Cardioprotective mechanisms targeting thiol redox homeostasis and mitochondrial bioenergetics

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Coronary heart disease is a leading cause of death in the United States, totaled mostly by deaths associated with myocardial infarction and fatal ventricular arrhythmias. The inability to predict the occurrence of these pathologies due to their sudden and transient etiologies has hindered successful translation of therapies to the clinic. Given the multi-billion-dollar economic burden that cardiovascular disease exerts, it would be beneficial to further our knowledge on ways to better treat acute coronary syndromes. The goal of this work is to determine how mitochondria impact cardiac ischemia/reperfusion (I/R) injury, and to identify potential mechanisms to therapeutically target. The studies within were conducted on treadmill-trained male rats, *ex vivo* heart preparations, isolated/cell cultures, and isolated mitochondria. Herein demonstrates a strong link between susceptibility to I/R injury and cardioprotection through the manipulation of mitochondrial thiol status. Hearts from exercised rats were better protected from ischemic insults, and this coincided with preserved thiol redox homeostasis and greater stability in mitochondrial bioenergetics. The maintenance of mitochondria thiol was demonstrated through preservation of glutathione, which is a key redox control point in cardiac bioenergetics. When the thiol pool becomes more oxidized following oxidative stress, loss of mitochondrial membrane potential and collapsed bioenergetics increase susceptibility to I/R injury. Glutathione reductase helps maintain cell redox homeostasis by maintaining glutathione in a reduced form, where it can be utilized in ROS scavenging and redox signaling. In cell models of hypoxia/reoxygenation, targeting glutathione reductase expression influences the cells

sensitivity to mitochondrial dysfunction. Several hallmark features of the cardioprotective phenotype include reductions in myocardial infarction, resistance to arrhythmic stimuli, lower ROS accumulation, and preserved mitochondrial function. Taken together, data from the studies suggest that targeting mitochondrial function during I/R, and more specifically, targeting mitochondrial thiol homeostasis, may have beneficial effects on treating coronary heart disease symptoms.

Cardioprotective mechanisms targeting thiol redox homeostasis and mitochondrial bioenergetics

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2-AAPA	2-Acetylamino-3-[4-(2-acetylamino-2 carboxyethylsulfanylthiocarbonylamino)phenylthiocarbamoylsulfanyl]propionic acid hydrate
ADP	Adenosine diphosphate
AMPK	Adenosine monophosphate-activated protein kinase
ATP	Adenosine triphosphate
A/R	Anoxia/reoxygenation
AF	Auranofin
BCNU	Bis-chloroethylnitrosourea
BN-PAGE	Blue-native polyacrylamide gel electrophoresis
BSA	Bovine serum albumin
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)-phenylhydrazone
IC_{50}	Concentration for 50% inhibition
CTRL	Control
CHD	Coronary heart disease
CVD	Cardiovascular disease
DMEM	Delbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
ECG	Electrocardiogram
ETS	Electron transfer system
EV	Empty Vector
Ex	Exercise
$FADH_2$	Flavin adenine dinucleotide
GR	Glutathione reductase enzyme

Gsr	Glutathione reductase gene
GSH	Glutathione (reduced form)
GSSG	Glutathione (oxidized from)
5-HD	5-Hydroxydecanoate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H_2O_2	Hydrogen peroxide
JH_2O_2	Hydrogen peroxide emission rate
H/R	Hypoxia/reoxygenation
IMAC	Inner-membrane anion channel
i.p.	intraperitoneal
I/R	Ischemia/reperfusion
LVDP	Left-ventricular developed pressure
MPG	N-2-mercaptoproprionyl glycine
MC540	Merocyanine 540
mRNA	Messenger RNA
min	Minute
MIM	Mitochondria isolation medium
$\Delta\Psi_m$	Mitochondrial membrane potential
mitoK_{ATP}	Mitochondrial potassium adenosine triphosphate
MnSOD	Manganese superoxide dismutase
MAO-A	Monoamine oxidase A
MI	Myocardial infarctio
MOI	Multiplicity of infection
NEM	N-ethylmaleimide
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenenine dinucleotide phosphate

NO	Nitric oxide
NOX	NADPH-oxidase
OPA	O-phthaldehyde
OCR	Oxygen consumption rate
OE	Overexpression
JO_2	Rate of oxygen consumption
PCI	Percutaneous coronary intervention
PTP	Permeability transition pore
KCN	Potassium cyanide
RIP	Remote ischemic preconditioning
RNA	Ribonucleic acid
Sed	Sedentary
shRNA	Short hairpin ribonucleic acid
STEMI	ST-segment elevation myocardial infarction
SOD	Superoxide dismutase
TMRM	Tetramethylrhodamine methyl ester
TNB	Thionitrobenzoic acid
Trx	Thioredoxin
TrxR	Thioredoxin reductase
VO_2 max	Maximal rate of oxygen consumption

Chapter 1: Introduction

Prevalence and economic burden of cardiovascular disease

Cardiovascular disease (CVD) remains one of the leading causes of death in North America, and although rates of death attributable to cardiovascular disease have declined in recent years, the burden of disease remains high (94). It is estimated that 85.6 million American adults have one or more types of cardiovascular disease, with coronary heart disease (CHD) making up more than half of all cardiovascular events in men and women <75 years of age (151). The indirect cost for all CVD is projected to increase from $202.5 billion to $308.2 billion between 2013 and 2030; a 52% increase in costs, and CHD is projected to account for ≈43% of the increase. Furthermore, CHD accounts for ≈50% of deaths that are attributed to CVD (*Figure* 1). Given the high prevalence and economic burden of CHD in the American population, there is a need for improved therapeutic interventional strategies targeting acute coronary syndromes.

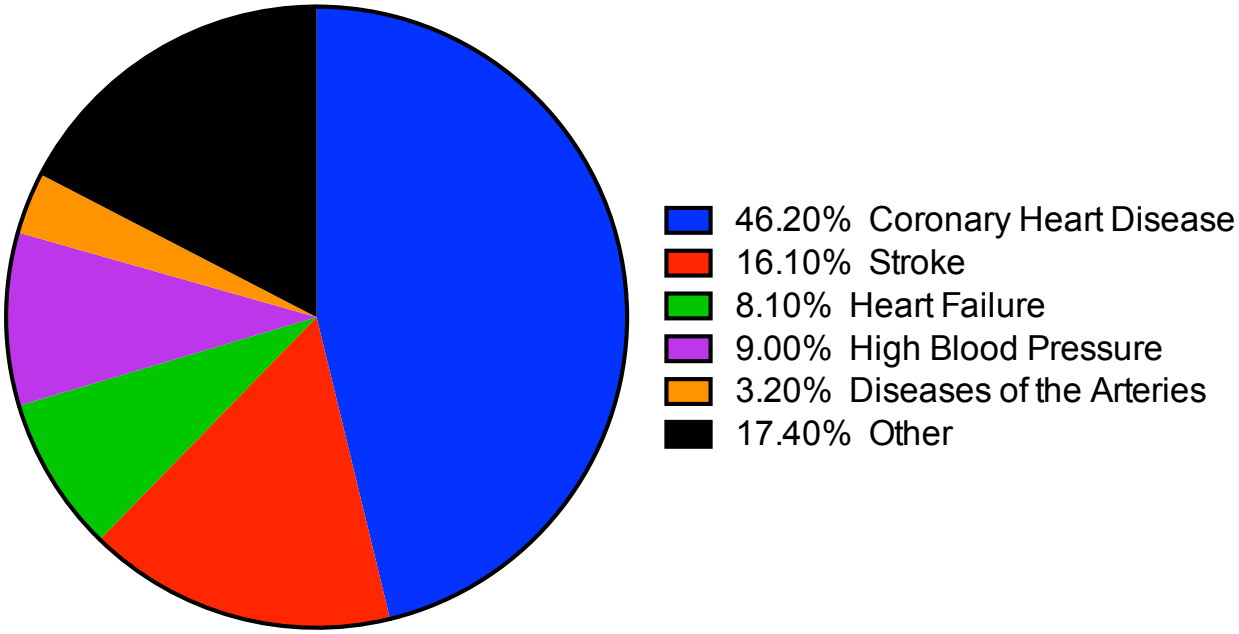

Figure 1. Breakdown of deaths attributable to CVD in the United States (2013).

Acute coronary syndromes and ischemia/reperfusion (I/R) injury increases the risk of myocardial infarction and arrhythmia (152), and will be the main focus of this dissertation.

Ischemia/Reperfusion injury in cardiac pathology

The heart demands a constant supply of energy due to its high metabolic activity and contractile function. Cellular energetic homeostasis is critical for normal cardiac physiology and when the heart becomes ischemic due to temporary or permanent occlusion of coronary arteries the cells energy stores become depleted (e.g. adenosine triphosphate (ATP) levels fall). Upon the onset of ischemia, mitochondrial oxidative phosphorylation decreases with the fall of cellular oxygen tension, leading to an increase in anaerobic metabolism and decrease in cellular pH.

After longer duration ischemia, the depletion of energy stores and accumulation of intracellular protons leads to altered ATP-dependent channel regulation. Sarcolemmal ATP-sensitive potassium (K_{ATP}) channels conduct an inward rectifying potassium current and contain ATP regulatory subunits. When ATP levels fall, sarcolemmal K_{ATP} channel open probability increases, allowing potassium to move down its concentration gradient and leave the cell. The accumulation of potassium in the extracellular space coupled with accumulation of intracellular sodium and calcium leads to altered action potential duration, and reduced cellular excitability. The decrease in ATP and altered ion homeostasis sets the stage for subsequent reperfusion injury.

In acute coronary syndromes the duration of ischemia is the most important variable as an independent predictor of infarct size (99). Therefore, prompt restoration of blood flow to the ischemic tissue is critical. This was emphasized in a recent meta analysis that found higher short-term mortality in ST-segment elevation myocardial infarction (STEMI) patients who presented off hours and had longer door-to-balloon times for percutaneous coronary intervention (PCI) (199).

Paradoxically, subsequent reperfusion of previously ischemic tissue exacerbates cardiac injury and accelerates tissue necrosis (110). Although the etiology of reperfusion injury is multifactorial, the generation of ROS upon the restoration of oxygen has been identified as a central mediator of reperfusion injury (26, 70, 150, 237). Several lines of evidence implicate mitochondria as central hubs for the generation of ROS during reoxygenation, as well as targets that mediate the downstream injury response (5, 225). To this end, targeting mitochondria therapeutically has come to the forefront in cardioprotective paradigms reaching clinical trials, and a schematic of candidate mechanisms is provided in *Figure* 2.

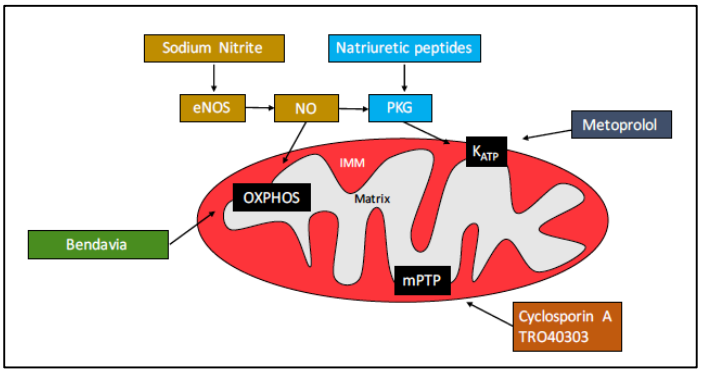

Figure 2. Mitochondrial therapies recently investigated in clinical studies to reduce myocardial infarction.

Mitochondria in health and disease

Mitochondrial bioenergetics is critical in maintaining energy homeostasis in cardiac tissue. Carbon substrates and intermediary metabolism of nutrients consumed in the diet provides reducing equivalents in the form of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucledotide ($FADH_2$). Oxidation of these reducing equivalents by the electron transport system (ETS) is coupled with proton pumping into the inter-membrane space, thereby generating an electro-chemical gradient across the inner-mitochondrial membrane. The electro-chemical gradient is mostly comprised of the mitochondrial membrane potential ($\Delta\Psi_m$), and ATP synthase utilizes the release in free energy to replenish cellular ATP through oxidative phosphorylation under increasing energetic demand.

Tight coupling of cardiac supply-demand matching allows for normal cardiac physiology. However, during metabolic stress, ROS production can induce oscillations in $\Delta\Psi_m$ and collapse

mitochondrial energetics (8, 161). When mitochondrial bioenergetics are compromised, cellular ATP levels fall, which can lead to sarcolemmal K_{ATP} channel opening and altered ion flux across the sarcolemmal membrane. As mentioned previously, sarcolemmal K_{ATP} channel opening leads potassium efflux and heterogeneity in cardiac action potential duration. Altered synchronicity of cardiac electrical activity is an arrhythmic substrate for the genesis of re-entrant ventricular arrhythmias (153, 161). Thus, oxidative stress and altered redox homeostasis is intimately linked with the stabilization of mitochondrial energetics.

Targeting thiol redox stress in cardioprotection

During I/R injury, ATP demand matching by the mitochondria falters, leading to adverse cardiac outcomes. However, cellular ATP hydrolysis is not the only system that operates in an "energetic" fashion. Thiol redox stress during metabolic insults, such as cellular hypoxia/reoxygenation (6), also requires energy as nicotinamide nucleotide transhydrogenase (NNT) utilizes $\Delta\Psi_m$ for reduction of NADPH and oxidation of NADH. Reduced glutathione (GSH), thioredoxin (Trx), NADPH, and NADH provide ROS buffering through enzyme mediated oxidation and reduction reactions (11, 90). These buffering systems are important links that integrate mitochondrial energetics and redox homeostasis (121). In cardiac myocytes a ROS-induced ROS release phenomenon has been described a mediator of mitochondrial permeability transition pore (PTP) opening (235). More recent studies demonstrate cross talk between mitochondrial thiol, ROS production, and mitochondrial membrane potential ($\Delta\Psi_m$) oscillations and depolarization (8, 198). The precise mechanism that links thiol redox stress with a collapse in mitochondrial energetics is not completely clear. However the two mitochondrial channels implicated in oxidative-stress induced mitochondrial dysfunction are the inner membrane anion channel (IMAC) and the mitochondrial permeability transition pore (PTP) (236). Both of which play prominent roles in mediating I/R injury.

Understanding exercise cardioprotection for therapeutic development

Exercise is a well-characterized cardioprotective model that is highlighted by enhanced antioxidant capacity. The cardioprotective mechanisms that trigger exercise-induced cardioprotection are thoroughly reviewed in Chapter 2. The adaptive phenotype ultimately reduces thiol stress during I/R, helping to preserve cardiac electrical synchrony as well as tissue necrosis (80). Redox biology in medicine has yet to reach its full potential as we have only reached the tip of the iceberg in our understanding of redox chemistry in a cellular context. Compartmentalization only increases the complexity of studying this phenomenon. And while the benefits to exercise are clear, the 2014 National Health Interview Survey data indicated only half of American adults met the current aerobic physical activity guideline, and that even this number is likely substantially overestimated according to self-reported physical activity studies (151). Therefore, a better understanding of cardioprotective models implicated in redox biology would be beneficial for the development of future therapies.

Central Hypothesis

The goal of this dissertation is to advance our understanding of cardiac mitochondria in I/R injury. The central hypothesis is that preservation of redox homeostasis during I/R injury minimizes the mitochondrial dysfunction and bioenergetic collapse mediated by ROS accumulation and thiol redox stress. Therapies aimed at preserving thiol redox homeostasis or stimulating enhanced redox control may prove beneficial in reducing acute coronary syndromes. This dissertation covers areas on how thiol redox homeostasis is linked to cardiac disease pathogenesis (Chapter 3), mechanisms involved in regulating redox homeostasis and mitochondrial function (Chapter 4), and how exercise-induced adaptations alter post-ischemic mitochondrial function (Chapter 5).

Chapter 2: Why does exercise "trigger" adaptive protective responses in the heart?

From **Alleman RJ**, Stewart LM, Tsang AM, Brown DA. Why does exercise "trigger" adaptive protective responses in the heart? Dose-Response, 2015; 13(1) (PMID 26674259).

Introduction

The beneficial effects of exercise on the cardiovascular system have been well characterized over the last several decades and it is now accepted that exercise can be used as primary prevention for cardiovascular disease (162). Manifestations of cardiovascular disease are blunted with exercise in experimental animal models, and epidemiological data in humans further support these findings (95, 217). Exercise-induced protection against acute coronary syndromes encompasses a reduction in myocardial infarction (35, 80, 129), arrhythmia (80, 81), and stunning (27, 131, 206, 207). While there is an abundance of literature on proposed mechanisms that seek to explain the protective effects of exercise (129, 202), a large portion of this research focuses on end points of protection as well as the downstream signaling events that protect the myocardium.

During exercise, an increase in cardiac output is warranted so that the heart can meet the demands of exercising muscles. Aside from matching cardiac output with peripheral blood supply, exercise also induces preconditioning whereby the heart is more resistant to injury even long after the exercise has ceased. The proverbial "triggers" that induce cardioprotective signaling are clearly multi-factorial, and include neural, endocrine, and paracrine factors, as well as autocrine signaling and adaptations that arise from within the heart itself.

Exercise can be thought of as eustress; positive stress that a cell responds to in a way that allows it to better cope with that stressor. The adaptive mechanisms associated with

exercise ultimately induce a cardioprotective phenotype, resulting in increased endogenous defenses against longer duration stressors (i.e. ischemia). Proposed triggers of exercise cardioprotection include: adenosine, opioids, adenosine monophosphate-activated protein kinase (AMPK), cytokines, mitochondrial and cytosolic derived reactive oxygen species (ROS), nitric oxide (NO), and adrenergic signaling. This review will focus on studies investigating cardioprotection induced by acute aerobic exercise regimens (i.e. days, weeks, and months of training) at moderate to high intensity. The windows of protection include an early window that occurs within the first hour after exercise, and a late window that typically lasts from 24 to 72 hours (33, 227). Studies that utilize different exercise regimens or include protection outside of these time points will be described in detail. We will start by briefly discussing epidemiological findings in humans pertaining to exercise duration and disease risk prevention, and then shift the focus to the various biological compounds that are responsible for cardioprotection. The main objective herein is to provide a review of the literature addressing the adaptive response to exercise that triggers the phenotypic-cardioprotective switch with different doses of exercise, and to shed light on gaps in the literature that may be hindering our understanding of exercise cardioprotection. The first half of our review will focus on circulating factors released during exercise that converge on the heart, and the latter portion of the review will focus on adaptations that occur within the heart during exercise.

What dose of exercise is needed for cardioprotection?

Although there are benefits of exercise across intensities, both epidemiological and animal studies suggest that moderate to high-intensity exercise is best for the heart. The dose-response aspect relating the quantity of exercise that results in a reduction in cardiovascular risk has been extensively investigated across a number of human epidemiological studies. In a longitudinal study Lee et al. tracked physical activity in 482 males (average 66 years of age) over a five year period and showed that energy expenditure was the key variable in reducing

coronary heart disease risk (128). They found shorter intervals of exercise at a higher intensity provides the same protective benefit as longer intervals of exercise at a lower intensity, as long as the overall energy expenditures were equal. The study also supports the idea that exercise intensity is an important determinant of cardioprotection following an acute exercise regimen (e.g. days to weeks), and that multiple small bouts of intense exercise may have the same net result as one extended bout of exercise. Mora et al. investigated differing levels of physical activity in a group of 27,055 healthy women, determined by kcal/wk expended (149). They showed a dose-dependent relationship with 200-599, 600-1499 and >1500 kcal/wk groups having a 27%, 32% and 41% reduction in cardiovascular disease risk respectively compared to the baseline group which expended less than 200 kcal/wk. Although the authors acknowledged more research was necessary to determine the exact biological mechanisms that resulted in this protection, they found that the reduction in risk seen with increasing levels of physical activity can be explained in large part by a reduction in inflammatory/hemostatic biomarkers. These findings provide evidence for systemic adaptations with chronic physical activity that contributes to reductions in cardiovascular mortality, which are multifactorial with extrinsic and intrinsic cardiac adaptations.

In animal studies, cardioprotection from I/R injury has been shown to occur after only a single bout of exercise and is sustainable if the exercise continues for many months (reviewed in (79, 175)). The majority of our focus herein is on factors released during exercise itself. Long-term chronic exercise is likely a combination of acute factors (reaping the benefits of each individual exercise session) and adaptations that include shifted autonomic nervous system activity, heightened levels of cardioprotective proteins (described below), and beneficial hypertrophy. In terms of acute exercise, cardioprotection (reductions in myocardial infarction) is observed after moderately high-intensity exercise (>70% VO_2 max) (33, 84, 104, 178, 227), consistent with the notion that higher intensity appears to be the most beneficial for the heart. In

the following sections, we will describe the different factors released during exercise that initiate the protective phenotypic shift.

Adenosine

Adenosine is a purine nucleoside molecule that has been identified as a trigger of exercise-induced adaptations within the myocardium. Signaling occurs through four cell-surface receptors distributed heterogeneously throughout regions of the myocardium: adenosine A_1, A_{2A}. A_{2B}, and A_3 receptors (83). Adenosine receptor activation signals through G-protein coupled receptors (G_i, G_s, G_o, and G_q) leading to the targeting of various downstream effectors and divergent regulation of cardiac function (44). During exercise, cardiac adenosine levels rise proportional to increasing heart rate (219). A potential interplay between heart rate and adenosine release in exercise cardioprotection was demonstrated in dogs where the infarct salvage observed following intermittent bouts of tachycardia was abolished with administration of an adenosine receptor blocker (66). Support for the cardioprotective effect of adenosine is also provided in non-exercise, non-I/R studies whereby treatment with adenosine leads to the activation of endogenous antioxidant defense systems, and the adenosine receptor antagonist theophylline abolishes this effect (107, 138). Similarly, adenosine receptor blockade during exercise exacerbates post-exercise oxidative stress biomarkers (107). Taken together, these findings indicate that the increase in heart rate during exercise leads to a transient oxidative stress which is blunted through adenosine-induced upregulation of the antioxidant defense system. However the intermediate signaling of adenosine that may be responsible for triggering exercise cardioprotection is less well defined. Non-exercise studies suggests that A_1 receptor activation reduces infarct size by priming the opening of mitochondrial potassium adenosine triphosphate (mitoK_{ATP}) channels, presumably through a PKC mediated mechanism (187). One study demonstrated that opening of mitoK_{ATP} channels may play a role in the early phase of exercise cardioprotection, as the early window of protection was abolished with channel

blockade during exercise (65). However, exercise-induced mitoK_{ATP} channel activity has not been linked to adenosine signaling and merits further research before conclusions can be drawn. Therefore, these results suggest that transient increases in adenosine levels are important for ROS buffering during acute exercise, but whether or not this is due to opening of mitoK_{ATP} channels is not known. While cardiac adenosine signaling following exercise seems to be important for the activation of redox networks, adenosine has not been established as being solely responsible for the increase in antioxidant capacity. In addition it is also unknown if adenosine receptor blockade during consecutive exercise bouts would mitigate the upregulation in antioxidant defense systems.

One of the limitations in our understanding of adenosine as a trigger for exercise cardioprotection is the lack of knowledge pertaining to the specificity of adenosine receptor activation following exercise. As mentioned previously, there are four different adenosine receptors, and the specific subtypes activated following exercise has not been well characterized. For example, pharmacological blockade of adenosine receptors with theophylline is thought to inhibit signaling through A_1 and A_{2A} receptors (109). Theophylline is commonly used as an adenosine receptor blocker (66, 107), but the specificity of their action and the downstream signaling events has not been tested in exercise-preconditioning studies. The use of non-specific pharmacological compounds is problematic from a mechanistic standpoint because adenosine receptor activation elicits divergent effects depending on the subtype of receptors activated. Further, adenosine receptors possess the ability to dimerize with other subtypes (83), leading to greater complexity in the biological actions of adenosine. Nonetheless, adenosine appears to exert a substantial effect on cardiac physiology and pathophysiology, but more research is needed to solidify adenosine as a required trigger for exercise cardioprotection.

Opioids

Opioids are another cell-surface signaling molecule that can trigger a protective phenotype. Endorphins, enkephalins, and dynorphins predominately signal through μ-, δ-, and κ-opioid receptors respectively, each with various subtypes distributed centrally and peripherally (211). Pharmacological activation of κ- and δ-opioid receptors reduces infarct size, with a 'second window of preconditioning' similar to what is seen with exercise (85, 190). Opioid-mediated signaling occurs throughout the nervous system, and there is evidence that striated muscle can produce preproenkephalin mRNA and peptide products (200, 221).

Several studies have examined opioids following exercise. A ten-fold increase in overall serum opioid activity immediately following exhaustive exercise has been observed in human (179, 191) and rodent models (60), with release of various endorphins being most prominent following high-intensity exercise (>90% VO_2 max). These data are particularly interesting from a dose-response standpoint, as the opioid release occurred following near-maximal exercise, and many studies find benefit after a sub-maximal exercise regimen (32, 36, 41, 79, 171, 175, 208).

Further support for the role of opioids in exercise-induced cardioprotection comes from studies examining blood-borne factors. Michelsen et al. recently observed infarct size reductions in isolated rabbit hearts that were perfused with human plasma dialysates conditioned by acute high-intensity exercise. Co-perfusion with a non-specific opioid antagonist reduced the infarct sparing effect (147), which is consistent with other studies where pre-exercise administration of the non-specific opioid antagonist naloxone/naltrexone abolished protection afforded by a 12-week exercise regimen (63, 86).

Like adenosine mediated protection, there is evidence that opioid signaling also acts through the mitoK_{ATP} channel. In non-exercise studies, protection observed following opioid receptor activation is abolished with the mitoK_{ATP} blocker 5-HD (Fryer et al. 1999).

Administration of 5-HD prior to I/R also abolishes the anti-arrhythmic effect of exercise, but opioid levels were not measured (178). However, unlike adenosine-mediated protection, opioid signaling may not exert its protective effects through the upregulation of antioxidant defense systems. Twenty-four hours after a five-day exercise regimen, mRNA levels of opioid precursors and receptors increased in unstressed hearts while there was no change in superoxide dismutase, HSPs, and catalase (63). Even though these specific antioxidant gene transcripts did not change, enhanced ROS buffering cannot be ruled out because antioxidant capacity was not comprehensively analyzed. Although we are still early in our understanding of how opioids are influencing exercise cardioprotection, these preliminary studies provide rationale for their release and biological activity following exercise.

Cytokines

Cytokine production during exercise is another putative triggering mechanism of exercise cardioprotection that has received less attention from the scientific community. During exercise contracting muscle acts as an endocrine organ by secreting various cytokines that can facilitate downstream biological actions (67). In non-exercise studies, early work demonstrated a cardioprotective role for cytokines in I/R injury that involved lower oxidative stress during the reperfusion period (37, 72). Subsequent studies by Yamashita et al. sought to determine how exercise-induced cytokine production influenced infarct salvage (227). They demonstrated that administration of TNF-α and IL-1 antibodies prior to a single exercise bout abolished the early- and late-windows of cardioprotection. However, aside from the cardioprotective effects that cytokines can exert on the myocardium, there are deleterious effects as well. The discrepant findings in the literature regarding adverse and cardioprotective actions of cytokines on I/R has been reviewed (185). The cardioprotective action of cytokines appears to occur at lower concentrations, whereas higher concentrations may exert harmful effects. Moving forward more

research is needed to uncover the divergent roles of cytokines on myocardial physiology before they can be implemented as therapeutic agents for I/R injury.

Adrenergic Signaling

The role of adrenergic receptor stimulation during exercise has become recognized as a part of exercise-induced cardioprotection. In response to systemic demand, β-adrenergic stimulation increases cardiac chronotropy, inotropy and lusitropy (reviewed in (203)). These effects are mainly attributed to the β_1-adrenergic receptor which is the predominant isoform in the heart, but β_3-adrenergic receptors appear to play a contradictory role, as stimulation leads to a negative inotropic response (156, 205). The negative inotropic effect is mediated through downstream activation of eNOS (89). However, the existence of a functional β_3-adrenergic receptor in the human heart has recently been called into question due to the lack of selectivity of pharmacological tools used to study its function and expression (reviewed in (146)). Nonetheless, it has been postulated that β-adrenergic stimulation may trigger exercise cardioprotection by increasing NO bioavailability. β-adrenergic stimulation of cardiac tissue via the sympathetic nervous system has been shown to be important in triggering the protective phenotype, as ablation of the cardiac sympathetic nerve with topical application of phenol abolishes the infarct salvage afforded by seven days of exercise in mice (4). The authors attributed these effects to a decrease in eNOS activity because an increase in eNOS phosphorylation was not observed in mice with cardiac sympathetic nerve ablation, but was increased with exercise alone. Interestingly, the transient oxidative stress observed with exercise was also absent with cardiac sympathetic nerve ablation, indicating interplay between adrenergic stimulation, NO, and ROS in exercise cardioprotection. In another study Calvert et al. also demonstrated that adrenergic receptors play an important role in exercise cardioprotection via interaction with the NOS isoforms (42). Plasma catecholamine and β_3-adrenergic receptor density increased following four weeks of voluntary wheel running, with no

changes in the β_1 and β_2 isoforms. The cardioprotection against myocardial infarction observed in the voluntary wheel running mice was abolished in β_3-adrenergic receptor deficient mice. Similar to the previous study that linked adrenergic signaling to increased eNOS phosphorylation, eNOS phosphorylation as well as cardiac NO metabolites were depressed in β_3-adrenergic receptor deficient mice exposed to voluntary wheel running. These findings implicate adrenergic signaling as a triggering mechanism during exercise. In this regard, Calvert et al. demonstrated that a single epinephrine bolus increased eNOS phosphorylation and heart NO metabolites. Importantly, infarct salvage following voluntary wheel running was lost when NO metabolites returned to normal levels after four weeks of exercise cessation. Taken together, there is strong evidence for a role of adrenergic signaling in the triggering phase of exercise cardioprotection and the subsequent upregulation of NO bioavailability. However, more research is needed to fully characterize the specific role of β_3-adrenergic receptor stimulation in NOS activation, especially in light of the fact that β_2-adrenergic receptor activation has also been shown to be cardioprotective and can increase eNOS activity and NO metabolites (18).

Nitric Oxide

NO was initially thought to act only through local mediation of vasodilation due to its short half-life and high reactivity with biological substrate (134), however more recent work implicates NO in downstream mechanisms distant from the site of production (41, 49, 73), as well as in cardiac myocytes themselves (reviewed in (23)). During exercise, blood flow and vascular shear stress are elevated in tissue beds with high metabolic activity, which leads to the activation of endothelial nitric oxide synthase (eNOS) and heightened release of NO (17, 193, 217, 231). NO metabolites such as nitrite, nitrate, and nitrosothiols were once thought of as inert, but are now widely accepted as storage forms of NO that undergo inter-conversion to exert biological effects (39, 41, 220, 238). In non-exercise studies, the molecular reduction of nitrite to NO and nitrosothiols during I/R is cardioprotective (38, 49, 220), which indicates that an

increase in NO bioavailability may be an important determinant of exercise-induced cardioprotection.

Following exercise, there is an increase in eNOS activation and NO metabolites (4, 41), and when eNOS is genetically knocked out, the infarct sparing effects after seven days of exercise is abolished (4). The study also demonstrated that the upregulation of eNOS during exercise was necessary for the subsequent increased activity of inducible NOS (iNOS) and the downstream infarct sparing effect of exercise (4). Others have observed an increase in iNOS activity following an acute bout of exercise, and when an iNOS inhibitor was administered prior to I/R the antiarrhythmic effect of exercise was abolished (15). However, the role of iNOS in exercise cardioprotection has been called into question due to the interspecies variability in expression patterns and a lack of increase following various exercise regimens (41, 174). More recently Farah et al. demonstrated a role for eNOS in exercise cardioprotection in rats after five weeks of training (73). Following the exercise regimen phosphorylation of eNOS was increased in the exercise group, as well as s-nitrosylated proteins and nitrite. Perfusion with a global NOS inhibitor prior to and immediately after I/R abolished the infarct sparing and mechanical recovery observed with exercise. They also demonstrated that eNOS uncoupling during the reperfusion period was required for the cardioprotection. However, not all groups demonstrate an essential role for NO in exercise cardioprotection. Taylor et al. administered a global NOS inhibitor prior to two days of exercise with the idea that cardioprotection would be lost. However, the beneficial effects of exercise on mechanical recovery and LDH release after I/R in rats were not different than with exercise alone (207). The main difference in these studies is the timing of NOS inhibition (before exercise vs before I/R), and the duration of the exercise regimen. The study by Taylor et al. provides evidence against a role for NO production during exercise as a triggering mechanism for cardioprotection. However, NO production during exercise may not be responsible for cardioprotection per se, rather the increase in NO bioavailability and increase in

eNOS activation (phosphorylation) seems to be more important in the cardioprotective phenotype. A mechanism whereby NO production can increase after exercise has been demonstrated. Following acute exercise, circulating bradykinin levels increase (21), stimulating the production of NO and NO metabolites (234). Furthermore, bradykinin has been demonstrated to mediate its anti-arrhythmic effects through liberation of NO during I/R (212). Given the discrepant findings, a few questions are left that need to be addressed moving forward. What is the locus of NO production that leads to an increase in NO metabolites (endothelium vs cardiac myocytes), what are the temporal characteristics of NO production during and/or following exercise, and when precisely does the cardioprotective phenotype become evident? In response to the latter, most studies indicate that storage forms of NO precipitate their cardioprotective effects during reperfusion. Clearly more work is needed to definitely determine the role of NO production during/after exercise and how this affects NO metabolite accumulation en route to cardioprotection.

Adenosine Monophosphate-Activated Protein Kinase

Cardiac myocytes are densely packed with mitochondria in order to support cellular energetic requirements. In the healthy heart, the heightened rate of ATP hydrolysis during exercise increases mitochondrial respiration, ultimately allowing healthy myocytes to efficiently match ATP generation to cardiac workload. While cellular ATP:ADP ratios remain constant, AMP levels are thought to rise with increasing exercise intensity, leading to the activation of AMPK in cardiac muscle (55, 82). In this context, the activation of AMPK stimulates catabolic processes and down regulates anabolism allowing the cell to regulate metabolism for the production of ATP (55). AMPK has been deemed as one of the energy sensors of the cell and its activity increases by phosphorylation within 10 minutes of the onset of moderate and high intensity exercise (55). AMPK has also been shown to be important in post-ischemic cardiac injury, with exacerbated injury in transgenic mice expressing a dominant negative kinase dead α

subunit of AMPK (183). Canonical AMPK signaling increases glucose and lipid oxidation, which is essential for replenishing ATP following an ischemic period.

In addition to increasing catabolism, AMPK has been shown to play a role in ischemic preconditioning by regulating sarcolemmal K_{ATP} channel trafficking and activity (204). These studies suggest an important role for AMPK activity following ischemia/reperfusion (I/R), but the extent to which AMPK influences exercise cardioprotection has received less attention. Although studies have consistently shown that exercise increases the phosphorylation of AMPK (55, 163), AMPK has not been shown to be crucial for exercise adaptations. Similar levels of exercise can be attained in transgenic mice expressing a cardiac-specific dominant-negative AMPKα2 subunit (155). Following 30 minutes of exercise, transgenic mice had similar cardiac glycogen and ATP levels as wild-type controls. A similar metabolic profile between the wild type and transgenic mice indicates that AMPK may not be crucial for enhanced cardiac metabolism, and that other overlapping pathways can help meet energy requirements during increased demand. Although AMPK is an attractive target for the cell to regulate its energy needs during metabolic stress, there is a gap in the literature linking exercise-induced AMPK activation with cardioprotection. More research is required to definitively determine if/how exercise influences AMPK activity in the heart, and whether or not these changes modify cardioprotection.

Reactive Oxygen Species

Cardiac ROS are another potential candidate involved in exercise cardioprotection, as well as other preconditioning stimuli such as ischemic and pharmacological preconditioning (88). A large body of literature suggests that exercise induces a transient oxidative stress that leads to upregulation in antioxidant defense systems; however the locus of ROS production and downstream effectors during exercise remains unclear. In this section we will focus on evidence for the role of mitochondrial ROS in exercise cardioprotection, and cytosolic ROS in the following section.

ROS have received considerable attention in the cardiac literature due to their role in pathologies like I/R injury, heart failure, and cardiomyopathies. However, a growing body of literature suggests that ROS exert hormesis, where transient bursts of ROS leads to favorable adaptive redox signaling. Cellular ROS can act as second messengers in downstream signaling by altering the activity of redox sensitive enzymes throughout the cytosol and/or mitochondria of cardiac myocytes (184). Similar mechanisms may occur when transient bursts of ROS are generated during exercise. (24, 59, 97). Following acute exercise there is an alteration in cellular redox status towards a more oxidized environment which may act as a signal to activate endogenous protective mechanisms (80, 157).

There is general consensus that an increase in antioxidant enzymes is responsible for a large portion of exercise cardioprotection, and transient oxidative stress with exercise may play a role in this adaptation. Evidence for this has been provided by several groups who have observed increases in key antioxidant enzymes following exercise (61, 80, 81, 96, 129, 178, 227). Studies in favor of this hypothesis have shown that administration of antioxidants prior to exercise abolishes infarct salvage (4, 227) and prevents exercise-induced improvements in cardiac performance (157). However, another study indicated that ROS generated during exercise were not required for functional recovery following I/R (208). The antioxidant frequently used in these studies was N-(2-mercaptopropionyl)glycine) (MPG), which was administered intraperitoneally 10-30 minutes prior to exercise. An important note to consider is that MPG has been shown to have higher specificity for hydroxyl radicals rather than hydrogen peroxide (H_2O_2) and superoxide (25), indicating that not all ROS signaling is abolished with treatment. In addition, MPG has a plasma half-life of approximately 7 minutes (103), making it difficult to interpret how effective the treatment was at scavenging ROS during hour-long exercise bouts. These methodological differences make it difficult to directly compare their results (i.e.

differences in species, duration of I/R, duration of exercise, measurement of injury, the timing of the administration of antioxidants, and in vivo versus ex vivo experiments).

Mitochondrial ROS. ROS signaling is a highly regulated and localized process, implying that the origin of ROS generated during exercise may be extremely important. Although mitochondrial ROS are thought to play a central role in ischemic preconditioning (16, 124, 166), there is a paucity of evidence suggesting a role in exercise preconditioning. Frasier et al. recently found that the locus of ROS production during exercise is not mitochondrial in origin (80). As shown in Figure 3, exercise cardioprotection was not lost when administering agents that reduce mitochondrial ROS prior to exercise (mito TEMPO and Bendavia). This indicates that extramitochondrial-derived ROS may be responsible for redox signaling following exercise.

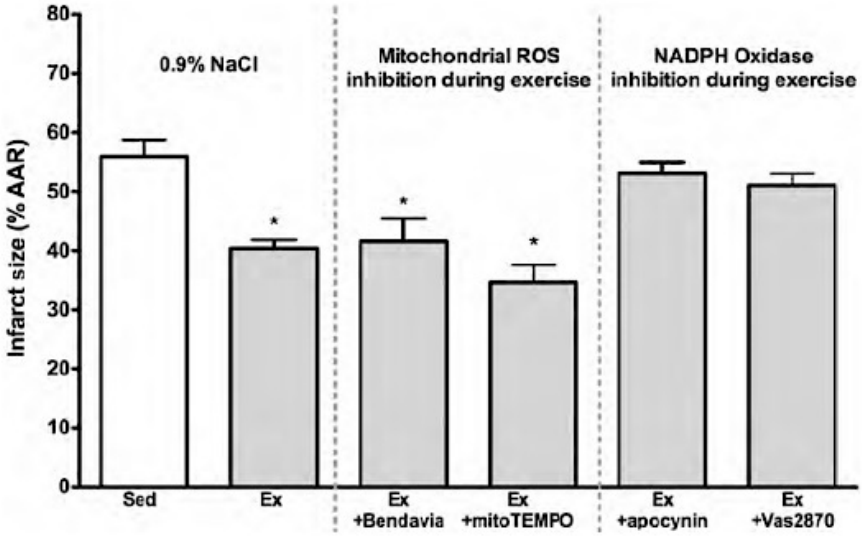

Figure 3. Reductions in infarct size are abolished by inhibiting NADPH Oxidase (with pre-exericse treatment of apocynin or VAS2870) during exercise. Inhibition of mitochondrial ROS during exercise (with pre-exercise administration of TEMPO or the mitochondria-targeting peptide Bendavia) had no effects on exercise cardioprotection. Figure reproduced from Frasier et al., Cardiovascular Research 2013, with permission (pending).

Monoamine oxidase-A (MAO-A) is another potential site for mitochondrial ROS production. MAO-A is located on the outer mitochondrial membrane and catalyzes the oxidative deamination of neurotransmitters such as norepinephrine and serotonin while generating H_2O_2

as a byproduct in the reaction. A recent review highlights the importance of MAO-A in pathological states such as heart failure and I/R (116). Accumulation of serotonin released by platelets during I/R can lead to the production of H_2O_2 and subsequent apoptotic signaling cascades (19). Recent findings indicate that exercise leads to down-regulation MAO-A (119), which may play a role in the attenuation of I/R damage associated with exercise cardioprotection. Moreover, these findings indicate that there is likely a *reduction* in mitochondrial ROS production with exercise, given the decrease in MAO-A expression and increases in the activity of key antioxidants such as MnSOD and glutathione reductase (GR) (80, 178). A decrease in cardiac mitochondrial MAO-A would theoretically dampen the oxidative burden imposed on the cell, not only during exercise, but also during thrombus formation and subsequent I/R injury. A mechanism for the decrease in MAO-A expression following exercise has not been investigated and therefore the triggering event for this adaptation is purely speculative. Perhaps acute increases in cardiac sympathetic nerve stimulation and increasing norepinephrine levels during exercise play a role in downstream silencing of MAO-A through non-canonical adrenergic pathways (215). Cardiac sympathetic stimulation increases contractility and myocardial stretch during exercise, which in and of itself may trigger a cardioprotective phenotype through elevated cytosolic ROS production (80, 218). Furthermore, cardiac sympathetic nerve ablation has been shown to abolish the infarct sparing effect of exercise, but this was not linked to silencing of MAO-A expression (4). A hypothetical adrenergic/MAO-A axis scenario opens up an exciting area of research to explore mechanisms controlling MAO-A expression in cardiac tissue during normal physiological as well as pathophysiological states. We will further expand on the topic of stretch-induced activation of cardioprotection in the next section.

Cytosolic ROS. Free-radical generating enzyme systems outside of the mitochondria have also received considerable interest in normal physiology as well as in pathological states such as I/R

injury (24, 144, 148) and heart failure (98, 210). Sources of extramitochondrial-derived ROS in cardiac myocytes include xanthine oxidase, NADPH oxidase, and uncoupled nitric oxide synthase. Of these, the NADPH oxidase (NOX2 in particular) complex generates ROS in a highly localized manner in the sarcolemmal and t-tubule membranes during physiological stretch (172, 186). Myocardial contraction and wall stress increases during exercise as a function of heart rate and adrenergic signaling. The increased inotropic and chronotropic state is an autoregulatory mechanism that allows for tight regulation of blood pressure and delivery of nutrients to metabolically active tissue. Recent work indicates that the sarcolemmal NOX2-generated ROS system plays a central role in this phenomenon. NOX2-generated ROS imposes redox signaling through ryanodine receptors leading to increased calcium release and subsequent contractile activity (68, 186). Stretch induction through the microtubule network and NOX2 activation has been termed X-ROS signaling (172). X-ROS signaling describes the transfer of a mechanical to a chemical signal throughout the heart via the microtubule system, leading to assembly of the NOX2 ROS generating complex. Recently, several independent groups have established a role for NOX2 as a potential trigger for the cardioprotective phenotype associated with exercise (80, 186).

As mentioned previously, a critical threshold of exercise intensity appears to be important for cardioprotection, and at higher exercise intensities myocardial contraction increases in conjunction. In line with the X-ROS signaling hypothesis, increased inotropy and myocardial stretch during exercise may lead to activation of NOX2 and perhaps downstream adaptations. We and others have demonstrated that inhibition of NOX2 prior to exercise abolishes the infarct salvage of early and late phases of exercise cardioprotection (80, 186). Furthermore, the upregulation of GR activity that is typically observed following exercise (81, 115, 180, 213) is also abolished immediately and 24 hours after the exercise bout when NOX2 is inhibited during exercise (80). GR is a central enzyme involved in cellular redox control by

utilizing NADPH to convert oxidized glutathione to the reduced form. Therefore, increasing GR activity allows the cell to maintain the glutathione pool in the reduced state, thus providing a greater buffering power during oxidative insults. During an exercise bout, mechanical stretch of the myocardium increases, leading to NOX2-generated ROS and activation of GR (80). ROS signaling through GR may be a mechanism where GR acts as a sensor during oxidative shifts of the redox environment, leading to upregulation of endogenous defense systems. Future studies examining the time frame of GR activation and sustainability of protection will shed light on signaling between NOX2 and GR during the cardioprotective window of exercise. Also, studies that determine the importance of GR compartmentalization, namely whether cytosolic and/or mitochondrial GR pools are involved in this adaptive signaling network (118). While it seems apparent that exercise upregulates redox buffering capacity, more research is needed to definitively determine if transient bursts of ROS during exercise act as a signal to trigger downstream cardioprotection.

Conclusions

We have described a number of circulating and intrinsic factors postulated to induce cardioprotective signaling with exercise. These factors converge on the myocardium, and result in downstream adaptations that characterize the protective phenotype. Subsequent investigation into these downstream effects using novel approaches will greatly advance the field. For example, ROS production during exercise is an intriguing factor that leads to both post-translational modifications to existing proteins in the short-term, as well as altered protein expression on a longer time-scale. Given that 21,000 to 42,000 thiols in the proteome can contribute to the integration of metabolic function through redox signaling (112), further exploration of the redox hypothesis in the context of exercise adaptations is warranted. The convergent effects of cellular ROS production and elevated levels of cell-signaling molecules such as adenosine, NO, cytokines, and catecholamines during elevated workloads transduce

the exercise stimulus that culminates into a hormetic cardiac response. Inhibition of any one of these putative triggers can dampen the cardioprotective phenotypic switch observed with exercise, but ultimately, these adaptations lead to tolerance to I/R injury characterized by lower arrhythmia and decreased myocardial infarction. Given that exercise is known to confer protection in humans, future studies that continue to advance our understanding of the intrinsic factors responsible for evoking this protective phenotype may ultimately pave the way for novel therapies to reduce the burden of acute coronary syndromes.

Chapter 3: Exercise-induced protection against reperfusion arrhythmia involves stabilization of mitochondrial energetics

From Alleman RJ, Tsang AM, Ryan TE, Patteson DJ, McClung JM, Spangenberg EE, Shaikh SR, Neufer PD, Brown DA. Exercise-induced protection against reperfusion arrhythmia involves stabilization of mitochondrial energetics, 2016. (PMID 26945082).

Introduction

Cardiovascular disease remains a leading cause of death in the industrialized world (94, 158). One manifestation of cardiovascular disease is sudden cardiac death, which has been estimated to account for approximately 1 death per 1000 in the general population (78). Several factors are known to influence the susceptibility to arrhythmia, including various genetic abnormalities, channelopathies, compromised autonomic function, left ventricular hypertrophy, and acute coronary syndromes (78, 159, 216). During acute coronary syndromes, the reperfusion of previously ischemic tissue leads to a burst in reactive oxygen species (ROS), a significant contributor to electromechanical dysfunction (3, 26, 141, 237).

Exercise is known to protect against arrhythmia (81, 105, 178), as well as other post-ischemic damage such as myocardial stunning (27, 132, 207) and infarction (32, 80, 176, 177). Despite the clear beneficial effect, the underlying cellular mechanisms are not completely understood. The high oxidative environment during reperfusion collapses mitochondrial energetics and alters cardiac action potential duration, which is known to be arrhythmogenic (3, 8, 9, 31). Among their many functions, mitochondria are centrally involved in both ATP production and free radical detoxification through redox reactions, both of which ultimately rely on mitochondrial membrane potential ($\Delta\Psi_m$). Collapses in $\Delta\Psi_m$ are known to be associated with

the onset of arrhythmia, and pharmacological interventions that preserve $\Delta\Psi_m$ have been shown to stabilize sinus rhythm (31, 198). Whether the preservation of $\Delta\Psi_m$ is an endogenous adaptation involved in exercise-induced protection has never been determined.

We recently observed that exercise delayed the onset of arrhythmia and decreased the incidence of ventricular fibrillation (VF) through better preservation of redox homeostasis (81). This was attributed to enhanced glutathione reductase (GR) activity, which was essential for cardioprotection (80). While exercise-induced cardioprotection has been repeatedly shown to augment endogenous myocardial antioxidant capacity (80, 81, 129, 178, 227), there is a lack of evidence demonstrating how these adaptations directly protect against reperfusion arrhythmia. Therefore, the objective of the present study was to determine if exercise decreases reperfusion arrhythmia by preserving mitochondrial bioenergetics. Using several different experimental models, we employed a vertically integrated approach to test the hypothesis that exercise protects against reperfusion arrhythmia via better maintenance of $\Delta\Psi_m$, lower mitochondrial ROS production, and preserved redox homeostasis.

Methods

Animals

Male Sprague Dawley rats (250-350 g) were housed on a 12-hour light/dark cycle with food and water provided ad libitum. All experiments were conducted in accordance with guidelines established by the NIH (2012 edition) and the AVMA (2013 Edition:https://www.avma.org/KB/Policies/Documents/euthanasia.pdf), and approved by East Carolina University's Animal Care and Use Committee. For all experiments rats were anesthetized using a ketamine/xylazine mixture (90mg/kg ketamine, 10mg/kg xylazine, i.p.), and hearts were excised via midline thoracotomy after animals reached a surgical plane of anesthesia. Hearts were placed briefly in 0.9% saline (4ºC) and used for isolated heart studies, myocyte isolations, or mitochondrial experiments.

Exercise protocol

Rats were randomly assigned to exercise (Ex) or sedentary (Sed) groups and exposed to daily exercise or control handling using established protocols (81). Briefly, rats were acclimated to the treadmill at 15 m/min over a 3-day period, increasing the time of exercise from 5, 10, and 15 min each day. Ex rats underwent 10 days of consecutive treadmill running at 6% grade for 60 min per day, in intervals broken up to 15 m/min for 15 min, 30 m/min for 30 min, and 15 m/min for 15 min. Sed rats were placed on the non-moving treadmill for 5 min each day. This exercise protocol mimics a moderate- to high-intensity exercise regimen, characterized by training adaptations with little/no indication of systemic stress (34). All experiments were performed 24 hours after the last bout of exercise or handling.

Isolated heart preparation and assessment of arrhythmia

Excised hearts were rapidly cannulated by the aorta per our established methods (80, 81) and retrograde-perfused on a modified Langendorff apparatus with gassed (95%O_2,

5%CO_2) Krebs Henseleit buffer (KHB) containing (mM): 118 NaCl, 24 $NaHCO_3$, 4.8 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, and 10 glucose (37ºC), at a constant pressure of 75 mmHg. Coronary flow was monitored throughout the protocol with a Transonic flow probe connected in series proximal to the cannula. All measurements were recorded on Lab Chart 7.0 software (A.D. Instruments) and stored on a personal computer for subsequent analysis. The definition of ventricular arrhythmia was used in accordance with the methods described by the Lambeth Convention (58).

Two-photon microscopy whole heart imaging during ischemia/reperfusion

Using slight modifications of our previous techniques (31), instrumented hearts (n=18) were imaged using two-photon microscopy (Olympus FV 1000 multiphoton microscope; Spectra-Physics Maitai Deepsee laser) with a 30X silicon objective lens (UPLSAPO, NA 1.05). Hearts were mounted in a 100mm glass-bottom dish (MatTek) maintained at 37ºC for imaging, with ECG obtained via volume-conductance recordings using electrodes placed in the bath. Hearts were enclosed by an on-board incubator maintained at 37ºC and imaged at a depth of 800nm. 640x640 pixel resolution images were obtained each minute at 2µs/pixel with low laser power (6.5%) for the duration of the protocol. The left ventricle was imaged within 2mm of the left anterior descending coronary artery on the MatTek dish and stabilized by applying a glass coverslip over the heart to minimize artifacts induced from vibrations.

Isolated hearts were loaded with 100nM tetramethylrhodamine methyl ester (TMRM; Molecular Probes, Inc.) for 15 min to measure $\Delta\Psi_m$ per our established methods (31). Our preliminary experiments showed this concentration of TMRM to be optimal in order to observe collapses in $\Delta\Psi_m$ with the mitochondrial uncoupler carbonyl cyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP). TMRM was excited at 800nm and emission collected at 495-540nm using a 2-channel filter cube (FV10-MRG/R). After TMRM loading hearts were perfused with KHB+blebbistatin (10µM) to inhibit contraction. Once the image stabilized, a baseline image

was captured and followed immediately by global, no-flow ischemia/reperfusion (40 min/10 min). To control for unequal fluorophore loading, TMRM fluorescence was normalized to baseline (F_0; prior to ischemia). All images were analyzed using ImageJ, and mean TMRM fluorescence was calculated after thresholding to exclude background for areas not containing sheets of myocardial cells. Four hearts were excluded from the imaging analysis due to technical difficulties during image acquisition (Sed n=2 and Ex n=2).

Glutathione levels in cardiac tissue following Langendorff ischemia/reperfusion

Reduced glutathione (GSH) and glutathione disulfide (GSSG) were measured using high performance liquid chromatography (HPLC) (74, 93, 117). Left ventricular cardiac tissue was snap frozen in liquid nitrogen after 20 min ischemia and 2 hours of reperfusion in a subset of rats. Left ventricular tissue was homogenized in a buffer containing 50mM Trizma base supplemented with 20mM boric acid, 20mM L-serine and 10mM N-ethylmaleimide (NEM). NEM is an alkylating agent that will both conjugate GSH and inhibit GR, which is added to the homogenization buffer to limit auto-oxidation effects during sample preparation. The tissue homogenate is then split into two derivatization pathways for the detection of GSH and GSSG. For GSH derivatization, 280µl of the homogenate was deproteinated with 1:10 (v/v) 15% trichloroacetic acid, and then centrifuged for 5 minutes at 20,000xg. The supernatant was transferred to an autosampler vial for processing in the HPLC equipment. GSH samples were run on freshly made mobile phase containing 91% of a 0.25% (v/v) glacial acetic acid mixed with 9% pure HPLC grade acetonitrile. Samples were run using a Shimadzu Prominence HPLC system equipped with a Premier C18 column (4.6 x 150mm. 5µm, Shimadzu Part # 220-91199-12) at flow rate of 1.0ml/min. GSH-NEM conjugate was detected by UV chromatography at a wavelength of 265nM (Shimadzu SPD-20A) (93). Samples were quantified using standards prepared under identical conditions and normalized to the protein content measured in the muscle homogenate by BCA assay.

For GSSG derivatization, 200µl of the homogenate was deproteinized in 200ul 15% perchloric acid, and then centrifuged for 5 minutes at 20,000xg. The resulting supernatant (200µl) was next diluted in 1000µl of 0.1M NaOH twice to ensure proper pH (~12) is reached before reacting with 0.1% 0-phthaladehyde (OPA). OPA will react with GSSG at high pH (~12) to form a fluorescent product detectable at excitation/emission wavelengths 350/420 (Shimadzu RF-20A xs) (117). GSSG samples were processed using a 25mM sodium phosphate buffer containing 15% HPLC grade methanol at pH of 6. Samples were run through a Shimadzu Prominence HPLC system equipped with a Purospher STAR RP-18 endcapped column (4.6 x 150mm. 3µm, EMDmillipore) at flow rate of 0.5ml/min. Samples were quantified using standards prepared under identical conditions and normalized to the protein content measured in the muscle homogenate by BCA assay.

Cardiac myocyte cell isolation

Cardiac ventricular myocytes were isolated using previously published methods with slight modifications (30). Hearts were digested enzymatically on a modified Langendorff apparatus using 1mg/ml collagenase (Type 2 Worthington) and 0.15 mg/ml protease (type XIV Sigma) dissolved in Tyrodes solution. After 8-12 min of digestion, hearts were cut down, minced in Tyrodes solution, and passed through a nylon mesh filter. Cells were allowed to gravity precipitate and resuspended in Tyrodes with increasing titrations of calcium up to a final concentration of 1.8 mM. Isolated myocytes were incubated (95% O_2, 37°C) in DMEM and used for experiments within 8 hours of dispersion.

Cardiac myocyte imaging during hypoxia/reoxygenation

Myocytes were loaded on a perfusion chamber housed on the confocal microscope stage and enclosed in glass to minimize oxygen diffusion from room air. The chamber was connected to an in-line solution heater that delivers the superfusate via laboratory tubing with low oxygen permeability (Tygon F-4040-A), and equipped with heating filaments for

maintenance at 37°C. Pacing electrodes were utilized for field stimulation for the duration of the hypoxia/reoxygenation protocol (4-ms duration, 1-Hz frequency, 10-V amplitude). Myocytes were perfused with Tyrodes solution gassed with 100% O_2 containing (in mM): 140 NaCl, 10 HEPES, 5 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, and 10 glucose (pH 7.4, 37°C). For hypoxic Tyrodes solution, glucose was excluded, the solution was gassed with 100% argon continuously, and the pH was decreased to 6.5 in an attempt to mimic the *in vivo* cellular environment during ischemia.

Myocytes were incubated for 15 min with TMRM (10nM) and CellTracker Blue CMAC (1μM) (Molecular Probes, Inc.) for fluorescent imaging of $\Delta\Psi_m$ and cellular GSH, respectively. CellTracker Blue CMAC is a GSH sensitive dye that has been shown to have better cell retention than monochlorobimane in primary cardiac myocytes (122). Myocytes were incubated on a glass coverslip coated with poly-d-lysine and allowed 15 min to adhere, followed by 5 min of baseline perfusion. Only rod-shaped myocytes that responded to field stimulation were utilized in the experiments. Preliminary control experiments indicated that a low concentration of TMRM (5nM) in the Tyrodes solutions was required to maintain a stable fluorescent signal for the duration of the protocol. After 5 min of baseline perfusion, the solution was switched to the hypoxic Tyrodes solution. After 20 min of hypoxia the superperfusate was switched back to normoxic Tyrodes for reoxygenation (30 min or until cell death). At the end of each experimental protocol myocytes were perfused with the mitochondrial uncoupler FCCP (1μM) to verify mitochondrial TMRM specificity. A 60X water immersion objective lens was used to image myocytes every minute using 408nm and 559nm argon lasers, and emissions were collected using a 430-470 and 575-675 band pass filter, respectively. Images were analyzed with NIH ImageJ (http://imagej.nih.gov) in 8-bit following background subtraction (rolling ball radius 50) with regions of interest drawn around individual cells. NIH “Fire” and “Blue” look-up tables (LUTs) were used for all $\Delta\Psi_m$ and GSH images respectively.

Mitochondria isolation

Cardiac mitochondria were isolated from Ex or Sed hearts twenty-four hours following the last exercise bout (or handling) using similar previously published methods (197). Briefly, hearts were excised and minced on ice and trypsin-digested in mitochondria isolation medium (MIM) containing (in mM): 300 sucrose, 10 sodium-HEPES, and 1 EGTA. After 2 min of digestion, 10 ml of MIM with BSA (1mg/ml), and trypsin inhibitor (100 mg/ml) was added and allowed to gravity pellet for 8 min. The digested tissue was then homogenized and centrifuged at 800xg for 10 min. Supernatant was collected and centrifuged at 12,000xg for 10 min to pellet mitochondria. The pellet was rinsed to remove debris and impurities, suspended in fresh MIM and centrifuged at 12,000xg for 10 min. The final pellet was re-suspended in MIM and kept on ice for experiments.

Mitochondrial O_2 consumption and H_2O_2 emission measurements

Rates of O_2 consumption (JO_2) and H_2O_2 emission (JH_2O_2) were measured simultaneously using the Oroboros high-resolution respirometry oxygraph-2k equipped with a custom-made stopper to accommodate a fiber-optic cable for fluorescence measurements (Fluoromax 3, HORIBA Jobin Yvon, Edison, NJ, USA). Mitochondria were energized using complex I and complex II substrates: glutamate (10mM), malate (2mM), pyruvate (2mM), and succinate (5mM), and assayed at 37°C in 2.5 ml of Buffer Z assay medium containing (mM): 110 K-Mes, 35 KCl, 1 EGTA, 5 K_2HPO_2 , 3 $MgCl_2$-$6H_2O$, 0.5 mg/ml BSA, and 25 creatine monohydrate. The rate of H_2O_2 emission was quantified using Amplex UltraRed (25μM) and horseradish peroxidase (4U/mL), added to the assay buffer. Exogenous superoxide dismutase (SOD, 30U/mL) was added in order to convert all generated superoxide to H_2O_2. The hexokinase/2-deoxyglucose (2U/mL/5mM) "ADP clamp" was used to mimic *in vivo* conditions. These conditions keep mitochondria in a submaximal phosphorylating state at a fixed $\Delta\Psi_m$ by recycling ATP back to ADP (75μM) (229). Anoxia was "self-induced" for 25 min by allowing

mitochondria to consume all of the O_2 in the chamber. Following anoxia, mitochondria were reoxygenated by injecting pure O_2 into an air bubble above the solution in the chamber. The chamber was then sealed allowing for JO_2 and JH_2O_2 to be measured during the reoxygenation phase.

The contribution of thioredoxin reductase (TrxR) or GR to mitochondrial ROS production was ascertained in parallel experiments. Mitochondria were energized with succinate (10mM) and treated with either 1μM auranofin (AF) or 100μM bis-chloroethylnitrosourea (BCNU) to inhibit the thioredoxin and glutathione redox buffering systems, respectively. Endogenous mitochondrial ROS production was monitored as described above.

Statistics

Data are presented as mean ± standard error. Arrhythmia analysis was performed using a chi-square test. Mean fluorescence during reperfusion and respiratory control ratios were analyzed using unpaired Student's t-test. Imaging data were analyzed with an ANOVA for reperfusion or reoxygenation using the least significant difference test for matched time comparisons between Ex and Sed. All JO_2 and H_2O_2 data were analyzed using a two-way ANOVA with Tukey's post-hoc test. Statistical significance was established when $P<0.05$. All data were analyzed and graphed using GraphPad Prism software.

Results

Exercise decreases arrhythmia and preserves $\Delta\Psi_m$ during ischemia/reperfusion

Hearts from Ex rats experienced a significantly decreased incidence of ventricular arrhythmia, with 33% of Ex hearts vs 88% of Sed hearts transitioning to ventricular tachycardia (VT) and/or fibrillation (VF) during early reperfusion ($P<0.05$, n=8 per group, *Figure* 4A). In our two-photon studies, non-ischemic control hearts showed a stable TMRM fluorescent signal 30 minutes following TMRM loading, indicating that TMRM washout was not a major contributor to declines in the TMRM signal (data not shown). There was no difference in TMRM signal during ischemia between groups (*Figure* 4B). However, Ex hearts better maintained $\Delta\Psi_m$ than Sed

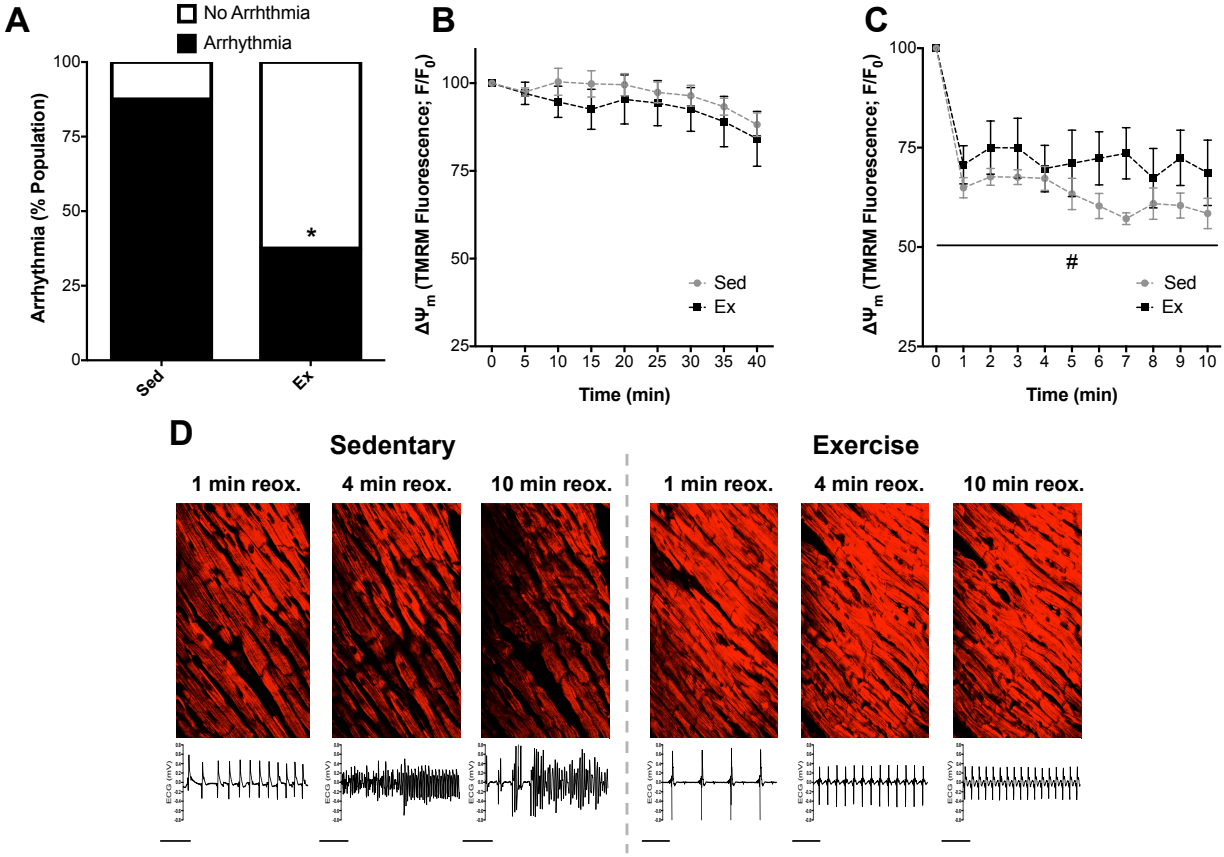

Figure 4. Arrhythmia and simultaneous two-photon imaging of mitochondrial membrane potential (ΔΨm) in isolated hearts during ischemia/reperfusion. (A) The percentage of Ex and Sed hearts that transitioned to arrhythmia (VT/VF) following 40 min of ischemia. (B&C) Baseline TMRM fluorescence ($\Delta\Psi_m$) values were used to normalize all data (F/F_0) during ischemia (B) and reperfusion (C). (D) Representative images of $\Delta\Psi_m$ in the ventricular free-wall and simultaneous ECG recordings during reperfusion for Sed and Ex. Data are shown as % of population for arrhythmia and mean ± SEM for all other data, n=7-8 per group. * $p<0.05$ vs Sed; # $p<0.05$ vs Sed main effect.

hearts over the course of reperfusion, which coincided with a decrease in arrhythmia ($P<0.05$, *Figure 4*). The transition to arrhythmia in Sed hearts was often accompanied by loss of $\Delta\Psi_m$, which was better preserved in Ex hearts that did not transition to arrhythmia during the reperfusion period (*Figure* 4D).

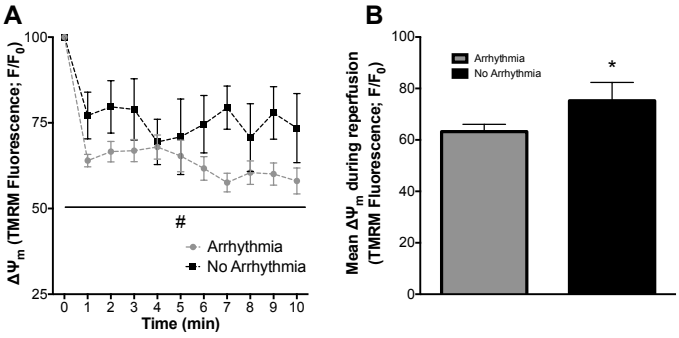

Figure 5. Mitochondrial membrane potential ($\Delta\Psi_m$) in isolated hearts that transitioned to arrhythmia vs no arrhythmia during reperfusion. (A) $\Delta\Psi_m$ was was better maintained in hearts that did not transition to arrhythmia. (B) Mean $\Delta\Psi_m$ fluorescence values during reperfusion. Data are mean ± SEM * $p<0.05$ vs Arrhythmia; # $p<0.05$ vs Arrhythmia main effect.

Underscoring the importance of maintaining $\Delta\Psi_m$ during reperfusion, pooled data for all hearts (regardless of Sed vs Ex group) corroborated the association between $\Delta\Psi_m$ loss and electrical dysfunction, with maintenance of $\Delta\Psi_m$ associated with protection against arrhythmia (*Figure 5*).

Glutathione and $\Delta\Psi_m$ dynamics

In cardiac myocytes exposed to *in vitro* hypoxia/reoxygenation (H/R) (20 min/30 min), myocytes from Ex hearts maintained higher levels of GSH during reoxygenation and showed an enhanced ability to replenish GSH levels compared to Sed (*Figure* 6A and B). In a more quantitative approach, GSH was

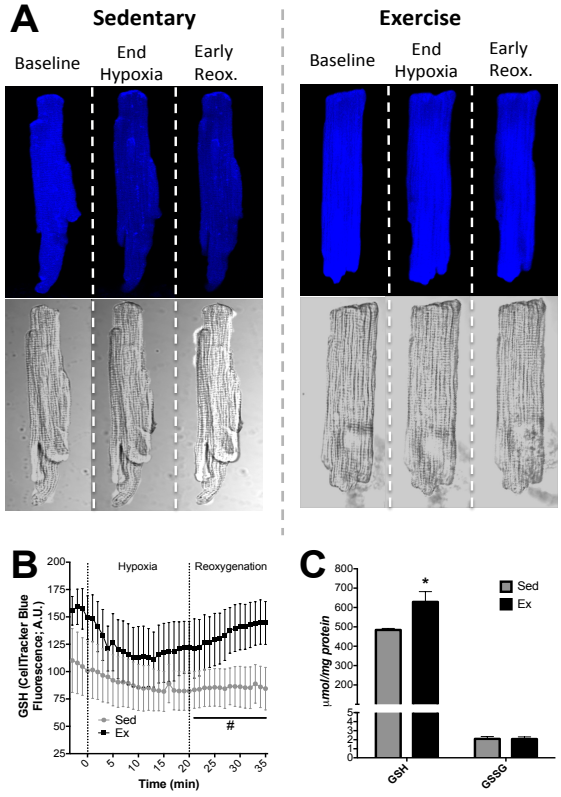

Figure 6. Cardiac glutathione (GSH) during cellular hypoxia/reoxygenation or cardiac ischemia/reperfusion. (A) Representative primary cardiac myocyte fluorescent images for Sed and Ex during baseline, at the end of hypoxia, and 6 minutes into reoxygenation. (B) Quantification of glutathione levels as measured by CellTracker Blue fluorescence. (C) HPLC quantification of reduced (GSH) and oxidized (GSSG) glutathione in hearts following ischemia/reperfusion. Data are shown as mean ± SEM, * $p<0.05$ vs Sed; # $p<0.05$. vs Sed main effect.

measured in whole hears exposed to ischemia/reperfusion injury (*Figure 6C)*. GSH was significantly higher in Ex hearts (*Figure* 6C), further demonstrating adaptive maintenance of redox control following a hypoxic or ischemic insult. The attenuated GSH replenishment in Sed cardiac myocytes coincided with collapse of $\Delta\Psi_m$ during reoxygenation, while the enhanced ability of Ex cardiac myocytes to replenish GSH translated into $\Delta\Psi_m$ stability during reoxygenation (*Figure* 6 and 7). There was a slight decrease in $\Delta\Psi_m$ during hypoxia as shown in Figure 7B, particularly during late hypoxia, but $\Delta\Psi_m$ depolarization was more evident during reoxygenation, which is consistent with our observations in whole heart experiments. The time-lapse images of $\Delta\Psi_m$ in paced myocytes exposed to H/R were consistent with the whole heart two-photon data demonstrating more energetically stable mitochondrial networks in Ex hearts.

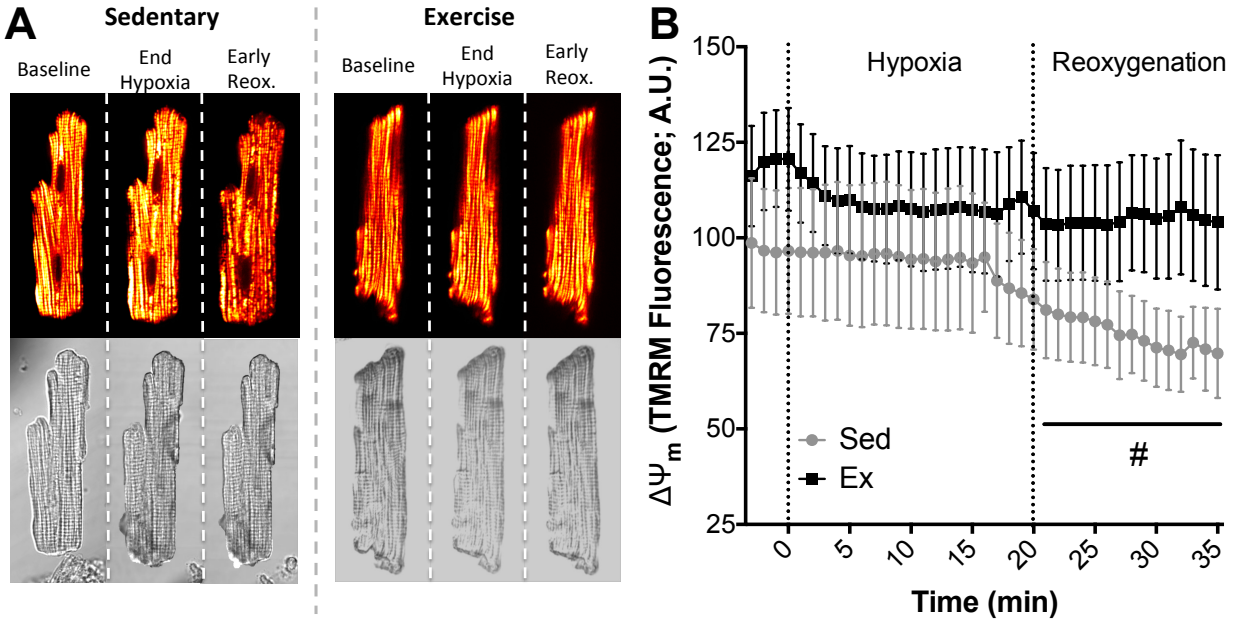

Figure 7. Mitochondrial membrane potential ($\Delta\Psi_m$) during cardiac myocytes hypoxia/reoxygenation (H/R). (A) Representative images of Sed and Ex cardiac myocytes during H/R. Depolarized mitochondrial networks and collapses in $\Delta\Psi_m$ are shown during reoxygenation as a transition in color from yellow to red and black. (B) Quantification of TMRM fluorescence during H/R. Data are shown as mean ± SEM. # p<0.05 vs Sed main effect.

Rates of mitochondrial O_2 consumption and H_2O_2 emission during hypoxia/reoxygenation

The quality of mitochondria was similar between the groups following isolation as assessed by the RCR (Sed 5.1± 0.4 vs Ex 5.2± 0.1). JO_2 at a submaximal ADP (75μM)

concentration was not different between Ex and Sed mitochondria prior to anoxia when respiring on complex I and II substrate (*Figure 8*A). The decrement in JO_2 immediately following anoxia was blunted in mitochondria from Ex animals ($p<0.05$, *Figure 8*B). Baseline state 3 JH_2O_2 was not different between Ex and Sed before the onset of anoxia (*Figure 8*C). JH_2O_2 was

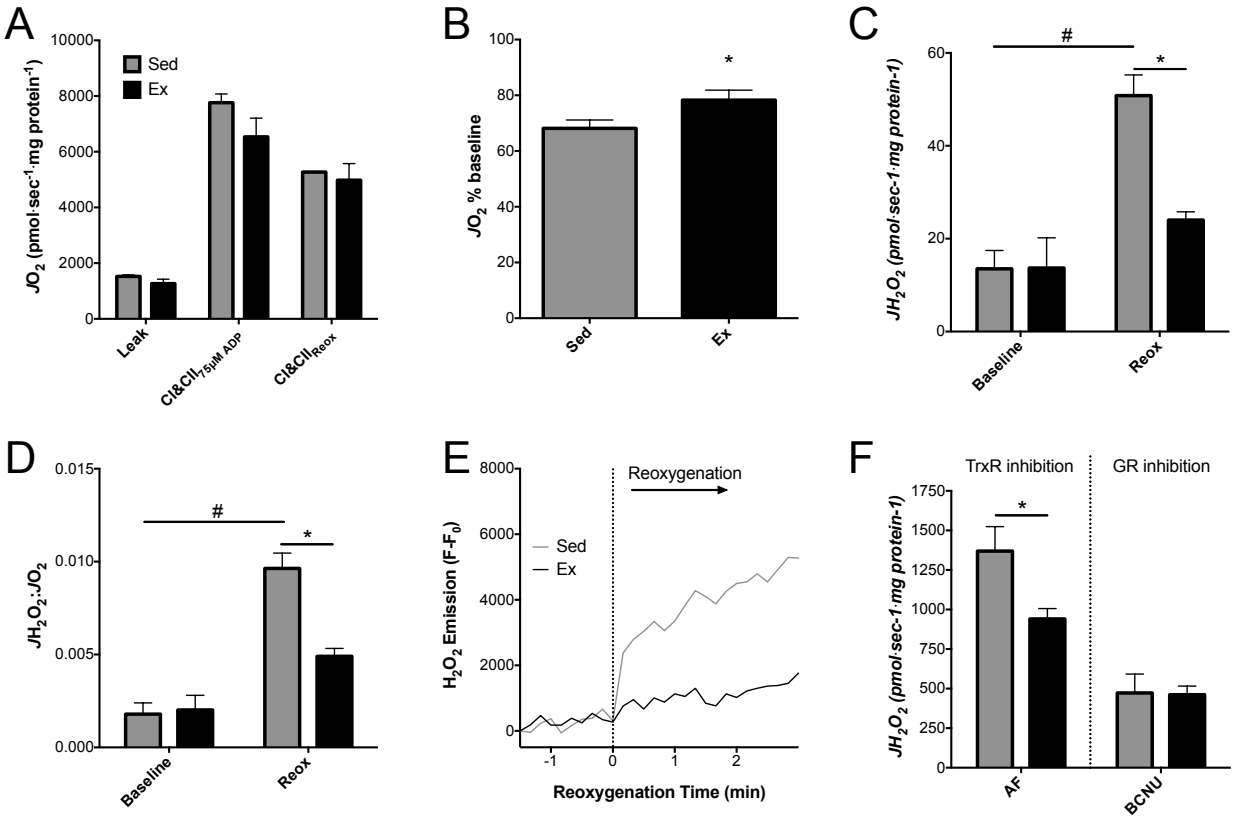

Figure 8. Reactive oxygen species (ROS) and isolated mitochondrial energetics during anoxia/reoxygenation (A/R). Sed is gray and Ex is black in all graphs. O_2 consumption rate (JO_2) and H_2O_2 emission rate (JH_2O_2) was measured in isolated mitochondria from Sed and Ex hearts. (A) JO_2 was similar at baseline between Ex and Sed isolated mitochondria respiring on glutamate + malate, pyruvate, and succinate, and ADP clamped at 75 μM (state 3). (B) Impairments in state 3 JO_2 following A/R was determined by comparing relative decreases from baseline for Sed and Ex. (C) State-3 JH_2O_2 before and after A/R. (D) The JH_2O_2:JO_2 ratio demonstrates impaired mitochondrial function in Sed mitochondria following A/R. (E) A representative experiment showing a trace of resorufin fluorescence used to calculate JH_2O_2 during A/R. For clarity, data were transformed by subtracting the anoxic fluorescent value recorded prior to reoxygenation. (F) JH_2O_2 in isolated mitochondria in the presence of either thioredoxin reductase inhibitor (AF) or glutathione reductase inhibitor (BCNU). Data are shown as mean ± SEM. * $p<0.05$ vs Sed main effect ; # $p<0.05$ vs Sed baseline.

significantly higher following A/R compared to pre-A/R values only for Sed mitochondria ($p<0.05$ vs Sed baseline), while Ex attenuated this increase and was significantly lower than Sed following A/R ($p<0.05$ vs Sed reox, *Figure 8*C). Post-A/R JH_2O_2:JO_2 was significantly higher than baseline only for Sed mitochondria following A/R ($p<0.05$ vs Sed baseline, *Figure 8*D), and

significantly higher than Ex mitochondria following A/R ($p<0.05$ vs Sed reox, *Figure 8*D). A representative trace for JH_2O_2 is shown in *Figure 8*E and demonstrates the lower ROS burst during reoxygenation observed in mitochondria from Ex hearts.

In parallel experiments, the contributions of the thioredoxin and glutathione redox systems to ROS scavenging were investigated separately. Mitochondria generated H_2O_2 was measured under state 4 conditions with succinate, incubated with the TrxR inhibitor AF, or the GR inhibitor BCNU. Ex and Sed mitochondrial JH_2O_2 were similar after inhibition with BCNU, where as Ex JH_2O_2 was significantly lower than Sed only after inhibition with AF ($p<0.05$, Figure 8F).

Discussion

The objective of the present study was to determine the effect of exercise-induced cardioprotection on mitochondrial bioenergetics and redox homeostasis during reperfusion-induced arrhythmia. Our findings indicate that exercise decreases arrhythmia through mitochondria-dependent mechanisms, including better maintenance of $\Delta\Psi_m$ and lower ROS production. Several aspects of the present study provide novel insight into mechanisms of exercise cardioprotection. First, the simultaneous recording of $\Delta\Psi_m$ and ECG in the intact heart provides crucial confirmation that mitochondria from exercised hearts have a protective phenotype *in situ*. We have directly demonstrated that this phenotype correlates with cardiac electrical stability. Second, the continuous, simultaneous recording of mitochondrial O_2 consumption and ROS during *in vitro* hypoxia/reoxygenation allows us to determine the exact time and nature of bioenergetic dysfunction during the metabolic insult itself (as opposed to after the injury has occurred). The present study, and our previously published data indicate that the maintenance of redox homeostasis through GR is an exercise-induced adaptation that helps sustain energetic and electrical coupling in the heart (80, 81). Finally, our vertically integrated approach using intact hearts, isolated ventricular myocytes, and isolated mitochondria provides comprehensive insight into the endogenous changes that occur in exercised hearts, indicating that stabilization of mitochondrial energetics is centrally involved in the anti-arrhythmic effects of exercise.

Maintenance of $\Delta\Psi_m$ and lower reperfusion arrhythmia following exercise-induced cardioprotection

The maintenance of $\Delta\Psi_m$ is an important determinant of ischemia-reperfusion injury, cell death, and arrhythmia (3, 31, 56, 123). Under conditions of metabolic stress, collapses of $\Delta\Psi_m$ are known to induce oscillations in cardiac action potential duration due to transient increases in sarcolemmal ATP-sensitive K^+ channel currents (3, 9, 161, 228). Lability in K^+ current during the

repolarization phase of the cardiac cycle can alter the spatiotemporal organization of cardiac electrical activity and increase the susceptibility to abnormal cardiac rhythms (233).

In the present study, exercise-induced cardioprotection led to better preservation of $\Delta\Psi_m$ in the intact heart during early reperfusion with a concomitant decrease in arrhythmia. We also observed a more robust preservation of $\Delta\Psi_m$ when hearts were pooled for those that transitioned to arrhythmia vs. no arrhythmia. Heterogeneous collapses in $\Delta\Psi_m$ in intact hearts during ischemia/reperfusion have been previously observed using two-photon microscopy (143, 198) or optical mapping (137). Studies that have looked at ischemia and reperfusion often see more robust collapses in $\Delta\Psi_m$ at the onset of reoxygenation (143, 198), when ROS levels surge and ATP demands resume with the recovery of excitation-contraction coupling (ischemic tissue does not contract and thus has lower energy demands). Our findings here are in line with these observations as we saw the most robust decline in $\Delta\Psi_m$ in cells and hearts at the onset of reoxygenation and reperfusion, respectively. While this represents the first direct demonstration of preserved $\Delta\Psi_m$ in exercised hearts, our results are consistent with previous studies showing better maintenance of energetics (reviewed in (79)) and delayed opening of ATP-sensitive potassium channels (111) in exercise-conditioned hearts.

The overall reduction in oxidant stress with exercise and maintenance of $\Delta\Psi_m$ that we observed are likely inter-related. In beating hearts, $\Delta\Psi_m$ helps to sustain redox homeostasis through replenishing endogenous antioxidants via the nicotinamide nucleotide transhydrogenase. The cellular redox environment is then regulated by ROS detoxifying enzymes (e.g. GR and TrxR) whose activity and subcellular localization controls redox-sensitive protein networks. Through second-messenger signaling and post-translational modifications, redox chemistry has been implicated in cardiac hypertrophy, remodeling, apoptosis, autophagy, and cell death (1, 160, 182). In our study the overall stabilization of energetics contributes to improved cardiac function observed after exercise. The redox status of proteins following

exercise, such as eNOS (4) and GR (80), appear to play an important role in this protective phenotype. Consistent with our findings are other physiological observations following exercise, whereby redox modification to the ryanodine receptor enhances SR calcium release (186). Furthermore, enhanced redox control may also prevent aberrant SR calcium ATPase activity and calcium reuptake by decreasing the oxidation of regulatory thiol-containing residues (173).

Preservation of cellular GSH and $\Delta\Psi_m$ during ischemia/reperfusion with exercise

The GSH pool is an essential part of redox homeostasis and an intricate antioxidant system used in the scavenging of ROS (188). Recent work implicates the cellular redox state in mitochondrial physiology and susceptibility to arrhythmia (8, 31, 81). $\Delta\Psi_m$ collapses have been observed when GSH levels become oxidized to a critical level, leading to ROS-induced ROS release that can scale to depolarize mitochondrial networks (8, 31, 235). Decreasing the cellular oxidative burden during an oxidative challenge with perfusion of scavengers or a GSH analog prevents the collapse of $\Delta\Psi_m$ (87), attenuates shortening of the action potential duration (2), and preserves mitochondrial function when isolated from post-ischemic hearts (43).

Exercise cardioprotection against arrhythmia has been shown to be dependent on enhanced ROS scavenging through several different endogenous mechanisms acting in parallel (31, 97, 178). Antisense treatment against MnSOD has been shown to abolish the antiarrhythmic effect of exercise (97), corroborating a number of studies that implicate heightened MnSOD in exercise-induced cardioprotection (reviewed in (79)). Since the product of the dismutase reaction, H_2O_2, must be further processed to keep overall ROS levels low, detoxification by the GSH pool, the largest capacity thiol buffer in heart, is also involved. Most studies find no basal differences in total GSH or the ratio of reduced/oxidized glutathione (GSH/GSSG) after exercise, (81, 108, 114, 130) and our work corroborate these findings (although there was a trend for myocytes from exercised hearts to have increased basal GSH, it did not reach statistical significance).

Although basal GSH/GSSG changes are rarely observed after exercise, the ability to replenish the GSH pool during an oxidative insult does appear to be involved. In this study the recovery of GSH levels during early reoxygenation correlated with maintained $\Delta\Psi_m$ in both heart cells and intact hearts. Myocardial GSH levels were also better preserved in hearts as assessed with HPLC (Figure 6C). The GSSG content was not significantly different in Ex versus Sed at the end of reperfusion, which we also saw in an earlier study (81). This is likely due to heightened cell permeability of GSSG (209), ostensibly diffusing out of the tissue during the reoxygenation window. We previously showed that GR activity, which replenishes GSH, was enhanced after exercise (80, 81). As pharmacological inhibition of GR during ischemia/reperfusion abolished the anti-arrhythmic phenotype of exercise (80), the ability to replenish the cellular glutathione pool appears to be centrally involved in exercise cardioprotection. Our observation herein that isolated cardiac myocytes exposed to H/R displayed enhanced GSH replenishment is also consistent with previous studies in intact hearts/cells where Ex protected against injury after perfusion with the thiol-oxidizing agent diamide (81).

Exercise causes intrinsic mitochondrial adaptations that preserve post-ischemic function

We used simultaneous acquisition of mitochondrial O_2 consumption and H_2O_2 emission *during* A/R to determine the extent and time-course of endogenous mitochondrial dysfunction. Similar studies using electroparamagnetic spin trapping on isolated mitochondria have shown that A/R results in a significant rise in superoxide production during the reoxygenation phase with concomitant declines in respiratory function (70). Therefore, the A/R insult allows one to remove cytosolic and compartmentalized cellular defense systems, unmasking mitochondria-specific adaptations.

Several studies have investigated the effect of A/R on mitochondrial function (70, 71, 222), but only one following exercise training. Ascensao et al (14). exercised male Wistar rats

for 14 weeks at a similar intensity as our protocol, and exposed isolated mitochondria to A/R 24 hours after the last exercise bout. The exercise group maintained higher post-A/R state 3 respiratory rates compared to sedentary controls. However, they reported no difference in the magnitude of decline in respiration between the two groups (63% and 60% of baseline following A/R in Ex and Sed groups respectively). Our study demonstrates that Ex maintains mitochondrial energetics following a metabolic insult, assessed by higher percentage of JO_2 recovery following A/R (78% and 68% of baseline for Ex and Sed, respectively). Seeking to mimic the in vivo conditions, we used complex I- and II-linked substrates (glutamate, malate, pyruvate, and succinate) and physiologically clamped ADP levels (75μM), while Ascensao et al. used only complex I substrate and ~400μM ADP, which more closely approaches V_{max} and may not be as physiologically relevant (53). Recent findings implicate post-ischemic succinate accumulation as a driver of mitochondrial ROS production through reverse electron transfer, or RET (50) in early reperfusion. The inclusion of succinate in our mitochondrial buffers may also explain the differences observed in this study versus previous work (13).

Isolated mitochondria from the Ex group had lower JH_2O_2 emission following A/R compared to Sed, which was especially prominent in early reoxygenation. These findings are consistent with observations that exercise lowers cardiac ROS accumulation during ischemia/reperfusion (81), protecting against oxidative stress and subsequent collapses in mitochondrial bioenergetics. Furthermore, the JH_2O_2:JO_2 ratio was ~2-fold higher in the Sed vs Ex group, implying that exercise induces endogenous mitochondrial adaptations that result in a lower oxidative burden relative to O_2 consumption.

Direct demonstration that mitochondria from exercised animals experience lower levels of oxidative stress corroborates recent findings. Lee et al. reported that exercise significantly decreased the production of H_2O_2 in actively respiring mitochondria following ischemia-reperfusion (129). However, Lee et al. isolated mitochondria from the myocardium after the

ischemia/reperfusion insult, and one cannot ascertain if better mitochondrial function was a cause or a consequence of exercise-induced protection.

Exercise-induced adaptations enhances GSH replenishment through glutathione reductase

We determined if lower ROS bursts following A/R was due to improved scavenging by GR and/or TrxR. Inhibition of GR abolished the exercise-induced reduction in ROS, but the exercise effect still persisted when TrxR was pharmacologically blocked. These data are in line with our previously published data (80, 81) implicating mitochondrial GR in enhanced redox control and stabilization of mitochondrial energetics following exercise-induced cardioprotection. Although not many studies have examined TrxR in exercise cardioprotection, the lack of contribution for this scavenging mechanism we observed is consistent with previous studies (62).

Measurement of ROS in living systems often represents the net balance between mitochondrial production and scavenging. Improved endogenous scavenging in the heart following exercise is clear (5, 79). It is plausible that Ex mitochondria also produce less ROS. Mitochondrial ROS production occurs at several different sites along the Krebs Cycle and electron transport system (164). Mitochondrial Complexes I, III, and supercomplexes can all promote formation of reactive intermediates, especially during pathological conditions (142, 154). Future studies will continue to advance our understanding of how Ex leads to both augmented scavenging, and perhaps lower ROS emission, in cardiac mitochondria.

Although exercise studies indicate a role for mitochondrial adaptations in the cardioprotective phenotype, there are clear areas that require further investigation. For example, the energy-sensing mitochondrial K_{ATP} channel has been implicated in exercise cardioprotection, and channel blockade abolishes the anti-arrhythmic effect of exercise (178). Determining if mitochondrial K_{ATP} channel function directly affects $\Delta\Psi_m$ and/or cellular redox status represents an exciting area for future research.

Limitations

Although there are several limitations in our study, we tried to address these shortcomings with experiments at different levels of tissue organization. First, we used blebbistatin for the whole heart imaging experiments to limit motion artifacts. Blebbistatin inhibits actin-myosin interactions, but has no effect on calcium cycling and the cardiac action potential, which allows for electrical activity to be recorded (69). Still, the clear limitation is that $\Delta\Psi_m$ is assessed in a model where the energetic demand of contraction is substantially blunted. For this reason, we used field-stimulated cardiac myocytes as an additional measurement of mitochondrial function during metabolic insult. Two photon studies are also confounded by the limitation that global, no-flow ischemia provides consistent ventricular arrhythmia, but that the restoration of coronary flow at reperfusion induces movement artifact as the coronary bed is replenished with fluid. This prevents the continuous monitoring of the same section of ventricular muscle through ischemia and reperfusion, providing relative signal over time. We also used a mixed population of mitochondria for our experiments, and acknowledge that the sub-sarcolemmal and intermyofibrillar mitochondria may have divergent responses to the ischemic insult (114, 120, 129).

Conclusions

In summary, our findings demonstrate that exercise helps sustain post-ischemic mitochondrial bioenergetics and redox homeostasis, which is associated with preserved $\Delta\Psi_m$ and protection against reperfusion arrhythmia. This builds on a growing body of literature that indicates a close relationship between the redox environment and stability of $\Delta\Psi_m$. Future work aimed at determining the evolution of specific mitochondrial adaptations may assist in developing therapeutic targets that mimic the adaptive response to exercise-induced cardioprotection.

Chapter 4: *G*lutathione reductase and redox homeostasis *modulates* mitochondrial bioenergetics following metabolic stress

Introduction

Mitochondria are central hubs for the regulation of cellular redox- and energetic-homeostasis and have been linked to mechanisms of exercise cardioprotection against reperfusion arrhythmia (6, 177, 178) and myocardial infarction (129, 170, 201, 227). Glutathione reductase is a key enzyme involved in mitigating oxidative stress by maintaining the glutathione pool in the reduced state, and our 10-day cardioprotective-exercise model in rats demonstrates enhancements in the heart's capacity to replenish GSH (80, 81). The GSH pool also represents a major marker of cellular redox homeostasis and is involved in an intricate antioxidant system used in the scavenging of ROS (188). In Chapter 3 I have demonstrated that cell redox status plays a critical role in mitochondrial dynamics and susceptibility to reperfusion injury (8, 31, 81). Oxidative shifts in the redox environment during I/R leads to opening of inner mitochondrial ion channels and mitochondrial permeability transition (9, 10, 52, 235). Subsequent instability in mitochondrial membrane potential ($\Delta\Psi_m$) and a collapse in mitochondrial bioenergetics leads to ventricular arrhythmia and cell death, both of which are attenuated with exercise (80, 81).

Modulation of oxidative stress and redox homeostasis through pharmacological and/or genetic manipulations has potential for therapeutically treating conditions of I/R (31, 80, 97), hypertension (64), and heart failure (54, 195). Therefore, uncovering mechanisms to restore redox homeostasis remains a high priority in the development of treatments of cardiovascular disease. Using exercise as a model of cardioprotection we note significant enhancements in cardiac glutathione redox regulation, however the extent to which glutathione reductase (GR) contributes to preservation of mitochondrial energetics and cardioprotection is unknown. The

objective of this study was to utilize pharmacological and genetic modifications of GR to determine its impact on cellular bioenergetics under conditions of oxidative stress.

Methods

Cell Culture

The H9c2 rat myocardial cell line was obtained from Sigma and cultured in DMEM with 10% fetal bovine serum, 1% (v/v) penicillin/streptomycin and 0.25 µg/ml amphotericin B at 37ºC with 5% CO_2. Cells below passage 20 were used experimentally in this study. To induce differentiation and myotube formation, cells were switched to 1% fetal bovine serum and supplemented daily with 10nM *all-trans*-retinoic acid (Sigma) for 5 days unless otherwise stated.

shRNA plasmid and adenovirus vector production

Double-stranded 58-mer oligonucleotides encoding shRNAs targeting the glutathione reductase gene (Gsr) mRNA (*Figure 9*) were inserted in place of the 1.9 Kb stuffer region between Age I and Eco RI sites of the AddGene pLKO.1 TRC vector (Sigma), under the transcriptional control of the human U6 polymerase III promoter. Invitrogen's shRNA generation program was used to obtain target sequences for the Gsr shRNA (shgsr). A panel of the target sequences screened is shown in Table 1. For screening of shRNAs, H9c2 cells were grown to 70% confluence and transfected in Opti-MEM with 3µg DNA using the Fugene (Promega) transfection reagent at a 3:1 ratio (Fugene/DNA). Cells were incubated in transfection media for 24hr and then transfected again for another 24hr. Cells were harvested 48hr post transfection and GR protein content was determined using Western blots. After identification of positive targets, the insert and expression cassette from the pLKO.1 vector was PCR amplified using CloneAmp HiFi Premix for cloning into the pAdenoX adenoviral system. The purified PCR product was then cloned into a linearized pAdenoX vector using In-Fusion cloning. The In-Fusion reaction mixture was used to transform Stellar competent *E.coli* cells, and positive colonies were screened using Terra PCR and observed on agarose gel. Positive clones were

cultured overnight and purified using the Nucleobond Xtra Plasmid kit. pAdenoX DNA was then digested with PacI to expose the inverted terminal repeats (ITRs) for adenoviral DNA replication. Adeno-X 293 cells were transfected with CalPhos Mammalian Transfection kit. Upon visual cytopathic effects, Adeno-X 293 cells were harvested and exposed to three freeze thaw cycles. The supernatant of freeze-thawed cells containing recombinant virus were then amplified one time and viral titer was estimated using CloneTech GoStix.

Glutathione reductase overexpression and adenovirus vector production

Sprague Dawley rat Gsr mRNA was PCR amplified from cardiac tissue using Tri reagent (Sigma). mRNA was converted into cDNA using the Invitrogen Super Script III First Strand Synthesis system and polyA enriched RNA was selected for cloning. Gsr primers were used to PCR amplify the cDNA using CloneAmp HiFi mix. PCR products were verified by ethidium-bromide stained on agarose gel and visualized under UV light. The Gsr gene was cloned into the pCMV5 shuttle vector using restriction site cloning. Stellar competent cells were transformed and selected for positive colonies. The pCMV5 + gsr overexpression cassette was cloned into the pAdenoX vector to generate adenovirus (Gsr OE) using the same cloning strategies as above. An empty vector expressing GFP was also generated for a vector control (EV-GFP).

Glutathione reductase shRNA knockdown and overexpression in H9c2 cells

H9c2 cells were grown overnight to 70% confluence and then switched to differentiation media supplemented with 6μg/mL of polybrene upon adenoviral infection. A multiplicity of infection (MOI) of 50 was used to infect H9c2 cells with the shRNA adenoviruses, and a MOI of 100 was used for the Gsr overexpressing or empty vector adenoviruses. Following 12hr of virus exposure cells were switched back to differentiation media and cultured for 4 more days to allow changes in gene expression to occur.

Hypoxia /reoxygenation in H9c2 cells

H9c2 cells were seeded at 10,000 cells per well in 96-well Seahorse plates and switched to differentiation media 12 hours later. After 5 days of differentiation, cells were exposed to an established model of cellular hypoxia/reoxygenation (12). Cells were subjected to 0% O_2 and deprived of nutrients in Hank's balanced salt solution (HBSS), and kept in an incubator at 5% CO_2 for the duration of hypoxia.

Measurement of respiration in H9c2 cells following hypoxia/reoxygenation

Following hypoxia cells were returned back to growth media and allowed to recover for 2hr, or immediately switched to Seahorse XF assay medium and immediately exposed to a mitochondrial stress test in a Seahorse XF96 using an injection strategy that included oligomycin A (1 µg/ml), followed by FCCP (4µM), then antimycin A (2µM). Seahorse XF assay medium was supplemented with pyruvate (10mM), glucose (10mM), and glutamax (2mM).

Mitochondrial bioenergetics following pharmacological inhibition of glutathione reductase

The diothiocarbamate derivative 2-Acetylamino-3-[4-(2-acetylamino-2 carboxyethylsulfanylthiocarbonylamino)phenylthiocarbamoylsulfanyl]propionic acid hydrate (2-AAPA) was used as a pharmacological model of thiol oxidative stress in H9c2 cells. 2-AAPA has previously been used in H9c2 cells to show potent inhibition of Gsr and thioredoxin reductase (TrxR) activity, in addition to oxidative shifts in the reduced GSH to oxidized glutathione ratio (GSH/GSSG) (226). H9c2 cells were exposed to 2-AAPA for 20min followed by bioenergetics analysis. H9c2 cells were seeded at a density of 20,000 cells per well in 96-well Seahorse plates 24 hours prior to 2-AAPA exposure. Following a 20 min exposure to 2-AAPA cells were washed with Seahorse XF Assay Medium and immediately exposed to a mitochondrial stress test in a Seahorse XF96 using the same injection strategy as above.

Measurement of mitochondrial membrane potential and cell viability following 2-AAPA exposure

In parallel experiments mitochondrial membrane potential and cell viability were determined in H9c2 cells exposed to 2-AAPA. Cells were seeded at a density of 30,000 cells/well in a 96-well plate and allowed to attach for 24hr. Cells were then treated in triplicate with DMSO, FCCP, or increasing concentrations of 2-AAPA (25μM, 50μM, or 100μM) and incubated at 37°C in 5% CO_2 for 20 minutes. Mitochondrial membrane potential ($\Delta\Psi_m$) was assessed by tetramethylrhodamine, methyl ester (TMRM). TMRM is a membrane-permeant cationic fluorescent dye that equilibrates with Nernstian behavior and is sequestered by mitochondria. Cells were incubated for 20 minutes in XF Assay medium and 200nM TMRM. . Following TMRM loading, cells were washed with Hank's balanced salt solution and fluorescence intensity was measured on a Cytation5 multi-mode microplate reader (BioTek) using excitation/emission wavelengths of 540/590nm. In separate experiments, viability was determined using PrestoBlue viability reagent (ThermoFisher Scientific) using the manufacturers recommended protocol in a Cytation5 multi-mode microplate reader (BioTek).

Statistics

All data are presented as mean ± standard error. Seahorse adenovirus knockdown experiments were compared against vector controls using a student's t-test. Mean fluorescence during reperfusion and respiratory control ratios were analyzed using unpaired Student's t-test. Seahorse 2-AAPA data were analyzed with an ANOVA followed by a Dunnett's post-hoc test. Statistical significance was established when $P<0.05$. All data were analyzed and graphed using GraphPad Prism software.

Results

Gsr knockdown and overexpression adenovirus

Invitrogen's shRNA generation program was used to obtain candidate target sequences for Gsr mRNA transcripts. The target sequences from Table 1 were cloned into the AddGene pLKO.1 vector and further tested for knockdown of Gsr expression.

Table 1: 58-mer oligonucleotide sequences tested for Gsr knockdown

shgsr	Construct sequence
#1	5' CCCAAATTCTAAGGGCCTGAA-CTCGAG-TTCAGGCCCTTAGAATTTGGG 3'
#2	5' GCTCCAAGACGTCTCTTATGA-CTCGAG-TCATAAGAGACGTCTTGGAGC 3'
#3	5' GGATTCAGACTGATGACAAAG-CTCGAG-CTTTGTCATCAGTCTGAATCC 3'

The optimal concentration of DNA for transfection was empirically determined (3µg), with shgsr target #3 as the best candidate for efficient knockdown (*Figure 9*A and B). The pAdeno-X system was used to generate four different adenoviruses using the expression cassettes depicted in *Figure 9*C. The shRNA vectors were under control of a human U6 promoter (hU6), and the overexpression vectors were controlled by a CMV promoter. Differentiated H9c2 cells show high adenovirus expression 96hr post infection (*Figure 9*D), with time-matched changes in Gsr protein expression (*Figure 9*E).

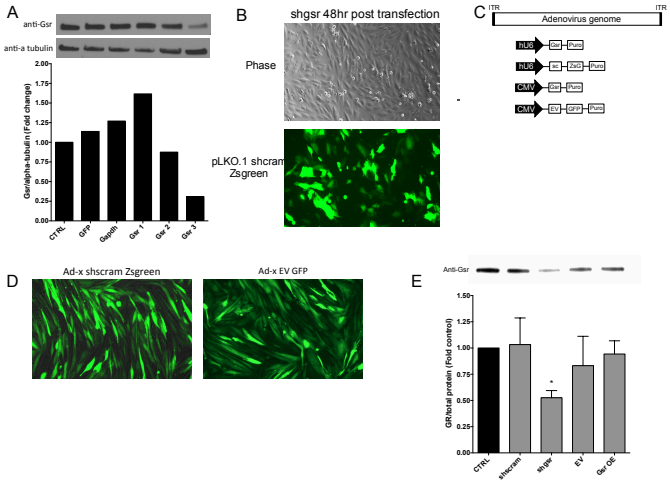

Figure 9. Gsr knockdown and overexpression in H9c2 cells. (A) Western blot and densitometry after 48hr transfection with shRNA cloned into the pLKO.1 vector. (B) GFP and phase images 48hr post transfection. (C) Illustration of adenovirus expression cassettes of shgsr, shscram, Gsr overexpression (Gsr OE), and empty vector control (EV). (D) 96hr post infection fluorescent images of differentiated H9c2 cells, and (E) Western blot of glutathione reductase protein content.

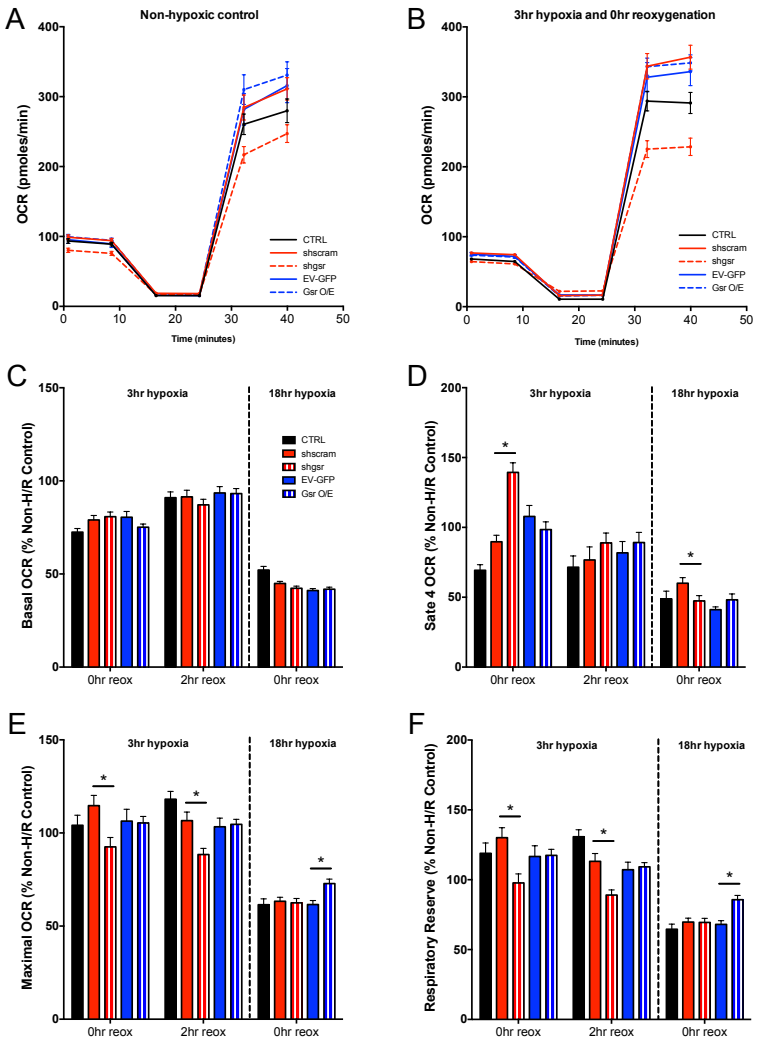

Figure 10. Mitochondrial bioenergetic analysis in H9c2 cells exposed to hypoxia/reoxygenation. (A) Seahorse trace of mitochondrial stress test protocol following a 20min 2-AAPA exposure. All data were transformed by subtracting out the antimycin A respiratory rate. (B) Basal respiration. (C) Maxmial uncoupled respiration with FCCP (E) ATP-dependent respiration (Basal respiration – State 4). Data in C-E are expressed as percent of Non-H/R control for each time point. *p<0.05 vs vector control.

Mitochondrial bioenergetics were studied in differentiated H9c2 cells exposed to various durations of hypoxia/reoxygenation (H/R) using glutathione reductase gain- and loss-of-function studies. Non-hypoxic control plates for each hypoxic time point were used to normalize the data as percent of non-hypoxic control (n=16-24 per group). *Figure 10*A and 10B show examples of the mitochondrial stress test raw data from an experiment for the 3hr/0hr H/R protocol. There was no difference in basal respiration following 3hr or 18hr hypoxia across groups (*Figure 10*C). State 4 leak respiration following inhibition of complex V with oligomycin was significantly higher in shgsr after 3hr/0hr H/R, but was significantly lower after 18hr H/R ($p<0.05$ vs shscram, *Figure 10*D). Maximal respiration was significantly lower in shgsr after 3hr hypoxia at both 0hr and 2hr reoxygenation, but this was not observed after 18hr H/R ($p<0.05$ vs shscram, *Figure 10*E). Interestingly Gsr OE had no rescue affect following short

term 3hr H/R, but at 18hr H/R, maximal respiration and respiratory reserve were significantly higher than the EV-GFP control ($p<0.05$ vs EV-GFP, *Figure 10*F). In contrast, respiratory reserve in shgsr following 3hr H/R was significantly lower, and this effect was lost at 18hr H/R ($p<0.05$ vs shscram, *Figure 10*F).

Pharmacological thiol redox stress and mitochondrial function

To determine the relationship between acute thiol oxidative stress and mitochondrial function induced by glutathione reductase inhibition oxygen consumption rate (OCR) was measured in H9c2 cells following a 20min dose-response exposure to 2-AAPA (n=8-24 per group). OCR was measured using a mitochondrial stress test on the Seahorse XF96 and XF96e. Traces of respiratory rates obtained from the mitochondrial stress test are shown in *Figure 11*A, and were transformed by subtracting the average antimycin A rate for each group. Basal respiratory rates were significantly higher than controls at the lower concentrations of 2-

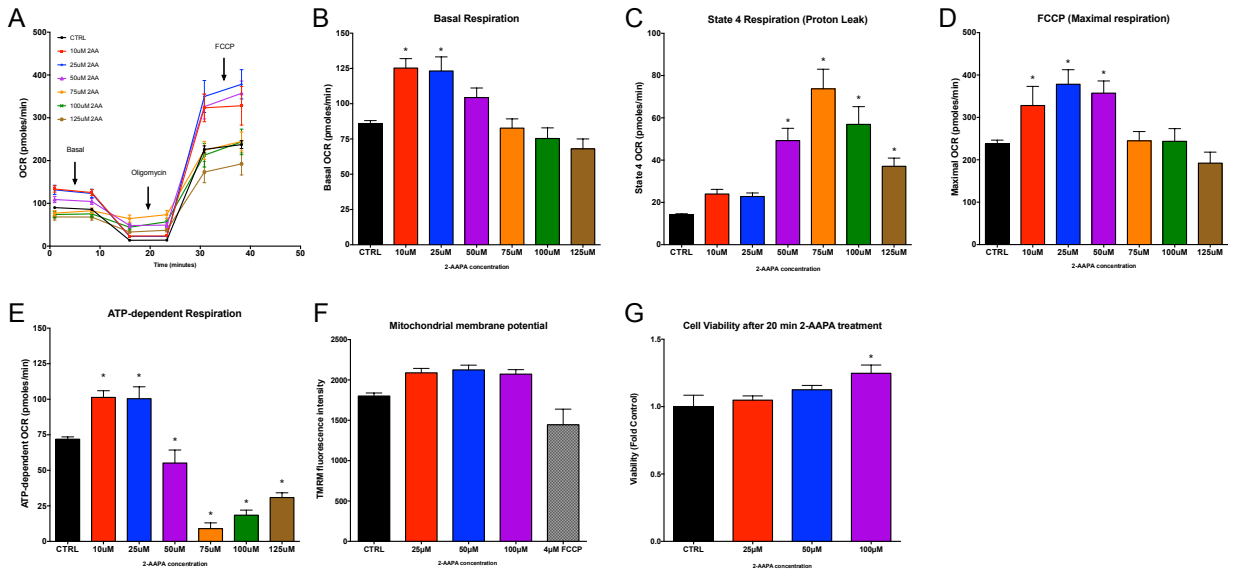

Figure 11. Mitochondrial bioenergetic analysis in H9c2 cells following pharmacological induction of thiol redox stress. (A) Seahorse trace of mitochondrial stress test protocol following a 20min 2-AAPA exposure. All data were transformed by subtracting out the antimycin A respiratory rate. (B) Basal respiration. (C) Maximal uncoupled respiration with FCCP (E) ATP-dependent respiration (Basal respiration – State 4). (F) Mitochondrial membrane $\Delta\Psi_m$ with TMRM and (G) cell viability following 2-AAPA exposures. *$p<0.05$ vs CTRL (ANOVA followed by Dunnett's post-hoc test).

AAPA, and this response was blunted at the higher concentrations ($p<0.05$ vs CTRL, *Figure*

*11*B). Proton leak under state 4 conditions increases in a dose-dependent fashion up to 75μM 2-AAPA ($p<0.05$ vs CTRL, *Figure 11C).* Maximal respiratory rates increase when exposed to lower 2-AAPA concentrations, and this increase is blunted at higher doses ($p<0.05$ vs CTRL, *Figure 11*D). There was a significant decrease in ATP-dependent respiration at the higher concentrations of 2-AAPA exposure, which is opposite compared to the lower concentrations of 2-AAPA where OCR was significantly higher than control ($p<0.05$ vs CTRL, Figure 11E). Interestingly, the energetic collapse is independent of decreases in $\Delta\Psi_m$ (*Figure 11*F) and cell viability (*Figure 11*E) with acute 2-AAPA exposures. In fact, cell viability actually increases at 100μM 2-AAPA, which may be an experimental artifact.

Discussion

The main findings from this study are that glutathione redox potential and thiol redox stress play a profound role in mitochondrial dysfunction in acute pathological states. Cellular redox homeostasis not only influences ROS scavenging, but also energetic homeostasis and cell survival (224). This is supported by a growing appreciation for post-translational redox modifications under physiological and pathological conditions (139), and the role of mitochondrial function in cardiac physiology. The principle findings from this work are that: 1) Gsr deficiency exacerbates mitochondrial dysfunction following a hypoxic insult; 2) Gsr overexpression mitigates mitochondrial dysfunction after more severe hypoxic insults; and 3) Thiol redox stress modulates mitochondrial function via increasing respiration at lower levels and collapsing energetics at higher levels of thiol redox stress.

Essential to our ability to better treat cardiac I/R injury, is the fundamental role of oxidative stress on cellular energetics and mitochondrial dysfunction. In the myocardium transient shifts in the redox environment can lead to a perpetual cycle whereby ROS-induced ROS release and altered ion homeostasis during I/R increases the susceptibility to a collapse in $\Delta\Psi_m$, mitochondrial PTP opening, and cell death (9, 235). Improving our understanding on how altered redox homeostasis influences mitochondrial function and myocardial energetics will accelerate the development of therapeutics aimed at mitigating damage from ischemic heart disease.

Glutathione reductase and mitochondrial dysfunction following hypoxia/reoxygenation

Gsr gain- and loss-of-function experiments were employed to determine the impact of Gsr on mitochondrial dysfunction following simulation of I/R injury. Cellular GSH levels are important mediators of redox homeostasis and have been implicated in several disease models including I/R injury (198), insulin resistance (77), and Parkinson's disease (135). In the present study impeding GSH replenishment through an shRNA targeting Gsr was shown to sensitize

mitochondria to metabolic insults such as H/R. Following a more sever hypoxic insult mitochondria function was partially restored by Gsr overexpression, as demonstrated by preserved respiratory capacity and heightened maximal respiration. This demonstrates that redox homeostasis is an important pivoting point between maintenance of mitochondrial bioenergetics and susceptibility to I/R injury.

Early work on the role of ROS production in I/R injury implicates the importance of GSH in the cardioprotective phenotype, as GSH supplementation prevented myocardial dysfunction following short periods of ischemia in isolated Langendorff perfused hearts (22). Furthermore, cardiac GSH depletion exacerbates myocardial infarction and recovery of contractile function, while intravenous GSH infusion reverses these defects (196). These findings support the results herein by demonstrating that enhanced redox control leads to preservation of mitochondrial function and that the recovery of post-ischemic cardiac dysfunction is intimately related to the maintenance of GSH/GSSG redox potential (140).

Pharmacological thiol redox stress leads to decompensated mitochondrial function

In the present study the use of a dithiocarbamate derivative, 2-AAPA, was used to induce thiol redox stress in H9c2 cells to better understand the acute consequences of altered redox homeostasis on mitochondrial function. Upon low levels of acute thiol redox stress, basal and maximal respiratory rates increase above that of control levels. However, after more severe redox stress, respiratory function declines and mitochondria become uncoupled as shown by elevated leak respiration and significant reductions in ATP-dependent respiration. Interestingly, the energetic collapse was independent of detectable changes in $\Delta\Psi_m$ and viability, indicating that redox stress and subsequent mitochondrial dysfunction play a larger role upstream in the development of I/R injury.

2-AAPA is a potent inhibitor of GR and has been shown to be more specific than other GR inhibitors with an IC_{50} of 50μM (192). A 20min exposure to 2-AAPA in H9c2 cells leads to a significant reduction in GSH/GSSG, ranging from ≈40% reduction with 25μM 2-AAPA to ≈80% reduction with 100μM 2-AAPA (226). The dose and exposure times of 2-AAPA used in their studies are consistent with the present study and demonstrate that mitochondria are extremely sensitive to acute alterations of the redox environment and that mitochondrial respiration is highly responsive to the thiol redox status of the cell.

Directly altering the thiol redox status of the cell through the GSH/GSSG ratio allows one to isolate the response of oxidative phosphorylation machinery to redox stress. Although the mechanisms and physiological significance of altered protein thiol modifications are uncertain and remain an area of active investigation. The role of ROS in the damage associated with I/R injury has been well established. And while much of the early work focused on the antioxidant properties of GSH as a function of the redox environment, it has now become clear that redox reactions through GSH go far beyond the scavenging of ROS. But less clear are the consequent changes to the redox environment (i.e. GSH/GSSG) and alterations to protein thiol redox states.

Mitochondrial proteins contain many cysteine thiols that act as redox switches involved in mitochondrial bioenergetics. Reactive protein thiol groups can undergo a number of redox modifications to either enhance or suppress enzymatic activity, including direct oxidation, thiol-disulfide exchange with GSSG, and sulfenic acid intermediates among others (91). Redox-glutathionylation modifications to complex I (46) and II (47) have been found to play protective roles in the post-ischemic myocardium by limiting ROS production and preserving enzymatic activity. The reversible formation of protein mixed disulfide via glutathionylation may serve to protect electron transfer in response to oxidative stress (45). In the present study, the increase in maximal mitochondrial respiration with lower concentrations of 2-AAPA (*Figure 11*) is

attributed to increased glutathionylation of Complex I, as 2-AAPA has been found to increase glutathionylation of proteins in a dose-dependent manner (226). Furthermore, *in vitro* studies using a different GR inhibitor, BCNU, have reported similar increases in mitochondrial respiratory capacity, which coincided with increased Complex I glutathionylation.

Limitations

One of the limitations of this study is the absence of an energetic demand from excitation-contraction coupling, which when inhibited in the myocardium, alters the susceptibility to I/R injury (6). Important to this study is the role of oxidative stress on mitochondrial bioenergetics, however one cannot rule out the effect of calcium on alterations in mitochondrial respiration during oxidative stress. This is especially noted because in primary cardiomyocytes calcium homeostasis plays a significant role in cardiac I/R pathologies (236). Furthermore, several groups have established that calcium kinetics are intricately linked with mitochondrial bioenergetics and oxidative stress through regulatory thiol groups on the ryanodine receptor being susceptible to oxidation and increasing open probability of the channel (68, 173, 232).

Conclusions

In conclusion this study demonstrates that endogenous redox regulatory mechanisms can be targeted to influence the sensitivity of mitochondria to hypoxic insults. This provides insight into how an altered redox environment may affect mitochondrial function during the first few minutes of reperfusion when the production of ROS and cellular oxidative stress leads to myocardial injury. These studies implicate the targeting of GR as a potential therapeutic target for the treatment of I/R injury.

Chapter 5: The effects of NADPH-oxidase generated ROS during exercise on post-ischemic mitochondrial function

Introduction

Cardiovascular disease remains one of the leading causes of death in North America, and although rates of death attributable to cardiovascular disease have declined in recent years, the burden of disease remains high (94). It has been estimated that 1,000,000 Americans suffer acute coronary syndromes annually (152), which increases ones risk for myocardial infarction and fatal ventricular arrhythmia in particular**.** The cardioprotective effect of exercise on decreasing severity of myocardial infarction and arrhythmia has been well documented (27, 32, 80, 132, 176, 177, 207), however, cellular mechanisms are not fully understood. Exercise-induced cardioprotection is most likely a result of increased cardiac antioxidant capacity (80, 81, 97), decreased sensitivity to apoptotic stimuli (120, 176), and conservation of mitochondrial function (28, 125, 129). Recent clinical trials investigating mitochondrial-targeted therapeutics demonstrate that mitochondrial-medicine remains a high priority as a viable treatment for ischemia/reperfusion (I/R) injury (57, 92, 145, 169). Accordingly, identifying the underlying mechanisms responsible for exercise cardioprotection merits further investigation, as it may foster new treatments that evoke sustainable cardiac protection against acute coronary syndromes.

We previously demonstrated that NADPH-oxidase (NOX) generated ROS play a critical role in exercise cardioprotection (80). Activation of NOX-generated ROS during exercise was essential for the hormetic response, leading to an increase in glutathione reductase (GR) activity, and decreasing myocardial infarction following I/R. GR is an important component to the

oxidative stress defense system by maintaining the GSH pool and preserving redox homeostasis (6). Furthermore, oxidative stress from mitochondrial derived reactive oxygen species (ROS) have been linked to post-ischemic cardiac dysfunction and cell death (26, 80, 214, 235). An overwhelming oxidative stress has a negative effect on mitochondrial energetics, and can also damage mitochondrial inner-membrane lipids. Mitochondrial membranes are rich in phospholipids and are susceptible to oxidative modifications that can alter the biophysical properties of the membrane, which can have a profound effect on protein activity and ultimately mitochondrial function (48, 194, 230). To date, studies have characterized how exercise attenuates defects of the mitochondrial electron transport system after ischemia/reperfusion (29, 129), yet few studies have examined this in the context of altered redox homeostasis and subsequent changes to the mitochondrial membrane lipid environment. The objective of this study was to test the hypothesis that exercise cardioprotection through NOX-generated ROS leads to preserved fluidity of the inner mitochondrial membrane, enhanced supercomplex assembly, and better post-ischemic mitochondrial function.

Methods

Animals

Male Sprague Dawley rats (300-350g) were housed on a 12/12 hour light/dark cycle with food and water provided *ad libitum*. Experiments were conducted in accordance with NIH guidelines and approved by East Carolina University's Animal Care and Use Committee (Internal Animal Use Protocol #Q279a and #Q279b). Rats were anesthetized by injecting ketamine/xylazine i.p. (90mg/kg ketamine, 10mg/kg xylazine). Following absence of toe reflex, hearts were excised via midline thoracotomy, placed briefly in 0.9% saline (4ºC) and used for isolated heart studies.

Exercise protocol and drug treatment

Rats were injected 30 minutes prior to each exercise bout with the NADPH-oxidase inhibitor VAS2870 (2mg/kg) or DMSO as a vehicle control (Exercise+DMSO or Exercise+VAS). Rats were acclimated to the treadmill over three days, running at a speed of 15 m/min, and increasing the duration to 5, 10, and 15 min each day. Rats in the exercise group underwent 10 days of consecutive treadmill running at 6% grade for 60 min per day, in intervals broken up to 15 m/min for 15 min, 30 m/min for 30 min, and 15 m/min for 15 min. Sedentary rats were injected with DMSO or VAS2870 and placed on a non-moving treadmill for 5 min each day (Sedentary+DMSO or Sedentary+VAS). All hearts were used experimentally 24 hours after completion of the 10-day protocol or after the time-matched sedentary control.

Whole heart Langendorff experiments

Isolated hearts (n=4/group) were perfused in Langendorff mode with gassed (95%O_2, 5%CO_2) Krebs Henseleit buffer at 37ºC containing (mM): 118 NaCl, 24 $NaHCO_3$, 4.8 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, and 10 glucose (37ºC). A Transonic flow probe was used to monitor coronary flow during the protocol. Cardiac function parameters including heart rate

(HR), left-ventricular developed pressure (LVDP), and the rate of pressure development over time (+/- *d*P/*d*T) were monitored and recorded. A latex balloon was inserted into the left ventricle for isovolumic LVDP recordings. Electrocardiogram (ECG) recordings were obtained via volume-conductance using electrodes placed in a water-jacketed bath maintained at 37ºC. All parameters were recorded and analyzed using Lab Chart 7.0 software (A.D. Instruments). After a 10 min baseline and stabilization period, hearts were exposed to 20 min of global no-flow ischemia, followed by 2 hours or reperfusion. Myocardial infarct size and arrhythmia was assessed following reperfusion as previously described (80).

Mitochondria isolation

A subset of hearts were exposed to I/R and cardiac mitochondria were immediately isolated following the protocol to assess mitochondrial function. Following the protocol the left ventricle was dissected and minced on ice in mitochondrial isolation medium (MIM) + BSA containing (in mM): 300 sucrose, 10 sodium-HEPES, 1 EGTA, and 1mg/ml BSA. The minced heart was then homogenized followed by centrifugation at 800xg for 10 min. The supernatant was centrifuged at 12,000xg for 10 min to pellet mitochondria. The pellet was then resuspended in MIM and kept on ice until experimentation.

Mitochondrial O_2 consumption rate and H_2O_2 emission rate

Mitochondrial function (n=4/group, run in duplicate) was measured polargraphically and fluorometrically using the Oroboros high-resolution respirometry oxygraph-2k (o2k) with the fluorescent module. Mitochondria were injected into the o2k in Buffer Z + Amplex UltraRed assay medium containing (in mM): 110 K-Mes, 35 KCl, 1 EGTA, 5 K_2HPO_2 , 3 $MgCl_2$-$6H_2O$, 0.5 mg/ml BSA, and 25 creatine monohydrate. The rate of H_2O_2 emission was quantified using Amplex UltraRed (25µM), horseradish peroxidase (4U/mL), and superoxide dismutase (SOD, 30U/mL). First mitochondria were energized with glutamate (10mM), malate (2mM), and

pyruvate (5mM) to assess complex I-dependent respiration with maximal adenosine diphosphate (ADP) stimulation (4mM). Succinate (10mM) was then injected to assess complex I + II respiration. Complex II-dependent respiration was measured by the addition of rotenone (1μM), which blocks complex I activity. To ensure equal amounts of mitochondrial protein loading across experiments, citrate synthase activity was measured by following TNB spectrophotometrically using previously published methods (127).

Mitochondrial enzyme kinetics

Frozen mitochondria from I/R hearts were utilized for mitochondrial enzyme kinetic analysis using previously published methods with slight modifications (194). Activities were determined spectrophotemetrically in triplicate at 37°C in 1mL total reaction volume. Citrate synthase activity was determined in frozen mitochondria (10μg total protein) in hypotonic medium containing: 10mM Tris (pH 7.5), 0.31mM acetyl-CoA, 0.1mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), and 0.25% Triton X-100. The reaction was initiated with the addition of freshly prepared oxaloacetate (0.5mM), and the reduction of DTNB was monitored at 412nm for 3 min. Citrate synthase activity (n=4/group; run in triplicate) was calculated using an ε_{412} 14140 $M^{-1}cm^{-1}$. Averages of citrate synthase specific activities were used to normalize the specific activity of Complex I.

Complex I (NADH: decylubiquinone oxidoreductase) specific activity (n=4/group; run in triplicate) was determined in frozen mitochondria that underwent two 30-sec freeze thaw cycles in hypotonic medium. Mitochondria (40μg total protein) were added to a working reagent containing: 50mM Tris (pH 8.0), 5mg/mL BSA, 240μM potassium cyanide (KCN), 4μM Antimycin A, and 100μM decylubiquinone, and the reaction was initiated with the addition of 0.08mM NADH. The oxidation of NADH to NAD^+ was followed at 340nm for 3 min and an ε_{340} of 6220 $M^{-1}cm^{-1}$ was used to calculate activity of complex I. Separate experiments were carried out

in the presence of rotenone (4μM) to ensure that the rotenone-sensitive catalytic oxidation of NADH was being measured.

Mitochondrial membrane phospholipid packing following ischemia/reperfusion

Mitochondrial membrane lipid packing was assessed in mitochondria (n=4/group; run in duplicate) from post-ischemic hearts using Merocyanine 540 (MC540). Fluorescence spectra were recorded using a Jobin Yvon Fluorolog spectrofluorometer at 37ºC. Excitation was set at 495nm, and emission spectra were obtained from 540-660nm using 5nm steps. MC540 fluorescence measures the degree of phospholipid packing in model and biological membranes (126), with increasing fluorescence upon the gel to liquid phase transition. Isolated mitochondria (0.2mg/ml) were added to MIM + MC540 (75nM) and incubated for 10 minutes. Spectra recordings were made prior to the addition of mitochondria and were subtracted from the spectra after the addition of mitochondria.

Respiratory supercomplex analysis following ischemia/reperfusion

Mitochondria from post-ischemic hearts (n=4/group; run in duplicate) were isolated and supercomplexes were measured via Blue Native-Polyacrylamide Gel Electrophoresis (BN-PAGE) using published protocols with slight modifications (189). Mitochondria were solubilized (4°C for 15min, digitonin:protein ratio of 8:1), and the lysate centrifuged for 30min at 16873 x g (4°C). Protein quantification (BCA assay) was performed on the supernatant, after which 36μg was loaded into a 3-12% gradient gel. Samples were run at 150V for 3 hours, after which the gels were fixed and then washed overnight at 4°C to remove background staining. Supercomplexes were quantified using densitometry using NIH image J software.

Statistics

All data are presented as mean ± standard error. All data were compared to vehicle controls using a student's t-test. Statistical significance was established when $P<0.05$. All data

were analyzed and graphed using GraphPad Prism software.

Results

Exercise reduces ischemia/reperfusion injury through an NADPH-oxidase dependent mechanism

Hearts from rats (n=5 to 7 per group) in the Exercise+DMSO (ED) group experienced a reduced infarct size by 31% compared to Sedentary+DMSO (SD) ($P<0.05$, *Figure 12*A). The administration of VAS2870 prior to each exercise bout (EV) blunted exercise-induced cardioprotection against myocardial infarction and arrhythmia (*Figure 12*A and 12B). The anti-arrhythmic effect of exercise was evident during early reperfusion (first 15 minutes) as shown by the significant reduction in arrhythmia score (ED, 1.6±0.4 vs SD, 3.1±0.6; $P<0.05$, *Figure 12*B), while exercised animals administered VAS2870 demonstrated only a modest non-significant reduction in arrhythmia compared to controls (EV, 2.3±0.6 vs SV, 3.0±0.4; $P>0.05$, *Figure 12*B). The time to ischemic contracture was also blunted in ED animals compared to SD ($P<0.05$, *Figure 12*C), and this effect was lost with VAS2870 administration.

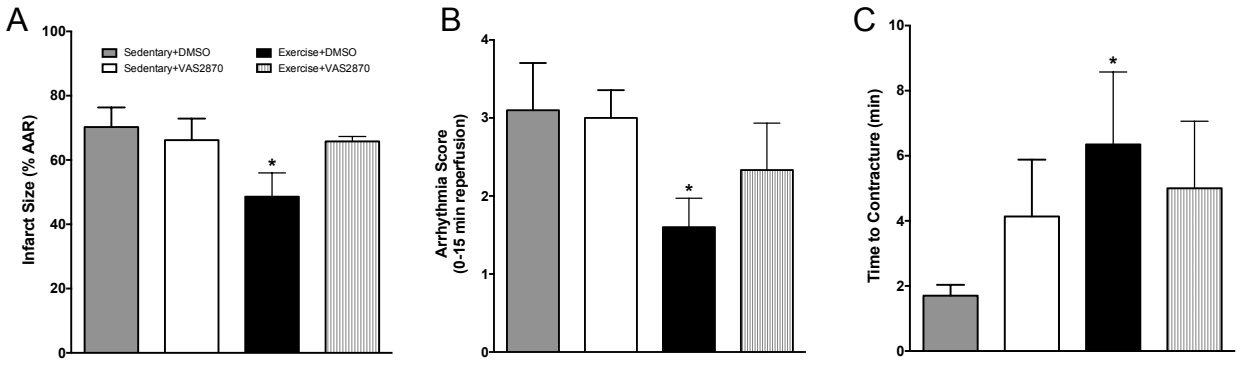

Figure 12. Infarct size and arrhythmia in isolated hearts following ischemia/reperfusion. (A) Infarct size for isolated rat hearts following 20 minutes of ischemia and 2 hours of reperfusion. (B) Arrhythmia score following the first 15 minutes of reperfusion. (C) The time to contracture was determined by calculating the amount of time elapsed during ischemia leading to a 5mmHg increase in end-diastolic pressure (EDP). All data are mean ± SEM, n=5-7 per group. * $P<0.05$ vs SD.

Post-ischemia/reperfusion mitochondrial O_2 consumption rate and H_2O_2 emission rate

Following I/R, a subset of hearts was used experimentally to isolate and study mitochondrial function (Figure 13). Rates of O_2 consumption (JO_2) and H_2O_2 emission (JH_2O_2) were measured simultaneously using the Oroboros high-resolution respirometry oxygraph-2k. There was no difference in JO_2 under state 4, non-phosphorylating conditions with glutamate, malate, and pyruvate (GMP) (*Figure 13*A). JO_2 with complex I substrate under saturating ADP conditions was lower in exercised rats compared to sedentary controls, however statistical

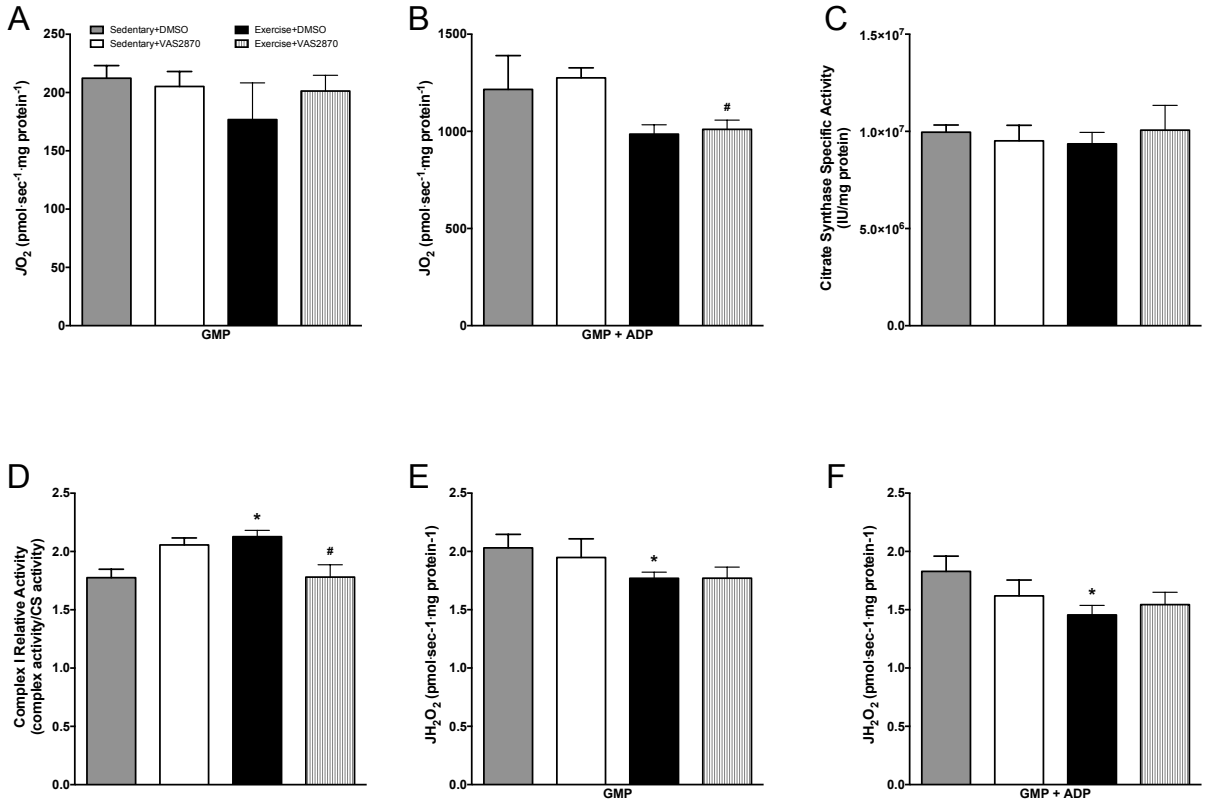

Figure 13. Mitochondrial function following ischemia/reperfusion injury. (A) O_2 consumption rate (JO_2) in isolated mitochondria under state 4 conditions (no ADP) with glutamate (10mM), malate (2mM), and pyruvate (5mM). (B) JO_2 under state 3 conditions with saturating ADP (4mM). (C) Citrate synthase activity. (D) Relative specific activity of complex I normalized to the respective citrate synthase activity. (E) H_2O_2 emission rate (JH_2O_2) under state 4 conditions (same substrate as *Figure* 2A). (F) JH_2O_2 with saturating ADP. All data are mean ± SEM, n=4 per group, JO_2 and JH_2O_2 assays were run in duplicate and kinetic assays were run in triplicate. * P<0.05 vs SD, # P<0.05 vs SV.

significance was only reached in the EV group (P<0.05 vs SV, *Figure 13*B). Citrate synthase activity in isolated mitochondria was determined by monitoring the reduction of DTNB following the addition of oxaloacetate and was not different across groups following I/R (*Figure 13*C).

Relative specific activity of complex I was determined by following NADH oxidation to NAD^+ after addition of the electron accepter, decylubiquinone. Complex I activity was significantly higher in ED vs SD and VAS2870 administration prior to exercise reversed this ($P<0.05$ vs SD, *Figure 13*D). JH_2O_2 was lower in ED vs SD under state 4 conditions, and this differences was lost with VAS2870 administration prior to exercise ($P<0.05$ vs SD, *Figure 13E)*. Similarly, JH_2O_2 was significantly lower only in ED vs SD under state-3 respiration ($P<0.05$ vs SD, *Figure 13*F).

Exercise cardioprotection is independent of altered post-ischemic inner mitochondrial membrane lipid packing and supercomplex assembly

Mitochondria were isolated following I/R and used in parallel experiments to study the biophysical properties of the inner mitochondrial membrane using MC540 and respiratory supercomplex assembly via BN-PAGE (*Figure* 14). Lipid packing of the mitochondrial membrane was observed by recording MC540 fluorescence spectra following 10 minutes of incubation at 37ºC. There was no difference in the MC540 spectra between groups (*Figure 14*A and 14B). Similarly, there was no difference in peak fluorescence between groups indicating a similar degree of lipid packing following I/R (*Figure 14*C). Mitochondrial supercomplex assembly (complexes I, III_2, and IV) was determined using BN-PAGE and was not different between groups (*Figure 14*C and 14D)

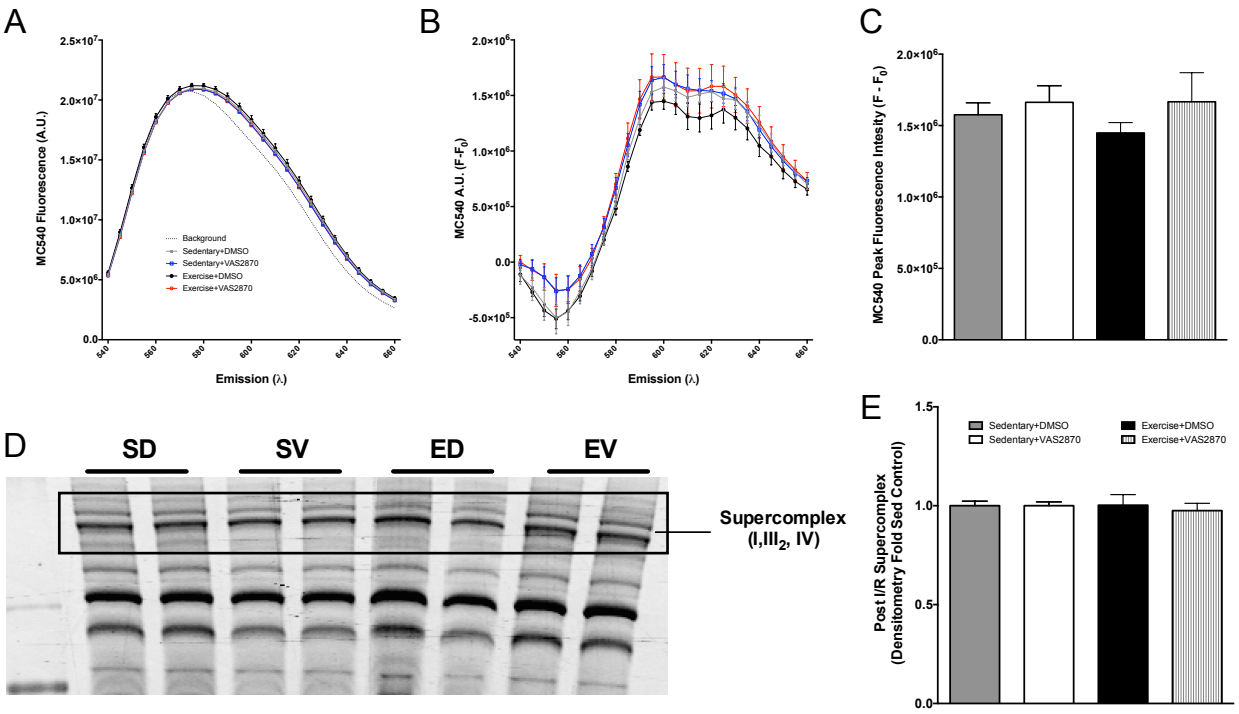

Figure 14. Mitochondrial membrane lipid properties and respiratory supercomplex assembly. (A) Merocyanine 540 (MC540) fluorescent spectra before and after the addition of mitochondria. Note the peak fluorescence red shift of the spectra indicates a lipid phase (B) Relative shift in MC540 fluorescence following the addition of mitochondria ($F-F_0$). C) Peak fluorescence intensity at 600nm. D) Representative image of supercomplex density via Blue-native PAGE; n=4 per group run in duplicate. The black box encloses supercomplex I, III_2, IV and used for quantification. E) Quantification of densitometry from supercomplex gel, normalized to control. All data are mean ± SEM, n=4 per group.

Discussion

The objective of the present study was to determine the role of NOX in exercise cardioprotection and the resultant phenotype of post-ischemic mitochondria. The main findings are: 1) Exercise cardioprotection against myocardial infarction and arrhythmia are abolished when cardiac NOX enzymes are inhibited during exercise with VAS2870, 2) post-ischemic mitochondrial complex I activity was higher, and H_2O_2 production was lower in isolated mitochondria from exercised hearts, and 3) exercise-induced cardioprotection occurs through a mechanism that is independent of changes in mitochondrial membrane lipid packing and respiratory supercomplex assembly.

Based on our previous work, the heart's adaptive response to exercise appears to be closely linked with improved maintenance of cellular redox homeostasis and preservation of mitochondrial membrane potential ($\Delta\Psi_m$) during ischemia/reperfusion injury (6, 80). These studies suggested that a stimulus ROS produced during exercise, and acute alterations to the redox environment, leads to an adaptive/protective phenotype with exercise training. However, the localization and compartmentalization of cellular redox biology increases the complexity of exercise-generated ROS in cardioprotective signaling. In an earlier study we injected rats with apocynin, a NOX-2 inhibitor, prior to exercise to demonstrate that NOX-generated ROS during exercise leads to protection against myocardial infarction. This adaptation was associated with the transient bouts of thiol stress experienced during exercise (80). However, apocynin has been questioned for its specificity and has been shown to act as a ROS scavenger in vascular systems (101). Also, several studies have demonstrated that VAS2870 is superior for its specificity of NOX inhibition (101, 181), and thus was chosen herein to further characterize the role of NOX-generated ROS in the mechanism of exercise cardioprotection.

Inhibition of NADPH-oxidase during exercise abolishes exercise cardioprotection

Administration of VAS2870 abolished the protective effect of exercise as measured by myocardial infarct size, arrhythmia, and time to contracture (*Figure* 12). The reduction in infarct size corroborates the loss of exercise-induced protection that we previously reported in exercised rats administered the NOX inhibitors (apocynin or VAS2870) prior to exercise (80), and builds on this by demonstrating that inhibition of NOX prevents the anti-arrhythmic phenotype observed with exercise. An increase in time to contracture during ischemia has previously been used as an indicator of preserved ATP homeostasis (113), and is consistent with the idea that exercise preserves cellular ATP and delays the onset of diastolic contracture . Furthermore, these findings support the hypothesis that mitochondria play a role in the protective phenotype, and that targeting mitochondrial function can influence the severity of pathology.

Isolated mitochondrial function from the post-ischemic heart

Mitochondrial function and ROS production has been shown to be a major determinant of cardiac ischemia/reperfusion injury (7, 26). Given the importance of ROS signaling in mediating mitochondrial adaptations in various cardioprotective paradigms we determined how inhibiting NOX-generated ROS affected post-ischemic mitochondrial function. Contrary to our hypothesis, an interesting finding from the study was that isolated mitochondria from exercised hearts exposed to I/R had lower mitochondrial respiratory rates under state-3 conditions when respiring on complex-I substrate (*Figure* 13B). Although not statistically significant in the ED vs SD groups, there was a trend for lower respiration, and this was statistically significant in the EV vs SV groups. This is not the first time we have observed similar complex-I linked respiratory rates in isolated mitochondria from post-ischemic hearts of sedentary and exercised rats (Rick Alleman unpublished data). These findings contrast those of Lee et al. who observed significantly higher complex-I dependent respiration from isolated mitochondria of exercised

rats. However they used a 40 min ischemia and 45 min reperfusion protocol in a Langendorff working-heart model (129), which may explain the discrepant findings. Our findings demonstrate that cardioprotection can be achieved in the absence of higher mitochondrial respiratory rates by complex I. However, this is not unprecedented, as other studies have noted little to no difference in complex-I linked mitochondrial respiration with remote ischemic preconditioning (RIP), another well-established cardioprotective model (75).

After observing lower complex-I linked respiratory rates in mitochondria from exercised rats we wanted to determine the activity of citrate synthase, as this has been used as a quantitative marker of intact mitochondria (102, 127). The activity of citrate synthase was not different across groups, confirming the loading of equal mitochondria in respiratory experiments. An interesting finding however, was that complex I activity was significantly higher only in the ED group (*Figure* 13D). While it is difficult to explain the discrepancy in complex I respiration vs complex I activity, one hypothesis is that mitochondria from exercised rats are more efficient and/or have lower demand by other energy consuming pathways such as the nicotinamide nucleotide transhydrogenase (NNT). Merit to this hypothesis comes from studies demonstrating that NNT can act as an "energy-consuming redox circuit" due to NADPH oxidation in redox reactions and subsequent reduction through NNT utilization of $\Delta\Psi_m$ (136). In addition, several redox-sensitive sites on complex I have been shown to decrease enzyme activity under heightened redox stress (106). Therefore, it is plausible that heightened complex I activity in the ED group is a marker for mitochondrial reserve capacity to scavenge ROS and metabolic demand matching of the cell. This hypothesis is in line with our data showing lower post-ischemic mitochondrial H_2O_2 emission in the ED group following I/R, and VAS2870 administration abolished the lower H_2O_2 emission observed with exercise alone (*Figure* 13E and 13F). Furthermore, the lower H_2O_2 emission observed in ED mirrored the reduction in infarction and protection against arrhythmia, both of which were abolished with VAS2870. Next, we

sought to determine if the lower H_2O_2 emission rates and heightened enzyme activity with exercise were due to a more fluid inner-mitochondrial membrane, and preserved assembly of supercomplexes. Both of which have been implicated in the cardioprotective mechanism of the mitochondrial targeting peptide, SS-31 (Unpublished data).

Post-ischemic mitochondrial membrane lipid packing and supercomplex assembly following exercise cardioprotection

Following I/R injury, no differences in lipid packing in cardiac mitochondria were observed between groups using MC540 (*Figure* 14C). We hypothesized that lower H_2O_2 production and preserved complex I activity in isolated mitochondria from exercised rats was due to a more fluid mitochondrial membrane and preserved respiratory supercomplex assembly. However, we also did not detect any differences in supercomplex assembly across groups (*Figure* 14D and 14E). A decrease in lipid packing was expected in the ED group, as this would indicate preservation of membrane polyunsaturated acyl chains and perhaps more abundant cardiolipin content. The structure, assembly, and composition of mitochondrial membrane phospholipids plays a significant role in mitochondrial bioenergetics under normal and pathological states (51). Altering the lipid composition of membranes can affect its biophysical properties, including membrane microviscosity as well as lateral diffusion and protein interaction.

The inner-mitochondrial membrane is mostly composed of three different phospholipids: phosphatidylcholine, phosphatidylethanolamine, and cardiolipin (165). Of these phospholipids, cardiolipin imparts a unique characteristic to the lipid environment due to its bicyclic structure and acyl chain orientation (133). Furthermore, cardiolipin has been shown to be important for respiratory supercomplex assembly (168) and optimal mitochondrial respiratory function (20). During I/R injury total cardiolipin content appears to decrease, which mirrors decrements in complex I activity and increased H_2O_2 production (167). Paradies et al. 2004 found that fusion of

exogenous cardiolipin with mitochondria from post-I/R hearts restored complex I activity to control non-ischemic levels (167). These previous studies demonstrate the importance of cardiolipin in mitochondrial function, yet it is unknown how exercise affects cardiolipin content in the post-ischemic heart.

Preserved mitochondrial membrane fluidity following I/R has been demonstrated using steady state fluorescence anisotropy of DPH in a cardioprotective remote ischemic preconditioning model (75). However as mentioned previously, this study also found no difference between RIP and I/R alone on state 3 mitochondrial respiration. In a more recent study by the same group, RIP once again preserved mitochondrial membrane fluidity following I/R using DPH (76). These studies support the idea of targeting the inner-membrane in acute coronary syndromes.

Limitations

In the present study non-I/R controls were not utilized for comparisons, however we have previously observed decrements in mitochondrial membrane fluidity, supercomplex breakdown, and perturbations in post-ischemic mitochondrial function (unpublished data). The MC540 probe was used this study because it has been extensively characterized in model membrane systems where it localizes to membranes and orients perpendicular to phospholipid chains (223, 230). However, one limitation of this technique is its localization to the membranes outer leaflet, and superficial positioning in relation to the inner membrane, where more exaggerated changes may occur. In addition, the interpretation of membrane properties must be done cautiously as changes in lipid packing with cardiolipin can induce divergent MC540 fluorescent spectra in the presence of different mixtures of lipid species (230). While we did not detect any differences across groups using MC540, complimentary measurements of fluorescence anisotropy using diphenylhexatriene (DPH) and/or fluorescence resonance energy transfer (FRET) probes would help determine the extent to which exercise-induced

cardioprotection involves alterations in biophysical properties of the inner-mitochondrial membrane.

Conclusions

It is widely accepted that exercise helps sustain mitochondrial function and cellular ATP levels during I/R (28, 129, 178). In the present study exercise cardioprotection resulted in higher complex I activity and lower H_2O_2 production in isolated mitochondria from post I/R hearts and this cardioprotective phenotype is lost with VAS2870 administration. While it seemed plausible that supercomplex assembly would be preserved in exercise as well, we did not observe any differences in respiratory supercomplex assembly across groups following I/R. Although this is not surprising based on the analysis of the mitochondrial lipid environment. Given this, it seems more likely that exercise-induced cardioprotection is a result of preserved biochemical mitochondrial adaptations, rather than biophysical alterations. However more research is needed to definitively determine if lipid microdomains are influenced by exercise, and to what extent these changes have on the cardioprotective phenotype.

Chapter 6: Integrated discussion

Major Findings

The central hypothesis of this work is that exercise-induced cardioprotection is a result of stabilization of mitochondrial bioenergetics and preservation of cellular thiol redox status. The findings from this work establish that the interdependence of cellular redox control and mitochondrial function is an important mediator of exercise-induced cardioprotection. Furthermore, modulation of glutathione reductase has a profound effect on cellular response to metabolic insult, and targeting this system is an attractive therapeutic target. Together these studies provide unprecedented insight into the metabolic abnormalities mitigated through exercise cardioprotection.

The work in this dissertation indicates that oxidative stress during I/R injury leads to mitochondrial dysfunction and a collapse in cellular energetics. In Chapter 3 it was shown that exercise prevents the collapse in energetics by decreasing the oxidative burden on the cell. Maintenance of GSH and lower production of ROS during early reperfusion was shown to be critical in stabilizing energetics and mediating the antiarrhythmic effect of exercise (*Figure* 3). The maintenance of GSH through enhanced redox buffering was linked to GR activity as it has previously been demonstrated that exercise increases cellular GR activity (80). In Chapter 4 the importance of GR on mitochondrial function was assessed through pharmacological inhibition or through adenovirus mediated alterations in Gsr expression. These studies provide unprecedented insight into the role of redox regulation on mitochondrial function during pathological oxidative stress conditions. Finally, in Chapter 5 we determined how inhibiting the cardioprotective ROS stimulus during exercise impacted post-ischemic mitochondria. We demonstrate that inhibition of NADPH oxidase (NOX) abolished exercise cardioprotection (*Figure* 12). However, the protective phenotype was not a result of alterations to the mitochondrial lipid environment, or preservation of supercomplex assembly. Rather, limiting the

production of ROS and maintenance of redox homeostasis through exercise-induced adaptations appeared to be more responsible for the protection against arrhythmia and infarct sparing observed with exercise.

The finding that exercise-induced cardioprotection does not operate through stabilization of mitochondrial supercomplexes (*Figure* 14) demonstrates divergent strategies for the protection of myocardium during I/R injury, as recent studies with mitochondrial targeting peptides have been hypothesized to protect the myocardium by stabilizing supercomplex assembly and preserving mitochondrial energetics (unpublished data). The discovery of potential therapeutics for the treatment of acute coronary syndromes is important with very few drugs reaching primary endpoints in clinical trials, and even fewer translating into clinical practice (100). Therefore, furthering our understanding on how redox biology regulates mitochondrial function is a critical step in advancing our knowledge on the treatment of I/R injury.

Future Directions

The studies presented herein demonstrate how the redox environment alters mitochondrial function, and that enhanced redox control may be a key underlying factor in the cardioprotective effect of exercise. In Chapter 3 it was demonstrated that maintenance of GSH was strongly associated with the stabilization of energetics and protection against arrhythmia, however it would be interesting to determine the extent to which exogenous GSH administration affects the observed collapse in $\Delta\Psi_m$ during reperfusion.

To address the role of the redox environment on mitochondrial energetics, in Chapter 4 we engineered adenovirus constructs aimed at altering Gsr expression in order to better understand the consequences of oxidative stress on the cardioprotective phenotype. Our preliminary studies demonstrate promising results in regard to modulating endogenous redox control mechanisms for the treatment of I/R injury. While these studies were conducted *in vitro*,

it is critical to better understand how manipulation of redox control through this approach translates to *in vivo* cardioprotection. Given the importance of the redox environment and GSH homeostasis on modulating cellular energetics, it will be interesting to determine if modulation of Gsr expression in animals exposed to exercise training are still able to maintain the cardioprotective phenotype observed under normal conditions.

Chapter 5 investigated how inhibiting NOX during exercise affected post-ischemic mitochondria, as we have previously observed a loss of exercise-induced cardioprotection with the NOX inhibitors apocynin and VAS2870 (80). This earlier study characterized how apocynin prevented the upregulation in GR activity observed with exercise, and did not fully characterize the affect of VAS2870 on GR activity. While VAS2870 has been shown to be a more specific inhibitor of NOX compared to apocynin (101, 181), it was assumed to have the same affect as apocynin on preventing the upregulation of GR activity following exercise. It is important to fully characterize the mechanism by which VAS2870 prevents the cardioprotective phenotype afforded by exercise. This may provide further insight into the "triggering" mechanisms of exercise cardioprotection, potentially leading to a new area of investigation.

While these studies implicate redox control in cardioprotective models, it is imperative to further our knowledge on how redox biology affects mitochondrial function, and what mechanisms may be at play in mediating the protection afforded by preserved redox homeostasis. Chapter 3 sought to address the mechanistic link between preserved redox homeostasis and maintenance of electrical activity in the post-ischemic myocardium, however more research is needed to fully characterize how loss of $\Delta\Psi_m$ scales to affect electrical conductivity in the intact heart. It is presumed that maintenance of energetics preserves cellular ATP levels, where ATP-regulatory sites on energy sensitive ions channels then mediate the protective phenotype. However, these assumptions need to be substantiated in the intact

myocardium in order to push the field toward more targeted therapeutics in the treatment of cardiovascular disease.

References

1. ***Ahsan MK, Lekli I, Ray D, Yodoi J, and Das DK***. *Redox regulation of cell survival by the thioredoxin superfamily: an implication of redox gene therapy in the heart. Antioxidants & redox signaling 11: 2741-2758, 2009.*

2. ***Aiello EA, Jabr RI, and Cole WC***. *Arrhythmia and delayed recovery of cardiac action potential during reperfusion after ischemia. Role of oxygen radical-induced no-reflow phenomenon. Circulation research 77: 153-162, 1995.*

3. ***Akar FG, Aon MA, Tomaselli GF, and O'Rourke B***. *The mitochondrial origin of postischemic arrhythmias. The Journal of clinical investigation 115: 3527-3535, 2005.*

4. ***Akita Y, Otani H, Matsuhisa S, Kyoi S, Enoki C, Hattori R, Imamura H, Kamihata H, Kimura Y, and Iwasaka T***. *Exercise-induced activation of cardiac sympathetic nerve triggers cardioprotection via redox-sensitive activation of eNOS and upregulation of iNOS. American journal of physiology Heart and circulatory physiology 292: H2051-2059, 2007.*

5. ***Alleman RJ, Katunga LA, Nelson MA, Brown DA, and Anderson EJ***. *The "Goldilocks Zone" from a redox perspective-Adaptive vs. deleterious responses to oxidative stress in striated muscle. Frontiers in physiology 5: 358, 2014.*

6. ***Alleman RJ, Tsang AM, Ryan TE, Patteson DJ, McClung JM, Spangenburg EE, Shaikh SR, Neufer PD, and Brown DA***. *Exercise-induced protection against reperfusion arrhythmia involves stabilization of mitochondrial energetics. American journal of physiology Heart and circulatory physiology ajpheart 00858 02015, 2016.*

7. ***Aluri HS, Simpson DC, Allegood JC, Hu Y, Szczepanek K, Gronert S, Chen Q, and Lesnefsky EJ***. *Electron flow into cytochrome c coupled with reactive oxygen species from the electron transport chain converts cytochrome c to a cardiolipin peroxidase: role during ischemia-reperfusion. Biochimica et biophysica acta 1840: 3199-3207, 2014.*

8. ***Aon MA, Cortassa S, Maack C, and O'Rourke B***. *Sequential opening of mitochondrial ion channels as a function of glutathione redox thiol status. The Journal of biological chemistry 282: 21889-21900, 2007.*

9. ***Aon MA, Cortassa S, Marban E, and O'Rourke B***. *Synchronized whole cell oscillations in mitochondrial metabolism triggered by a local release of reactive oxygen species in cardiac myocytes. The Journal of biological chemistry 278: 44735-44744, 2003.*

10. **Aon MA, Cortassa S, and O'Rourke B**. *Percolation and criticality in a mitochondrial network. Proceedings of the National Academy of Sciences of the United States of America 101: 4447-4452, 2004.*

11. **Aon MA, Stanley BA, Sivakumaran V, Kembro JM, O'Rourke B, Paolocci N, and Cortassa S**. *Glutathione/thioredoxin systems modulate mitochondrial H2O2 emission: an experimental-computational study. The Journal of general physiology 139: 479-491, 2012.*

12. **Arany Z, Foo SY, Ma Y, Ruas JL, Bommi-Reddy A, Girnun G, Cooper M, Laznik D, Chinsomboon J, Rangwala SM, Baek KH, Rosenzweig A, and Spiegelman BM**. *HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1alpha. Nature 451: 1008-1012, 2008.*

13. **Ascensao A, Ferreira R, and Magalhaes J**. *Exercise-induced cardioprotection--biochemical, morphological and functional evidence in whole tissue and isolated mitochondria. International journal of cardiology 117: 16-30, 2007.*

14. **Ascensao A, Magalhaes J, Soares JM, Ferreira R, Neuparth MJ, Marques F, Oliveira PJ, and Duarte JA**. *Endurance training limits the functional alterations of rat heart mitochondria submitted to in vitro anoxia-reoxygenation. International journal of cardiology 109: 169-178, 2006.*

15. **Babai L, Szigeti Z, Parratt JR, and Vegh A**. *Delayed cardioprotective effects of exercise in dogs are aminoguanidine sensitive: possible involvement of nitric oxide. Clinical science 102: 435-445, 2002.*

16. **Baines CP, Goto M, and Downey JM**. *Oxygen radicals released during ischemic preconditioning contribute to cardioprotection in the rabbit myocardium. Journal of molecular and cellular cardiology 29: 207-216, 1997.*

17. **Barbosa VA, Luciano TF, Marques SO, Vitto MF, Souza DR, Silva LA, Santos JP, Moreira JC, Dal-Pizzol F, Lira FS, Pinho RA, and De Souza CT**. *Acute exercise induce endothelial nitric oxide synthase phosphorylation via Akt and AMP-activated protein kinase in aorta of rats: Role of reactive oxygen species. International journal of cardiology 167: 2983-2988, 2013.*

18. **Bhushan S, Kondo K, Predmore BL, Zlatopolsky M, King AL, Pearce C, Huang H, Tao YX, Condit ME, and Lefer DJ**. *Selective beta2-adrenoreceptor stimulation attenuates myocardial cell death and preserves cardiac function after ischemia-reperfusion injury. Arteriosclerosis, thrombosis, and vascular biology 32: 1865-1874, 2012.*

19. **Bianchi P, Kunduzova O, Masini E, Cambon C, Bani D, Raimondi L, Seguelas MH, Nistri S, Colucci W, Leducq N, and Parini A**. *Oxidative stress by monoamine oxidase mediates receptor-independent cardiomyocyte apoptosis by serotonin and postischemic myocardial injury. Circulation 112: 3297-3305, 2005.*

20. **Birk AV, Chao WM, Bracken C, Warren JD, and Szeto HH**. *Targeting mitochondrial cardiolipin and the cytochrome c/cardiolipin complex to promote electron transport and optimize mitochondrial ATP synthesis. British journal of pharmacology 171: 2017-2028, 2014.*

21. **Blais C, Jr., Adam A, Massicotte D, and Peronnet F**. *Increase in blood bradykinin concentration after eccentric weight-training exercise in men. Journal of applied physiology 87: 1197-1201, 1999.*

22. **Blaustein A, Deneke SM, Stolz RI, Baxter D, Healey N, and Fanburg BL**. *Myocardial glutathione depletion impairs recovery after short periods of ischemia. Circulation 80: 1449-1457, 1989.*

23. **Bloch W, Addicks K, Hescheler J, and Fleischmann BK**. *Nitric oxide synthase expression and function in embryonic and adult cardiomyocytes. Microscopy research and technique 55: 259-269, 2001.*

24. **Bolli R**. *Oxygen-derived free radicals and postischemic myocardial dysfunction ("stunned myocardium"). Journal of the American College of Cardiology 12: 239-249, 1988.*

25. **Bolli R, Jeroudi MO, Patel BS, Aruoma OI, Halliwell B, Lai EK, and McCay PB**. *Marked reduction of free radical generation and contractile dysfunction by antioxidant therapy begun at the time of reperfusion. Evidence that myocardial "stunning" is a manifestation of reperfusion injury. Circulation research 65: 607-622, 1989.*

26. **Bolli R, Jeroudi MO, Patel BS, DuBose CM, Lai EK, Roberts R, and McCay PB**. *Direct evidence that oxygen-derived free radicals contribute to postischemic myocardial dysfunction in the intact dog. Proceedings of the National Academy of Sciences of the United States of America 86: 4695-4699, 1989.*

27. **Bowles DK, Farrar RP, and Starnes JW**. *Exercise training improves cardiac function after ischemia in the isolated, working rat heart. The American journal of physiology 263: H804-809, 1992.*

28. **Bowles DK, and Starnes JW**. *Exercise training improves metabolic response after ischemia in isolated working rat heart. Journal of applied physiology 76: 1608-1614, 1994.*

29. **Bowles DK, and Starnes JW**. *Exercise training improves metabolic response after ischemia in isolated working rat heart. Journal of applied physiology 76: 1608-1614, 1994.*

30. **Brown DA, Aon MA, Akar FG, Liu T, Sorarrain N, and O'Rourke B**. *Effects of 4'-chlorodiazepam on cellular excitation-contraction coupling and ischaemia-reperfusion injury in rabbit heart. Cardiovascular research 79: 141-149, 2008.*

31. **Brown DA, Aon MA, Frasier CR, Sloan RC, Maloney AH, Anderson EJ, and O'Rourke B**. *Cardiac arrhythmias induced by glutathione oxidation can be inhibited by preventing mitochondrial depolarization. Journal of molecular and cellular cardiology 48: 673-679, 2010.*

32. **Brown DA, Chicco AJ, Jew KN, Johnson MS, Lynch JM, Watson PA, and Moore RL**. *Cardioprotection afforded by chronic exercise is mediated by the sarcolemmal, and not the mitochondrial, isoform of the KATP channel in the rat. The Journal of physiology 569: 913-924, 2005.*

33. **Brown DA, Jew KN, Sparagna GC, Musch TI, and Moore RL**. *Exercise training preserves coronary flow and reduces infarct size after ischemia-reperfusion in rat heart. J Appl Physiol (1985) 95: 2510-2518, 2003.*

34. **Brown DA, Johnson MS, Armstrong CJ, Lynch JM, Caruso NM, Ehlers LB, Fleshner M, Spencer RL, and Moore RL**. *Short-term treadmill running in the rat: what kind of stressor is it? J Appl Physiol 103: 1979-1985, 2007.*

35. **Brown DA, Lynch JM, Armstrong CJ, Caruso NM, Ehlers LB, Johnson MS, and Moore RL**. *Susceptibility of the heart to ischaemia-reperfusion injury and exercise-induced cardioprotection are sex-dependent in the rat. The Journal of physiology 564: 619-630, 2005.*

36. **Brown DA, and Moore RL**. *Perspectives in innate and acquired cardioprotection: cardioprotection acquired through exercise. Journal of applied physiology 103: 1894-1899, 2007.*

37. **Brown JM, White CW, Terada LS, Grosso MA, Shanley PF, Mulvin DW, Banerjee A, Whitman GJ, Harken AH, and Repine JE**. *Interleukin 1 pretreatment decreases ischemia/reperfusion injury. Proceedings of the National Academy of Sciences of the United States of America 87: 5026-5030, 1990.*

38. **Bryan NS, Calvert JW, Elrod JW, Gundewar S, Ji SY, and Lefer DJ**. *Dietary nitrite supplementation protects against myocardial ischemia-reperfusion injury. Proceedings of the National Academy of Sciences of the United States of America 104: 19144-19149, 2007.*

39. ***Bryan NS, Fernandez BO, Bauer SM, Garcia-Saura MF, Milsom AB, Rassaf T, Maloney RE, Bharti A, Rodriguez J, and Feelisch M****. Nitrite is a signaling molecule and regulator of gene expression in mammalian tissues. Nature chemical biology 1: 290-297, 2005.*

40. ***Bulluck H, Yellon DM, and Hausenloy DJ****. Reducing myocardial infarct size: challenges and future opportunities. Heart 102: 341-348, 2016.*

41. ***Calvert JW, Condit ME, Aragon JP, Nicholson CK, Moody BF, Hood RL, Sindler AL, Gundewar S, Seals DR, Barouch LA, and Lefer DJ****. Exercise protects against myocardial ischemia-reperfusion injury via stimulation of beta(3)-adrenergic receptors and increased nitric oxide signaling: role of nitrite and nitrosothiols. Circulation research 108: 1448-1458, 2011.*

42. ***Calvert JW, and Lefer DJ****. Role of beta-adrenergic receptors and nitric oxide signaling in exercise-mediated cardioprotection. Physiology 28: 216-224, 2013.*

43. ***Ceconi C, Curello S, Cargnoni A, Ferrari R, Albertini A, and Visioli O****. The role of glutathione status in the protection against ischaemic and reperfusion damage: effects of N-acetyl cysteine. Journal of molecular and cellular cardiology 20: 5-13, 1988.*

44. ***Chandrasekera PC, McIntosh VJ, Cao FX, and Lasley RD****. Differential effects of adenosine A2a and A2b receptors on cardiac contractility. American journal of physiology Heart and circulatory physiology 299: H2082-2089, 2010.*

45. ***Chen CL, Zhang L, Yeh A, Chen CA, Green-Church KB, Zweier JL, and Chen YR****. Site-specific S-glutathiolation of mitochondrial NADH ubiquinone reductase. Biochemistry 46: 5754-5765, 2007.*

46. ***Chen J, Chen CL, Rawale S, Chen CA, Zweier JL, Kaumaya PT, and Chen YR****. Peptide-based antibodies against glutathione-binding domains suppress superoxide production mediated by mitochondrial complex I. The Journal of biological chemistry 285: 3168-3180, 2010.*

47. ***Chen YR, Chen CL, Pfeiffer DR, and Zweier JL****. Mitochondrial complex II in the post-ischemic heart: oxidative injury and the role of protein S-glutathionylation. The Journal of biological chemistry 282: 32640-32654, 2007.*

48. ***Chicco AJ, and Sparagna GC****. Role of cardiolipin alterations in mitochondrial dysfunction and disease. American journal of physiology Cell physiology 292: C33-44, 2007.*

49. ***Chouchani ET, Methner C, Nadtochiy SM, Logan A, Pell VR, Ding S, James AM, Cocheme HM, Reinhold J, Lilley KS, Partridge L, Fearnley IM, Robinson AJ, Hartley RC,***

Smith RA, Krieg T, Brookes PS, and Murphy MP. *Cardioprotection by S-nitrosation of a cysteine switch on mitochondrial complex I. Nature medicine 19: 753-759, 2013.*

50. ***Chouchani ET, Pell VR, Gaude E, Aksentijevic D, Sundier SY, Robb EL, Logan A, Nadtochiy SM, Ord EN, Smith AC, Eyassu F, Shirley R, Hu CH, Dare AJ, James AM, Rogatti S, Hartley RC, Eaton S, Costa AS, Brookes PS, Davidson SM, Duchen MR, Saeb-Parsy K, Shattock MJ, Robinson AJ, Work LM, Frezza C, Krieg T, and Murphy MP***. *Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. Nature 515: 431-435, 2014.*

51. ***Claypool SM, and Koehler CM***. *The complexity of cardiolipin in health and disease. Trends in biochemical sciences 37: 32-41, 2012.*

52. ***Cortassa S, Aon MA, Winslow RL, and O'Rourke B***. *A mitochondrial oscillator dependent on reactive oxygen species. Biophysical journal 87: 2060-2073, 2004.*

53. ***Cortassa S, O'Rourke B, and Aon MA***. *Redox-optimized ROS balance and the relationship between mitochondrial respiration and ROS. Biochimica et biophysica acta 1837: 287-295, 2014.*

54. ***Costa S, Reina-Couto M, Albino-Teixeira A, and Sousa T***. *Statins and oxidative stress in chronic heart failure. Revista portuguesa de cardiologia : orgao oficial da Sociedade Portuguesa de Cardiologia = Portuguese journal of cardiology : an official journal of the Portuguese Society of Cardiology 35: 41-57, 2016.*

55. ***Coven DL, Hu X, Cong L, Bergeron R, Shulman GI, Hardie DG, and Young LH***. *Physiological role of AMP-activated protein kinase in the heart: graded activation during exercise. American journal of physiology Endocrinology and metabolism 285: E629-636, 2003.*

56. ***Crompton M***. *The mitochondrial permeability transition pore and its role in cell death. The Biochemical journal 341 (Pt 2): 233-249, 1999.*

57. ***Cung TT, Morel O, Cayla G, Rioufol G, Garcia-Dorado D, Angoulvant D, Bonnefoy-Cudraz E, Guerin P, Elbaz M, Delarche N, Coste P, Vanzetto G, Metge M, Aupetit JF, Jouve B, Motreff P, Tron C, Labeque JN, Steg PG, Cottin Y, Range G, Clerc J, Claeys MJ, Coussement P, Prunier F, Moulin F, Roth O, Belle L, Dubois P, Barragan P, Gilard M, Piot C, Colin P, De Poli F, Morice MC, Ider O, Dubois-Rande JL, Unterseeh T, Le Breton H, Beard T, Blanchard D, Grollier G, Malquarti V, Staat P, Sudre A, Elmer E, Hansson MJ, Bergerot C, Boussaha I, Jossan C, Derumeaux G, Mewton N, and Ovize M***. *Cyclosporine before PCI in Patients with Acute Myocardial Infarction. The New England journal of medicine 373: 1021-1031, 2015.*

58. **Curtis MJ, Hancox JC, Farkas A, Wainwright CL, Stables CL, Saint DA, Clements-Jewery H, Lambiase PD, Billman GE, Janse MJ, Pugsley MK, Ng GA, Roden DM, Camm AJ, and Walker MJ**. *The Lambeth Conventions (II): guidelines for the study of animal and human ventricular and supraventricular arrhythmias. Pharmacology & therapeutics 139: 213-248, 2013.*

59. **Dabkowski ER, Williamson CL, and Hollander JM**. *Mitochondria-specific transgenic overexpression of phospholipid hydroperoxide glutathione peroxidase (GPx4) attenuates ischemia/reperfusion-associated cardiac dysfunction. Free radical biology & medicine 45: 855-865, 2008.*

60. **Debruille C, Luyckx M, Ballester L, Brunet C, Odou P, Dine T, Gressier B, Cazin M, and Cazin JC**. *Serum opioid activity after physical exercise in rats. Physiological research / Academia Scientiarum Bohemoslovaca 48: 129-133, 1999.*

61. **Demirel HA, Powers SK, Zergeroglu MA, Shanely RA, Hamilton K, Coombes J, and Naito H**. *Short-term exercise improves myocardial tolerance to in vivo ischemia-reperfusion in the rat. Journal of applied physiology 91: 2205-2212, 2001.*

62. **Demirel HA, Powers SK, Zergeroglu MA, Shanely RA, Hamilton K, Coombes J, and Naito H**. *Short-term exercise improves myocardial tolerance to in vivo ischemia-reperfusion in the rat. J Appl Physiol 91: 2205-2212, 2001.*

63. **Dickson EW, Hogrefe CP, Ludwig PS, Ackermann LW, Stoll LL, and Denning GM**. *Exercise enhances myocardial ischemic tolerance via an opioid receptor-dependent mechanism. American journal of physiology Heart and circulatory physiology 294: H402-408, 2008.*

64. **Dikalova AE, Bikineyeva AT, Budzyn K, Nazarewicz RR, McCann L, Lewis W, Harrison DG, and Dikalov SI**. *Therapeutic targeting of mitochondrial superoxide in hypertension. Circulation research 107: 106-116, 2010.*

65. **Domenech R, Macho P, Schwarze H, and Sanchez G**. *Exercise induces early and late myocardial preconditioning in dogs. Cardiovascular research 55: 561-566, 2002.*

66. **Domenech RJ, Macho P, Velez D, Sanchez G, Liu X, and Dhalla N**. *Tachycardia preconditions infarct size in dogs: role of adenosine and protein kinase C. Circulation 97: 786-794, 1998.*

67. **Donges CE, Duffield R, Smith GC, Short MJ, and Edge JA**. *Cytokine mRNA expression responses to resistance, aerobic, and concurrent exercise in sedentary middle-aged men.*

Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme 39: 130-137, 2014.

68. ***Donoso P, Finkelstein JP, Montecinos L, Said M, Sanchez G, Vittone L, and Bull R****. Stimulation of NOX2 in isolated hearts reversibly sensitizes RyR2 channels to activation by cytoplasmic calcium. Journal of molecular and cellular cardiology 68: 38-46, 2014.*

69. ***Dou Y, Arlock P, and Arner A****. Blebbistatin specifically inhibits actin-myosin interaction in mouse cardiac muscle. American journal of physiology Cell physiology 293: C1148-1153, 2007.*

70. ***Du G, Mouithys-Mickalad A, and Sluse FE****. Generation of superoxide anion by mitochondria and impairment of their functions during anoxia and reoxygenation in vitro. Free radical biology & medicine 25: 1066-1074, 1998.*

71. ***Du G, Willet K, Mouithys-Mickalad A, Sluse-Goffart CM, Droy-Lefaix MT, and Sluse FE****. EGb 761 protects liver mitochondria against injury induced by in vitro anoxia/reoxygenation. Free radical biology & medicine 27: 596-604, 1999.*

72. ***Eddy LJ, Goeddel DV, and Wong GH****. Tumor necrosis factor-alpha pretreatment is protective in a rat model of myocardial ischemia-reperfusion injury. Biochemical and biophysical research communications 184: 1056-1059, 1992.*

73. ***Farah C, Kleindienst A, Bolea G, Meyer G, Gayrard S, Geny B, Obert P, Cazorla O, Tanguy S, and Reboul C****. Exercise-induced cardioprotection: a role for eNOS uncoupling and NO metabolites. Basic research in cardiology 108: 389, 2013.*

74. ***Ferdaoussi M, Dai X, Jensen MV, Wang R, Peterson BS, Huang C, Ilkayeva O, Smith N, Miller N, Hajmrle C, Spigelman AF, Wright RC, Plummer G, Suzuki K, Mackay JP, van de Bunt M, Gloyn AL, Ryan TE, Norquay LD, Brosnan MJ, Trimmer JK, Rolph TP, Kibbey RG, Manning Fox JE, Colmers WF, Shirihai OS, Neufer PD, Yeh ET, Newgard CB, and MacDonald PE****. Isocitrate-to-SENP1 signaling amplifies insulin secretion and rescues dysfunctional beta cells. The Journal of clinical investigation 125: 3847-3860, 2015.*

75. ***Ferko M, Kancirova I, Jasova M, Carnicka S, Murarikova M, Waczulikova I, Sumbalova Z, Kucharska J, Ulicna O, Ravingerova T, and Ziegelhoffer A****. Remote ischemic preconditioning of the heart: protective responses in functional and biophysical properties of cardiac mitochondria. Physiological research / Academia Scientiarum Bohemoslovaca 63 Suppl 4: S469-478, 2014.*

76. ***Ferko M, Kancirova I, Jasova M, Waczulikova I, Carnicka S, Kucharska J, Ulicna O, Vancova O, Murarikova M, Ravingerova T, and Ziegelhoffer A****. Participation of heart mitochondria in myocardial protection against ischemia/reperfusion injury: benefit effects of*

short-term adaptation processes. Physiological research / Academia Scientiarum Bohemoslovaca 64 Suppl 5: S617-625, 2016.

77. ***Fisher-Wellman KH, and Neufer PD****. Linking mitochondrial bioenergetics to insulin resistance via redox biology. Trends in endocrinology and metabolism: TEM 23: 142-153, 2012.*

78. ***Fishman GI, Chugh SS, Dimarco JP, Albert CM, Anderson ME, Bonow RO, Buxton AE, Chen PS, Estes M, Jouven X, Kwong R, Lathrop DA, Mascette AM, Nerbonne JM, O'Rourke B, Page RL, Roden DM, Rosenbaum DS, Sotoodehnia N, Trayanova NA, and Zheng ZJ****. Sudden cardiac death prediction and prevention: report from a National Heart, Lung, and Blood Institute and Heart Rhythm Society Workshop. Circulation 122: 2335-2348, 2010.*

79. ***Frasier CR, Moore RL, and Brown DA****. Exercise-induced cardiac preconditioning: how exercise protects your achy-breaky heart. Journal of applied physiology 111: 905-915, 2011.*

80. ***Frasier CR, Moukdar F, Patel HD, Sloan RC, Stewart LM, Alleman RJ, La Favor JD, and Brown DA****. Redox-dependent increases in glutathione reductase and exercise preconditioning: role of NADPH oxidase and mitochondria. Cardiovascular research 98: 47-55, 2013.*

81. ***Frasier CR, Sloan RC, Bostian PA, Gonzon MD, Kurowicki J, Lopresto SJ, Anderson EJ, and Brown DA****. Short-term exercise preserves myocardial glutathione and decreases arrhythmias after thiol oxidation and ischemia in isolated rat hearts. Journal of applied physiology 111: 1751-1759, 2011.*

82. ***Frederich M, and Balschi JA****. The relationship between AMP-activated protein kinase activity and AMP concentration in the isolated perfused rat heart. The Journal of biological chemistry 277: 1928-1932, 2002.*

83. ***Fredholm BB, AP IJ, Jacobson KA, Linden J, and Muller CE****. International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors--an update. Pharmacological reviews 63: 1-34, 2011.*

84. ***French JP, Hamilton KL, Quindry JC, Lee Y, Upchurch PA, and Powers SK****. Exercise-induced protection against myocardial apoptosis and necrosis: MnSOD, calcium-handling proteins, and calpain. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 22: 2862-2871, 2008.*

85. ***Fryer RM, Hsu AK, Eells JT, Nagase H, and Gross GJ****. Opioid-induced second window of cardioprotection: potential role of mitochondrial KATP channels. Circulation research 84: 846-851, 1999.*

86. ***Galvao TF, Matos KC, Brum PC, Negrao CE, Luz PL, and Chagas AC****. Cardioprotection conferred by exercise training is blunted by blockade of the opioid system. Clinics 66: 151-157, 2011.*

87. ***Ganitkevich V, Reil S, Schwethelm B, Schroeter T, and Benndorf K****. Dynamic responses of single cardiomyocytes to graded ischemia studied by oxygen clamp in on-chip picochambers. Circulation research 99: 165-171, 2006.*

88. ***Garlid AO, Jaburek M, Jacobs JP, and Garlid KD****. Mitochondrial reactive oxygen species: which ROS signals cardioprotection? American journal of physiology Heart and circulatory physiology 305: H960-968, 2013.*

89. ***Gauthier C, Leblais V, Kobzik L, Trochu JN, Khandoudi N, Bril A, Balligand JL, and Le Marec H****. The negative inotropic effect of beta3-adrenoceptor stimulation is mediated by activation of a nitric oxide synthase pathway in human ventricle. The Journal of clinical investigation 102: 1377-1384, 1998.*

90. ***Gauthier LD, Greenstein JL, Cortassa S, O'Rourke B, and Winslow RL****. A computational model of reactive oxygen species and redox balance in cardiac mitochondria. Biophysical journal 105: 1045-1056, 2013.*

91. ***Ghezzi P****. Regulation of protein function by glutathionylation. Free Radic Res 39: 573-580, 2005.*

92. ***Gibson CM, Giugliano RP, Kloner RA, Bode C, Tendera M, Janosi A, Merkely B, Godlewski J, Halaby R, Korjian S, Daaboul Y, Chakrabarti AK, Spielman K, Neal BJ, and Weaver WD****. EMBRACE STEMI study: a Phase 2a trial to evaluate the safety, tolerability, and efficacy of intravenous MTP-131 on reperfusion injury in patients undergoing primary percutaneous coronary intervention. European heart journal 2015.*

93. ***Giustarini D, Dalle-Donne I, Milzani A, Fanti P, and Rossi R****. Analysis of GSH and GSSG after derivatization with N-ethylmaleimide. Nature protocols 8: 1660-1669, 2013.*

94. ***Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, Dai S, Ford ES, Fox CS, Franco S, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Huffman MD, Judd SE, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Mackey RH, Magid DJ, Marcus GM, Marelli A, Matchar DB, McGuire DK, Mohler ER, 3rd, Moy CS, Mussolino ME, Neumar RW, Nichol G, Pandey DK, Paynter NP, Reeves MJ, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Wong ND, Woo D, Turner MB, American Heart Association Statistics C, and Stroke Statistics S****. Executive summary: heart disease and stroke statistics--2014 update: a report from the American Heart Association. Circulation 129: 399-410, 2014.*

95. **Hamalainen H, Luurila OJ, Kallio V, and Knuts LR**. *Reduction in sudden deaths and coronary mortality in myocardial infarction patients after rehabilitation. 15 year follow-up study. European heart journal 16: 1839-1844, 1995.*

96. **Hamilton KL, Powers SK, Sugiura T, Kim S, Lennon S, Tumer N, and Mehta JL**. *Short-term exercise training can improve myocardial tolerance to I/R without elevation in heat shock proteins. American journal of physiology Heart and circulatory physiology 281: H1346-1352, 2001.*

97. **Hamilton KL, Quindry JC, French JP, Staib J, Hughes J, Mehta JL, and Powers SK**. *MnSOD antisense treatment and exercise-induced protection against arrhythmias. Free radical biology & medicine 37: 1360-1368, 2004.*

98. **Harzand A, Tamariz L, and Hare JM**. *Uric acid, heart failure survival, and the impact of xanthine oxidase inhibition. Congestive heart failure 18: 179-182, 2012.*

99. **Hasche ET, Fernandes C, Freedman SB, and Jeremy RW**. *Relation between ischemia time, infarct size, and left ventricular function in humans. Circulation 92: 710-719, 1995.*

100. **Hausenloy DJ, and Yellon DM**. *Myocardial ischemia-reperfusion injury: a neglected therapeutic target. The Journal of clinical investigation 123: 92-100, 2013.*

101. **Heumuller S, Wind S, Barbosa-Sicard E, Schmidt HH, Busse R, Schroder K, and Brandes RP**. *Apocynin is not an inhibitor of vascular NADPH oxidases but an antioxidant. Hypertension 51: 211-217, 2008.*

102. **Hood DA, Zak R, and Pette D**. *Chronic stimulation of rat skeletal muscle induces coordinate increases in mitochondrial and nuclear mRNAs of cytochrome-c-oxidase subunits. European journal of biochemistry / FEBS 179: 275-280, 1989.*

103. **Horwitz LD, Fennessey PV, Shikes RH, and Kong Y**. *Marked reduction in myocardial infarct size due to prolonged infusion of an antioxidant during reperfusion. Circulation 89: 1792-1801, 1994.*

104. **Hoshida S, Yamashita N, Otsu K, and Hori M**. *Repeated physiologic stresses provide persistent cardioprotection against ischemia-reperfusion injury in rats. Journal of the American College of Cardiology 40: 826-831, 2002.*

105. **Hull SS, Jr., Vanoli E, Adamson PB, Verrier RL, Foreman RD, and Schwartz PJ**. *Exercise training confers anticipatory protection from sudden death during acute myocardial ischemia. Circulation 89: 548-552, 1994.*

106. **Hurd TR, Requejo R, Filipovska A, Brown S, Prime TA, Robinson AJ, Fearnley IM, and Murphy MP**. *Complex I within oxidatively stressed bovine heart mitochondria is glutathionylated on Cys-531 and Cys-704 of the 75-kDa subunit: potential role of CYS residues in decreasing oxidative damage. The Journal of biological chemistry 283: 24801-24815, 2008.*

107. **Husain K, and Somani SM**. *Interaction of exercise and adenosine receptor agonist and antagonist on rat heart antioxidant defense system. Molecular and cellular biochemistry 270: 209-214, 2005.*

108. **Husain K, and Somani SM**. *Response of cardiac antioxidant system to alcohol and exercise training in the rat. Alcohol 14: 301-307, 1997.*

109. **Jacobson KA, Balasubramanian R, Deflorian F, and Gao ZG**. *G protein-coupled adenosine (P1) and P2Y receptors: ligand design and receptor interactions. Purinergic signalling 8: 419-436, 2012.*

110. **Jennings RB, Sommers HM, Smyth GA, Flack HA, and Linn H**. *Myocardial necrosis induced by temporary occlusion of a coronary artery in the dog. Archives of pathology 70: 68-78, 1960.*

111. **Jew KN, and Moore RL**. *Exercise training alters an anoxia-induced, glibenclamide-sensitive current in rat ventricular cardiocytes. J Appl Physiol 92: 1473-1479, 2002.*

112. **Jones DP**. *Radical-free biology of oxidative stress. American journal of physiology Cell physiology 295: C849-868, 2008.*

113. **Jones RN, Attarian DE, Currie WD, Olsen CO, Hill RC, Sink JD, and Wechsler AS**. *Metabolic deterioration during global ischemia as a function of time in the intact normal dog heart. The Journal of thoracic and cardiovascular surgery 81: 264-273, 1981.*

114. **Judge S, Jang YM, Smith A, Selman C, Phillips T, Speakman JR, Hagen T, and Leeuwenburgh C**. *Exercise by lifelong voluntary wheel running reduces subsarcolemmal and interfibrillar mitochondrial hydrogen peroxide production in the heart. American journal of physiology Regulatory, integrative and comparative physiology 289: R1564-1572, 2005.*

115. **Kakarla P, Vadluri G, Reddy KS, and Leeuwenburgh C**. *Vulnerability of the mid aged rat myocardium to the age-induced oxidative stress: influence of exercise training on antioxidant defense system. Free Radic Res 39: 1211-1217, 2005.*

116. **Kaludercic N, Carpi A, Menabo R, Di Lisa F, and Paolocci N**. *Monoamine oxidases (MAO) in the pathogenesis of heart failure and ischemia/reperfusion injury. Biochimica et biophysica acta 1813: 1323-1332, 2011.*

117. **Kand'ar R, Zakova P, Lotkova H, Kucera O, and Cervinkova Z**. *Determination of reduced and oxidized glutathione in biological samples using liquid chromatography with fluorimetric detection. Journal of pharmaceutical and biomedical analysis 43: 1382-1387, 2007.*

118. **Kang PT, Chen CL, Zen P, Guarini G, and Chen YR**. *BCNU-Induced gR2 DEFECT mediates S-glutathionylation OF Complex I and RESPIRATORY uncoupling in myocardium. Biochemical pharmacology 2014.*

119. **Kavazis AN, Alvarez S, Talbert E, Lee Y, and Powers SK**. *Exercise training induces a cardioprotective phenotype and alterations in cardiac subsarcolemmal and intermyofibrillar mitochondrial proteins. American journal of physiology Heart and circulatory physiology 297: H144-152, 2009.*

120. **Kavazis AN, McClung JM, Hood DA, and Powers SK**. *Exercise induces a cardiac mitochondrial phenotype that resists apoptotic stimuli. American journal of physiology Heart and circulatory physiology 294: H928-935, 2008.*

121. **Kembro JM, Aon MA, Winslow RL, O'Rourke B, and Cortassa S**. *Integrating mitochondrial energetics, redox and ROS metabolic networks: a two-compartment model. Biophysical journal 104: 332-343, 2013.*

122. **King N, Korolchuk S, McGivan JD, and Suleiman MS**. *A new method of quantifying glutathione levels in freshly isolated single superfused rat cardiomyocytes. Journal of pharmacological and toxicological methods 50: 215-222, 2004.*

123. **Kloner RA, Hale SL, Dai W, Gorman RC, Shuto T, Koomalsingh KJ, Gorman JH, 3rd, Sloan RC, Frasier CR, Watson CA, Bostian PA, Kypson AP, and Brown DA**. *Reduction of ischemia/reperfusion injury with bendavia, a mitochondria-targeting cytoprotective Peptide. Journal of the American Heart Association 1: e001644, 2012.*

124. **Kloner RA, and Jennings RB**. *Consequences of brief ischemia: stunning, preconditioning, and their clinical implications: part 2. Circulation 104: 3158-3167, 2001.*

125. **Kraljevic J, Marinovic J, Pravdic D, Zubin P, Dujic Z, Wisloff U, and Ljubkovic M**. *Aerobic interval training attenuates remodelling and mitochondrial dysfunction in the post-infarction failing rat heart. Cardiovascular research 99: 55-64, 2013.*

126. **Krumova SB, Koehorst RBM, Bóta A, Páli T, van Hoek A, Garab G, and van Amerongen H**. *Temperature dependence of the lipid packing in thylakoid membranes studied by time- and spectrally resolved fluorescence of Merocyanine 540. Biochimica et Biophysica Acta (BBA) - Biomembranes 1778: 2823-2833, 2008.*

127. **Kuznetsov AV, Strobl D, Ruttmann E, Konigsrainer A, Margreiter R, and Gnaiger E**. *Evaluation of mitochondrial respiratory function in small biopsies of liver. Analytical biochemistry 305: 186-194, 2002.*

128. **Lee IM, Sesso HD, and Paffenbarger RS, Jr.** *Physical activity and coronary heart disease risk in men: does the duration of exercise episodes predict risk? Circulation 102: 981-986, 2000.*

129. **Lee Y, Min K, Talbert EE, Kavazis AN, Smuder AJ, Willis WT, and Powers SK**. *Exercise protects cardiac mitochondria against ischemia-reperfusion injury. Medicine and science in sports and exercise 44: 397-405, 2012.*

130. **Leeuwenburgh C, Hollander J, Leichtweis S, Griffiths M, Gore M, and Ji LL**. *Adaptations of glutathione antioxidant system to endurance training are tissue and muscle fiber specific. The American journal of physiology 272: R363-369, 1997.*

131. **Lennon SL, Quindry J, Hamilton KL, French J, Staib J, Mehta JL, and Powers SK**. *Loss of exercise-induced cardioprotection after cessation of exercise. Journal of applied physiology 96: 1299-1305, 2004.*

132. **Lennon SL, Quindry JC, Hamilton KL, French JP, Hughes J, Mehta JL, and Powers SK**. *Elevated MnSOD is not required for exercise-induced cardioprotection against myocardial stunning. American journal of physiology Heart and circulatory physiology 287: H975-980, 2004.*

133. **Lewis RN, and McElhaney RN**. *The physicochemical properties of cardiolipin bilayers and cardiolipin-containing lipid membranes. Biochimica et biophysica acta 1788: 2069-2079, 2009.*

134. **Liu X, Miller MJ, Joshi MS, Sadowska-Krowicka H, Clark DA, and Lancaster JR, Jr.** *Diffusion-limited reaction of free nitric oxide with erythrocytes. The Journal of biological chemistry 273: 18709-18713, 1998.*

135. **Lopert P, Day BJ, and Patel M**. *Thioredoxin reductase deficiency potentiates oxidative stress, mitochondrial dysfunction and cell death in dopaminergic cells. PloS one 7: e50683, 2012.*

136. **Lopert P, and Patel M**. *Nicotinamide nucleotide transhydrogenase (Nnt) links the substrate requirement in brain mitochondria for hydrogen peroxide removal to the*

thioredoxin/peroxiredoxin (Trx/Prx) system. The Journal of biological chemistry 289: 15611-15620, 2014.

137. ***Lyon AR, Joudrey PJ, Jin D, Nass RD, Aon MA, O'Rourke B, and Akar FG****. Optical imaging of mitochondrial function uncovers actively propagating waves of mitochondrial membrane potential collapse across intact heart. Journal of molecular and cellular cardiology 49: 565-575, 2010.*

138. ***Maggirwar SB, Dhanraj DN, Somani SM, and Ramkumar V****. Adenosine acts as an endogenous activator of the cellular antioxidant defense system. Biochemical and biophysical research communications 201: 508-515, 1994.*

139. ***Mailloux RJ, and Willmore WG****. S-glutathionylation reactions in mitochondrial function and disease. Frontiers in cell and developmental biology 2: 68, 2014.*

140. ***Mallet RT, and Sun J****. Antioxidant properties of myocardial fuels. Molecular and cellular biochemistry 253: 103-111, 2003.*

141. ***Manning A, Bernier M, Crome R, Little S, and Hearse D****. Reperfusion-induced arrhythmias: a study of the role of xanthine oxidase-derived free radicals in the rat heart. Journal of molecular and cellular cardiology 20: 35-45, 1988.*

142. ***Maranzana E, Barbero G, Falasca AI, Lenaz G, and Genova ML****. Mitochondrial respiratory supercomplex association limits production of reactive oxygen species from complex I. Antioxidants & redox signaling 19: 1469-1480, 2013.*

143. ***Matsumoto-Ida M, Akao M, Takeda T, Kato M, and Kita T****. Real-time 2-photon imaging of mitochondrial function in perfused rat hearts subjected to ischemia/reperfusion. Circulation 114: 1497-1503, 2006.*

144. ***McCord JM****. Oxygen-derived free radicals in postischemic tissue injury. The New England journal of medicine 312: 159-163, 1985.*

145. ***Mewton N, Croisille P, Gahide G, Rioufol G, Bonnefoy E, Sanchez I, Cung TT, Sportouch C, Angoulvant D, Finet G, Andre-Fouet X, Derumeaux G, Piot C, Vernhet H, Revel D, and Ovize M****. Effect of cyclosporine on left ventricular remodeling after reperfused myocardial infarction. Journal of the American College of Cardiology 55: 1200-1205, 2010.*

146. ***Michel MC, Harding SE, and Bond RA****. Are there functional beta(3)-adrenoceptors in the human heart? British journal of pharmacology 162: 817-822, 2011.*

147. **Michelsen MM, Stottrup NB, Schmidt MR, Lofgren B, Jensen RV, Tropak M, St-Michel EJ, Redington AN, and Botker HE**. *Exercise-induced cardioprotection is mediated by a bloodborne, transferable factor. Basic research in cardiology 107: 260, 2012.*

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

149. **Mora S, Cook N, Buring JE, Ridker PM, and Lee IM**. *Physical activity and reduced risk of cardiovascular events: potential mediating mechanisms. Circulation 116: 2110-2118, 2007.*

150. **Motloch LJ, Hu J, and Akar FG**. *The mitochondrial translocator protein and arrhythmogenesis in ischemic heart disease. Oxidative medicine and cellular longevity 2015: 234104, 2015.*

151. **Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, Das SR, de Ferranti S, Despres JP, Fullerton HJ, Howard VJ, Huffman MD, Isasi CR, Jimenez MC, Judd SE, Kissela BM, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Magid DJ, McGuire DK, Mohler ER, 3rd, Moy CS, Muntner P, Mussolino ME, Nasir K, Neumar RW, Nichol G, Palaniappan L, Pandey DK, Reeves MJ, Rodriguez CJ, Rosamond W, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Woo D, Yeh RW, Turner MB, American Heart Association Statistics C, and Stroke Statistics S**. *Executive Summary: Heart Disease and Stroke Statistics-2016 Update: A Report From the American Heart Association. Circulation 133: 447-454, 2016.*

152. **Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, de Ferranti S, Despres J, Fullerton HJ, Howard VJ, Huffman MD, Judd SE, Kissela BM, Lackland DT, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Matchar DB, McGuire DK, Mohler ER, 3rd, Moy CS, Muntner P, Mussolino ME, Nasir K, Neumar RW, Nichol G, Palaniappan L, Pandey DK, Reeves MJ, Rodriguez CJ, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Willey JZ, Woo D, Yeh RW, and Turner MB**. *Heart Disease and Stroke Statistics-2015 Update: A Report From the American Heart Association. Circulation 2014.*

153. **Muntean DM, Kiss L, Jost N, and Baczko I**. *ATP-sensitive potassium channel modulators and cardiac arrhythmias: an update. Current pharmaceutical design 21: 1091-1102, 2015.*

154. **Murphy MP**. *How mitochondria produce reactive oxygen species. The Biochemical journal 417: 1-13, 2009.*

155. **Musi N, Hirshman MF, Arad M, Xing Y, Fujii N, Pomerleau J, Ahmad F, Berul CI, Seidman JG, Tian R, and Goodyear LJ**. *Functional role of AMP-activated protein kinase in the heart during exercise. FEBS letters 579: 2045-2050, 2005.*

156. **Napp A, Brixius K, Pott C, Ziskoven C, Boelck B, Mehlhorn U, Schwinger RH, and Bloch W**. *Effects of the beta3-adrenergic agonist BRL 37344 on endothelial nitric oxide synthase phosphorylation and force of contraction in human failing myocardium. Journal of cardiac failure 15: 57-67, 2009.*

157. **Nelson MJ, Harris MB, Boluyt MO, Hwang HS, and Starnes JW**. *Effect of N-2-mercaptopropionyl glycine on exercise-induced cardiac adaptations. American journal of physiology Regulatory, integrative and comparative physiology 300: R993-R1000, 2011.*

158. **Nichols M, Townsend N, Scarborough P, and Rayner M**. *Cardiovascular disease in Europe 2014: epidemiological update. European heart journal 35: 2950-2959, 2014.*

159. **Nieminen T, Scirica BM, Pegler JR, Tavares C, Pagotto VP, Kanas AF, Sobrado MF, Nearing BD, Umez-Eronini AA, Morrow DA, Belardinelli L, and Verrier RL**. *Relation of T-wave alternans to mortality and nonsustained ventricular tachycardia in patients with non-ST-segment elevation acute coronary syndrome from the MERLIN-TIMI 36 trial of ranolazine versus placebo. The American journal of cardiology 114: 17-23, 2014.*

160. **Nishida K, and Otsu K**. *Autophagy during cardiac remodeling. Journal of molecular and cellular cardiology 2015.*

161. **O'Rourke B, Ramza BM, and Marban E**. *Oscillations of membrane current and excitability driven by metabolic oscillations in heart cells. Science 265: 962-966, 1994.*

162. **Oberman A**. *Exercise and the primary prevention of cardiovascular disease. The American journal of cardiology 55: 10D-20D, 1985.*

163. **Ogura Y, Iemitsu M, Naito H, Kakigi R, Kakehashi C, Maeda S, and Akema T**. *Single bout of running exercise changes LC3-II expression in rat cardiac muscle. Biochemical and biophysical research communications 414: 756-760, 2011.*

164. **Orr AL, Ashok D, Sarantos MR, Shi T, Hughes RE, and Brand MD**. *Inhibitors of ROS production by the ubiquinone-binding site of mitochondrial complex I identified by chemical screening. Free radical biology & medicine 65: 1047-1059, 2013.*

165. **Osman C, Voelker DR, and Langer T**. *Making heads or tails of phospholipids in mitochondria. The Journal of cell biology 192: 7-16, 2011.*

166. **Pain T, Yang XM, Critz SD, Yue Y, Nakano A, Liu GS, Heusch G, Cohen MV, and Downey JM**. *Opening of mitochondrial K(ATP) channels triggers the preconditioned state by generating free radicals. Circulation research 87: 460-466, 2000.*

167. ***Paradies G, Petrosillo G, Pistolese M, Di Venosa N, Federici A, and Ruggiero FM****. Decrease in mitochondrial complex I activity in ischemic/reperfused rat heart: involvement of reactive oxygen species and cardiolipin. Circulation research 94: 53-59, 2004.*

168. ***Pfeiffer K, Gohil V, Stuart RA, Hunte C, Brandt U, Greenberg ML, and Schagger H****. Cardiolipin stabilizes respiratory chain supercomplexes. The Journal of biological chemistry 278: 52873-52880, 2003.*

169. ***Piot C, Croisille P, Staat P, Thibault H, Rioufol G, Mewton N, Elbelghiti R, Cung TT, Bonnefoy E, Angoulvant D, Macia C, Raczka F, Sportouch C, Gahide G, Finet G, Andre-Fouet X, Revel D, Kirkorian G, Monassier JP, Derumeaux G, and Ovize M****. Effect of cyclosporine on reperfusion injury in acute myocardial infarction. The New England journal of medicine 359: 473-481, 2008.*

170. ***Powers SK, Demirel HA, Vincent HK, Coombes JS, Naito H, Hamilton KL, Shanely RA, and Jessup J****. Exercise training improves myocardial tolerance to in vivo ischemia-reperfusion in the rat. The American journal of physiology 275: R1468-1477, 1998.*

171. ***Powers SK, Smuder AJ, Kavazis AN, and Quindry JC****. Mechanisms of exercise-induced cardioprotection. Physiology 29: 27-38, 2014.*

172. ***Prosser BL, Ward CW, and Lederer WJ****. X-ROS signaling: rapid mechano-chemo transduction in heart. Science 333: 1440-1445, 2011.*

173. ***Qin F, Siwik DA, Lancel S, Zhang J, Kuster GM, Luptak I, Wang L, Tong X, Kang YJ, Cohen RA, and Colucci WS****. Hydrogen peroxide-mediated SERCA cysteine 674 oxidation contributes to impaired cardiac myocyte relaxation in senescent mouse heart. Journal of the American Heart Association 2: e000184, 2013.*

174. ***Quindry JC, French J, Hamilton KL, Lee Y, Selsby J, and Powers S****. Exercise does not increase cyclooxygenase-2 myocardial levels in young or senescent hearts. The journal of physiological sciences : JPS 60: 181-186, 2010.*

175. ***Quindry JC, and Hamilton KL****. Exercise and cardiac preconditioning against ischemia reperfusion injury. Current cardiology reviews 9: 220-229, 2013.*

176. ***Quindry JC, Hamilton KL, French JP, Lee Y, Murlasits Z, Tumer N, and Powers SK****. Exercise-induced HSP-72 elevation and cardioprotection against infarct and apoptosis. Journal of applied physiology 103: 1056-1062, 2007.*

177. **Quindry JC, Miller L, McGinnis G, Kliszczewicz B, Irwin JM, Landram M, Urbiztondo Z, Nanayakkara G, and Amin R**. *Ischemia reperfusion injury, KATP channels, and exercise-induced cardioprotection against apoptosis. Journal of applied physiology 113: 498-506, 2012.*

178. **Quindry JC, Schreiber L, Hosick P, Wrieden J, Irwin JM, and Hoyt E**. *Mitochondrial KATP channel inhibition blunts arrhythmia protection in ischemic exercised hearts. American journal of physiology Heart and circulatory physiology 299: H175-183, 2010.*

179. **Rahkila P, Hakala E, Alen M, Salminen K, and Laatikainen T**. *Beta-endorphin and corticotropin release is dependent on a threshold intensity of running exercise in male endurance athletes. Life sciences 43: 551-558, 1988.*

180. **Ramires PR, and Ji LL**. *Glutathione supplementation and training increases myocardial resistance to ischemia-reperfusion in vivo. American journal of physiology Heart and circulatory physiology 281: H679-688, 2001.*

181. **Rehman AU, Dugic E, Benham C, Lione L, and Mackenzie LS**. *Selective inhibition of NADPH oxidase reverses the over contraction of diabetic rat aorta. Redox biology 2C: 61-64, 2013.*

182. **Richters L, Lange N, Renner R, Treiber N, Ghanem A, Tiemann K, Scharffetter-Kochanek K, Bloch W, and Brixius K**. *Exercise-induced adaptations of cardiac redox homeostasis and remodeling in heterozygous SOD2-knockout mice. Journal of applied physiology 111: 1431-1440, 2011.*

183. **Russell RR, 3rd, Li J, Coven DL, Pypaert M, Zechner C, Palmeri M, Giordano FJ, Mu J, Birnbaum MJ, and Young LH**. *AMP-activated protein kinase mediates ischemic glucose uptake and prevents postischemic cardiac dysfunction, apoptosis, and injury. The Journal of clinical investigation 114: 495-503, 2004.*

184. **Sadoshima J**. *Redox regulation of growth and death in cardiac myocytes. Antioxidants & redox signaling 8: 1621-1624, 2006.*

185. **Saini HK, Xu YJ, Zhang M, Liu PP, Kirshenbaum LA, and Dhalla NS**. *Role of tumour necrosis factor-alpha and other cytokines in ischemia-reperfusion-induced injury in the heart. Experimental and clinical cardiology 10: 213-222, 2005.*

186. **Sanchez G, Escobar M, Pedrozo Z, Macho P, Domenech R, Hartel S, Hidalgo C, and Donoso P**. *Exercise and tachycardia increase NADPH oxidase and ryanodine receptor-2 activity: possible role in cardioprotection. Cardiovascular research 77: 380-386, 2008.*

187. **Sato T, Sasaki N, O'Rourke B, and Marban E**. *Adenosine primes the opening of mitochondrial ATP-sensitive potassium channels: a key step in ischemic preconditioning? Circulation 102: 800-805, 2000.*

188. **Schafer FQ, and Buettner GR**. *Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free radical biology & medicine 30: 1191-1212, 2001.*

189. **Schagger H**. *Respiratory chain supercomplexes of mitochondria and bacteria. Biochimica et biophysica acta 1555: 154-159, 2002.*

190. **Schultz Je-J, Hsu AK, Nagase H, and Gross GJ**. *TAN-67, a delta 1-opioid receptor agonist, reduces infarct size via activation of Gi/o proteins and KATP channels. The American journal of physiology 274: H909-914, 1998.*

191. **Schwarz L, and Kindermann W**. *Changes in beta-endorphin levels in response to aerobic and anaerobic exercise. Sports medicine 13: 25-36, 1992.*

192. **Seefeldt T, Zhao Y, Chen W, Raza AS, Carlson L, Herman J, Stoebner A, Hanson S, Foll R, and Guan X**. *Characterization of a novel dithiocarbamate glutathione reductase inhibitor and its use as a tool to modulate intracellular glutathione. The Journal of biological chemistry 284: 2729-2737, 2009.*

193. **Sessa WC, Pritchard K, Seyedi N, Wang J, and Hintze TH**. *Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. Circulation research 74: 349-353, 1994.*

194. **Shaikh SR, Sullivan EM, Alleman RJ, Brown DA, and Zeczycki TN**. *Increasing mitochondrial membrane phospholipid content lowers the enzymatic activity of electron transport complexes. Biochemistry 53: 5589-5591, 2014.*

195. **Shiomi T, Tsutsui H, Matsusaka H, Murakami K, Hayashidani S, Ikeuchi M, Wen J, Kubota T, Utsumi H, and Takeshita A**. *Overexpression of glutathione peroxidase prevents left ventricular remodeling and failure after myocardial infarction in mice. Circulation 109: 544-549, 2004.*

196. **Singh A, Lee KJ, Lee CY, Goldfarb RD, and Tsan MF**. *Relation between myocardial glutathione content and extent of ischemia-reperfusion injury. Circulation 80: 1795-1804, 1989.*

197. **Sloan RC, Moukdar F, Frasier CR, Patel HD, Bostian PA, Lust RM, and Brown DA**. *Mitochondrial permeability transition in the diabetic heart: contributions of thiol redox state and*

mitochondrial calcium to augmented reperfusion injury. Journal of molecular and cellular cardiology 52: 1009-1018, 2012.

198. ***Slodzinski MK, Aon MA, and O'Rourke B****. Glutathione oxidation as a trigger of mitochondrial depolarization and oscillation in intact hearts. Journal of molecular and cellular cardiology 45: 650-660, 2008.*

199. ***Sorita A, Ahmed A, Starr SR, Thompson KM, Reed DA, Prokop L, Shah ND, Murad MH, and Ting HH****. Off-hour presentation and outcomes in patients with acute myocardial infarction: systematic review and meta-analysis. Bmj 348: f7393, 2014.*

200. ***Springhorn JP, and Claycomb WC****. Preproenkephalin mRNA expression in developing rat heart and in cultured ventricular cardiac muscle cells. The Biochemical journal 258: 73-78, 1989.*

201. ***Starnes JW, Barnes BD, and Olsen ME****. Exercise training decreases rat heart mitochondria free radical generation but does not prevent Ca2+-induced dysfunction. Journal of applied physiology 102: 1793-1798, 2007.*

202. ***Starnes JW, and Taylor RP****. Exercise-induced cardioprotection: endogenous mechanisms. Medicine and science in sports and exercise 39: 1537-1543, 2007.*

203. ***Steinberg SF****. The molecular basis for distinct beta-adrenergic receptor subtype actions in cardiomyocytes. Circulation research 85: 1101-1111, 1999.*

204. ***Sukhodub A, Jovanovic S, Du Q, Budas G, Clelland AK, Shen M, Sakamoto K, Tian R, and Jovanovic A****. AMP-activated protein kinase mediates preconditioning in cardiomyocytes by regulating activity and trafficking of sarcolemmal ATP-sensitive K(+) channels. Journal of cellular physiology 210: 224-236, 2007.*

205. ***Tavernier G, Toumaniantz G, Erfanian M, Heymann MF, Laurent K, Langin D, and Gauthier C****. beta3-Adrenergic stimulation produces a decrease of cardiac contractility ex vivo in mice overexpressing the human beta3-adrenergic receptor. Cardiovascular research 59: 288-296, 2003.*

206. ***Taylor RP, Harris MB, and Starnes JW****. Acute exercise can improve cardioprotection without increasing heat shock protein content. The American journal of physiology 276: H1098-1102, 1999.*

207. **Taylor RP, Olsen ME, and Starnes JW**. *Improved postischemic function following acute exercise is not mediated by nitric oxide synthase in the rat heart. American journal of physiology Heart and circulatory physiology 292: H601-607, 2007.*

208. **Taylor RP, and Starnes JW**. *Reactive oxygen species are not a required trigger for exercise-induced late preconditioning in the rat heart. American journal of physiology Regulatory, integrative and comparative physiology 303: R968-974, 2012.*

209. **Tritto I, Duilio C, Santoro G, Elia PP, Cirillo P, De Simone C, Chiariello M, and Ambrosio G**. *A short burst of oxygen radicals at reflow induces sustained release of oxidized glutathione from postischemic hearts. Free radical biology & medicine 24: 290-297, 1998.*

210. **Tsutsui H, Kinugawa S, and Matsushima S**. *Oxidative stress and heart failure. American journal of physiology Heart and circulatory physiology 301: H2181-2190, 2011.*

211. **van den Brink OW, Delbridge LM, Rosenfeldt FL, Penny D, Esmore DS, Quick D, Kaye DM, and Pepe S**. *Endogenous cardiac opioids: enkephalins in adaptation and protection of the heart. Heart, lung & circulation 12: 178-187, 2003.*

212. **Vegh A, and Parratt JR**. *A common mechanism in the protective effects of preconditioning, cardiac pacing and physical exercise against ischemia and reperfusion-induced arrhythmias. Experimental and clinical cardiology 10: 200-205, 2005.*

213. **Venditti P, and Di Meo S**. *Antioxidants, tissue damage, and endurance in trained and untrained young male rats. Archives of biochemistry and biophysics 331: 63-68, 1996.*

214. **Venditti P, Masullo P, and Di Meo S**. *Effects of myocardial ischemia and reperfusion on mitochondrial function and susceptibility to oxidative stress. Cellular and molecular life sciences : CMLS 58: 1528-1537, 2001.*

215. **Vidal M, Wieland T, Lohse MJ, and Lorenz K**. *beta-Adrenergic receptor stimulation causes cardiac hypertrophy via a Gbetagamma/Erk-dependent pathway. Cardiovascular research 96: 255-264, 2012.*

216. **Wachtell K, Okin PM, Olsen MH, Dahlof B, Devereux RB, Ibsen H, Kjeldsen SE, Lindholm LH, Nieminen MS, and Thygesen K**. *Regression of electrocardiographic left ventricular hypertrophy during antihypertensive therapy and reduction in sudden cardiac death: the LIFE Study. Circulation 116: 700-705, 2007.*

217. **Wang J, Wolin MS, and Hintze TH**. *Chronic exercise enhances endothelium-mediated dilation of epicardial coronary artery in conscious dogs. Circulation research 73: 829-838, 1993.*

218. ***Ward CW, Prosser BL, and Lederer WJ***. *Mechanical stretch-induced activation of ROS/RNS signaling in striated muscle. Antioxidants & redox signaling 20: 929-936, 2014.*

219. ***Watkinson WP, Foley DH, Rubio R, and Berne RM***. *Myocardial adenosine formation with increased cardiac performance in the dog. The American journal of physiology 236: H13-21, 1979.*

220. ***Webb A, Bond R, McLean P, Uppal R, Benjamin N, and Ahluwalia A***. *Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage. Proceedings of the National Academy of Sciences of the United States of America 101: 13683-13688, 2004.*

221. ***Weil J, Zolk O, Griepentrog J, Wenzel U, Zimmermann WH, and Eschenhagen T***. *Alterations of the preproenkephalin system in cardiac hypertrophy and its role in atrioventricular conduction. Cardiovascular research 69: 412-422, 2006.*

222. ***Willet K, Detry O, and Sluse FE***. *Resistance of isolated pulmonary mitochondria during in vitro anoxia/reoxygenation. Biochimica et biophysica acta 1460: 346-352, 2000.*

223. ***Wilson-Ashworth HA, Bahm Q, Erickson J, Shinkle A, Vu MP, Woodbury D, and Bell JD***. *Differential detection of phospholipid fluidity, order, and spacing by fluorescence spectroscopy of bis-pyrene, prodan, nystatin, and merocyanine 540. Biophysical journal 91: 4091-4101, 2006.*

224. ***Won SJ, Kim JE, Cittolin-Santos GF, and Swanson RA***. *Assessment at the single-cell level identifies neuronal glutathione depletion as both a cause and effect of ischemia-reperfusion oxidative stress. The Journal of neuroscience : the official journal of the Society for Neuroscience 35: 7143-7152, 2015.*

225. ***Xie C, Kauffman J, and Akar FG***. *Functional crosstalk between the mitochondrial PTP and KATP channels determine arrhythmic vulnerability to oxidative stress. Frontiers in physiology 5: 264, 2014.*

226. ***Xie J, Potter A, Xie W, Lynch C, and Seefeldt T***. *Evaluation of a dithiocarbamate derivative as a model of thiol oxidative stress in H9c2 rat cardiomyocytes. Free radical biology & medicine 70: 214-222, 2014.*

227. ***Yamashita N, Hoshida S, Otsu K, Asahi M, Kuzuya T, and Hori M***. *Exercise provides direct biphasic cardioprotection via manganese superoxide dismutase activation. The Journal of experimental medicine 189: 1699-1706, 1999.*

228. ***Yan XS, Ma JH, and Zhang PH***. *Modulation of K(ATP) currents in rat ventricular myocytes by hypoxia and a redox reaction. Acta pharmacologica Sinica 30: 1399-1414, 2009.*

229. ***Yu L, Fink BD, Herlein JA, and Sivitz WI***. *Mitochondrial function in diabetes: novel methodology and new insight. Diabetes 62: 1833-1842, 2013.*

230. ***Zeczycki TN, Whelan J, Hayden WT, Brown DA, and Shaikh SR***. *Increasing levels of cardiolipin differentially influence packing of phospholipids found in the mitochondrial inner membrane. Biochemical and biophysical research communications 450: 366-371, 2014.*

231. ***Zhang QJ, McMillin SL, Tanner JM, Palionyte M, Abel ED, and Symons JD***. *Endothelial nitric oxide synthase phosphorylation in treadmill-running mice: role of vascular signalling kinases. The Journal of physiology 587: 3911-3920, 2009.*

232. ***Zhou L, Aon MA, Liu T, and O'Rourke B***. *Dynamic modulation of Ca2+ sparks by mitochondrial oscillations in isolated guinea pig cardiomyocytes under oxidative stress. Journal of molecular and cellular cardiology 51: 632-639, 2011.*

233. ***Zhou L, Cortassa S, Wei AC, Aon MA, Winslow RL, and O'Rourke B***. *Modeling cardiac action potential shortening driven by oxidative stress-induced mitochondrial oscillations in guinea pig cardiomyocytes. Biophysical journal 97: 1843-1852, 2009.*

234. ***Zhou M, Widmer RJ, Xie W, Jimmy Widmer A, Miller MW, Schroeder F, Parker JL, and Heaps CL***. *Effects of exercise training on cellular mechanisms of endothelial nitric oxide synthase regulation in coronary arteries after chronic occlusion. American journal of physiology Heart and circulatory physiology 298: H1857-1869, 2010.*

235. ***Zorov DB, Filburn CR, Klotz LO, Zweier JL, and Sollott SJ***. *Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. The Journal of experimental medicine 192: 1001-1014, 2000.*

236. ***Zorov DB, Juhaszova M, and Sollott SJ***. *Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. Physiological reviews 94: 909-950, 2014.*

237. ***Zweier JL, Flaherty JT, and Weisfeldt ML***. *Direct measurement of free radical generation following reperfusion of ischemic myocardium. Proceedings of the National Academy of Sciences of the United States of America 84: 1404-1407, 1987.*

238. ***Zweier JL, Wang P, Samouilov A, and Kuppusamy P***. *Enzyme-independent formation of nitric oxide in biological tissues. Nature medicine 1: 804-809, 1995.*

Appendix A: Animal care and use protocol approval

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

August 7, 2012

David Brown, Ph.D.
Department of Physiology
Brody 6N-98
ECU Brody School of Medicine

Dear Dr. Brown:

Your Animal Use Protocol entitled, "Cardiac Ischemic Injury in Rat Myocardium: Influence of Exercise Training and Sex on Mitochondrial Function" (AUP #Q279a) was reviewed by this institution's Animal Care and Use Committee on 8/7/12. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

enclosure

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

June 10, 2015

David Brown, Ph.D.
Department of Physiology
ECHI 4th floor
ECU Brody School of Medicine

Dear Dr. Brown:

Your Animal Use Protocol entitled, "Cardiac Ischemic Injury in Rat Myocardium" (AUP #Q279b) was reviewed by this institution's Animal Care and Use Committee on 6/10/15. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure

Appendix B: Figure reproduction

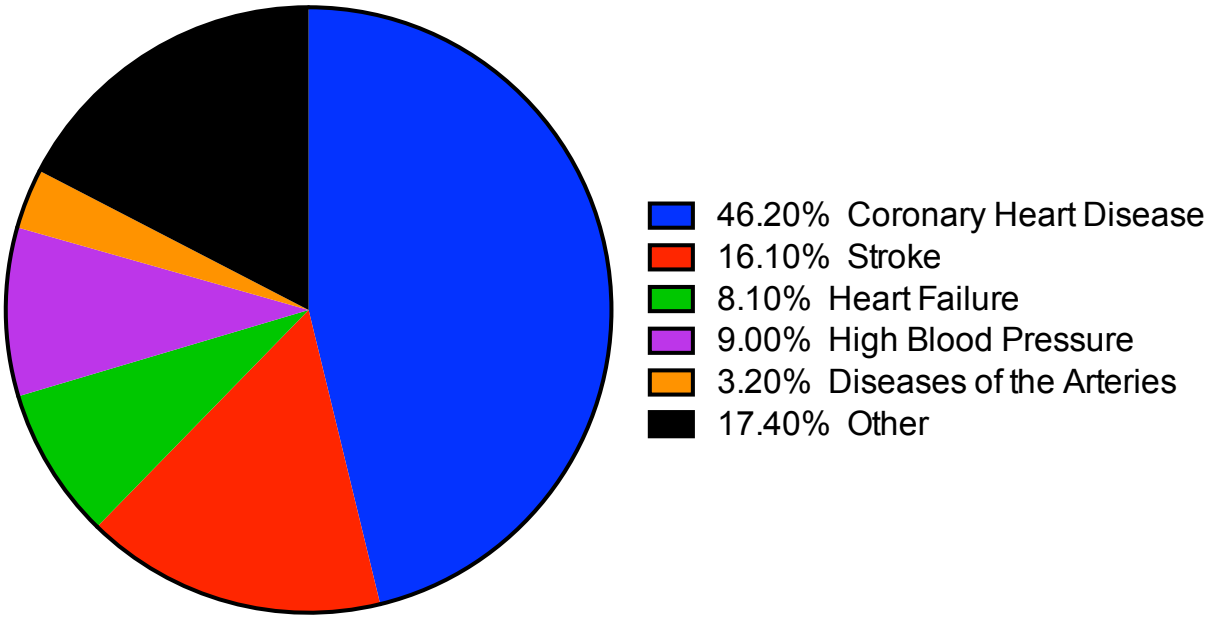

Figure 1. Breakdown of deaths attributable to CVD in the United States (2013).

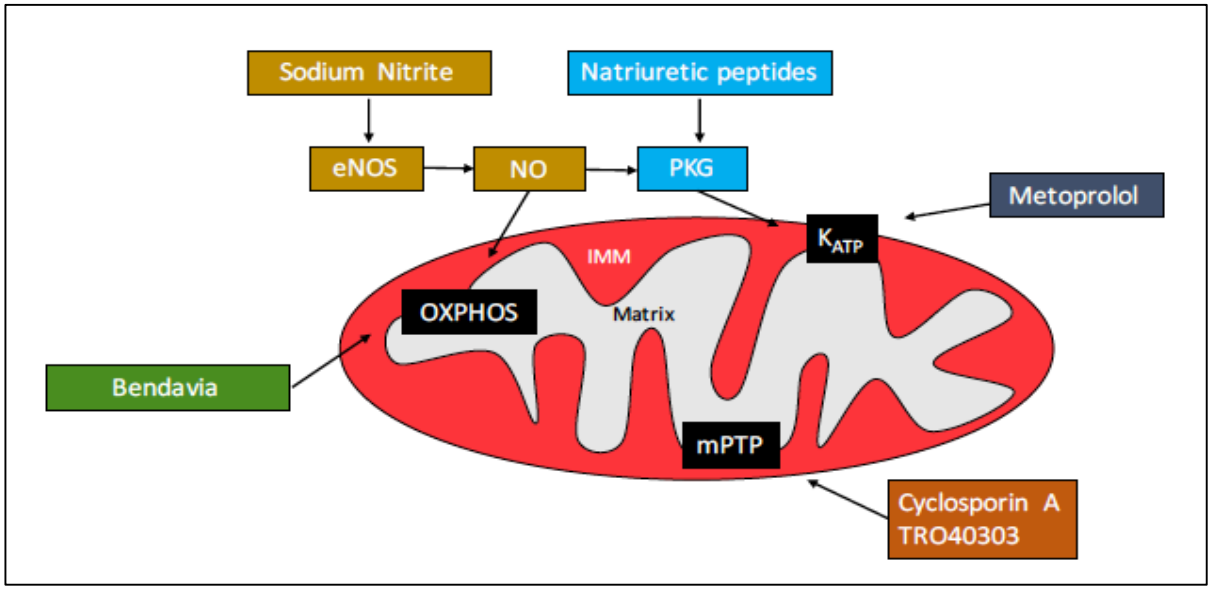

Figure 2. Mitochondrial therapies recently investigated in clinical studies to reduce myocardial infarction.

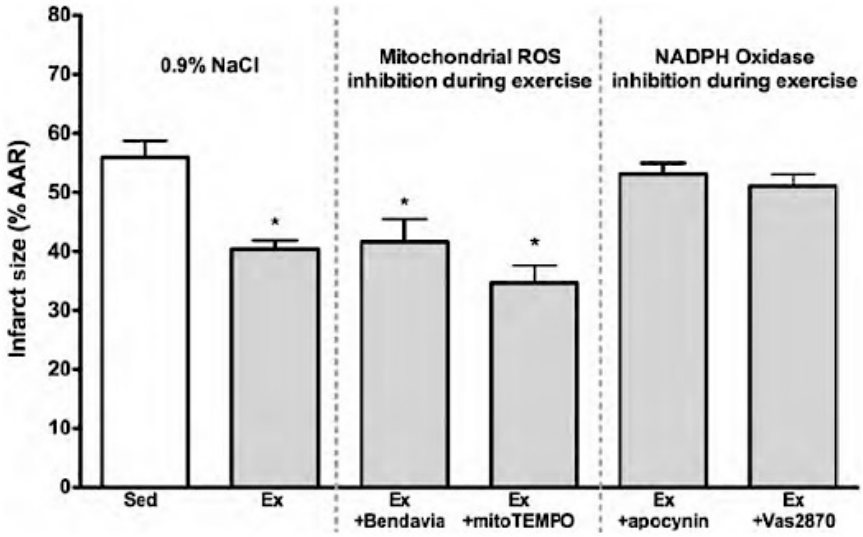

Figure 3. Reductions in infarct size are abolished by inhibiting NADPH Oxidase (with pre-exericse treatment of apocynin or VAS2870) during exercise. Inhibition of mitochondrial ROS during exercise (with pre-exercise administration of TEMPO or the mitochondria-targeting peptide Bendavia) had no effects on exercise

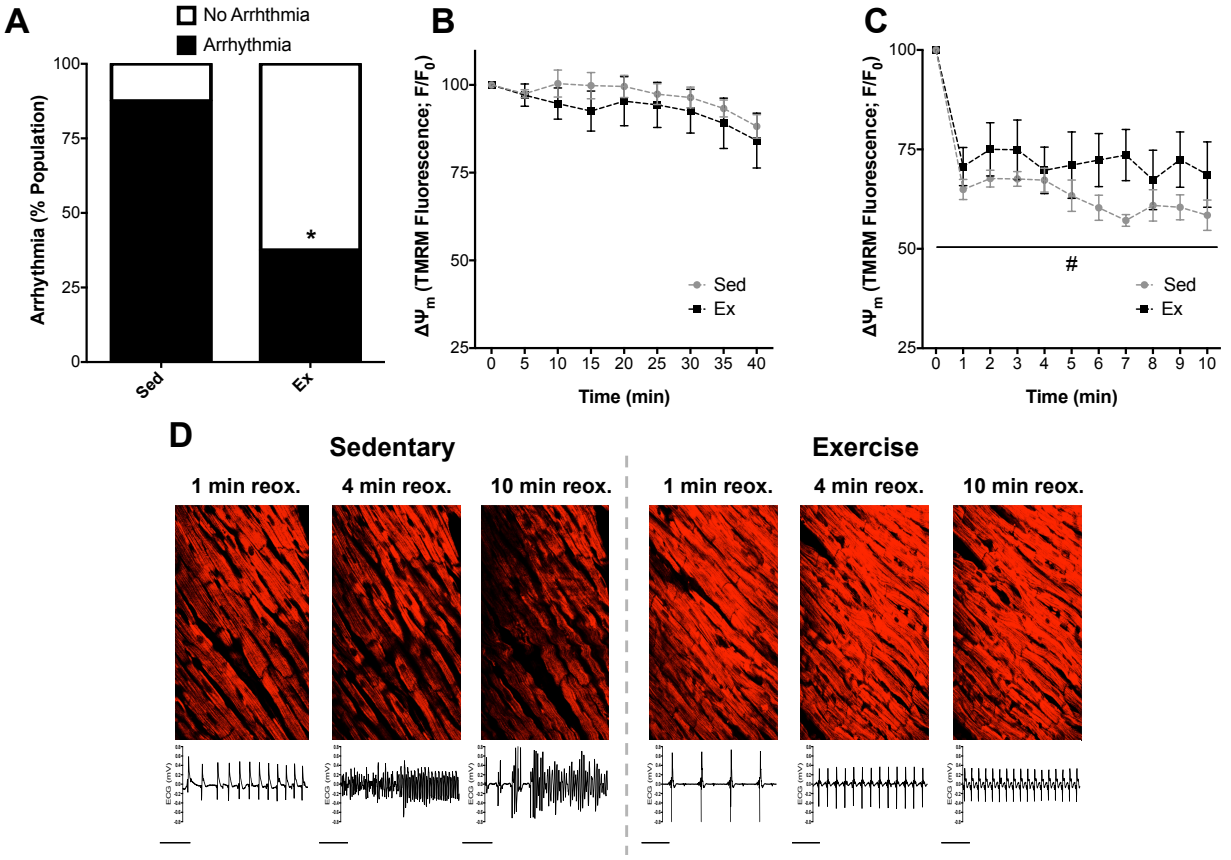

Figure 4. Arrhythmia and simultaneous two-photon imaging of mitochondrial membrane potential ($\Delta\Psi_m$) in isolated hearts during ischemia/reperfusion. (A) The percentage of Ex and Sed hearts that transitioned to arrhythmia (VT/VF) following 40 min of ischemia. (B&C) Baseline TMRM fluorescence ($\Delta\Psi_m$) values were used to normalize all data (F/F_0) during ischemia (B) and reperfusion (C). (D) Representative images of $\Delta\Psi_m$ in the ventricular free-wall and simultaneous ECG recordings during reperfusion for Sed and Ex. Data are shown as % of population for arrhythmia and mean ± SEM for all other data, n=7-8 per group. * $p<0.05$ vs Sed; # $p<0.05$ vs Sed main effect.

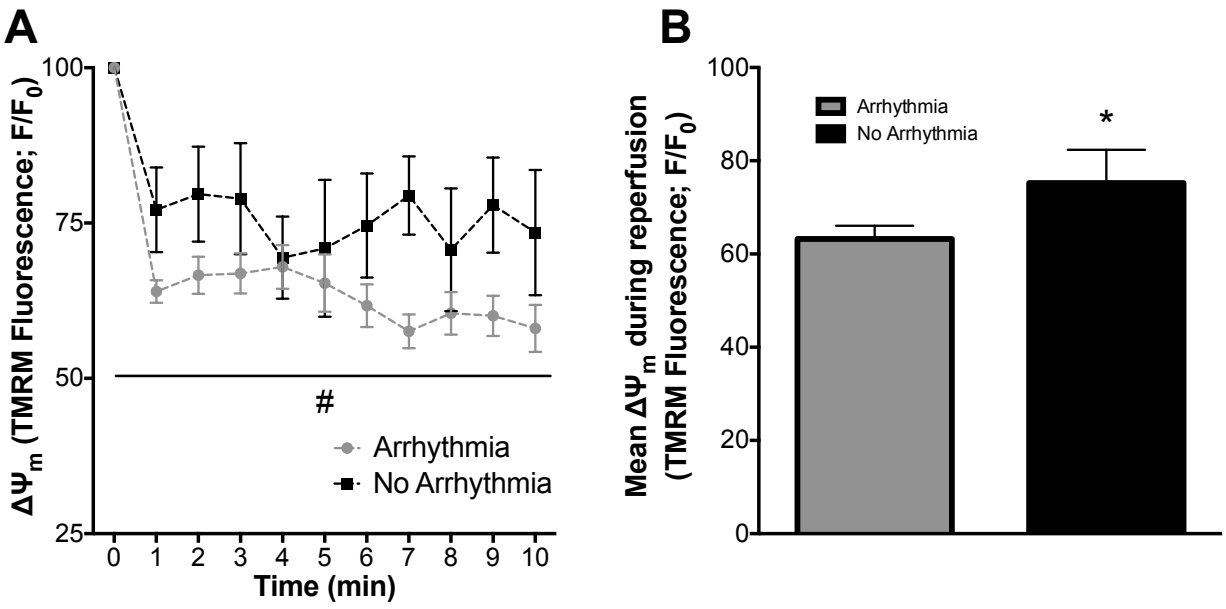

Figure 5. Mitochondrial membrane potential ($\Delta\Psi_m$) in isolated hearts that transitioned to arrhythmia vs no arrhythmia during reperfusion. (A) $\Delta\Psi_m$ was was better maintained in hearts that did not transition to arrhythmia. (B) Mean $\Delta\Psi_m$ fluorescence values during reperfusion. Data are mean ± SEM * $p<0.05$ vs Arrhythmia; # $p<0.05$ vs Arrhythmia main effect.

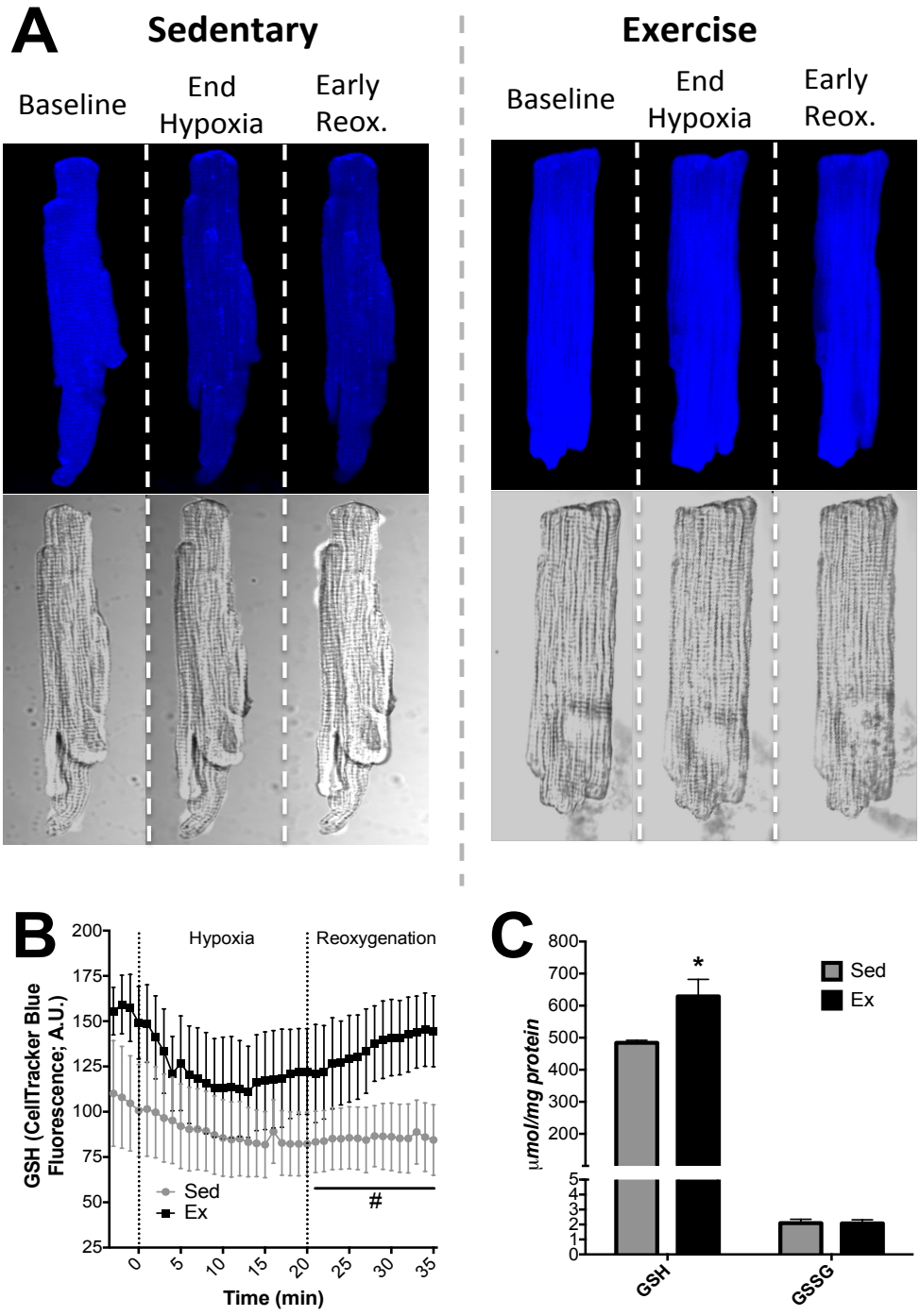

Figure 6. Cardiac glutathione (GSH) during cellular hypoxia/reoxygenation or cardiac ischemia/reperfusion. (A) Representative primary cardiac myocyte fluorescent images for Sed and Ex during baseline, at the end of hypoxia, and 6 minutes into reoxygenation. (B) Quantification of glutathione levels as measured by CellTracker Blue fluorescence. (C) HPLC quantification of reduced (GSH) and oxidized (GSSG) glutathione in hearts following ischemia/reperfusion. Data are shown as mean ± SEM, * $p<0.05$ vs Sed; # $p<0.05$. vs Sed main effect.

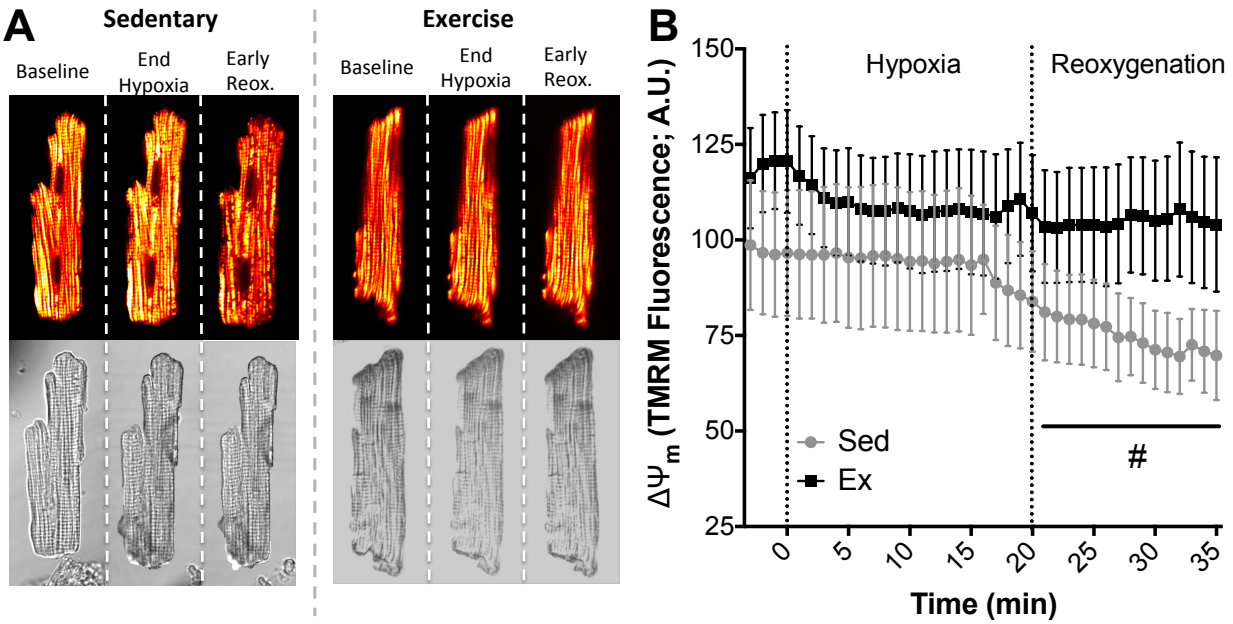

Figure 7. Mitochondrial membrane potential ($\Delta\Psi_m$) during cardiac myocytes hypoxia/reoxygenation (H/R). (A) Representative images of Sed and Ex cardiac myocytes during H/R. Depolarized mitochondrial networks and collapses in $\Delta\Psi_m$ are shown during reoxygenation as a transition in color from yellow to red and black. (B) Quantification of TMRM fluorescence during H/R. Data are shown as mean ± SEM. # p<0.05 vs Sed main effect.

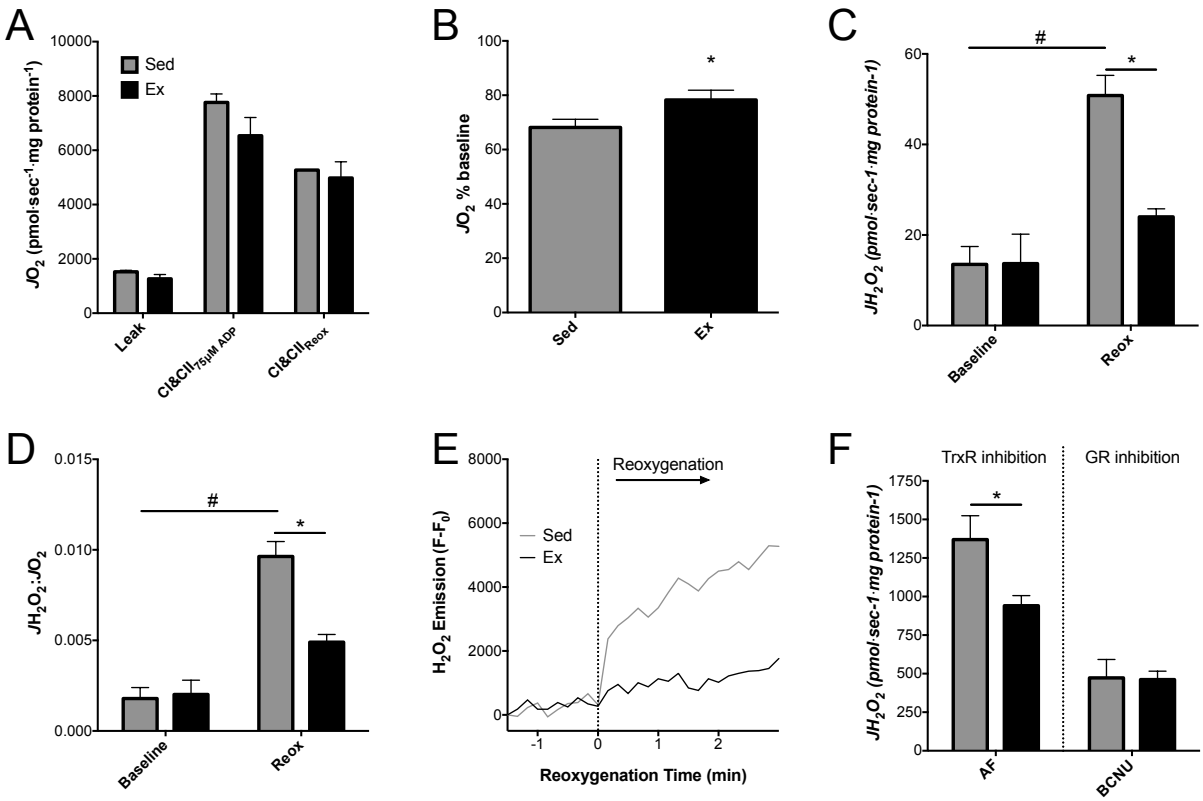

Figure 8. Reactive oxygen species (ROS) and isolated mitochondrial energetics during anoxia/reoxygenation (A/R). Sed is gray and Ex is black in all graphs. O_2 consumption rate (JO_2) and H_2O_2 emission rate (JH_2O_2) was measured in isolated mitochondria from Sed and Ex hearts. (A) JO_2 was similar at baseline between Ex and Sed isolated mitochondria respiring on glutamate + malate, pyruvate, and succinate, and ADP clamped at 75 μM (state 3). (B) Impairments in state 3 JO_2 following A/R was determined by comparing relative decreases from baseline for Sed and Ex. (C) State-3 JH_2O_2 before and after A/R. (D) The JH_2O_2:JO_2 ratio demonstrates impaired mitochondrial function in Sed mitochondria following A/R. (E) A representative experiment showing a trace of resorufin fluorescence used to calculate JH_2O_2 during A/R. For clarity, data were transformed by subtracting the anoxic fluorescent value recorded prior to reoxygenation. (F) JH_2O_2 in isolated mitochondria in the presence of either thioredoxin reductase inhibitor (AF) or glutathione reductase inhibitor (BCNU). Data are shown as mean ± SEM. * $p<0.05$ vs Sed main effect ; # $p<0.05$ vs Sed baseline.

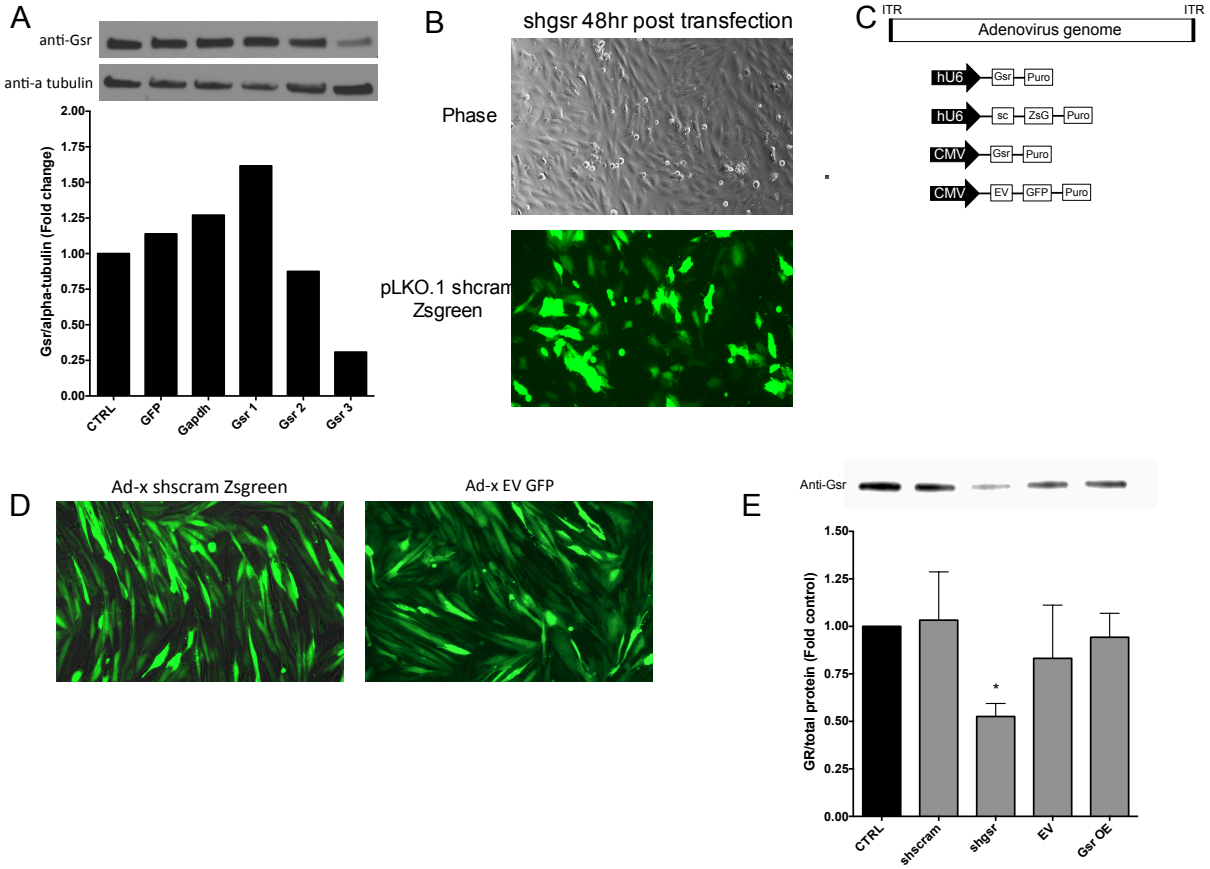

Figure 9. Gsr knockdown and overexpression in H9c2 cells. (A) Western blot and densitometry after 48hr transfection with shRNA cloned into the pLKO.1 vector. (B) GFP and phase images 48hr post transfection. (C) Illustration of adenovirus expression cassettes of shgsr, shscram, Gsr overexpression (Gsr OE), and empty vector control (EV). (D) 96hr post infection fluorescent images of differentiated H9c2 cells, and (E) Western blot of glutathione reductase protein content.

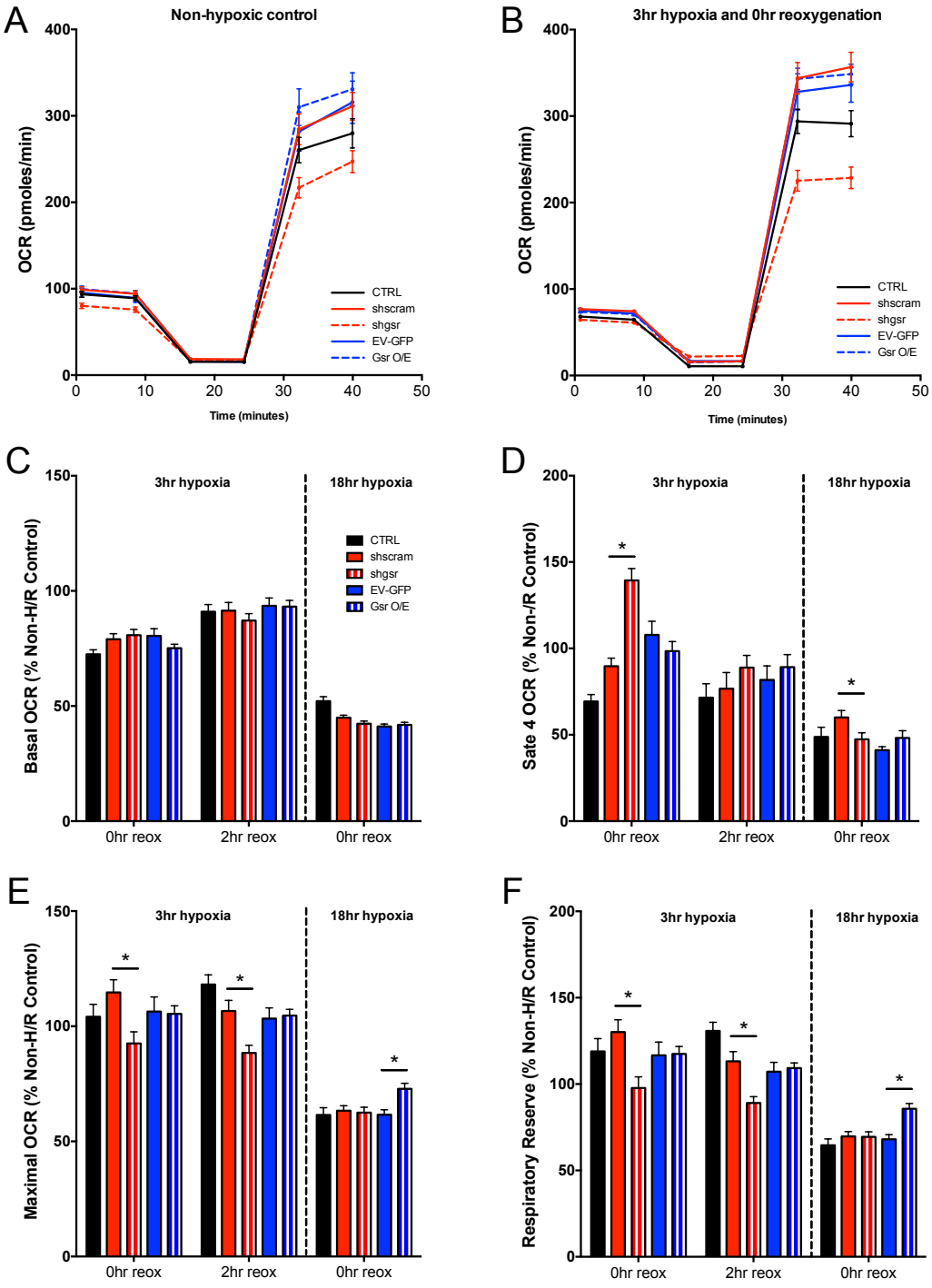

Figure 10. Mitochondrial bioenergetic analysis in H9c2 cells exposed to hypoxia/reoxygenation. (A) Seahorse trace of mitochondrial stress test protocol following a 20min 2-AAPA exposure. All data were transformed by subtracting out the antimycin A respiratory rate. (B) Basal respiration. (C) Maxmial uncoupled respiration with FCCP (E) ATP-dependent respiration (Basal respiration – State 4). Data in C-E are expressed as percent of Non-H/R control for each time point. *p<0.05 vs vector control.

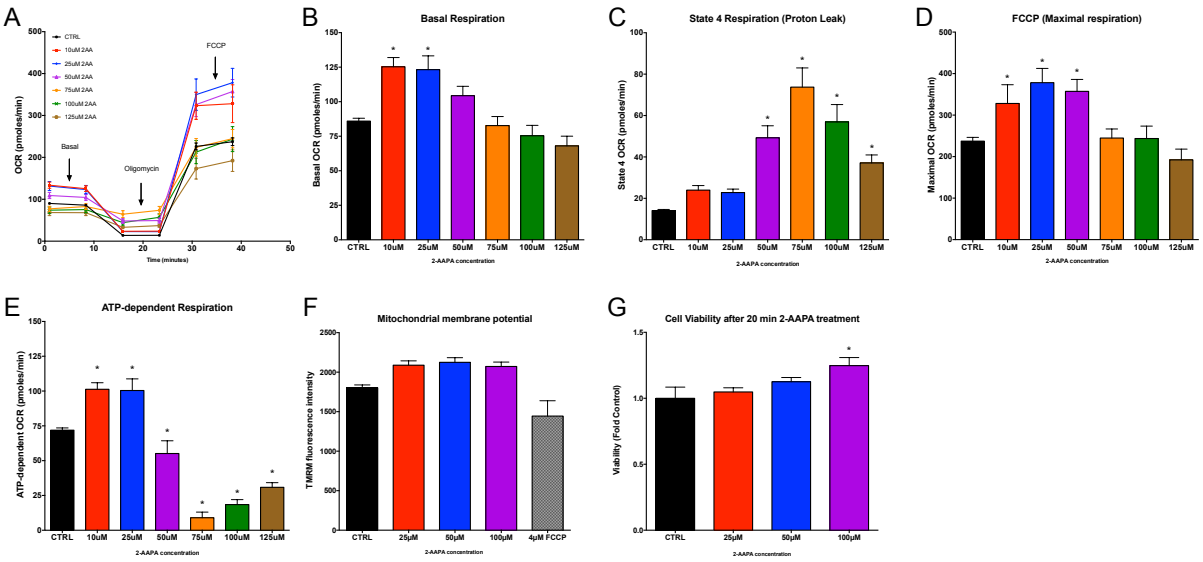

Figure 11. Mitochondrial bioenergetic analysis in H9c2 cells following pharamacological induction of thiol redox stress. (A) Seahorse trace of mitochondrial stress test protocol following a 20min 2-AAPA exposure. All data were transformed by subtracting out the antimycin A respiratory rate. (B) Basal respiration. (C) Maxmial uncoupled respiration with FCCP (E) ATP-dependent respiration (Basal respiration – State 4). (F) Mitochondrial membrane $\Delta\Psi_m$ with TMRM and (G) cell viability following 2-AAPA exposures.*p<0.05 vs CTRL (ANOVA followed by Dunnett's post-hoc test).

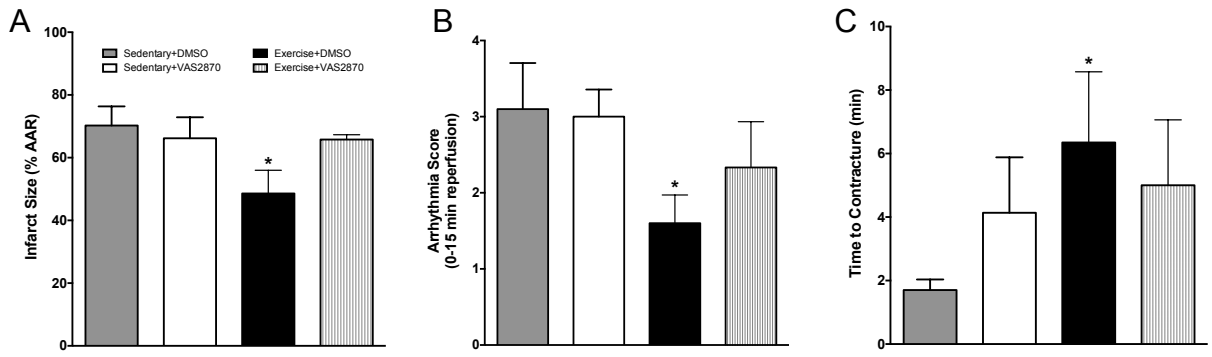

Figure 12. Infarct size and arrhythmia in isolated hearts following ischemia/reperfusion. (A) Infarct size for isolated rat hearts following 20 minutes of ischemia and 2 hours of reperfusion. (B) Arrhythmia score following the first 15 minutes of reperfusion. (C) The time to contracture was determined by calculating the amount of time elapsed during ischemia leading to a 5mmHg increase in end-diastolic pressure (EDP). All data are mean ± SEM, n=5-7 per group. * $p<0.05$ vs SD.

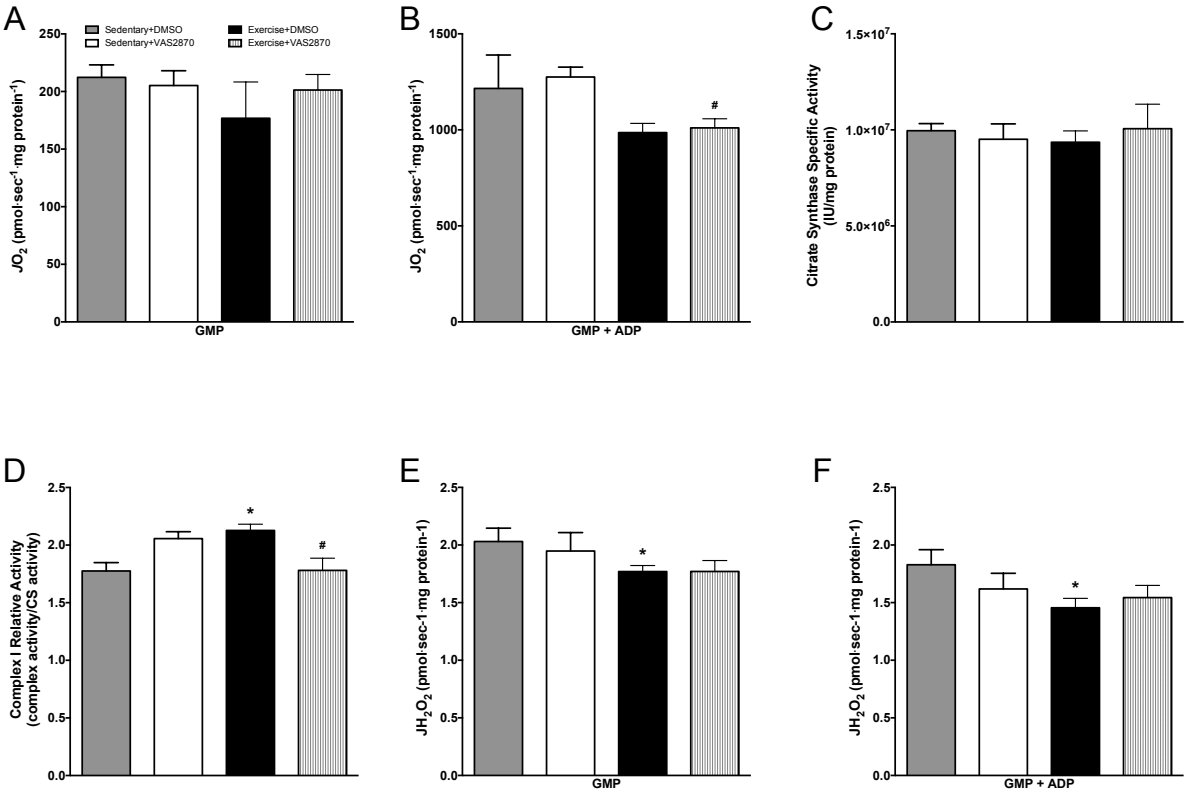

Figure 13. Mitochondrial function following ischemia/reperfusion injury. (A) O_2 consumption rate (JO_2) in isolated mitochondria under state 4 conditions (no ADP) with glutamate (10mM), malate (2mM), and pyruvate (5mM). (B) JO_2 under state 3 conditions with saturating ADP (4mM). (C) Citrate synthase activity. (D) Relative specific activity of complex I normalized to the respective citrate synthase activity. (E) H_2O_2 emission rate (JH_2O_2) under state 4 conditions (same substrate as *Figure* 2A). (F) JH_2O_2 with saturating ADP. All data are mean ± SEM, n=4 per group, JO_2 and JH_2O_2 assays were run in duplicate and kinetic assays were run in triplicate. * P<0.05 vs SD, # p<0.05 vs SV.

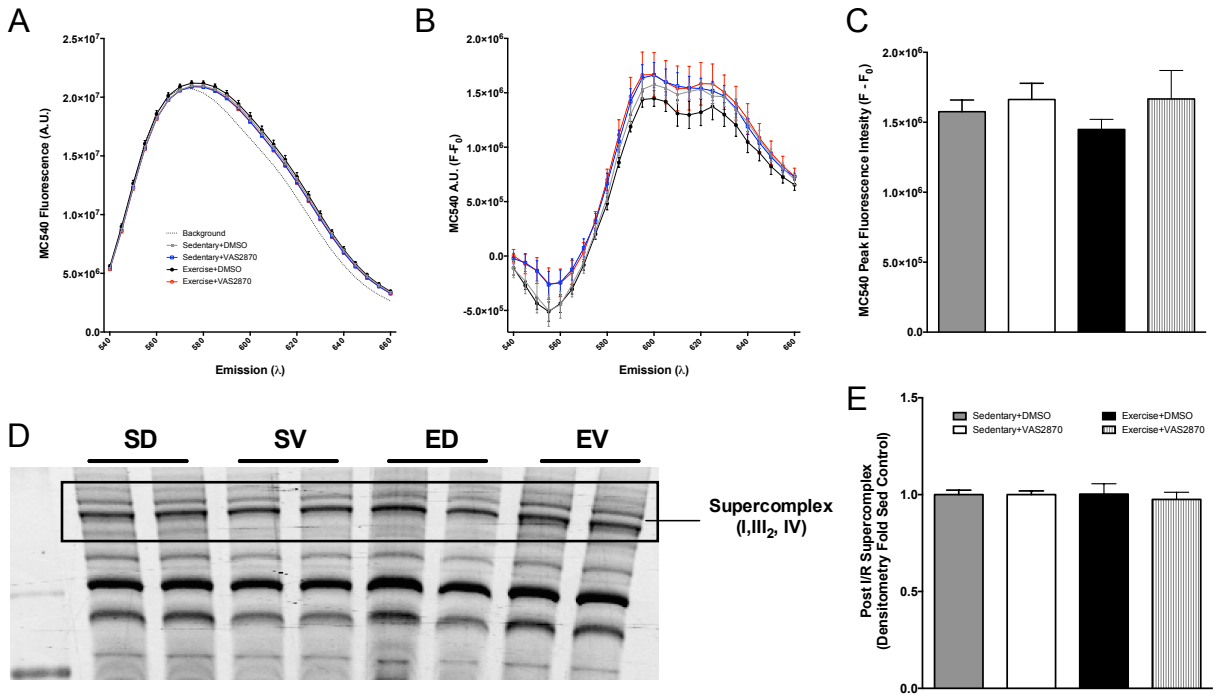

Figure 14. Mitochondrial membrane lipid properties and respiratory supercomplex assembly. (A) Merocyanine 540 (MC540) fluorescent spectra before and after the addition of mitochondria. Note the peak fluorescence red shift of the spectra indicates a lipid phase (B) Relative shift in MC540 fluorescence following the addition of mitochondria ($F\text{-}F_0$). C) Peak fluorescence intensity at 600nm. D) Representative image of supercomplex density via Blue-native PAGE; n=4 per group run in duplicate. The black box encloses supercomplex I, III_2, IV and used for quantification. E) Quantification of densitometry from supercomplex gel, normalized to control. All data are mean ± SEM, n=4 per group.