mM
9	Inject	25 µL	Azide (6M)	15 mM

Measurement of H_2O_2 cell production using The O2k-Fluorescence LED2-Module

Measurement of ROS generation from H9c2 cells exposed to 10 min. hypoxia in Oroboros was done based on the Amplex Red method. This method is based on the hydrogen peroxide dependent oxidation of Amplex UltraRed (AmR) to the red fluorescent compound resorufin catalyzed by the enzyme enzyme horseradish peroxidase (HRP) and an increase in fluorescence output related to the reaction flux of hydrogen peroxide production.

Amplex UltraRed 5 µM and add 1 U/ml HRP stock and 5 U/ml superoxide dismutase (SOD) was added followed by 15 minutes to obtain a stability of oxygen and H_2O_2 flux in MiR05. H9c2 cells were added and incubated 5-10 min. to stabilize cell respiration. H9c2 cells were exposed to 10 min. hypoxia in the presence of complex I substrate (10 mM Glutamate (G), 5 mM Pyruvate (P) and 2 mM Malate (M)) to obtain C I –linked LEAK respiration and hydrogen peroxide generation. ADP 1 mM was used to induce C I -linked OXPHOS. H_2O_2 production was measured at the onset of the reoxygenation under ADP stimulated conditions.

Cell Viability assay

To assess viability in H9c2 cells, two complimentary assays were performed (154). Briefly, cells translocate membrane phosphatidylserine (PS) from the inner face of the plasma membrane to the cell surface. PS is detected by staining with Annexin V. Cells ($3x10^4$ cells/ml) were seeded in $25cm^2$-flasks after reaching 70% confluency. Cells were exposed to simulated ischemia for 6 hours as described above. Reoxygenation was performed by replacing ischemia

buffer with complete DMEM media under aerobic culture conditions described above and continued for 24 hours. Metformin treatment (1mM) was used at the onset of reoxygenation and continued for either 30 min then replace the media with untreated DMEM or continued treatment for 24 hours. At the end of the incubation, cells were gently trypsinized and washed twice with phosphate buffered saline (PBS). Cells were collected by centrifugation and resuspended in 500 µl of 1x Binding Buffer. Five µl of Annexin V-FITC and 5 µl of propidium iodide were added. The suspension was incubated at room temperature for 5 min. in the dark followed by quantification with flow cytometry.

In parallel experiments, the Mitochondrial ToxGlo assay (Promega, Madison, WI) was used to measure dead-cell protease activity. This assay uses a fluorogenic peptide substrate (bis-alanyl-alanyl-phenylalanyl-rhodamine 110) that labels cells that have lost membrane integrity (155). Using a 96 well plate seeded with 5000 cells per well, separate plates were used in parallel; one plate for cells incubated under normoxic condition as a control in parallel with additional plates of cells that underwent 6 hours simulated ischemia followed by 24 hours reoxygenation. Cells were treated with or without metformin (1 mM) at the onset of reoxygenation. At the end of the experiment, CytoTox-Fluor™ Cytotoxicity Assay Reagent (5x) was added to all wells (20 µl per well) followed by brief mixing using orbital shaking, then incubated for 30 min. at 37°C followed by measurement of resulting fluorescence (485nmEx/520nmEm).

Protease Viability Marker Assay

A cell permeable fluorogenic protease substrate (glycylphenylalanyl-aminofluorocoumarin; GF-AFC) was used as a marker of cell viability by measurement of a conserved protease activity within live cells. The GF-AFC substrate can penetrate live cells

where cytoplasmic aminopeptidase activity removes the gly and phe amino acids to release aminofluorocoumarin (AFC) and produce a fluorescent signal related to the number of viable cells.

Estimation of reactive oxygen species

The redox-sensitive dye 5-(and-6)-chloromethyl-2,7dichlorodihydrofluorescein diacetate acetyl ester (H_2DCFDA) was used to assess ROS (156). H_2DCFDA is a cell-permeant indicator that is oxidized in the presence of ROS such as H_2O_2 thereby emitting green fluorescence. Five thousand H9c2 cells were seeded in wells of a 96 well plate in phenol-free medium and following overnight normoxic incubation, cells underwent 6 hours simulated ischemia by changing the media to ischemic buffer under conditions of 1% O2 , 5% CO2. followed by 24 hours reoxygenation (with or without metformin 1 mM). Wells were then loaded with H_2DCFDA (20 μmol for 45 min at 37°C in phenol-free medium). The wells were then washed one time with 1x PBS after plates were measured in the fluorimeter using the setting for excitation at 485 nm and emission at 535 nm wavelength, the results were normalized to the numbers of the cell viability in each well.

Caspase 3/7 activity

The Caspase-Glo 3/7 Assay depends on generation of a stable luminescent signal based on the luciferase reaction in response to caspase-3/7 activities. To measure caspase-3/7 activities, 5,000 cells were seeded in a 96 well plate incubated overnight then exposed to simulated ischemia for 6 hours by changing the media to ischemic buffer under conditions of 1% O2 , 5% CO2. This was followed by incubation for 24 hours under normoxic conditions with replacement of the buffer with complete DMEM media. Caspase 3/7 activity was tested by Caspase-Glo 3/7 Assay (G8090,Promega) according to the manufacturer's instructions(157).

Cy3 labelling

Using Oroboros, H9c2 (2.5 million cells) were loaded in each chamber. Cells were permeabilized by digitonin in the presence of complex I and complex II substrate, Glutamate, Pyruvate and Malate for Complex I and Succinate for complex II as described. Hypoxia was reached by allowing the cells to consume all the oxygen inside the chamber. Hypoxia was allowed to persist for 25 minutes followed by reoxygenation by opening the tightly closed chamber and allowing the oxygen content to increase. At the onset of reoxygenation alamithicin (10 ug/ml) was added to permeabilized the inner mitochondrial membrane and Cy3 40 µM supplied to labelled free unmodified thiol groups.

Detection of S-Nitrosylation

Samples from H9c2 cells used to investigate S-Nitrosylation. There were three groups control, simulated ischemia-reoxygenation and metformin treated group at the onset of reoxygenation. Briefly, H9c2 cells were exposed to 6 hours simulated ischemia and 24 hours reoxygenation. Metformin treatment was used at the onset of reoxygenation. We investigated the effect of metformin treatment on global thiol nitrosylation modification by using Pierce S-Nitrosylation Western Blot Kit (Thermo Fisher, US). Free sulfhydryl cysteines were blocked by methyl methanethiosulfonate (MMTS), which generates a mixed disulfide. S-nitrosylated cysteins were then selectively reduced with ascorbate in HENS Buffer (100 mM HEPES pH 7.8, 1 mM EDTA, 0.1 mM Neocuproine, and 1% SDS) for specific labeling with iodoTMTzero Reagents, which irreversibly bind to the cysteine thiol, Detection of the TMT reagent-modified proteins was achieved by using an anti-TMT antibody and by western blot.

Immunofluorescence staining

H9c2 cells were plated on a 2-well glass chamber slides (Nunc® Lab-Tek® Chamber Slide) at a density of 10,000 cells/well and allowed to grow for 24 hours. Cells wre then treated with metformin (1mM) for 24 hours. Cells were then fixed in 4 % paraformaldehyde for 15 minutes at room temperature and washed three times with 1x PBS with 10 mM glycine. Cells were then permeabilized with 0.5% Triton X-100 in PBS-glycine for 3 min. followed by washing twice time with 1x PBS-glycine. Cells were then incubated with primary antibodies (Santa Cruz, 2 µg/ml final concentration) in PBS-glycine with 1% BSA and incubated at least 20 minutes at room temperature. Thereafter, slides were washed three times in 1x PBS for 5 minutes each and then incubated with secondary antibodies (ThermoFisher 5 µg/ml final concentration) in PBS-glycine with 1% BSA at least 20 min. The secondary antibodies were cross-adsorbed to ensure species specificity; Alexa-488 anti-mouse (OPA1, green), Alexa-555 anti-rabbit (Tom20, red), and Alexa-633 anti-goat (cytochrome C, blue). The slides were subjected to three additional washings in 1x PBS after which cover slips were mounted in 100% glycerol with 10 mM n-propylgallate. Cells were visualized using a Zeiss LSM 700 confocal microscope with sequential scanning to prevent crossover between fluorescence channels.

Preparation of mouse hearts for perfusion

The study was approved by The Animal Care and Use Committees of the McGuire VA Medical Center and Virginia Commonwealth University. Hearts from male C57BL/6 mice (2-3 mo. and 18 mo. age) were excised and perfused retrograde via the aorta in the Langendorff mode with modified Krebs-Henseleit (K-H) buffer (115 mM NaCl, 4.0 mM KCl, 2.0 mM $CaCl_2$, 26 mM $NaHCO_3$, 1.1 mM $MgSO_4$, 0.9 mM KH_2PO_4, and 5.5 mM glucose), gassed with 95% O_2-5% CO_2 to adjust pH to 7.35–7.45 (6).

Hearts were paced via epicardial leads at 420 beats per min during the equilibration period. Pacing was suspended during ischemia and during the initial 20 min. of reperfusion. Pacing was restarted following 20 min. reperfusion. Cardiac function was monitored with a balloon inserted into the left ventricle, and data were recorded digitally with Powerlab (AD Instruments, Colorado Springs, CO). In the untreated ischemia-reperfusion group, hearts underwent for 15 min. equilibration with K-H buffer, followed by 25 minutes global ischemia at 37°C and 60 min. reperfusion. In the metformin treatment group, metformin (2 mM final concentration) was perfused for 5 min. at the onset of reperfusion followed by normal buffer for the remainder of reperfusion. Time control hearts were perfused without ischemia. Myocardial infarct size was measured at 60 minutes of reperfusion to assess the extent of cardiac injury. Hearts were frozen at -20°C for 20 minutes and sectioned into 2-mm-thick slices. Hearts were then incubated in 1% 2,3,5,-triphenyltetrazolium chloride for 20 min at 37°C, and storage in 10% formalin for 24 hours. The infarct area was measured using Image J software. Infarct size was expressed as percentage of the entire myocardium.

Isolation of mouse heart mitochondria

Mouse hearts at the end of the experiment were quickly placed in cold buffer A (composition in mM: 100 KCl, 50 MOPS [3-(N-morpholino)propanesulfonic acid], 1 EGTA, 5 $MgSO_4$, and 1 mM ATP). The heart was blotted dry, weighed, and homogenized using a polytron tissue homogenizer at 10,000 rpm for 2.5 sec. in the presence of trypsin (5 mg/g tissue). The homogenate was incubated for 15 min. at 4^{o}C, then the same volume of buffer B (buffer A + 0.2% bovine serum albumin (BSA)) was added and the mixture was centrifuged at 500 × g for 10 min. The supernatant was again centrifuged at 3000 × g to pellet mitochondria. The mitochondrial pellet was first washed with buffer B, resuspended in KME (100 mM KCl, 50 mM MOPS, 0.5 mM EGTA), and centrifuged at 3000 × g to yield the final mitochondrial pellet. Mitochondria were resuspended in KME for study (158). Protein content was measured using the Lowry method. Mitochondria were kept on ice and used within 4 hours.

NADH-ferricyanide reductase (NFR)

NADH-ferricyanide reductase was used to measure activity of the NADH dehydrogenase activity of complex I with non-covalently bound flavin-mononucleotide (FMN) molecule This activity includes the proximal portion of the complex, with the NFR activity was recorded as a decrease in absorbance at 340 nm because of the oxidation of added NADH. The reaction is carried out in 1ml buffer at pH 7.4 containing 50 mM KH_2PO_4, 0.1 mM EDTA, 0.2% de-fatted BSA (w/v), 0.015% sonicated asolectin, 0.66 mM $K_3Fe(CN)_6$ (an artificial electron acceptor from flavin-mononucleotide), 2 mM NaN_3 and 0.2 mM NADH. After established the baseline reading, the reaction was started by addition of mitochondrial protein and the NFR activity was calculated using an extinction coefficient of 6.22 mM-1cm-1 for NADH. The NFR

actual rate was obtained after subtracting the baseline rate obtained in the absence of mitochondria.

Blue Native-PAGE

NativePAGE Novex Bis-Tris Gel System was used for protein electrophoresis in the native state, according to the manufacturer's instructions with modifications. Briefly, mitochondria were suspended in SET buffer (0.25 M sucrose,0.2 mM EDTA, 50 mM Tris–HCl) with pH 8.0 supplemented with 1mM malonate and 5mM $MgCl_2$ and incubated at 35 °C for 30 minutes with constant shaking. We used this treatment to obtain D-Form of complex I. To obtain complex I in active A-Form complex I was re-activated in the presence of 400 μM NADH and an NADH regenerating system (0.1 mg/ml alcohol dehydrogenase from Saccharomyces cerevisiae,1% ethanol) with constant stirring for 20 minuts at room temperature. Then, mitochondria samples were solubilized in cold 1x NativePAGE Sample Buffer containing DDM (n-dodecyl-D-maltoside). After incubating the suspension on ice for 10 minutes, the samples were centrifuged at 16,000 x g for 30 minutes at 4 ºC and supernatants transferred to new Eppendorf tubes. The protein concentration of the lysates was determined using Lowry protein assay. Blue Native-PAGE was run using 3 – 12% gradient NativePAGE Bis-Tris. The upper chamber contained dark blue cathode buffer (NativePAGE running buffer mixed with 0.02% Coomassie blue G-250 dye) which was exchanged after the dye front reached 1/3rd of the gel into the light blue cathode buffer (0.002% G-250).The electrophoresis run was done in the cold room at 4 ºC, 150 V constant for 60 minutes after which the voltage was increased to 250 V constant. After electrophoresis we measured in gel complex I activity by Incubate the gel in 20 ml of the Complex I substrate solution (2 mM Tris-HCl pH 7.4 , 0,1 mg/ml NADH and 2,5

mg/ml Nitrotetrazolium Blue chloride). The presence of violet bands is indicative of Complex I activity and was measured using Image J software.

Statistical analysis

Statistical analysis was made with GraphPad Prism 7.0 (Graphpad Software Inc.). Data are presented as mean ± SEM. One-way ANOVA was used when comparing more than two groups followed by Newman–Keuls post hoc test for pair-wise comparison. Statistical comparisons between two groups were achieved with the unpaired Students t-test. A $p < 0.05$ was considered to be statistically significant.

CHAPTER 3: Results

Partial inhibition of complex I by treatment with metformin

We first sought if metformin (1 mM) affects complex I mediated respiration in H9c2 cardiomyoblast cells. Cells were incubated with metformin for 24 hours under normoxic condition. Metformin decreased the rate of state 3 respiration oxidizing complex I substrates compared to untreated cells. Metformin did not alter respiration using complex II substrates or complex IV substrates. Thus, inhibition of respiration is selective to complex I.

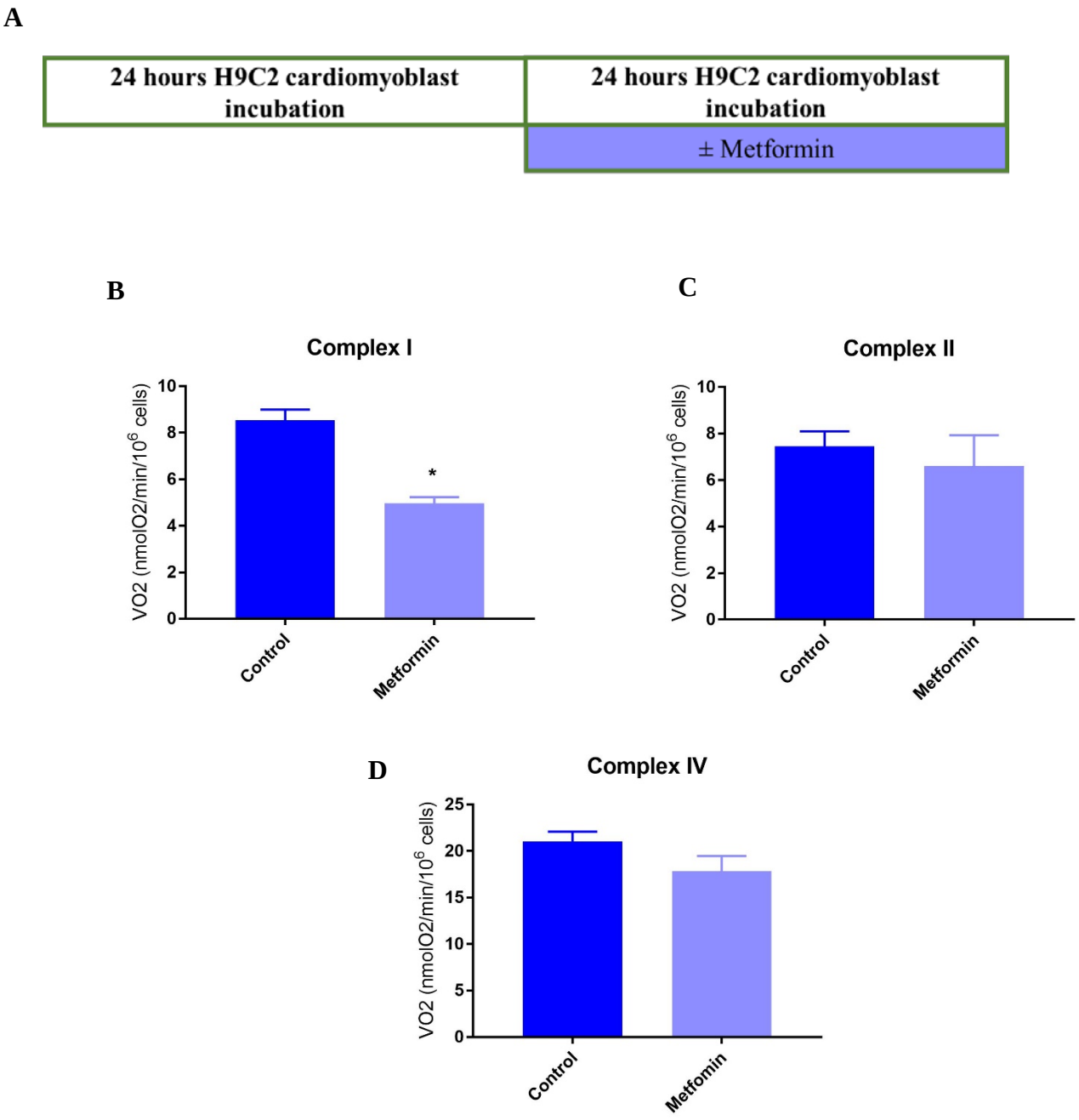


Figure 4. Metformin selectively inhibits complex I, but not CII or CIV activity

(A) Experimental protocol. H9c2 cells grown for 24 hours followed by I mM treatment with metformin for 24 hours. (B) Metformin exposure decreased respiration using complex I substrates. (C) Metformin did not affect complex II respiration (D) Metformin did not inhibit Complex IV respiration in H9c2 cells. Mean ± SEM, n=3, *p<0.05 vs. untreated control.

Acute metformin treatment selectively inhibits complex I function during reoxygenation after 10 minutes of hypoxia

Since the ETC is a major source of cardiac injury during ischemia-reperfusion, we asked if metformin exerted a selective inhibition at complex I in the early reperfusion period. H9c2 cells were exposed to 10 minutes of hypoxia followed by reoxygenation in the Oroboros. Metformin specifically inhibited complex I respiration in the reoxygenation phase (Table 2). However, there was no significant inhibition of respiration with complex II or complex IV substrates. These findings indicate that acute metformin treatment selectively inhibits the ETC at complex I during early reperfusion phase. Additionally, the same concentration of metformin did not *acutely* inhibit complex I during normoxia. Thus, metformin only inhibits complex I in cells following hypoxia (Table 2).

Table 2. Metformin selectively inhibits complex I respiration following hypoxia and reoxygenation.

	Complex I	Complex II	Complex IV
HRO	0.59 ± 0.05*	0.81±0.03	0.67±0.02*
HRO + MET	0.34 ± 0.1†*	0.85±0.01	0.68±0.06*
Normoxia	1.0	1.0	1.0
Normoxia + MET	0.90 ± 0.03	0.99±0.01	0.87±0.03*

Metformin (MET, final concentration 1 mM) was added after the addition of complex I substrate and ADP in digitonin permeabilized cells. MET was present during hypoxia (H). The rate of respiration during normoxia was taken as control (1.00). Respiration was again measured during reoxygenation (RO). Mean± SEM, n=3; * $p \leq 0.05$ vs. normoxia control; † $p \leq 0.05$ MET vs. corresponding hypoxia-reoxygenation (HRO) untreated.

Metformin selectively inhibits complex I after 25 minutes of hypoxia

We next asked if an increase in the time of hypoxic incubation will affect the response of complex I to metformin. Acute metformin treatment selectively inhibited complex I in the reoxygenation phase after 25 minutes of hypoxia (Figure 5). There was no significant inhibition of respiration using complex II or complex IV substrates. These findings indicate that metformin selectively inhibits the ETC at complex I in the early reoxygenation phase. Therefore, these results after both 25 minutes and 10 minutes hypoxia, show that acute metformin treatment inhibits complex I with greater affinity to inhibit complex I after hypoxia. (Figure 5).

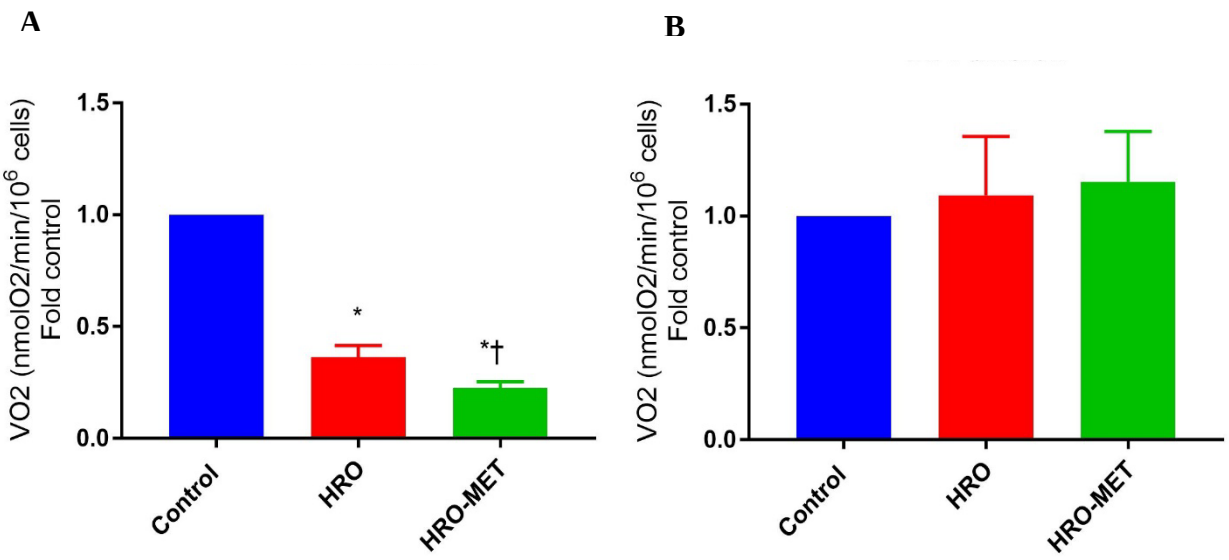


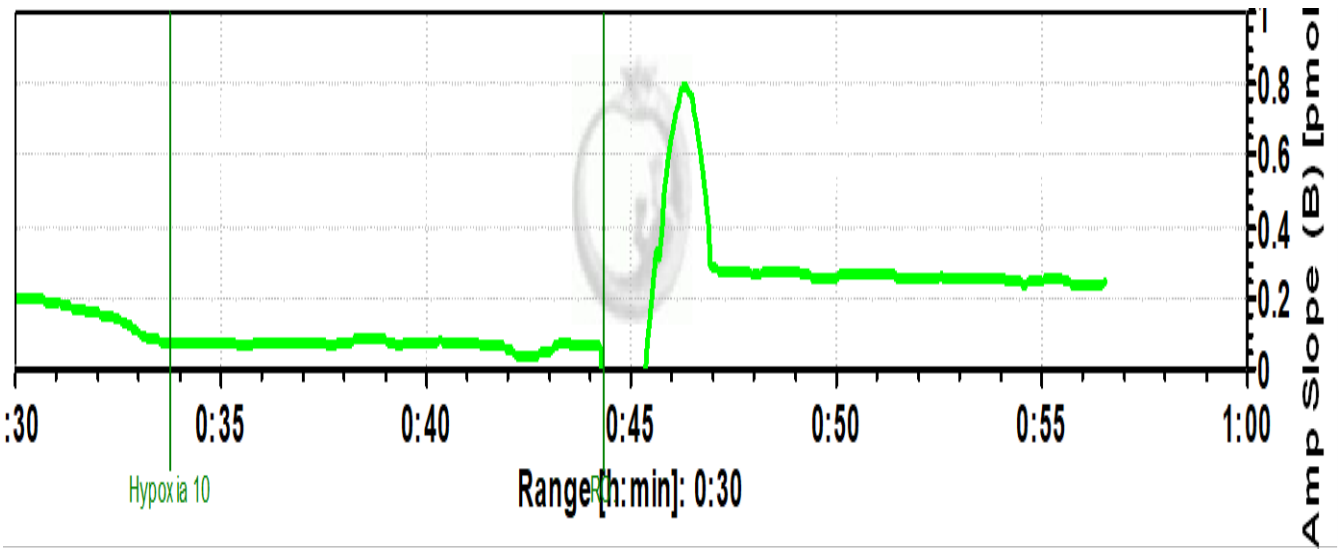
Figure 5. Metformin selectively inhibits complex I after 25 minutes of hypoxia

(A) Complex I respiration for H9c2 cells (B) Complex II respiration for H9c2, cells Data are plotted as mean ± SEM, n=4. *$p<0.05$ vs. non-metformin treated cells, † $p\leq0.05$ MET vs. corresponding hypoxia-reoxygenation (HRO) untreated.

Metformin reduced the burst of ROS generation at the onset of reoxygenation.

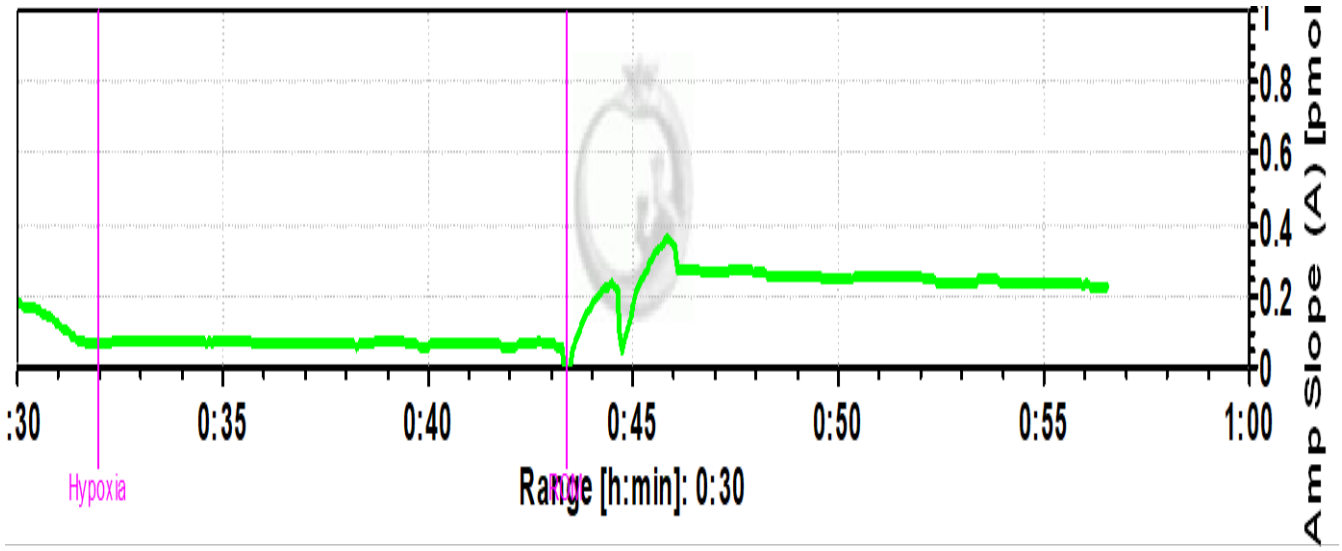
The expected benefit effect of early reperfusion on tissue recovery after ischemia can cause a paradoxical augmentation of the injury response following reperfusion or reoxygenation of ischemic or hypoxic tissue (159,160). The generation of ROS in cardiac myocytes exposed to simulated ischemia and reoxygenation (SI/RO) show augmented ROS production and increased cell death (161). The rapid restoration of molecular oxygen to ischemic tissue results in a distinctive type of injury reaction known by reperfusion injury (162). We asked if the selective inhibition of complex I by metformin decreased ROS production at the onset of reoxygenation. H9c2 cells in the Oroboros were exposed to 10 minutes hypoxia in the presence of complex I substrate followed by reoxygenation. Our results showed that metformin attenuated the increased production of reactive oxygen species generated at the onset of reoxygenation (Figure 6).

A



Hypoxia **Reoxygenation**

B



Hypoxia **Reoxygenation**

C

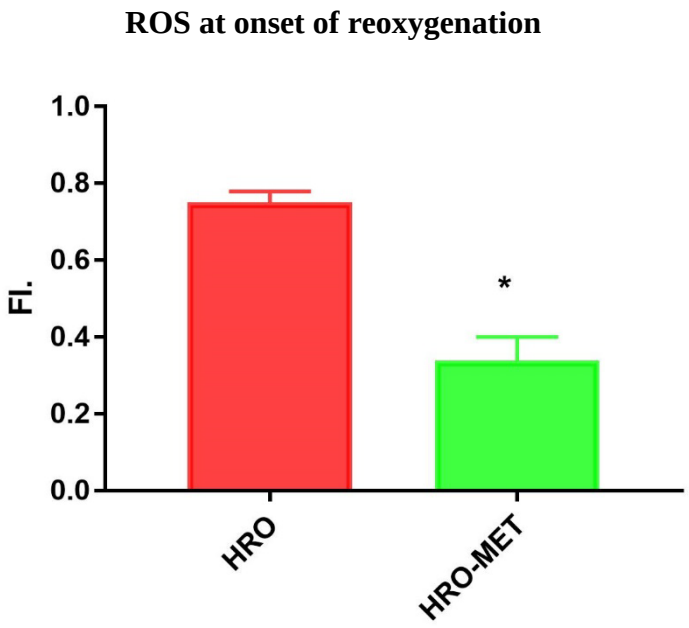


Figure 6. Metformin decrease ROS generation at the onset of reoxygenation.

(A) Representative tracing of fluoresce intensity (FI) for H_2O_2 production in H9c2 cells at reoxygenation without treatment. (B) Fluoresce intensity for H_2O_2 production for H9c2 with 1 mM metformin treatment is shown. (C) H_2O_2 production at onset of reoxygenation in H9c2 cells. Mean ± SEM, n=3 in each group; *$p<0.05$ vs. non-metformin treated cells,

Metformin did not affect cell viability during normoxia

Next, we investigated the effects of metformin treatment at the base line during normoxia. H9c2 cells were incubated with and without metformin (1mM) for 24 hours, followed by assessment of cell death and morphology. There was no significant difference between cells treated with metformin and control. Therefore, the extent of inhibition of complex I by metformin had no effect on cell viability (Figure 7).

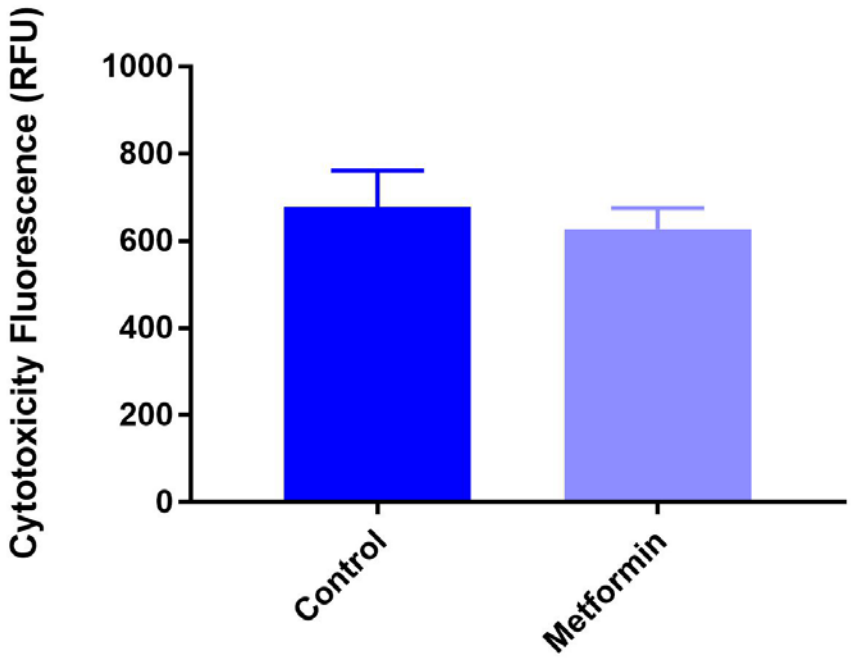


Figure 7. Metformin does not alter cell viability under normoxic conditions

Metformin does not affect H9c2 cell viability under normoxic condition, H9c2 cells were incubated 24 hours with or without metformin. Cell death was measured by fluorescence cytotoxicity assay. Data are plotted as mean ± SEM, n=3; p=NS metformin treated cells vs. control.

Metformin does not affect mitochondria morphology under normoxic condition

To test that there were no substantial effect on mitochondria shape and morphology in cells after 24 hours metformin treatment, we used confocal microscopy and staining for OPA1 (Optic atrophy 1) is a mitochondrial inner membrane protein that has an interesting role in mitochondrial fusion and structural integrity (163) (green), Tom20 (translocase of outer membrane 20) is a receptor subunit of the Tom complex responsible for the translocation of cytosolically synthesized mitochondrial proteins (164) (red), and is evolutionarily conserved mitochondrial protein critical in mitochondrial electron transport and intrinsic apoptosis (165) (blue). Cells were visualized after 24 hours of treatment. Results showed there was no specific effect of metformin treatment on mitochondrial shape and integrity (Figure 8).

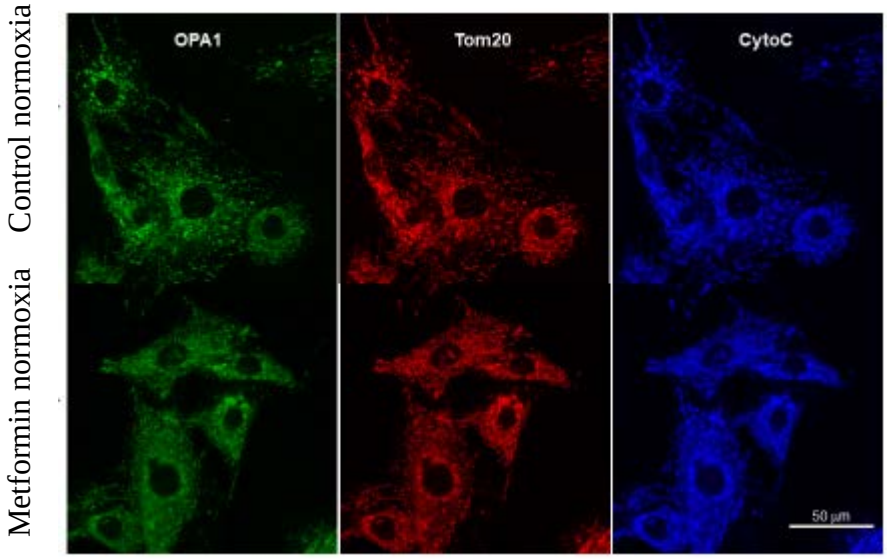


Figure 8. Metformin does not affect mitochondria morphology under normoxic conditions

Representative confocal microscopy images of mitochondria morphology after treatment. Metformin does not affect mitochondrial shape or cyt c content under normoxic condition, H9c2 cells were incubated 24 hours with or without metformin.

Metformin reduced cell death in H9c2 cells exposed to 24 hours chemical hypoxia

Cyanide (KCN) inhibits cytochrome oxidase. This leads to decreased respiration and increased reactive oxygen species (ROS) generation with decreased production of ATP. In addition, KCN leads to the depolarization of the $\Delta\Psi_m$ (166). H9c2 cells were exposed to chemical hypoxia by 2 mm potassium cyanide (KCN) for 24 hours. Metformin treatment was used along with cyanide for 24 hours. We found that treatment with metformin significantly reduced necrosis induced by chemical hypoxia. (mean ± SEM, 4.25 ± 2.2 vs. KCN 10.66±2.12, $n=\geq 4$, $p<0.05$). Quantification assay for cell death was evaluated by Annexin V-FITC-PI flow cytometry assay (Figure 9).

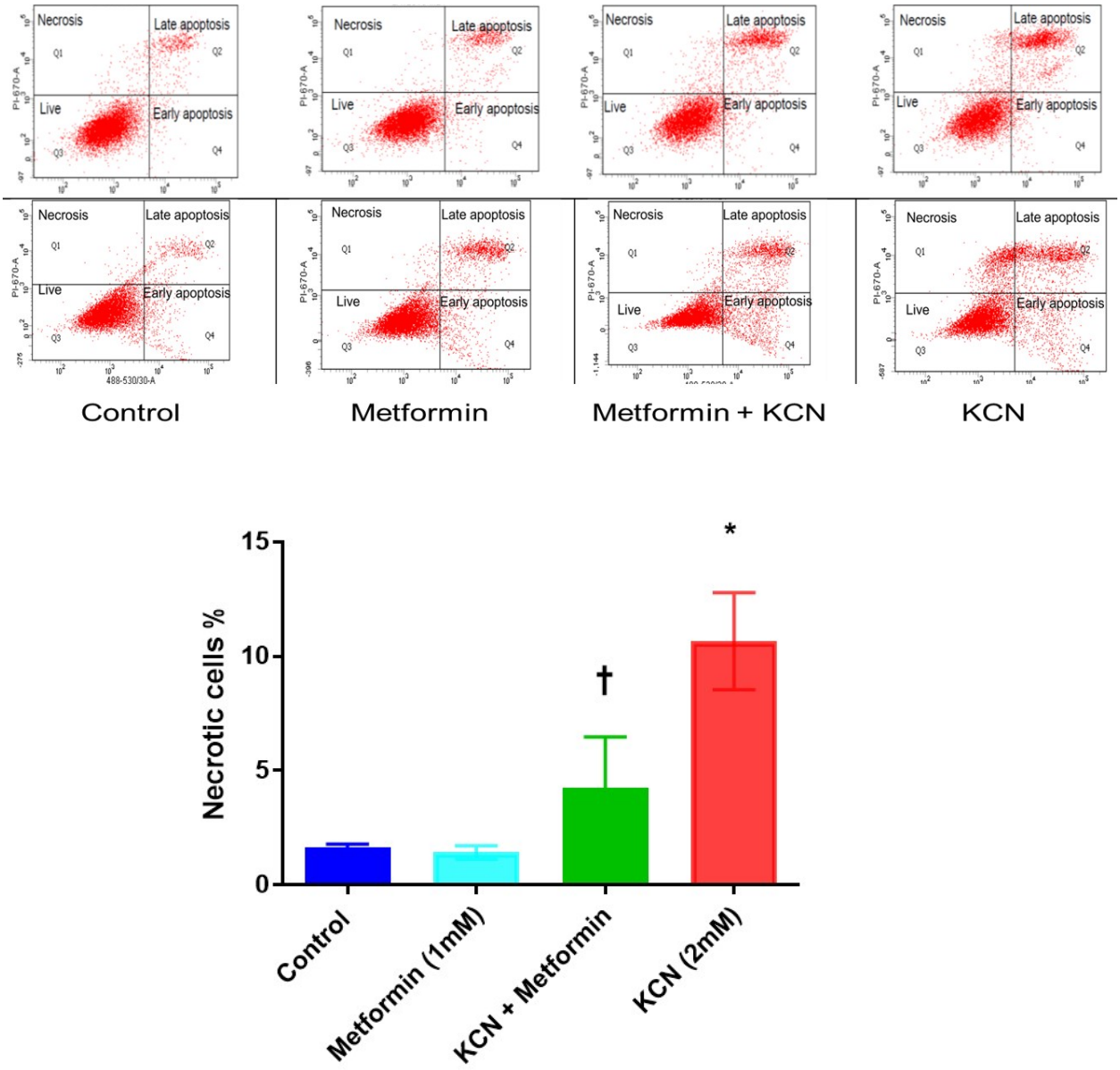


Figure 9. Metformin significantly reduced necrosis induced by 24 hours KCN.

Quantification for cell death was evaluated by Annexin V-FITC-PI flow cytometry assay.

Mean ± SEM *p<0.05 vs. control; † p<0.05 KCN alone n=≥4.

KCN treatment activate AMPK in H9c2 cells

AMPK is an essential regulator of cellular metabolism and acts as an important sensor of cell energetic homeostasis. AMPK is phosphorylated and becomes active by low cellular energy levels and increased ROS generation (167). Phosphorylated AMP-activated kinase (P.AMPK) plays an important role in preserving energy homeostasis in the heart and skeletal muscle (168,169). The chemical hypoxia system was chosen as a likely AMPK activated system to test the role of metformin when AMPK is already activated. We found that AMPK was in the phosphorylated state (P.AMPK Thr172) in H9c2 cells exposed to chemical hypoxia with KCN for 24 hours. Phosphorylation was significantly greater compared to control cells without exposure to KCN (Figure 10).

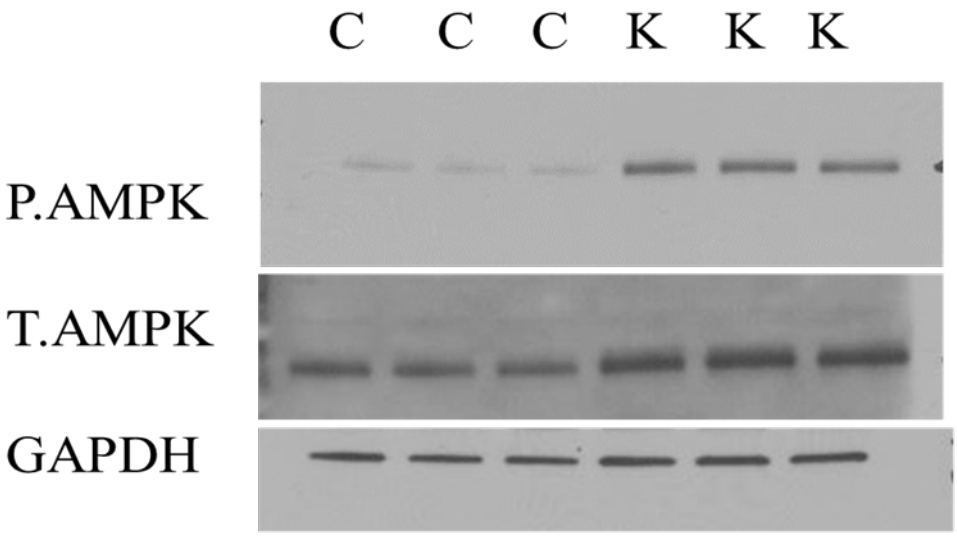


Figure 10. KCN treatment activate AMPK in H9c2 cells

AMPK activation in three independent samples of H9c2 cells exposed to chemical hypoxia with 2 mM KCN for 24 hours. C is control, K is KCN.

Metformin protected H9c2 cells against SI/RO-Induced cardiac cell injury.

Next, a cellular model of ischemia, rather than chemical hypoxia was used to assess the potential role of metformin on cell death. H9c2 cells were exposed to 6 hours simulated ischemia (SI) followed by 24 hours of reoxygenation (RO) with or without treatment of 1mM metformin at the onset of the reoxygenation (Figure 11). We chose the time of treatment by metformin at the onset of reoxygenation. Production of reactive oxygen species was markedly increased at this critical time. H9c2 cell death was measured by cell membrane integrity assessed by evidence of presence or absence of protease activity related with necrosis by using a bis-AAF-R110 a fluorogenic peptide substrate (Figure 11). Metformin treatment at the onset of reoxygenation reduced cell death compared to untreated cells.

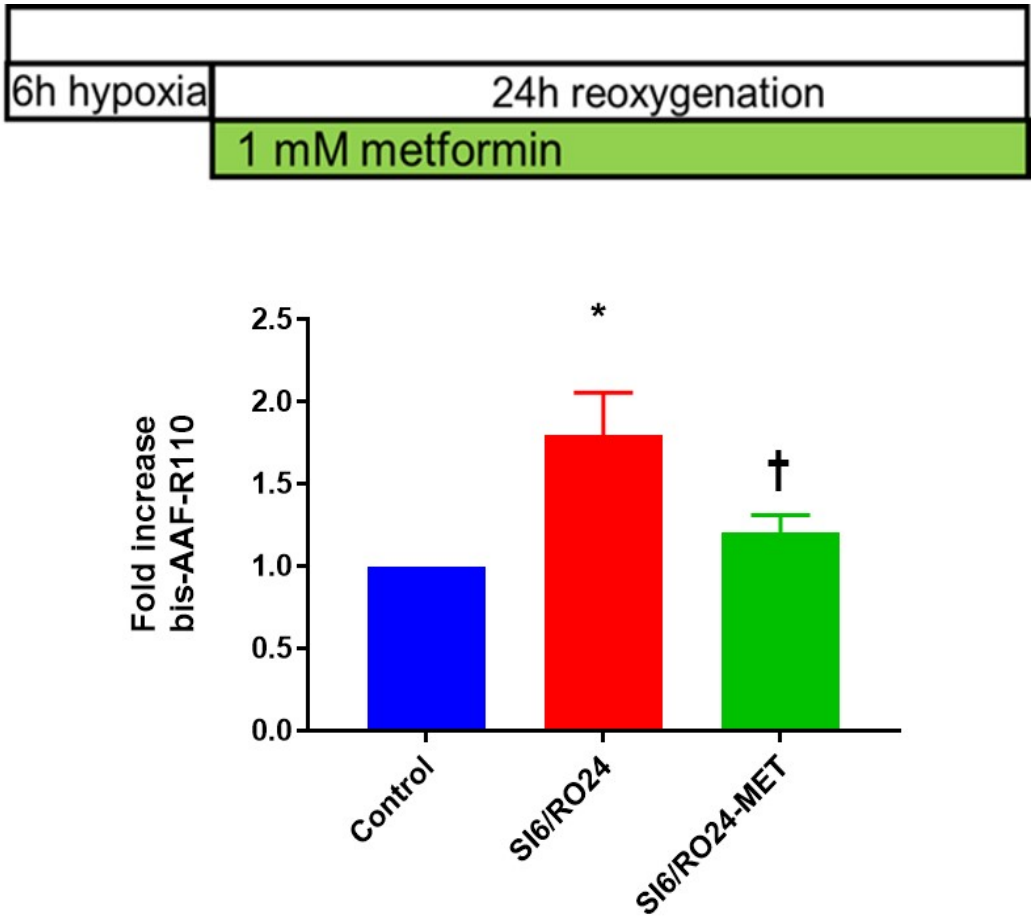


Figure 11. Metformin protected H9c2 cells against SI/RO Induced cardiac cell injury

Measure of cell death in metformin treated and untreated samples exposed to simulated ischemia-reoxygenation compare to time control. Mean ± SEM; n=5 in each group. *p<0.05 SI vs. control; †p<0.05 vs SI/RO24 alone

Metformin reduced apoptosis and total cell death

To understand more about the mechanism of the protection provided by metformin, the PI-Annexin V assay by flow cytometry was used in this experiment to measure total cell death and the apoptosis component. H9c2 cells were exposed to 6 hours simulated ischemia and 24 hours reoxygenation. Metformin was added at the onest of reoxygenation and continued for 24 hours. The results showed that metformin treated cells exhibited significantly less apoptosis and total cell death compare to the untreated simulated ischemia reoxygenation group. Metformin again significantly decreased H9c2 cell death. The summation of quadrants two and four (late and early apoptosis, respectively) was calculated for the results of apoptosis. Total cell death was calculated as the summation of the quadrants one, two and four (Figure 12).

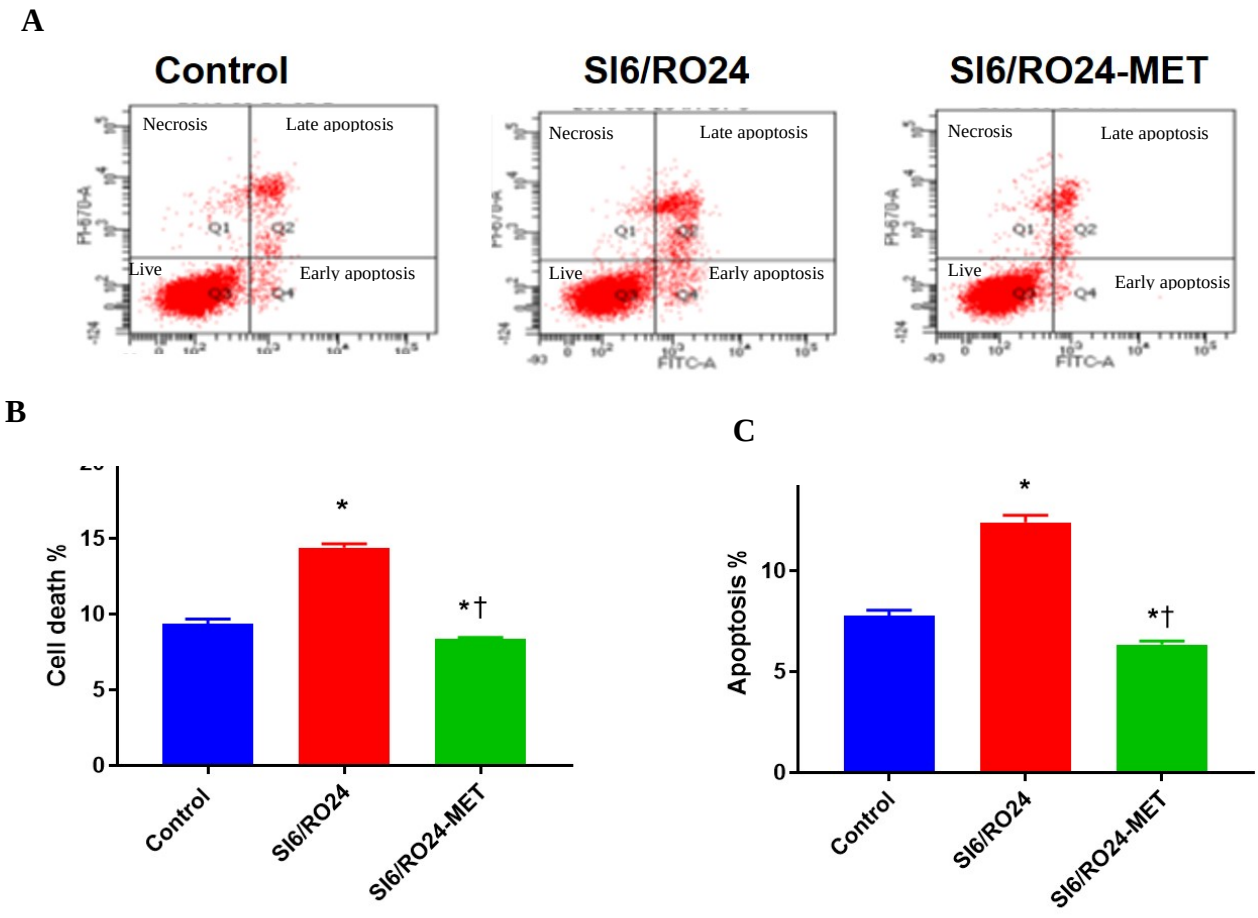


Figure 12. Metformin reduced Apoptosis and total cell death

A. Representative figures of flow cytometry. The PI-Annexin V assay by flow cytometry was used in this experiment to measure total cell death and the apoptosis component. **B.** Metformin decreased total H9c2 cell death. **C.** Apoptosis cell death; the summation of quadrants two and four was calculated for the Apoptosis calculation, n=3 in each group, *p<0.05 vs. control; †p<0.05 metformin + SI/RO vs SI/RO alone.

Metformin decreased caspase 3/7 activity

Since metformin decreased the contribution of apoptosis to total cell death, the activation of caspase 3/7 was assessed. Metformin treatment significantly reduced the activity of caspase 3/7 compared to untreated simulated ischemia-reoxygenation cells. This result shows that metformin decreased the activation of apoptosis signaling pathways in H9c2 cells following simulated ischemia reoxygenation (Figure 13).

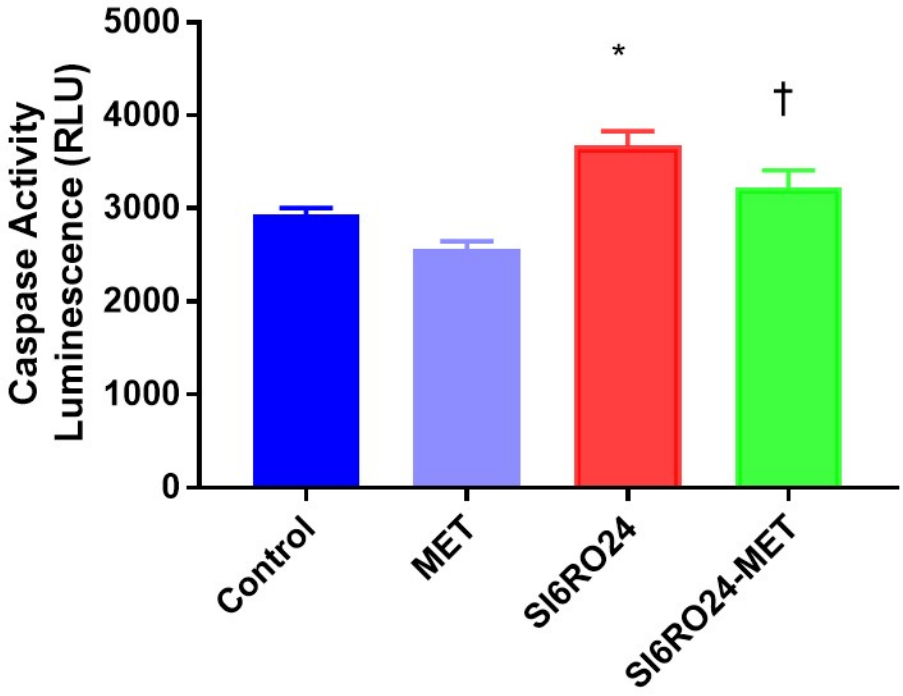


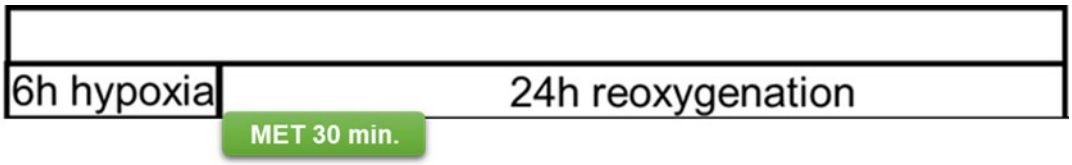
Figure 13. Metformin decreased Caspase3/7 activity

H9c2 cells groups were normoxic time control, normoxic metformin treated (MET) and simulated ischemia-reoxygenation metformin treated (SI6RO24-MET) or untreated (SI6RO24). Mean ± SEM; n=4 in each group. *p<0.05 SI vs. control; †p<0.05 metformin + SI/RO vs SI/RO alone

Short time metformin treatment decreased cell death induced by simulated ischemia-reoxygenation.

We established that 24 hours metformin treatment is protective. Next, we asked if a decrease in the time of treatment to 30 minutes of metformin treatment can protect cells during simulated ischemia reoxygenation as this short time can have more clinical relevance than 24 hours treatment. This treatment period focuses on the time when the acute modulation of complex I is needed for cytoprotection. H9c2 cells were exposed to 6 hours simulated ischemia then 24 hours of reoxygenation. Metformin (1mM) was used at the onset of the reoxygenation for 30 minutes then the media was changed. Cells were cultured in normal media for the remainder of the 24 hours. H9c2 cell death was assessed by cell membrane integrity assay by evidence of the presence or absence of protease activity related with necrosis by the use of bis-AAF-R110. The short time period of metformin treatment was also protective, as in 24 hours metformin treatment (Figure 14).

A



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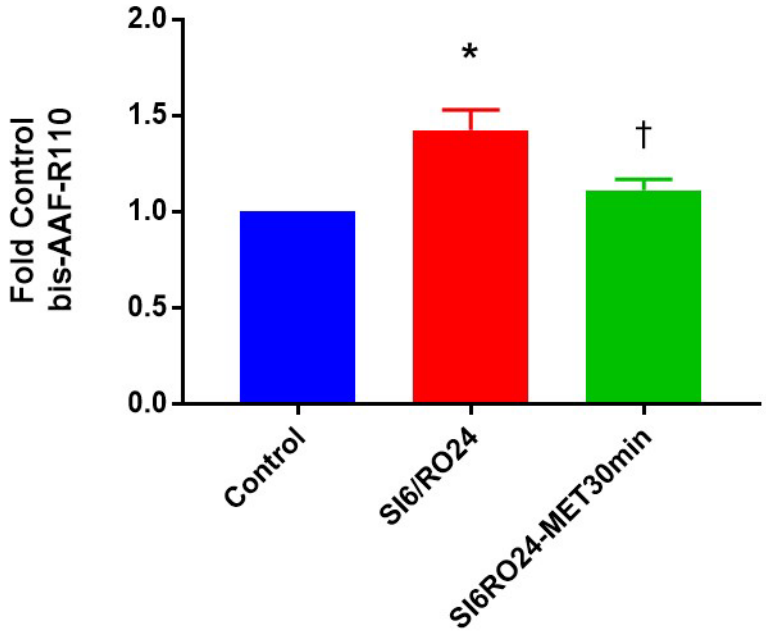


Figure 14. Metformin 30 min. treatment protected against simulated ischemia-reoxygenation induced cardiac cell injury.

Experimental protocol is shown. H9c2 cells groups were normoxic time control, and simulated ischemia-reoxygenation metformin treated for 30 minutes at onset of reoxygenation (SI6RO24-MET30min) or untreated (SI6RO24). H9c2 cell death was assessed by cell membrane integrity by measure protease activity related to necrosis by used a bis-AAF-R110 a fluorogenic peptide substrate. B. Metformin decreased cell death. Mean ± SEM, n=3 in each group; *p<0.05 SI vs. control; †p<0.05 metformin + SI/RO vs SI/RO alone

AMPK activation in simulated ischemia is independent of metformin

AMPK was phosphorylated (P.AMPK Thr172) in H9c2 cells exposed to simulated ischemia for 6 hours. AMPK was still phosphorylated in H9c2 cells exposed to six hours simulated ischemia followed by reoxygenation for 24 hours with a significant difference compared to control cells not exposed to simulated ischemia (Figure 15). Therefore, metformin treatment at reoxygenation is delivered into an AMPK activated system. Thus, it is likely that metformin protection is not fully dependent on AMPK activation. AMPK phosphorylates ACC1 on Ser79 therefore P-ACC is a specific readout for AMPK activity, our results indicated that ACC was strongly active by phosphorylation at Ser79 this finding indicates that AMPK activity was pervasively induced.

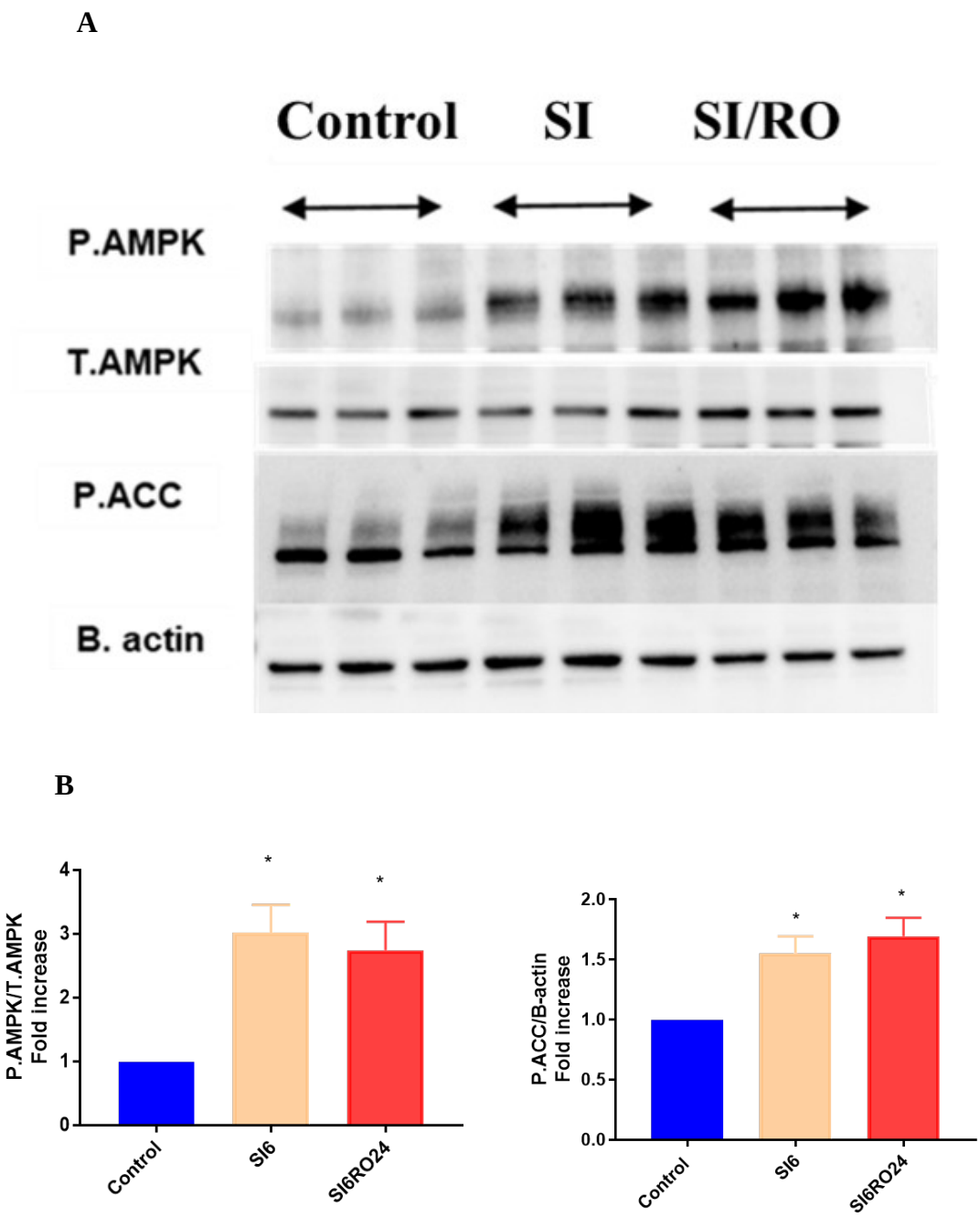


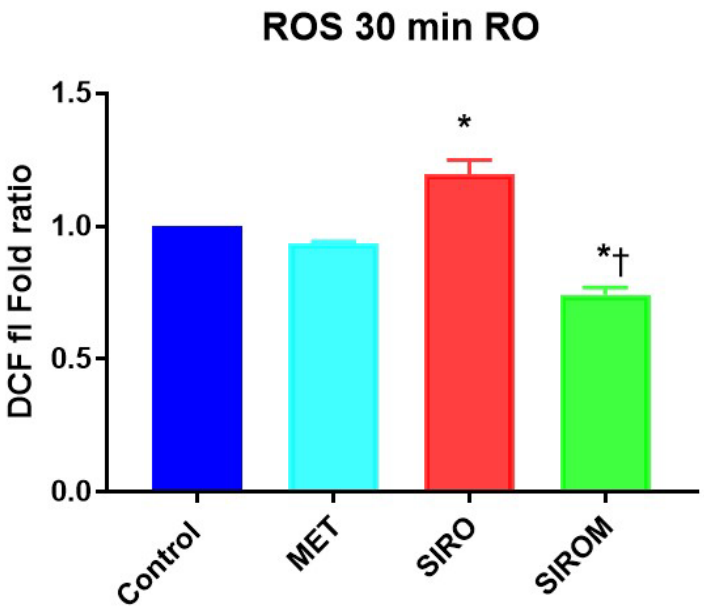
Figure 15. AMPK activation in simulated ischemia independent of metformin

A. Blot for triplicate samples control, simulated ischemia six hours(SI) and simulated ischemia 6 hours followed by reoxygenation for 24 hours (SI/RO) **B.** Quantitation for triplicate samples for H9c2 cells blot in A. Mean ± SEM *p<0.05 SI6 and SI6/RO24 vs. control.

Blockade of complex I by metformin during reoxygenation decreases ROS generation

We studied whether metformin caused decreased cellular ROS generation by using peroxide-sensitive fluorescent probe 2,7-dichlorofluorescein diacetate (DCFH-DA). Metformin treatment at the onset of the reoxygenation after 6 hours simulated ischemia (SI) decreased cellular ROS generation compared to cells exposed to simulated ischemia-reoxygenation without metformin (Figure 16) Reactive oxygen species were assessed at 30 minutes and 24 hours of metformin treatment after reoxygenation. Metformin protects against the burst of reactive oxygen species at the onset of reoxygenation as well as the longer time associated increased in ROS production.

A



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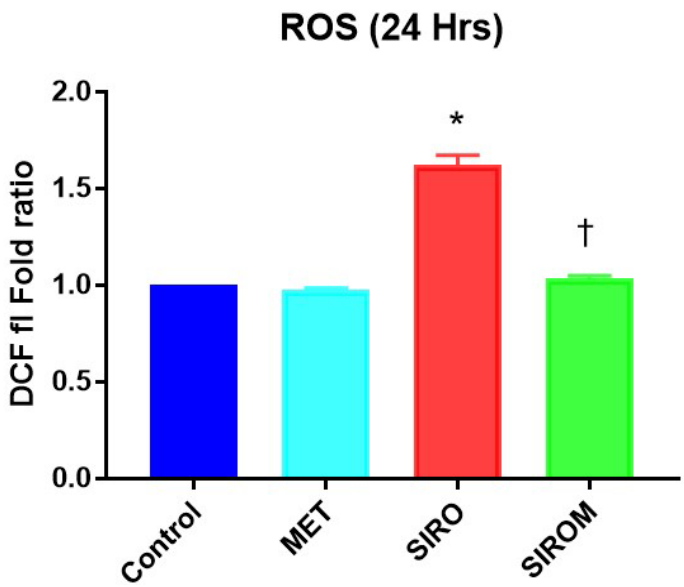


Figure 16. Blockade of complex I by metformin during Ischemia decrease ROS generation

H9c2 cells groups were normoxic time control, normoxic metformin treated (MET) and simulated ischemia-reoxygenation metformin treated (SI6RO24-MET) or untreated (SI6RO24). **A** ROS were measured after 30 min of RO with or without metformin. **B.** ROS were measured after 24 hours of RO with or without metformin. Mean ± SEM, n=3 in each group, *p<0.05 vs. control; †p<0.05 metformin + SI6RO24 vs SI6RO24 alone

Metformin treatment reduced CHOP ER stress marker in H9c2 cells

Several studies report the importance role of endoplasmic reticulum (ER) stress associated with the ischemic insult leading to apoptosis in several cell types (170). Hypoxia (1% O_2) intensely increased reactive oxygen species production. Mitochondrial reactive oxygen species specifically regulate expression of the CHOP (171). CHOP [C/EBP homologous protein; also known as GADD153 (growth arrest and DNA damage 153)] is considered to have a critical role in ER-stress-induced apoptosis (172). CHOP mediates downregulation of Bcl-2 and increases proapoptotic proteins including Bax with elevated oxidative stress, causes ER-stress-dependent apoptosis (173). Metformin significantly decreased the protein level of CHOP, a conspicuous ER stress marker that induces apoptotic signaling pathways (Figure 17). The decrease in CHOP content as an index of ER stress is in line with decreased ROS production.

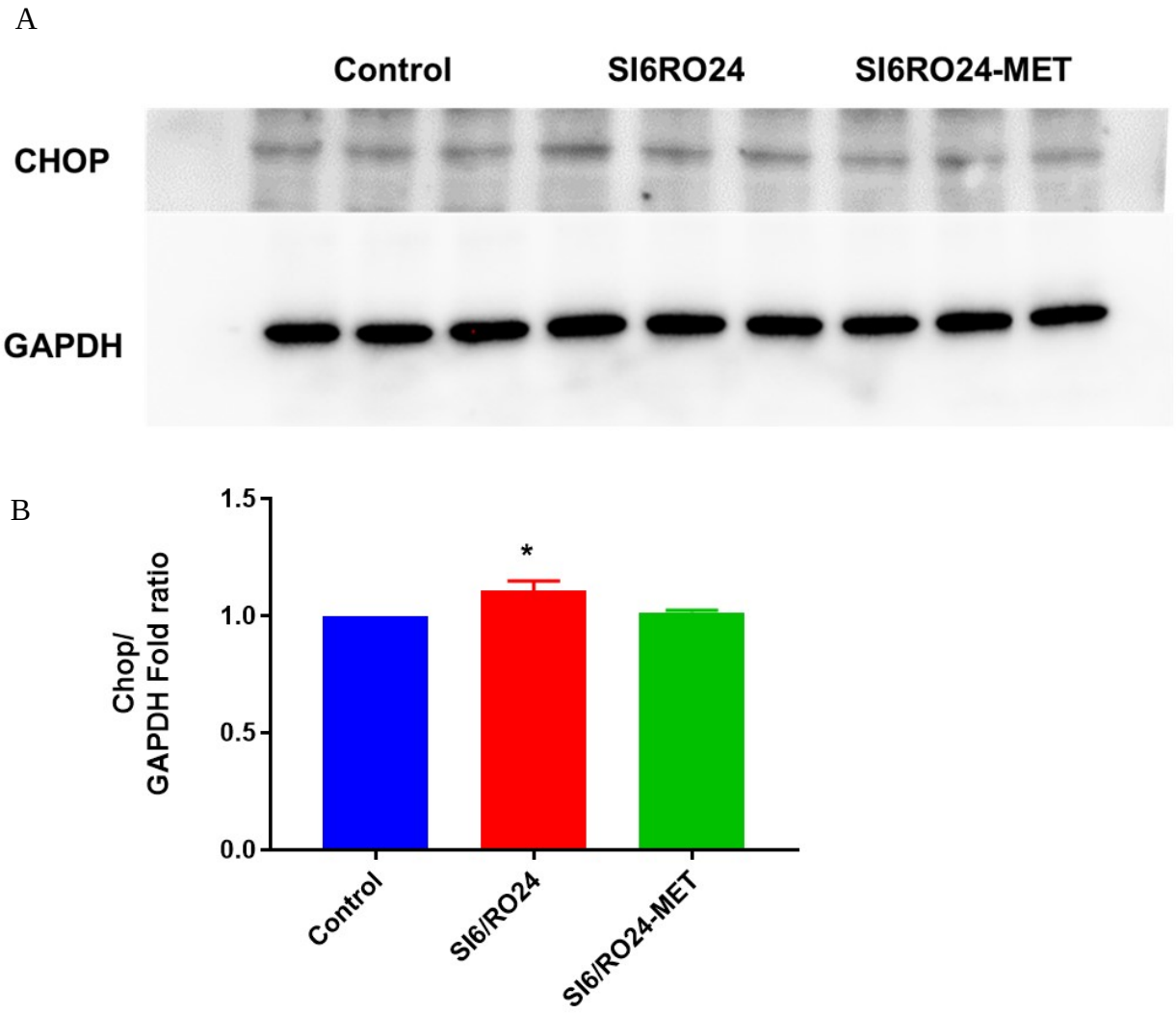


Figure 17. Metformin treatment reduced CHOP ER stress marker in H9c2 cells

A. Western blot for CHOP protein for H9c2 cells triplicate samples of control, SI6RO24 and SI6RO24 treated with metformin at the onset of reoxygenation. **B.** Quantitative results of A. Mean ± SEM, n=3 in each group; *p<0.05 SI6 and SI6/RO24 vs. control.

Metformin treatment in AMPK downregulated systems

To investigate the AMPK role in metformin cellular protection, we wished to study metformin in AMPK downregulated models. First we used Compound C, a known AMPK activation inhibitor. Compound C is the only available agent that is used as a cell-permeable selective AMPK inhibitor (174). We found that 20 μM of compound C was the optimal concentration to inhibit AMPK activation in H9c2 cardiomyoblast cells. Unfortunatly, our initial results indicated that Compound C caused high cytotoxicity in normoxia samples with significant increase in cell death compared to the vehicle-treated control (Figure 18). Thus, Compound C was not suitable for use in our cytoprotection studies. We therefore utilized knockdown of AMPK isoforms.

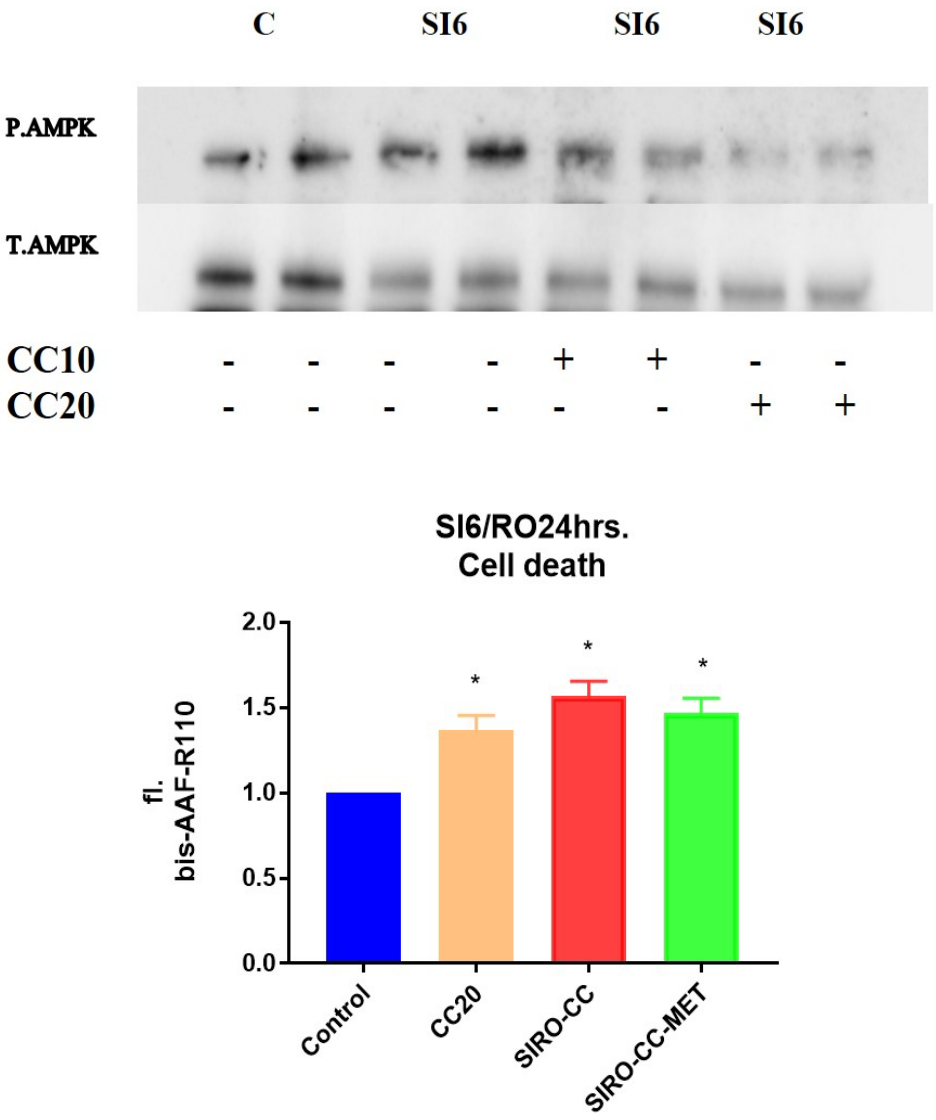


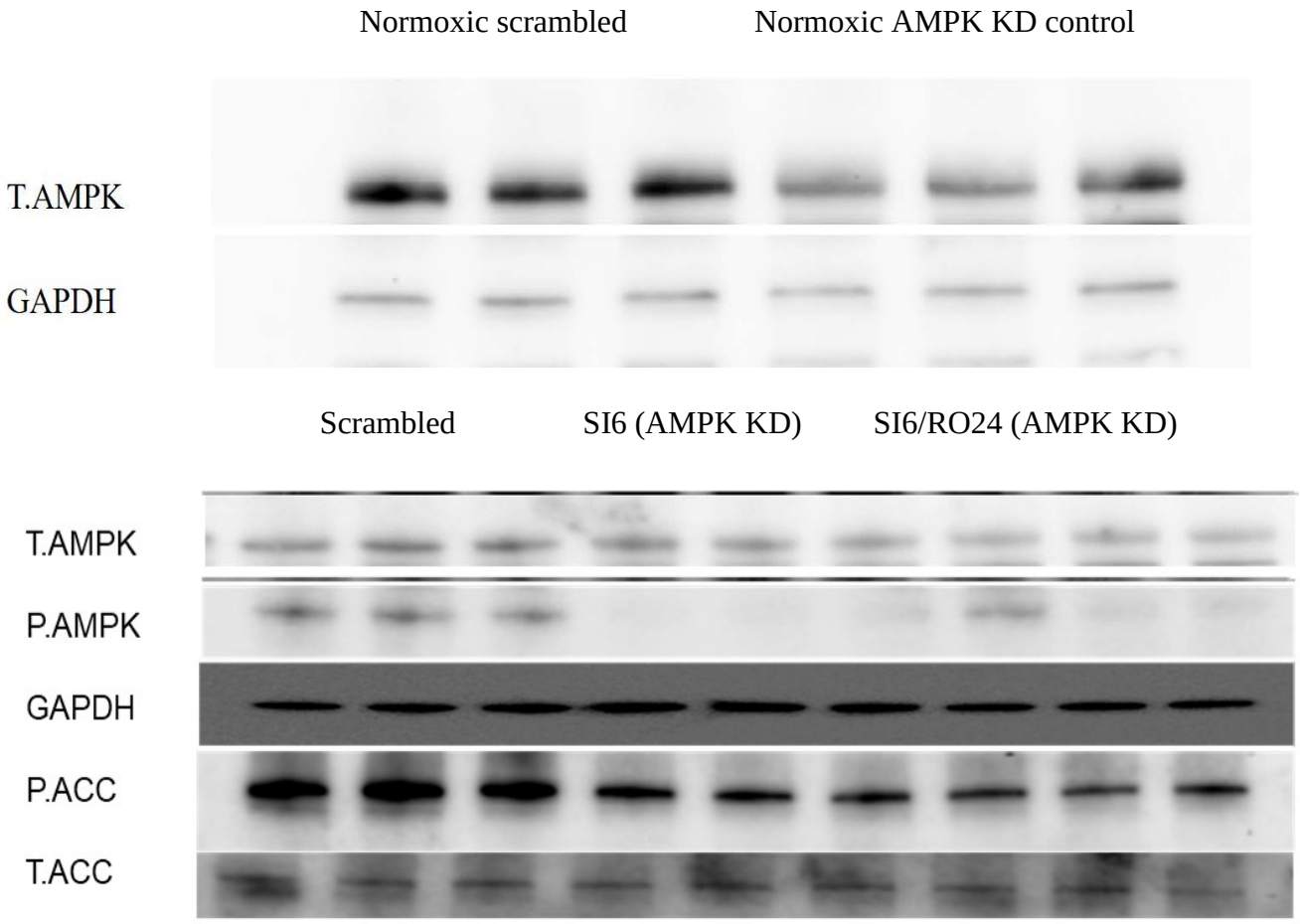
Figure 18. Metformin treatment in AMPK downregulated systems

A. Compound C (CC) used to downregulate AMPK activity. **B.** Measure effects of CC 20 µM in normoxic condition and SI6/RO24 with or without metformin, n=3 in each sample, Mean ± SEM *p<0.05 vs. control. H9c2 groups were vehicle control, compound C 20 µM treated normoxic (CC20) and simulated ischemia reoxygenation treated with compound C ± metformin

Metformin protects against SI/RO causing cell death in AMPK KD cells.

We sought if metformin can protect against cell death during simulated ischemia and reoxygenation independent of AMPK. AMPK Silencer pre-designed siRNA was used to knock down the AMPK alpha 2 catalytic subunit. AMPK alpha 2 is the dominant AMPK subunit in heart. Our initial finding showed that AMPK phosphorylation and the direct downstream of AMPK activation P.ACC were decreased following knockdown which confirming impact of the knockdown. Metformin still protected AMPK alpha 2 knockdown cells following 6 hours of simulated ischemia and 24 hours of reoxygenation .(Figure 19). This finding strongly supports that protection by metformin involves a contribution by a non – AMPK mediated mechanism. These findings are consistent with the previous observations that addition of metformin at reoxygenation into an already activated AMPK-system, nevertheless exerts substantial additional cytoprotection.

A


Normoxic scrambled
Normoxic AMPK KD control
T.AMPK
GAPDH
Scrambled
SI6 (AMPK KD)
SI6/RO24 (AMPK KD)
T.AMPK
P.AMPK
GAPDH
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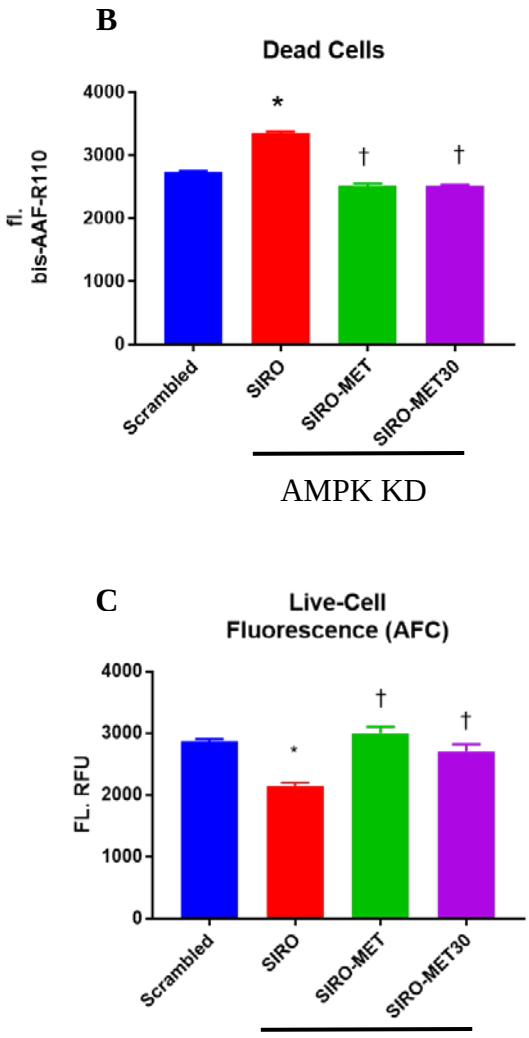


Figure 19. Metformin treatment in AMPK downregulated systems.

A. Upper blot AMPK KD with SiRNA for AMPK alpha 2 in normoxic condition control, lower blot scrambled, SI6 hours and SI6RO24 hours triplicate samples each. **B.** Measurement of cell death for simulated ischemia-reoxygenation AMPK KD samples treated or untreated with metformin treatment at onset of reoxygenation for 30 minutes or 24 hours. **C.** Cell viability Mean ± SEM, n=3; *p<0.05 vs. control. †p<0.05 vs. SI/RO alone

Metformin decreased ROS generation from AMPK KD cells following simulated ischemia and reoxygenation.

We studied the potential contribution of metformin treatment to cellular ROS levels in AMPK KD H9c2 cells by using the peroxide-sensitive fluorescent probe 2,7-dichlorofluorescein diacetate (DCFH-DA). Metformin treatment at the onset of the reoxygenation after 6 hours simulated ischemia decreased cellular ROS generation compared to cells exposed to simulated ischemia roxygenation without treatment (Figure 20). These results are consistent with previous findings linking decreased ROS production to modulation of complex I and cytoprotection. Importantly, this finding is observed in AMPK KD cells, supporting an AMPK-independent component of action.

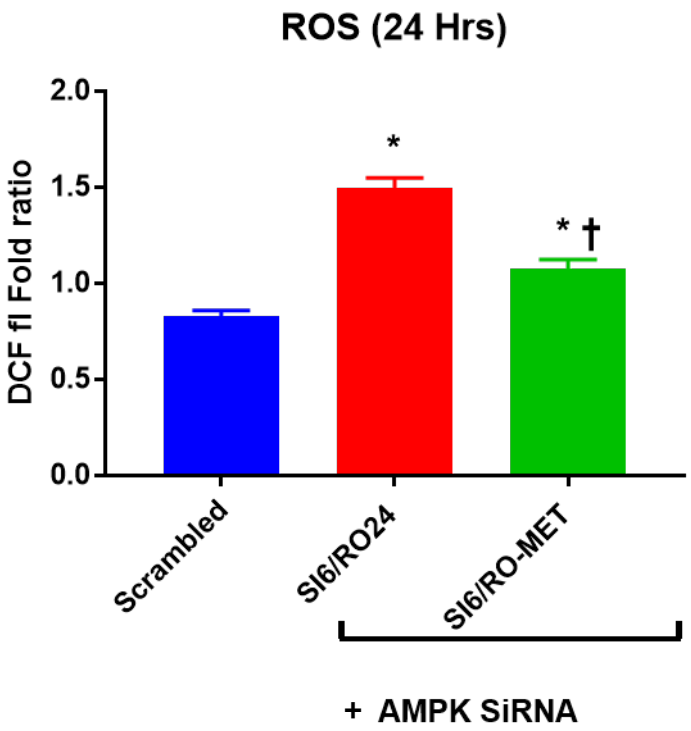


Figure 20. Metformin decreased ROS generation from AMPK KD cells following simulated ischemia and reoxygenation

Measure ROS generation was measured, H9c2 groups were simulated ischemia-reoxygenation with AMPK KD treated or untreated with metformin at the onset of reoxygenation compared to scrambled normoxic control. n=3, Mean ± SEM. *p<0.05 vs. control. †p<0.05 metformin + SI/RO vs SI/RO alone

Influence of metformin on autophagy

We assessed the effect of metformin on stimulation of autophagy in H9c2 cells exposed to six hours of simulated ischemia and 24 hours reoxygenation. Metformin or vehicle was added at the onset of reoxygenation and continued for 24 hours. We measured the autophagy marker Beclin-1. The protein content of Beclin-1 was similar in simulated ischemia reoxygenation group versus control. Intriguingly, the effect of metformin on autophagy activity was augmented indicated by increased Beclin-1 protein expression (Figure 21). Beclin -1 is a regulatory protein that is essential for the autophagosome formation and upregulation of Beclin 1 is associated with autophagy that protects heart against myocardial infarction injury (175).

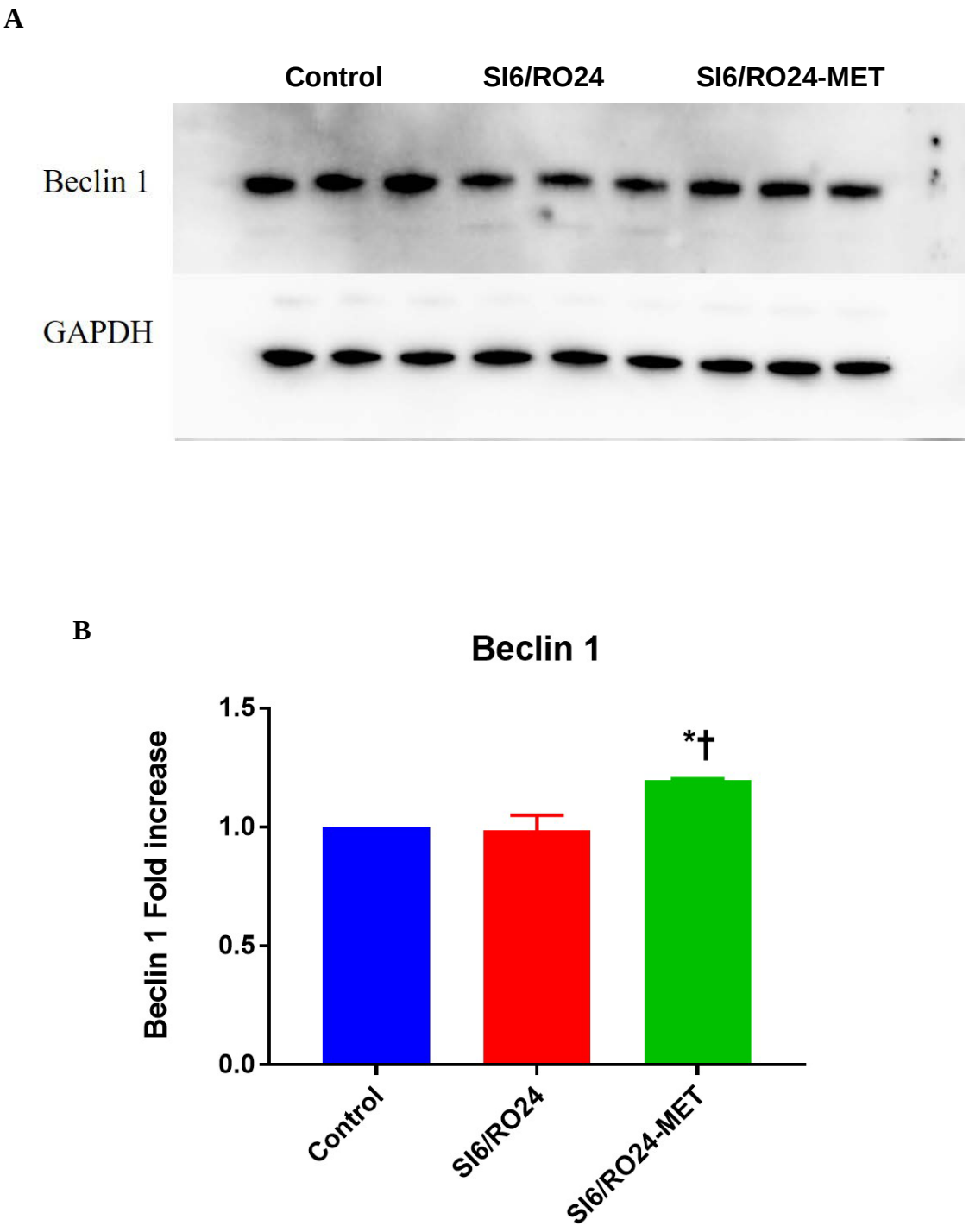


Figure 21. Influence of metformin on autophagy activity

Beclin-1 blot for control, simulated ischemia roxygenation treated with or without metformin. **B**. Beclin-1 results graph bar. n=3, Mean ± SEM. *p<0.05 vs. control. †p<0.05 metformin + SI/RO vs SI/RO alone.

Metformin reduced p53 protein expression

p53 translocates into mitochondria and could be related to impaired mitochondria function leading to cell death (176). Moreover, there is an interaction between p53 and cyclophillin D that can favor mitochondria permeability transition pore opening following cerebral ischemia/reperfusion (92). A decrease in p53 expression was associated with protection against stroke injury (92). In our current study we sought effects of metformin treatment at the onset of reoxygenation on the p53 protein content. Surprisingly, we found that metformin treatment successfully downregulated p53 compared to untreated simulated ischemia reoxygenation. Metformin treatment preserved p53 content with no significant difference compared to the normoxic control (Figure 22). Thus, the downregulation of complex I respiration with decreased ROS production may decrease cell death in part by downregulation p53 protein content as a downstream effector mechanism.

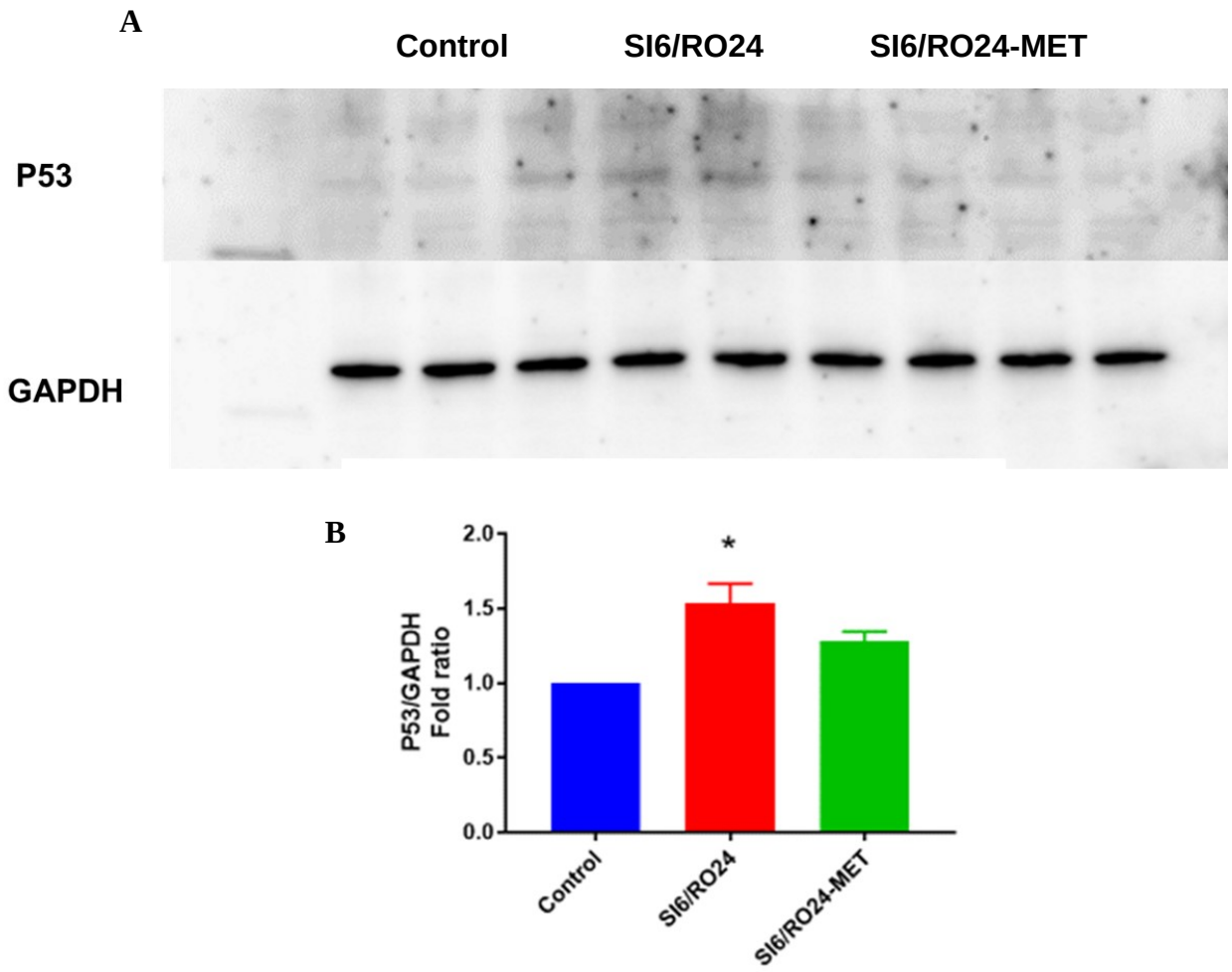


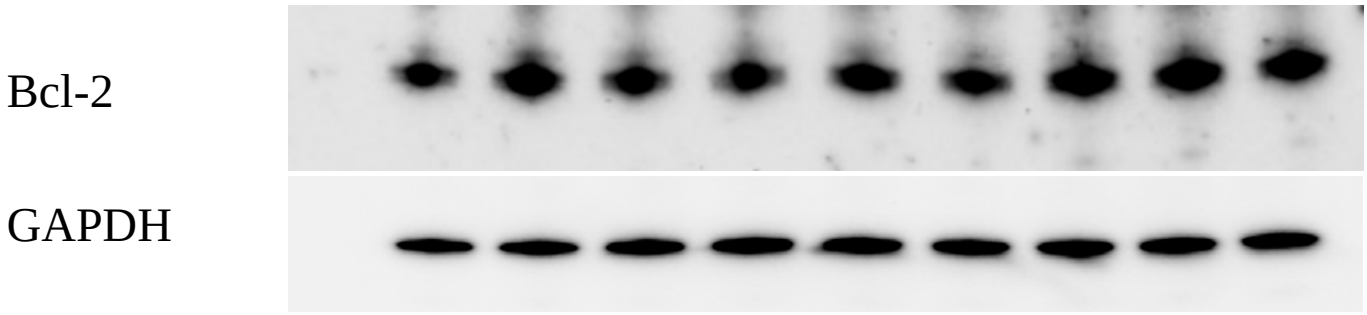
Figure 22. Metformin reduced p53 protein expression

A. p53 blot for control, simulated ischemia roxygenation treated with or without metformin. **B.** Measurement p53 in metformin treated and untreated samples exposed to SI6/RO24 compare to time control. n=3, Mean ± SEM. *p<0.05 vs. control

Metformin increased Bcl-2 protein expression

Bcl-2 family proteins contribute an important role in cell survival versus death pathway. We consequently examined the effects of simulated ischemia and reoxygenation on the expression of the antiapoptotic protein Bcl-2 in H9c2 cells. Increased expression of bcl-2 blocks lipid peroxidation and decreased cell death in cardiomyocytes exposed to hypoxia and reoxygenation (177). The changes in the protein level of Bcl-2 in response to simulated ischemia-reoxygenation are shown (Figure 23). Our results from immunoblotting showed that the expression of the anti-apoptotic protein Bcl-2 was decreased following simulated ischemia-reoxygenation without metformin treatment. Metformin treatment increased expression of Bcl-2 compared to non-ischemic control and simulated ischemia-reoxygenation untreated H9c2 cardiomyoblast (Figure 23).

A



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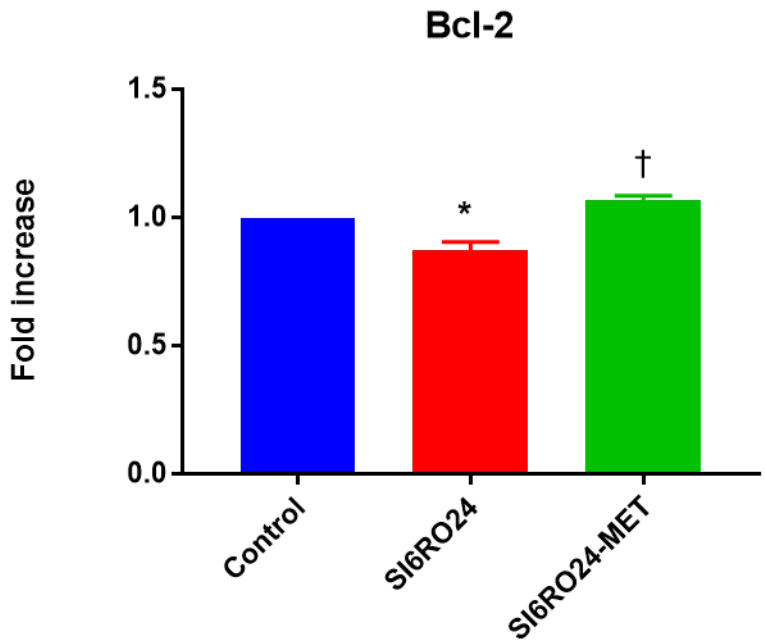


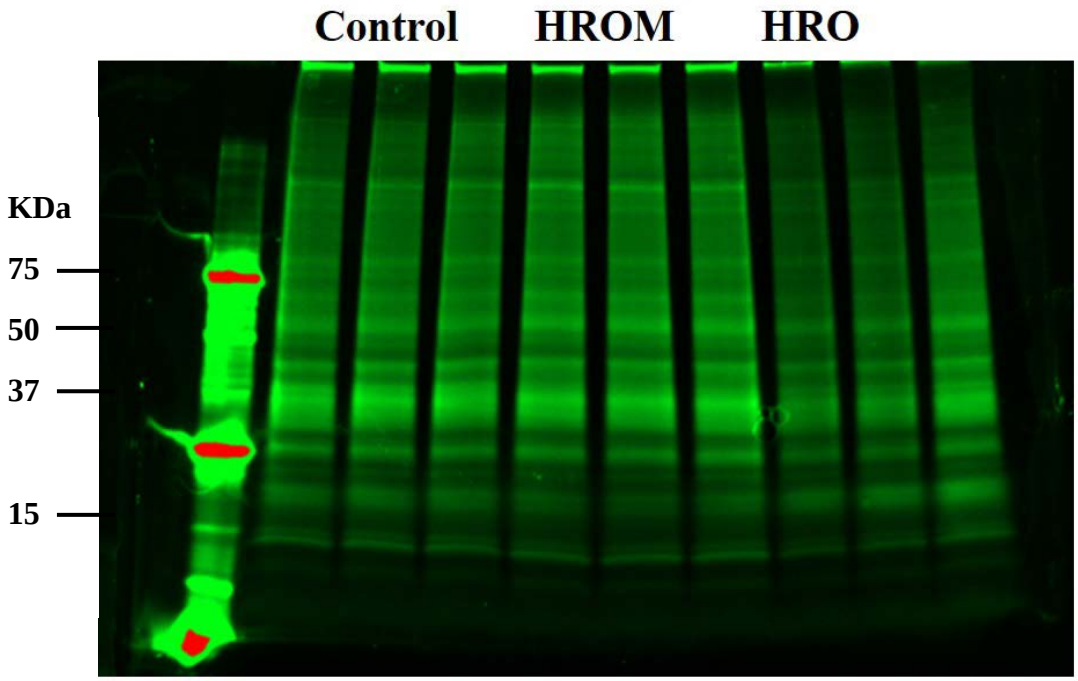
Figure 23. Metformin increased Bcl-2 protein expression

A. Bcl-2 blot for normoxic control and simulated ischemia roxygenation treated with or without metformin. **B**. Measure Bcl-2 protein expression in metformin treated and untreated samples exposed to SI6/RO24 compare to time control. n=3, Mean ± SEM. *p<0.05 vs. control. †p<0.05 metformin + SI/RO vs SI/RO alone

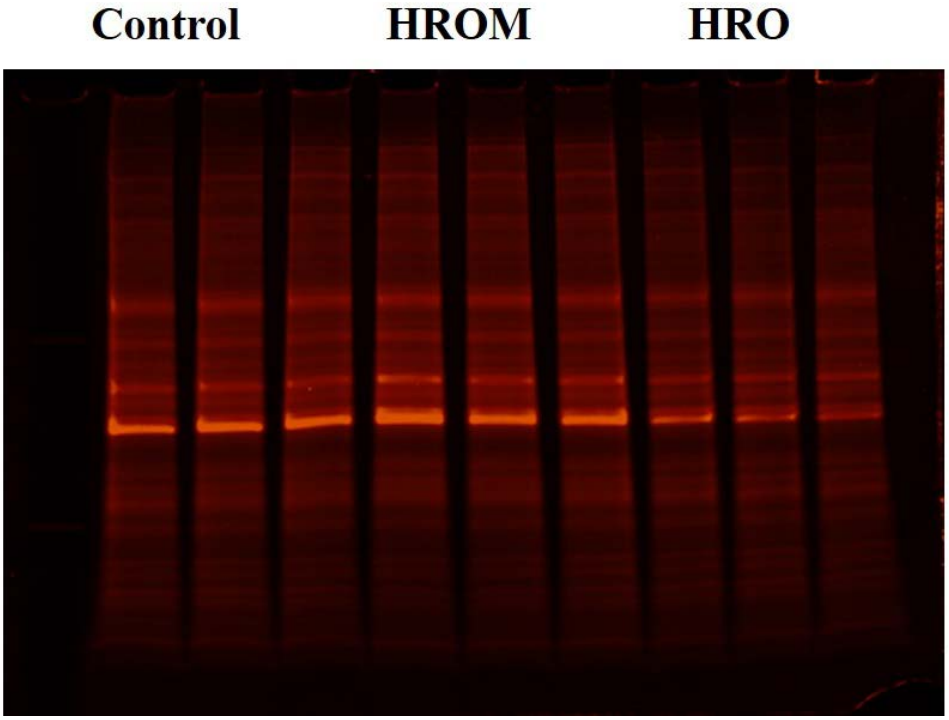
Metformin protected exposed thiol groups against modification

As an index of oxidative modification during early reoxygenation, we sought an effect of metformin treatment on the modification of thiol groups. H9c2 cells were incubated with complex I and complex II substrates (Glutamate, Pyruvate, Malate and Succinate). The addition of succinate as complex II substrate was designed to maximized oxidative stress to include that generated through reverse electron transport. H9c2 cells reached hypoxia through normal respiration by consuming all the oxygen inside the Oroboros chamber. After 25 minutes of hypoxia in the Oroboros chamber with or without metformin treatment, Cyanine 3 water soluble fluorescent label dye at the final concentration of 40µM was added at the onset of reoxygenation with the addition of Alamithicin 10 ug/ml to permeabilize the inner mitochondrial membrane. Cy3 was able to label multiple protein bands. There is a highly decreased sulfhydryl labelling with Cy3 in the untreated hypoxia reoxygenation group. Cy3 results were normalized to the total protein stain gel by SYPRO® Ruby Protein Gel Stain. SYPRO Ruby is highly sensitive fluorescent stain used to detect of proteins separated by polyacrylamide gel electrophoresis (PAGE). We measured the individual thirteen KD protein band which possibly belongs to ND3 mitochondria complex I subunits. This result supports that hypoxia reoxygenation leads to increased production of ROS (shown above) that results in sulfhydryl oxidation. Metformin likely by partial inhibition of complex I with a decrease in ROS generation as previously shown resulted in less sulfhydryl modification. The preservation of native sulfhydryl groups led to enhanced Cy3 labelling of native sulfhyryls, as seen in metformin treated cells that underwent hypoxia reoxygenation.

Cy3



Sypro Ruby



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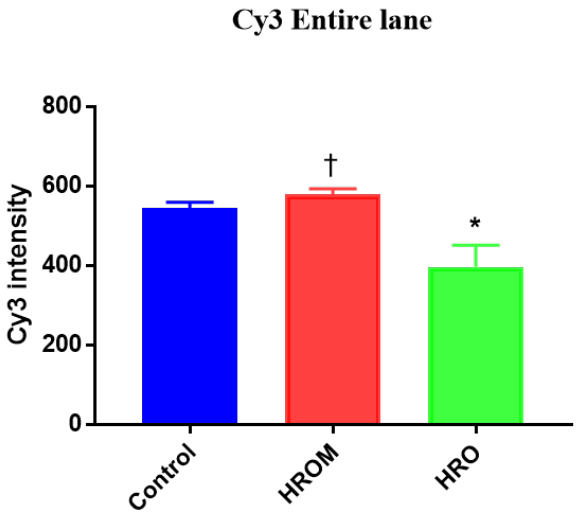

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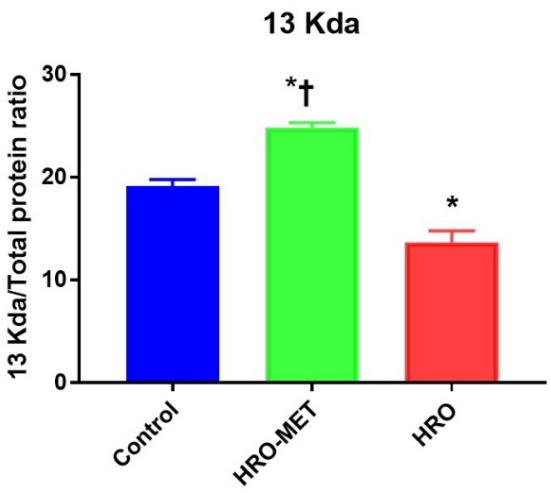
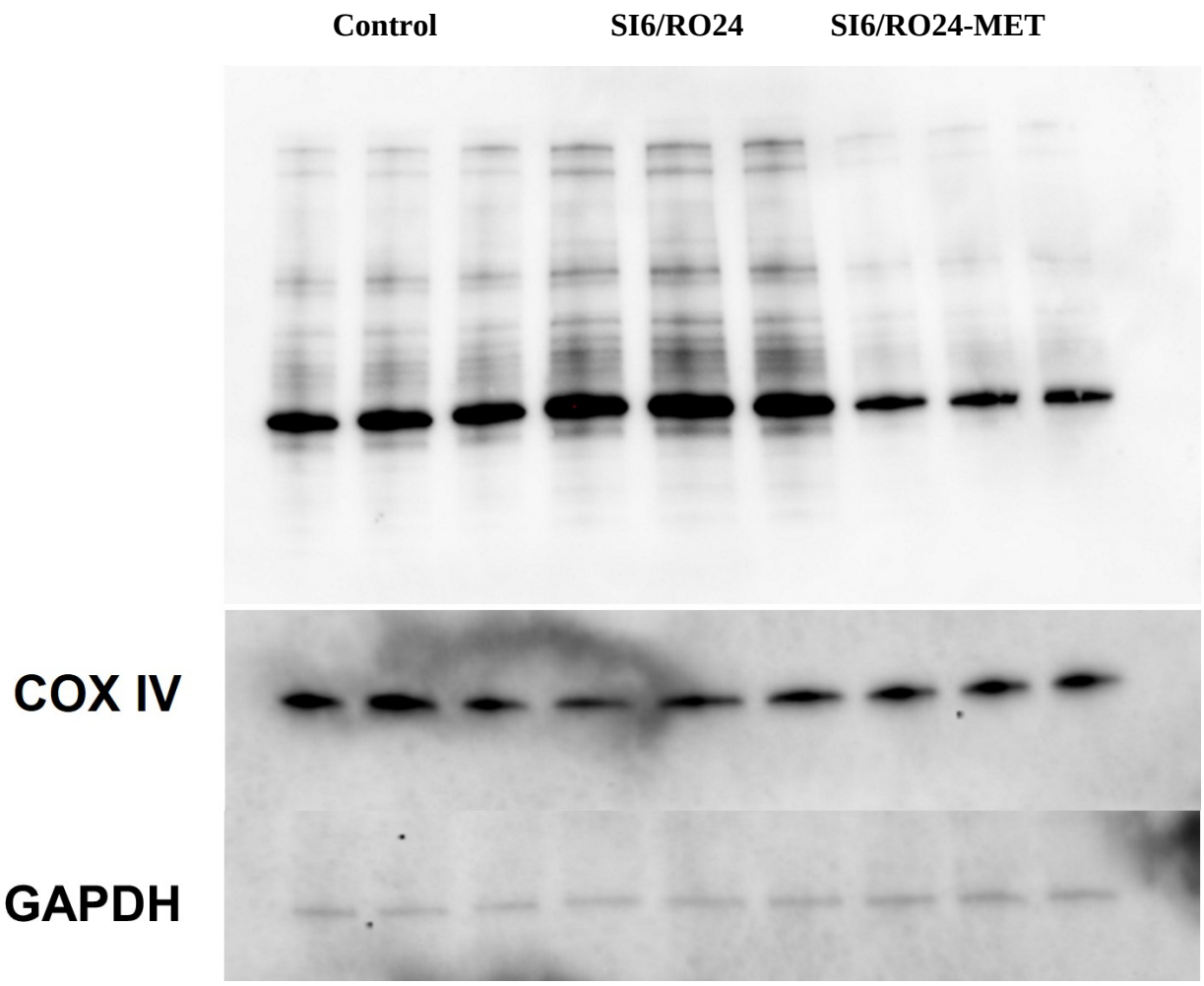


Figure 24. Metformin protects exposed thiol group against modification

A. Measurement of exposed free thiol groups in metformin treated and untreated samples exposed to 25 minutes hypoxia and 30 minutes reoxygenation in Oroboros **B**. Measurement of 13 KDa Cy3 labelled protein band, n=3, Mean ± SEM. *p<0.05 vs. control. †p<0.05 metformin + HRO vs HRO alone

Metformin decreased the global *S*-nitrosylation of proteins

One mechanism of sulfhydryl group modification is modification to form S-NO derivatives. Global S-NO modification was studied. Generation of NO or peroxynitrite can modify sulfhydryl groups. We studied S-NO modification using Thermo Scientific Pierce S-Nitrosylation Western Blot Kit which provide sensitive recognition of protein S-nitrosocysteine post-translational modifications. Reduced S-nitrosylated cysteines was specifically labelled with iodoTMTzero™ reagent that binds irreversibly to the cysteine thiol in the samples which became easy to detect it by anti-TMT antibody. Metformin decreased the S-NO content and protected against a global S-nitrosylation of proteins. These findings are consistent with the preservation of native free sulfhydryl groups by metformin treatment during hypoxia and reoxygenation, likely through decreased oxidative stress via metformin-induced modulation of complex I.


Control
SI6/RO24
SI6/RO24-MET
COX IV
GAPDH

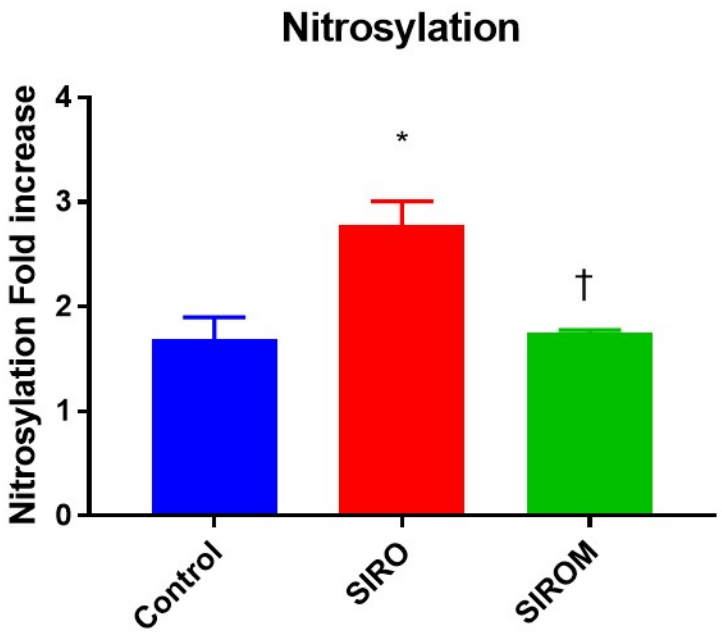


Figure 25. Metformin reduce a global S-nitrosylation of proteins

Measure nitrosylation thiol group modification in metformin treated and untreated samples exposed to SI6/RO24 compare to time control. Indirect labeling of S-NO adducts has been achieved by blocking free thiols, *S* -nitrosylated cysteines are then selectively reduced with ascorbate and then the resulting free thiols are labeled with a iodoTMTzero reagent, which irreversibly binds to the cysteine thiol that was *S* -nitrosylated, n=3, Mean ± SEM. *$p<0.05$ vs. control. †$p<0.05$ metformin + SI6/RO24 vs SI6/RO24 alone. Quantitative results were normalized to COX IV mitochondria protein.

Metformin significantly protects against ischemia induced cardiac injury in 18 month aging mice

To translate our observation of protection in the cellular system to the intact heart, we studied the potential of metformin to protect the *ex vivo* isolated, perfused heart from aging mice, Elderly 18 month old mice were studied and compared to 3 month old controls. Metformin or vehicle control was administrated at the onset of reperfusion for 5 minutes in buffer-perfused mouse hearts, Metformin treatment at reperfusion decreased the infarct size (Figure 26). These results support that transient, high dose metformin treatment at reperfusion decreases cardiac injury assessed following the early phase of reperfusion in the intact mouse heart, including in the aged mouse heart. Experiment done by Ms. Ying Hu and Dr. Qun Chen.

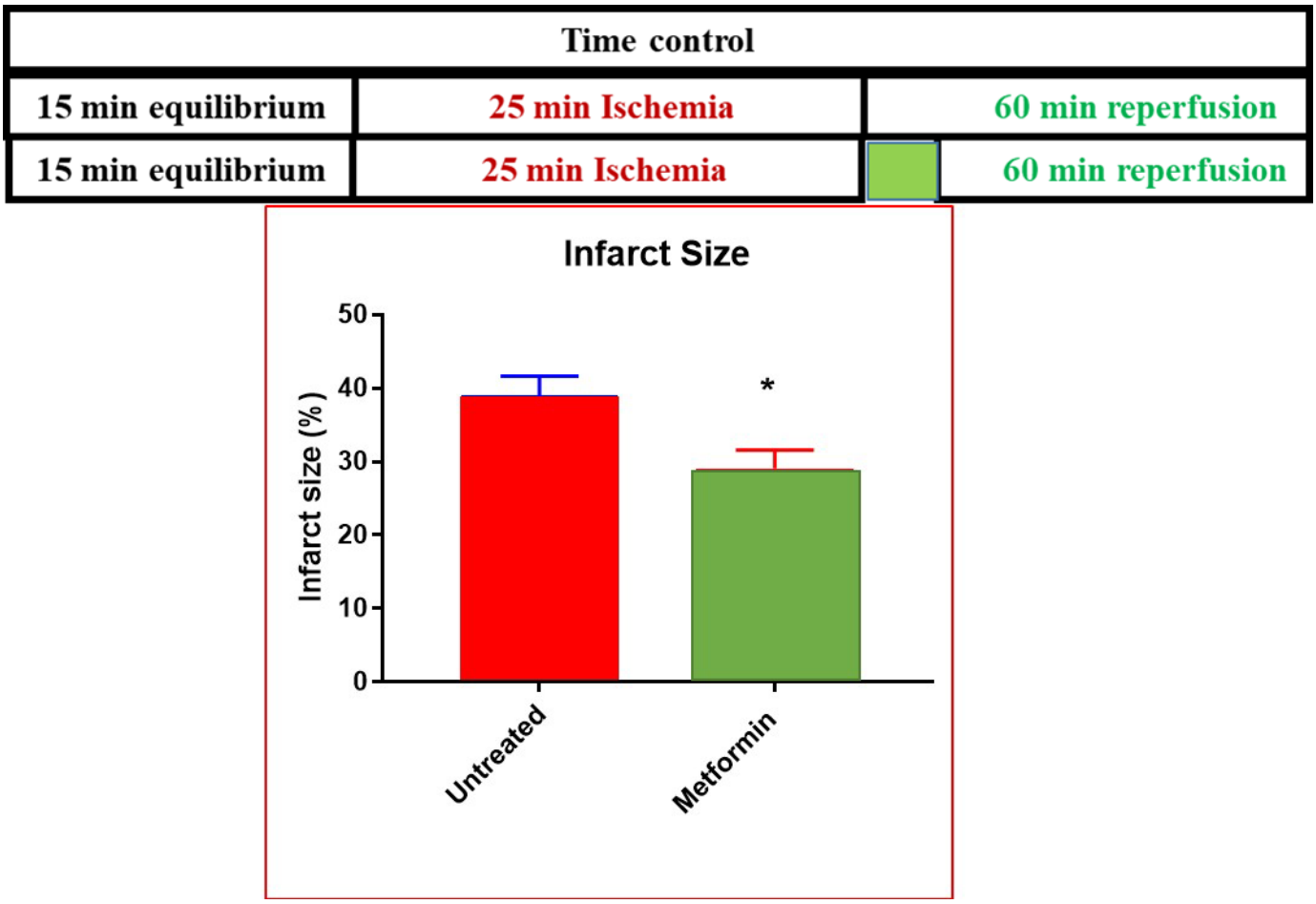


Figure 26. Metformin treatment only during early reperfusion significantly protects against cardiac injury in 18 month aging mice

Myocardial infarct siz**e** was measured after 60 minutes reperfusion and expressed as percent of the affected area. n=3, Mean ± SEM. *p<0.05 vs. metformin untreated group.

AMPK is robustly active in the cytosol from ischemic mouse hearts.

In line with the observation in the cell model, at the end of 25 minutes ischemia in the isolated perfused mouse heart, in cytosol from mouse hearts, AMPK was robustly phosphorylated (Figure 27). This finding again supports that metformin-mediated cardiac protection from delivery of metformin at reperfusion is in part via an AMPK-independent mechanism. AMPK is robustly activated before metformin is administrated.

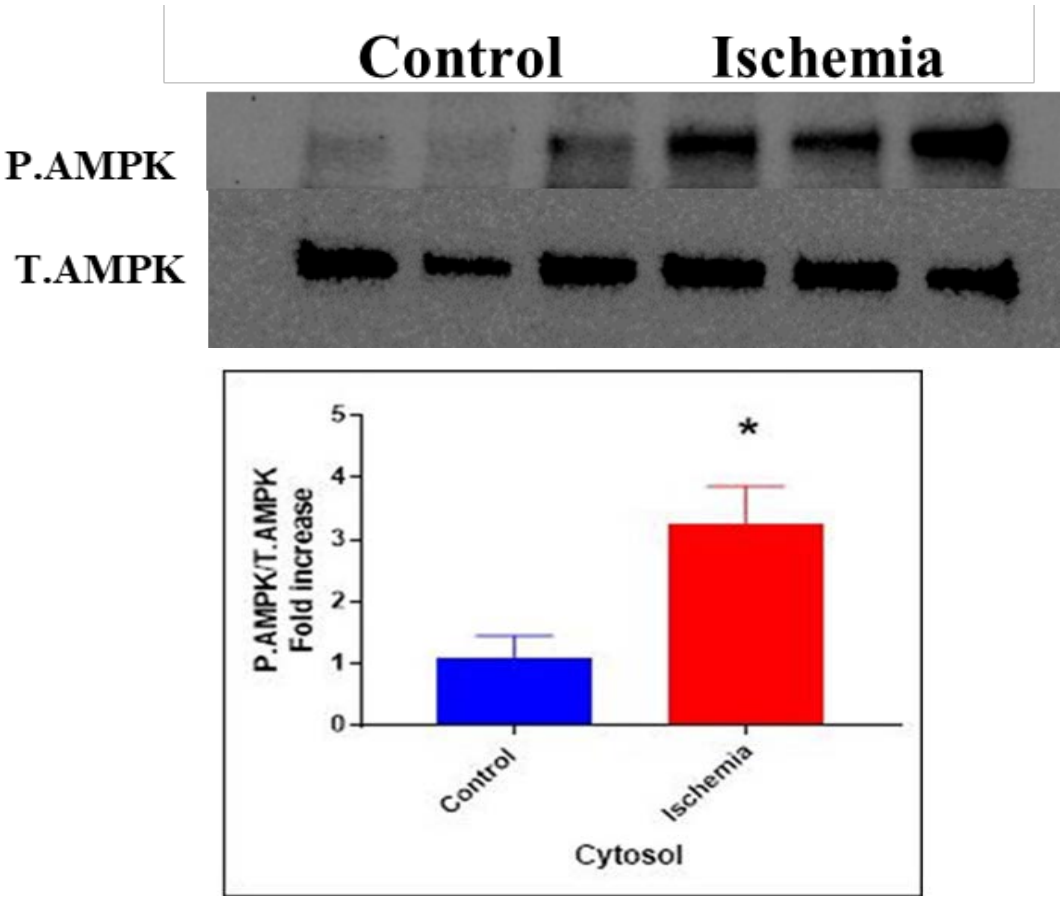


Figure 27. AMPK is robustly active in the cytosol from ischemic heart.

Measurement of AMPK activation in the the cytosol samples from 25 minutes ischemic and non-ischemic hearts. Mean ± SEM. N=3; *$p<0.05$ vs. control.

Metformin inhibited complex I activity in ischemia-damaged mitochondria

Compared to control, 25 minutes global ischemia significantly decreased complex I activity in mouse heart mitochondria (Figure 28). Ischemia did not alter the activities of complex II or the mitochondrial marker enzyme citrate synthase. Metformin treatment of isolated mitochondria did not decrease complex I activity in control mitochondria from non-ischemic hearts, but it further decreased complex I activity in ischemic-damaged mitochondria (Figure 28). Metformin did not decrease complex II activity in neither control nor ischemic-damaged mitochondria (Figure 28). These results indicate that ischemia-damaged mitochondria are more sensitive to metformin inhibition of complex I. The inhibition by metformin was performed in mitochondria following isolation from control or ischemic (non-reperfused) hearts.

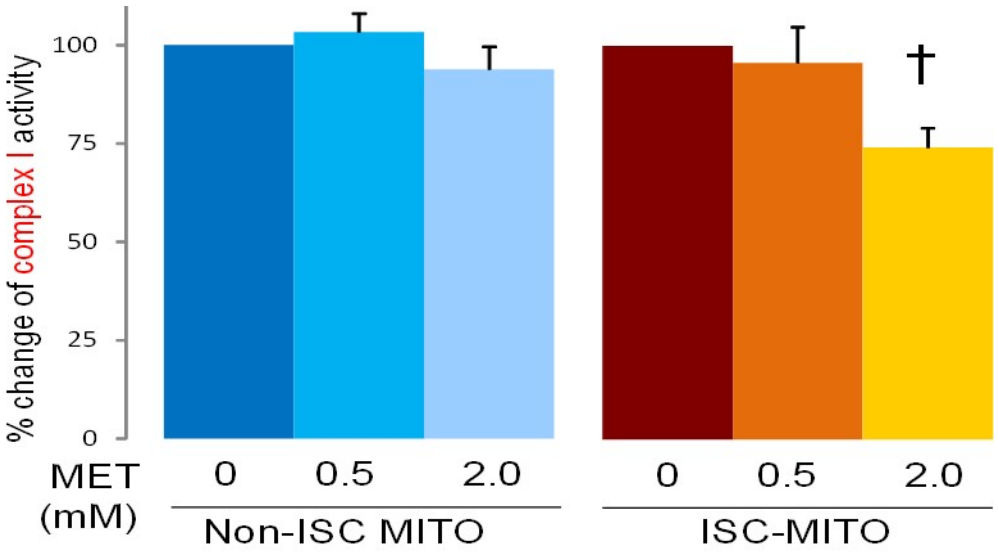


Figure 28. Metformin inhibited complex I activity selectively in ischemia-damaged mitochondria

Complex I activity was measured in mitochondria isolated from control or ischemic hearts.

Mean ± SEM. † $p<0.05$ metformin vs. non-metformin-treated mitochondria. n=4 in each group

Metformin does not inhibit the NADH oxidation site associated with the flavin mononucleotide (FMN)

Metformin does not inhibit the NADH oxidation site associated with the flavin mononucleotide (FMN) cofactor that is located proximal in the path of electron transport. There is no difference between activity of both active (A-form) and deactive (D-form) in activity associated with FMN electron transport as we showed in the results of rotenone-insensitive NADH:tetrazolium reaction for Blue Native in-gel activity for mitochondria samples induced A and D-form as explained in method also the results from NADH:ferricyanide reductase (NFR) for mitochondria samples from 25 minutes ischemia and 60 minutes reperfusion mouse hearts and metformin treatment for 5 minutes at the onset of reoxygenation. Non-significant results for complex I NFR activity between metformin treated and untreated mitochondria (Figure 29).

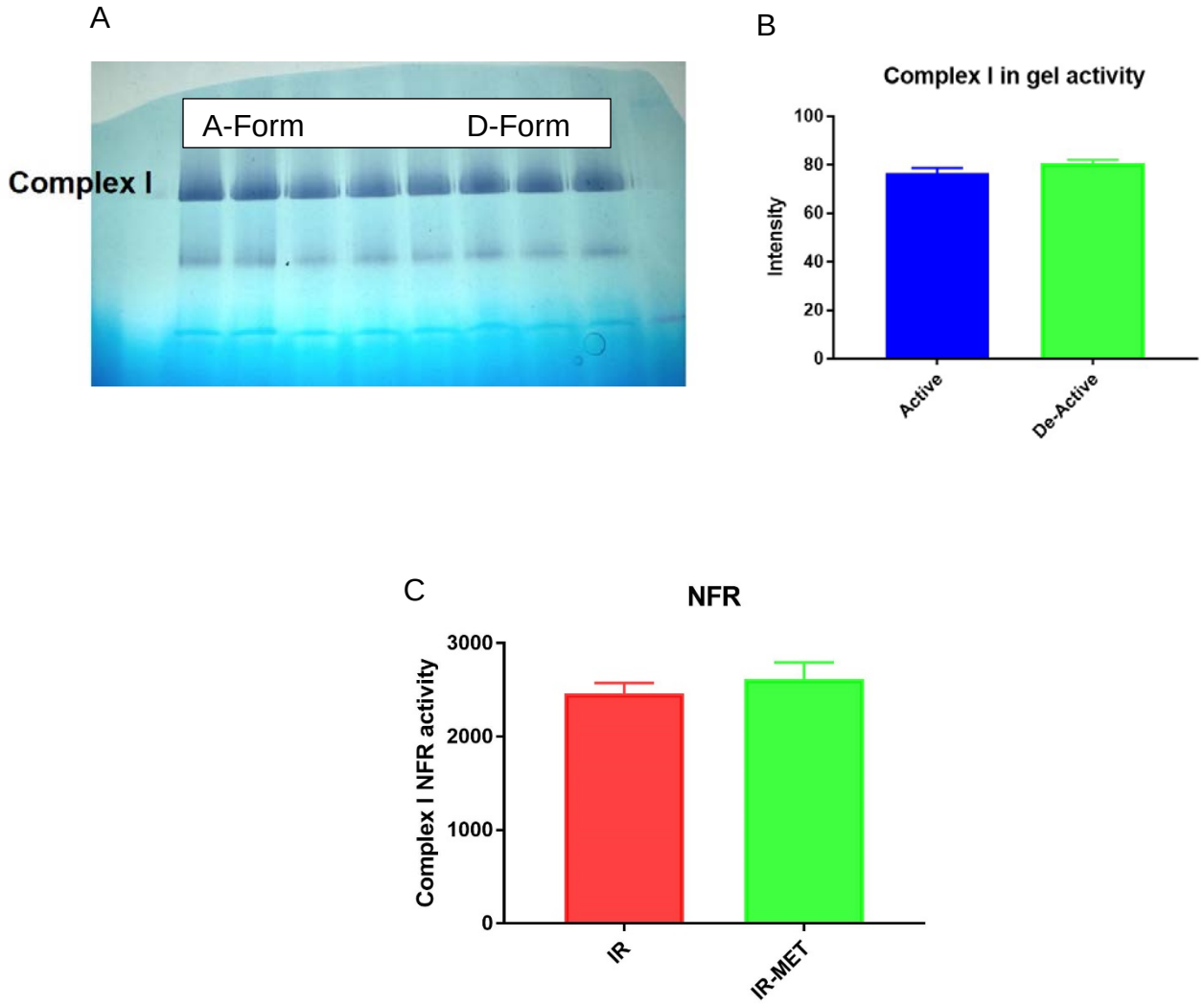


Figure 29. Metformin does not inhibit the NADH oxidation site associated with the flavin mononucleotide (FMN)

A. Complex I in gel activity for mitochondria in A-Form and D-Form. B. In gel activity results. Mean ± SEM. n=4 in each group. C. NADH dehydrogenase part of complex I (NFR) for mitochondria derived from 25 minutes ischemic heart and 30 minutes REP, metformin was treated at the onset of REP.

CHAPTER 4: Discussion

Treatment at the onset of reperfusion following ischemic stress with acute, high dose metformin substantially decreases cardiac injury. The study of in situ mitochondria in cells and isolated mitochondria supports that this high dose treatment regimen selectively inhibits the D-form of complex I. Although chronic therapy with metformin is known to decrease cardiac injury in models of cardiac stress through activation of AMPK (133,178), *acute* metformin treatment at the onset of reperfusion occurs at a time when AMPK is already robustly activated by the preceding ischemia. Furthermore, in support of AMPK-independent mechanisms of acute protection, high dose metformin treatment reduced cell death in vitro in H9c2 AMPK knock down cells. Thus, protection by the acute, early reperfusion high dose treatment paradigm is not mediated solely via activation of AMPK. Using our novel treatment approach with high dose metformin at the onset of reperfusion, cytoprotection was achieved in vitro in cells and in isolated hearts. Thus, we found that using high dose metformin at the onset of reperfusion is cardioprotective through the AMPK-independent mechanism of partial inhibition of complex I. Metformin can decrease cardiac injury during ischemia and reperfusion in an integrative manner through complimentary AMPK-independent mechanism from acute therapy to inhibit complex I followed by canonical AMPK-dependent mechanisms.

Chronic, standard dose metformin therapy is generally thought to activate AMPK by decreasing the cellular ATP content through partial inhibition of complex I (178). Metformin has decreases complex I activity in submitochondrial particles and purified complex I (179). Bridges et al. shows that high dose of metformin (mM range) inhibits complex I activity (NADH:ubiquinone oxidoreductase) (179). In the diabetic patient receiving chronic metformin therapy, the metformin level usually is in micromolar range (180,181). In order to achieve mM content within mitochondria, inner mitochondrial membrane potential is required for metformin to accumulate within mitochondria (179). In the present study, H9c2 cells demonstrated acute inhibition of complex I respiration by metformin was greater following hypoxia than during normoxia. Although there was no acute inhibition by metformin when used during normoxia, we found significant inhibition by metformin after 24 hours incubation with H9c2 cells. These results indicate that metformin has a specific effects to inhibit D-form of mitochondria complex I. The D-form is the predominant one in loss of oxygen conditions such as ischemia. Inhibition of the D-form by metformin at the onset of reoxygenation will prevent the rapid reactivation of complex I at reoxygenation that also decreases the burst reactive oxygen species generated from forward and reverse electron transport. Furthermore, acute metformin therapy rapidly decreased complex I activity in ischemia-damaged mouse heart mitochondria, but not in mitochondria from non-ischemic control hearts. The results in isolated mitochondria were consistent with and confirm the results in H9c2 cells and again support the likely metformin inhibition of the D-form of complex I. These findings are particularly intriguing, since they raise the potential for a reperfusion intervention that selectively targets the ischemia-damaged mitochondria that perpetrate cardiac injury during early reperfusion.

The majority of damage to mitochondria, including the ETC, occurs during ischemia (49,51,113,118,119). Ischemic damage to mitochondria occurs in part via the pathologic action of the ETC during ischemia under conditions of low oxygen, favoring the production of ROS that damage the inner mitochondrial membrane and the ETC itself (65,66). The damaged ETC in turn, augments cardiac injury during reperfusion (6,51). Although treatment during reperfusion cannot restore ischemia-mediated damage to mitochondria, modulation of the ischemia-damaged ETC during early reperfusion does attenuate the ETC-driven injury to the myocyte (10,110,182). Modulation of ETC activity at complex I reduces injury not only by attenuating the production of ROS (183), but also attenuates ETC effector mechanisms of opening of the permeability transition pore (184), activation of mitochondrial proteases (158), leading to activation of programmed cell death (185). The optimal modulation of complex I appears to be a partial inhibition of the complex that does not lead to depolarization of mitochondrial membrane potential, yet blunts ROS production (123), striking a key balance between less reactive oxygen species production without substantially impairing ATP synthesis.

Complex I couples electron flux to vectoral proton translocation in order to generate an electrochemical gradient across the inner mitochondrial membrane (186,187). This process harnesses chemical energy in a form that is used by mitochondria to phosphorylate ADP. Ischemia causes a functional defect within complex I that disrupts electron flow through the complex (7,83,118,119,188). The site was localized to a portion of the complex near the co-enzyme Q binding site that likely involves iron-sulfur centers of complex I (7,118,119). Recent studies show that complex I exists in two different conformations, the ‘A-form’ and the ‘D-form’ conformations (55,118,119,181,189). Complex I converts to the D-form with oxygen deprivation and is reactivated during reoxygenation of the ischemic tissue with increased production of ROS.

(190,191) The D-form of complex I is increased during ischemia (55,118,119). Metformin does not inhibit the NADH oxidation site associated with the flavin mononucleotide (FMN) cofactor that is located proximal in the path of electron transport because there is no difference between activity of both active and deactive forms. This was shown by the results of rotenone-insensitive NADH:tetrazolium reaction for Blue Native in-gel activity. Thus, our results are consistent with the notion that the mechanism of complex I inhibition by metformin involves metformin-induced stabilization of the D-Form of complex I.

Treatment with high dose metformin at the onset of reperfusion decreased cardiac injury, consistent with previous studies that reversible inhibition of complex I decreases cardiac injury during reperfusion (10,96,110,182). Metformin treatment at reperfusion does not improve oxidative phosphorylation since most damage to the ETC occurred during ischemia. The key findings are that metformin treatment to modulate electron transport chain led to decreased production of ROS. Increase ROS production during early reperfusion can occurs by forward electron flow from complex I into complex III (121,122). ROS production also occurs at the onset of reperfusion via reverse electron flow from complex II into complex I in the presence of accumulated succinate (112,117,192) supported by the presence of a mitochondrial membrane potential. Although the relative contribution of forward and reverse electron transport is likely to vary based upon the presence of high concentration of succinate (onset of reperfusion), extent of mitochondrial damage (ability to support a membrane potential adequate to support reverse electron transport), issues which dynamically change as reperfusion continues, inhibition of complex I at the site distal in the complex attenuates ROS production from both forward and reverse electron transport. The deactive form of complex I blocks ROS production via both

forward and reverse electron transport (83,112,192). Thus, inhibition of complex I by metformin will protect against ROS production by both mechanisms.

An increase in mitochondria permeability transition pore (MPTP) opening augments cardiac injury during reperfusion and is attenuated by transient blockade of electron transport at complex I (184,193). Oxidative stress is a key mechanism contributing to MPTP opening during reperfusion (193). Recent work found that reverse electron transport mediated ROS generation sensitizes to opening of the MPTP (194). Thus, metformin may decrease MPTP opening through reduction of ROS generation via complex I inhibition, protecting against reverse electron flow-mediated ROS generation at the onset of reperfusion and likely forward electron transport mediated ROS from complex III as reperfusion continues.

Simulated ischemia in H9c2 cells and stop flow ischemia in mouse hearts results in robust AMPK phosphorylation before the onset of reoxygenation/reperfusion. Thus, metformin therapy is delivered into an already active AMPK system. Metformin activates AMPK by increasing the phosphorylation of AMPKα at Thr-172 (195-197). We found that metformin protects cardiomyocytes and intact hearts against injury although AMPK is already active. This finding strongly supports that the protective mechanism for metformin against early reperfusion is likely an AMPK-independent modulation of complex I as discussed above. Previous studies confirm that metformin metabolic action in H9c2 cells can occur without AMPK activation (198).

Multiple previous studies found that various treatment paradigms, most involving chronic pretreatment before ischemia with chronic low dose metformin protect against aspects of ischemia-reperfusion injury. Metformin decreased the incidence of ischemia-reperfusion induced apoptosis in rat hearts (199), and oxidative stress–induced cardiomyocyte apoptosis in

dogs (200). Metformin protects both the diabetic and non-diabetic heart during ischemia and reperfusion (133). It is of interest that treatment at the onset of reperfusion with lower dose metformin than in the present study was protective in the previous work, although in an AMPK-dependent fashion, since protection was not observed in the AMPK kinase dead mouse (133). In contrast, treatment at the onset of reperfusion in the current study with high dose metformin protected in an AMPK-independent fashion, protecting the AMPK knockdown H9c2 cells. Taken together, these results support a key potential advantage in cardioprotection of integrated therapy with metformin during reperfusion. Acute, high dose metformin therapy can be deployed as in the current study to attenuate the early ETC-driven cardiac cell death that occurs early in the course of reperfusion. The cardioprotection of this treatment can be consolidated with the continued use of standard dose metformin during the more prolonged period of reperfusion to continue with AMPK activation to recruit AMPK mediated cardioprotection during the remodeling/recovery phase of reperfusion to blunt the transition to post-infarct ischemic cardiomyopathy. Beneficial longer term impacts of AMPK-mediated signaling during reperfusion include the shift of substrate utilization toward glucose oxidation from fatty acid oxidation (197,201), the potential attenuation of endoplasmic reticulum stress (202), the activation of protective autophagy/mitophagy and the potential activation of mitochondrial biogenesis (196). We observed initial evidence of several of these processes by 24 hours of reoxygenation.

We observed that expression of p53 was markedly upregulated after six hours simulated ischemia and twenty four hours of reoxygenation in H9c2 cells. p53 gene deletion enhanced cardiac function after myocardial infarction (203). There was a decreased expression of anti-apoptotic Bcl-2 protein. Metformin was able to downregulate of p53 expression. Bcl-2 content

was greater in metformin treated samples compared to simulated ischemia reoxygenation samples. Studies in mice found that upregulation of anti-apoptotic Bcl-2 protein or genetic deletion of proapoptotic Bax protein inhibits apoptosis and decreased infarct size (204). Studies showed that hypoxia-induced p53 apoptosis in cardiac myocytes (205). Consequently, other interesting studies showed that inhibition of tumor suppressor p53 is cardioprotective against ischemic injury (206,207).

To initially assess the effect of metformin in the autophagy process after SI6/RO24, we measured Beclin-1, the mammalian homolog of yeast Atg6, which is a crucial protein in the initiation of autophagosome formation (208). Previous studies revealed that Beclin1 expression is increased in hibernating myocardium (209) and throughout reperfusion, where increased autophagy (210) and is likely to be cardioprotective (211).

We found that simulated ischemia-reoxygenation treated with metformin at the onset of reoxygenation led to an increase in Beclin -1 content. This finding suggests that metformin favored the formation of autophagosome by upregulation of Beclin -1. Hypoxic stress induces reactive oxygen species generation, which plays an essential role in negatively regulating autophagy through activation of p53 (212). Based on these findings and the previous reports it can be speculated that oxidative stress by increased production of reactive oxygen species triggers upregulation of p53 that mediated inhibition of myocyte autophagy leading into subsequent apoptosis.

Cysteine thiol modifications are increasingly known to occur under both physiological and pathophysiological conditions like aging and ischemia-reperfusion. Modification of reactive cysteine thiols alters the function of proteins through modulation of enzymatic activity and may be irreversibly oxidized by pathological conditions, and thus affect protein function (213-215).

Modification of cysteine residues by reactive oxygen species (ROS), reactive nitrogen species (RNS) and their interaction has appeared as a important means of changing the structure and function of various proteins (216) including mitochondrial protein complexes. These modification can alter mitochondrial complex activity (42). Thiols can react with ROS/RNS. Cysteine thiolate anion can be oxidized to nitrosothiols (SNO) and sulfoxidation products, sulfenic (SOH), sulfinic (SO_2H), and sulfonic (SO_3H) acids. Some of these reactions are reversible nitrosothiols; this reversible reaction can cause reversible modification in protein function (217).

We found that there is highly decreased sulfhydryl labelling with Cy3 in untreated hypoxia reoxygenation group which reflect the modification of the thiol group resulted from oxidation and nitrosylation stress during hypoxia-reoxygenation. Metformin protects against thiol getting modification by decrease ROS generation and nitrosylation. This result is consistent with the increased ROS generation and nitrosylation in H9c2 cells exposed to simulated ischemia-reoxygenation. Reaction with ROS could cause modification of the thiol group. Thus a decrease in reactive oxygen species production from metformin – induced modulation of complex I could be preserve and protect against modification. The preservation of native sulfhydryl groups led to strong Cy3 labelling of sulfhydryls, as seen in non-hypoxic control cells. Cy3 results were normalized to the total protein stain gel by SYPRO® Ruby Protein Gel Stain which is a highly sensitive fluorescent stain. We performed analysis on the individual thirteen KD protein band molecular weight of the ND3 mitochondria complex I subunits, Thiol stained at 13 Kda band in metformin treated samples versus untreated hypoxia-reoxygenation thus it is possible that the exposed ND3 subunit in mitochondria complex I remains unmodified and is

labelled with cy3. In contrast, untreated hypoxia reoxygenation samples there is decreased labeling of this band with cy3 indicating this subunit has undergone sulfhydryl modification.

We studied the type of modification to the protein thiol group could occur in the hypoxia reoxygenation model in response to increased oxidative stress that results from increase reactive oxygen species generation leading to increase thiol modification. Our results are in line with the above cy3 labeling results. We found that metformin protects against S-nitrosylation modification while there was a high general S-nitrosylation modification in samples from untreated hypoxia reoxygenation. Metformin inhibits The D-form of complex I causing decreased reactive oxygen species generation at the onset of reoxygenation, since reactive oxygen species and nitric oxide (·NO) interact together to produce various reactive nitrogen species (RNS) such as peroxynitrite ($ONOO^-$) and nitrogen dioxide (NO_2) leading to increased cell stress (oxidative and nitrosative stress) (218).

Cysteine thiols are modified to produce S-nitrosothiol (SNO) (219). The increase of modified proteins is an essential hallmark associated with oxidative and nitrosative stress in pathological conditions like ischemia (220).

The present study is built upon previous biochemical work that demonstrated the cardioprotective utility of the modulation of the ischemia-damaged ETC during early reperfusion (51,113). The inhibition of ischemia-altered complex I with high dose, acutely administered metformin, in relative preference to undamaged complex I, opens a new potential paradigm of targeted treatment of reperfusion injury. Next, a currently approved agent is used that has the potential to be repurposed for treatment of reperfusion injury. Lastly, the present study opens a novel therapeutic paradigm for the treatment of reperfusion injury that will address both the early phase mitochondrial driven cell death with the potential of continued chronic therapy to activate

canonical AMPK cytoprotective mechanisms of autophagy and decreased apoptosis during the later phases of reperfusion (167,196,197,221). Thus, surprisingly with the same agent, the acute phase of reperfusion and the recovery/remodeling phase of reperfusion injury can be addressed. It is likely that successful clinical intervention against reperfusion injury will require such complimentary therapy that approaches differing mechanisms of cardiac injury throughout the time course of reperfusion.

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List of References

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VITA

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Positions/Employment, Memberships and Honors

2001 – 2004	Technical Trainer: in College of Health &Medical Tech. / 2001-2004.
2004-2013	Lecturer in College of Health &Medical Tech.
2016	Member of the American Heart Association (AHA)
2017	Served as the student representative on an Assistant professor to Associate Professor promotion committee

Peer Reviewed Publications and Presentation

1- Ahmed Mohsin; Estimation of some physical and chemical parameters for water in selected Baghdad areas; Uruk Scientific J. 2011, 4(1):27-31.

2- Mohsin, A., Chen, Q., Quan, N., Maceyka, M., Samidurai, A., Thompson, J., Hu, Y., Li, J., and Lesnefsky, E. J. (2016) Abstract 19919: Cardioprotection During Early Reperfusion via Complex I Inhibition by Metformin. Circulation 134, A19919-A19919.

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