The role of cardiac mitochondria in myocardial ischemia/reperfusion injury

by

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Cardiovascular disease (CVD) exerts economic and humanitarian costs that are unparalleled by any other disease. Of the many etiologies of CVD, myocardial infarction accounts for over 50% of the associated mortality and anything that can decrease the extent of infarction could drastically impact the burden of CVD. The purpose of this work was to further our understanding of the role of cardiac mitochondria in ischemia/reperfusion injury. Herein I found that physiologic (exercise) and pharmacologic (Bendavia) interventions that lessen the oxidative burden during ischemia/reperfusion have the potential to limit myocardial infarction. Under conditions of oxidative stress, animals who received short term exercise (Ex) were better able to maintain the glutathione couple in a reduced state, likely through an increase in glutathione reductase (GR) activity. This phenotypic change was associated with decreased reactive oxygen species (ROS) accumulation and a lower incidence of fatal ventricular arrhythmias. Furthermore, I found that ROS generated within the cytosol, and not the mitochondria, during bouts of Ex are important signaling molecules that increase GR activity and this increased activity may be responsible for the

cardioprotective effects observed with Ex. Finally, I found that treatment with the mitochondrially-targeted peptide Bendavia was successful at lowering infarct size in isolated guinea pig hearts, due to an ability to decrease ROS accumulation and maintain mitochondrial energetics. Taken together, these studies suggest that therapies aimed at decreasing mitochondrial ROS and/or maintaining mitochondrial energetics during ischemia/reperfusion may have significant clinical impact.

The role of cardiac mitochondria in myocardial ischemia/reperfusion injury

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List of symbols and abbreviations

$\pm$ dP/dt	Maximal rate of contraction and relaxation
ADP	Adenosine diphosphate
Akt	Protein Kinase B
AMPK	AMP-activated protein kinase
ANT	Adenine nucleotide transferase
ATP	Adenosine triphosphate
BDW	Body weight
bpm	Beats per minute
Ca	Calcium
CM-DCF	5-(6)-chloromethyl-2,7-dichlorohydrofluorescein diacetate
COX2	Cyclooxygenase-2
CsA	Cyclosporine A
CVD	Cardiovascular disease
DMEM	Dulbecco's Modified Eagle Medium
DTT	Dithiothreitol

ECG	Electrocardiogram
Ex	Exercise
GLUT	Glucose transporter
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSK3	Glycogen synthase kinase 3
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HW	Heart weight
IL-1	Interleukin 1
i.p.	Intraperitoneal
IPC	Ischemic preconditioning
I/R	Ischemia-reperfusion
K_{ATP}	ATP-sensitive potassium channel

K_{ir}	Inward-rectifier potassium channel
LV	Left ventricle
LVDP	Left ventricular developed pressure
MPG	N-2-mercaptopropionyl glycine
VO_2	Oxygen consumption
Na	Sodium
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NOS	Nitric oxide synthase
O_2	Molecular oxygen
PKC	Protein kinase C
PTP	Permeability transition pore
ROS	Reactive oxygen species
SED	Sedentary
SOD	Superoxide dismutase
Sur	Sulfonylurea receptor

SR	Sarcoplasmic reticulum
tBOOH	t-butyl hydroperoxide
TEE	Tris, EDTA, EGTA Buffer
TNF	Tumor necrosis factor
TTC	Triphenyltetrazolium chloride
VF	Ventricular fibrillation
VT	Ventricular tachycardia
$\Delta\Psi_p$	Sarcolemmal membrane potential
$\Delta\Psi_m$	Mitochondrial membrane potential

Chapter 1: Introduction

Prevalence of cardiovascular disease

Cardiovascular disease (CVD) exerts economic and humanitarian costs that are unparalleled by any other disease. Ischemic heart disease costs an estimated $165 billion per year, with physical inactivity accounting for approximately 10% of this cost (162), making a sedentary lifestyle a multi-billion dollar problem and highlighting the need to promote exercise as medicine within populations at risk. In 2005, approximately 1 out of every five deaths in the United States was due to coronary heart disease (162). Since CVD represents such a burden on the well being of the human population, further investigation into how to decrease either the prevalence or mortality associated with CVD is of grave importance.

Coronary heart disease accounts for the majority of deaths due to CVD (Figure 1), and ischemia/reperfusion injury resulting from treatment for coronary heart disease is the focus of this dissertation.
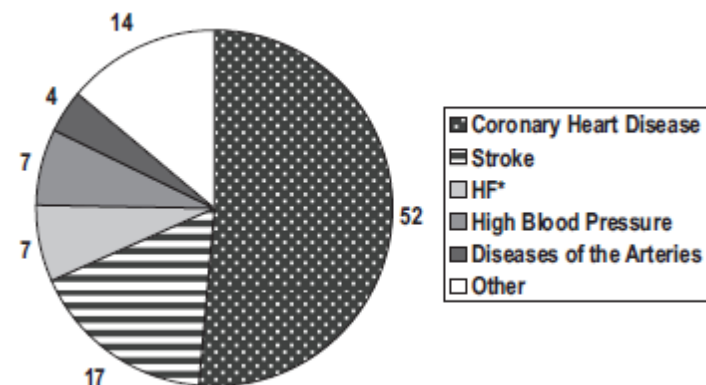
Figure 1: Percentage breakdown of deaths due to CVD (United States: 2006, preliminary). Taken from (162).

Ischemia/reperfusion injury: What is it?

Myocardial ischemia occurs when the blood supply to the myocardium is insufficient to match the metabolic demand. Ischemia is an appropriate term as it stems from the Greek *ischo* (to hold back) and *haima* (blood). The primary event that leads to

obstruction of a coronary artery is generally due to thrombus formation over a preexisting atherosclerotic plaque. The formation of the atherosclerotic plaque and development of coronary artery disease are beyond the scope of this work and are described in detail elsewhere (226). This thrombotic blockage prevents blood flow from reaching downstream tissues and predisposes them to cell death. The onset of cellular death during ischemia can begin as early as 15 minutes from the onset of symptoms, and as duration of ischemia increases, the probability of cell death also rises (111). Importantly, both short- and long-term mortality are inversely proportional to the size of the infarction (21, 97, 98, 113, 114). Various mechanisms are involved in cellular death during ischemia and reperfusion and are discussed below (see also Figure 2 and Figure 4).
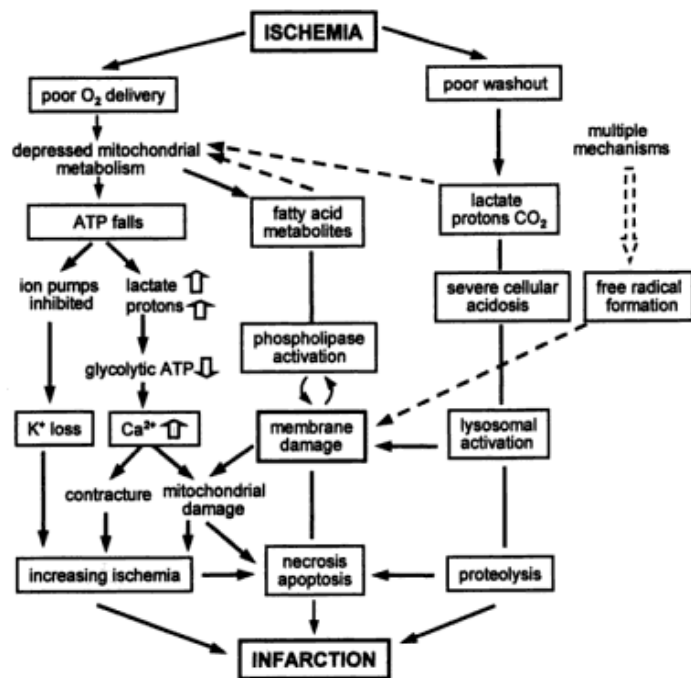
Figure 2: Mechanisms by which myocardial ischemia can lead to cell death and myocardial infarction. From (200)

During ischemia the heart tries to compensate by matching its demand with the decreased supply by decreasing contraction and switching from aerobic to anaerobic metabolism. However, due to the decreased coronary flow, metabolite washout is diminished in the ischemic tissue. This combination of increased anaerobic metabolism and decreased washout leads to a net increase in proton concentration and CO_2 within

cardiac tissue, effectively lowering intracellular pH (62). This decrease in pH lowers the myofilaments sensitivity to calcium and decreases contractile force (6, 84, 151).

During ischemia, mitochondrial metabolism is diminished (due to decreased oxygen delivery required for oxidative phosphorylation) and ATP levels being to fall, When this occurs, ATP-sensitive potassium (K_{ATP}) channels on the sarcolemmal membrane open, allowing K^+ to freely leave the cell. Coupled with decreased metabolite washout, K^+ accumulates in the extracellular space, decreasing the Nernst potential for K^+, depolarizing the membrane potential and shortening of the action potential duration. Intracellular calcium levels also rise during ischemia, likely due to a combination of decreased reuptake into the SR (because of diminished ATP levels) with no change in its release from the ryanodine receptor (6, 151). This increased calcium can lead to ischemic contracture and an increase in the likelihood that the mitochondria will undergo permeability transition.

Currently the best treatment for myocardial ischemia is prompt reperfusion. However, reestablishing coronary flow can lead to further injury, such as myocardial stunning, fatal ventricular arrhythmias, and further cell death. These etiologies may be due, in part, to the "bursts" of reactive oxygen species (ROS) that occur in the early stages of reperfusion (283, 284). In Chapter 5 (see Figure 22 below) I show that, in untreated cells, ROS accumulation is observed prior to cell death during reoxygenation and that this reoxygenation injury can be prevented if mitochondrial ROS accumulation is prevented. The ability to decrease ROS accumulation during reperfusion is associated with decreased injury and is the focus of this dissertation.

Role of the mitochondria in health and disease

Mitochondria are important regulators of cell life and death that respond to a wide variety of stress signals, including loss of growth factors, hypoxia, oxidative stress, and DNA damage (101). The main role of the mitochondria is to generate enough ATP through oxidative phosphorylation and the mitochondria has thus earned the nickname "The Powerhouse of the Cell". In cardiomyocytes, mitochondria account for approximately 30% of the intracellular volume, presumably reflecting the high energy demand of contraction that the heart undergoes on a beat-to-beat basis. However, during oxidative phosphorylation approximately 0.4-4% of molecular oxygen (O_2) consumed is reduced by a single electron transfer to form superoxide (140).

During oxidative phosphorylation, mitochondria set up a membrane potential ($\Delta\Psi_m$) by pumping protons from the matrix to the inner membrane space. This $\Delta\Psi_m$ is what drives the ATP synthase to synthesize ATP. However, during metabolic stress, fluctuations in $\Delta\Psi_m$ have been observed in whole hearts (38, 237) and isolated myocytes (9). These depolarizations in $\Delta\Psi_m$ drop cellular levels of ATP and can induce action potential heterogeneity by opening sarcolemmal ATP-sensitive potassium (K_{ATP}) channels (3, 199). As action potential lability is a prime substrate for the formation of reentrant arrhythmias (3), interventions that stabilize $\Delta\Psi_m$ diminish electrical dysfunction during an oxidative challenge (38). In Chapter 3, I show that short-term exercise training is associated with a phenotype where glutathione is maintained in a reduced state due to an increase in glutathione reductase activity. It is expected that this would lead to improved maintenance of mitochondrial energetics and is responsible for the decrease in arrhythmogenic events.

Role of glutathione system

Glutathione (GSH) represents the largest capacity thiol buffer in the heart (229) and exerts a significant effect on mitochondrial function (9, 237). Under normal conditions, the balance of reduced (GSH) and oxidized (GSSG) highly favors the reduced state (69) and recent data implicates the glutathione redox couple as a "pivoting point" between ROS balance and mitochondrial dysfunction (9, 11, 143, 237). The importance of maintaining a high GSH:GSSG ratio has been highlighted elsewhere (38, 46), but it is interesting to note that the mitochondrial permeability transition pore (PTP) opening is sensitized during oxidative stress (172) and significant oxidation of the GSH couple (GSH:GSSG of < 50:1) has been shown to trigger PTP opening (9).

Despite the fact that several studies have shown that superoxide reduction to H_2O_2 through MnSOD is increased with Ex (41, 87, 220, 271), the effect of Ex on H_2O_2 scavenging by the glutathione system remains unclear (131, 221, 241). Recently, we showed that uncompensated oxidation of reduced GSH can induce catastrophic ventricular arrhythmias, even under normoxic conditions (38). In Chapter 3, I describe how Ex preserved GSH in a reduced state under similar conditions and protected hearts from fatal ventricular arrhythmias. This was associated with decreased ROS accumulation and an increase in GR activity. Interestingly, administration of exogenous glutathione (GSH) (232) or its precursor, N-acetyl cysteine (72), has resulted in decreased injury in animal models. Since there is little *de novo* synthesis of GSH within cardiac tissue (99) the ability of the mitochondria to replenish GSH is of significant importance and is the focus of Chapters 3 and 4.

To investigate if decreasing mitochondrial ROS during reperfusion presents a good pharmacological target, we utilized the novel mitochondrially-targeted peptide Bendavia. In Chapter 5, I describe that treatment with Bendavia decreased the ROS accumulation in cardiomyocytes, enhanced the ability to maintain mitochondrial energetics, and was associated with decreased cell death.

Exercise as a therapy

The ability for exercise to confer protection from I/R injury is described in detail in Chapter 2. Despite significant advances in our understanding of the preconditioning phenomenon, *none of the experimental preconditioning strategies put forth have improved therapeutic treatments for patients* (80). One potential reason for this lack of translation to clinical practice is the short-lived effectiveness of most preconditioning stimuli (60, 255). Exercise remains one of the few *sustainable* strategies shown to precondition the heart against ischemia/reperfusion injury. Data from our group (43, 44) and others (104, 220) have demonstrated that short term exercise is cardioprotective, and that this is sustained if exercise continues for months (41). We believe that by studying the mechanisms involved in exercise-induce preconditioning we can uncover novel and sustainable *preventative* treatments for those who cannot or will not adhere to an exercise regimen.

In the United States the proportion of individuals engaging in physical activity declines with age and physical inactivity is responsible for approximately 10% of the global burden of myocardial infarction after accounting for other CVD risk factors (162). Epidemiological evidence supports the notion that patients who exercise regularly are more prone to survive a myocardial infarction (183), and have a significantly lower

incidence of sudden cardiac death (103). This protection has also been well documented in animal models, as training confers resistance against several different indices of ischemia/reperfusion injury, including infarction (for review see (45, 87) and Chapter 2), stunning (35, 41, 106, 157), and arrhythmias (105, 118, 194, 220). While the cardioprotective effect of exercise are clear, the cellular mechanisms responsible for the protective phenotype have not been elucidated.

Central Hypothesis

The goal of this dissertation is to advance our understanding of the role that cardiac mitochondria play in exercise preconditioning. The overriding hypothesis is that ischemia/reperfusion injury is mediated by a mitochondrial overload of ROS that collapse mitochondrial energetics, and therapies that can reduce this oxidative burden will prevent cell death. In Chapter 3, I establish that maintenance of reduced glutathione through the glutathione reductase reaction may be the mechanism by which exercise confers protection from ventricular arrhythmias. Chapter 4 sheds valuable insight into the role of cytosolic ROS as signaling molecules that modify GR activity and confer protection from myocardial infarction. In Chapter 5, I describe the effects of a novel pharmacological treatment for I/R injury that decreases mitochondrial ROS accumulation.

Chapter 2: Exercise-induced cardiac preconditioning: How exercise mends your achy-breaky heart

Introduction

The benefits of exercise in promoting health are well documented throughout human history. Various forms of exercise were prescribed by the ancient Chinese, Indians, Greeks, and Romans, making exercise arguably the oldest therapeutic intervention for the treatment or prevention of disease (160). As our understanding of the cardiovascular system evolved, so too did the notion that the overall health of the cardiovascular system could be improved with exercise. Beginning as early as the 1850s exercise was prescribed specifically for the prevention of heart disease in Scotland and Scandinavia, and later in the mountain resorts of Germany (160). In the United States, the public health pioneer Dr. James M. Anders was among the first to recognize the beneficial effects of exercise medicine. In a 1904 speech, Anders noted that, "It should ever be a feature of our therapeutic creed, to give close attention to physiologic means and to recognize their superiority over drugs as curative agencies" (7).

Fast forward a century and one will find an obesity and diabetes epidemic sweeping through industrialized nations, with physical inactivity now recognized as a major risk factor for cardiovascular disease.   Recent estimates suggest that approximately 12% of the cost of cardiovascular disease can be attributed to physical inactivity (275), making physical inactivity a multi-billion dollar problem. A significant

amount of time, effort, and resources are being devoted to preventative methods seeking to reduce the burden that ischemic heart disease places on both our species and our health care system. In this review article, we seek to summarize the current literature regarding the ability of exercise to delay/reduce cardiac ischemia/reperfusion injury; in short, we will describe how exercise can make "achy" hearts a little less "breaky".

Before we begin, it should be noted that exercise is known to reduce arrhythmia (105, 194, 220), decrease myocardial stunning (35, 165), and improve coronary vascular reactivity (42, 147, 148) in hearts exposed to ischemia/reperfusion. Several recent papers have discussed exercise cardioprotection (14, 25, 215, 216, 242), and many of these articles have focused on other indices of ischemic injury besides cell death. In this mini-review, we will focus almost exclusively on the ability of exercise to confer resistance against infarction. In the first half of this review, we provide a comprehensive overview of the exercise type, duration, and intensity needed to protect the heart. In the second half, we discuss underlying cellular mechanisms responsible for exercise cardioprotection. We highlight new insights into how exercise may trigger and mediate protection against infarction, and we discuss the time-course of cellular events during ischemia and reperfusion that may be altered in the heart after exercise. Finally, throughout this review we point out areas where future research can augment our understanding of exercise-induced cardioprotection.

Can we really 'precondition' the heart against infarction?

In the scientific literature, a growing number of strategies have been found to protect the heart from ischemia/reperfusion injury. Among the first of these strategies is

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the "preconditioning" phenomenon, first noted by Murry et al. (186), where short ischemic episodes before a long index ischemia decreased infarction. A number of stimuli have subsequently shown to precondition the heart against injury (reviewed in (29)), along with the recent discovery that slowly bringing the heart out of ischemia with "post-conditioning" also salvages myocardium (261). Pre/post-conditioning delays the onset of ischemia/reperfusion injury, but the extent of protection depends critically on the establishment of reperfusion. In the clinic, prompt reperfusion remains the best treatment to salvage tissue, and in experimental settings pre/post-conditioning stimuli lose their efficacy to reduce injury with prolonged ischemia (186, 203, 273). However, the re-establishment of coronary flow induces problems of its own (reperfusion injury, discussed below), and we will address the etiology of injury during both ischemia and reperfusion.

Naturally, there is enormous interest in trying to 'mimic' ischemic pre/post-conditioning with a compound administered to patients hospitalized for ischemic events. Despite scores of potential treatments that are effective in experimental settings, to date *none* of the putative compounds have been incorporated into clinical standard of care. The reasons for the lack of translation have been well described elsewhere (80), but the correlation between humans who exercise and reduced morbidity/mortality after infarction is well documented.

What is the relationship between exercise and infarct size?

Epidemiological evidence has indicated that there is a strong correlation between individuals who exercise regularly and those who survive a myocardial infarction (183, 205-209). Elderly humans who are sedentary appear to lose the preconditioning

benefits of preinfarction angina, although the protection was seen in counterparts who exercised (2, 222). Given the relationship between infarct size and mortality (112, 178), exercise is postulated to promote survival by delaying cell death during metabolic challenge, reducing the mass of infarcted tissue.

Although direct confirmation of exercise-induced infarct sparing is difficult to measure in human hearts, several studies have provided indirect evidence of exercise cardioprotection in the human heart. Zdrenghea et al. found that exercise-induced ST-segment depression was significantly attenuated in high risk patients during the subsequent exercise bout (276). Lambiase and colleagues exercised patients with known coronary artery disease prior to percutaneous coronary intervention, and observed that the ST-segment deflection induced by 3-minute intracoronary balloon inflation was lessened in the exercised patients (145). Remote preconditioning may also protect human heart tissue, as forearm exercises improved ischemic function in isolated human atrial trabeculae (224).

Exercise-induced reductions in infarct size have been observed across animal models, corroborating human epidemiological data. The first observation of exercise cardioprotection was noted 8 years before the discovery of ischemic preconditioning (IPC) (176), and has been observed in both male (4, 44, 52, 55, 91, 117, 270, 271) and female (39, 42, 44, 55, 106) animals. Infarct salvage can be seen using both *in vivo* (4, 91, 106, 117, 271) and *ex vivo* (perfused heart) (39, 42, 44, 52, 55, 73, 270) preparations of ischemia/reperfusion, indicating that both systemic and intrinsic cardiac adaptations are likely responsible for the protective phenotype. Although most studies have used younger animals, the exercise-induced cardioprotection appears to be

upheld with aging (150, 218, 244). This distinction is important, as the vast majority of deaths from MI occur in humans over the age of 65.

On average, the magnitude of protection evoked by exercise preconditioning is a 30-40% reduction in injury (reviewed in more detail in (45)). This magnitude in infarct size reduction is consistent with many pharmacological treatments aimed to decrease injury, but does not appear to be as large in magnitude as classical ischemic preconditioning (273).

Is exercise really the same as 'preconditioning'?

There are a number of similarities between exercise-induced cardioprotection and other preconditioning stimuli. The time-course for protection is very similar across models, with a narrow first window of robust protection followed by a 'second window' of more modest protection (77, 271, 273). In both IPC and acute exercise preconditioning, infarct size is significantly lower within one hour of the stimulus, but this protection wanes for approximately 24 hours. A second window of protection is observed following both IPC and exercise preconditioning, and in both cases the second window reflects a much wider time frame to observe a preconditioning effect (approximately 24-36 hours after the preconditioning stimulus).

The only studies to examine the time-course for exercise preconditioning used a single bout of exercise (77, 271). Repetitive exercise training over weeks/months evokes a number of morphological/phenotypic changes in the myocardium, including resting bradycardia, hypertrophy of the left ventricle, cellular growth/adaptations in cardiac myocytes, and altered coronary vascular function (39, 42, 147, 168). These changes make it a little more difficult to compare a chronic stimulus with acute stimuli

such as classical ischemic preconditioning, and future studies looking to compare the time-course for protection following chronic training hope to provide insight into whether the protection is still characterized by two distinct windows of preconditioning.

Although exercise may share some of the mechanistic pathways with IPC (e.g. a role for reactive oxygen species; covered in detail below), there are clear distinctions. For example, phosphorylation of Akt or GSK-3β has been observed in a number of preconditioning models (reviewed in (185)), but neither Akt nor GSK-3β phosphorylation appear to be necessary for exercise cardioprotection (39, 61). Furthermore, increased cyclooxygenase-2 (COX2) is seen in several preconditioning models (31), but up-regulated COX2 was not found to be involved in exercise preconditioning (219).

Several other characteristics of exercise preconditioning distinguish this preconditioning stimulus from the others. First, any preventative treatment must be shown to be *sustainable* for long periods of time. Many experimental stimuli appear to "precondition" the heart when given one time, but unless our powers to forecast impending coronary events improve drastically, the clinical relevance of one-time administration of preventative measures must be questioned. As a preventative measure, exposure to exercise protects the heart against infarction after either 1 day, or many months of the exercise stimulus. Second, any potential therapy must be readily available to patients, and there is no treatment that is more readily available to patients (or more economically affordable) than exercise. Finally, as addressed above the epidemiological evidence is clear that exercise is also protective to human hearts. Given these distinctions from classical models of preconditioning, exercise is arguably the most clinically relevant preconditioning stimulus that has been studied to date. With

such enormous potential, we will now address how much of this stimulus is necessary to precondition the myocardium.

How much exercise is needed to protect the heart?

An interesting (and on-going) challenge is to determine exactly how much exercise is needed to evoke protection against I/R injury. By far, the most common exercise model in the literature is forced treadmill running in the rat. In most studies, 30-60 minutes of running at treadmill speeds of 27-33 meters per min is used as the exercise stimulus (often bookended by 10-15 min of lower intensity running at ~15 meters per min). Such protocols consistently confer protection against infarction (see below), and reflect an exercise intensity of approximately 75% of VO_2max (19). To the best of our knowledge, the influence of lower intensity treadmill running ($\leq$60% VO_2max) on infarct-salvage is not known. Studies examining post-ischemic mechanical recovery after lower intensity treadmill running protocols have found equivocal results, with some finding improved recovery with exercise (156), and others finding no effect (243). A direct relationship between exercise intensity/duration with infarct salvage must be addressed in future studies.

Unlike skeletal muscle, training adaptations to the heart following treadmill protocols are not normally characterized by increased Krebs Cycle intermediates (citrate synthase, for example, is not normally increased in trained hearts (146, 195, 213)), but there are training adaptations such as elevated anti-oxidants (covered in detail below) and left ventricular hypertrophy. The resting bradycardia induced by higher intensity treadmill running can even be observed in the isolated heart (39),

indicating that intrinsic adaptations to pacemaker currents may also accompany altered autonomic nervous system tone.

Our best insight into how much exercise is needed is provided by studies that have used different models, intensities, and durations of exercise and looked at increased expression of cardioprotective proteins (such as cellular anti-oxidants and/or heat shock proteins). An exercise intensity of $\geq$ 24 meters/min (with a treadmill incline $\geq$ 2%) appears to be necessary for the upregulation of myocardial heat shock proteins (179, 195), although several studies have dissociated the link between exercise-induced protection and elevated heat shock proteins (104, 243, 252). Increased manganese superoxide dismutase (MnSOD) is observed in young animals following exercise at intensities $\geq$ 27 meters/min with treadmill incline set at either 0% (92, 117, 220, 271) or 10% (42, 52). Increased cardiac MnSOD can be induced at lower treadmill speeds (20-25 meters/min) if a treadmill incline of $\geq$ 10% is applied (119, 221), but is not observed at treadmill speeds lower than 20 meters/min (54, 156).

Future research that determines how long exercise cardioprotection persists after cessation of an exercise regimen will also improve our understanding of exercise preconditioning. Although no study has directly determined how long exercise-induced infarct salvage lasts after stopping exercise, an interesting study from Powers' group investigated the effects of exercise cessation on functional recovery. In their study, Lennon et al. (155) exercised animals for a total of 8 days, with the final 3 days consisting of high intensity exercise (30 meters/min for 1 hour). The beneficial effects of exercise (as evidenced by post-ischemic recovery of cardiac work) persisted for 9

days after cessation of the exercise, but by 18 days post-exercise there was no longer an exercise effect.

While the cardioprotective benefits of treadmill running protocols are clear, the limitations of this model must be clearly recognized. Animals that do not run on the treadmill are often prodded, blasted with air, or shocked via an electrical grid. The caveat that animals may display a stress response following this 'motivation' must be acknowledged in forced treadmill studies, especially those where exercise only lasts a few days. Only a few investigators have attempted to address this issue, and in both cases there was evidence (especially in male animals) of systemic stress (reflected by adrenal hypertrophy, splenic atrophy, and increased circulating corticosterone) (43, 182). Admittedly, these data are subject to different interpretations (what is 'stress' versus 'exercise adaptations'?), and we hope that the dialogue regarding the best experimental exercise approach will continue among scientists in this field.

More work must be done to determine if voluntary wheel running protocols can protect the heart against infarction. Wheel running has the advantage that animals run ad libitum, with rats often covering several kilometers per day (153). The disadvantages of wheel running include a much lower intensity of exercise, and variability among the amount of exercise that each animal receives. Voluntary wheel running has been shown to improve survival after ischemia (67), but future studies are needed to determine if voluntary running can protect the heart to the same extent as higher intensity treadmill running.

A few studies have looked at the infarct sparing effects of swimming training. In these studies, animals were exposed to several hours of swimming per day for 7-8

weeks, and infarctions were significantly reduced in swim-trained animals compared to sedentary counterparts (91, 279). The limitations of swimming protocols must also be acknowledged, as the exercise is often characterized by diving reflexes, animal stress from borderline drowning, and intermittent hypoxia (22, 86).

What is it about exercise that triggers a protective phenotype?

We are only beginning to understand what exercise does to "trigger" a preconditioning response. Exercise-induced activation of adenosine or opioid receptors, transient ROS production, AMP kinase, and/or surges in inflammatory cytokines are candidate triggers for the protection (a hypothetical schematic is presented in Figure 3).

Several studies suggest that receptor-dependent signaling cascades are involved in triggering exercise cardioprotection. Adenosine receptor blockade abolished the cardioprotection evoked by intermittent bouts of tachycardia in paced dog

Figure 3: Putative sequence of events leading to exercise-induced cardioprotection. Postulated 'triggers' of exercise-induced cardioprotection are denoted in green, with end-effectors labeled in red font. Images of nucleus and myofilaments obtained from (24, 266) respectively.

hearts (78), suggesting that adenosine release at high heart rates initiates a cascade of events that confer resistance to infarction. The mechanism of action for adenosine

receptor activation is surely multi-factorial. Husain and Somani (121) recently found that the increase in myocardial anti-oxidant enzymes after acute exercise was abolished when exercise was performed in the presence of an adenosine receptor blocker. The authors did not investigate ischemia/reperfusion injury, and more work is needed to determine if adenosine receptors initiate cardioprotective signaling following repetitive bouts of exercise. This is especially important in light of observations where chronic administration of adenosine loses efficacy to precondition the myocardium (255).

Opioids may also play a role in triggering exercise cardioprotection. A recent study found that blocking opioid receptors during the exercise treatment abolished the infarct salvage (73). Endogenous opioids can be released from a number of tissues (including heart) (70, 75), and administration of both opioid peptides and opioid receptor agonist preconditions tissue against injury (74, 93). Exercise may increase endogenous opioids in the coronary circulation via endocrine/autocrine pathways, although the exact role of endogenous opioids in exercise preconditioning warrants further investigation.

An attractive hypothesis is that the activation of adenosine and/or opioid receptors during exercise leads to protection via protein kinase C (PKC)-dependent mechanisms. Adenosine and opioid receptor-signaling both converge on PKC (180, 260, 272), and the activation/translocation of PKC isoforms may induce a number of adaptive changes within the myocardium. The expression of several PKC isoforms is altered in the heart after repetitive exercise (49), and inhibiting PKC before exercise abolishes the infarct salvage (177, 270). Among the different PKC isoforms in the heart, several studies suggest a protective role for PKCε in particular (49, 177). It is not yet clear which cellular proteins "down-stream" of PKC may be activated to mediate

exercise preconditioning. PKC-dependent activation of heat shock protein 70 does not appear to be an absolute requirement for exercise cardioprotection (177), although PKC-dependent activation/trafficking of sarcolemmal ATP-sensitive potassium channel may be involved. Future research that elucidates down-stream PKC targets will improve our understanding of its role in exercise cardioprotection.

Another putative trigger of exercise preconditioning is the transient release of reactive oxygen species (ROS) during exercise. Several studies have noted that the infarct-salvaging affects of exercise, as well as exercise-induced improvements in cardiac function are abolished when anti-oxidants are given during the exercise (4, 191, 271). Exercise has been shown to increase the activity of myocardial NADPH oxidase, and inhibiting NADPH oxidase, a source of reactive oxygen species, abolished the protective effects of acute exercise (227). The notion that a small amount of reactive oxygen species may lead to cardiac adaptations that confer resistance to infarction has been put forth in other preconditioning models (138), although the mechanisms that ultimately lead to decreased I/R damage are yet to be determined. A small ROS burst may increase anti-oxidant buffering capacity by promoting gene expression and protein synthesis, similar to the hormesis observed in skeletal muscle (126). ROS-dependent (redox) modulation of the ryanodine receptor after exercise was recently found to decrease SR calcium leak (227), although it is not clear if this mechanism is involved in delayed exercise cardioprotection (i.e. 24 hours after exercise versus acute effects investigated by Sanchez et al. (227)).

Adenosine monophosphate-activated protein kinase (AMPK) is another candidate 'trigger' for exercise preconditioning. AMPK is believed to be quiescent in the

heart when energy supply-demand is in balance, but is activated during changing metabolic conditions (such as exercise or ischemia; reviewed in (12, 137)). Pharmacological activation of AMPK has been shown to reduce infarct size to a similar degree as exercise preconditioning (142). Cardiac AMPK is activated following treadmill running, with the AMPKα-2 isoform appearing to be the most responsive (61, 142, 187). Relating to the cardioprotective mechanism of action, AMPK activation is postulated to stimulate glucose/fat metabolism in the heart during metabolic stress (12, 137), and may promote translocation of cardiac ATP-sensitive potassium channel subunits ((246); described in more detail below). The ischemic heart switches to anaerobic glycolysis very quickly, and both glycogen storage and sarcolemmal glucose transport are stimulated by AMPK. During reperfusion, AMPK is believed to augment fatty acid uptake and oxidation, further augmenting substrate flux through re-energized mitochondria. AMPK activation after exercise may protect the heart through better preservation of cardiac energetics, and there is both direct and indirect evidence that cellular ATP content is better maintained after ischemia/reperfusion in exercised hearts ((36, 124); addressed in more detail below). Inhibiting AMPK activation has not yet been shown to abolish exercise preconditioning. Using an AMPKα-2 dominant negative mouse model, Musi et al. found that neither the ability of animals to exercise nor the maintenance of cardiac energy stores was altered with AMPKα-2 deficiency (187). Future experiments that directly examine exercise cardioprotection in AMPK knockout models, as well as development of more specific pharmacological tools that block AMPK activation will improve our understanding of AMPK in exercise cardioprotection.

The role of cytokines on cardiac ischemia/reperfusion injury in the literature is conflicting (65, 169). Regarding the involvement of cytokines in triggering exercise induced cardioprotection, Yamashita et al. found that antibodies directed at both TNFα and IL-1β abolished exercise-protection when given prior to a single bout of exercise, and one-time administration of TNFα has been shown to reduce injury (169, 271). Clearly more investigation is needed to clarify the role that cytokines play in exercise-induced cardioprotection, and whether cytokines such as TNFα are involved in long-term exercise adaptations (such as cardioprotection or hypertrophy).

Among the candidate triggers and mediators involved in exercise-induced infarct sparing, augmented collateral coronary circulation, elevated nitric oxide synthase, heat shock proteins, and ER stress proteins do not appear to be obligatory for protection. For the interested reader, several excellent review articles have articulated these studies in more detail (216, 242).

Is exercise-induced cardioprotection due to improved ability to scavenge ROS?

Superoxide Dismutase.

Exercise appears to improve at least some aspects of cellular ROS scavenging systems. Superoxide production by complexes I and III of the electron transport chain, NADPH oxidase, xanthine oxidase, and NOS is believed to contribute to ischemia/reperfusion injury in the heart (28). The "front-line" of cellular superoxide detoxification involves enzyme-catalyzed dismutation by superoxide dismutase (SOD). Cellular compartmentalization of SOD has led to several distinct isoforms including extracellular SOD, copper-zinc (Cu/Zn)SOD in the cytosolic compartment, and manganese (Mn)SOD in the mitochondrial matrix. Among these isoforms, increased

MnSOD in particular has been found in many studies to correlate with protection against infarction.

In most exercise preconditioning studies, upregulated MnSOD has been observed following exercise. The duration (number of consecutive days) of exercise appears to be a key determinant to altering cardiac MnSOD levels. As noted in Table 1, most (but not all) investigations have observed increased MnSOD activity following short-term (one to five days) exercise, but MnSOD mRNA (73) or protein (44) is not increased after acute exercise. In studies examining MnSOD with longer duration exercise protocols, there is a clear upregulation in both the activity (52, 119, 120, 131, 149, 213) and protein expression (42). This ability to augment MnSOD with exercise appears to be maintained in the aged heart (100, 131, 149). Supporting evidence for augmented MnSOD content and cardioprotection comes from mouse models where genetic over-expression of MnSOD protected against infarction (59), and genetic knockdown was associated with increased necrosis and impaired cardiac function (164).

Even within the same study, increased MnSOD activity can be observed without increased protein expression (271). Post-translational modification of MnSOD is known to affect enzyme activity (267), and future experiments will help to determine if post-translational modifications of MnSOD (such as de-phosphorylation) are involved in the exercise-induced increase in MnSOD activity. As with exercise cardioprotection, upregulation in either the activity or protein content of MnSOD appear to be critically dependent on the intensity of exercise, as low-intensity treadmill running (54, 156, 213)

and voluntary free wheel running (130) do not appear to increase myocardial MnSOD levels.

Pharmacological SOD mimetics show promise as a cardioprotective treatment across models of I/R (27, 53, 129, 136, 141). In recent human clinical trials, exogenous ROS scavengers decreased short-term injury (256), although there didn't appear to be a long-term benefit. Further development of SOD mimetics, especially mitochondria-specific ROS scavengers (248), has potential in mitigating cardiac I/R injury.

Table 1: Changes in myocardial MnSOD after exercise

Study (ref.)	Animal (sex)	Exercise Duration/type	Cardiac MnSOD change following exercise (method employed)
SHORT-DURATION EXERCISE STUDIES			
Yamashita et al. (271)	rat (M)	1 d treadmill	Early Phase: increased (activity), NC (protein)
			Late Phase: increased (activity and protein)
Dickson et al. (73)	rat (M)	1 d treadmill	NC (mRNA)
Hoshida et al. (117)	rat (M)	2 d treadmill	Increased (activity; early and late phase)
Quindry et al. (220)	rat (M)	3 d treadmill	Increased (activity)
Lennon et al. (156)	rat (M)	3 d treadmill	NC: low intensity running (activity)
			Increased: high intensity running (activity)
Demirel et al. (68)	rat (F)	3-5 d treadmill	Increased (activity)
French et al. (92)	rat (M)	5 d treadmill	Increased (activity)
Brown et al. (44)	rat (M and F)	1 or 5 d treadmill	NC (protein content)
LONG-DURATION EXERCISE STUDIES			
Husain (119)	rat (M)	8 wk treadmill	Increased (activity and protein)
Ramires and Ji (221)	rat (F)	10 wk treadmill	Increased (total SOD activity)
Chaves et al. (52)	rat (M)	10 wk treadmill	Increased (total SOD activity)
Kakarla et al. (131)	rat (F)	12 wk treadmill	Increased (total SOD activity)
Chicco et al. (54)	rat (M)	12 wk treadmill (low intensity)	NC (protein)
Lawler et al. (149)	rat (?)	12 wk treadmill	Increased (activity and protein)
Starnes et al. (241)	rat (M)	16 wk treadmill	NC (total mitochondrial SOD activity)
Brown et al. (42)	rat (F)	20 wk treadmill	Increased (protein content)
Gunduz et al. (100)	rat (M)	1 yr swimming	Increased (total SOD activity)
Vaanholt et al. (257)	mouse (M)	life-long wheel	NC (total SOD activity)
Judge et al. (130)	rat (M)	life-long wheel	Decreased (activity)

Abbreviations: NC, no change; SOD, superoxide dismutase; ?, sex not disclosed.

Augmented H_2O_2 Scavenging

Heightened SOD capacity appears to be involved in exercise cardioprotection, but there is little evidence of increased enzymes responsible for converting the SOD reaction product, H_2O_2, to water. In the myocardium, enzymatic detoxification of H_2O_2 depends on catalase, glutathione peroxidase, and thioredoxin. Only a few studies have noted increased catalase in the heart after exercise training (119), with most studies finding no difference in myocardial catalase activity after exercise (68, 92, 106, 130, 220, 221, 251). Most studies also find no change in glutathione peroxidase in the heart (68, 92, 106, 130, 220, 241, 251), consistent with observations where genetic over-expression of glutathione peroxidase did not confer protection against infarction (129). Finally, although there has not been as much investigation into cardiac thioredoxin with exercise, it also appears to be uninfluenced by exercise (68). Taken together, heightened enzymatic scavenging of H_2O_2 does not seem to be a requisite for exercise-induced cardioprotection.

Glutathione and Glutathione Reductase

Exercise has been shown to increase total cardiac glutathione content (221), although this too appears to be intensity-dependent as increased glutathione was not observed with life-long wheel running (130). It is not clear glutathione reductase is involved in exercise cardioprotection, with some studies finding that glutathione reductase increases with exercise (131, 221) and others finding no change (52, 68, 130). Further work, especially those comparing glutathione content during/after

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oxidative stress, may provide more insight into glutathione and exercise cardioprotection.

Are ATP-sensitive potassium channels involved in exercise cardioprotection?

A candidate protein complex that appears to be involved in exercise cardioprotection is the family of cardiac ATP-sensitive potassium (K_{ATP}) channels. In the heart, there is one family of K_{ATP} channels in the sarcolemmal membrane (sarcK_{ATP}), and another in the mitochondrial inner membrane (mitoK_{ATP}) (122, 196). SarcK_{ATP} channels couple the metabolic status of the cell to the electrical excitability, and may be part of a negative feedback mechanism utilized by cells to shorten the action potential and reduce excitability when energy supplies fall (see (198) for a review of K_{ATP} channels in cardiac preconditioning,). K_{ATP}-dependent truncation of the action potential is believed to reduce cellular calcium levels by reducing L-type calcium transients. Functional cardiac sarcK_{ATP} channels are believed to exist as hetero-octomers, with 4 pore-forming subunits and 4 accessory subunits inserted into the sarcolemma. Heart-specific isoforms were originally thought to consist of $K_{ir}6.2$ pore-forming subunits, and SUR2a accessory subunits, although recent findings indicate that the molecular identity of cardiac sarcK_{ATP} may be more complicated and include multiple isoforms of both pore-forming (K_{ir}) and accessory (SUR) subunits (278).

Several studies support a role for sarcK_{ATP} channels in exercise preconditioning. Genetic knockout of sarcK_{ATP} pore-forming subunits confers exercise intolerance (282), and upregulation of K_{ATP} channel subunits has been observed following both short-term and chronic exercise (39, 44). A confirmatory role for sarcK_{ATP} channels in exercise-induced cardioprotection is provided in both short-term (55) and long-term (39) exercise

studies where pharmacological block of sarcK$_{ATP}$ channels abolished the
cardioprotection. In isolated cardiac myocytes exposed to I/R, Libonati et al. showed

A.
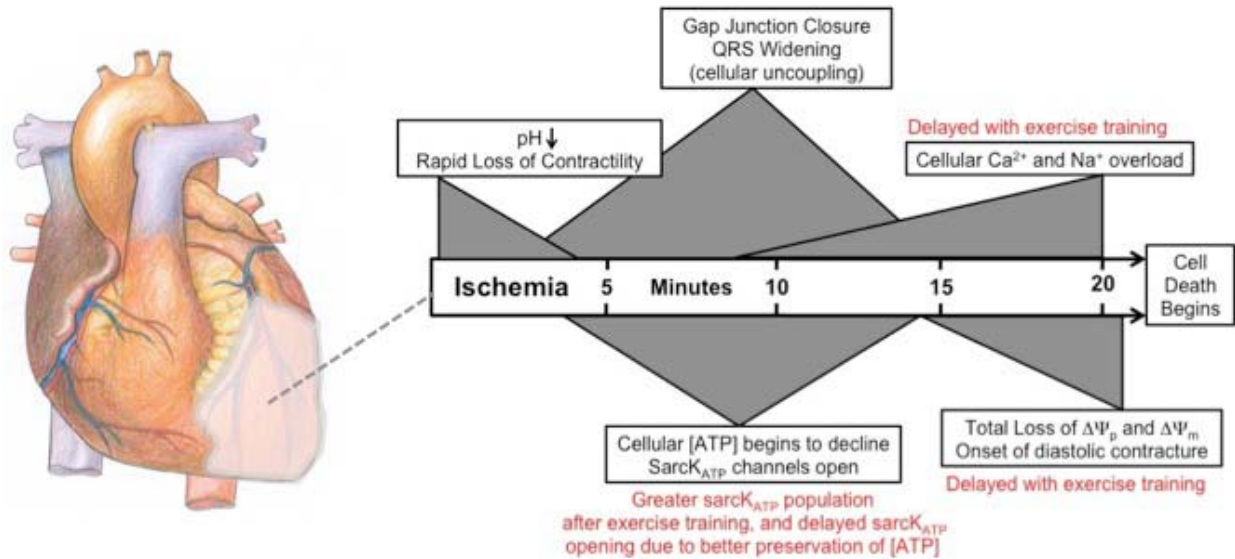
B.
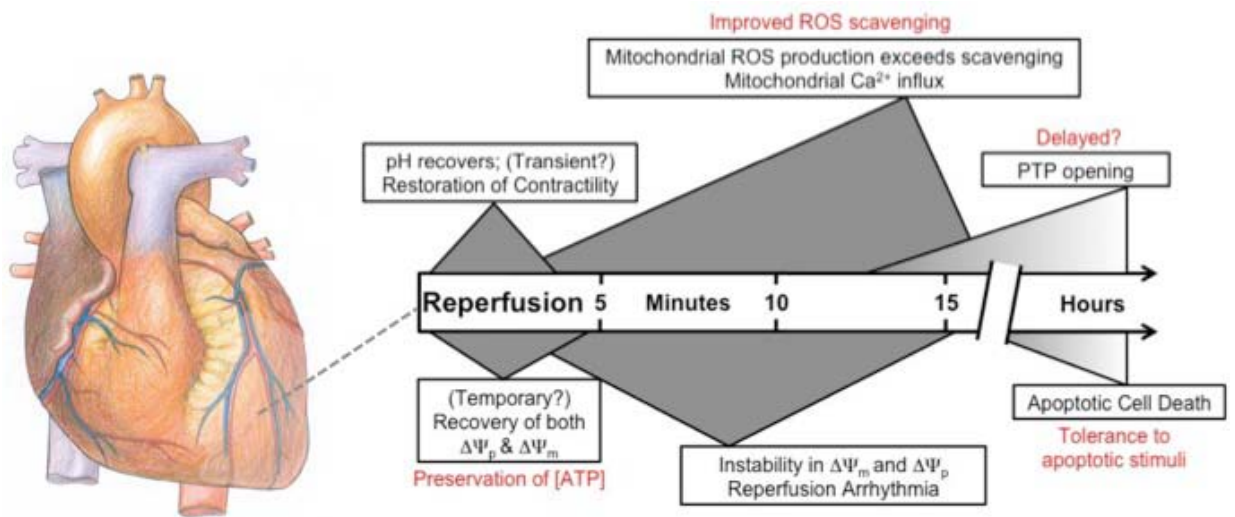
Figure 4: Pathophysiological changes in rodent cardiac tissue during ischemia (**A**) and reperfusion (**B**). Postulated mechanisms involved in exercise-preconditioning noted in red font. Heart image modified from (170). Abbreviations: $\Delta\Psi_p$, sarcolemmal membrane potential; $\Delta\Psi_m$, mitochondrial membrane potential; sarcK$_{ATP}$, sarcolemmal ATP-sensitive potassium channels.

that myocytes from trained animals had shorter action potentials than sedentary counterparts, consistent with the notion that exercise may protect the heart by augmenting the ability to repolarize (159). The issue of sarcK$_{ATP}$ channel subunit trafficking should be addressed in future exercise studies, as both PKC (82) and AMPK (246) have been shown to translocate sarcK$_{ATP}$ subunits to the sarcolemma and induce cardioprotection in other preconditioning models.

Sarcolemmal K$_{ATP}$ channel opening during ischemia appears to be the crucial time-point to protect tissue against infarction, as blocking the channels during reperfusion alone doesn't influence infarct size (127). During the ischemic period, there is probably a relatively short window for K$_{ATP}$ channels to protect the heart. In rodent hearts exposed to ischemia, electrical activity in ventricular tissue continues for approximately 15 minutes, although mechanical function stops within in the first 2 minutes. Conduction velocity slows during ischemia due to the closure of gap junctions ("cellular uncoupling") (50), and after approximately 15-20 minutes of ischemia cells become inexcitable due to run-down of sarcolemmal ion gradients and gradual sarcolemmal depolarization secondary to ATP depletion (see Figure 4). This loss of electrical excitability is best observed in global ischemia models (37), and is along the same time-line as the onset of ischemic contracture. Given the small window for sarcK$_{ATP}$-dependent protection, sarcK$_{ATP}$ opening likely reduces infarction by delaying the onset of injury, although this delay may be inconsequential after prolonged ischemia (203). While speculative, this would explain why K$_{ATP}$ channel block during longer ischemic bouts did not influence infarct size in sedentary male hearts (55, 127), as sedentary male rats are the most susceptible to injury and the window of protection from

K_{ATP} channel opening may have passed during prolonged injury. Despite a higher propensity for injury in male animals (127), exercised male hearts still show increased sarcK_{ATP} channel expression (44), and blocking sarcK_{ATP} channels during ischemia abolishes the exercise preconditioning in both sexes (39, 55).

Although the opening of K_{ATP} channels reduces cellular injury, the consequence of increasing K_{ATP} currents may introduce electrical heterogeneity in the heart and promote arrhythmia, especially during reperfusion (reviewed in (46)). Regarding the ischemic versus reperfusion opening of K_{ATP} channels, it seems plausible that blocking sarcK_{ATP} channels during ischemia delays calcium overload and decreases infarction (127), while blocking sarcK_{ATP} channels during reperfusion has negligible effect on infarction but maintains cardiac function secondary to preservation of electrical stability (as observed in (125)).

Unlike a number of other preconditioning models (reviewed in (197)), blocking the mitochondrial K_{ATP} channel during I/R did not influence exercise-induced protection from infarction (39). Interestingly, administration of a mitoK_{ATP} blocker during exercise (versus during I/R) does abolish the protection (77), indicating that mitoK_{ATP} activation might be involved in triggering the protective phenotype, but is not involved during the ischemic insult. It is important to note that the compound used as a 'specific' blocker of mitoK_{ATP} (5-hydroxydecanoate) is notoriously non-specific (39, 108-110). Future experiments using a better pharmacological approach, as well as improved insight into the molecular identity of mitoK_{ATP}, will advance our understanding regarding the involvement of mitoK_{ATP} in exercise preconditioning.

Does prior exercise protect against cellular calcium overload?

Cellular calcium overload is central to the etiology of ischemia/reperfusion damage (reviewed in (201)). There is surprisingly little information regarding the effects of exercise on myocardial calcium handling during pathological circumstances. Jew and Moore (125) found no difference in cellular calcium content between sedentary and trained hearts exposed to I/R, although the method employed indicated only the total calcium content (and does not indicate if compartmentalization of calcium, i.e. to the mitochondria, occurred). Consistent with these findings, Libonati et al. (159) found no difference in cellular calcium transients in myocytes from sedentary and trained animals, although these measurements were done in myocytes isolated after experimental I/R. Bowles and Starnes (36) used radiolabeled calcium isotopes and found that the exercise trained heart appeared to be less-susceptible to calcium overload after 30 minutes of reperfusion. One of the best physiological correlates to calcium overload is increased left ventricular diastolic pressure, and exercise does appear to protect the heart from diastolic dysfunction during I/R (42). Clearly much more investigation is warranted regarding the time-specific changes in cardiac calcium handling in hearts exposed to I/R to determine if prior exercise effectively delays the time-course for calcium overload after the onset of ischemia.

What is the role of cardiac mitochondria in exercise preconditioning?

Exercise evokes adaptations in cardiac mitochondria that likely contribute to exercise-induced cardioprotection. While increases in Krebs Cycle intermediates are not observed after exercise in the healthy heart, a number of exercise-induced changes in mitochondrial proteins associated with apoptosis and ROS scavenging have been

observed (134). Interestingly, the subsarcolemmal population of mitochondria appeared to have more changes than the interfibrillar population of mitochondria (133). While there is much work to be done to understand how the altered mitochondrial proteome leads to infarct sparing, there are clearly phenotypic changes to mitochondria following exercise training. Isolated mitochondria from exercised animals are more resistant to apoptotic stimuli (134, 279). Whether or not delayed permeability transition pore opening (PTP) is a characteristic of mitochondria from exercised animals is equivocal, with some investigators finding no change in mitochondrial calcium tolerance (241) and others finding that mitochondria from exercised animals have a greater calcium retention capacity (134, 171). A likely explanation underlying differences between studies is the experimental methods employed. Starnes et al. (241) found no differences in calcium tolerance after administering one calcium pulse (200nmol/mg mitochondrial protein) to de-energized mitochondria. Both Marcil et al. (171) and Kavazis et al. (134) found that PTP opening was delayed after training, but only when mitochondria were respiring on the complex II substrate succinate. Given the heightened ROS production with succinate-supported respiration, an improvement in endogenous ROS scavenging capacity is likely the culprit behind delayed PTP opening in these studies.

Taken together, mitochondria from exercised animals appear to be more resistant to injury, and it is likely that this healthier population of mitochondria is better able to preserve cellular energetics during oxidative stress (Figure 4). Preservation of energetics after exercise is reflected by a slower decline in myocardial ATP levels during ischemia/reperfusion (35), maintenance of cardiac oxygen consumption after

ischemia (36), prolonged time-course for sarcK$_{ATP}$ channel opening during cellular anoxia (124), and resistance to apoptotic stimuli (134).

Conclusion

Exercise training is one of the few preconditioning stimuli that evokes sustainable protection against cardiac ischemia/reperfusion injury. Improved oxidant buffering capacity, decreased cellular/mitochondrial calcium overload, and preservation of bioenergetics all appear to be involved in the underlying mechanisms. There is arguably no stimulus, whether pharmacological or physiological, that can promote such potent and long-lasting protection to hearts. Continued research into the mechanisms underlying exercise-induced cardioprotection, as well as novel pharmacological agents that are effective in exploiting these mechanisms, will improve our ability to treat those achy, breaky hearts.

Chapter 3: Short-term exercise decreases arrhythmias following thiol oxidation and ischemia in isolated rat hearts

Introduction

Sudden cardiac death due to sustained ventricular arrhythmia is a significant cause of mortality following a myocardial infarction, especially in patients with underlying cardiovascular disease (162). Epidemiological evidence indicates that humans who exercise regularly are more prone to survive a myocardial infarction (183), likely due to a significantly lower incidence of sudden cardiac death among exercised individuals (103). The cardioprotective effect of exercise training is also well documented in animal models, as exercise confers resistance against several different indices of ischemia/reperfusion injury including infarction (for review see (45, 88)), myocardial stunning (35, 41, 106, 157), and arrhythmia (reviewed in (25)). With regards to the anti-arrhythmic effects of exercise, several studies have noted that exercise evokes an anti-arrhythmic phenotype characterized by lower incidence of ventricular arrhythmia (25, 105, 118, 220) and increased ventricular fibrillation threshold (194). Despite the clear association between exercise and resistance to arrhythmia, the cellular mechanisms have not been fully elucidated.

Although prompt reperfusion remains the best treatment of an ischemic event, abrupt flow restoration is associated with a burst of reactive oxygen species (ROS) that

is believed to be partly responsible for reperfusion injury (245, 283). This ROS burst may lead to fatal arrhythmias (reviewed in (46)), and strategies that improve mitochondrial ROS scavenging have clear potential in mitigating electrical dysfunction. Exercise has been shown to decrease ROS-mediated myocardial damage (251), but an explanation of how improved tolerance to an oxidative insult protects exercised hearts against arrhythmia has not been provided.

Cardiac glutathione represents the largest capacity thiol buffer in the heart (229) and exerts a significant effect on mitochondrial function (3, 47). Recent data implicates the glutathione redox couple as a "pivoting point" between ROS balance and mitochondrial dysfunction (9, 11, 143, 237). During conditions when ROS generation exceeds scavenging capacity (such as early reperfusion), ROS-mediated opening of energy-dissipating ion channels in the inner mitochondrial membrane leads to instability in mitochondrial membrane potential ($\Delta\Psi_m$) (10). Oscillations in $\Delta\Psi_m$ activate sarcolemmal ATP-sensitive potassium (K_{ATP}) channels and can induce lability in the cardiac action potential (3, 10), a prime substrate for re-entrant arrhythmia (46). Previous investigations found that collapses in $\Delta\Psi_m$ occur when the reduced/oxidized glutathione ratio (GSH/GSSG) reaches a 'critical' level, and our previous work indicated that chemically oxidizing the cellular thiol pool induced ventricular arrhythmias under otherwise normoxic conditions (38). Pharmacological treatments that sustain GSH/GSSG have been shown to stabilize $\Delta\Psi_m$ (9, 38, 94), and prevent arrhythmias (38), but whether a physiological stimulus such as exercise can reduce arrhythmia by maintaining GSH/GSSG is not known.

Given the important influence that glutathione can exert on cardiac electrical activity, we conducted this study to determine if the anti-arrhythmic effects of short-term exercise were due to an improved tolerance to oxidative stress by the cardiac glutathione system. We hypothesized that 10 days of exercise would reduce arrhythmias evoked by either chemical oxidation of glutathione or ischemia/reperfusion. We also postulated that isolated myocytes from exercised animals would show diminished ROS emission and improved viability during cellular oxidative challenge, and that this protective phenotype would be associated with maintenance of ROS-buffering capacity by the glutathione system.

Methods

Experimental Animals

Female Sprague-Dawley rats (150-250g) were housed on a 12:12 hour light-dark cycle with food and water provided ad libitum. All experiments were conducted in accordance with guidelines established by the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996), and with prior approval from East Carolina University's Animal Care and Use Committee.

Exercise Protocol

Rats were randomly assigned into one of two experimental groups: sedentary control (Sed) or short-term exercise (Ex). Exercise was performed on a motorized treadmill similar to a well-established protocol (40). We chose an exercise protocol consisting of 10 days of running, because our previous work indicated that this protocol evokes both a cardioprotective phenotype and skeletal muscle adaptation to the exercise while minimizing the stress response in female rats (43). Following 3 days of acclimation to the treadmill (15m/min for 5, 10, and 15 minutes for days 1, 2, and 3, respectively), Ex animals received 10 consecutive days of treadmill running (6% grade) using the protocol in Figure 5.
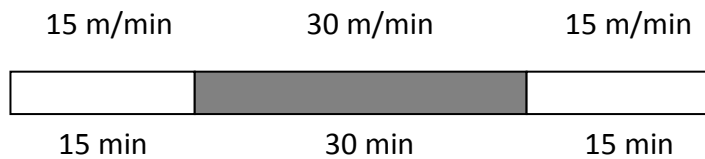
Figure 5: Exercise protocol used in this study.

Animals that did not run were placed back on the moving treadmill using prodding or a mild shock via the shock grid at the end of each running lane. Sed

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animals were handled and placed on the stationary treadmill for 5 minutes each day for 10 days.

Experimental Groups

Group 1 (N = 8 per group):

Baseline   200 µM Diamide until sustained arrhythmia

Group 2 (N = 8 Ex only):

Baseline   39 minutes of 200 µM Diamide

Group 3 (N = 7 per group):

Baseline   30 minutes 200 µM Diamide   20 minute Washout

Group 4 (N = 7 per group):

Baseline   30 minutes Global Ischemia   30 minutes Reperfusion
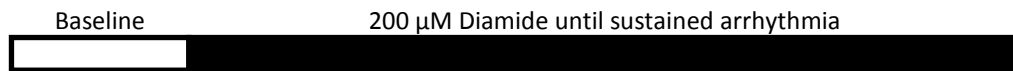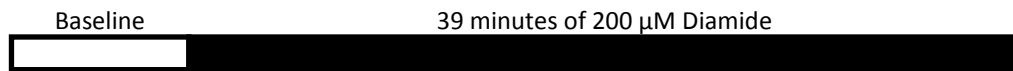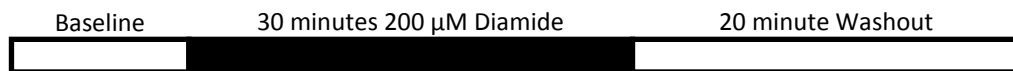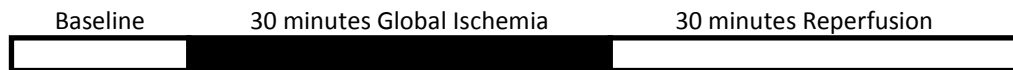
Figure 6: Protocols used in this study

A total of 71 rats were used in the study. Following 10-days of exercise (or handling control), experimental animals were used in one of the following six study arms: 1. Whole heart diamide perfusion until the heart went into a sustained (greater than 10 seconds) ventricular arrhythmia (n = 8 Sed and 8 Ex); 2. Whole heart diamide perfusion for 39 minutes (n = 8 for Ex only); 3. Whole heart diamide perfusion for 30 minutes followed by a 20 minute washout period (n = 7 Sed and 7 Ex); 4. Whole heart ischemia/reperfusion experiments (n=7 Sed and 7 Ex animals); 5. Whole-heart perfusion for 15 min (with no diamide) for 'untreated controls' (n = 6 Sed and 5 Ex animals), or 6: Isolated cardiac myocyte experiments (n=4 Sed and 4 Ex animals). Following each whole heart perfusion protocol, hearts were removed and the left ventricle frozen for further analysis. Each set of experiments is described in detail in Figure 6.

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Isolated Heart Perfusion

Twenty-four hours after the last bout of exercise (or handling control), rats were anesthetized using a ketamine/xylazine mixture (90 mg/kg ketamine, 10 mg/kg xylazine, i.p.). Upon the absence of animal response reflexes, hearts were removed via midline thoracotomy, placed briefly in 0.9% saline (4°C), and cannulated by the aorta on a modified Langendorff apparatus. Hearts were instrumented for the measurement of left ventricular function, coronary flow, and electrocardiogram as previously described (38, 236). Briefly, hearts were retrograde perfused with gassed (95%O_2 5%CO_2) Krebs buffer 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 (37^0C). The calcium content in the experimental buffers is on the high end of the physiological range, but normoxic hearts perfused under these experimental conditions retain function for at least 90 minutes with no loss of function (41), suggesting that calcium overload in our preparation (perfusion time ~60 min) is minimal in non-stressed hearts. A latex balloon (Harvard Apparatus) was inserted through the mitral valve into the left ventricle and inflated to a diastolic pressure of 4-7 mmHg for measurement of left ventricular pressures. ECG leads were placed in the bath for volume-conducted ECGs. Coronary flow was monitored throughout the protocol with a Transonic flow probe placed in series with the perfusion line proximal to the perfusion cannulas, and flow rates were normalized to heart wet weight. All measurements were recorded on a PowerLab System (A.D. Instruments) at a sampling rate of 1000Hz. Data were stored on a personal computer and subsequently analyzed using Chart 7.0 software (A.D. Instruments).

Diamide Experiments

Following instrumentation, 16 hearts (n = 8 Sed and 8 Ex) were allowed to equilibrate for 15 minutes and baseline values were recorded, after which the buffer was switched to Krebs buffer with the addition of 200µM diamide. Diamide perfusion was allowed to continue until the heart entered a sustained (greater than 10 seconds) ventricular arrhythmia. In a separate subset of Ex hearts (n = 8), diamide perfusion lasted 39 minutes, which was the average time it took for Sed hearts to go into an arrhythmia.

To determine if Ex improved the ability of hearts to recover following diamide treatment, 14 hearts (n=7 per group) were perfused with diamide for 30 minutes. After the 30 minute diamide perfusion, the buffer was switched back to the diamide-free Kreb's for a 20-minute washout period to determine the extent of recovery. The concentration of diamide used in this study has previously been shown to deplete the cardiac glutathione pool and induce cardiac arrhythmias (38). Immediately following perfusion, the left ventricle was isolated and frozen in liquid nitrogen for biochemical analysis.

Ischemia/Reperfusion Experiments

A subset of 14 hearts (n=7 and 7 for Sed and Ex groups, respectively) was subjected to 30 minutes of global no-flow ischemia by stopping flow to the heart. Following 30 minutes of ischemia, the static buffer was drained from the perfusion lines and flow was restored for 30 minutes. As with the other groups, the left ventricle was isolated and frozen at the end of reperfusion for biochemical studies.

Arrhythmia Assessment

Arrhythmias were scored in accordance with the Lambeth Conventions (263). Arrhythmias were scored for the diamide treatment and washout periods separately, and for the entire reperfusion period. The scoring system used was: 0 = 0-49 premature ventricular beats; 1 = > 50 premature beats; 2 = at least 1 episode of VT (regardless of duration); 3 = at least one episode of VF (regardless of duration) and 4 = fatal event.

Cardiac Myocyte Isolation

Hearts from Sed and Ex animals (n=4 per group) were excised after anesthesia as described above and promptly placed on a retrograde perfusion cannula. Isolated cardiac myocytes were prepared using methods similar to those previously described (37). Briefly, the hearts received 5 minutes of perfusion with calcium-free Tyrode's solution containing (in mM): 140 NaCl, 10 HEPES, 5 KCl, 1 $MgCl_2$, and 10 Glucose (pH = 7.4, 37°C). The solution was then switched to a digestion buffer consisting of Tyrode's solution plus 25µg/mL Liberase DH (Roche) and 20µM $CaCl_2$ for 22-26 minutes. The heart was cut down and the left ventricle minced in digestion buffer in a heated (37°C) dissection dish. Chunks were gently aspirated with pipettes of increasing resistance (25mL, 10 mL, and 5mL serological pipettes) for 5 minutes. The cell suspension solution was filtered through 0.25µm mesh and allowed to gravity precipitate for 12 minutes. Following gravity precipitation, cells were exposed to increasing amounts of calcium in Tyrode's (50, 100, 200, 400 µM and 1 mM), each followed by a 12 min gravity precipitation, before being put into Dulbecco's Modification of Eagle's Medium (DMEM) with 10% Fetal Bovine Serum. Once in DMEM, the cells were incubated (37°C, 5% CO_2, balance room air) and used within 8 hours post-dispersion.

Cellular ROS Fluorescence Measurements

At the time of each experiment, isolated myocytes were placed in Tyrode's solution containing 1.8mM $CaCl_2$, and cellular ROS production was measured with the fluorescent probe 5-(6)-chloromethyl-2,7-dichlorohydrofluorescein diacetate (CM-DCF, Invitrogen). CM-DCF fluorescence increases in proportion with cellular ROS production, specifically production of H_2O_2 and hydroxyl radical, but not superoxide (259). Ventricular cardiomyocytes from Sed and Ex animals were loaded with 500nM CM-DCF for 10 minutes and placed in a heated (37 $^{\circ}$C) flow-through perfusion chamber (Warner Instruments) housed on the stage of an inverted fluorescent microscope (Leica). CM-DCF fluorescence was evoked using light from a metal-halide lamp filtered to an excitation wavelength of 472nm (bandpass filter width 30nm), and emission was collected at 520nm (bandpass filter width 36nm). Emitted light was captured with a CCD camera, and images were acquired on a personal computer. To avoid photobleaching of the probe, sampling rate was set at 1 min intervals. Our preliminary data indicated that this sampling rate and flourophore concentration led to stable recordings in normoxic (non-stressed) myocytes for up to 50 minutes (data not shown). After 5 minutes of baseline imaging, the solution was switched to Tyrode's solution plus 200 μM diamide. Fluorescence was monitored every minute for 40 minutes or until cell death occurred, whichever came first.

Changes in fluorescence intensity were quantified for each time-point by subtracting the cell fluorescence (obtained via a region of interest drawn around the cell perimeter) from background fluorescence (obtained via a region of interest in an area adjacent to each myocyte). To account for unequal flourophore loading across cells, each cellular fluorescence trace was normalized to baseline fluorescence intensity (F_o)

(before diamide) for each cell. As expected, diamide treatment led to an increase in the CM-DCF signal over time, rapidly increasing after about 15 minutes of diamide treatment. Because some of our myocytes died during diamide treatment (depicted in Figure 11D), we were not able to plot the average fluorescence intensity increase over the time of treatment (as cell death precludes accurate fluorescence measurements). Therefore, we quantified an 'inflection point', when the fluorescence signal significantly increased from baseline. We defined the inflection point as the time point when the fluorescence slope (using a linear fit) increased more than 3 times from the mean slope during the first 5 minutes of treatment. At the beginning and end of each experiment, a bright-field image of each cardiac myocyte was obtained using differential interference contrast, and these images are presented along with fluorescence traces for improved clarity.

Myocardial Glutathione, Glutathione Peroxidase, and Glutathione Reductase

Myocardial glutathione was assessed as described previously (38). Briefly, total glutathione (GSHt) and oxidized glutathione (GSSG) were determined using a commercially available kit (Oxis International). Final concentrations were normalized to protein content using a BCA Protein Assay (Thermo Scientific).

For the determination of glutathione peroxidase (GPx) and glutathione reductase (GR) activities, 50-70 mg of frozen powdered tissue was homogenized in the presence of 0.3 mM 1-methyl-2-vinylpyridinium trifluoromethanesulfonate, and samples were diluted in ddH$_2$O so that 1.5 mg protein per sample was loaded into a 96-well plate. Glutathione reductase was measured similar to the method of Carlberg and Mannervik (48). Briefly, samples were loaded in triplicate on a 96-well plate containing 1 mM

GSSG and 0.5 mM NADPH in TEE buffer (10 mM Tris, 1 mM EDTA, 1mM EGTA, pH 7.4). Absorbance at 340nm was measured over 5 minutes and the rate of oxidation for 1μM NADPH/min is equivalent to 1 U of GR activity. A similar method was used for measurement of glutathione peroxidase, as described previously (250). Samples were loaded into 96-well plate along with 1 mM GSH, 100 mU/ml glutathione reductase, 0.5 mM NADPH, and tBOOH in TEE buffer. Absorbance at 340 nm was measured over 5 minutes and the rate of NADPH oxidation per minute was used to calculate the GPx activity.

Statistical Analysis

All data are expressed as mean ± S.E.M. Comparisons for dichotomous variables between Sed and Ex were done using a Student's t-test. Arrhythmia scores and glutathione content, between each group and baseline values, was analyzed with two-way ANOVA (group x treatment), followed by Newman-Keuls *post-hoc* tests. Between-group comparisons for incidence of ventricular fibrillation and fatal arrhythmias were made using a chi square test. Analysis of left ventricular developed pressure was determined via a two-factor (group x time) ANOVA with repeated-measures (time). Analysis of the Kaplan-Meier survival curve was done with a one-tailed Mantel-Cox test. For all comparisons, statistical significance was determined if $P < 0.05$.

Results

Animal Characteristics Following Exercise

Morphological data from animals in the study are presented in Table 2. Consistent with our previous work (43), our short-term exercise protocol did not lead to significant differences in body, adrenal, or spleen weights. We observed significant hypertrophy of the heart following 10 days of exercise. Baseline hemodynamic values are presented in Table 3, and we found no differences in cardiac function between Sed and Ex hearts during baseline recordings.

Table 2: Morphological data from experimental animals. Data are expressed as mean ± s.e.m. Abbreviations: HW, heart weight; BDW, body weight; LV, left ventricle.

	Sed	Ex	P Value
BDW (g)	220 ± 2	214 ± 6	0.441
Left Adrenal (mg)	35 ± 8	38 ± 7	0.321
Right Adrenal (mg)	34 ± 1	37 ± 8	0.929
Spleen (mg)	561 ± 83	599 ± 92	0.608
Heart Weight (mg)	858 ±25	954 ± 28 *	0.013
LV Weight (mg)	503 ± 17	602 ± 16 *	P<0.001
HW/BDW * 1000	4.0 ± 0.1	4.4 ± 0.2 *	0.02

Table 3: Baseline hemodynamic variables for hearts in the study. Data are expressed as mean ± s.e.m.

	Sed	Ex
LVDP (mmHg)	116 ± 8	114 ± 6
Coronary Flow (mL/min*g)	9.2 ± 1.2	10.0 ± 0.6
Heart Rate (bpm)	301 ± 13	314 ± 7
+dP/dt (mmHg/sec)	4312 ± 380	4728 ± 427
-dP/dt (mmHg/sec)	-2564 ± 240	-2903 ± 178

Incidence of Ventricular Arrhythmia Following Diamide Treatment

The time to the first sustained arrhythmia during diamide treatment is presented in Figure 7. Ex hearts had a delayed onset of ventricular arrhythmia when compared to their Sed counterparts (P<0.05; Figure 7). A sustained arrhythmia was defined as any ventricular arrhythmia occurring with a duration ≥ 10 seconds. The average time for Sed
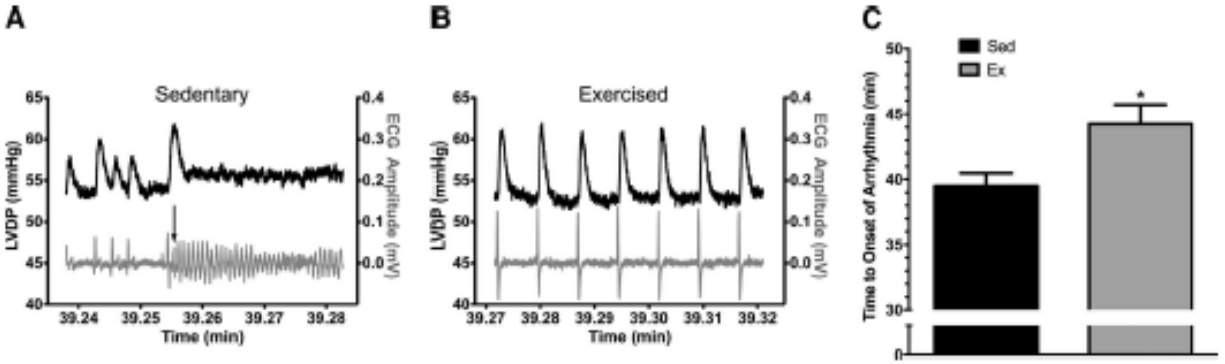
Figure 7: Exercise delays the onset of ventricular arrhythmias during sustained thiol oxidation. A. Representative left ventricular developed pressure (LVDP; black trace) and volume-conducted electrocardiogram (ECG; gray trace) recordings from a sedentary animal approximately 39 minutes into diamide treatment. The transition to ventricular arrhythmia and subsequent loss of pump function is noted with an arrow. B: Representative trace from an exercised animal during the same duration of diamide treatment as the sedentary trace. C Mean time to onset of ventricular arrhythmia following sustained diamide treatment; *, P<0.05 vs. Sed. N = 8 per group. Mean ± S.E.M.

animals to enter an arrhythmia was 39 minutes, and it should be noted that not one animal in the Ex group had a time to arrhythmia less than 39 minutes.

The extent and severity of cardiac arrhythmias for hearts in the group exposed to 30 minutes of diamide exposure and 20 minutes of washout are presented in Figure 8. As expected, during the shortened diamide treatment, the incidence and severity of arrhythmia was not significantly different between Sed and Ex. However, during the washout period, hearts from the Ex group had a significantly lower
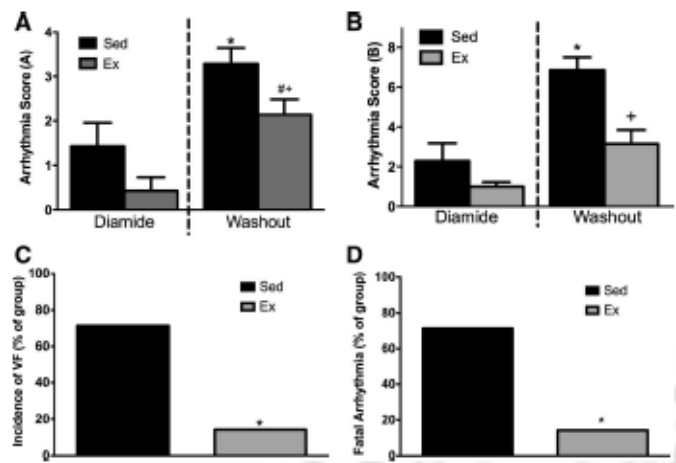
Figure 8: Ex decreases the severity of arrhythmias in the isolated rat heart exposed to reversible treatment with the thiol oxidant diamide (30 min), followed by a 30 minute washout period. **A:** Arrhythmia scores (calculated from System A) between Sed and Ex animals during/after the diamide treatment. **B:** Arrhythmia scores (calculated from System B) between Sed and Ex animals during/after the diamide treatment. **C:** Percentage of hearts in each group that experienced an episode of VF. **D:** Summary of hearts in each group that experienced a fatal (non-spontaneously reverting) ventricular arrhythmia; *, P<0.05 vs. Sed during diamide treatment; +, P<0.05 vs. Ex during diamide; #, P<0.05 vs. Sed during washout. N= 7 per group. Mean ± S.E.M.

incidence of arrhythmia, as reflected by a lower arrhythmia score and longer time until the first run of VT/VF during washout (P<0.05; Figure 8C and D). Ex significantly

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decreased the number of hearts that had an episode of VF (P<0.05; Figure 8E), and lowered the incidence of non-reverting (fatal) arrhythmia at the end of the protocol (P<0.05; Figure 8F).

Incidence of Arrhythmia Following Global Ischemia/Reperfusion

Reperfusion arrhythmias in hearts exposed to 30 minutes of global ischemia/reperfusion are presented in Figure 9. During reperfusion, hearts from Ex animals had a significantly lower arrhythmia score (P<0.05; Figure 9A). Ex also significantly decreased the number of hearts that experienced an episode of VF (P<0.05; Figure 9B).
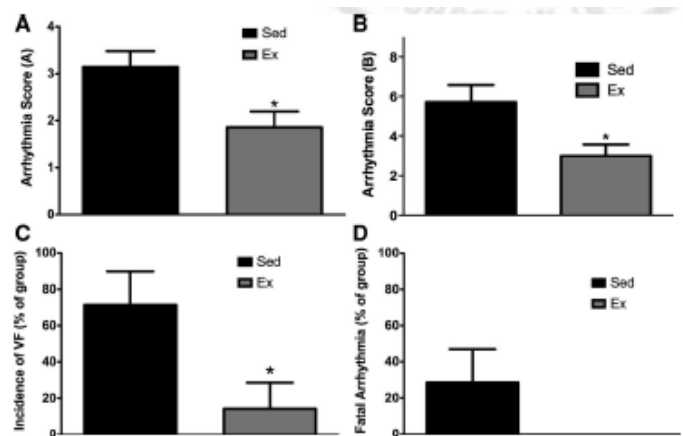
Figure 9: Incidence of reperfusion arrhythmias in hearts exposed to 30 minutes of global ischemia and 30 minutes of reperfusion. **A**: Arrhythmia scores (calculated from System A) for hearts 15 minutes into reperfusion. **B**: Arrhythmia scores (calculated from System B) for hearts 15 minutes into reperfusion. **C**. Summary of hearts in each group that experienced an episode of VF. **D**. Incidence of non-reverting (fatal) arrhythmia during reperfusion for hearts in the study. *, P<0.05 vs. Sed. N = 7 per group. Mean ± S.E.M.

The incidence of fatal arrhythmias was not significantly different between Sed and Ex animals, But none of the 7 Ex animals had a fatal arrhythmia, whereas 2 Sed animals were fatal at the end of the protocol (Figure 9 C; P=0.13).

Left Ventricular Function

Left ventricular developed pressure (LVDP) data from the study where functional recovery was under investigation are presented in Figure 10. Diamide treatment induced a steady decline in the pressure developed in the left ventricle. This decrease in LVDP was significantly attenuated in the Ex group (P<0.05). Developed pressure rebounded
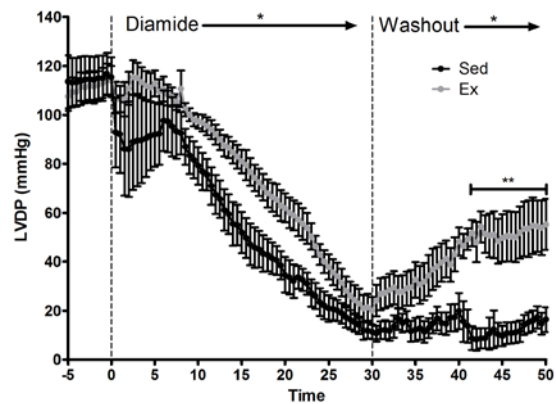
Figure 10: Left ventricular developed pressure (LVDP) during diamide treatment and washout for Sed (black circles) and Ex (gray circles) groups. Hearts were perfused with 200 μM diamide for 30 minutes, followed by a 20 minute washout; *, P<0.05 Main effect; **, P<0.05 Bonferonni post hoc for time. N= 7 per group. Mean ± S.E.M.

during the washout period in the Ex group and was significantly greater than the sedentary controls starting 11.5 minutes into the washout period and lasting throughout the remainder of the protocol (Figure 10; P<0.05). In the group exposed to ischemia/reperfusion there was no statistically significant differences in recovery of LV function between Sed and Ex animals (LVDP at the end of reperfusion was 18 ± 3 and 27 ± 4 mmHg, for Sed and Ex respectively; P=0.14).

Cellular ROS Production

Cellular ROS (specifically H_2O_2 and hydroxyl radical) production during diamide treatment was monitored in isolated ventricular myocytes with the flourophore CM-DCF, and representative images and traces are presented in Figure 11A and B. Black and white images represent bright-field images of the myocyte at the beginning (left) and end (right) of each experiment. The sudden increase in CM-DCF fluorescence after approximately 15 min of diamide has been previously observed in isolated cardiac
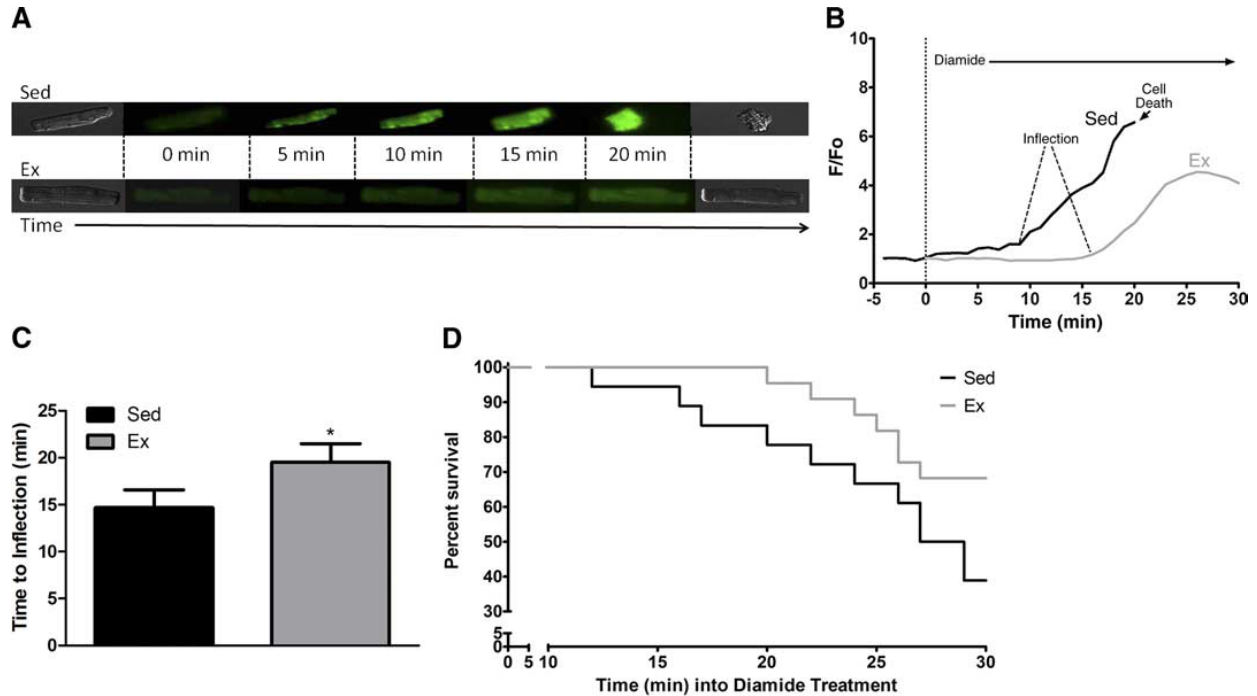
Figure 11: Ex delays the increase in ROS fluorescence and decreases cell death in isolated ventricular myocytes exposed to oxidative stress. **A**: Representative images over time for Sed and Ex myocytes. Black and white pictures represent bright field images of myocytes at the beginning and end of each experiment, and fluorescence traces in between are fluorescence images from cells loaded with the ROS sensor CM-DCF. **B**: Representative CM-DCF traces from Sed (black line) and Ex (gray line) myocytes. **C**: Average time to inflection for both groups. **D**: Survival curve for Sed (black) and Ex (gray); *, P<0.05 for Sed versus Ex myocyte survival. N = 18 (from 4 animals) for Sed and 25 (from 4 animals) for Ex. Mean ± S.E.M.

myocytes (9), and is consistent with the concept of mitochondrial criticality (for review, see (8)). This steep rise in H_2O_2 emission that preceded myocyte death was quantified using an inflection point, and the time to inflection was significantly delayed in Ex animals (Figure 11C; P <0.05). As diamide exposure often led to cell death in some cells (depicted in the Sed representative image in Figure 11A), we plotted a Kaplan-Meier survival curve for myocytes in the study (Figure 11D). Myocytes from Ex animals displayed both a delay in the onset of cell death, as well as a decrease in the total number of cells that died following diamide treatment.

Myocardial Glutathione, Glutathione Peroxidase, and Glutathione Reductase

Myocardial glutathione content data are presented in Figure 12. At baseline, there was no significant difference between Sed and Ex in total glutathione (GSHt), oxidized glutathione (GSSG), or the reduced/oxidized glutathione ratio (GSH/GSSG) (Figure 12A, B, and C respectively). In the Sed and Ex groups that were allowed to enter a sustained arrhythmia, perfusion with diamide led to a significant decrease in GSH/GSSG. In the Ex group perfused with diamide for only 39 minutes, there was also a significant decline in GSH/GSSG from baseline. However, this group had a significantly higher GSH/GSSG ratio than both the Sed and Ex groups that were allowed to continue until an arrhythmia occurred (Figure 12C).

GSHt in the Sed group decreased significantly in the washout and reperfusion groups, but did not significantly change in the Ex group (Figure 12A; $P<0.05$). GSSG levels increased in both the Ex and Sed groups entering a sustained arrhythmia as well as the Ex group receiving 39 minutes of diamide perfusion (Figure 12B).
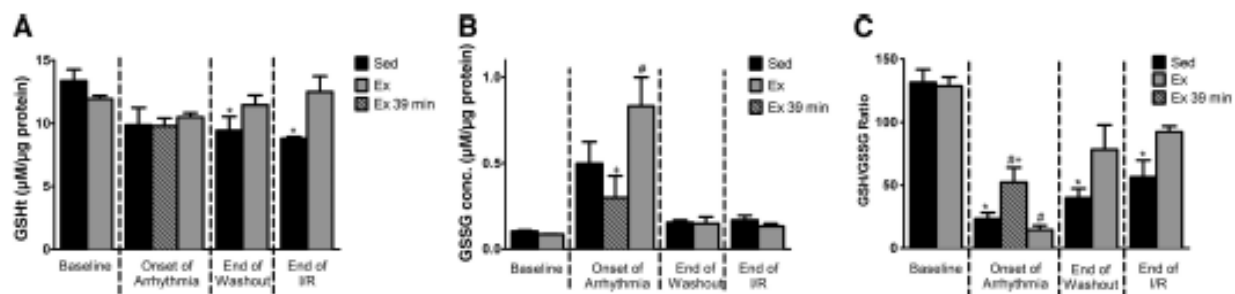
Figure 12: Myocardial content of total glutathione (GSHt), oxidized glutathione (GSSG), and ratio of reduced to oxidized glutathione (GSH/GSSG). All tissue collected immediately after the completion of each perfusion protocol. **A**: total glutathione (GSH$_t$) content. **B**: oxidized glutathione (GSSG) content. **C**: ratio of reduced to oxidized glutathione (GSH/GSSG) in hearts. *, $P<0.05$ vs. Sed at Baseline; #, $P<0.05$ vs. Ex Baseline; +, $P<0.05$ vs. Ex until arrhythmia; Mean ± S.E.M.

GSH/GSSG declined significantly following washout in the Sed hearts perfused with diamide for 30 minutes, but was not significantly decreased in the Ex group (Figure

12C). In the I/R group Sed hearts showed a decline in the ratio of GSH/GSSG, but remained unaltered in the exercise group (Figure 12C).

Glutathione reductase activities from diamide only treated hearts are presented in Figure 13. In hearts perfused until a ventricular arrhythmia occurred, there was no significant difference between Sed and Ex animals. If the perfusion was limited to
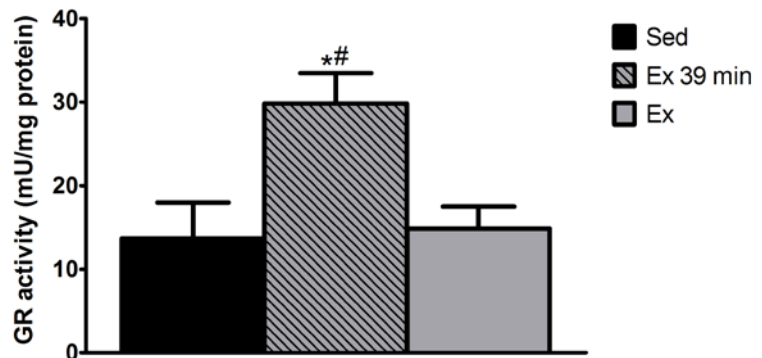
Figure 13: Glutathione reductase (GR) activity in hearts exposed to sustained thiol oxidation. Hearts were cut down after the onset of ventricular arrhythmia (time-points noted in Figure 1) and analyzed for GR activity. A subset of exercised hearts was cut down at the mean arrhythmia onset time for sedentary hearts (39 minutes). *, $P<0.05$ vs. Sed; #, $P<0.05$ vs. Ex. N = 8 per group. Mean ± S.E.M.

39 minutes in the Ex group, there was a significant preservation in glutathione reductase activity versus both the Sed and Ex groups ($P<0.05$; Figure 13).

Enzyme activities from untreated hearts are presented in Figure 14. There were no differences in the activity of myocardial GPx between Sed and Ex. On the other hand, GR activity was significantly greater in the hearts of Ex animals than in Sed animals (Figure 14; $P<0.05$).
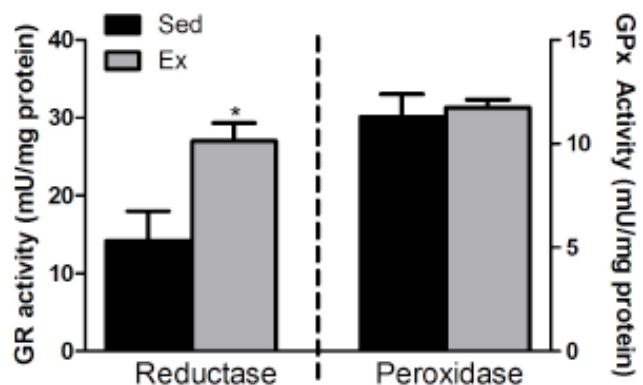
Figure 14: Basal changes in cardiac glutathione reductase (GR) and glutathione peroxidase (GPx) enzyme activity for sedentary (Sed) and exercise trained (Ex) hearts. *, $P<0.05$. N = 6 for Sed and 5 for Ex. Mean ± S.E.M.

Discussion

This study was conducted to determine if short-term exercise (Ex) protected hearts against arrhythmias during conditions of oxidative stress. Our results indicate that Ex delays the onset and decreases the incidence and severity of ventricular arrhythmias following thiol oxidation and ischemia, and that augmented ROS-buffering capacity by the cardiac glutathione system is associated with this anti-arrhythmic phenotype. To the best of our knowledge, several aspects of this work represent novel findings. First, we demonstrated that Ex confers an anti-arrhythmic phenotype in isolated female hearts exposed to two different oxidative challenges. Second, we observed that isolated myocytes from Ex animals display slower rises in cellular H_2O_2 levels and lower incidence of cell death during oxidative stress than sedentary cells. Third, Ex led to better maintenance of GSH/GSSG following thiol oxidation or ischemia. Finally, increased glutathione reductase activity is directly related to the preservation of the glutathione redox couple in a more reduced state during sustained oxidative stress. Taken together our results indicate that short-term exercise augments the ability of female hearts to replenish cardiac GSH through glutathione reductase reaction during an oxidative challenge leading to decreased incidence and severity of arrhythmias, improved cardiac function, and delayed cellular death.

Exercise-induced Protection Against Arrhythmia

Our observation that Ex reduced fatal ventricular arrhythmias confirms findings in both human epidemiological studies (103) and previous reports using animal models (105, 118, 194, 220). Using an isolated heart model, we found that Ex reduced the incidence and severity of ventricular tachycardia/fibrillation (VT/VF) either after

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overwhelming cellular anti-oxidant defenses with the thiol-oxidant diamide or following global ischemia/reperfusion. These findings are in agreement with other studies where exercise protected against arrhythmias induced by *in vivo* ischemia/reperfusion (105, 118, 220) or ectopic ventricular pacing protocols (194). By using the isolated female rat heart, we were able to corroborate earlier work (in male animals) (194) showing that exercise induces intrinsic adaptations in the heart that protect against arrhythmia.

In this study, we found that Ex delays the onset of ventricular arrhythmia during conditions of sustained oxidative stress. A likely explanation for this time-dependency is that exercise, like other preconditioning stimuli, *delays* the onset of injury but loses efficacy with prolonged insult (88, 186, 203, 273). Interestingly, when the diamide insult was shortened to investigate the ability of Ex hearts to recover we found that this protection from arrhythmias persisted, possibly due to the preservation of glutathione reductase reaction's ability to re-reduce oxidized glutathione. This Ex-induced decrease in arrhythmias also allowed for better recovery of cardiac function after diamide (consistent with several ischemia/reperfusion studies (35, 41, 157, 279)).

Role of the Cardiac Glutathione System in Arrhythmias

Glutathione is the largest capacity thiol buffer in the heart (229), and uncompensated oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) can induce catastrophic ventricular arrhythmias (for review see (8, 46)). In this study, we used two different models of glutathione oxidation to evoke arrhythmia: chemical oxidation of the glutathione pool with diamide, and ischemia/reperfusion. As expected, each intervention reduced both total glutathione and the reduced/oxidized glutathione

(GSH/GSSG) ratio in Sed animals (See Figure 12). These findings are in agreement with previous studies where ischemia/reperfusion depleted cardiac glutathione (154) and lowered GSH/GSSG (51, 210, 253, 265). In sedentary controls, the extent of glutathione depletion/oxidation with diamide was similar to what we observed following reperfusion (Figure 12) and the incidence and severity of arrhythmias was also comparable between these groups (see Figure 8 and Figure 9).

During conditions of oxidative stress, cardiac action potentials can become heterogeneous subsequent to the opening of sarcolemmal ATP-sensitive potassium (K_{ATP}) channels (3, 199). Oscillatory K_{ATP} currents occur in phase with fluctuations in mitochondrial membrane potential ($\Delta\Psi_m$) when cellular/mitochondrial anti-oxidants become overwhelmed (10). Collapses in $\Delta\Psi_m$ following GSH oxidation have been observed in intact hearts (38, 237) and isolated myocytes (9). This collapse in bioenergetics induced by GSH oxidation was associated with the onset of ventricular arrhythmias (38). Pharmacological treatments that maintain GSH/GSSG ratios during oxidative challenge sustain $\Delta\Psi_m$ (9, 94) and decrease cardiac arrhythmias (38). Of notable translational interest, improving the GSH buffering capacity with the glutathione precursor N-acetylcysteine was also effective in preventing arrhythmias in humans undergoing cardiac surgery (204).

Exercise-induced Preservation of Cell Viability and GSH/GSSG

We show for the first time that Ex leads to a phenotype that is resistant to arrhythmias through the preservation of GSH/GSSG. This is evidenced by the fact that both GSH/GSSG and glutathione reductase activity are higher in our Ex group

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subjected to only 39 minutes of diamide treatment when compared to the Sed group. This exercise-induced delay against GSH oxidation and subsequent arrhythmias in intact hearts was associated with improved cellular H_2O_2 buffering and significantly greater resistance to cell death during thiol-oxidation in isolated cardiac myocytes. To the best of our knowledge, this study is the first observation that exercise-induced protection against sustained oxidative stress has been shown in viable ventricular cardiomyocytes. During oxidative stress, there is a sharp increase in ROS emission observed when 'mitochondrial criticality' is reached (8). We observed a spike in cellular ROS along the same time-scale as previous work (10), and found that the time until this ROS surge was significantly prolonged in myocytes from Ex animals.

The depletion in cardiac GSH was attenuated in Ex hearts in the washout and I/R groups (Figure 12A). These results support our hypothesis that Ex delays the ROS surge because of preserved cardiac H_2O_2 scavenging capacity. Short-term exercise did not alter basal GSH/GSSG ratio or the GPx enzyme activity, consistent with several other reports (106, 220, 241). However, following either challenge, GSH/GSSG was much better maintained in Ex hearts. This suggests that the preservation of GSH in a more reduced state is more important in delaying H2O2 accumulation than scavenging of H_2O_2 by the GPx reaction.

Exercise-induced sustainment in GSH/GSSG was likely due to increased activity of glutathione reductase, which has been previously reported in female hearts after exercise (268). As the enzyme responsible for reducing GSSG back to GSH, these data suggest that it is not the ROS-scavenging ability of the glutathione system that is

protective following exercise (as there is no increase in GPx activity; see Figure 14), but rather the ability of the heart to replenish GSH. Given that the *de novo* synthesis of glutathione is very low in the heart (99), and that GSSG is released from the heart during reperfusion (254) the importance of GSH replenishment is obligatory for the heart's ability to withstand a sustained oxidative challenge. Of interest, the ability to regenerate GSH is enhanced by a polarized $\Delta\Psi_m$ to support NADPH regeneration by the mitochondrial NADH/NADPH transhydrogenase (229). While the high GSH/GSSG ratio that we observed in Ex hearts exposed to oxidative stress would likely be associated with a higher $\Delta\Psi_m$ (9), future experiments will provide insight into additive effects of enhanced glutathione reductase and better maintenance of $\Delta\Psi_m$ after Ex.

Although there may be variability across animal species, it is interesting to note that a GSH/GSSG ratio < 50 has been implicated as a threshold for the opening of the mitochondrial permeability transition pore (9), which induces necrotic and apoptotic cell death (102). Our observation that GSH/GSSG in animals that were perfused with diamide until an arrhythmia occurred had values below 50 while the Ex group that received only 39 minutes of perfusion remained higher than 50 and was absent of any sustained ventricular arrhythmias is of particular interest. Furthermore, Ex animals receiving only 30 minutes of diamide followed by a washout, exhibited a decrease in arrhythmia severity, improved cardiac function, and GSH/GSSG values above 50 in our Ex group versus lower values in their Sed counterparts.   This preservation of GSH/GSSG may explain the delayed production of ROS and lower propensity for cell death in Ex myocytes. While speculative, this would be in line with previous studies

indicating that Ex elicits a phenotype characterized by reduced opening of the mitochondrial permeability transition pore (134).

Our finding that Ex improves the ROS-scavenging ability of the heart through improved GSH replenishment compliments several studies examining other ROS-scavenging mechanisms within the myocardium. Using anti-sense oligonucleotides, Hamilton et al. (105) showed a role for manganese superoxide dismutase (MnSOD) in exercise-induced protection against arrhythmia, and a recent paper by Quindry's group also showed upregulated MnSOD activity following short-term exercise training that associated with the anti-arrhythmic phenotype (220). In both of these studies, there was no clear exercise-induced upregulation in either GPx or catalase, the major routes for the conversion of H_2O_2 to water. Given that the product of enzymatic superoxide dismutation is H_2O_2, the lack of heightened capacity to break down the H_2O_2 (augmented by upregulated MnSOD) left several unanswered questions. In this study, we offer a putative explanation that the ability of the Ex heart to scavenge H_2O_2 lies not in improved scavenging by GPx (seen herein and also in both (105, 220)), but by augmented replenishment of GSH through glutathione reductase. Indeed, other studies have shown that GR activity increased (in liver) proportionally with the amount of exercise (269). Although there are obvious limitations to this speculation, including sex-specific differences in MnSOD (44) and GPx (268), this viewpoint could provide a consensus into how Ex augments ROS-scavenging capacity from superoxide production through the complete conversion to water. Clearly, future experiments are warranted to support this notion.

Limitations

This study used 10 days of exercise to elicit a cardioprotective phenotype. Our previous work showed that 10 days of exercise in female rats evoked some early adaptations to training (such as increased skeletal citrate synthase activity), while minimizing markers of systemic stress (43). This model of exercise may be independent of changes seen with classical 'exercise training', where the exercise stimulus is carried out for many months and more robust training adaptations (such as resting bradycardia) are observed. Also, we obtained a volume-conducted ECG signal by placing the leads directly into the bath surrounding the heart. Volume-conducted ECGs are generally more variable than leads placed on the surface of the heart and thus did not allow for direct comparison of electrical vector between groups. We used 2.0 mM $CaCl_2$ in our Kreb's Henseleit buffer. This level of calcium may be considered hypercontractile, however all groups were exposed to the same levels of calcium.

Conclusions

In this study, Ex induced intrinsic changes to the female heart that reduced the susceptibility to arrhythmias during two distinct oxidative challenges. This cardioprotection was observed in intact hearts and isolated cardiac myocytes, and involved augmented ROS buffering capacity after exercise. Specifically, the ability to maintain a reduced glutathione environment through heightened glutathione reductase activity may be the underlying mechanism by which Ex confers protection. Our findings contribute to a growing body of literature describing the cardioprotective effects of exercise, a very inexpensive and widely available preventative strategy. Future studies examining pharmacological strategies designed to improve glutathione replenishment

have enormous potential as prophylactic therapies seeking to abrogate fatal ventricular

arrhythmias.

Chapter 4: Exercise-induced preconditioning relies on NAD(P)H oxidase, and not mitochondrial, derived reactive oxygen species in isolated rat hearts.

Introduction

Cardiovascular disease constitutes a significant amount of the mortality within the United States, with myocardial ischemia accounting for the largest portion of these deaths. Despite the fact that prompt reperfusion remains the best treatment for myocardial ischemia, restoration of coronary flow is associated with further cell death, likely due to an increase in reactive oxygen species at the onset of reperfusion (210, 254, 283). An improved capacity to buffer reactive oxygen species is centrally involved in exercise-induced protection from ischemia/reperfusion (I/R) injury (87).

Under pathological conditions, reactive oxygen species (ROS) are generally thought to exacerbate the insult. However, evidence is emerging the ROS signaling actually may play an important role in cell life as well as death (for review see (101, 116)). ROS have been implicated in the cardioprotective signaling cascade underlying ischemic preconditioning (79, 161, 247), heat stress (13), anesthesia (34, 66), and angiotensin II treatment (138). Interestingly, ROS signaling appears to be upstream of protective signaling mediated by PKC, implying that ROS may be one of the first steps of the signal transduction cascade leading to cardioprotection (79).

Exercise has been shown to be a potent and sustainable form of cardiac preconditioning by us and others (41, 44, 104, 251). In our previous work we demonstrated that prior exercise led to an increase in the ability of glutathione to buffer ROS and decrease ventricular arrhythmias (90). This was associated with an increase

in glutathione reductase (GR) activity and better maintenance of reduced to oxidized glutathione (GSH:GSSG). Similarly others have shown that increased antioxidant capacity following an exercise regimen is associated with a cardioprotective phenotype (105, 271), although the signaling processes involved in antioxidant upregulation are not fully understood.

A modest increase in ROS may play a vital role in cellular events leading to exercise preconditioning, and several studies suggest that ROS-scavengers may actually prevent the cardioprotective adaptations after exercise. During exercise ROS production has been shown to increase (for review see (123, 214)) and this transient redox shift can be blocked with administration of an antioxidant (5, 192). Using the NAD(P)H oxidase inhibitor apocynin, Sanchez and colleagues found that inhibiting the formation of NAD(P)H oxidase blunts the cardioprotective effects of exercise and tachycardia (227). Use of the general antioxidant N-2-mercaptopropionyl glycine (MPG) has also been shown to blunt the beneficial effects of exercise on myocardial infarction (5) and cardiovascular function (192). However, neither of these studies directly compared if the site of ROS generation are important for the signaling involved in exercise-induced preconditioning.

The purpose of this study was to investigate the importance and origin of ROS generated during exercise on exercise-induced preconditioning. Using Bendavia, a novel peptide that has been shown to decrease mitochondrial ROS (57, 248, 281), and apocynin (an inhibitor of NAD(P)H oxidase formation) we investigated two major sources of ROS generation within the myocardium. Use of Bendavia in an animal model of diabetes has previously been shown by our lab to be protective (235). Herein we find

that administration of Bendavia just prior to exercise did not blunt the protection, but administration of apocynin abolished the protective effects of exercise. Our data suggest that during exercise a transient increase in the oxidative burden occurs, increasing GR activity. This study is the first to directly investigate the compartmentalization of ROS generation in exercise. Furthermore, this study provides novel insight into how increased oxidative stress during an exercise bout can lead to post-translational modifications of GR.

Methods

Animals

All experiments were conducted in accordance with guidelines established by the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996), and with prior approval by East Carolina University's Animal Care and Use Committee. Female Sprague-Dawley rats (150-250g) were housed on a 12:12 hour light-dark cycle with free access to food and water. A total of 94 animals were used in this study.

Exercise Protocol

Rats were exercised daily as described previously (89). Briefly, rats were given a 3 day acclimation period on the treadmill. Ex animals then received 10 consecutive days of treadmill running for 60 minutes per day at 15 m/min for 15 minutes, 30 m/min for 30 minutes, and 15 m/min for 15 minutes. Animals that did not run were gently prodded or received a mild shock.

MTP-131 and Apocynin Injections

Rats were randomly assigned to receive an injection (i.p.) of either 1.5 mg/kg MTP-131 (n=7/group for Sed and Ex), 5 mg/kg apocynin (n=8 and 12 for Sed and Ex respectively), or 0.9% saline (n= n=12/group for Sed and Ex). The injection was given ten minutes prior to each bout of exercise or handling control.

Isolated Heart Experiments

Twenty four hours after the last bout of exercise rats were injected with ketamine/xylazine (90 mg/kg ketamine, 10 mg/kg xylazine, i.p.) and upon the absence of reflexes the hearts removed via midline thoracotomy and retrograde perfused on the cannula of a modified Langendorff apparatus as described previously (38, 236). Following a 15 minute baseline period global, no-flow, ischemia was induced for 25 minutes. After 25 minutes, the static buffer was drained from the lines and flow was re-established for two hours. Immediately following two hours of reperfusion the left ventricle was isolated and stained for infarct via TTC staining as described previously (236). In a separate subgroup of animals hearts were perfused for 10 minutes before being snap frozen for subsequent biochemical analysis (n=4/group for Sed, Ex, and Ex + Apocynin).

Glutathione Reductase Inhibition

In another subset of hearts, carmustine (BCNU) was used in the isolated heart model described above to inhibit glutathione reductase activity. Following a 10 minute baseline period the buffer was switched to a Kreb's buffer with 150 μM BCNU for 5 minutes before global ischemia induction (n=6/group for Sed, Sed + BCNU, Ex, and Ex + BCNU) and continued throughout the reperfusion period.

Arrhythmia Assessment

Arrhythmias were scored as described previously (90) based on the electrocardiogram signal according to the Lambeth Conventions (63, 263). The incidence of ventricular fibrillation (VF) and fatal arrhythmias was noted for each group.

Myocardial Glutathione Content

Glutathione content was measured as described previously (38, 89). To assess the amount of glutathione oxidation that occurs during exercise animals were trained as normal and immediately following the last bout animals were anaesthetized and left ventricle snap frozen (n=4/group for Sed, Ex, and Ex + Apo).

Glutathione Reductase Activity

Glutathione reductase activity was measured in tissue homogenates as described previously (89). To determine the redox sensitivity of glutathione reductase within cardiac tissue a subset of experiments were carried out in which tissue homogenates were incubated with DTT (2mM) or diamide (200 µM) at 4^0C for 20 minutes prior to testing the activity.

Immunoblot Analysis

Left ventricle homogenates were subjected to SDS-PAGE and subsequent transfer to a nitrocellulose membrane. Membranes were blocked for 1 hour at room temperature with Tris-buffered saline containing 0.1% Tween and 4% bovine serum albumin. Following blocking the membrane was incubated with an antibody for GR (1:1000; Invitrogen) overnight at 4°C. The membrane was then washed and incubated with an IR-Dye-conjugated secondary antibody (LiCor Biosciences). Membranes were scanned and quantified using the Odyssey Infrared Imaging system (LiCor Biosciences).

Statistical Analysis

All data are presented as mean ± S.E.M. A two-way ANOVA (training x treatment), followed by Newman-Keuls *post-hoc* test was used for all analysis. Between-group comparisons for the incidence of ventricular fibrillation and fatal arrhythmias were made using a chi square test. For all comparisons, statistical significance was determined when $P<0.05$.

Results

Animal Morphology and Baseline Characteristics

Rat morphological data is presented in Table 4. No differences were observed in body weight between groups. Furthermore, no indices of stress (as assessed by splenic atrophy or adrenal hypertrophy) were seen. However, there was a trend towards cardiac hypertrophy in our Ex animals following 10 days of exercise (P=0.07).

Table 4: Morphological Data from animals in this study. Data presented as mean ± S.E.M.

	Sed/Saline	Sed/Bendavia	Sed/Apocynin	Ex/Saline	Ex/Bendavia	Ex/Apocynin
BDW (g)	234±5	242±7	220±3	247±7	243±8	235±7
Spleen (mg)	610±23	614±31	653±28	641±28	629±27	639±40
L. Adrenal (mg)	31±2	32±2	29±1	34±2	34±2	30±2
HW/tibia (mg/mm)	30±1	28±1	26±1	32±1	30±1	30±1

Myocardial Infarction

Infarct size is presented in Figure 15. As expected, our Ex group had a significant reduction in infarct size (53 ± 3 vs. 40 ± 1% for Sed and Ex respectively; $P < 0.05$). Treatment with Bendavia had no effect on infarct size in our Ex group (42 ± 4%; $P > 0.05$), but treatment with apocynin abolished the protective effects of exercise (53 ± 2%; $P < 0.05$ vs. Ex/Saline). In our Sed animals apocynin treatment had no effect on

myocardial infarction (55 ± 3%; P > 0.05), but Bendavia treatment significantly

decreased the size of infarction (41 ± 3%; P <

0.05 vs. Sed/Saline).
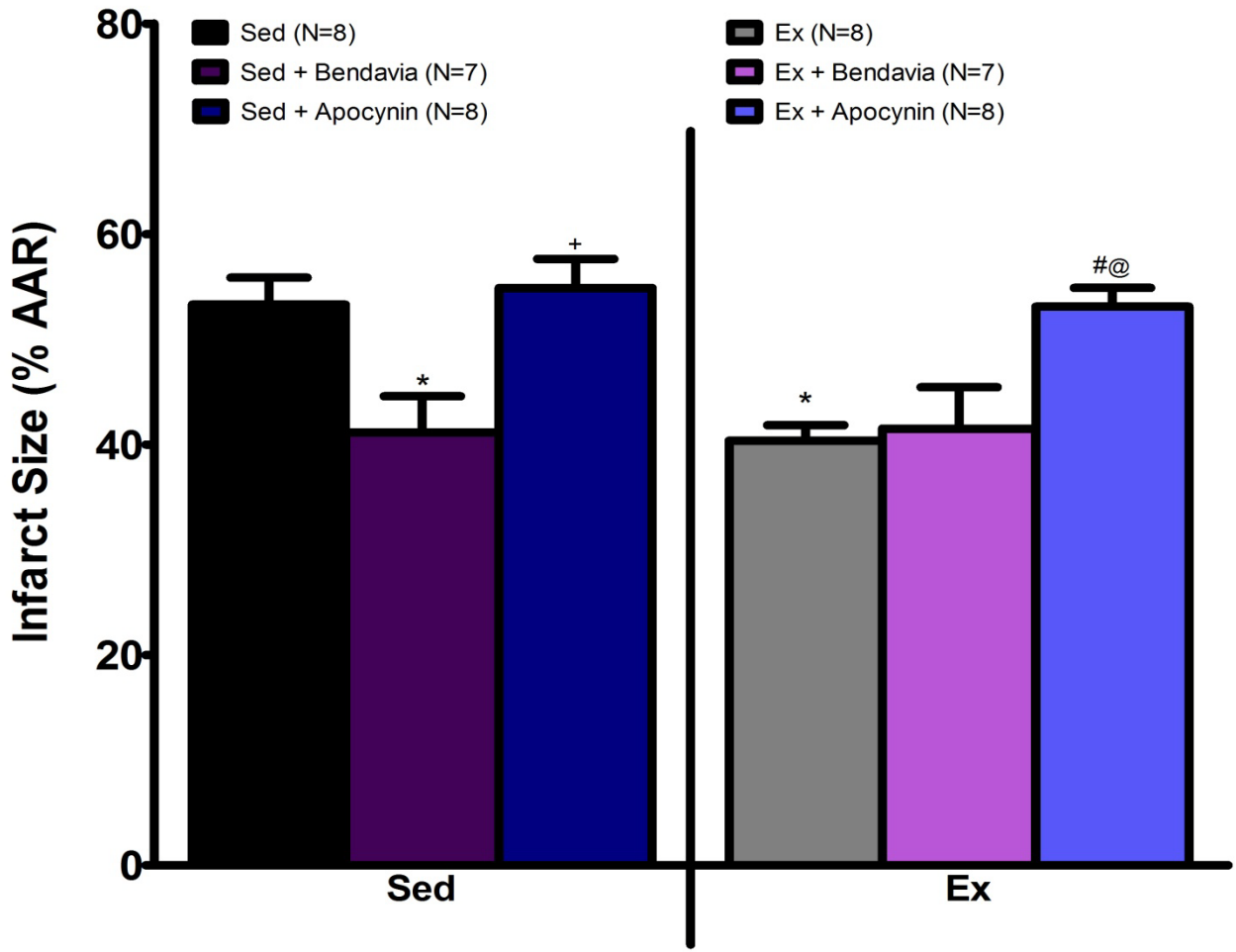
Figure 15: Exercise-induced preconditioning abolished with apocynin treatment. * P<0.05 vs. Sed, # P<0.05 vs. Ex, + P<0.05 vs. Sed and MTP, @ P<0.05 vs. Ex and MTP. Mean ± S.E.M.

Glutathione Reductase Expression and Activity

Corroborating our previous work (89) Ex animals had increased GR activity (20.3 ± 1.3 vs. 49.9 ± 7.5 mU/mg for Sed and Ex respectively; P < 0.05; Figure 16). This increase in activity was abolished in Ex animals treated with apocynin (26.4 ± 8.2 mU/mg; P < 0.05). If Ex samples were

Figure 16: Inhibiting NAD(P)H oxidase generated ROS prior to exercise blunts increases in glutathione reductase activity. * P<0.05 vs. Sed, # P<0.05 vs. Ex

treated with DTT, a thiol reductant, activity was reduced (26.3 ± 10.3 mU/mg; Figure 17). Furthermore, if Sed samples were treated with diamide, a thiol oxidant, GR activity was significantly increased (57.3 ± 6.2 mU/mg; P < 0.05). No difference was seen
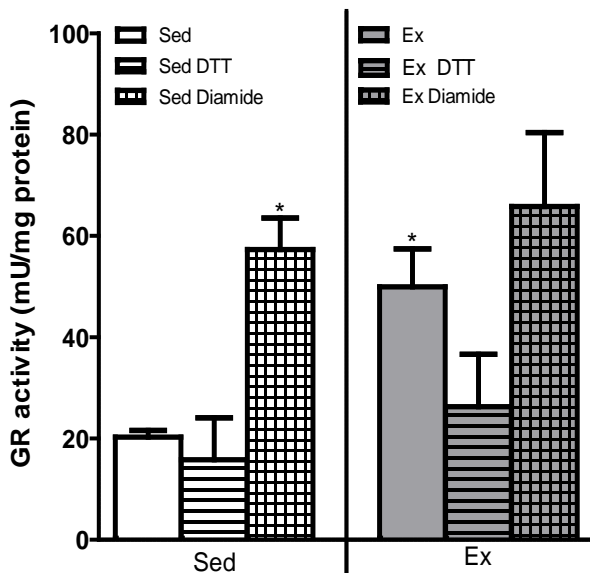
Figure 17: Redox modification of GR activity in Sed and Ex left ventricular homogenates. * P<0.05 vs. Sed.
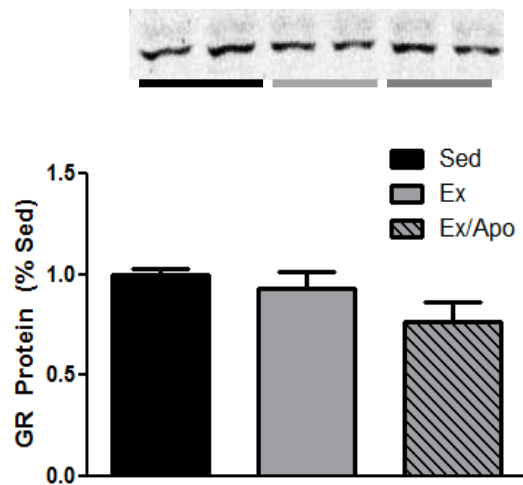
Figure 18: No differences in GR protein expression between groups.

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between groups in the expression of GR protein (P > 0.05; Figure 18).

Glutathione Reductase Inhibition

Inhibition of glutathione reductase with the pharmacological inhibitor BCNU abolished the protective effects of Ex from myocardial infarction (57 ± 1; P < 0.05; Figure 19A). Perfusion with BCNU also abolished the protection of exercise against ventricular arrhythmias (Figure 19B)

A.   **B.**
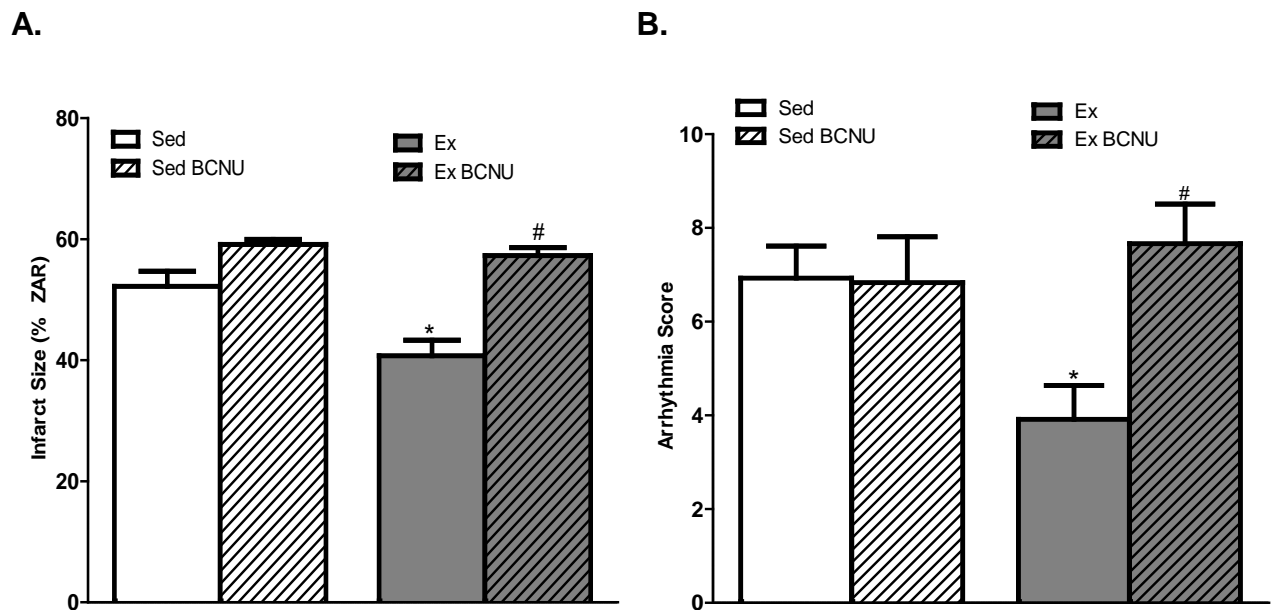
Figure 19: GR inhibition abolishes exercise-induced preconditioning from myocardial infarction and ventricular arrhythmias. Infarct size (**A**) and arrhythmia (**B**) data from isolated hearts perfused with BCNU *, P<0.05 vs. Sed; #, P<0.05 vs. ; Data presented as mean ± S.E.M.

Discussion

Main Findings

In this study we investigated if ROS produced during exercise act as signaling molecules that lead to exercise-induced preconditioning and if the site of this ROS generation plays a role. The major finding of this study is that ROS generated through NAD(P)H oxidase, and not within the mitochondria, act as signaling molecules involved in increasing the activity of glutathione reductase. We found that during exercise the oxidative burden on the cell transiently increases and may shift the oxidative state of GR, increasing its activity. Confirming the importance of GSH replenishment in exercise-induced preconditioning, we showed that pharmacological inhibition of GR abolished the beneficial effects of exercise.

This study is important in establishing that ROS can act as signaling molecules under physiological conditions and that GSH replenishment through the GR reaction are important steps in exercise-induced cardioprotection. To our knowledge this is the first study to investigate if the origin of ROS (i.e. mitochondrial vs. cytosolic) plays a role in exercise-induced preconditioning. Interestingly, a recent study found that cytosolic ROS, and not mitochondrial, may contribute to cardiac hypertrophy in a glucose transporter knockout mouse (158). Further investigations into the origin of ROS and their role in physiology/pathophysiology will help establish their role response to various stressors and may alter the way we view ROS.

The Role of GSH in Cardiac Health

During a bout of exercise reactive oxygen species are generated which lead to a transient oxidative shift in cellular thiols and in this study we establish that these ROS are generated through NAD(P)H oxidase and not the mitochondria. It is important to note that this transient increase in oxidative stress is protective, whereas others have found that when ROS are constitutively high some of the effects of exercise training are absent (223). The ability to maintain a high reduced to oxidized GSH:GSSG ratio is important for buffering ROS in the heart and is linked to improved cardiac function (38, 46). Herein we found that an improved ability to maintain GSH may be a requisite for exercise-induced preconditioning. This corroborates our previous work where we showed the Ex animals had an increase in GR activity which was associated with an improved ability to maintain GSH in a reduced state, a decrease in ROS accumulation, and a lower incidence of fatal ventricular arrhythmias (89).

Under physiological conditions, the heart's ROS production is countered by sufficient antioxidant defenses. However, during pathological conditions ROS production exceeds the intrinsic antioxidant defenses and leads to cell death (11) (for example, ROS generation is elevated in early reperfusion (30, 283, 284)) and an ability to decrease this oxidative burden has been associated with improved cardiovascular health. Improved ROS scavenging following exercise training is well established in the literature (for review see (87)), but the role of the glutathione system in exercise-induced preconditioning remains unclear (131, 221, 241). This study helps establish that increased GR activity and maintenance of GSH:GSSG is important to the beneficial effects of exercise.

Redox Modification of Glutathione Reductase

Since there is little *de novo* synthesis of GSH in the heart (99) the ability to replenish GSH is of significant importance. GSH represents the largest capacity thiol buffer in the heart (229) and is known to exert a significant effect on mitochondrial function (9, 237) and improving the heart's ability to maintain GSH in its reduced state is an area that warrants further study. Fortunately, the reduction of GSSG by GR is kinetically more favorable that its other fates with the rate of the GR reaction approximately 20 times higher than its utilization or loss from the cell (18, 58, 202).

The activity of GR has been shown to be affected by temperature, pH, and the redox status of the cell (211). Importantly, Edwards et al. found that GR does not exist in either a phosphorylated or carboxylated form (83), suggesting that post-translational modification of GR is primarily due to redox modification (95, 173, 211). In support of this, GR activity seems to be inversely proportional to the concentration of GSH (58). In mammals, where pH and temperature are held relatively constant under physiological conditions, redox modification of GR may be a pathway by which the heart increases its ability to replenish GSH under conditions of oxidative stress. In this study we found that Ex significantly increased GR activity and this increase in activity could be blunted if left ventricle homogenates from Ex animals were incubated with the thiol reductant, DTT (Figure 17). Similar to others (173), we also found that GR activity was significantly increased in our Sed group if samples were incubated with the thiol oxidant diamide, suggesting that redox modification of GR does occur within cardiac tissue. Although speculative, these data also seem to suggest that Ex animals have significantly more

oxidation of GR than Sed counterparts and future studies will be needed to validate this hypothesis.

Physiological Role for NAD(P)H Generated ROS

In this study we investigated the two major sites of ROS production within cardiac tissue: the mitochondria and NAD(P)H oxidase and found that NAD(P)H oxidase generated ROS are potent signaling molecules in exercise-induce preconditioning. In cardiac tissue NAD(P)H oxidase exist in two isoforms, NOX2 and NOX4 (47) and appears to be located in T-tubules, in close proximity to the sarcoplasmic reticulum (SR) (115, 277). An interesting observation is that NOX4 may generate ROS within a cellular compartment that is not readily accessible to current probes for superoxide, but once it is converted to hydrogen peroxide it is easily detected (76, 233). ROS produced in close proximity to the SR may play a role in modification of the ryanodine receptor and increasing calcium release (20, 115). Although speculative, ROS generated through NAD(P)H oxidase may provide an alternative mechanism to increase intracellular calcium during a bout of exercise and this physiological role for ROS generated within the cytosol (vs. the mitochondria) may explain their importance in exercise-induced preconditioning.

Conclusions

The results of this study include several novel findings that are important to the field of exercise-induced preconditioning. First, ROS generated through NAD(P)H oxidase, and not within the mitochondria, act as signaling molecules in the cardioprotective pathway. Second, an ability to increase GR activity seems to be a

requisite for decreasing cellular injury. Third, this study establishes that transient increases in ROS production occur during exercise and shift GSH:GSSG to a more oxidized state. This decreased ratio of GSH:GSSG may in turn act as a mechanism by which thiols on GR are modified and activity is increased.

Chapter 5: Reduction of ischemia/reperfusion injury with Bendavia, a novel mitochondrial-targeting cytoprotective peptide.

Introduction

Early and successful myocardial reperfusion with primary percutaneous coronary intervention remains the most effective strategy for reducing the size of a myocardial infarct and improving clinical outcome. Reperfusion injury remains a major issue in patients who receive percutaneous coronary intervention, thrombolysis or have spontaneous reperfusion for ST-segment myocardial infarction. Given that long-term prognosis has been linked to both size of infarction(112, 114, 178), strategies aimed to decrease infarct size have significant clinical potential.

Reactive oxygen species are centrally involved in the development of myocardial infarction. Augmented production of reactive oxygen species (ROS) during early reperfusion contributes to myocyte death (175, 228). Elevated ROS increases the open probability of the mitochondrial permeability transition pore (PTP)(102), which is followed by bioenergetic collapse and ultimately cell death. Given that mitochondrial ROS production is high in early reperfusion, therapies that directly (and effectively) target mitochondrial ROS production are ideal in this setting. Recently, a novel class of cell permeable small peptides has been developed that selectively target mitochondria. The Szeto-Schiller (SS) peptides are relatively small (<10 amino acids), water soluble molecules that contain a similar structural motif of alternating basic (Arg, Lys) and aromatic (Phe, Tyr, Dmt (2'6'-dimethyltyosine)) residues which allows them to freely cross cell membranes (despite a 3+ net charge at physiological pH)(280). Studies with fluorescent and radiolabeled SS peptides indicate that they localize to mitochondria and

concentrate at the inner mitochondrial membrane (IMM) (281). One particular peptide, Bendavia (also called SS-31) has been shown to significantly reduce ROS levels (248) and protected hearts from injury in a rodent model of ischemia/reperfusion(56).

The purpose of the present study was to determine whether the mitochondrial-targeting cytoprotective peptide Bendavia could protect the myocardium from reperfusion injury. We discovered that Bendavia decreases infarct size in an isolated heart model. This was corroborated with work done in isolated cardiomyocytes showing that Bendavia may abolish cell death due to ROS accumulation during early reperfusion and lead to better maintenance of bioenergetics.

Methods

      The animals used in these studies were maintained in accordance with the policies and guidelines of the Position of the American Heart Association on Research Animal Use (American Heart Association, 1985) and the Guide for Care and Use of Laboratory Animals (1996). Protocols received prior approval by The Institutional Animal Care and Use Committee (East Carolina University).

Ischemia/Reperfusion Injury in Guinea Pigs

      Adult male guinea pigs (200-300g) were anesthetized with a ketamine/xylazine cocktail (85/15 mg/kg, respectively; i.p.). Upon the absence of reflexes to ensure a deep plane of anesthesia, hearts were excised via midline thoracotomy and immersed in ice-cold saline. Guinea pig hearts were placed on a modified Langendorff apparatus and instrumented for the observation of electromechanical function as previously described (37, 38, 89). After a 10-minute equilibration period, hearts were divided into the following treatment groups: **1**. Control followed by global ischemia/reperfusion (I/R); **2**. Administration of 1nM Bendavia in the perfusate beginning 10 minutes before index ischemia and for the entire reperfusion; **3**. Post-ischemic administration of 1nM Bendavia for the duration of reperfusion; **4**. Post-ischemia administration of 0.2μM cyclosporine A. Hearts were exposed to global no-flow ischemia by stopping perfusion for 20 minutes. At the end of the 120-minute reperfusion period, infarct size and arrhythmia scores were assessed as previously described (37, 38, 236).

Myocyte Hypoxia/Reoxygenation Experiments

      Guinea pig left ventricular myocytes were isolated by enzymatic digestion for hypoxia/reoxygenation studies using our established protocols(89). Isolated primary cardiomyocytes were incubated (95% O_2 balance room air, 37 C) for 2-8 hours post-

dispersion. For the drug treatment groups, myocytes were either incubated for 10 minutes with 1nM Bendavia prior to being placed in the perfusion chamber (Bendavia group), or received no drug treatment (Control group). Cells were placed into a custom-built perfusion chamber housed on the stage of an inverted fluorescence microscope for the study of cell survival during cellular hypoxia/reoxygenation. For each myocyte experiment, cells were allowed to settle on the glass coverslips for at least 20 minutes before the initiation of perfusion. More information on the construction of the perfusion chamber is provided in the Methods Supplement.

Myocytes were superfused for a 5 minute baseline period with Tyrode's solution containing (in mM): 140 NaCl, 10 HEPES, 5 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, and 10 glucose (pH = 7.4, 37°C), delivered via an in-line solution heater a rate of 1.0-1.2mL/min. Myocytes were paced (4ms duration, 1Hz frequency, 10V amplitude) for the duration of the hypoxia-reoxygenation protocol. Our decision to pace during hypoxia is based on observations indicating that guinea pig myocardium maintains electrical activity through 20 minutes of ischemia(3). After 5 minutes of baseline perfusion, the solution was switched to Tyrode's solution gassed with 100% argon (hypoxia solution) for 20 minutes. Following 20 minutes of perfusion with hypoxia solution, the superfusate was switched back to normoxic Tyrode's to initiate reoxygenation. Cells were reoxygenated for 30 minutes or until cell death, whichever came first. When appropriate, the time of myocyte death during hypoxia/reoxygenation was noted by complete transition from rod-shaped to rounded, necrotic cell morphology. Differential interference contrast images of the myocytes were obtained at the beginning and end of each experiment.

Subsets of myocytes that underwent hypoxia-reoxygenation were loaded with

one of two fluorescent probes to monitor either cellular ROS levels or mitochondrial membrane potential ($\Delta\Psi_m$) during the protocol (described below). Another cohort of cells was exposed to hypoxia/reoxygenation with no fluorescent probe to determine if the flourophores themselves influenced cell survival. Since the different flourophores did not influence the proclivity to cell death during the protocol (when compared to unloaded cells), the data were pooled for the survival plot to include all cells exposed to hypoxia/reoxygenation.

Myocyte ROS Levels During Hypoxia/Reoxygenation

For the determination of cellular ROS production, guinea pig ventricular cardiomyocytes were loaded with 500 nM of the fluorescent ROS probe 5-(6)-chloromethyl-2,7-dichlorohydrofluorescein diacetate (CM-DCF, Invitrogen) for 10 minutes in the incubator prior to imaging as described previously (89). CM-DCF fluorescence intensity was captured every 30 seconds throughout the hypoxia/reoxygenation experiments.

Mitochondrial Membrane Potential ($\Delta\Psi_m$) During Hypoxia/Reoxygenation

Separate experiments were conducted to determine the influence of Bendavia on $\Delta\Psi_m$ during cellular hypoxia/reoxygenation. Isolated guinea pig ventricular myocytes were loaded with the $\Delta\Psi_m$ sensor tetramethylrhodamine, methyl ester (TMRM; 25nM, 'non-quench mode') for 10 minutes prior to imaging. TMRM fluorescence intensity was captured every 60 seconds throughout hypoxia and reoxygenation or until cell death, whichever came first.

Statistical Analyses

The myocyte survival curve was analyzed using a Logrank (Mantel-Cox) test

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based on the *a prioi* hypothesis that Bendavia would improve cell survival. Infarct Size and Bendavia Uptake data for the Guinea Pig hearts were analyzed using an ANOVA followed by Newman-Keuls Post-Hoc tests for between-group comparisons. For all comparisons, significance was noted if $P<0.05$.

Results

Effect of Bendavia on Ischemia/Reperfusion Injury

The effect of Bendavia on infarct size is presented in Figure 20. Following the ischemia/reperfusion protocol, untreated (control) guinea pig hearts had infarct sizes of 50 ± 4 % of the area at risk (AAR; n = 14, Figure 1). Administration of 1nM Bendavia, either before ischemia (n = 9) or at the onset of reperfusion only (n = 9), significantly reduced infarct size to 30 ± 5 and 31 ± 6 %
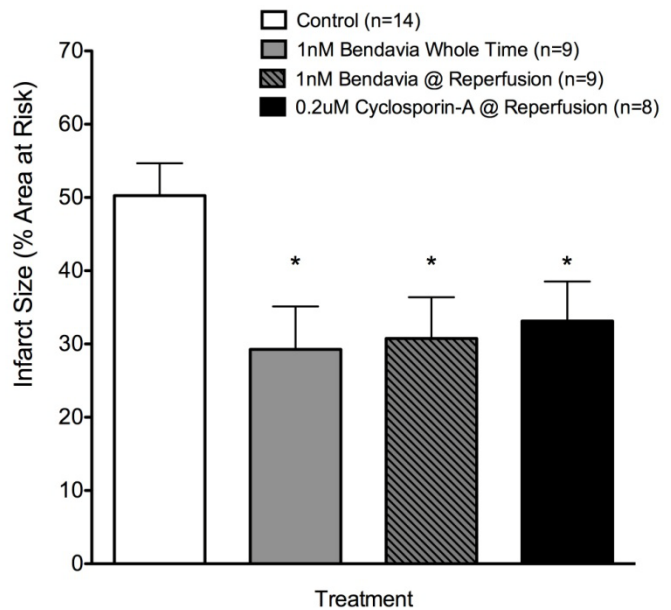
Figure 20: Infarct size in isolated guinea pig hearts exposed to 20 minutes of ischemia and 2 hours of reperfusion. Whole time = compound administered both before and after ischemia; @ reperfusion = compound administered only during reperfusion; $P < 0.05$ versus control (ANOVA).

AAR, respectively (p<0.05 versus control, ANOVA). Treatment with 0.2µM CsA also significantly reduced infarct size to 33 ± 5 % of the AAR (p<0.05 versus control, ANOVA; n = 8). There were no differences in infarct size among treatment groups.

Effects of Bendavia on Post-Ischemia Hemodynamic Recovery and Arrhythmia

Bendavia had no major effects on the recovery of cardiac electromechanical function after ischemia/reperfusion. Neither the incidence of arrhythmia nor the extent of recovery of left ventricular function was influenced by Bendavia treatment. Arrhythmia scores for the 2h reperfusion period were 4.9 ± 0.4 in the control group (n = 14). Administration of Bendavia, either before ischemia or at the onset of reperfusion, had no

effect on arrhythmia scores (5.7 ± 0.7 and 4.4 ± 0.4, respectively, n = 9 and 9, respectively; P > 0.05, ANOVA). CsA treatment also had no significant effect on the incidence of arrhythmia (arrhythmia score of 4.0 ± 0.5, n = 8; P > 0.05, ANOVA). Left ventricular developed pressure at the end of the 2 hour protocol was 22 ± 5 mmHg in the control group (n = 14), and 24 ± 6 mmHg when Bendavia was administered before and after ischemia (n = 9). Administration of Bendavia or CsA at the onset of reperfusion also had no effect on recovery of developed pressure (39 ± 5 and 34 ± 6 mmHg, n = 9 and 8, respectively; P > 0.05, ANOVA).

Guinea Pig Myocyte Survival During Hypoxia/Reoxygenation

In this study, a total of 78 guinea pig cardiac myocytes were exposed to cellular hypoxia/reoxygenation. Since cells died during both the hypoxic and reoxygenation periods, a cell survival plot is presented in Figure 21. A total of 43 cells were exposed to hypoxia/reoxygenation under control (no drug) conditions, with another 35 cells that were treated with 1nM Bendavia. At the end of the 20 minute hypoxia period, 65% (28 of 43 total) of control cells and 74% (26 of 35
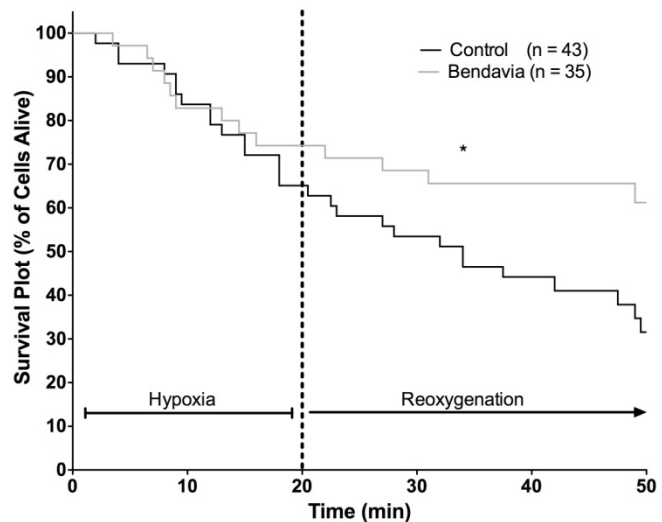
Figure 21: Bendavia significantly abolished ROS-dependent cell death, but the extent of cell death during hypoxia as well as ROS-independent cell death during reperfusion was similar to control. Survival plot for myocytes in the study exposed to hypoxia and reoxygenation. Each cell death event is noted as a downward step in the survival curve. *, P<0.05 versus control for the reoxygenation period.

total) of Bendavia-treated cells were still alive (no drug effect during hypoxia, P>0.05,

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Mantel-Cox test). Bendavia specifically prevented death associated with reoxygenation. Following reoxygenation there was a significant decrease in cell death evoked by Bendavia. Of the cells that were alive at the onset of reoxygenation, only 54% of control cells survived until the end of reoxygenation, compared to 85% of cells that were treated with Bendavia (P<0.05 for survival during reoxygenation).

Role of Mitochondrial ROS in Cell Death

A subset of myocytes (n = 35 total) exposed to hypoxia/reoxygenation was loaded with the ROS sensor CM-DCF during the protocol to monitor ROS production with (n=18) or without (n=17) Bendavia pre-treatment. Representative images and traces of cells are presented in Figure 22. The vast
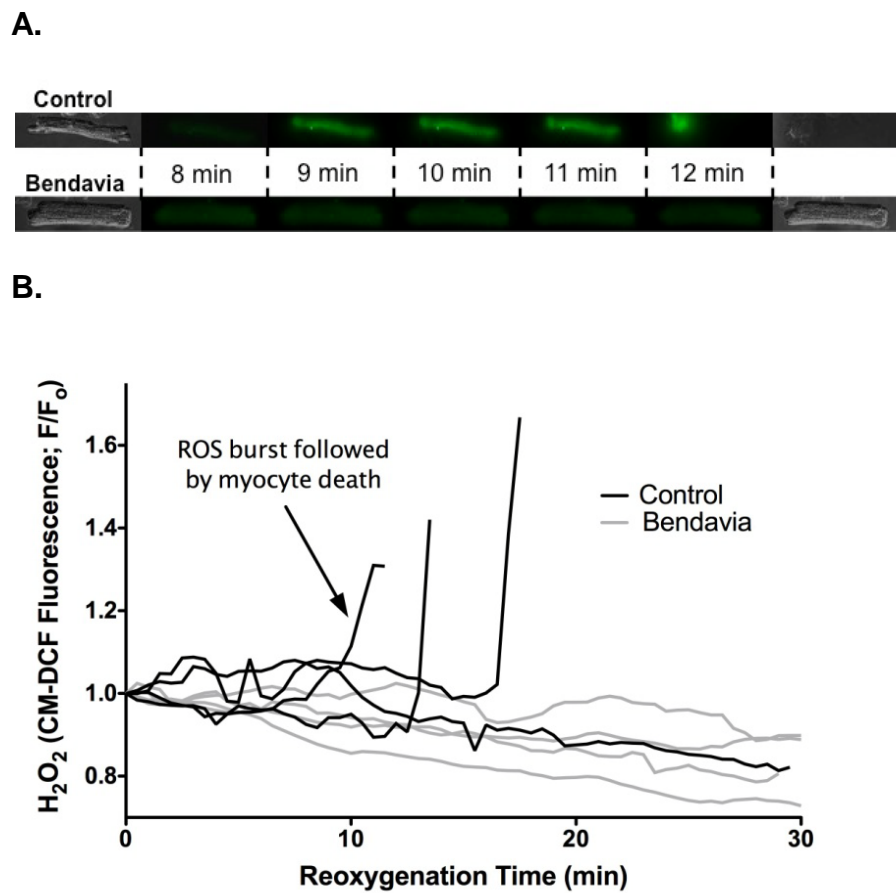
Figure 22: Cellular ROS production during hypoxia/reoxygenation. A: Representative fluorescence images of cardiac ventricular myocytes loaded with the ROS sensor CM-DCF. ROS bursts during reoxygenation preceded cell death, and Bendavia treatment prevented ROS-induced cell death. B: Representative fluorescence intensity traces for cells in the study. CM-DCF fluorescence is normalized to the basal fluorescence (F_o) for each cell at the end of hypoxia.

majority of cell death in the control group was preceded by a burst of ROS during the reoxygenation period. The ROS-dependent cell death was completely prevented in myocytes treated with Bendavia (Figure 23). ROS-independent
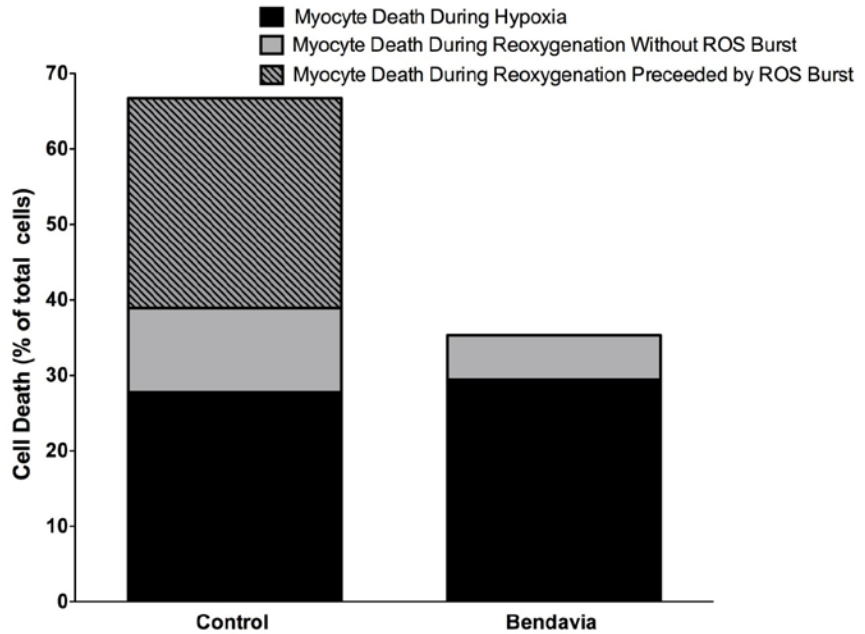
Figure 23: Contribution of ROS bursts to myocyte death during hypoxia/reoxygenation.

cell death during hypoxia or reoxygenation was similar between the control and Bendavia-treated groups.

Maintenance of Mitochondrial Membrane Potential ($\Delta\Psi_m$)

A different subset of myocytes (n = 37 total) were exposed to hypoxia/reoxygenation while loaded with the $\Delta\Psi_m$ indicator TMRM (presented in Figure 24). Myocytes treated with Bendavia were protected against cell death during reoxygenation, and specifically against cell death that was preceded by a collapse of $\Delta\Psi_m$, suggesting that the treatment prevented the opening of the mitochondrial permeability transition pore and subsequent loss of $\Delta\Psi_m$.

A.

Control

Reoxygenation

Bendavia

B.
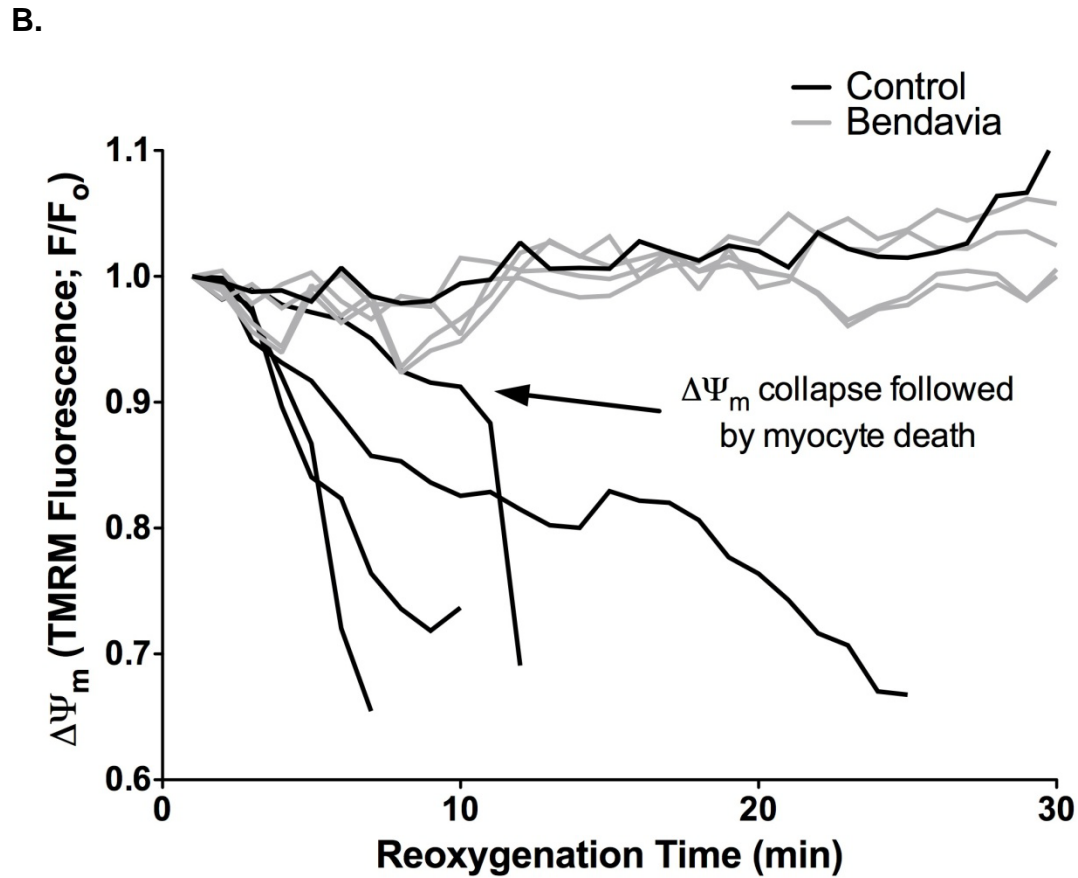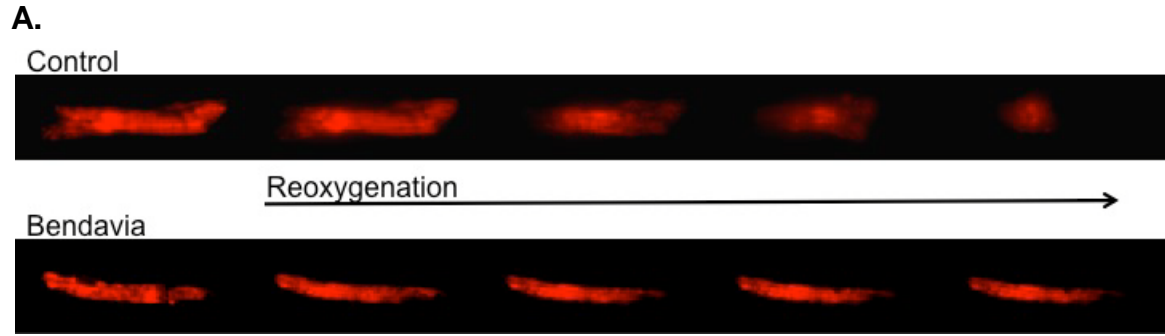
Figure 24: Mitochondrial membrane potential ($\Delta\Psi_m$) in myocytes during cellular hypoxia/reoxygenation. A. Representative fluorescence images of myocytes loaded with the $\Delta\Psi_m$ sensor TMRM. $\Delta\Psi_m$ collapse often preceded cell death, and treatment with Bendavia improved the capacity to maintain $\Delta\Psi_m$. B. Representative fluorescence intensity traces for cells in the study.

Discussion

Key Findings

The present studies show that Bendavia, administered *after* the onset of ischemia, demonstrated cardioprotective effects. Bendavia reduced myocardial infarct size in isolated guinea pig hearts exposed to ischemia/reperfusion. Bursts of reactive oxygen species were blunted by Bendavia, resulting in better maintenance of mitochondrial energetics and reduced cell death during reoxygenation.

Numerous agents have been tested as adjunctive therapy for reperfusion injury in the setting of acute myocardial infarction. At the present time early reperfusion via thrombolytic therapy or percutaneous coronary intervention (including percutaneous transluminal coronary angioplasty) are the only accepted definitive therapies for acute myocardial infarction(231). Early reperfusion limits myocardial infarct size and improves survival. However, not all patients receive early coronary artery reperfusion, and at the time of reperfusion some degree of injury may occur due to reperfusion itself.

Cardioprotection at Reperfusion: Salvaging Cells that are Salvageable

Cell death during ischemia and reperfusion is multi-factorial, and is generally attributed to a combination of necrosis, apoptosis, and autophagy (185). Although a matter of some debate, necrotic cell death is probably the predominant cause of death in ischemic myocardium (17). During early ischemia, insufficient electron flow down the mitochondrial electron transport chain shifts cellular ATP production away from oxidative phosphorylation. Cells increase rates of glycogen breakdown and transition to anaerobic glycolysis, and contraction rapidly ceases as intracellular pH decreases. As ischemia progresses, declining ATP levels lead to cellular sodium and calcium overload

(Figure 4). Necrotic cell death ensues, exacerbated by the development of ischemic contracture which strains cellular structural integrity.

While reperfusion is requisite to salvage tissue, prompt reperfusion can also injure cells that are hovering between life and death. Elevated intracellular levels of calcium, sodium, and inorganic phosphate, an alkaline shift towards physiological pH, and production of reactive oxygen species are all noted in early reperfusion (185). These factors promote the opening of energy-dissipating channels in the inner mitochondrial membrane. In particular, the open probability of both the mitochondrial permeability transition pore (reviewed in (102)) and the inner membrane anion channel (reviewed in (46)) is greatly enhanced by reactive oxygen species. Oxidant-induced pore/channel opening collapses mitochondrial membrane potential ($\Delta\Psi_m$), leading to cessation of ATP production and ultimately cell death.

The Mitochondria as a Therapeutic Target

In the clinical realm, heightened reactive oxygen species production is implicated in the development of reperfusion injury (46). There is obviously a great deal of interest in decreasing reperfusion injury with compounds that scavenging reactive intermediates and/or directly blocking the permeability transition pore. Among the candidate radical scavengers tested to date, early efforts focused primarily on superoxide dismutase mimetics and catalase. These approaches reduced infarct size in some (27, 53, 136, 141) (but not all (217)) animal studies. Despite promising results in animal studies, these strategies do not appear to translate to beneficial effects in clinical trials (85, 256). The reasons for the lack of translation to the clinic have been described in detail elsewhere (80, 139), but likely involve cell permeability concerns and findings that non-

specific radical scavengers must be used in very high doses to see efficacy. Further, superoxide dismutase mimetics scavenge only superoxide anion, while a significant portion of tissue injury may arise from radical-independent redox signaling (i.e. oxidative shifts in intracellular thiols)(128).

Direct permeability transition pore blockers have recently shown potential in reducing reperfusion injury in animal and human studies. CsA, which inhibits the association of cyclophilin D with the mitochondrial permeability transition pore, has been shown to reduce cardiac ischemia/reperfusion injury ((81, 234) and Figure 20). In a recent small clinical trial (212), cyclosporine given at the time of percutaneous coronary intervention significantly reduced infarct size in humans (assessed using both MRI and enzymatic markers in serum), corroborating previous data from animal studies. A multicenter clinical trial is currently underway in Europe, investigating cyclosporine in a larger population of myocardial infarct patients. While these early results are promising, the use of cyclosporine may be confounded by a narrow therapeutic window (189), non-specific effects on other cellular cyclophilins/calcineurin (262), and reports of cyclosporine-induced vasoconstriction(26).

Reduction of Infarction with Bendavia

There is a clear need for cytoprotective compounds that freely cross the sarcolemma, are effective across low doses, and target specifically to the region within the cell where sites of oxidant production is high (specifically, the mitochondria). Drs. Hazel H. Szeto and Peter W. Schiller developed a new class of peptides that concentrate within mitochondria and reduce intracellular ROS generation(249). Bendavia (D-Arg- Dmt-Lys-Phe-NH2; analogous to SS-31 in the literature) is a

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tetrapeptide that crosses cell membranes and targets to the inner mitochondrial membrane. From a therapeutic standpoint, a promising property of Bendavia is that mitochondrial accumulation of Bendavia appears to be independent of the $\Delta\Psi_m$. $\Delta\Psi_m$ collapses during ischemia and the recovery of $\Delta\Psi_m$ at reperfusion is very heterogeneous in the myocardium (174, 237). Treatment strategies that require $\Delta\Psi_m$ for mitochondrial delivery (such as anti-oxidants conjugated to triphenylphosphonium cations, i.e. MitoQ, MitoE, MitoSOD (238)) may only be targeting cells that are already on their way to recovery. In our study, myocardial uptake of Bendavia was observed even in early reperfusion when $\Delta\Psi_m$ may be compromised, consistent with studies in isolated cells where accumulation was not markedly affected by chemical uncouplers of mitochondria (281). Furthermore, the $\Delta\Psi_m$-depolarizing effects of compounds tethered to triphenylphosphonium makes them self-limiting and translates to a very narrow therapeutic window of efficacy (248). Bendavia treatment has no noticeable effect on basal $\Delta\Psi_m$ when assessed by either TMRM fluorescence (281) or triphenylphosphonium uptake (Unpublished observations), allowing for a very wide therapeutic range and cardioprotection at/below nanomolar concentrations ((281) and herein).

In this study, we show for the first time that Bendavia treatment reduces cellular reactive oxygen species generation and helped sustain $\Delta\Psi_m$ in primary cardiac myocytes exposed to hypoxia/reoxygenation. In particular, Bendavia reduced oxidant-dependent cell death during the reoxygenation period, yet had no effect on myocyte survival during hypoxia. This finding supports our hypothesis that this compound is most effective when production of reactive oxygen species is high. In our cellular

studies, it seems plausible that Bendavia reactive oxygen species sustained $\Delta\Psi_m$ by reducing the open probability of energy-dissipating ion channels in the inner membrane (such as the permeability transition pore and/or the inner membrane anion channel). Our myocyte data corroborate previous work in cell culture models, where Bendavia lowered the levels of reactive oxygen species and promoted cellular survival in neuronal cells exposed to t-butylhydroperoxide (281). Further studies are needed to determine the exact mechanism by which this peptide reduces reactive oxygen species levels and stabilizes mitochondrial energetics.

The cardioprotection that we observed in isolated guinea pig cells translated to infarct-size reduction in the isolated heart. Importantly, this protection was observed if Bendavia was given after the onset of ischemia. These data support the concept that damage to the mitochondria at the time of reperfusion is a therapeutic target. These studies confirm a previous study showing that the agent reduced infarct size in an acute rat model of myocardial infarction (56). However, in Cho's study, Bendavia was administered both prior to coronary occlusion as well as prior to reperfusion. In all of the models presented here, Bendavia worked when given after coronary artery occlusion, including studies in which the drug was given just at reperfusion, supporting the concept that it reduced reperfusion injury.

Summary

In conclusion, the mitochondria-targeting agent, Bendavia, demonstrated cardioprotective properties in *in vitro* and *in vivo* experimental models when administered prior to reperfusion. It protected cardiomyocytes, it limited myocardial infarct size, and for the first time was shown to reduce limit no reflow. Bendavia

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protected cells from reactive oxygen species damage and preserved mitochondrial potential. Bendavia is an attractive candidate for clinical studies in cardiac ischemia/reperfusion injury.

Chapter 6: Integrated discussion

Major Findings

The overriding hypothesis of this work is that ischemia/reperfusion injury is characterized by mitochondrial overload of ROS that collapses mitochondrial energetics and leads to cellular injury. These studies help to establish the importance of cardiac glutathione in decreasing this ROS burden. Furthermore, they establish that maintaining reduced glutathione through the glutathione reductase reaction may be an important factor in exercise-induced preconditioning. Finally, treatment of cardiac tissue with Bendavia led to decreased ROS accumulation and decreased myocardial infarction. Taken together the work presented in this dissertation provide novel insights into physiological and pharmacological approaches that decrease oxidative stress and cell death during ischemia/reperfusion injury.

In Chapter 3 I showed that Ex led to a phenotype that maintained GSH in a reduced state during conditions of thiol oxidation and ischemia/reperfusion. This was likely mediated by an intrinsic increase in the GR activity. Chapter 4 goes one step further by showing that pharmacological inhibition of GR abolishes protection. Furthermore, it showed that ROS generated through NAD(P)H oxidase act as signaling molecules that may post-translationally modify GR, thereby increasing its activity. Lastly, Chapter 5 investigated a pharmacological approach aimed at decreasing myocardial infarction. We found that Bendavia decreased mitochondrial ROS accumulation, preserved mitochondrial energetics, and led to decreased cellular injury. Given that we found that cytosolic ROS may be involved in preconditioning signaling invoked by Ex and that the role for mitochondrial ROS may be limited to the pathology

associated with reperfusion injury, these opposing functions may be part of the reason antioxidant treatments have translated poorly into the clinic (1, 167, 274).

One interesting finding of this study is that exercise-induced preconditioning and Bendavia treatment may converge on the same mechanism. Although speculative, this convergence may occur at the ability to buffer mitochondrial ROS during reperfusion. This is substantiated by several findings: 1) in isolated hearts there was no difference in the extent of protection if Bendavia was given either before ischemia or at the onset of reperfusion (Figure 20); 2) administration of Bendavia via i.p. injection for 10 days led to increased protection in our Sed animals (Figure 15); and 3) There was no additive effects on exercise with Bendavia treatment. Since both exercise and Bendavia were shown to decrease ROS accumulation (Figure 11 and Figure 22 respectively) this is likely the site of convergence, and may help explain why there was no additive effects on Ex.

Future Directions

The studies presented within this dissertation describe how glutathione regulation may be an underlying factor involved in the preconditioning effects of exercise. Chapter 4 establishes that ROS generated through NAD(P)H oxidase during exercise act as signaling molecules that increase GR activity. Although the data presented within strongly suggest that GR activity is vital to the cardioprotective phenotype, the post-translational modifications of GR that lead to this increase in activity need to be further elucidated.

Figure 17 shows that post-translational modification do occur through redox sensitive mechanisms, most probably through thiol-thiol interactions. This is consistent

with the literature where it has been shown that GR lacks candidate sites for phosphorylation or carboxylation (83) and that GR activity is related to glutathione concentrations and the general redox status of the cell (58, 95, 173, 211). Directly demonstrating that Ex leads to a decrease in the amount of free thiols on GR would help to definitively determine if this is how ROS signaling modifies the GR protein.

Another interesting study would be to investigate the redox status of GSH in the cardiac tissue immediately following exercise. This would give us an index of the extent of oxidative shift that occurs during exercise, and if this is blunted when cytosolic ROS formation is inhibited. With this knowledge we could directly investigate if physiological shifts in the oxidative state of the glutathione couple could directly affect the activity of GR in vitro.

The final chapter of this work utilized a pharmacologic approach to decrease oxidative stress during ischemia/reperfusion. Although the results are promising for this compound, there are several questions left to answer. First, we saw that cardiomyocytes treated with Bendavia were better able to maintain mitochondrial energetics during hypoxia/reoxygenation (Figure 24). Since we think that this compound is decreasing mitochondrial ROS, it would be interesting to see if cardiomyocytes treated with Bendavia were less susceptible to permeability transition during hypoxia/reoxygenation. Another important area left open to further study is if the timing of Bendavia administration affects ischemia/reperfusion injury. Given that we saw the same extent of protection regardless of whether hearts were treated before ischemia or at the onset of reperfusion (Figure 20). It would be interesting to see if Bendavia has a

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"therapeutic window" wherein it has to be administered after the re-establishment of coronary flow.

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Appendix A: Animal care and use protocol approvals

m̲ East Carolina University.
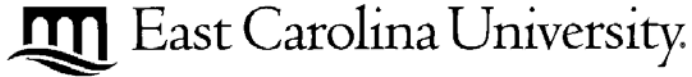
**Animal Care and
Use Commitee**

212 Ed Warren Life
Sciences Building
East Carolina University
Greenville, NC 27834

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

July 19, 2011

American Heart Association
Mid-Atlantic Affiliate Research Programs

The following application submitted to the American Heart Association was reviewed
and approved by this institution's Animal Care and Use Committee:

Title of Application: "The Role of Cardiac Mitochondria in Exercise-Induced
                            Cardioprotection"

Name of Principal Investigator: Chad Frasier

Name of Institution: East Carolina University

Date of Approval: July 18, 2011

This institution is fully accredited by AAALAC and has an Animal Welfare Assurance on
file with the Office of Laboratory Animal Welfare. The Assurance Number is A3469-01.

Sincerely yours,

Scott E. Gordon, Ph.D.
Chairman, Animal Care and Use Committee

SEG/jd

East Carolina University.

May 20, 2010

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

Dear Dr. Brown:

The Amendment to your Animal Use Protocol entitled, "Cardiac Arrhythmias and Reactive Oxygen Species Production in Guinea Pig Myocardium", (AUP #Q269) was reviewed by this institution's Animal Care and Use Committee on 5/20/10. The following action was taken by the Committee:

"Approved as amended"

A copy of the Amendment 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,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

Appendix B: Effects of rat estrous cycle on myocardial ischemia/reperfusion injury

Introduction

Epidemiological evidence suggests that women are less at risk for a cardiovascular event prior to undergoing menopause (258). Recent evidence has implicated that this may be associated with increased circulating levels of estrogen (107). The effect of menstrual cycle on cardiovascular disease has been less studied in humans than in animals, probably due to the rare nature of these events in a younger population. In fact a recent study found that cardiovascular events in menstruating women accounts for only 1% of all events (184). However, there is evidence that the phase of the menstrual cycle can have profound effects on cardiovascular function. Moran et al. have shown that blood pressure and heart rate can vary between phases (181). It's also been shown that women early in the menstrual cycle have more risk for angina (135), supraventricular tachycardia (188, 225), and exercise induced ST depression (163). SVT episodes were also linked to decreased levels of circulating estradiol and increased levels of progesterone (225). Interestingly, females who were irregular in their cycle are more susceptible to future coronary heart disease (23, 239) that can be extended to postmenopausal years (15). Since animal research is a potent tool for investigating cardiovascular disease, further investigation is warranted into the effects that the estrous cycle may have on cardiovascular health.

In animal models the cardioprotective phenotype of premenopausal females has been shown in mice, rats, rabbits and dogs (44, 64, 144, 152, 264). Although several mechanisms have been suggested for the possible underlying mechanism, none of these studies have delved into the changes that may occur to cardiac tissue during

specific phases of the estrous cycle. Unfortunately, to date nobody has examined if the effects of fluctuating hormone levels during the estrous cycle have any effect on cardiac tissue and most of what has been shown is the effects of sex hormones on vascular function (258).

Unlike humans, rodents do not undergo a menstrual phase where the uterine lining sloughs off and is expelled through the vagina, but rather the lining degenerates back to the normal size in an estrous cycle (166). The estrous cycle (in rats) is defined by four separate phases: proestrus, estrus, metestrus, and diestrus. Proestrus is characterized by increasing levels of estrogen. At the end of proestrus, ovulation (signaled by luteinizing hormone) occurs and marks the beginning of the estrus phase. During metestrus and diestrus the uterine lining degenerates back to normal and the cycle starts again (166, 230).

Since no study to date has investigated the role of estrous cycle phase on the rat heart, this study was performed to see if the effects of fluctuating hormones during the estrous cycle have acute changes on cardiac tissue. By utilizing both in vivo and ex vivo models we will also be able to discern if the effects of hormones have acute affects or are limited to the circulation. Here we find that the estrous phase does not have any effect on the level of myocardial infarction in rat hearts. The level of myocardial infarction was not correlated with serum levels of 17-β estradiol or progesterone adding to our conclusion that estrous cycle phase has no bearing on the extent of injury following ischemia/reperfusion. No differences in the severity of arrhythmias or incidence of ventricular fibrillation (VF) with cycle phase were observed.

Methods

Animals

Female Sprague-Dawley rats were obtained and kept on a 12:12 light dark cycle with access to food and water *ad libitum*. Rats were ordered so that they were over 50 days old at the onset of experiments. Typically female rats will automatically begin cycling immediately after the vaginal orifice opens at around 32-36 days (96, 166). To ensure a more regular cycle, Rats were housed in a room with at least one male rat for the duration of the study (96).Research was performed in an AAALAC-accredited facility and animals use adhered to the principles stated in the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85–23, revised 1996) and was approved by the East Carolina University IACUC.

Vaginal Cytology Examination

To evaluate estrous stage in the rats, vaginal cytology was performed daily at 0900. A sterile cotton-tipped swab moistened with sterile saline was inserted into the rat's vaginal opening. The swab was rotated gently against the vaginal wall and removed. The swab was immediately rolled onto a glass slide, then the smear was fixed by the use of a spray fixative (Safetex-Cytology Spray Fixative, Andwin Scientific, Woodland Hills, CA) and then stained with Dip Quick Stain Preparation (JorVet, Loveland, CO). Each slide was dipped into the three solutions 10 times, as this was determined to produce optimal staining quality. On the day of experiments, vaginal cytology was performed just prior to heart excision in order to get the most accurate reading of the phase each animal was in. Slides were analyzed by 2 independent reviewers for estrous phase. In the event that the 2 reviewers could not agree a third independent reviewer was brought in. Estrous stage was determined using the

following criteria: Estrous > 75% squamous cells; Proestrus, mix of basal and squamous cells accounting for over 80% of slide; Diestrus > 80% neutrophils; Metestrus, mix of all three cell types. To confirm that our rats were cycling vaginal swabs were taken from animals every morning over the course of 8 days and they all displayed either a 4 day or 5 day cycle (Figure 25).
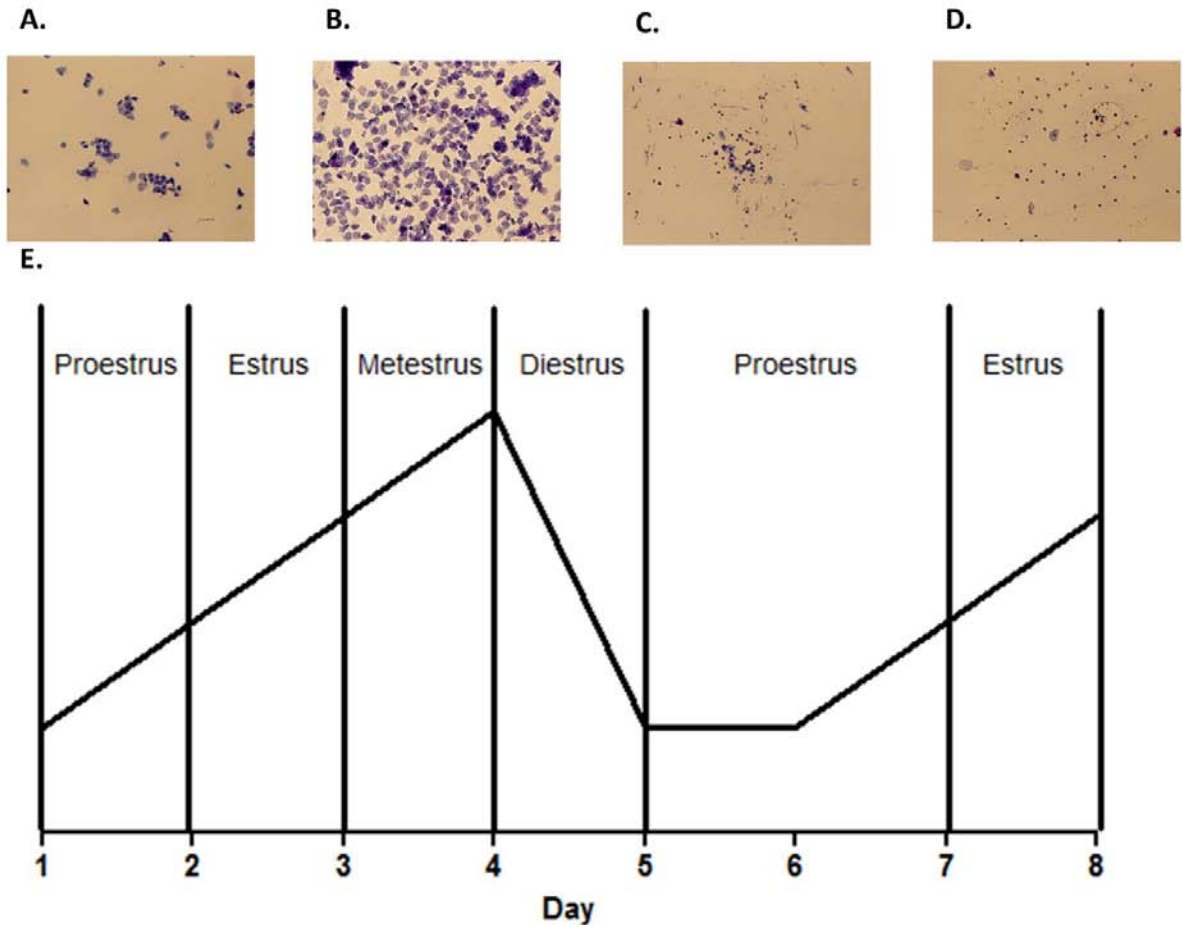
Figure 25: Representative images from vaginal cytology for Proestrus **(A)**, Estrus **(B)**, Metestrus **(C)**, and Diestrus **(D)** phases of the estrous cycle. **(E)** Daily changes in the phase of estrous in our animals.

In Vivo Preparation

Animals were anesthetized with ketamine/xylazine (90 mg/kg ketamine, 10 mg/kg xylazine, i.p.) and upon the absence of reflexes a midline tracheotomy was performed. The animals were intubated with PE-90 tubing and mechanically ventilated.

Supplemental injections of ketamine/xylazine (i.p.) were given as needed to maintain a surgical plane of anesthesia. A circulating water heating pad was use to maintain body temperature at 37°C.

Animals were given a 10-min equilibration period on the ventilator. Then the thorax was opened via a left parasternal incision, the pericardium was removed from the heart, and the left anterior descending coronary artery (LAD) was ligated by using a reversible snare applied 4 mm distal to the origin between the conus arteriosus and the left atrium. LAD occlusion was confirmed with the appearance of myocardial cyanosis distal to the occlusion. Following 25 min of occlusion, the ligature was released, and reperfusion ensued for 2 h. To minimize desiccation the chest walls were approximated with Parafilm.

To determine the area at risk (AAR) was risk the LAD was religated at the original point of occlusion immediately following 2 hours of reperfusion and a 1% Evans blue solution was infused through the aorta. After Evans blue staining, infarct size was determined via TTC staining as described previously (236) and expressed as a percent of the AAR.

Ex Vivo Preparation

Animals were injected with ketamine/xylazine mix (90 mg/kg ketamine, 10 mg/kg xylazine, i.p.) and upon the absence of reflexes the hearts removed via midline thoracotomy and retrograde perfused on the cannula of a modified Langendorff apparatus as described previously (38, 236). Following a 5 minute baseline period global, no-flow, ischemia was for 25 minutes. After 25 minutes flow was re-established

and reperfusion ensued for two hours. Immediately following reperfusion the left ventricle was isolated and stained for infarct via TTC staining as described above.

Assessment of Arrhythmias

Arrhythmias were scored as described previously and in accordance with the Lambeth Conventions (38, 263) from the ECG signal as follows: $0 = 0 - 49$ premature ventricular beats; $1 = 50\text{-}499$ premature ventricular beats; $2 = > 500$ premature ventricular beats and/or 1 episode of spontaneously reverting ventricular tachycardia (VT) or fibrillation (VF) less than 30 sec in total duration; $3 = > 1$ episode of reverting VT/VF that is < 60 sec total duration; $4 = >1$ episode of reverting VT/VF that was 61 to 119 seconds in total duration; $5 = \text{VT/VF}$ that is > 119 seconds in combined duration; $6 = \text{fatal (non-reverting) VT/VF that began} > 15$ min into treatment; $7 = \text{fatal VT/VF that}$ began between 4 minutes and 15 minutes min into treatment; $8 = \text{fatal VT/VF that}$ began between 1 and 4 minutes into treatment; $9 = \text{fatal VT/VF that began within the}$ first 59 seconds of treatment.

Hormone Concentrations

Rat serum was collected from the body cavity immediately following heart excision. Levels of 17-β estradiol and progesterone were determined using a commercially available kit (Cayman Chemicals, Ann Arbor, MI).

Statistical Analysis

Analysis was done with either GraphPad (Prism) or SPSS software. One way ANOVA with Tukey's post hoc test.

Results

Baseline Characteristics

	Proestrus	**Estrus**	**Metestrus**	**Diestrus**
Body Weight (g)	204 ± 2	200 ± 4	205 ± 4	201 ± 4
Heart Weight (g)	0.93 ± 0.03	0.99 ± 0.04	0.95 ± 0.03	1.00 ± 0.03
LVDP (mmHg)	117.6 ± 0.5	107.0 ± 0.6	124.1 ± 0.4	127.7 ± 0.5
Heart Rate (bpm)	233 ± 3	187 ± 3	193 ± 2	209 ± 2
Coronary Flow (mL/min/g)	11.73 ± 0.05	10.05 ± 0.07	10.48 ± .04	11.06 ± 0.06

Table 5: Baseline characteristic of animals in this study.

Animal morphological data and baseline cardiac function (Langendorff prep) data are presented in Table 5. No significant differences were observed in animal body weight or heart weight across the phases of estrous. Furthermore, there were no differences in baseline left ventricular developed pressure (LVDP), coronary flow, or heart rate.

Infarct Size

Infarct size data are presented in Figure 26. There were no differences observed in the infarcted area of the tissue based on the phase of the estrous cycle in isolated hearts(Proestrus, 42 ± 6%; Estrus, 49 ± 4%; Metestrus, 40 ± 9%; Diestrus, 47 ± 9%; $P=0.77$; Figure 26A). Furthermore no differences were seen between phases for the in vivo preparation (Proestrus, 32 ± 6%; Estrus, 29 ± 8%; Metestrus, 28 ± 4%; Diestrus, 34 ± 5%; $P=0.87$; Figure 26B). There was also no correlation observed between the levels of circulating estradiol or progesterone at the time of heart excision and infarct size (Figure 26A and B respectively).
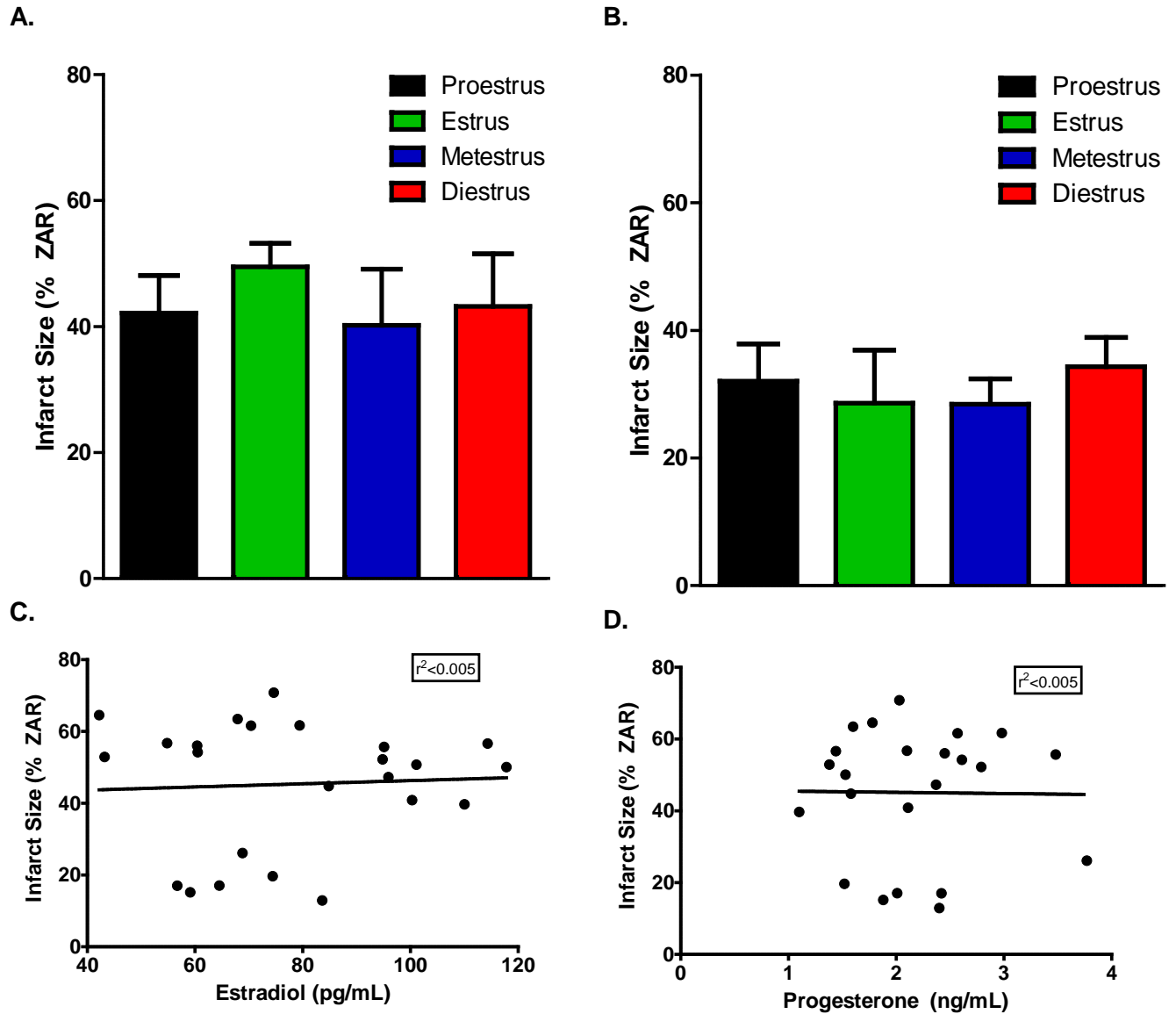
Figure 26: No difference in infarct size between the phases of the estrous cycle. Data from the ex vivo (**A**) and in vivo (**B**) hearts. There was also no correlation between infarct size and serum levels of estradiol (**C**) or progesterone (**D**) in isolated rat hearts.

Arrhythmias

Arrhythmias were scored for the first 15 minutes of reperfusion in isolated hearts. There were no differences observed in the severity of arrhythmias (as evident from our arrhythmia score) (P=0.32; Figure 27A) nor the incidence of VF (Figure 27B) and there were also no difference in the likelihood of an isolated heart to experience VF with the

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phase of estrous. The severity of these arrhythmias was not correlated with the levels of estradiol or progesterone (Figure 27Cand D respectively).
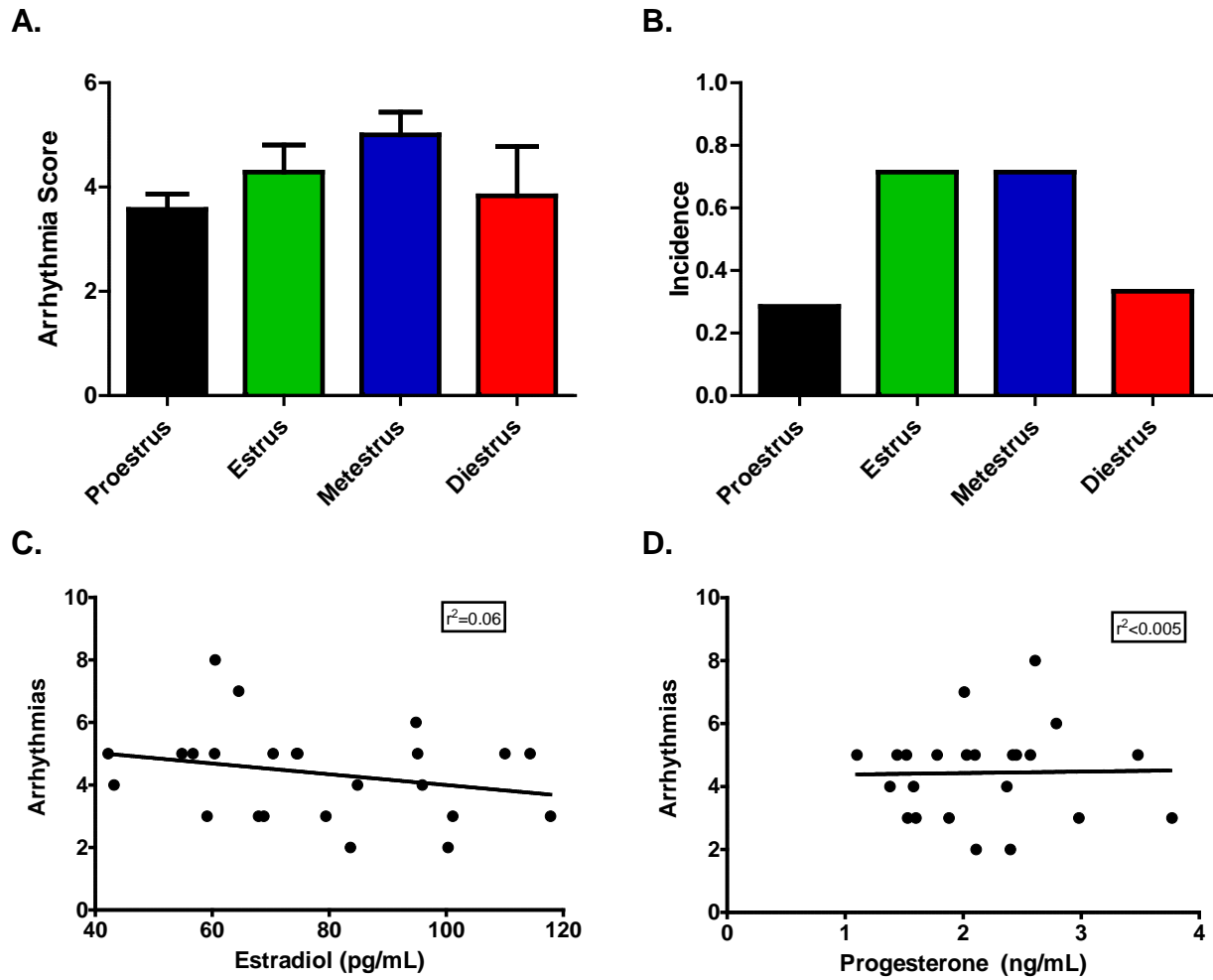
Figure 27: No differences were seen between groups in the severity or incidence of ventricular arrhythmias in isolated rat hearts. Arrhythmia scores (**A**) and incidence of VF (**B**) were used to assess the severity of arrhythmias. There was also no correlation between the arrhythmia score and serum levels of estradiol (**C**) or progesterone (**D**) in isolated rat hearts.

Discussion

It has been widely demonstrated in animal models that females exhibit a cardioprotective phenotype during their reproductive years (44, 64, 144, 152, 264). This has become a widely studied area with several different hypotheses as to how this occurs. However, there may be inherent variability in the data collected in these studies that arise from animals being in different phases of the estrous cycle. The goal of this study was to investigate if the estrous cycle has any effect on cardiovascular health and function in rat hearts during ischemia/reperfusion. Herein, we describe for the first time that the phase of the estrous cycle does not have any effect on the size of myocardial infarction or ventricular arrhythmias in rat hearts. Furthermore, we describe that there is no correlation between serum levels of 17-β estradiol or progesterone and infarction or arrhythmias. This study is important in establishing that the phase of estrous does not alter the results obtained in the isolated heart from female animals in studies where estrous cycle has not or cannot be standardized.

Several studies have investigated the role of exogenous estradiol on ischemia/reperfusion injury with several distinct mechanism put forth as possible explanations (for review see (32, 71)). In our study we saw no differences in the extent of injury with estrous phase or the amount of circulating estradiol. Although speculative, this may be because the effects of estrogen last longer than the duration of the estrous cycle in rats. Perhaps in larger animals, where cycle length is longer, differences may be observed.

Several studies have shown that estradiol both augments activity of the PI3K/Akt pathway. Inhibition of PI3K and PKC has been shown to diminish cardiac function and

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increase infarct size in female rats, but not in males (16). Using isolated hearts, Wang et al. found that female mice lacking estrogen receptor β (ERβ) exhibit diminished protection from myocardial infarction, inferring that increased activation of PI3K/Akt and decreased apoptosis may be responsible for the cardioprotective phenotype (264). Further demonstrating the possible anti apoptotic effects of estrogen, activation of a G-protein coupled receptor that binds directly to estrogen has been shown to decrease mitochondrial permeability transition(33).

It has also been shown that acute pharmacological doses of exogenous estradiol lead to cardioprotection in the isolated heart and that lower, more physiological levels of estradiol have no acute effect (240). The fact that we saw no difference in infarct size or correlation between infarct size and estradiol levels substantiates this observation. Debates within the literature as to the protective effects of exogenous estradiol administration may be due to the supraphysiological vs. physiological doses administered. The benefits to human health may also be limited as studies on hormone replacement therapy and morbidity have yielded conflicting results (for review see (190)).

Although studied less than estrogens and estradiol, progesterone is a major sex hormone that may affect cardiovascular function. Despite the fact that following the follicular phase, as progesterone levels increase, the risk of angina also increases (135) and is maintained after progesterone levels have dropped. High levels of progesterone have also been shown to be correlated with increased reactive oxygen species production in skeletal muscle (132). However, the fact that high progesterone levels may be associated with the development chronic heart failure, but not associated with

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myocardial infarction (193) argues against a role for progesterone in ischemia/reperfusion injury. The inability to distinguish between the effects of estradiol and progesterone hinders investigators from concluding if their results are due to the protective effects of estrogen or the negative effect of progesterone.

Conclusions

Despite the fact that several studies have shown that levels of estradiol and progesterone can have profound effects on cellular function, no one to date has investigated if circulating hormones affect ischemia/reperfusion injury. This study presents several novel findings, 1) circulating hormone levels during the estrous cycle do not influence cardiac ischemia/reperfusion injury and 2) serum levels of estradiol and progesterone are not correlated with infarct size or arrhythmia. These findings were confirmed in both the in-vivo and ex-vivo model, demonstrating that the effects of circulating hormones do not influence ischemia/reperfusion injury in rat hearts. This study is important in establishing the validity of data obtained from female rats in studies where estrous cycle was not determined.

Appendix C: Figure reproduction
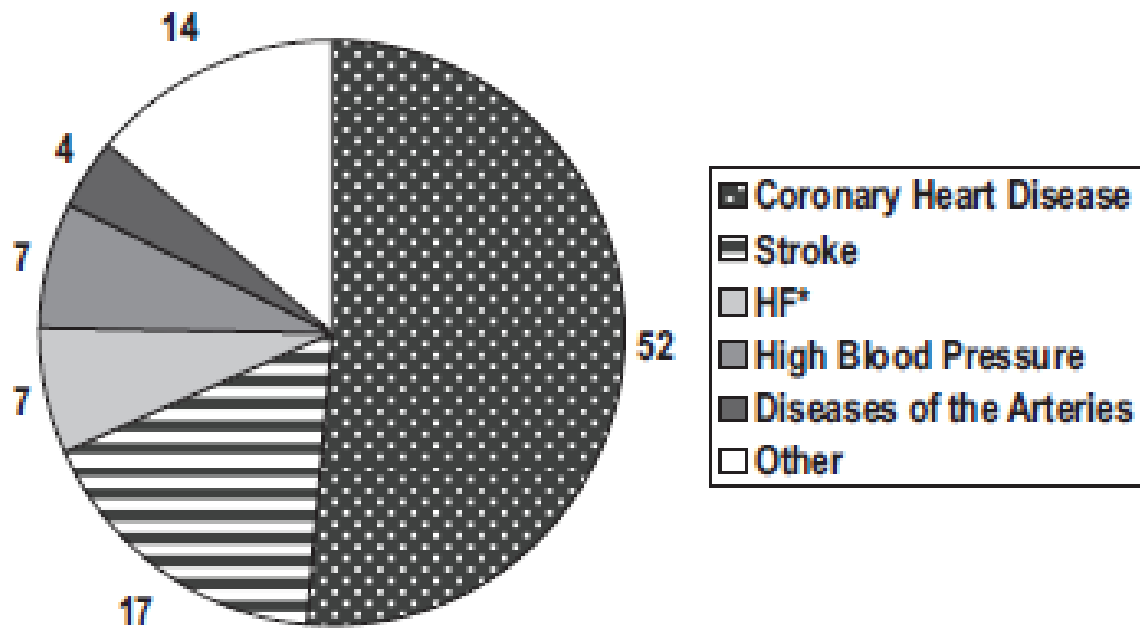
Figure 28: Percentage breakdown of deaths due to CVD (United States: 2006, preliminary). Taken from (162).
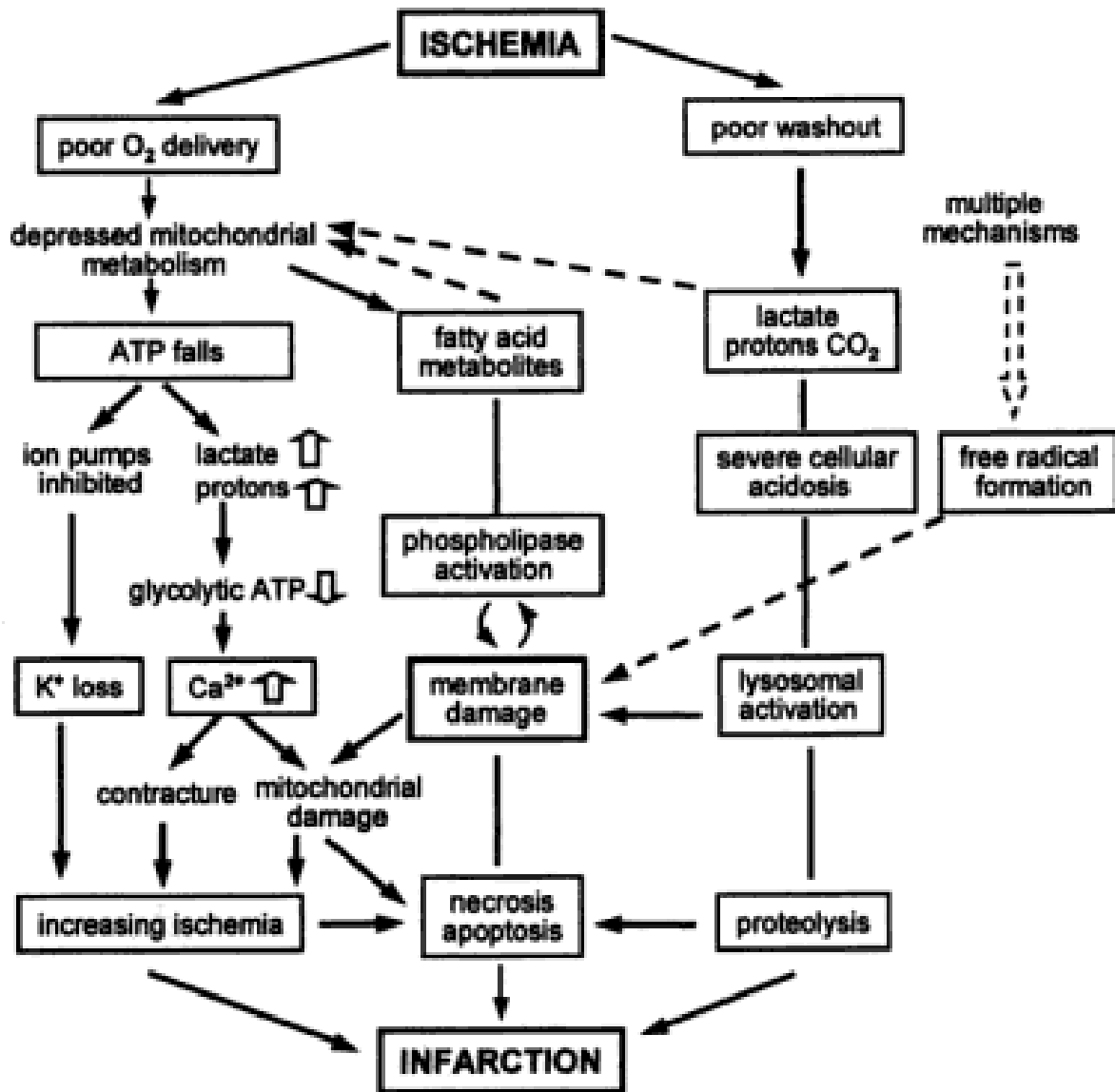
Figure 29: Mechanisms by which myocardial ischemia can lead to cell death and myocardial infarction. Taken from (200).
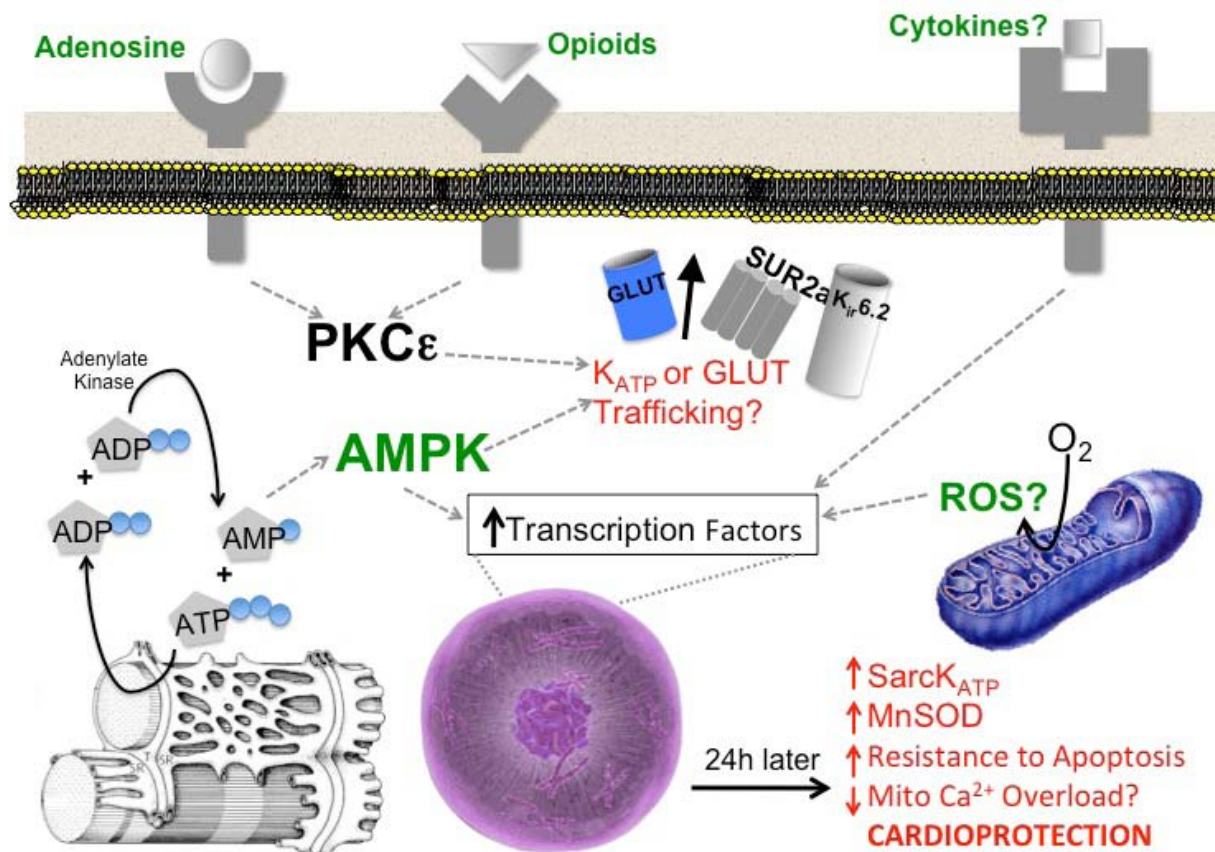
Figure 30: Putative sequence of events leading to exercise-induced cardioprotection. Postulated 'triggers' of exercise-induced cardioprotection are denoted in green, with end-effectors labeled in red font. Images of nucleus and myofilaments obtained from (24, 266) respectively.

A.
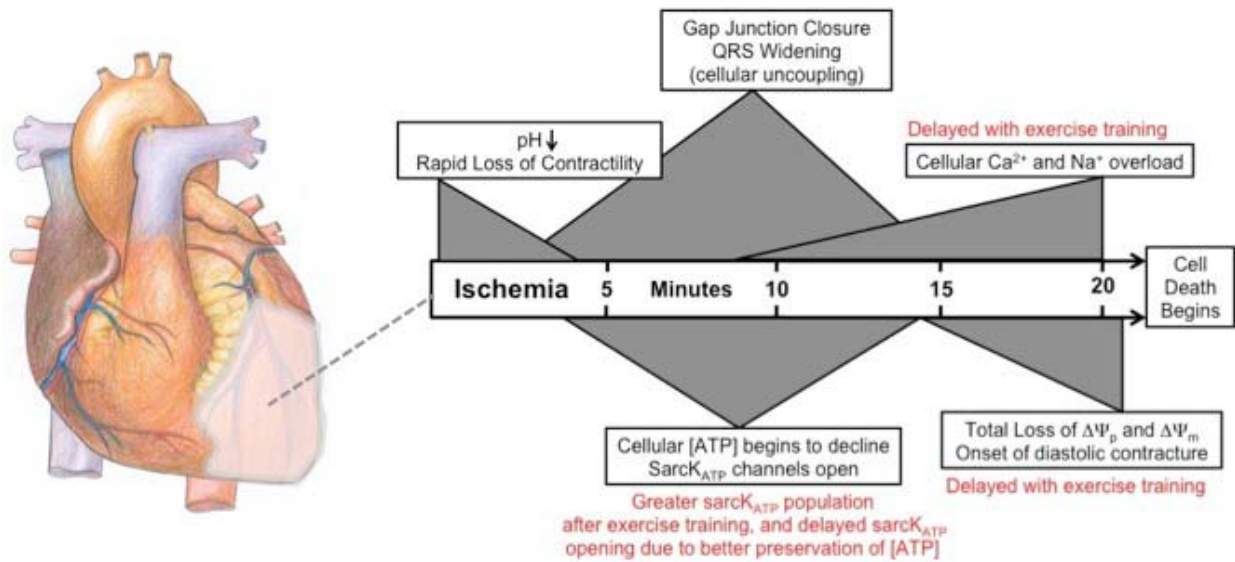
B.
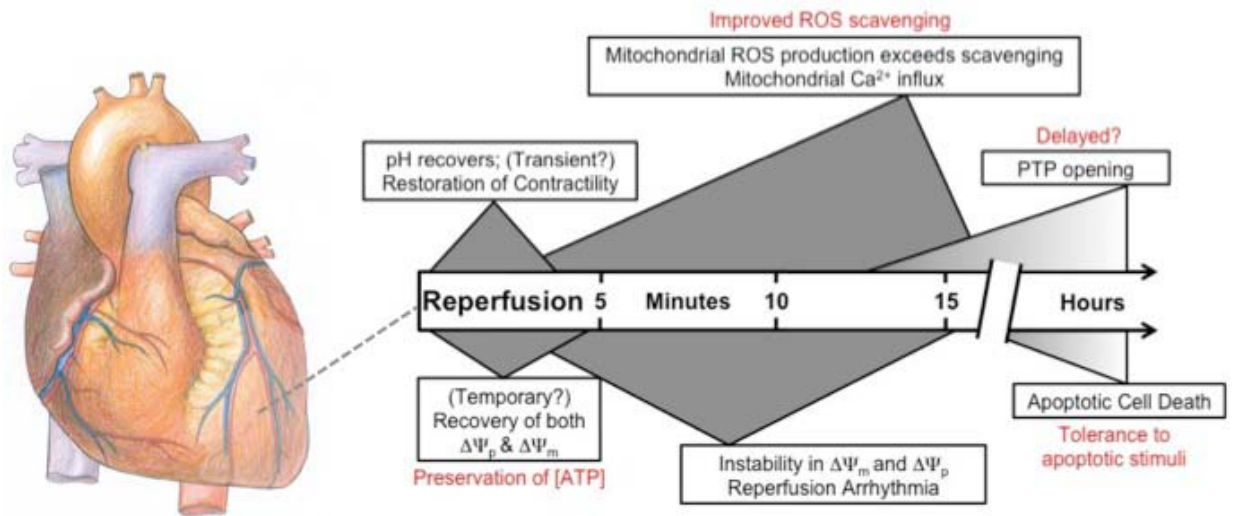
Figure 31: Pathophysiological changes in rodent cardiac tissue during ischemia (**A**) and reperfusion (**B**). Postulated mechanisms involved in exercise-preconditioning noted in red font. Heart image modified from (170). Abbreviations: $\Delta\Psi_p$, sarcolemmal membrane potential, $\Delta\Psi_m$, mitochondrial membrane potential; sarcK$_{ATP}$, sarcolemmal ATP-sensitive potassium channels.
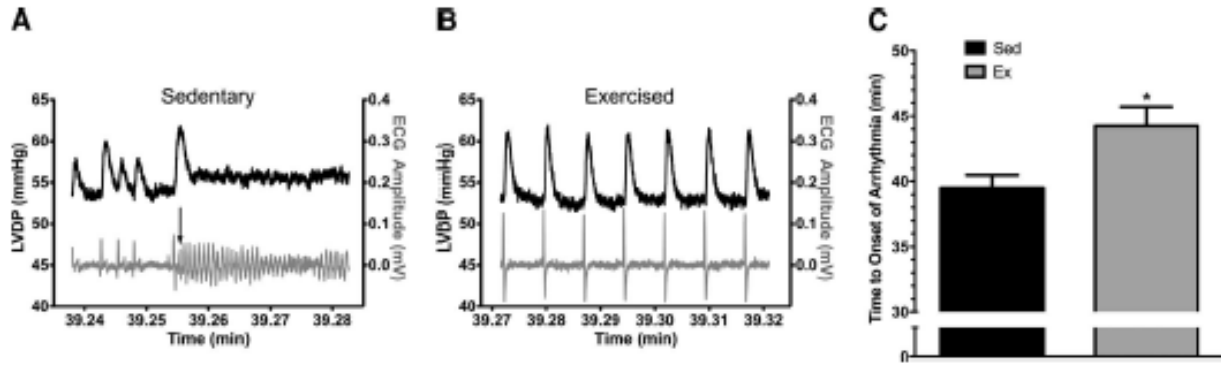
Figure 32: Exercise delays the onset of ventricular arrhythmias during sustained thiol oxidation. A. Representative left ventricular developed pressure (LVDP; black trace) and volume-conducted electrocardiogram (ECG; gray trace) recordings from a sedentary animal approximately 39 minutes into diamide treatment. The transition to ventricular arrhythmia and subsequent loss of pump function is noted with an arrow. B: Representative trace from an exercised animal during the same duration of diamide treatment as the sedentary trace. C Mean time to onset of ventricular arrhythmia following sustained diamide treatment; *, $P<0.05$ vs. Sed. N = 8 per group. Mean ± S.E.M.
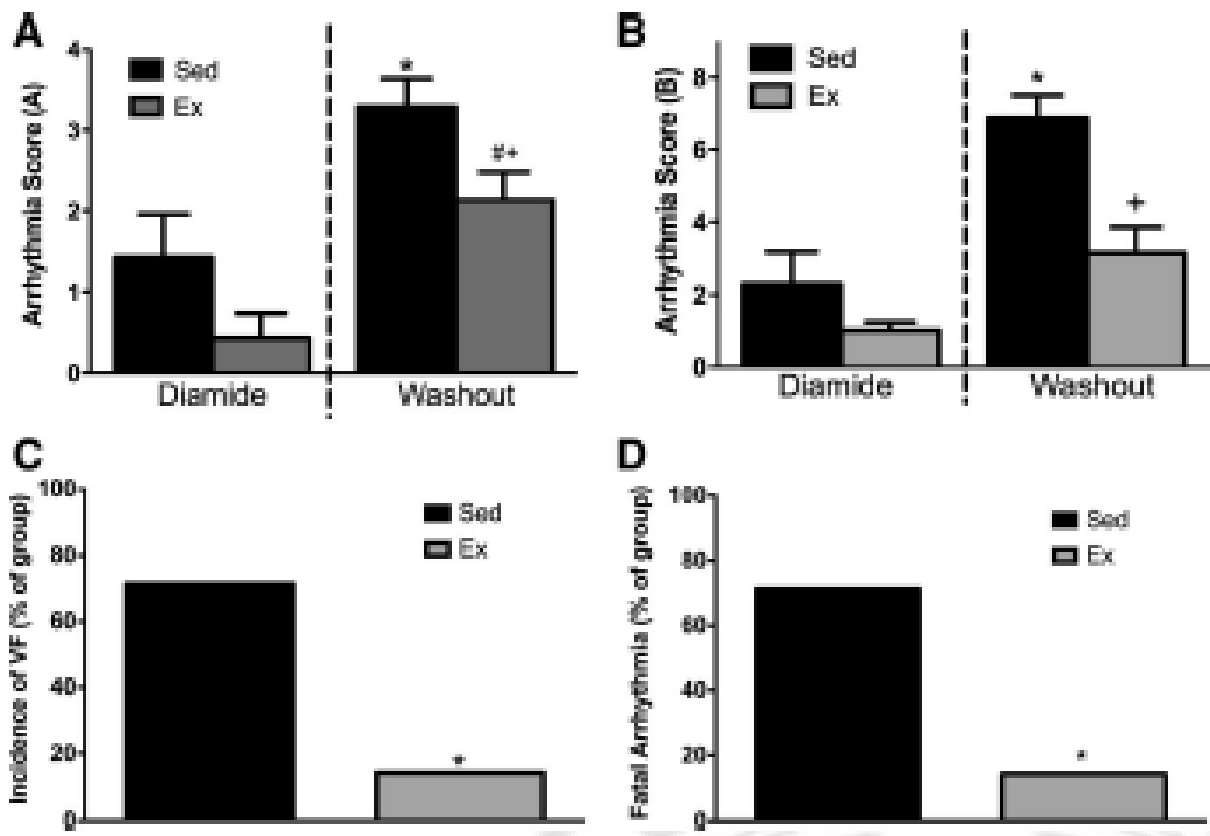
Figure 33: Ex decreases the severity of arrhythmias in the isolated rat heart exposed to reversible treatment with the thiol oxidant diamide (30 min), followed by a 30 minute washout period. **A:** Arrhythmia scores (calculated from System A) between Sed and Ex animals during/after the diamide treatment. **B:** Arrhythmia scores (calculated from System B) between Sed and Ex animals during/after the diamide treatment. **C:** Percentage of hearts in each group that experienced an episode of VF. **D:** Summary of hearts in each group that experienced a fatal (non-spontaneously reverting) ventricular arrhythmia; *, P<0.05 vs. Sed during diamide treatment; +, P<0.05 vs. Ex during diamide; #, P<0.05 vs. Sed during washout. N= 7 per group. Mean ± S.E.M.

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Figure 34: Incidence of reperfusion arrhythmias in hearts exposed to 30 minutes of global ischemia and 30 minutes of reperfusion. **A**: Arrhythmia scores (calculated from System A) for hearts 15 minutes into reperfusion. **B**: Arrhythmia scores (calculated from System B) for hearts 15 minutes into reperfusion. **C**. Summary of hearts in each group that experienced an episode of VF. **D**. Incidence of non-reverting (fatal) arrhythmia during reperfusion for hearts in the study. *, $P<0.05$ vs. Sed. N = 7 per group. Mean ± S.E.M.
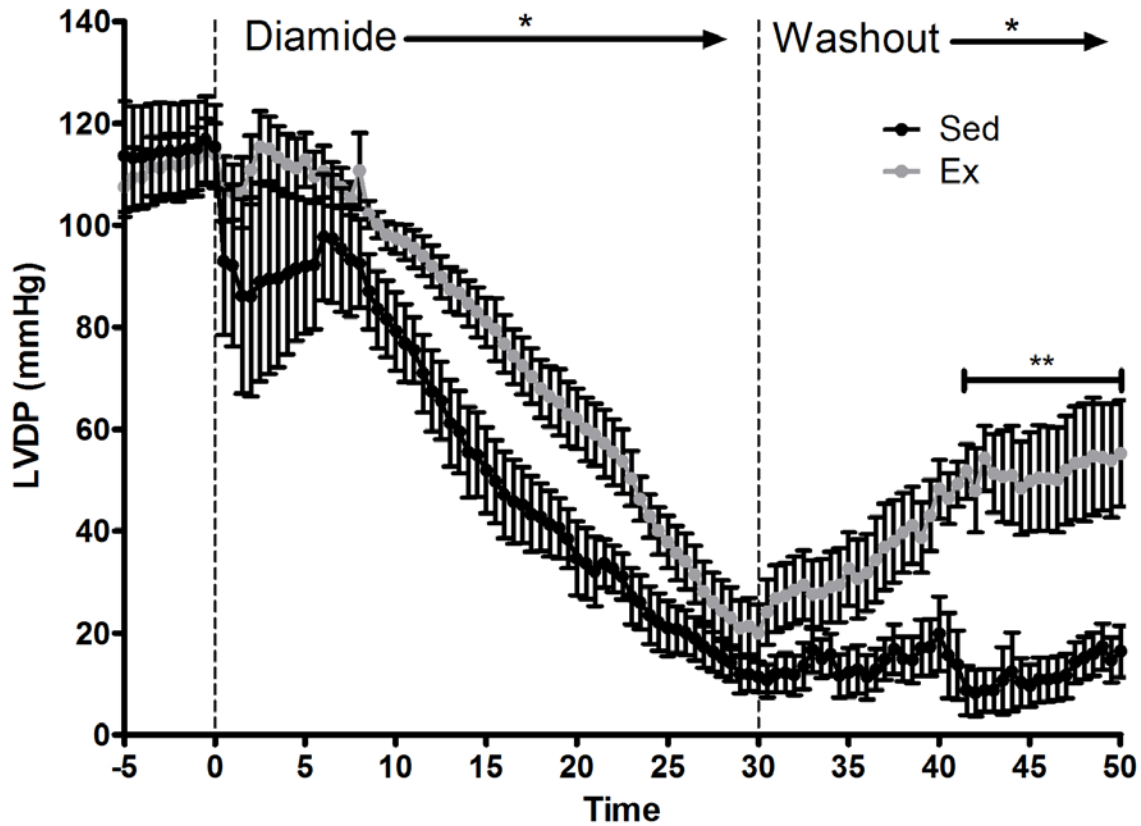
Figure 35: Left ventricular developed pressure (LVDP) during diamide treatment and washout for Sed (black circles) and Ex (gray circles) groups. Hearts were perfused with 200 µM diamide for 30 minutes, followed by a 20 minute washout; *, $P<0.05$ Main effect; **, $P<0.05$ Bonferonni post hoc for time. N= 7 per group. Mean ± S.E.M.
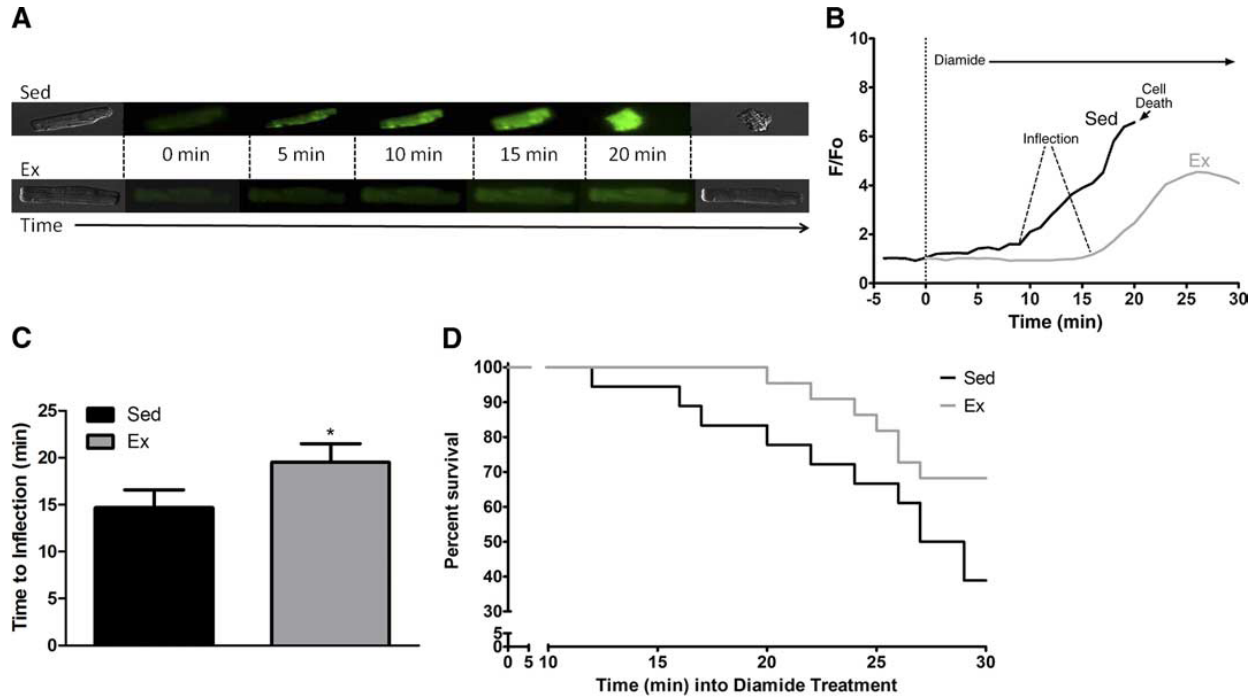
Figure 36: Ex delays the increase in ROS fluorescence and decreases cell death in isolated ventricular myocytes exposed to oxidative stress. **A**: Representative images over time for Sed and Ex myocytes. Black and white pictures represent bright field images of myocytes at the beginning and end of each experiment, and fluorescence traces in between are fluorescence images from cells loaded with the ROS sensor CM-DCF. **B**: Representative CM-DCF traces from Sed (black line) and Ex (gray line) myocytes. **C**: Average time to inflection for both groups. **D**: Survival curve for Sed (black) and Ex (gray); *, $P<0.05$ for Sed versus Ex myocyte survival. N = 18 (from 4 animals) for Sed and 25 (from 4 animals) for Ex. Mean ± S.E.M.

Figure 37: Myocardial content of total glutathione (GSHt), oxidized glutathione (GSSG), and ratio of reduced to oxidized glutathione (GSH/GSSG). All tissue collected immediately after the completion of each perfusion protocol. **A**: total glutathione (GSH$_t$) content. **B**: oxidized glutathione (GSSG) content. **C**: ratio of reduced to oxidized glutathione (GSH/GSSG) in hearts. *, P<0.05 vs. Sed at Baseline; #, P<0.05 vs. Ex Baseline; +, P<0.05 vs. Ex until arrhythmia; Mean ± S.E.M.
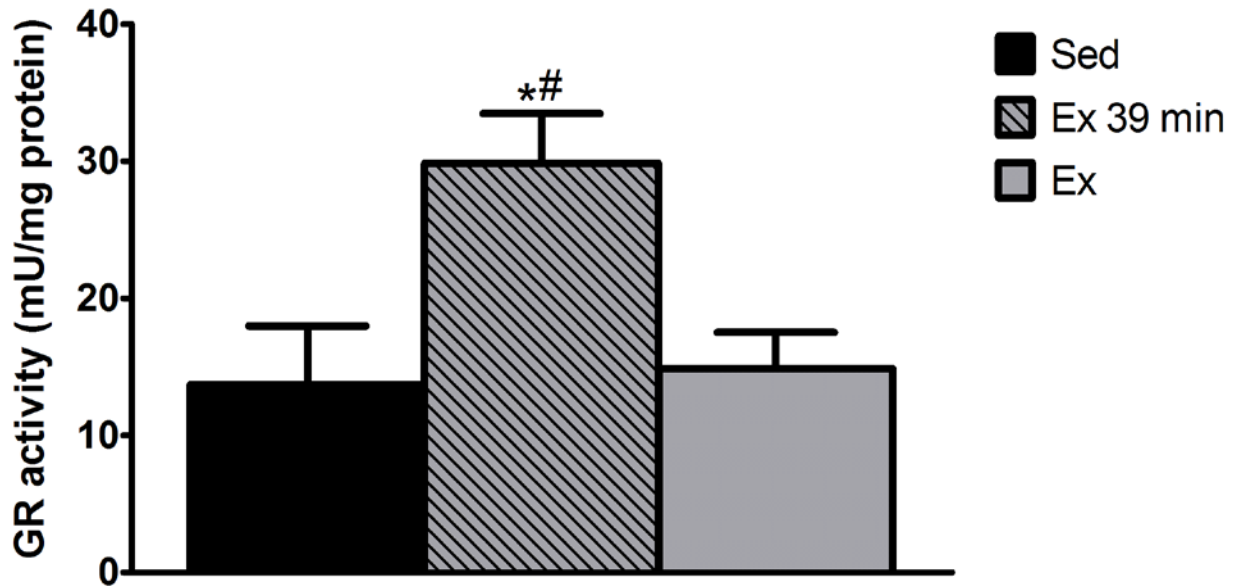
Figure 38: Glutathione reductase (GR) activity in hearts exposed to sustained thiol oxidation. Hearts were cut down after the onset of ventricular arrhythmia (time-points noted in Figure 1) and analyzed for GR activity. A subset of exercised hearts was cut down at the mean arrhythmia onset time for sedentary hearts (39 minutes). *, $P<0.05$ vs. Sed; #, $P<0.05$ vs. Ex. N = 8 per group. Mean ± S.E.M.
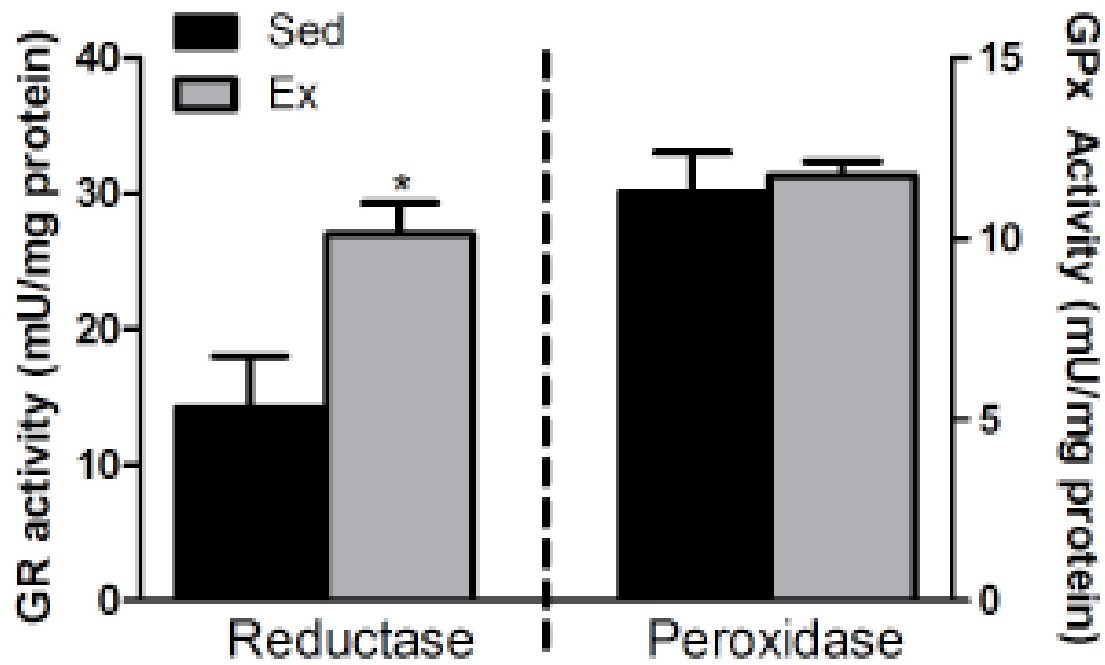
Figure 39: Basal changes in cardiac glutathione reductase (GR) and glutathione peroxidase (GPx) enzyme activity for sedentary (Sed) and exercise trained (Ex) hearts. *, P<0.05. N = 6 for Sed and 5 for Ex. Mean ± S.E.M.
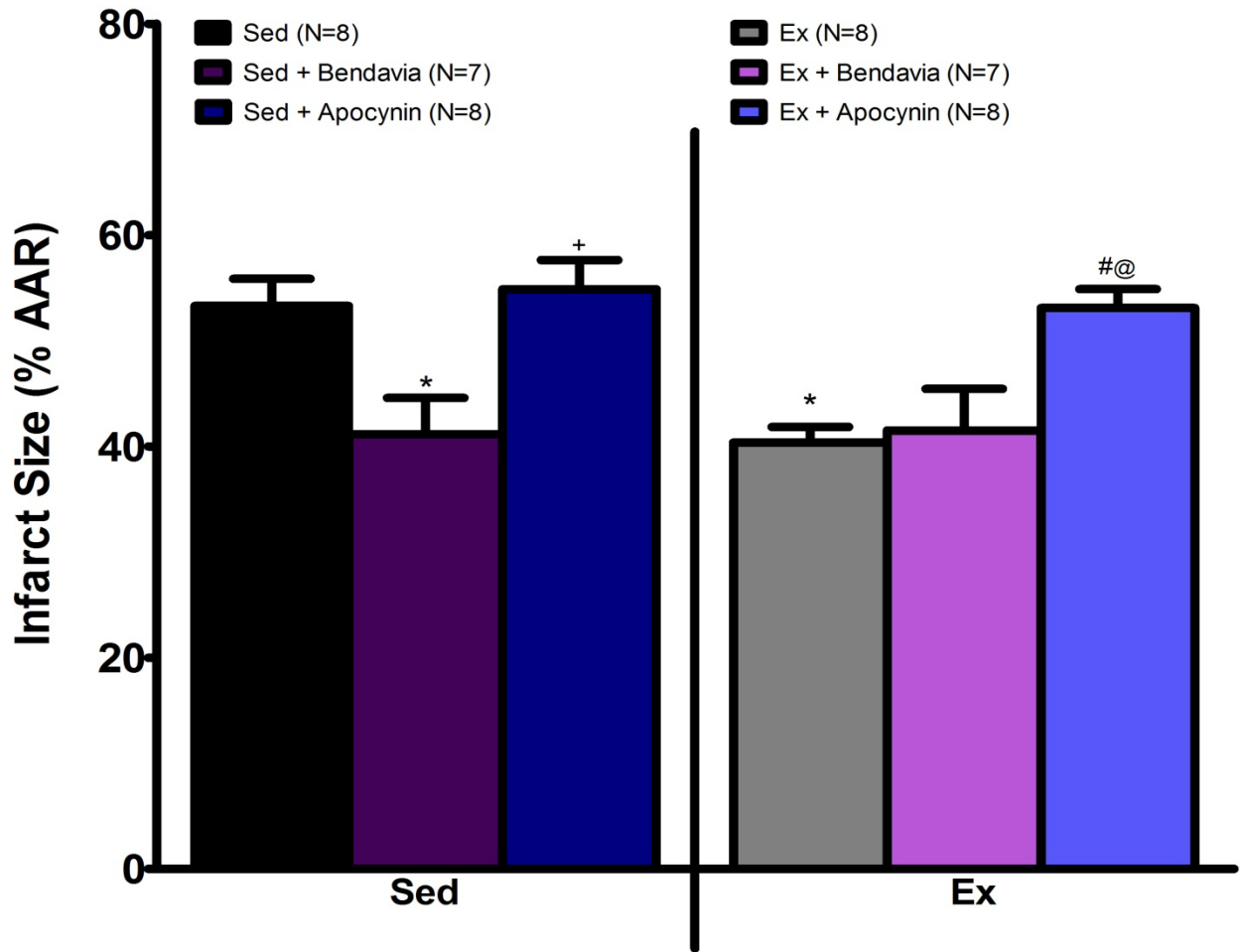
Figure 40: Exercise-induced preconditioning abolished with apocynin treatment. * P<0.05 vs. Sed, # P<0.05 vs. Ex, + P<0.05 vs. Sed and MTP, @ P<0.05 vs. Ex and MTP. Mean ± S.E.M.
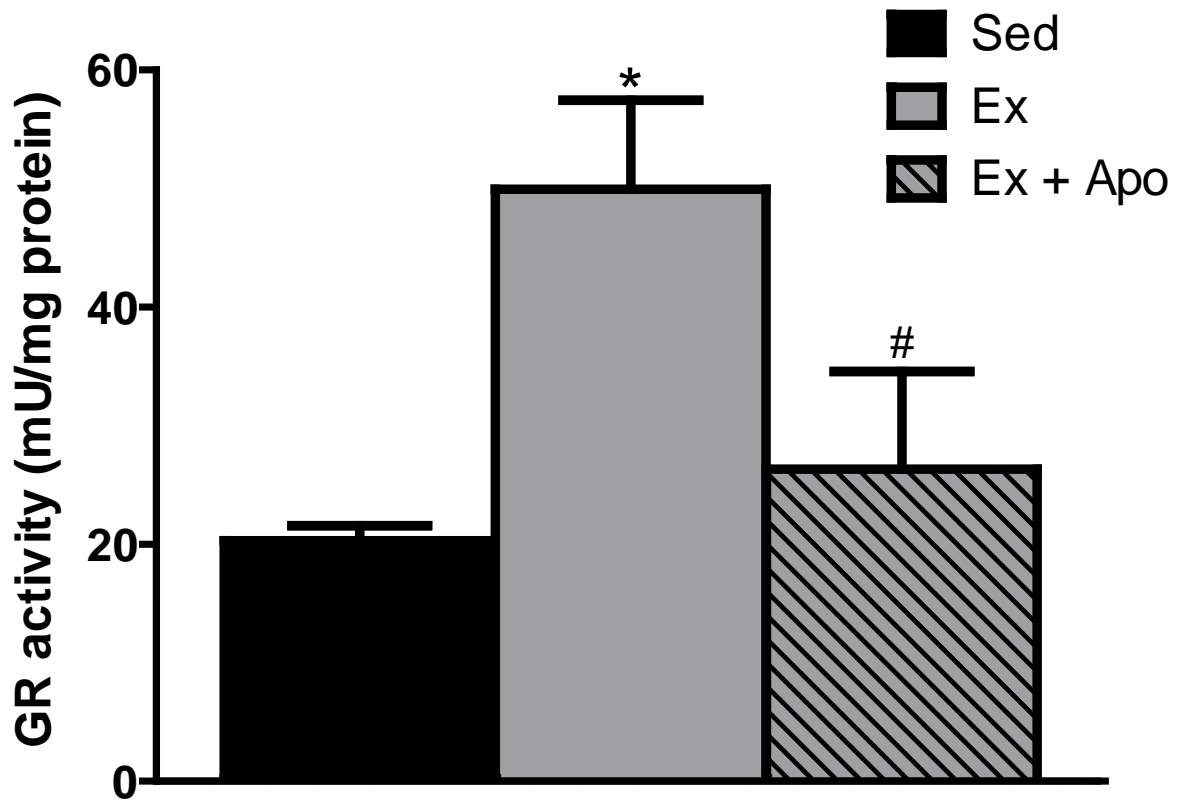
Figure 41: Inhibiting NAD(P)H oxidase generated ROS prior to exercise blunts increases in glutathione reductase activity. * $P<0.05$ vs. Sed, # $P<0.05$ vs. Ex
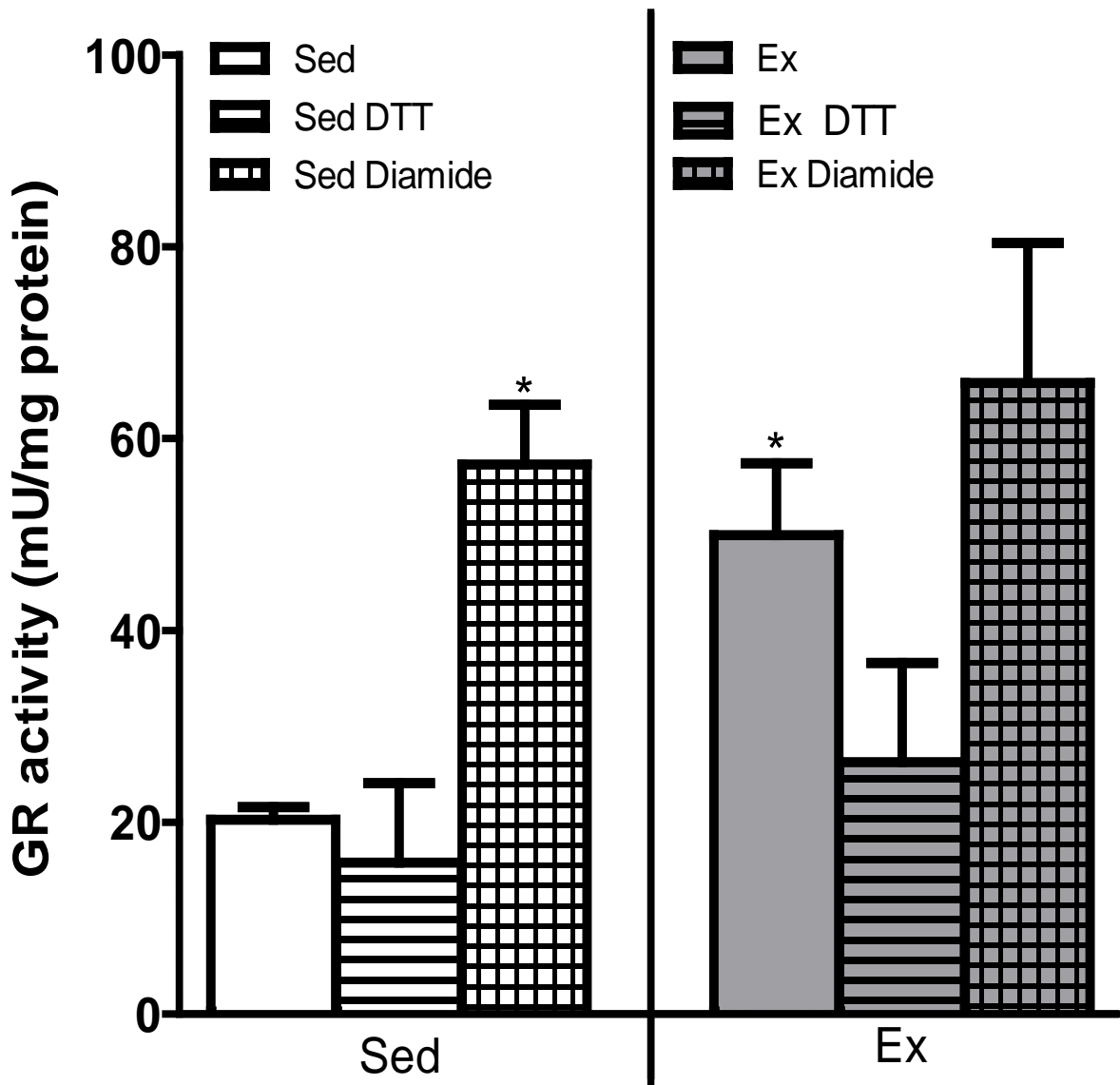
Figure 42: Redox modification of GR activity in Sed and Ex left ventricular homogenates. * $P<0.05$ vs. Sed.
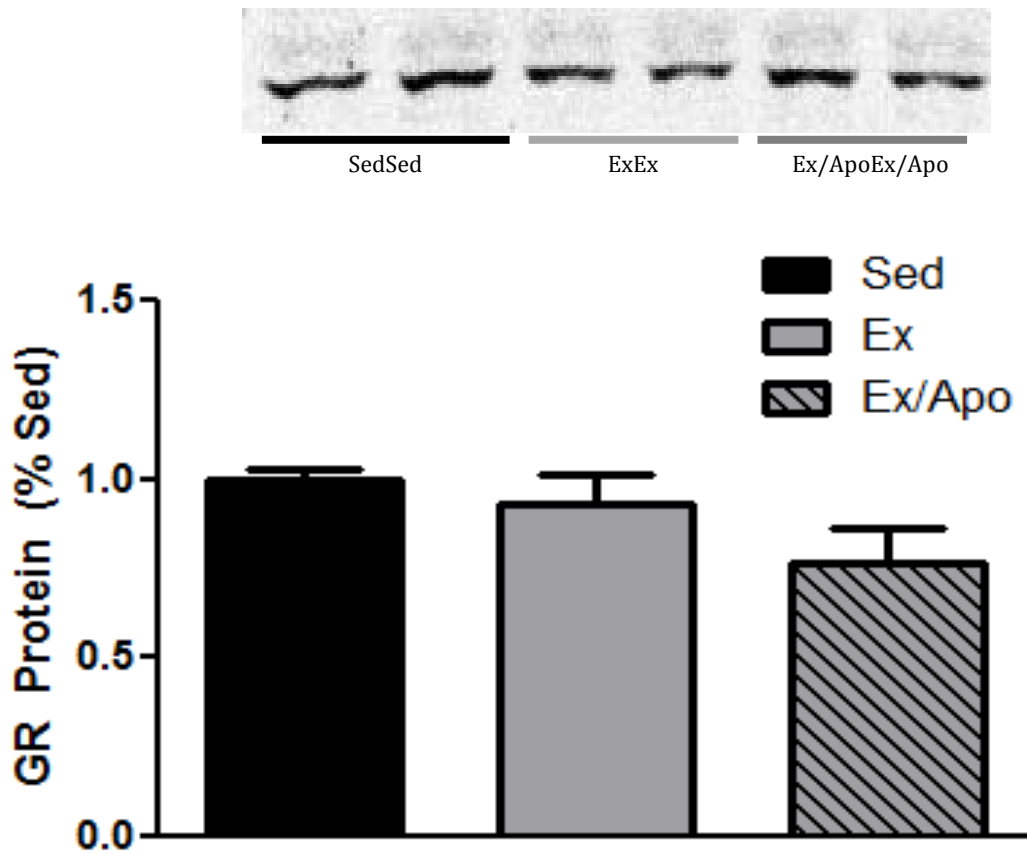
Figure 43: No differences in GR protein expression between groups.

A.

B.
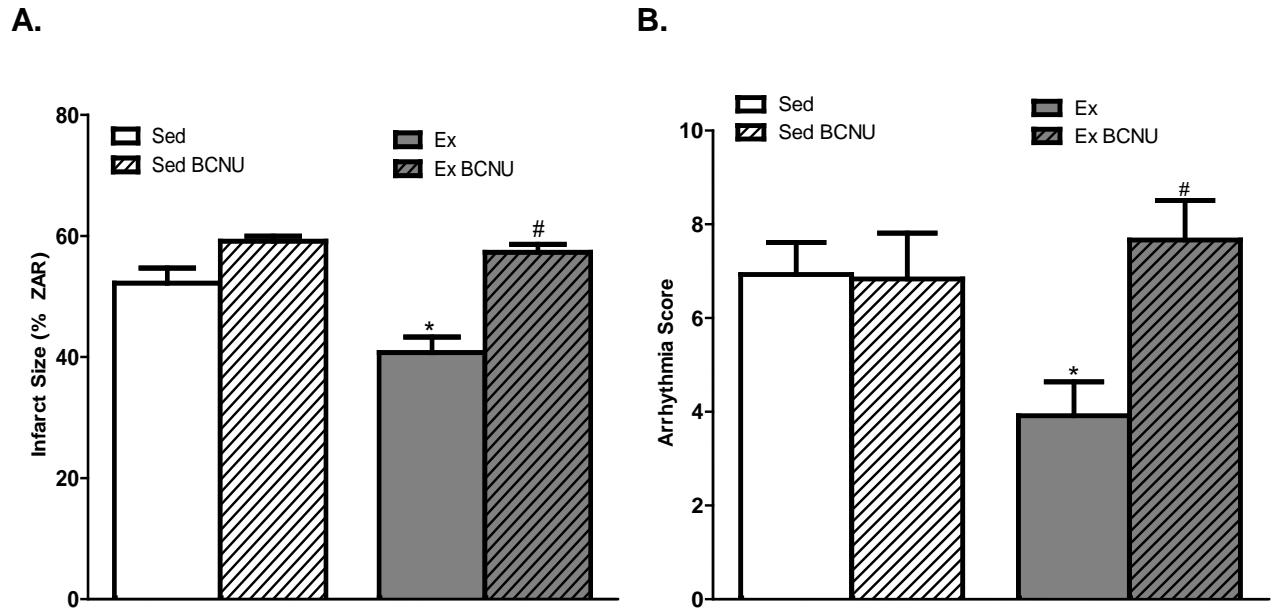
Figure 44: GR inhibition abolishes exercise-induced preconditioning from myocardial infarction and ventricular arrhythmias. Infarct size (**A**) and arrhythmia (**B**) data from isolated hearts perfused with BCNU *, P<0.05 vs. Sed; #, P<0.05 vs. ; Data presented as mean ± s.e.m.
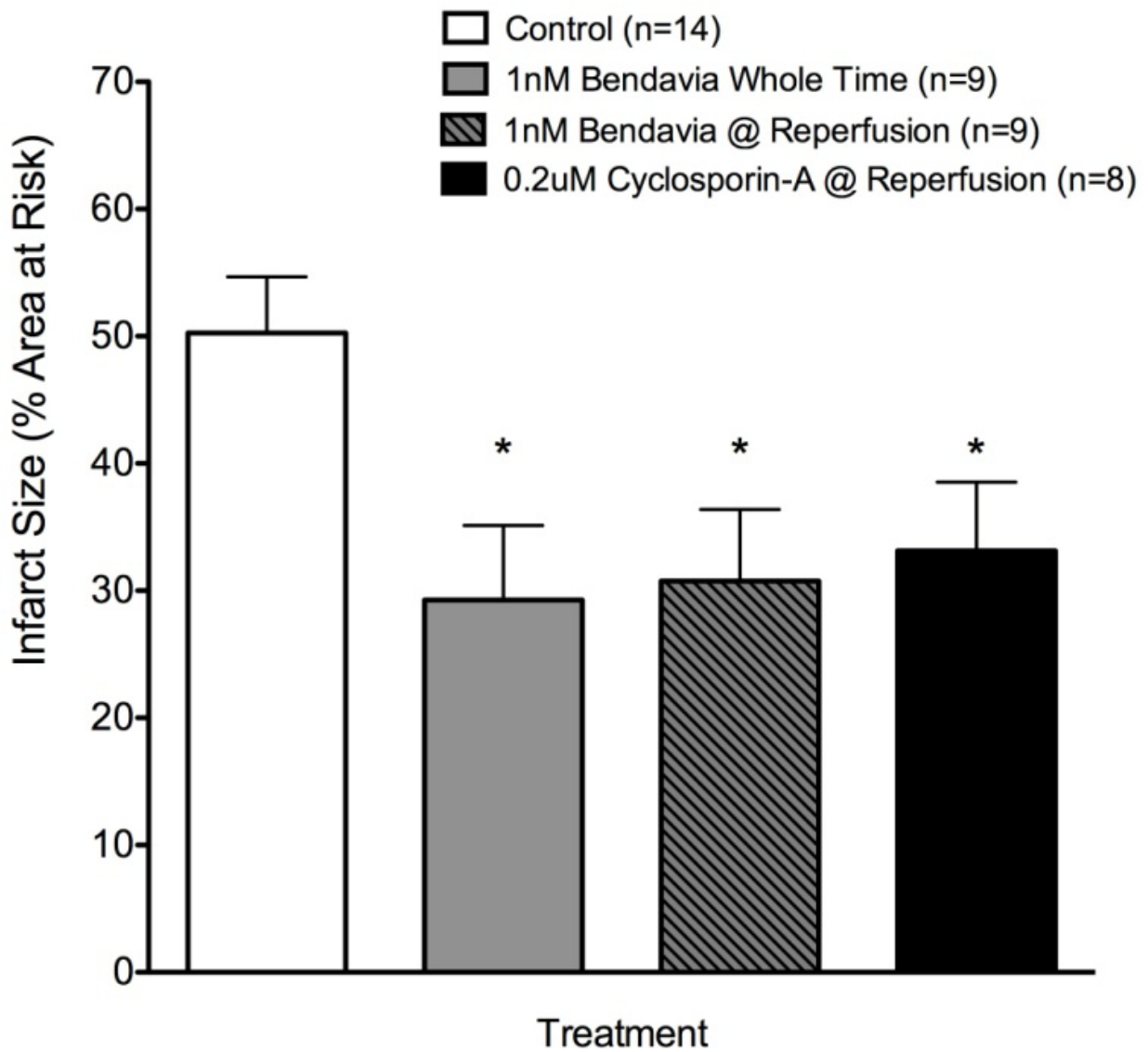
Figure 45: Infarct size in isolated guinea pig hearts exposed to 20 minutes of ischemia and 2 hours of reperfusion. Whole time = compound administered both before and after ischemia; @ reperfusion = compound administered only during reperfusion; P < 0.05 versus control (ANOVA).
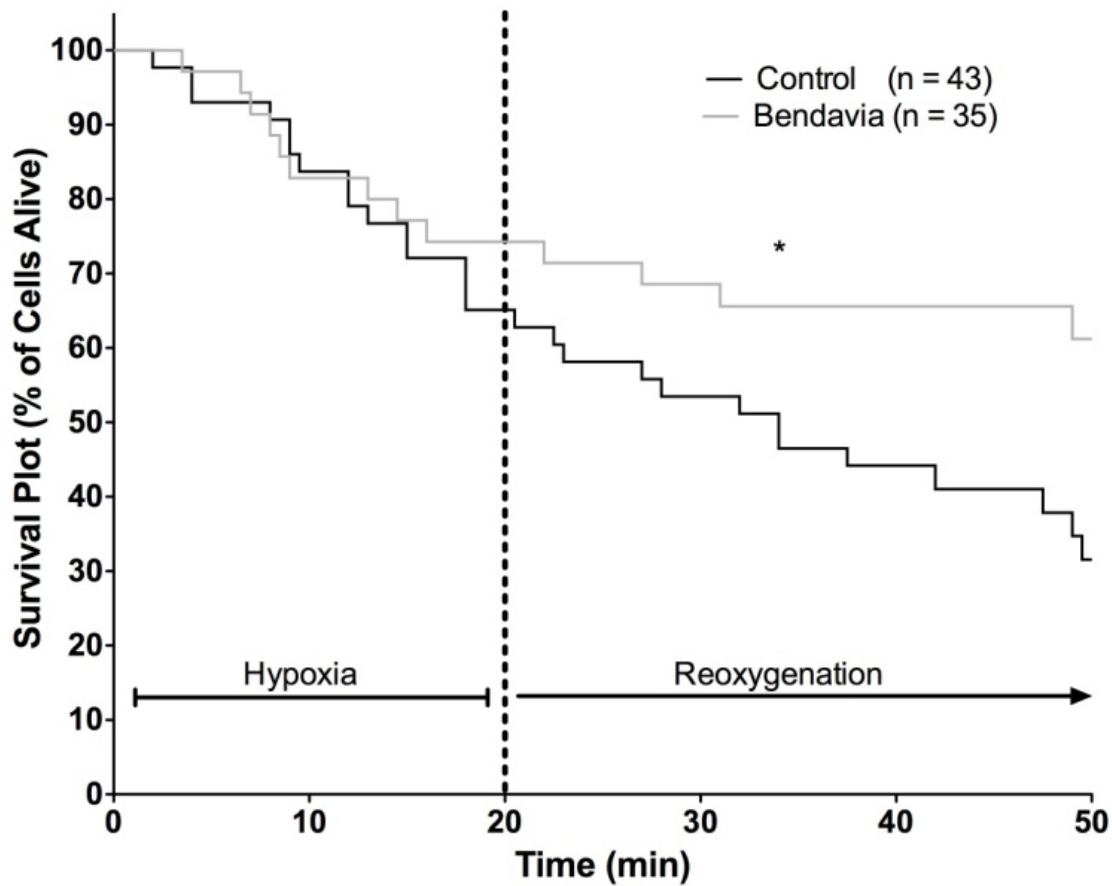
Figure 46: Bendavia significantly abolished ROS-dependent cell death, but the extent of cell death during hypoxia as well as ROS-independent cell death during reperfusion was similar to control. Survival plot for myocytes in the study exposed to hypoxia and reoxygenation. Each cell death event is noted as a downward step in the survival curve. *, P<0.05 versus control for the reoxygenation period.
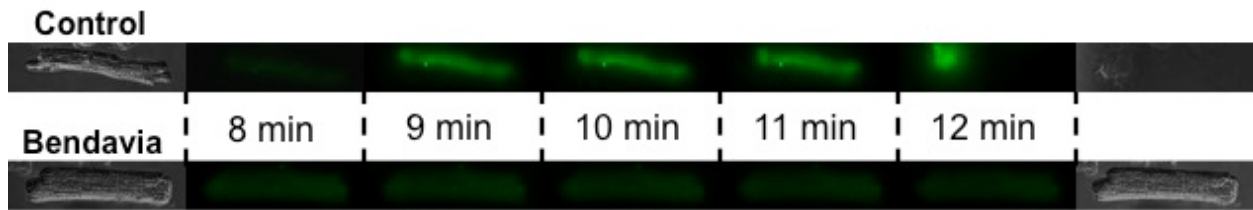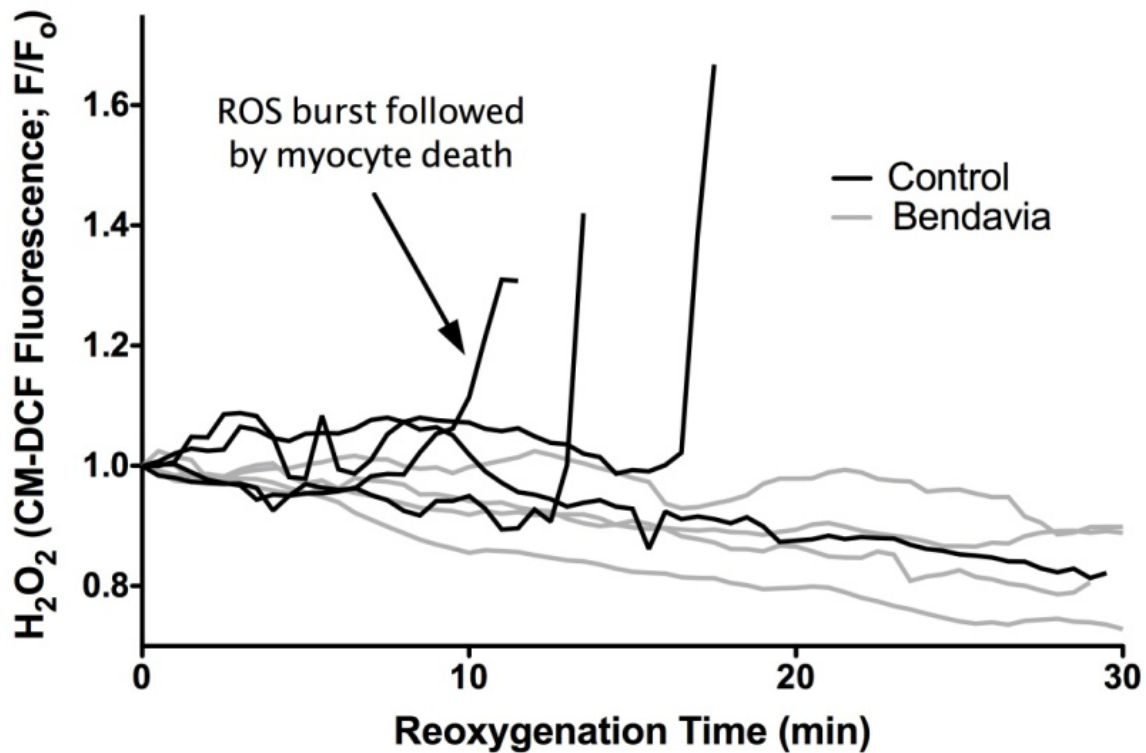
Figure 47: Cellular ROS production during hypoxia/reoxygenation. A: Representative fluorescence images of cardiac ventricular myocytes loaded with the ROS sensor CM-DCF. ROS bursts during reoxygenation preceded cell death, and Bendavia treatment prevented ROS-induced cell death. B: Representative fluorescence intensity traces for cells in the study. CM-DCF fluorescence is normalized to the basal fluorescence (F_o) for each cell at the end of hypoxia.
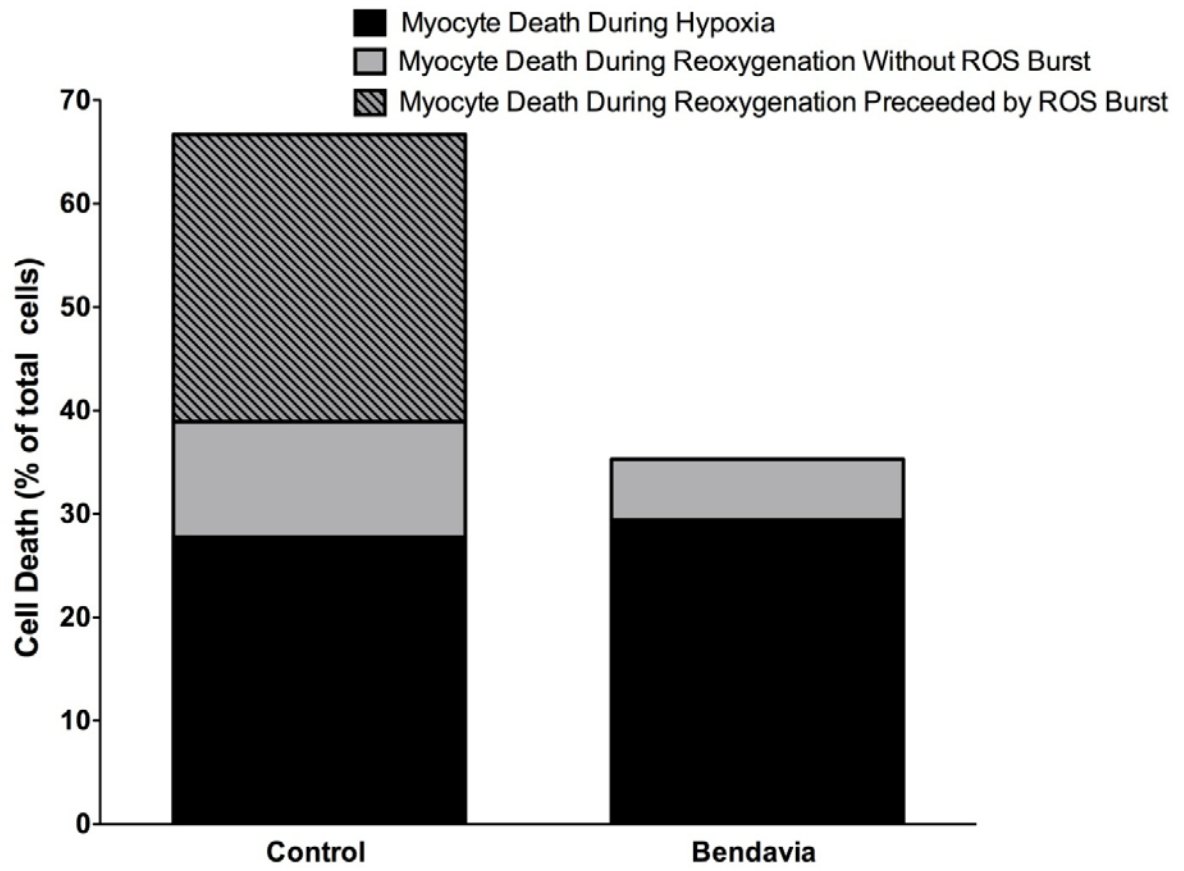
Figure 48: Contribution of ROS bursts to myocyte death during hypoxia/reoxygenation.

A.

Control

Reoxygenation

Bendavia

B.

$\Delta\Psi_m$ collapse followed
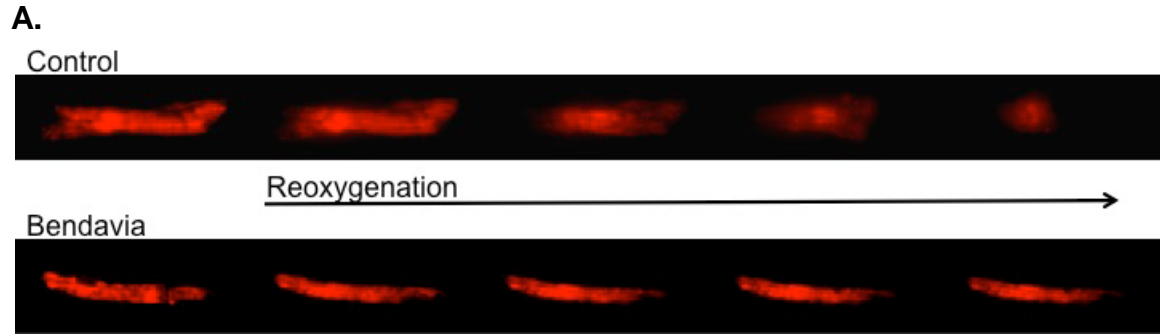by myocyte death
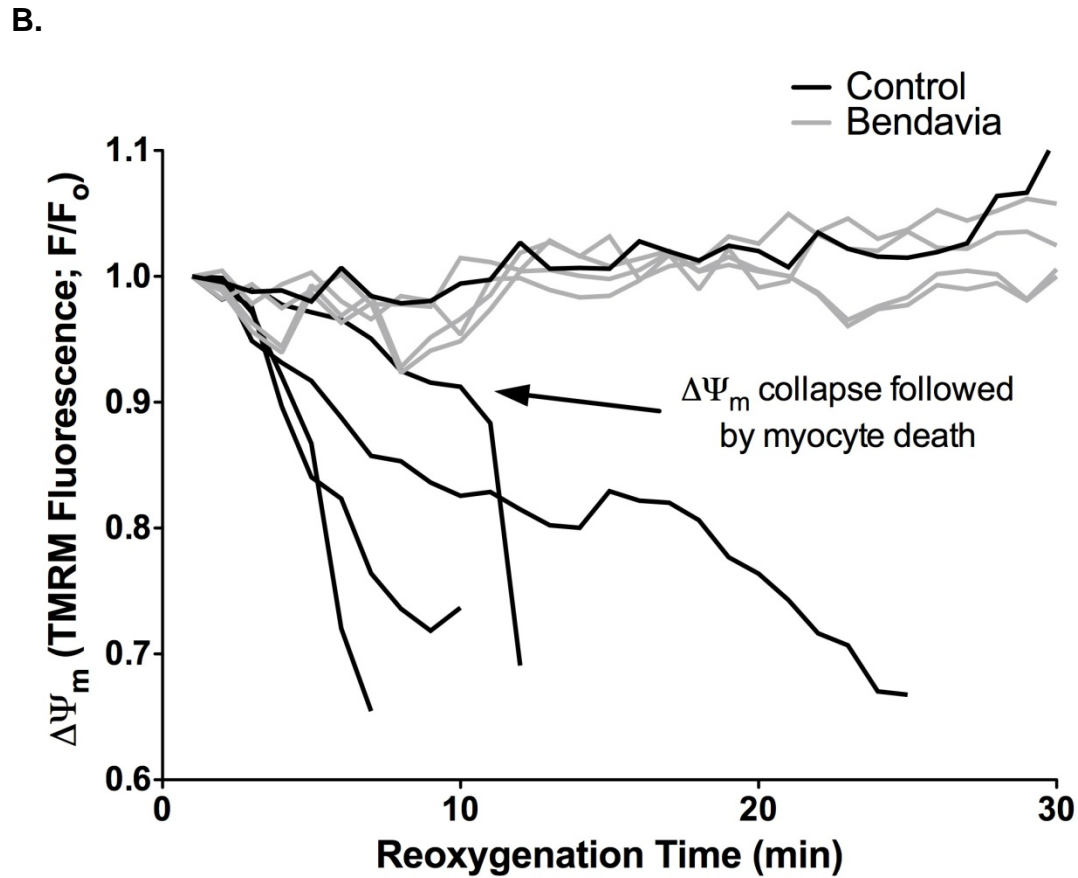
Figure 49: Mitochondrial membrane potential ($\Delta\Psi_m$) in myocytes during cellular

hypoxia/reoxygenation. A. Representative fluorescence images of myocytes loaded with

the $\Delta\Psi_m$ sensor TMRM. $\Delta\Psi_m$ collapse often preceded cell death, and treatment with

Bendavia improved the capacity to maintain $\Delta\Psi_m$. B. Representative fluorescence
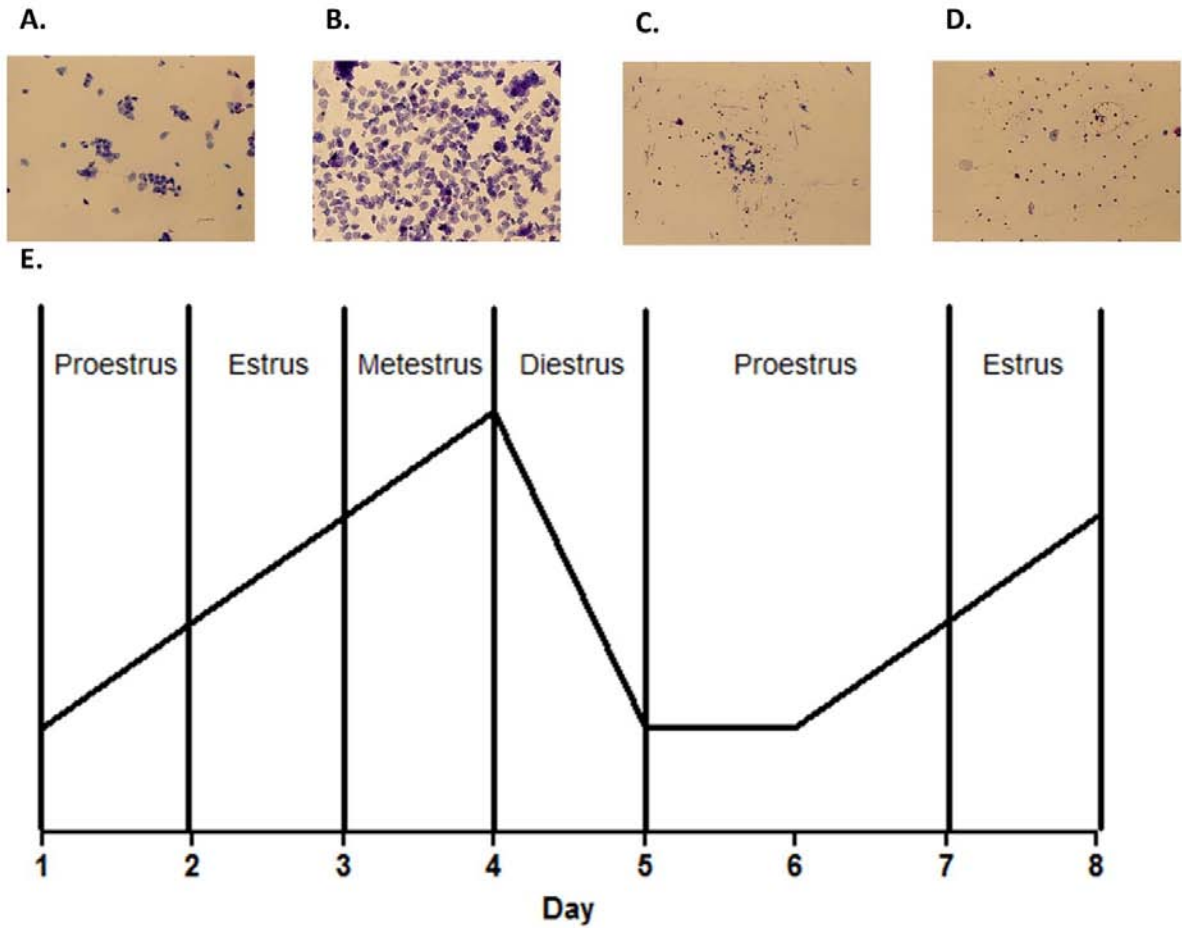
intensity traces for cells in the study.

Figure 50: Representative images from vaginal cytology for Proestrus **(A)**, Estrus **(B)**, Metestrus **(C)**, and Diestrus **(D)** phases of the estrous cycle. **(E)** Daily changes in the phase of estrous in our animals.
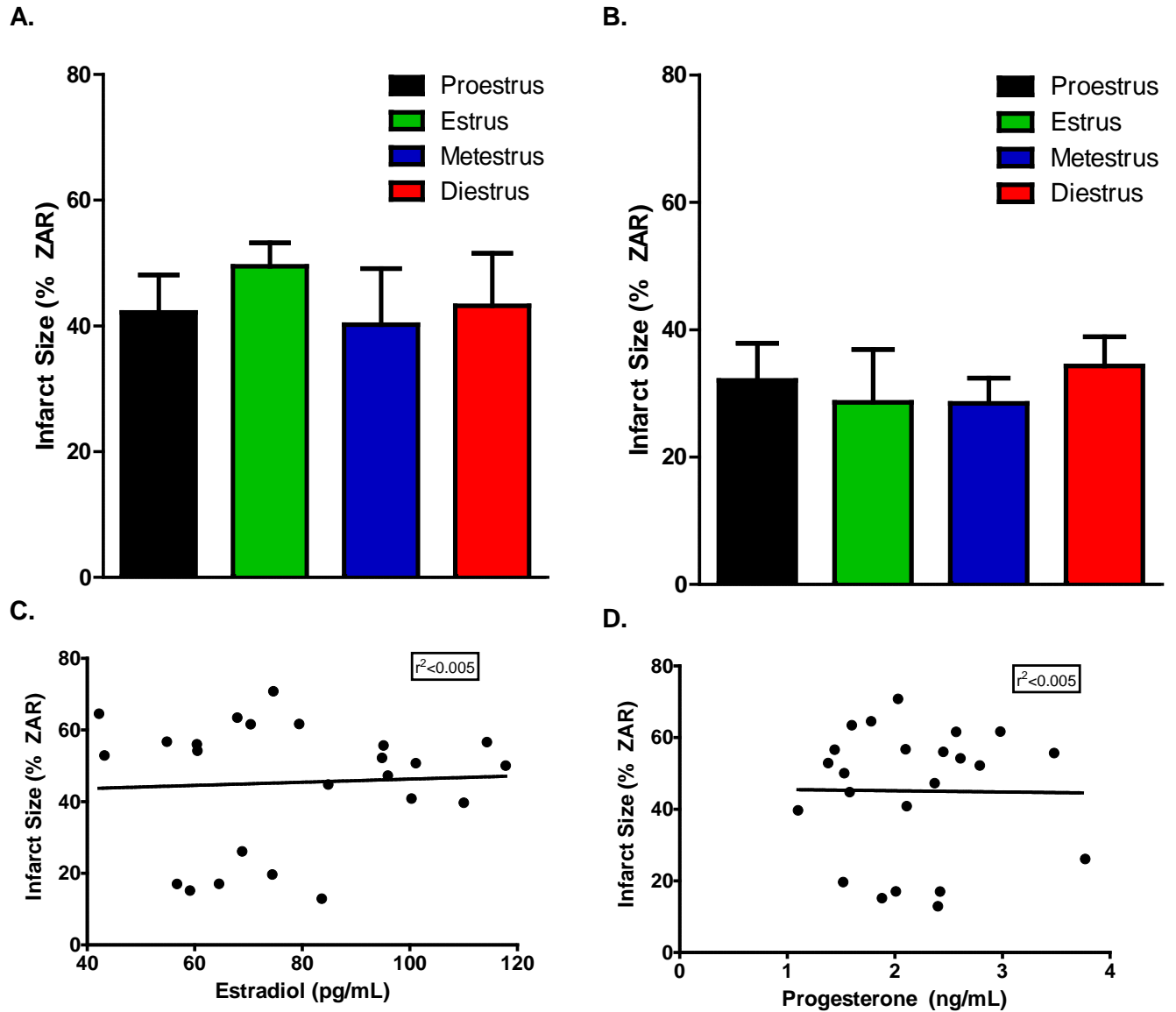
Figure 51: No difference in infarct size between the phases of the estrous cycle. Data from the ex vivo (**A**) and in vivo (**B**) hearts. There was also no correlation between infarct size and serum levels of estradiol (**C**) or progesterone (**D**) in isolated rat hearts.
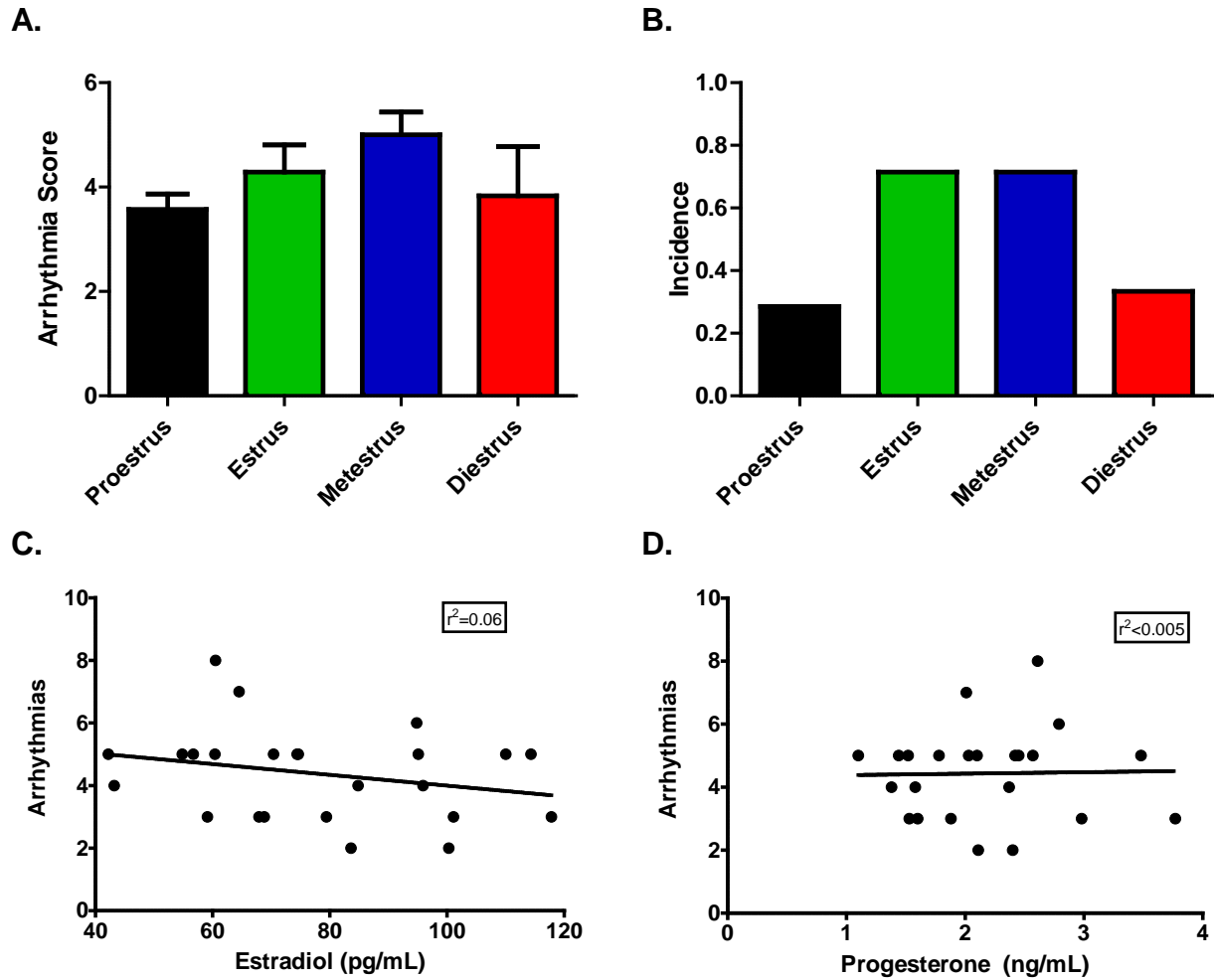
Figure 52: No differences were seen between groups in the severity or incidence of ventricular arrhythmias in isolated rat hearts. Arrhythmia scores (**A**) and incidence of VF (**B**) were used to assess the severity of arrhythmias. There was also no correlation between the arrhythmia score and serum levels of estradiol (**C**) or progesterone (**D**) in isolated rat hearts.