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The myocardial Na^+/H^+ exchanger-1 (NHE1) plays a major role in regulation of intracellular pH and its upregulation has been implicated in increased ischemia-reperfusion injury and other pathologies. Hydrogen peroxide (H_2O_2) is a major by-product of reperfusion and is known to enhance NHE1 activity via ERK1/2 signaling. However, neither NHE1 activity nor its regulation by H_2O_2 has been examined in response to exercise. Therefore, we examined the effect of aerobic exercise training on intrinsic NHE1 activity, H_2O_2-mediated activation of NHE1, and H_2O_2-induced phosphorylation of ERK1/2 using isolated adult ventricular myocytes. Adult female Sprague-Dawley rats were randomly divided into sedentary (S, n=10) and exercise-trained (E, n=8) groups. Heart weight:body weight and plantaris muscle cytochrome *c* oxidase activity was significantly greater by 6.8% and 1.9 fold, respectively, in E animals compared to S animals. NHE1 activity was determined in cells loaded with the pH fluoroprobe 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF). NHE1 activity was 161% greater in E myocytes compared to S myocytes (0.57 ± 0.10 *v* 1.49 ± 0.18 fmol/min), which is attributed to the 55% greater cell volume (22.2 ± 0.6 *v* 34.3 ± 1.1 pL) and 48% greater buffering capacity (28.79 ± 0.72 *v* 42.65 ± 0.79 mM/pH unit) of E cells. Stimulation with H_2O_2 enhanced NHE1 activity in S and E but to a significantly lower level in E myocytes (1.55 *v* 0.64 fold). As assessed by Western blotting, H_2O_2 stimulation also increased phosphorylation of ERK1/2. There was no difference in the density of H_2O_2-stimulated phosphorylation between S and E; however, E myocytes were

observed to have significantly less total ERK1/2. No difference was observed in either cardiomyocyte NHE1 content or catalase and glutathione peroxidase activity. Our data indicate that aerobic exercise training increases NHE1 activity at physiological intracellular pH and attenuates the H_2O_2-mediated activation of NHE1 activity.

THE EFFECT OF EXERCISE ON MYOCARDIAL Na^+/H^+ EXCHANGER-1 (NHE1)

by

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APPROVAL PAGE

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LIST OF ABBREVIATIONS

NHE1	Na^+/H^+ exchanger-1
IR	Ischemia-Reperfusion
Ca^{2+}_i	intracellular calcium
Na^+_i	intracellular sodium
pH_i	intracellular pH
H_2O_2	hydrogen peroxide
NK ATPase	Na^+/K^+ ATPase
NCX	Na^+/Ca^{2+} exchanger
SERCA2a	sarco-endoplasmic reticulum Ca^{2+} ATPase 2a
ER	endoplasmic reticulum
CK	creatine kinase
LDH	lactate dehydrogenase
MI	myocardial infarction
MPTP	mitochondrial permeability transition pore
ERK1/2	extracellular signal-regulated kinase 1/2
ROS	reactive oxygen species
MAPK	mitogen activated protein kinase
$sarcoK_{ATP}$	sarcoplasmic ATP-sensitive K^+ channel
$mitoK_{ATP}$	mitochondrial ATP-sensitive K^+ channel
HSP	heat shock protein
ATP	adenosine triphosphate

ADP	adenosine diphosphate
RyR	ryanodine receptor
iNOS	inducible nitric oxide synthase
COX-2	cyclooxygenase-2
GSH	reduced glutathione
GPx	glutathione peroxidase
GSSG	oxidized glutathione
MnSOD	manganese superoxide dismutase
FBS	fetal bovine serum
J_{H+}	proton flux
CJ_{H+}	corrected proton flux
BCECF	2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein

CHAPTER I

INTRODUCTION

Statement of Problem.

Nearly 2,300 Americans die of cardiovascular diseases each day – one person every 38 seconds. The most common type of cardiovascular disease and the leading cause of death in America is coronary heart disease. Ischemia-reperfusion (IR) injury (commonly referred to as a heart attack) is the result of a coronary event and remains an important clinical problem following heart surgery (132). IR is the lack of blood flow and the subsequent return of blood flow to a tissue, which can result in secondary cell death. Chronic aerobic exercise stimulates the heart cell to change in a way that better protects it from the consequences of a heart attack and other major pathologies. Although exercise-induced protection exists, the various mechanisms are not well understood. One candidate mechanism that involves heart cell death is the role of the sodium-hydrogen exchanger (NHE1). There is strong evidence that NHE1 plays a critical role in not only IR injury but also a number of pathologies including diabetes, hypertension, and pathological hypertrophy due to its role in intracellular Ca^{2+} overload, a primary factor of cell death. Increased expression and activity of NHE1 is tightly linked with greater IR injury and is consistently present in other cardiac pathologies (109). Furthermore, NHE1 activity is regulated by specific proteins activated during IR; these proteins are termed ERK1/2. Conversely, inhibition and knock-out of NHE1 significantly reduces IR-induced

injury (207,208) and pathological hypertrophy (110). Thus, previous results indicate that NHE1 suppression is cardioprotective. We have shown that exercise training significantly reduces NHE1 expression in heart muscle (67); however, it is not yet known whether this change has a role in exercise-induced cardioprotection. Therefore, investigating the NHE1 mechanism in response to exercise training will advance the knowledge of exercise-induced cardioprotective mechanisms and our understanding of exercise as a clinically-relevant measure for heart attack injury prevention. The *long-term goal* of this research is to understand the mechanisms by which aerobic exercise leads to cardioprotection. The *overall objective* of this proposal is to investigate a possible cardioprotective mechanism conferred by exercise on adult rat heart cells against a free radical (H_2O_2) insult; an H_2O_2 insult represents the oxidative stress suffered in the cell during IR. ***Our central hypothesis is that exercise reduces myocardial cell death by decreasing NHE1 protein content and secondary Ca^{2+} overload.*** We base our hypothesis on our prior work demonstrating that aerobic exercise training can decrease cardiac NHE1 protein content (67) and reduce heart cell injury upon exposure to H_2O_2 (191). Our *approach* will be to measure changes in NHE1 protein expression and activity, and concurrent changes in ERK1/2 regulation of NHE1 in rat myocardium following 5 weeks of aerobic exercise training. Isolated cardiomyocytes from the same area of myocardium will be stimulated by H_2O_2 to show the cardioprotective impact of changes in NHE1 following exercise training, which will be revealed by the amount of intracellular Ca^{2+} overload and the extent of cell death. Our *rationale* for this approach is that H_2O_2 activates ERK1/2, which activates NHE1; increased NHE1 activity leads to

cell death through intracellular Ca^{2+} overload and increased free radical production – two major contributors to IR-induced cell death. Upon completion of this work, our expectation is that we will be able to demonstrate the relationship between exercise training, NHE1, and H_2O_2 insult, which in turn will enhance our knowledge of the role of aerobic exercise training in generating protective mechanisms.

Aims and Hypotheses.

We will test our overall hypothesis by pursuing the following aims.

Aim 1: To determine the extent of change in NHE1 protein expression and activity induced by a 5-week aerobic exercise training program.

Working hypotheses: in response to chronic aerobic exercise training *1)* NHE1 protein expression will decrease, *2)* basal and H_2O_2-induced increase in ERK1/2 activity will not change, and *3)* basal and H_2O_2-induced increase in NHE1 activity will decrease in a manner proportional to the decrease in expression.

Aim 2: To determine the effect of exercise training on Ca^{2+} overload and myocardial apoptosis in response to an H_2O_2 insult.

Working hypotheses: chronic aerobic exercise training will *1)* decrease H_2O_2-induced Ca^{2+} overload and *2)* attenuate the H_2O_2-mediated activation of the cell death effector caspase-3.

Given the severity of coronary heart disease and its resulting IR injury, identifying protective mechanisms against this insult is necessary to improve treatments that will prevent cell death. This work is innovative in that it will be the first to examine aerobic exercise-induced downregulation of NHE1 as a natural cardioprotection.

Ultimately our goal is to improve the health and well being of those at risk for a heart attack and cardiovascular disease through cardioprotective exercise.

CHAPTER II

REVIEW OF THE LITERATURE

Cardiovascular disease is a major source of IR injury.

Nearly 2,300 Americans die of cardiovascular diseases each day – one person every 38 seconds (1). Coronary heart disease is the most common type of heart disease and the leading cause of death in America. The result of a coronary event, and still a clinical problem following heart surgery, is ischemia-reperfusion (IR) injury (132). This two part cellular event results in marked damage to downstream tissue. The ischemic phase represents when blood flow to an area of tissue significantly decreases or stops, markedly reducing ATP production by usual aerobic means; the rapid decline in energy inhibits specific ATP-dependent membrane proteins leading to intracellular ion imbalances, and shifts ATP production toward anaerobic glycolysis, which can result in decreased intracellular pH (pH_i). The lower pH_i alters enzyme activity and causes secondary ion changes. Moreover, most of the damage typically representing IR injury occurs during the reperfusion phase when blood flow is re-introduced to the area of tissue. The presented oxygen in combination with other cellular factors, some newly produced during ischemia, creates an environment well suited for cell damage, cell death, and arrhythmias – all of which are common developments preceding major declines in heart performance.

NHE1 is a critical element of IR injury.

The sodium-hydrogen exchanger-1 (NHE1) is an essential regulator of pH_i and has been implicated as a critical element of IR injury and other cardiac pathologies. During ischemia, the low oxygen saturation severely reduces the ability of oxidative phosphorylation to produce ATP, which switches energy production to anaerobic glycolysis. As glycolysis struggles to provide proper ATP levels, intracellular H^+ (H^+_i) concentration rises and ATP-sensitive proteins are inhibited. The inhibition of these proteins, specifically Na^+/K^+ ATPase (NK ATPase), results in intracellular Na^+ (Na^+_i) accumulation. Upon reperfusion, NHE1 acts as a primary source to the growing Na^+_i pool as it attempts to regulate the glycolysis-induced decline in pH_i by extruding one H^+_i in exchange for one extracellular Na^+ (213). The accumulation of Na^+_i activates the sarcolemmal Na^+/Ca^{2+} exchanger (NCX) leading to intracellular Ca^{2+} (Ca^{2+}_i) overload, which causes damaging effects such as cell death, contracture, and arrhythmias (73) (73). Additionally, the Ca^{2+}_i imbalance produces oxidative stress that contributes to these detrimental effects (154). The accumulation of both Na^+_i and Ca^{2+}_i beyond the normal range has harmful consequences on the cell. Ca^{2+} accumulation activates proteases (i.e. calpain) that can degrade regulatory elements (i.e. sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA2a)) that perpetuate the Ca^{2+}_i imbalance (77). Moreover, the high Ca^{2+}_i can overwhelm the mitochondria, which causes swelling of the organelle and initiation of the mitochondrial-mediated cell death cascade (199). On the other hand, Na^+_i can disrupt necessary Ca^{2+} entry into the mitochondria via the mitochondrial NCX. A lack of Ca^{2+} in the mitochondria slows Krebs Cycle dehydrogenase activity and subsequent NADH

production, which attenuates ATP production and glutathione restoration; decreased glutathione levels allows for greater ROS formation, which augments cellular damage (115). *These cellular events clearly illustrate the critical involvement of NHE1 in IR injury, and support that altering NHE1 in such a way that leads to a reduction in cell damage following IR (i.e. possibly via its downregulation) could be a potential mechanism for cardioprotection.*

Changes in NHE1 protein content and activity affect severity of injury.

Increased expression and activity of the NHE1 protein, associated with a number of pathologies including diabetes, hypertension, and pathological hypertrophy (109), results in greater IR-induced damage and eventual cell death. Because NHE1 is upregulated in many pathological settings, transgenic mice have been used to help determine the effects of this upregulation in relation to IR and hypertrophy, and to establish whether an increase in either expression, activity, or both play a central role in the effects.

NHE1 genetic manipulation has consistently proven to increase NHE1 levels by 2-fold or greater compared to controls (9,44,98). Imahashi et al. used a α-MHC promoter to create a N-line and a K-line. The N-line contained an overexpression of wild-type (WT) NHE1, while the K-line was overactive due to point mutations on the calmodulin-binding domain that produced an alkaline shift to the working pH of NHE1 (98); NHE1 is normally inactive near its set-point of pH 7.4 (46). No effect on basal contractile or metabolic function was observed following genetic manipulation; indices of contractile

function were LVDP, HR and +dP/dt, and indices of metabolic function were [ATP], [PCr], and pH_i. During 20 min of ischemia, neither pH_i nor Na_i were different among groups, which included wild-type, N-line, and K-line mice. After reperfusion, transgenic mice had an improved rate-pressure product (RPP) – heart rate X systolic blood pressure. The drawn conclusion was an overexpression of NHE1 above endogenous levels was not harmful at rest or in response to IR. This idea was supported by Baczko et al. who used WT and N-line (overexpressed NHE1) mice and showed no change in basal contractile function observed in isolated myocytes. Moreover, after metabolic inhibition (using NaCN and 2-deoxyglucose) and recovery, N-line mice had increased fractional cell shortening and diastolic Ca^{2+}_i levels. These increases were speculated to be the cause of the enhanced contractility following IR, as seen by Imahashi et al (98).

These mouse genetic studies were somewhat counterintuitive compared to the many NHE1 inhibition studies that had demonstrated cardiac protection in rats (169,185,199,210,225). Thus, Cook et al. thought examining the systemic cellular effects of the genetic manipulation of NHE1 on other cellular components was necessary. To date, no peripheral changes to other ion regulatory channels had been observed, such as NK ATPase, SERCA, NCX (98,143), NBC (Na-bicarbonate cotransporter), AE3 (anion exchanger type 3) (98), and calsequestrin (143). Again, overexpression of NHE1 provided protection against 20 or 30 min of ischemia and 120 min of reperfusion in 3-4 month old mice. Interestingly, this age point presented an increase of incomplete NHE1 precursors in the ER, as was an increase in ER stress proteins – presumably from an unfolded protein response. The upregulation of ER stress proteins calreticulin and protein

disulfide isomerase was later corroborated by multiple studies (108,139). Were these unintended changes of genetic manipulation? Were these NHE1-independent effects responsible for the protection initially prescribed to NHE1 overexpression? Martindale et al. showed that the unfolded protein response activates the upregulation of ER stress proteins, which reduce IR injury (129) and could have contributed to the observed protection. Possibly even more interesting was the mortality observed with aging in the transgenics. At 8 months, a dramatic separation of survival curves existed (44,139). At 12 months, there was an increase in apoptosis in left ventricular myocardium compared to wild-type animals. In support of this, another transgenic mouse model presented with disrupted ER function and increased left ventricular apoptosis, implicating unrecovered ER stress as a cause of pathology (88). At 15 months, 90% of transgenic mice were dead, whereas 95% of WT mice were alive (44). In light of these findings, the altered Ca^{2+}_i handling, initially seen in the genetic lines as a mechanism providing contractile benefits, could be viewed as a result of ER stress and as another contributor to the heart failure phenotype (9). To that end, the discovery of ER stress protein upregulation caused the focus of transgenic NHE1 overexpression to shift away from mechanisms of IR protection to how overexpression produced the pathological phenotype.

Cardiac hypertrophy and heart failure were the resultant phenotype of a study using mice with hyperactive NHE1 (K-line) (143). This study helped to elucidate the mechanism behind early-onset hypertrophy. An increase in the calcineurin-NFAT pathway was observed, which led to the speculation that NHE1 overexpression altered the expression of hypertrophy-associated genes (143). Again, was there a difference

between WT NHE1 and hyperactive NHE1 overexpression? Both WT overexpression and hyperactive NHE1 significantly changed the expression of genes associated with cardiac hypertrophy, necrosis/cell death, and infarction; however, the greatest response was from mice with hyperactive NHE1 (216). In fact, Mraiche et al. showed activity was necessary to induce cardiac hypertrophy in mice by separating the effect of the two transgenes (139). Mraiche et al. used point mutations on the C-terminal to show that the NHE1 activity was necessary for more severe effects of NHE1 overexpression, as K-line (hyperactive NHE1) mice displayed greater heart weights and performed significantly more poorly in a treadmill test compared to both N-line and control mice. This group also noticed that the effects of Nakamura et al. were more drastic than those of Cook et al. and attributed it to NHE1 activity, since Nakamura deleted a large piece of the C-terminal (amino acids 637-656) to create a hyperactive NHE1, whereas Cook et al. simply overexpressed the WT NHE1. The mice with WT overexpression took longer to present major pathology than those with hyperactive NHE1. This information aligns nicely with the fact that human hearts with chronic end-stage heart failure did not have greater NHE1 content compared to unused donor hearts, but instead had NHE-1 proteins with increased activity (219).

The transgenic mouse initially presented controversy over the effect of NHE1 overexpression; the protein was upregulated as in pathological conditions, yet was providing protection during IR. The genetic manipulation was uncovered to be the ultimate precursor of protection as an increase in unfolded NHE1 in the ER produced an upregulation of ER stress proteins and subsequent IR protection (9,129). Still, more work

should be performed to clarify these results, such as inhibiting ER stress proteins to observe whether protection remains. The transgenic mouse model is definitely useful for delineating the mechanisms behind NHE1-induced hypertrophy; however, the abundance of literature displaying IR protection conferred by NHE1 inhibition (as discussed below) trumps the evidence suggesting that NHE1 upregulation is a cell-mediated protective mechanism. The increased NHE1 present in diseased myocardium is attributed to a chronic stress, not ordinarily seen in healthy myocardium, and is most likely a response by the cell to better regulate pH_i in the face of altered substrate metabolism.

Many NHE1 inhibitors have been synthesized in order to elucidate the role of NHE1 in IR cardioprotection. Cariporide is the most well-known and most-used NHE1 inhibitor (48,63,132,134,170,179,199,225); though, in recent years, many new inhibitors have been made in hopes of providing greater organ specificity and potency (114). Regardless of the specific inhibitor used, inhibition of the NHE1 has resulted in protection against IR injury (106,134). During and following IR, protection has been indicated by decreased release of creatine kinase (48,147) and lactate dehydrogenase (48,106,147,225) from the cell, improved recovery of left ventricular end-diastolic pressure (41,160), reduced infarct size (119,134,147,167), improved coronary flow (41,225), and decreased cytosolic and/or mitochondrial Ca^{2+} overload (48,106,174,199,207). Furthermore, NHE1 inhibition has attenuated caspase-3 activation, Na^{+}_{i} and Ca^{2+}_{i} accumulation, mitochondrial Ca^{2+} overload and membrane potential following an H_2O_2 insult (195). Additionally, NHE1 inhibition results in decreased H^{+} extrusion from the cytosol leading to a maintained acidic pH_i, which is associated with

protection from mitochondrial permeability transition pore (MPTP) opening. The MPTP, a pore crossing the mitochondrial membranes that releases signals to the cell that activate cell death, is inhibited as the cytosolic pH drops below 7 (87). In sum, the cytoprotective effect of NHE1 inhibitors in basic models is quite evident; unfortunately, clinical trials have failed to garner a similar reputation.

Only cariporide has been able to display beneficial effects in human patients; both zoniporide and eniporide failed to show significant differences regarding clinical outcomes (i.e. death, myocardial infarction, and arrhythmia) in non-cardiac vascular surgeries (70), thrombolytic therapies, and primary angioplasties (221). In 2000, cariporide decreased CK and LDH release and improved ejection fraction and end-systolic volume in patients with acute myocardial infarction (MI) treated with percutaneous transluminal coronary angioplasty (172). That same year, cariporide was deemed safe when given to a wide range of at-risk clinical situations; unfortunately, protection was limited to patients undergoing bypass surgery (196). Subsequent clinical trials noted protection from cariporide in patients undergoing high-risk coronary artery bypass graft (CABG) surgery. One such trial, GUARd During Ischemia Against Necrosis (GUARDIAN), infused cariporide 1 hr before surgery and every 8 hrs for 2-7 days following surgery. A 120 mg dose reduced the risk of MI on post-operative day 1 and decreased MI and all-cause mortality at day 36 (24). The first phase III trial, EXchange inhibition to Prevent coronary Events in acute cardiac conDITIONs (EXPEDITION), to use cariporide on CABG patients saw that at 5 days post-operation the incidence of death or MI significantly decreased 20.3% compared to the placebo of 16.6%. Unfortunately,

cerebrovascular events associated with cariporide caused mortality to significantly increase 2.2% compared to the placebo of 1.5%. Additionally, no significance was observed at 6 months (131). Thus, NHE1 inhibition through pharmacological inhibitors does hold therapeutic promise, but more work needs to be completed to examine whether a more specific inhibitor would be more effective or if inhibition of regulators of NHE1 are critical to protection. Nonetheless, basic models have shown that NHE1 inhibition results in a decrease in Ca^{2+}_i overload, inhibition of MPTP opening, and a decrease in apoptosis (199,225) ultimately leading to cardioprotection. Because human clinical trials using NHE1 inhibitors have not produced their anticipated outcomes, *the rationale to explore alternative approaches that provide IR injury protection through NHE1 is strengthened*.

The transgenic overexpression data suggest that activation of the proteins regulating NHE1 activity (and not the protein itself) may be more critical to producing the harmful effects mediated by NHE1, since the hyperactive NHE1 was more closely associated with the IR protection (98,140) and cardiac hypertrophy (139) in this model. Additionally, post-translational regulation may likely play a role (219). Therefore, determining the roles of specific NHE1 regulators could be the key to future clinical therapies.

The main regulators of NHE1 activity are ERK1/2.

The mitogen-activated protein kinases (MAPK) are cell signaling proteins involved in the regulation of cell proliferation, differentiation, metabolism, motility,

survival and apoptosis. The MAPKs are divided into subfamilies: extracellular signal-regulated kinases (ERK1/2), c-jun NH2-terminal kinases (JNK), p38 kinase, and big MAPK (BMK or ERK5). These proteins are activated by dual phosphorylation of a Thr-X-Tyr motif in a regulatory loop, where X is either Gly, Pro, or Glu. Activation typically occurs from a proximal cascade of proteins: MAPK kinase kinase → MAPK kinase → MAPK. The MAPK can then use ATP to phosphorylate its target protein on Ser or Thr residues. The MAPK subfamilies have a great deal of specificity based on activating mitogens and upstream and downstream modulators; however, subfamilies also can share upstream and downstream activators, which allows for potential cross-talk and feedback between them (168). Specifically, ERK1/2 is a pair of proteins with the molecular weights of 44 kD and 42 kD for ERK1 and ERK2, respectively. They are 83% identical in homology, but are not completely redundant in action. Both assist in regulation of cell cycle progression, proliferation, transcription, differentiation, senescence, cell death, and cell adhesion – just to name a few actions – through their ability to phosphorylate over 100 possible substrates in the cytoplasm or nucleus, including transcription factors (220). ERK1/2's major role in cardiovascular disease highlights why understanding the response of ERK1/2 to exercise and its putative role in exercise-induced cardioprotection can shed light on how to better manipulate the protein for improved therapies. Focus is given to ERK1/2, because it has proven to be a major regulator/activator of NHE1 activity in response to IR and ROS (137,173,185,210). In fact, p38 has no effect on NHE1 activity (185), JNK is not activated from short-term ischemia (210), and ERK5 has not been investigated.

The acidosis and ROS production that accompany IR are major mediators of IR-induced ERK1/2 and NHE1 activation. The regulation of ERK1/2 on NHE1 has been observed in whole-hearts during episodes of IR (137), and in cardiomyocytes subjected to sustained acidosis (91,92) or H_2O_2 (173,185,210). In response to IR, in-gel kinase assays displayed that proteins with the molecular weights of 90, 44 and 42 kDa phosphorylated the carboxy-terminal 178 amino acids of NHE1; these molecular weights refer to p90rsk, ERK1 and ERK2, respectively. Western blots following immunoprecipitation showed that both ERK1/2 and NHE1 were phosphorylated. MEK is the MAPK kinase of ERK1/2 and is inhibited by PD98059. PD98059 decreased the activation of ERK1/2 and NHE1, as observed by Western blots, and this corresponded to a significantly reduced rate of acid recovery and a lower steady state pH following recovery, supporting ERK1/2-induced activation of NHE1 to be necessary for greater NHE1 activity (137).

During IR, pH_i levels can decrease to 5.5-6.0 after 30 min (187), which activates NHE1 because NHE1 is less active at basal pH_i. NHE1 activity increases with increasing duration of acidosis in both neonatal and adult ventricular myocytes (92). The time course for acidosis-induced ERK1/2 activation is consistent with that of NHE1 (91,92); max activation occurs after 4 min of sustained acidosis (91). PD98059 or U-0126 (both inhibitors of MEK, the MAPK kinase of ERK1/2) abolished ERK1/2 activation by sustained acidosis, as did use of a dominant negative (DN) MEK (DN-D208A-MEK1) (91). In order to determine where on the C-terminal ERK1/2 was phosphorylating NHE1 in response to acidosis, Malo et al. developed a group of human-NHE-1 mutants in AP-1 cells that lacked an endogenous NHE1. The mutants had an alanine residue replace

different serine/threonine residues. The group with the S766A/S770A/S771A was not stimulated by acidosis. Following mutation of the individual amino acids, S770 and S771 were deemed responsible for acidosis-induced ERK-dependent NHE1 activation (42,125).

Hydrogen peroxide is a major free radical, and has been shown to activate ERK1/2 and induce NHE1 phosphorylation in a concentration- and time-dependent manner (173,210). Using a range of 1-200 uM H_2O_2 for 20 min on neonatal cardiac myocytes, Sabri et al. evaluated the dose-dependent activation of H_2O_2 on ERK2; ERK2 was the main isozyme evaluated because it displayed detectable basal phosphorylation (173). Western blot analysis revealed that phosphorylation of ERK2 peaked with 50 uM H_2O_2, which is consistent with other research showing phosphorylation peaks above 10 uM (185). Using 100 uM H_2O_2, Sabri et al. evaluated the time-dependent action of H_2O_2 on ERK1/2. Activation was significant at 5 min of exposure and peaked at 20 min, which also is consistent with the time course of another study showing that >6 min is necessary for ERK1/2 activation (185). Furthermore, H_2O_2-induced NHE1 activation was blocked by PD98059 (173) and the antioxidant N-(2-mercaptopropionyl)-glycine (MPG) (81). These observations were corroborated in adult rat ventricular myocytes (210). Because many oxygen-derived free radicals exist in the IR environment, a series of experiments were used to determine if H_2O_2 truly was the ROS responsible for ERK2 activation. Catalase, presented to the cell 10 min before H_2O_2, completely abolished ERK2 activation at all time points, suggesting H_2O_2 or ^{-}OH was responsible for the activation. Additionally, neither presentation of superoxide generating systems (xanthine and

xanthine oxidase; LY83683 used in conjunction with normal cellular NADPH metabolism) nor pretreatment with SOD was able to significantly activate the MAPK. Together this evidence suggests that H_2O_2 mediates increases in NHE1 activity through ERK1/2. *However, the exercise-induced alteration of ERK1/2 and its potential compensatory changes on NHE1 activity have not been investigated.*

Other mediators of NHE1 activity have been identified such as Akt (184), RhoA/p160ROCK (200,206), calmodulin (15,203), PKC (204), and ezrin, radixin, and moesin proteins (50). Most notably, the protein $p90^{rsk}$ has been shown to be an effector of ERK1/2 and phosphorylate NHE1 on Ser703 independent from ERK1/2 (Haworth 2003; Cuello 2007); however, data suggests that $p90^{rsk}$ is mostly dependent upon ERK1/2 activation since inhibition with PD98059 (ERK1/2 inhibitor) blocked $p90^{rsk}$ activation of NHE1 (136). Generally, research with other possible NHE1 regulators is inconclusive and not all have been studied in cardiomyocytes. Research surrounding ERK1/2 is more conclusive, has been performed extensively in cardiomyocytes, and has been better studied in conjunction with products of IR injury. Just as direct inhibition of the NHE1 has proved beneficial at attenuating the ramifications of IR injury, so has inhibition of the ERK1/2 pathway. Many studies have demonstrated that by inhibiting ERK1/2 (with PD98059 on MEK), NHE1 activity is decreased or there is a reduced ability to recover from an acid load (92,137,169,173,186,206,210). *Not only does this support the regulatory effect of ERK1/2 on NHE1 activity, but also justifies the importance of further examining how possible exercise-induced alterations in the ERK1/2 pathway may change NHE1 activity and the consequences of that change. Additionally, the fact that ERK1/2*

has been deemed a critical factor in the reperfusion injury salvage kinase protein pathway, which is activated in ischemic preconditioning and postconditioning, supports the rationale for the need to investigate ERK1/2's regulation of NHE1 as a mediator of exercise-induced cardioprotection.

As of February 1, 2012, a PubMed search of "heart, exercise, MAPK" returned 14 articles of which only one (examined below) truly detailed the effect of exercise on ERK1/2 alterations in the heart. A subsequent query of "heart, exercise, MAPK, ischemia reperfusion" returned zero articles. This demonstrates the lack of information between ERK1/2, exercise-induced cardioprotection, and pathways of IR injury. Iemitsu et al. performed a comprehensive study that observed changes in the basal phosphorylation and exercise-induced phosphorylation status of ERK1/2. Initially, rats were run acutely for 30 min at 30 m/min to obtain a pre-training assessment of ERK1/2 during and post-exercise (97). In the untrained heart, ERK1/2 activation peaked at 15 min of exercise and remained significantly elevated by 2-fold at 30 min, suggesting during exercise ERK1/2 is stimulated. Post-exercise, ERK1 remained elevated for 1 hr, suggesting ERK1/2 from sedentary rats is sensitive to the stress of exercise. The trained groups were subjected to 4, 8, and 12 weeks of running training. At 4 weeks, ERK1/2 still displayed significant phosphorylation at 30 min of exercise compared to resting values; however, at 12 weeks, ERK1/2 was no longer stimulated at 30 min of exercise. Furthermore, the trained heart displayed no difference in basal ERK1/2 phosphorylation compared to sedentary controls. *These data demonstrate that basal ERK1/2 activation is not increased following chronic exercise training and that ERK1/2's sensitivity to exercise may be blunted as a*

result of training in cardiac muscle. Potentially, the blunted response of ERK1/2 activation following endurance training observed by Iemitsu et al. could be due to lower H_2O_2 production or decreased sensitivity of ERK1/2 to H_2O_2. In contrast, a 10-week, chronic training study of C57BL/10 mice observed a significant increase in the basal phosphorylation of ERK1/2 in the exercised group; however, this study did not control for acute training effects since they sacrificed immediately after the last training bout (142), which diminishes the ability to associate any observed changes to chronic exercise training. *The lack of studies displaying exercise-induced alterations in ERK1/2 activity validates the need for further investigation.*

A critical by-product of IR is H_2O_2, a compound highly implicated in the damage associated with IR injury. In fact H_2O_2 has been shown to cause damage by activating NHE1. ERK1/2 is a necessary mediator of H_2O_2-induced activation of NHE1, because ERK1/2 can directly phosphorylate NHE1 (185). The data presented demonstrate how IR- and H_2O_2-induced activation of ERK1/2 regulates NHE1 activity; however, *the exercise-induced alteration of ERK1/2 and its potential compensatory changes on NHE1 activity have not been investigated*. In fact, both ERK1/2 and NHE1 have been neglected from the entire exercise-induced cardioprotection field. Though many mechanisms have been examined, understanding how ERK1/2 and NHE1 fit into the picture will help to fully explain the exercise-induced cardioprotection framework.

Mechanisms of exercise-induced cardioprotection.

The protective effect of exercise on the heart has been studied for decades. Sound evidence has displayed that pathological hypertrophy, impaired glucose metabolism, and IR injury (21,22) are all attenuated by exercise training. With focus given to IR injury, the mechanisms by which exercise confers this protection are not completely clear; however, some have provided more compelling evidence than others (as noted in Figure 1). Mechanisms such as antioxidants and heat shock proteins have received a great deal of attention, while other probable mechanisms such as NHE1 have been unexplored. Recent findings help show that most of the protection may be directly afforded by non-mitochondrial mechanisms; however, the mitochondrion is a central focus of the damage created by IR injury due to its role in mediating cell death. Regardless of definitive protective mechanisms, the exercise trained cardiac phenotype presents with improved hemodynamics and cell function following an ischemic insult compared to non-trained hearts. This review seeks to shed light on where the protection predominates.

Non-mitochondrial mechanisms of exercise-induced cardioprotection.

Thirty percent of the heart cell is occupied by mitochondria, which supply the vast majority of energy to the cell. When the mitochondria are not supplied with oxygen to accept the final electron, the coupling between oxidation of the electron transport chain and phosphorylation of ADP is ultimately stopped. The cell switches its energy reliance to glycolysis, which supplies much less ATP per glucose molecule, thereby lowering the energy state of the cell. Processes like inter-membrane ion homeostasis and sarcoplasmic

reticulum (SR) Ca^{2+} uptake use the ATP-dependent proteins Na-K ATPase and SERCA2a, respectively. The marked reduction of ATP causes these proteins to fail. Decreasing the activity of Na-K ATPase produces an increase in intracellular Na^+ (Na^+_i) and extracellular K^+. The increase in Na^+_i can lead to increased intracellular Ca^{2+} (Ca^{2+}_i) (as discussed below), while the increase in extracellular K^+ can lead to decreased cell excitability and arrhythmia (68). In fact, IR injury is associated with depressed Na-K ATPase activity (180). When that activity is restored using oubain preconditioning, the heart is protected from injury (157). Interestingly, the hearts of high capacity runners have enhanced Na-K ATPase activity (34). This altered activity could be due to reduced regulatory inhibition by phospholemman, a regulatory protein of the Na-K ATPase; the phosphorylation of phospholemman removes its inhibitory control on the ATPase, meaning an increased constitutive phosphorylated state could be responsible for the enhanced Na-K ATPase activity. Five days of swimming training in Wistar rats increased the phosphorylation of phospholemman (80), but this exercise effect was not seen in humans after 10 days of training (13).

SERCA2a is responsible for removing free Ca^{2+}_i from the cytosol by loading it into the SR. The lowered energy state of ischemia decreases the activity of SERCA2a leading to a rise in Ca^{2+}_i. Additionally, SERCA2a content is attenuated in diseased hearts (4). The large Ca^{2+}_i concentration is a product of the decline in activity and content, and precedes mitochondrial Ca^{2+} overload – a critical point in the fate of the cell. Again, exercise seems to create an adaptation designed for protection, since expression of SERCA2a is increased in high exercise capacity rats compared to standard exercise

capacity rats (162) and in rats that displayed protection from myocardial infarction following exercise training (4). Additionally, exercise prevented the decline in SERCA2a content during heart failure progression in dogs (123). SERCA2a is regulated by phospholamban, which removes its inhibition upon phosphorylation. SERCA2a activity is not altered in rats after 12 weeks of training (54); however, Kemi et al. observed in adult female mice that 6 weeks of high-intensity exercise increased the activity of SERCA2a, partly due to a decrease in the phospholamban-to-SERCA2a ratio (112). A decrease in this ratio could prove beneficial since ischemia induces greater inhibition of SERCA2a via dephosphorylation of phospholamban in rats (178). Deductive reasoning suggests that exercise-induced protection against IR may be mediated partly by its effect on these ATP-dependent proteins; however, these exercise-induced changes have not been studied in the ischemic heart, creating an incomplete picture of their relationship with exercise.

The decreased energy state during ischemia triggers an increase in glycolysis for greater ATP production. The increased glycolytic flux coupled with depressed mitochondrial function leads to an increase in lactic acid production via lactate dehydrogenase. The accumulation of metabolic by-products in addition to the oxygen deprivation is what separates the detriment of ischemia from that of anoxia with sufficient blood flow (58). The increased H^+ content reduces the pH of the cell (to levels between 5.5 and 6) (183) and is a stimulator of the Na^+-H^+ exchanger-1 (NHE1). This sarcolemmal protein responds to the change in pH by extruding one H^+ in exchange for one Na^+ ion, which leads to an accumulation of Na^+_i. NHE1 has been implicated as a

point in the calcium-overload cascade, since the increased Na^+_i leads to cytosolic calcium accumulation via the Na^+-Ca^{2+} exchanger (NCX). Specifically, overexpression of NHE1 is associated with a number of pathologies including diabetes, hypertension, and pathological hypertrophy (109), resulting in greater IR-induced damage and eventual cell death.

NHE1 transcript has been observed to increase following exercise training (214); however, recent evidence from our lab shows that NHE1 protein content is decreased following treadmill training in adult female Sprague-Dawley rats (67). This decrease could prove beneficial by decreasing Na^+_i accumulation during reperfusion. Additionally, a decrease in H^+ extrusion from the cytosol leads to a maintained intracellular acidic pH, and the mitochondrial permeability transition pore is inhibited as the cytosolic pH drops below 7 (87). Supporting this idea is the cell survival and preserved cardiac function following an IR insult in the presence of an NHE1 inhibitor. During and following IR, protection from inhibition has been indicated by a decrease in the release of creatine kinase (48,147) and lactate dehydrogenase (48,106,147,225) from the cell, improved recovery of left ventricular end-diastolic pressure (41,160), reduced infarct size (119,134,147,167), greater coronary flow (41,225), and decreased cytosolic and/or mitochondrial Ca^{2+} overload (48,106,174,199,207). Additionally, NHE1 inhibition has attenuated caspase-3 activation, Na^+ and Ca^{2+}_i accumulation, mitochondrial Ca^{2+} overload and membrane potential depolarization following an H_2O_2 insult (195). These noted protective effects transcend model type and duration of IR. One human study using the NHE1 inhibitor cariporide displayed a decreased incidence of death and non-fatal

myocardial infarction; unfortunately, no clinical trial has been able to substantiate the use of cariporide due to elevated cerebrovascular events associated with the drug (131). Nonetheless, basic models have shown that NHE1 inhibition leads to cardioprotection (199,225). This evidence strongly supports why a decreased expression of NHE1 could potentially provide protection through similar mechanisms. Future studies need to focus on exercise-induced alterations to NHE1 activity, since a few studies have shown that NHE1 activity may be more critical to cellular effects than NHE1 content (139).

The NCX is normally involved in cellular depolarization as it creates an electrogenic potential across the cell membrane by extruding Ca^{2+} from the cytosol; as the NCX works in the forward-mode by extruding one Ca^{2+} for three Na^{+}, the cell progressively becomes more positive. Unfortunately, during IR when Na^{+}_{i} concentrations rise due to depressed NK ATPase activity and enhanced NHE1 activity, the NCX works in reverse-mode to extrude the Na^{+}_{i}. The Ca^{2+} brought in by this protein is a major source of the Ca^{2+} overload during IR. Inhibition of NCX attenuated diastolic Ca^{2+} overload and cardiac dysfunction associated with 8 min of ischemia in rat ventricular myocytes (209). The effect of exercise on this protein is unclear, however. In 1989, Tibbits et al. showed that 11 weeks of exercise training in adult female Sprague-Dawley rats decreased the Km of NCX for Ca^{2+}, which was thought to enhance the uptake of Ca^{2+} for the contractile elements, providing a mechanism for improved contractile performance during exercise. Evidence supported that the role NCX played in Ca^{2+} entry was critical to the enhanced stroke volume accompanying exercise (198). In support of the importance of NCX on contractile performance, exercise and adenoviral gene transfer increased NCX current

(224) and protein content (188), respectively, to rescue the contractile dysfunction produced from myocardial infarction. Evidence becomes muddled in the light that two other studies demonstrated that chronic training produced a decrease in NCX current (150) and no change in either NCX current or protein content (124). A decrease in NCX reverse-mode current would decrease Ca^{2+} entry during reperfusion. If exercise produces no change in NCX current or content, then exercise-induced alterations upstream (i.e. NHE1) could prove to be a vital site of protection. Further research determining the effect of exercise-induced changes of NCX (content and activity) in relation to IR is warranted.

During non-pathological situations, a major mechanism of Ca^{2+} entry is the dihydropyridine receptor (DHPR) located on the plasma membrane. Though, during IR this receptor plays no significant mechanism in Ca^{2+} overload (209). Wei et al. independently and dually inhibited NCX and DHPR and observed that inhibition of DHPR produced no significant difference from control diastolic Ca^{2+} values, while inhibition of NCX caused a marked reduction. Furthermore, exercise produced no change in DHPR protein content (135), suggesting that DHPR is not a mediator of protection and supporting that NCX likely is a more significant mediator of IR-induced damage.

ATP-sensitive potassium (K_{ATP}) channels exist on both the cardiac sarcolemmal and mitochondrial membranes, and are responsible for matching membrane excitability with the cellular energy status. These heteromultimers have two types of subunits – inward rectifiers, KIR6.2, and sulofnuryl receptors, SUR2A; a 1:1 stoichiometry between the two subunits is sufficient for channel formation and assembly. The SUR2A subunit responds to the cellular ATP/ADP ratio by affecting the opening or closing of the KIR6.2

subunit (3). The inhibition ATP imposes on the channel is offset by the presence of ADP, supporting why the nucleotide ratio provides better control than either compound independently (62). When ATP/ADP falls during ischemia and the channel opens, the cell is hyperpolarized by shifting the membrane potential toward that of the K^+ equilibrium potential (3). Channel opening shortens the cardiac action potential, limiting Na^+ and Ca^{2+} entry into the cell, thereby reducing energy requirements for ionic homeostasis (69). In addition, reducing Ca^{2+} uptake is a well-known solution for decreasing IR-related injury (22).

The sarcolemmal ATP-sensitive potassium channel (sarcoK_{ATP}) presently is one of the mechanisms of major focus in the exercise-induced cardioprotection literature, and evidence has supported its role in providing protection. A KIR6.2 knockout proved that this channel is necessary to acquire the benefits of training, since knockout mice displayed impaired cardiac contractile function during and following the exercise protocol (107). Additionally, recent work by Zingman et al. showed that the exercise-induced increase in sarcoK_{ATP} content enhanced the rate and magnitude of action potential shortening during increasing intensities, which significantly reduced cardiocyte energy consumption (227). Chronic exercise training has been shown to increase the protein content of both KIR6.2 and SUR2A subunits following both short- and long-term training (27,29). The increased content of these subunits was highly correlated to decreased infarct size following 1 hr ischemia and 2 hr reperfusion, and this protection was abolished following pre-treatment with the sarcoK_{ATP} inhibitor HMR1098 (a cardioselective K_{ATP} channel blocker that works on both KIR6.2 and Sur2a) (27,29,37).

Furthermore, pharmacological opening of the sarcoK_{ATP} displayed cardioprotection during an IR insult (85). Opposing the protective effect of the sarcoK_{ATP} is a study by Quindry et al. (166) that showed HMR1098 did not reduce the anti-arrhythmic protection conferred by short-term exercise. Interestingly, Quindry et al. provided HMR1098 at a time point outside that which has been shown protective and in a manner inconsistent with that which was referenced (79). Both Brown (27) and Quindry (166) used short-term exercise training at comparable intensities; however, a major difference was between the IR protocols – 60/120 min to 20/30 min – suggesting a time-dependent protection provided by the channel. For example, one channel may better protect the cell from arrhythmia whereas the other better protects from death. The disparity between results and IR model support the need for further investigation.

Heat shock proteins (HSP) provide transport, compartmentalization, and proper folding stability to proteins within the cell and assist in cell signaling (145). These proteins were named due to their upregulated expression in response to temperature increases, and they are classified based on their molecular weight. For instance, HSP70 refers to the family of HSPs with a molecular weight in the 70 kDa range. HSPs range from small (i.e. aB-crystallin, HSP10, HSP25, HSP27) to large (i.e. HSP60, HSP72, HSP90, HSP110) proteins, and are dispersed throughout the nucleus, cytosol, and mitochondria; specifically, HSP75, 60, and 10 have been observed in the mitochondria. The upregulation of these proteins in response to stress and their protection against IR injury has been noted for many years (194,218).

Possible mediators of the HSP response to exercise are stretch of stretch-activated channels, ATP depletion, oxidative stress, and hyperthermia (145). Activation of stretch-activated channels increases activation of heat shock factor-1 (HSF1), the transcription factor involved in hsp transcription and necessary for upregulating HSP protein content. (33). The increased myocardial stretch associated with exercise makes this a relevant mediator. Moderate decreases in ATP during ischemia correlate with HSF1-DNA binding, suggesting a critical ATP level is needed for HSF1 activation (32). However, in the non-ischemic exercising myocardium, ATP/ADP levels do not markedly change since the heart is truly never at rest (11), thus diminishing this mechanism as a mediator for the HSP response from exercise. Oxidative stress increases HSF1-DNA binding (145). For instance, GSH depletion, a product of ischemia, produces a marked increase in the oxidized state of the cell and, independent from exercise, causes an increase in HSF1-DNA binding (151). However, the heart does not produce significant increases in ROS during exercise (201), minimizing the role of oxidative stress as a mediator to the exercise-induced HSP increase. Both heat shock and exercise increased HSF1-DNA binding in male and female adult rats were compared to control, but this study did not tease apart heat stress from the exercise stress.

Many authors have discovered that exercise produces a stress that elicits changes in multiple HSPs. Specific to mitochondria, changes to HSP60 and HSP10 are unclear. Studies have shown that exercise training causes a decrease, increase or no change in mitochondrial HSPs (8,89,128,165). The most concrete evidence for a change in a HSP favors cytosolic HSP72 – the inducible form of HSP70. Exercise increases HSP72 after

short- and long-term training at moderate-to-high intensity (89,90,192) and long-term, moderate intensity (128); the threshold intensity for HSP70 upregulation in the adult rat heart appears to be at or above 24 m/min (133). This increase in HSP72 is a result of the increased core temperature associated with exercise but is not required for exercise-induced protection. Our lab was the first to show that when rats' core temperatures were not allowed to increase by exercising them with wet fur in a cold room, HSP72 was not elevated, yet improved recovery following IR was still observed (192,193). Cold running produced no upregulation of HSP72, yet pump function following IR was still significantly enhanced compared to controls (90,192). This finding was confirmed by studies from Powers' lab (89). The fact that heat shock produced similar protection and an increase in HSP72, supports that exercise mediates protection through other cardioprotective factors and is not dependent upon HSP72 (192). HSP72 is not discounted as a protective adaptation resulting from endurance training due to its many beneficial effects in the cell and integration with other mechanisms (55), but the present findings gives support to other adaptations within the heart being key to its protection by exercise.

Proteases are enzymes involved in cleavage and degradation of proteins. Calpains are Ca^{2+} activated proteases implicated in the degradation of cytosolic Ca^{2+} regulating proteins (78) and mitochondrial proteins necessary for cell function (36). IR significantly increases calpain activity and impairs cardiac work in sedentary control rats, and exercise training attenuates this response in a manner comparable to calpain inhibition. The work by French et al. (77,78) indicates that this is not due to an exercise-induced decrease in

calpain or an increase in calpastatin, an endogenous inhibitor of calpain (78). Instead, they concluded that the attenuated increase in calpain activity following IR in the exercise trained heart was mediated indirectly through increased MnSOD, suggesting oxidative stress may enhance calpain activation. In support of this, the antioxidant trolox abolished the activation of calpain (211). Furthermore, taurine administration inhibited NADPH oxidase-induced calpain activation (122). Although an elevation of MnSOD could attenuate calpain-assisted Ca^{2+} overload by decreasing calpain activation, there are other adaptations that directly decrease Ca^{2+} overload and several studies have reported that MnSOD was not elevated in the heart by exercise training.

The iNOS-COX-2 combination has proved to be a major mechanism by which ischemic preconditioning protects the heart (18,191). iNOS is upstream of COX-2 and produces nitric oxide (NO), which can protect the myocardium possibly by inhibiting Ca^{2+} influx, decreasing myocardial oxygen demands, and opening the mitochondrial K_{ATP} channel. NO also activates COX-2, which provides protection via its prostanoid products, PGI_2 and PGE_2. The combined effect of iNOS and COX-2 results in ischemic preconditioning of the heart (51). Although chronic exercise training increases COX-2 mRNA levels (128), exercise does not increase COX-2 protein content or activity in young or aged hearts (164). Additionally, iNOS does not appear to play a significant role in exercise-induced preconditioning against IR injury in rats since this enzyme is not elevated in the heart by chronic exercise programs (47,95), and inhibition of iNOS by L-NAME did not alter the improved recovery following IR (193).

Though research is not completely conclusive, exercise produces alterations to non-mitochondrial mechanisms that appear to create an environment more resistant to the stress of IR. The increased NK-ATPase activity, increased SERCA2a expression, decreased NHE1 content, neutral in NCX, increased sarcoK_{ATP}, and increased HSPs comprise a list of exercise-induced adaptations theoretically designed to protect the cell from the very detrimental IR-induced Ca^{2+} overload and protein misfolding and aggregation. Not all of these mechanisms have been examined against IR, but some have been observed to provide protection against IR injury.

Mitochondrial mechanisms of exercise-induced cardioprotection.

Mitochondria produce the greatest source of reactive oxygen species in the body (121). Estimates of 2-5% of the oxygen used during normal mitochondrial respiration have been reported to contribute to the formation of ROS, such as $O2^-$ and H_2O_2 (20). Although 2-5% may be an overestimation, the production of free radicals during ATP production cannot be argued (25). In skeletal muscle, an increase in oxygen consumption due to exercise causes an increase in ROS production (10), which has been indirectly supported by increased lipid peroxidation (52) and exhaled pentane levels during exercise (59). In the heart, however, Traverse et al. collected blood samples from the region of heart supplied by the left anterior descending artery and used electron paramagnetic resonance to show ROS production was not increased during exercise compared to rest (201). This suggests that the basal level of release of ROS is lower in the heart compared

to the skeletal muscle and/or the heart is more effective than skeletal muscle at controlling ROS accumulation during exercise using endogenous antioxidants.

Evidence exists for exercise-induced changes in the electron transport chain that result in less mitochondrial ROS production (103,189). Male 24-month old Fischer 344 rats displayed decreased H_2O_2 production in both the IMF and SS mitochondrial subfractions following lifelong voluntary wheel running, though this change could have been age related since no young rats were included (103). Marcil et al. used young female Sprague-Dawley rats but observed no change in ROS production in trained mitochondria compared to their sedentary counterparts, suggesting this specific adaptation may be strain, gender, and/or age dependent (127). Our lab used young male Fischer 344 rats to determine if exercise trained cardiac mitochondria produced lower H_2O_2 levels in response to a Ca^{2+} load compared to sedentary mitochondria (189). The trained isolated mitochondria, energized by succinate at Complex II, produced less H_2O_2 in the presence and absence of a Ca^{2+} load compared to sedentary mitochondria. Interestingly, the difference in H_2O_2 production between the trained and sedentary was abolished upon the addition of the Complex I inhibitor rotenone, suggesting that an adaptation at Complex I is responsible for the decreased H_2O_2 production. Additionally, this evidence supports that this adaptation may be gender and/or strain dependent. During reperfusion, an overload of mitochondrial Ca^{2+} can inhibit Complex I activity, which may possibly negate this adaptation from the cardioprotective equation. Understanding whether the mitochondrion adapts by reducing free radical generation at other complexes, especially

those downstream of the ubisemiquinone radical, would add support to whether the exercise phenotype is truly a result of the mitochondrion.

Evidence also exists that, compared to skeletal muscle, the heart has a more effective antioxidant system. Mitochondrial antioxidants MnSOD and glutathione peroxidase (GPx) play a role in protecting the mitochondria and the cell from oxidative stress, but their role in exercise-induced cardioprotection remains unclear. MnSOD is a predominant antioxidant in the mitochondria. An increase in MnSOD observed with exercise training is associated with reduced infarct size, percent of apoptotic cells, and oxidation of Ca^{2+} handling proteins following an ischemic insult (77). However, even with no change in any major antioxidant enzyme after exercise, protection following IR is still observed in the rat heart (29,191,193). The studies by Taylor et al. (193) and French et al. (77) both ran young adult male rats at an intensity ~70% VO_{2max} and used similar IR times; however, Taylor et al. ran Fischer 344 rats for 2 days while French et al. ran Sprague-Dawley rats for 5 days, suggesting MnSOD upregulation is strain dependent and/or time sensitive. Interestingly, Brown et al. (29) ran young adult male Sprague-Dawley rats for 5 days at ~70% VO_{2max} – the same as French et al. (77) – and saw an exercise-mediated decrease in infarct size without a change in MnSOD protein content. Unfortunately, the comparison is not completely conclusive since enzyme activity and protein content were being separately evaluated.

MnSOD is upregulated in response to oxidative stress or an increase in ROS (61). If ROS are not appreciably increased during exercise, then what is the stimulus for an increase in MnSOD upregulation? Studies where rats were run in the cold show no

evidence of changes in MnSOD, suggesting that temperature may be causal. The studies discussed above used short-term training, but even long-term training has not consistently upregulated MnSOD; exercise protocols longer than 9 weeks produce no observable changes to the enzyme (90,120,138). Most chronic training protocols use a constant intensity that becomes relatively easier over time, attenuating the stimulus over the training period and possibly diminishing the stimulus for MnSOD upregulation. A 10-week treadmill protocol that maintained relative intensity at 75% VO_{2max} displayed a continual increase in MnSOD (158). However, the same exercise protocol produced no change in MnSOD when performed by Demirel and colleagues (56). An inconsistency between the sensitivity of the measures used to detect MnSOD changes could be a reason for this conflict. A discrepancy of MnSOD changes exists at all possible levels of exercise stimuli, weakening the argument for this enzyme as a critical axis of exercise-induced cardioprotection. French et al. (77) even conceded that MnSOD alone could not be the only mediator of protection since loss of MnSOD via antisense oligonucleotides did not completely abolish the exercise-induced protection. This argument opens the door for other antioxidants.

Glutathione peroxidase converts H_2O_2 to H_2O using reduced glutathione (GSH). Depletion of GSH levels is highly implicated in the contractile dysfunction and arrhythmogenesis associated with IR. Maintaining GSH levels and GPx activity during IR can attenuate damage. Transgenic overexpression of GPx in the mitochondria of mice improved developed pressure and peak systolic pressure following 20 min of global no-flow ischemia (49). The glutathione oxidant diamide decreases the GSH-to-GSSG ratio

by both increasing GSSG (oxidized glutathione) and decreasing GSH levels. Application of diamide to isolated cardiocytes induced ventricular tachycardia and ventricular fibrillation (26). Moreover, diamide experiments displayed that even a 30% reduction in GSH-to-GSSG can trigger mitochondrial membrane potential oscillation (182). These data elegantly display the critical nature of maintaining antioxidant reducing potential. Unfortunately, exercise research markedly shows that GPx levels are not altered due to exercise training from short-term to lifelong wheel running (22,56,57,90,103,164,189). One study saw an increase in GSH levels between an exercise group and sham group, but the difference was negated after a bout of IR significantly reduced glutathione levels in both groups (57). In contrast, another study observed no difference in baseline GSH-to-GSSG levels between exercised and sedentary rats, but these levels were better preserved in exercise groups receiving both diamide and IR (76). The majority of evidence further attenuates the support for a mitochondrial antioxidant-mediated role for exercise-induced protection.

The fact that exercise training reduces the oxidative stress presented to the cell in normal situations and during IR is well established. What is not currently known is how the cell, specifically the mitochondria, reduces this stress. The study by our lab (189), displaying a decrease in mitochondrial H_2O_2 production, measured an exercise-induced increase in catalase activity, an antioxidant of H_2O_2. Had antioxidants played a role in the decline of H_2O_2 production, then a decrease would have been apparent without the Complex I inhibitor rotenone, thus providing evidence that the exercise-induced decrease in H_2O_2 is not purely a result of antioxidants. Judge et al. (103) observed that lifelong

wheel running reduced mitochondrial H_2O_2 production and that MnSOD decreased in runners, which reflects and further supports that mitochondria reduce free radical generation. Finally, a decrease in lipid peroxidation following IR was observed in female Sprague-Dawley rats after 10 weeks of running, and this was accompanied by no change in either GPx or MnSOD activities. The compiled evidence supports that a decrease in ROS production by the exercised mitochondria may play a greater role in reducing the oxidative stress presented to the cell rather than an increase in mitochondrial antioxidants.

ROS production can loop back to cytosolic Ca^{2+} pools, thereby affecting intracellular Ca^{2+} levels. For example, RyR thiol groups can be oxidized resulting in altered Ca^{2+} release and increased cytosolic Ca^{2+} concentrations (12,66,116). Cytosolic Ca^{2+} enters the mitochondria via the Ca^{2+} uniporter (mCU) by the electrochemical gradient of ~-180mV across the inner mitochondrial membrane (175). As the positively charged Ca^{2+} enters the matrix, the polarity between the cytosol and mitochondria is dissipated, which then decreases Ca^{2+} influx. In a physiological setting where mitochondria partly buffer cytosolic Ca^{2+} transients, this depolarization is offset by normal respiration and transfer of H^+ to the intermembrane space (146). During ischemia, however, decreased oxygen levels reduce electron flow and H+ transfer, allowing unopposed influx of Ca^{2+} into the mitochondrial matrix. Upon reperfusion and subsequent electron flow, the potential is re-established allowing for greater increases in mitochondrial Ca^{2+} (202). This mitochondrial Ca^{2+} overload can lead to MPTP opening (155).

A mitochondrion more tolerant to these increases in Ca^{2+} would better protect the cell from MPTP opening. The Ca^{2+} uniporter is the major mechanism by which Ca^{2+} enters the matrix during normal oscillations (146). Ischemic preconditioning results in decreased mitochondrial Ca^{2+} content following IR by attenuating Ca^{2+} influx through the Ca^{2+} uniporter (223). However, little research has observed the effect of exercise on this channel. Only indirect evidence is available from Marcil et al. (127), who saw that trained mitochondria did not have different Ca^{2+} uptake kinetics compared to their sedentary counterparts, suggesting that mitochondria are not protected from Ca^{2+} influx via this mechanism. In fact, mitochondria may not be better protected or more resilient to given Ca^{2+} loads as a result of exercise training. Our lab (189) demonstrated that MPTP opening (measured by decreases in light scattering) was similar between trained and sedentary mitochondria in response to a given Ca^{2+} load. Furthermore, the Ca^{2+} retention capacity of mitochondria from exercised animals was significantly lower than that of sedentary animals following 10 days of exercise training (75). Conversely, Marcil et al. (127) showed that trained mitochondria displayed an increased tolerance to Ca^{2+}-induced MPTP opening using both light scattering and Calcium Green 5N. Whether or not mitochondria are more tolerant to Ca^{2+}-induced MPTP opening is still somewhat under question. Based on the studies mentioned, a gender difference could be speculated as cause for mitochondrial Ca^{2+} tolerance. However, would the mitochondria need to be more tolerant to Ca^{2+} if the cell took-up less cytosolic Ca^{2+} during an IR insult? The evidence so far suggests that mitochondria may indirectly protect themselves from extra-

mitochondrial stresses by producing fewer free radicals, which would assist in reducing $Ca^{2+}{}_i$ levels.

The mitochondrial K_{ATP} (mitoK_{ATP}) has been speculated to provide protection to the mitochondria against Ca^{2+} overload during IR. Opening of the mitoK_{ATP} provides protection by partial depolarization of the mitochondrial membrane potential; this results in a decrease in mitochondrial Ca^{2+} influx and attenuates Ca^{2+} overload. Whether mitoK_{ATP} opening mediates exercise-induced cardioprotection is still of debate. Quindry et al. (164) inhibited the mitoK_{ATP} before 20 min of ischemia and observed that the protection against arrhythmia was abolished. Brown et al. (27), however, inhibited the mitoK_{ATP} before 60 min of ischemia but did not see an abolition of exercise's protection against infarct sparing. Again, there may be a IR time threshold for mitoK_{ATP} protection as discussed with sarcoK_{ATP}. This picture becomes more muddled since dogs that exercised immediately or 24-hours before coronary occlusion exhibited a significant decrease in infarct size following 60 min of ischemia, and mitoKATP inhibition abolished this protection (60,152). Additionally, the requirement and ability of mitoK_{ATP} to protect the heart from aortic constriction and H_2O_2 stimulation has been displayed (141,217), which lends support to the significant role of both K_{ATP} channels and the need for further research.

To date, the evidence remains unclear as to whether mitochondria are directly morphologically altered to protect themselves from Ca^{2+}-induced MPTP opening or are indirectly protected via decreased ROS production. Ciminelli et al. (38) used the 2-deoxy [^{3}H]glucose ([^{3}H]DOG) entrapment method to determine if exercise training protected

the mitochondria from MPTP opening. These authors observed that short-term exercise (5 days at ~75%$VO2_{max}$) significantly decreased pore opening in response to 30 min of ischemia and 40 min of reperfusion. However, this study failed to uncover any mechanisms of protection (38). Possible mechanisms include those already mentioned (i.e. ROS, AOX, and mitochondrial membrane channels); however, extramitochondrial proteins involved in regulation of MPTP opening or downstream effectors of the death signal present interesting options as well.

Bcl-2 and Bax proteins assist in controlling pore opening and regulating mitochondrial dysfunction. Activation of the anti-apoptotic factor Bcl-2 has been shown to increase the Ca^{2+} threshold necessary for MPTP opening (226), and to inhibit ATP hydrolysis by F1-F0 ATPase during ischemia (99). On the other hand, activation of the pro-apoptotic factor Bax causes its translocation from the cytosol to the mitochondria, where it inserts into the mitochondrial membranes and forms a protein-permeable pore (117). Bcl-2 prevents pore permeabilization by inhibiting Bax (86). Thus, decreases in the pro-apoptotic and increases in the anti-apoptotic proteins of the cell attenuate mitochondrial dysfunction and cell death. Evidence exists that exercise increases the Bcl-2-to-Bax ratio (53,156,181); a decrease in this ratio is highly correlated to an increase in the apoptotic threshold (53). Moderately intense treadmill exercise of at least 8 weeks decreased the Bcl-2-to-Bax ratio by either increasing Bcl-2 levels or decreasing Bax content (53,156,181). The only group not to see a difference in the ratio was Kwak et al. (118) but they used Fischer 344 rats, which was a different strain than the other studies that saw a significant change and suggests a possible strain difference. The Bcl-2 and

Ca^{2+} threshold relationship observed by Zhu et al. (226) could allow one to speculate that the exercise-induced increase in the Bcl-2-to-Bax ratio is a potential mediator of protection from mitochondrial Ca^{2+} overload; however, experiments discerning this relationship have not been performed.

Following MPTP opening, there is a release of pro-apoptotic factors from inside the matrix, which initializes a deleterious cascade. The most well-known scenario is the release of cytochrome c and the eventual activation of caspase-3. Exercise does not seem to alter the factors AIF, caspase-3 or caspase-9 following at least 8 weeks of moderately-intense treadmill exercise (53,156,181); however, this lack of effect does not seem critical since inhibition of the caspases does not prevent cell death. Cell death is only delayed because upstream mitochondrial membrane potential loss is not inhibited (23). This evidence further emphasizes the importance of the pore regulators, the Bcl-2 family of proteins, which are implicated in alterations of the mitochondrial membrane potential. It also further emphasizes the importance of mechanisms involved in preventing Ca^{2+}_{i} overload. Additionally, studies observing whether trained isolated mitochondria have an increased Ca^{2+}-induced pore opening threshold would serve well from determining altered levels Bak, a pro-apoptotic protein that is already present in the mitochondria as a membrane protein and does not have to be translocated from the cytosol like Bax (117). To our knowledge, exercise studies have only observed changes to Bcl-2 and Bax; further research should include other Bcl-2 family proteins.

Regardless of whether a change in the apoptotic threshold exists as a result of training, exercise still affords protection to the cardiocyte following an ischemic insult.

This would suggest that exercise may be affecting the activity of proteases involved in the apoptotic cascade. The protection has been observed in the form of decreased MPTP opening (38), cytochrome c release (111), caspase-3 activity (77,163,222), CK and LDH release (222), percent apoptotic cells (77,163,165,222), and infarct size (77,165,222). The protection observed biochemically is translated functionally as improved systolic (222) and diastolic function (22,28,222), cardiac output (21,22), coronary flow (22,28), and cardiac work (22) following an ischemic insult. Zhang et al. (222) swam adult male Sprague-Dawley rats for 8 weeks to induce cardiac hypertrophy. After hearts were subjected to 30 min of ischemia, trained hearts demonstrated reduced caspase-3 activity, and a decrease in percent of apoptotic cells and infarct size. Trained hearts also developed improved systolic and diastolic function post-reperfusion. To delineate a potential cellular mechanism involved in this cardioprotection, the PI3K inhibitor wortmannin was added to the hearts 15 min pre-reperfusion; the addition of wortmannin abolished protection, suggesting that protection is partly afforded by PI3K-Akt mechanism. More work is needed to uncover the signaling pathways responsible for the anti-apoptotic protection that exercise confers. In fact, none of the MAPKs have been examined in relation to exercise and IR injury. Examining the signaling pathways that regulate some of the cardioprotective proteins will unravel new questions about how exercise confers protection to the heart.

Protecting mitochondria from damage is critical to protecting the cell from damage and death. Some of the mitochondrial mechanisms contributing to the exercise-induced cardioprotective phenotype include increased antioxidants, decreased ROS

formation, increased mitoK_{ATP} content, and increased Bcl-2-to-Bax ratio. An increase in antioxidants may possibly be a key component of this exercise phenotype, but MnSOD data has not been consistent and the majority of studies report glutathione does not change in response to training. Evidence surrounding mitoK_{ATP} has not been conclusive; the use of a more consistent model may help. Data suggests that the increased Bcl-2-to-Bax ratio observed in the trained animal could be a contributor to protection, but more work in relation to IR injury is needed. We believe the main adaptation contributing to mitochondria's self-protection is the reduction in ROS production, specifically through alterations made to complex I. The decreased ROS alleviates one contribution to Ca^{2+} overload, since oxidation of key Ca^{2+}-regulating proteins is reduced. Because exercised mitochondria are not more tolerant to Ca^{2+} loads, we believe not only that an adaptation that reduces ROS production may be the most relevant, but also that the non-mitochondrial exercise-induced adaptations that reduce intracellular Ca^{2+} overload predominate in the heart cell's ability to protect itself from IR.

The relationship between exercise, NHE1, and IR injury.

Skeletal muscle has been shown to increase its NHE1 content in response to exercise in humans (96,105) and rats (104). Eight weeks of high-intensity knee-extensor exercise and 4 weeks of sprint training displayed increases of 116 (105) and 30% (96) in NHE1, respectively. Cells respond to a chronic acid load by increasing NHE1 mRNA (205). The high-intensity training results in large amounts of H^+ production secondary to lactic acid formation, thereby stimulating NHE1 production. This result is expected from

fiber types more glycolytic than type 1; although, type 1 fibers showed increased NHE1 expression as well. Skeletal muscle blood flow lags in response to the need of oxygen requirement at the start of high-intensity exercise, as metabolites and H^+ leak into the blood to cause dilation of terminal arterioles. This lag time transiently forces even oxidative fibers to produce H^+ by way of anaerobic glycolysis, thereby plausibly stimulating an increase in NHE1. On the other hand, the heart's response to an increase in stimulation is flow-mediated, meaning oxygen is delivered to heart cells through the coronary circulation in a parallel fashion to that of cardiac output. Cardiac myocytes, therefore, do not produce an intracellular environment as acidic as skeletal muscle in response to increased stimulation. This concept supports the fact that the cardiac NHE1 does not have to be upregulated in response to exercise in cardiocytes.

The only study to examine the effects of exercise on cardiac NHE1 observed increases in NHE1 mRNA levels. The training, which was intense enough to induce physiological hypertrophy, increased mRNA 130% acutely and 20% at 2 weeks; weeks 4 and 13 saw trends of upregulation but they were not significant (214), suggesting NHE1 message may decline back to sedentary levels with more chronic training. Nonetheless, these mRNA changes may not have translated into functioning protein due to post-transcriptional modification. This study did not address the relationship of these alterations with IR.

One of the most detrimental factors of IR is the production of Ca^{2+}_i overload. Literature has consistently displayed that NHE1 inhibition is protective to the cell, mainly due to a reduction in Ca^{2+}_i overload. Conversely, diseased hearts typically present with

increased NHE1 content and activity and elevated Ca^{2+}_i concentrations. We have shown that exercise training decreases NHE1 content by 38%; however, still unknown is whether this change confers protection from IR injury, and whether exercise causes a change in NHE1 activity. The fact that NHE1 activity may be more important to the damage presented to the cell supports why investigating the effect of exercise on NHE1 activity is necessary (140). Additionally, because non-mitochondrial adaptations from exercise seem to be most critical to protection, understanding how exercise affects the response of NHE1 to IR will help to clarify and strengthen the framework of the exercise-induced cardioprotection picture. We believe that NHE1 could be a critical axis of the exercise-induced cardioprotective phenotype.

Figure 2.1. Exercise-induced cardioprotective mechanisms.

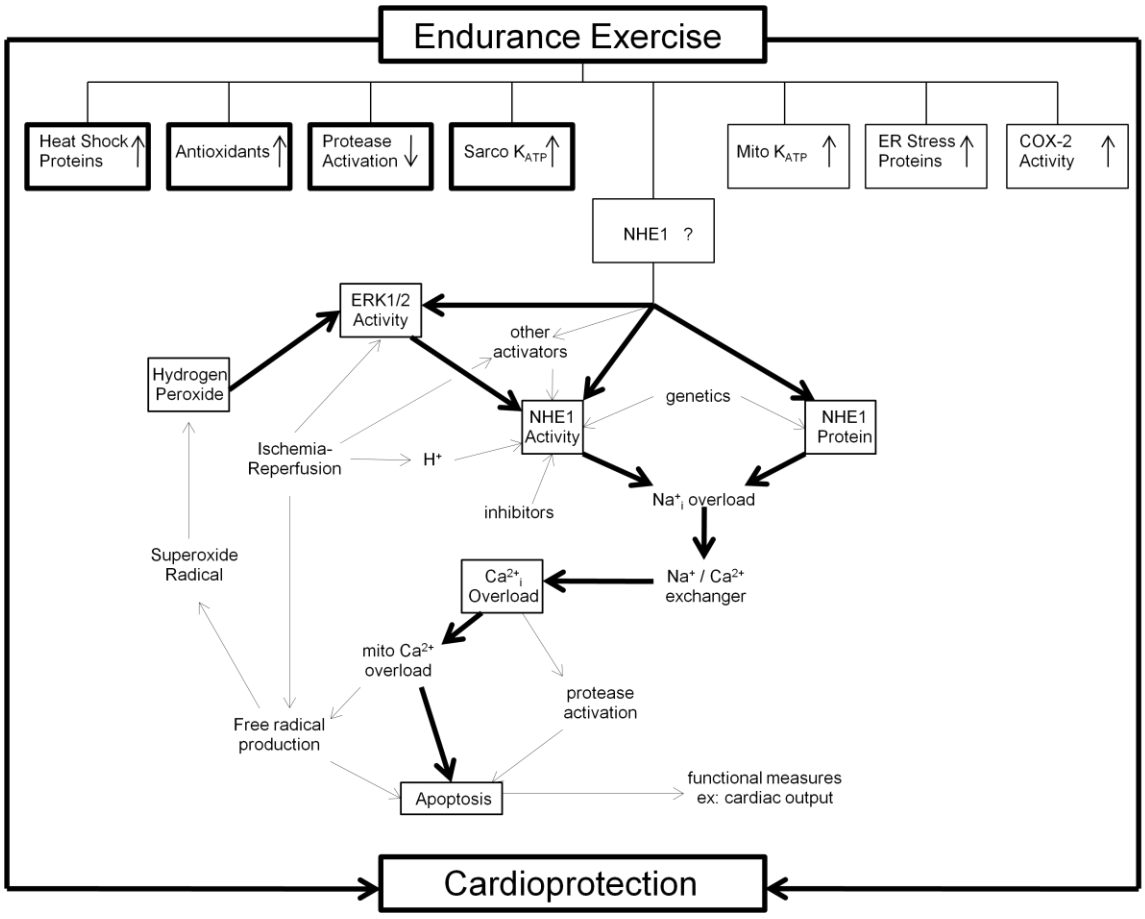

Figure 2.1. Exercise-induced cardioprotective mechanisms. The supported (dark boxes) and unsupported (normal boxes) mechanisms of exercise-induced cardioprotection. The unexplored NHE1 mechanism is unfolded to display its possible pathways. The bold arrows represent the pathways considered in this project, while the boxes represent the features of those pathways under review.

CHAPTER III

MYOCARDIAL NA^+/H^+ EXCHANGER-1 (NHE1) CONTENT IS DECREASED BY EXERCISE TRAINING

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Running Head:

NHE1 content following exercise training

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Abstract.

The effect of exercise training on myocardial NHE1 protein expression was examined. Adult female Sprague-Dawley rats were randomly divided into sedentary (S, n=8) and exercised (E, n=8) groups. Twenty-four hours after the last exercise bout, hearts were weighed and connected to an isolated perfused working heart apparatus for evaluation of cardiac functional performance. Heart weight and heart weight/body weight from E rats was significantly increased by 7.1 and 7.2% ($P < 0.05$), respectively, compared to S hearts. The E hearts displayed 15% greater cardiac output and 35% external cardiac work compared to the S group at both low and high workloads ($P < 0.05$ for both parameters). Ventricular tissue from the same hearts was homogenized and NHE1 and Na^+/Ca^{2+} exchanger (NCX) content determined by Western blotting. E hearts had a 38% ($P < 0.001$) reduction in NHE1 content related to S hearts; however, there was no difference in NCX content between groups. Cytochrome *c* oxidase activity in plantaris from E group increased by 100% ($P < 0.05$), and was assessed as a marker of mitochondria content and to verify training status. Our data indicate that exercise training at an intensity that results in cardiac hypertrophy and improved performance is accompanied by decreased NHE1 content in heart.

Key words: Sodium calcium exchanger; Isolated perfused heart; Cardioprotection; Anti-remodeling; Physiological cardiac hypertrophy

Introduction.

The Na^+/H^+ exchanger-1 (NHE1) is a cardiac and skeletal muscle sarcolemmal protein that plays a key role in the regulation of cell pH by extruding one intracellular H^+ in exchange for one extracellular Na^+. Upregulation of NHE1 has been extensively studied as a central component to the generation of cardiac hypertrophy (for review see (39)). Cardiac growth or hypertrophy can be generally classified as either pathological or physiological. Pathological hypertrophy includes growth in a diseased state (i.e. hypertension and aortic stenosis) and is associated with cell death, reduced cardiac function, and heart failure (43). On the other hand, physiological hypertrophy includes growth of the heart during postnatal development and in response to chronic exercise training and is associated with normal or improved cardiac function (65). In the pathological setting, NHE1 activity is increased by increased expression and phosphorylation initiated by such stimulants as Angiotensin-II, aldosterone, Endothelin-1, and reactive oxygen species (ROS) (39). NHE1 activation subsequently results in Na^+-dependent Ca^{2+} overload via the sarcolemmal Na^+/Ca^{2+} exchanger (NCX) (92). The increased intracellular Ca^{2+} is thought to then initiate hypertrophy through activation of the Ca^{2+}/calmodulin-dependent protein kinase II-histone deacetylase pathway (143) or calcineurin/nuclear factor of activated T cell transcription factors (212). Increased NHE1 also makes the heart more vulnerable to a variety of stresses including ischemia-reperfusion (IR) injury. The decrease in cytosolic pH that occurs during an ischemic event increases NHE1 activity leading to intracellular Na^+ accumulation. In response, NCX works in reverse mode causing Ca^{2+} overload and ensuing contractile dysfunction

and cell death. Several studies have shown that NHE1 genetic elimination or inhibition of NHE1 activity is protective to the myocardium during IR injury (6,98,101,102,208). Overall, an increase in NHE1 in the heart can be considered to be maladaptive.

Interestingly, the interaction of NHE1 and exercise-induced physiological hypertrophy has not been explored. In skeletal muscle this protein aids in attenuating exercise-induced cellular acidosis, a contributing factor to muscular fatigue [22]. Moderate to high-intensity exercise protocols are reported to increase NHE1 level in skeletal muscles (96,104,105) thereby enhancing their ability to tolerate an acid load. The impact of exercise training on myocardial NHE1 content is uncertain. Intense exercise has been reported to acutely increase myocardial NHE1 mRNA expression in rats, but the mRNA returns toward sedentary control levels within four weeks as the animals adapt to the exercise protocol (214). However, mRNA content does not always correspond to expression of its protein and myocardial NHE1 protein content has not been measured in response to exercise training.

Although NHE1 increases in skeletal muscle with exercise and in heart with pathological hypertrophy, we hypothesize that it will not increase in the exercise-induced hypertrophied heart because the heart is not expected to experience an acid load during exercise and because exercise training is reported to decrease ROS production (103,159,189). Many investigators, including ourselves, have demonstrated that exercise participation provides intrinsic cardioprotection against a variety of pathological challenges (30,90,159). Although the cardioprotective mechanisms are not completely understood, it seems doubtful that exercise-induced adaptations would include those that

are maladaptive such as an elevation of NHE1. If NHE1 does increase in the exercise trained heart, then other protective adaptations must occur to counter the potential harmful impact of overexpressed NHE1. To test our hypothesis NHE1 protein expression was determined in rat hearts after completing an exercise program previously established to produced physiological cardiac hypertrophy (90,144).

Methods.

Animals and training. Female, 5-6 month old, Sprague-Dawley rats were obtained from the breeding colony maintained by the University of Texas Animal Resource Center. Animals were kept on a 12:12-h light-dark cycle and fed ad libitum. This investigation was approved by the University's Institutional Animal Care and Use Committee and conforms to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996). This population has at least four advantages relevant to this study: *1)* they have reached a stable weight, thus exercise capacity will not change due to age; *2)* they have much more "enthusiasm" for running on the treadmill than do adult male rats, thus assuring that more animals will complete the exercise training protocol without physical or psychological injury; *3)* they are adults, which is consistent with most people presenting with coronary artery disease, and; *4)* they have been used successfully for this training protocol previously (192). Rats were randomly assigned to one of two groups: sedentary (S) or exercised on a treadmill for 5 days/wk up a 6° incline for 5 wks (E). The exercise training protocol was modified from a previously used protocol that produced significant cardiac hypertrophy with enhanced function and a gene expression profile consistent with

physiological hypertrophy (144). Briefly, rats were habituated to treadmill running for 1 week. During this week animals ran on a 6° grade gradually increasing the time and speed so that at the end of the week they were running for 30 minutes at 25 m/min. Following habituation animals began running in the cold (8˚C) at 25 m/min with a fan blowing on them to minimize the normal exercise-induced increase in core temperature (144). The time was gradually prolonged so that the animals were running for 1 hour per day by the end of the first week. Speed was gradually increased until reaching 30m/min and then maintained at that speed for the final week of exercise. All exercised animals were housed at 23˚C when not running.

Isolated heart perfusions. Myocardial performance was evaluated 24 hours after the last treatment using an isolated, working heart preparation as previously described (144). Briefly, rats were anesthetized with an intraperitoneal injection of 40 mg/kg pentobarbital sodium and hearts were excised, weighed, and mounted on the perfusion apparatus. The perfusate was a modified Krebs-Henseleit buffer containing (in mM): 10 glucose, 3.0 $CaCl_2$, 118.5 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 24.7 $NaHCO_3$, 0.5 EDTA, and 12 IU/l insulin. It was gassed with 95% O_2-5% CO_2 and maintained at 37°C. Myocardial function was evaluated while working against a low afterload set by an 80 cm high aortic column (internal diameter 3.18 mm) and against a high afterload created by inserting a 1.5 inch 23-gauge needle into the aortic column. Atrial filling pressure was maintained at 12.5 mmHg throughout. Hearts were electrically paced at 300 beats/min with an electrode placed at or near the SA node except for four hearts in S and three hearts in E because their intrinsic rate was at or above the target rate. This procedure maintained heart rates

within a tight range and avoided problems associated with trying to forcefully slow a heart beating at slightly faster than 300 beats/min. The resulting heart rates for the groups were (in beats/min): 313.5 ± 7.1 for S and 308.3 ± 4.5 for E during the low afterload, and 315.5 ± 6.5 for S and 306.9 ± 3.4 for E during the high afterload. Coronary flow (CF) and aortic flow (AF) were determined by weighing timed collection of the effluent from the pulmonary artery and aortic column overflow, respectively. Cardiac output (CO) was determined as the sum of CF and AF, and cardiac external work (COxSP) was defined as the product of CO and peak systolic pressure (SP). Oxygen content in the coronary effluent was continuously monitored with an in-line oxygen electrode for the determination of myocardial oxygen consumption (VO_2). Myocardial efficiency (EFF) was calculated by dividing external work by VO_2. Hearts were allowed to function at the low workload for approximately 25 minutes to assure stability and then switched to the high workload challenge for an additional 5 minutes. The beating hearts were then freeze-clamped and stored at -80°C until analysis.

Western Immunoblotting: Myocardial left ventricular samples were homogenized in 50 mM KH_2PO_4, 0.1 mM EDTA, pH 7.4 with a Protease Inhibitor Cocktail (Sigma # P8340) using a Teflon-glass Potter-Elvehjem B homogenizer. Samples were centrifuged at 10,000 x*g* for 15 min, supernatants were collected as membranous fractions, and protein concentrations of the homogenates were determined by the Biuret method described by Cooper (45) with BSA as a standard in order to establish sample loading volume. All samples were then mixed with Laemmli sample buffer, boiled for 5 min, and 100 ug of total protein were subjected to SDS-PAGE electrophoresis on a 10% resolving

gel until the desired molecular weight bands were separated. Thereafter, proteins were transferred onto a nitrocellulose (NHE1) or a PVDF (NCX) membrane and incubated at room temperature in 5% evaporated milk. The membranes were immunoblotted overnight at 4 °C with the following primary antibodies: mouse anti-NHE-1 (BD Biosciences, #611774), mouse anti-Actin Ab-5 (BD Biosciences, #612656), rabbit anti-NCX1 (Millipore, #AB3516P), and rabbit anti-Actin (CellSignal, #4967). Membranes were then incubated at room temperature with the following secondary antibodies: HRP-linked goat anti-mouse Ig (BD Biosciences, #554002) for 2 hr or HRP-linked anti-rabbit IgG (CellSignal, #7074) for 1 hr. After detection of NHE1 and NCX, membranes were stripped and reprobed for Actin. Proteins were detected with SuperSignal West Pico chemiluminescent substrate in a BioRad Chemidoc Station and band density was assessed using QuantityOne software.

Cytochrome c oxidase. Left ventricular tissue and whole plantaris muscle were homogenized in 20 volumes of 50 mM KH_2PO_4, 0.1 mM EDTA, 0.1% (v/v) Triton X-100, pH 7.4, centrifuged at 1,500xg for 10 minutes, and the supernatant analyzed. Cytochrome *c* oxidase activity, a marker of mitochondria content, was determined polarographically using a Clark-type oxygen electrode as described previously (171). All tissue preparation steps were carried out at ice-cold temperatures and the enzyme assay run at 25^{o}C.

Statistics. An unpaired Student's t-test was used to determine significant differences between the sedentary and exercise groups. A significance of $p < 0.05$ was considered significant for all tests.

Results.

Animal characteristics. Animal body weights, heart weights, and heart-to-body weight ratios are displayed in Table 1. The exercise training protocol increased the heart weight by 7.1% and the heart weight/body weight ratio by 7.2% ($P<0.05$). There was no significant change in body weight from training. The effectiveness of the exercise protocol was verified by observing an exercise-induced increase ($P<0.05$) in cytochrome *c* oxidase activity in the plantaris muscle (100%). No exercise-induced change was observed for heart Cytox ($P>0.05$), which is typical for this extremely aerobic muscle.

Heart performance. Hemodynamic data at both the low and high workloads provide evidence that the hypertrophy was indeed physiological. Even when normalized for heart weight, hearts from exercised animals performed significantly better ($P<0.05$) for all variables displayed (Table 2). For example, during the high workload normalized cardiac output and external work performed by E was higher than S by 17% and 35%, respectively. The greater work performed by E also resulted in higher VO_2 compared to S. However, the energy required to accomplish a given amount of work actually decreased in E so that their work efficiency increased.

NHE1 content. As displayed in Figure 1, myocardial NHE1 content decreased 38% in exercise vs. sedentary ($P<0.001$). There was no change in the content of actin, which was used as a control for protein loading.

NCX content. Figure 2 shows that exercise training did not affect NCX protein expression within the heart ($P=0.50$).

Discussion.

The study of pathological and physiological hypertrophy has resolved different mechanisms responsible for the induction of cell growth (39,161). NHE1 overexpression has been consistently implicated in the pathological cell growth and diminished function of the heart, whereas inhibition of NHE1 has been proven as antihypertrophic and cardioprotective (35,40,64). The present paper is the first to investigate the relationship of NHE1 protein expression and physiological cardiac hypertrophy. We hypothesized that NHE1 would not increase in the exercise-induced hypertrophied heart. The results of the present study indicate that not only does myocardial NHE1 content not increase, it actually decreases following a five-week exercise protocol (Fig. 1)! This decrease could be a major mechanism by which exercise allows for the anti-remodeling of pathological hypertrophy. Additionally, myocardial NHE1 can be modulated by a myriad of factors; therefore, its regulation in the normal state versus the pathological state and compared to the skeletal muscle during exercise needs to be considered.

Exercise has been shown to allow for anti-remodeling of pathological hypertrophy (82,148); however, the complete mechanisms for this alteration are not fully known. Garciarena et al. (82,148) exercise trained spontaneously hypertensive rats (SHR) by exercising them 90 min/day, 5 day/wk for 60 days. Exercised SHRs displayed even greater hypertrophy compared to control SHRs, yet cardiac performance was significantly improved. Understanding that the NCX1 is implicated in hypertrophy due to its role in Ca^{2+} dysregulation, they examined its protein expression but found no exercise-induced alteration, which is consistent with the findings of other investigators (124). The

content of NHE1 was not examined. We believe that the exercise-induced downregulation of NHE1 could be a predominant mechanism explaining the anti-remodeling effect of exercise that was overlooked at that time. Our data showing that NCX1 protein expression is not altered following exercise (Figure 2) coincides with their evidence. Recently, downregulation in NHE1 protein expression has afforded protection against pathological hypertrophy. Suppression of NHE1 protein content was shown to reverse isoproterenol-induced hypertrophy; chronic inhibition of NHE1 for 7 days attenuated the isoproterenol-induced increase in NHE1 transcript and protein content (177). Additionally, Kilic et al. displayed the anti-hypertrophic effect of suppressing NHE1 protein expression back to control levels using a chimera of C-type natriuretic peptide and urodilatin (113). These results support that a downregulation of myocardial NHE1 content is cardioprotective and may partly underlie the anti-remodeling effect of exercise against pathological hypertrophy.

In the pathological hypertrophy setting ROS have been shown to at least partially mediate the upregulation of NHE1 promoter activity and protein expression (5) and the increase in NHE1 activity (92). The increased NHE1 activity is through ROS-induced ERK1/2 activation (92). This increase in NHE1 content and activity sets up a vicious cycle because the augmented $[Na^+]_i$ causes a further increase in ROS production (115). Consistent with this, the antihypertrophic and cardioprotective effects of NHE1 inhibition have been attributed to the concomitant decrease in ROS production via a decrease in Na^+ influx (100). Therefore, previous findings allow us to examine the mechanistic difference regarding mechanisms that stimulate NHE1 content changes between pathological

hypertrophy and exercise-induced hypertrophy. First, chronic exercise training decreases ROS production from the mitochondria through an adaptation to complex I (189) and reduces overall myocyte hydrogen peroxide production (103). Second, exercise-induced ERK1/2 phosphorylation declines with the development of physiological hypertrophy, suggesting it becomes less sensitive to the stress (97). Third, exercise protocols that are sufficiently intense result in an increase in antioxidant capacity (159). We can speculate that decreasing two major initiators of NHE1 expression and activation (i.e. ROS and ERK1/2 activity) will result in a reduction of these characteristics. Indeed, we have shown that exercise training reduces NHE1 expression.

The sarcolemmal NHE1 helps maintain the intracellular steady-state basal pH (~7.25) by extruding an intracellular H^+ in exchange for an extracellular Na^+. This pH regulating function of NHE1 is severely important when intracellular acidosis occurs in a cell and sustained intracellular acidosis is a major regulator of NHE1 (92). Skeletal muscle NHE1 content increases in response to relatively intense exercise training and thereby helps these cells tolerate the stress of an exercise-induced acid load associated with intracellular lactic acid buildup (96,104,105). However, the healthy heart does not experience an acid load at high exercise intensities because it functions aerobically and without significant lactic acid buildup under all workloads. Indeed, direct measurement of pH_i in intact hearts demonstrates that pH_i does not change as workload increases (190). Thus, our finding that myocardial NHE1 is not increased in the exercise trained animals is consistent with the fact that the acid load in the heart does not increase during even vigorous exercise. While a downregulation of NHE1 may affect the maximum capacity

of the heart to maintain pH, decreases (or increases) in NHE1 alone do not affect pH_i under physiological conditions because other transporters also assist with pH regulation (98,153).

The integration of the physiological systems provides a multifaceted environment to study cell function. The addition of exercise adds a level of complexity as the whole-body stress causes a divergence from homeostasis. During exercise and in states of pathology, the heart is constantly under non-homeostatic intrinsic and extrinsic control. Myocardial NHE1 is stimulated by a variety of extrinsic, neurohumoral stimuli such as alpha-adrenergic agonists, Angiotensin-II, Endothelin-1, aldosterone, and atrial natriuretic peptide (39). These stimuli accompany the chronic pressure overload generated by hypertension and are major players in the cardiomyocyte remodeling process associated with heart failure. Interestingly, these stimuli are also increased during exercise in an attempt to regulate blood volume and cardiac output; however, these compounds return to normal levels shortly after the cessation of exercise (126). Having an adequate recovery period between exercise bouts allows the heart to adapt and remodel in such a way that it becomes able to better handle and recover from subsequent stresses. Exercise also activates the insulin-like growth factor 1-phosphoinositide-3 kinase pathway, which remains activated for some time after exercise and is considered the primary regulator of physiological hypertrophy (130). Whether the exercise-induced decline in NHE1 facilitates the development of physiological hypertrophy is worthy of further investigation.

In summary, we have found that NHE1 is downregulated in exercise-induced hypertrophy. This is a compelling finding because of the widely reported involvement of NHE1 upregulation to pathological hypertrophy and vulnerability to cell death. In animal models pharmacological suppression of NHE1 has consistently been reported to reverse pathological hypertrophy and provide protection against infarction following ischemia-reperfusion. However in humans, clinical trials have resulted in only modest success and some significant detrimental side effects. For example, infarct size was decreased in one study but there was a concomitant increase in mortality associated with cerebrovascular events (131). These drug therapies have not produced their anticipated outcomes, thereby strengthening the rationale to explore alternative approaches that provide cardioprotection through NHE1 suppression. The well-known cardioprotective effects of exercise coupled with the large decrease in NHE1 observed herein suggests that exercise may be a clinically relevant means to lower NHE1 and provide cardioprotection.

Table 3.1. Animal characteristics.

Group (n)	Body Wt (g)	Heart Wt (mg)	Heart/Body (mg/g)	Plantaris Cytox ($\mu molO_2/min/g$)	Heart Cytox ($\mu molO_2/min/g$)
Sedentary (8)	268 ± 6	952 ± 15	3.56 ± 0.01	17.4 ± 1.8	115.6 ± 5.0
Exercise (9)	267 ± 3	1020 ± 13*	3.82 ± 0.06*	34.7 ± 4.1*	122.4 ± 5.6

Values are means ± SE. Abbreviations: Cytox, cytochrome *c* oxidase. *Significantly different from Sedentary ($P<0.05$).

Table 3.2. Heart performance.

Group (n)	Cardiac Output (ml/min/g)	Systolic Pressure (mmHg)	CO x SP (ml/min/g x mmHg)	VO_2 (µmolO2/min/g)	Efficiency (COxSP/ VO2)
			Low workload		
Sedentary (8)	54.1 ± 2.0	107.3 ± 2.4	5,813 ± 279	12.3 ± 0.4	472 ± 14
Exercise (9)	67.5 ± 2.0*	116.1 ± 2.1*	7,834 ± 274*	15.3 ± 0.4*	513 ± 10*
			High workload		
Sedentary (8)	37.0 ± 1.6	158.7 ± 6.0	5,930 ± 440	20.3 ± 0.8	289 ± 13
Exercise (9)	43.1 ± 1.2*	184.1 ± 6.5*	7,986 ± 476*	25.1 ± 0.9*	317 ± 12*

Values are means ± SE. Abbreviations: CO, cardiac output; SP, systolic pressure; VO_2, oxygen consumption.
*Significantly different from Sedentary at same workload ($P<0.05$).

Figure 3.1. Myocardial NHE1 content.

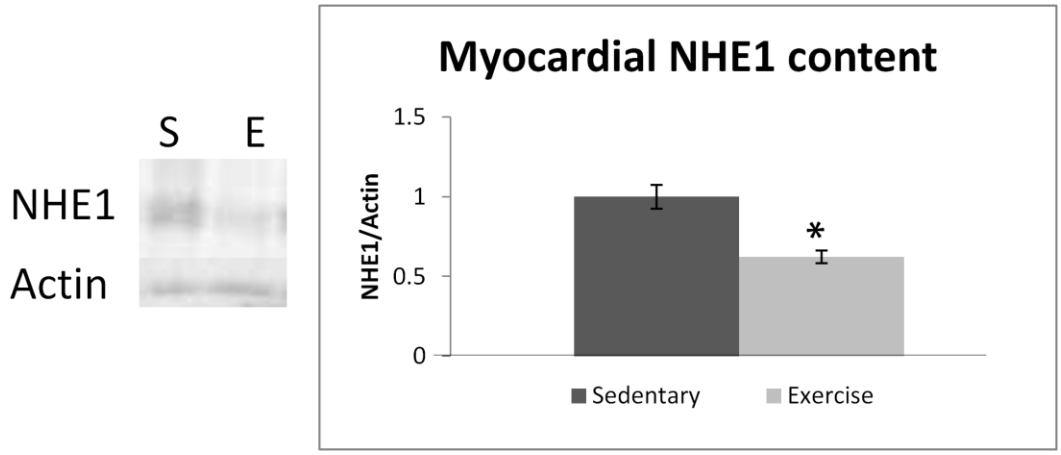

Figure 3.1. Western blot analysis displaying NHE1 expression in sedentary (S) and exercise (E) groups. * E is significantly lower than S ($P<0.001$).

Figure 3.2. Myocardial NCX content.

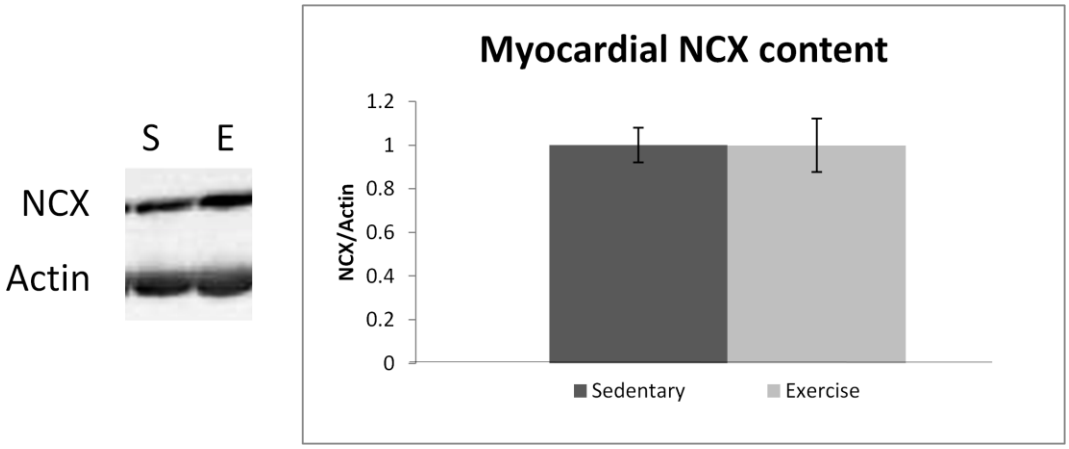

Figure 3.2. Western blot analysis displaying NCX expression in sedentary (S) and exercise (E) groups. There is no significant difference between groups.

CHAPTER IV

EXERCISE ALTERS THE REGULATION OF MYOCARDIAL NA^+/H^+ EXCHANGER-1 (NHE1) ACTIVITY

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NHE1 in exercise-trained cardiomyocytes

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Abstract.

The myocardial Na+/H+ exchanger-1 (NHE1) plays a major role in regulation of intracellular pH and its upregulation has been implicated in increased ischemia-reperfusion injury and other pathologies. Hydrogen peroxide (H_2O_2) is a major by-product of reperfusion and is known to enhance NHE1 activity via ERK1/2 signaling. However, neither NHE1 activity nor its regulation by H_2O_2 has been examined in response to exercise. Therefore, we examined the effect of aerobic exercise training on intrinsic NHE1 activity, H_2O_2-mediated activation of NHE1, and H_2O_2-induced phosphorylation of ERK1/2 using isolated adult ventricular myocytes. Adult female Sprague-Dawley rats were randomly divided into sedentary (S, n=10) and exercise-trained (E, n=8) groups. Heart weight:body weight and plantaris cytochrome *c* oxidase activity was significantly greater by 6.8% and 1.9 fold, respectively, in E animals compared to S animals. NHE1 activity was determined in cells loaded with the pH fluoroprobe BCECF. NHE1 activity was 161% greater in E myocytes compared to S myocytes (0.57 ± 0.10 *v* 1.49 ± 0.18 fmol/min), which is attributed to the 55% greater cell volume (22.2 ± 0.6 *v* 34.3 ± 1.1 pL) and 48% greater buffering capacity (28.79 ± 0.72 *v* 42.65 ± 0.79 mM/pH unit) of E cells. Stimulation with H_2O_2 enhanced NHE1 activity in S and E but to a significantly lower level in E myocytes (1.55 *v* 0.64 fold). As assessed by Western blotting, H_2O_2 stimulation also increased phosphorylation of ERK1/2. There was no difference in the density of H_2O_2-stimulated phosphorylation between S and E; however, E myocytes were observed to have significantly less total ERK1/2. No difference was observed in either cardiomyocyte NHE1 content or catalase and

glutathione peroxidase activity. Our data indicate that aerobic exercise training increases NHE1 activity at physiological intracellular pH and weakens the H_2O_2-mediated regulation of NHE1 activity.

Keywords: Na^+/H^+ exchanger-1 (NHE1); Extracellular signal-regulated kinase (ERK1/2); Aerobic exercise training; Internal pH; Hydrogen peroxide (H_2O_2)

Introduction.

The myocardial sarcolemmal Na^+/H^+ exchanger-1 (NHE1) plays a major role in regulation of intracellular pH (pH_i) by extruding one intracellular H^+ in exchange for one extracellular Na^+. The upregulation of this protein's content and/or activity increases ischemia-reperfusion (IR) injury and is highly implicated in other pathological states such as diabetes, hypertrophy, and hypertension due to its role in Ca^{2+} overload (72). Hydrogen peroxide (H_2O_2) is a major reactive oxygen species produced during reperfusion (83) and increases NHE1 activity in a time- and concentration-dependent manner in rat cardiomyocytes (185). Enhanced NHE1 activity leads to intracellular Na^+ accumulation beyond normal levels, which activates the sarcolemmal Na^+/Ca^{2+} exchanger (NCX1) in reverse mode resulting in detrimental Ca^{2+} overload (71). Even low levels of H_2O_2 can enhance NHE1 activity and sensitize the cell to Ca^{2+}-induced damage (169). On the other hand, inhibition of NHE1 activity or knockout of its content are well known to provide protection against IR injury, H_2O_2-induced apoptosis, and pathological hypertrophy in animal models (7,35,64,94,101,195,208). Thus, suppression of NHE1

seems to be cardioprotective against the oxidative stress of ischemia-reperfusion. We recently reported that aerobic exercise training can lower NHE1 content in the whole heart (67). However, the effect of exercise training on NHE1 activity and its regulation by H_2O_2 are unknown.

The extracellular signal-regulated kinase 1/2 (ERK1/2) are major regulators of NHE1 activity, since ERK1/2 can directly phosphorylate NHE1 on the cytosolic tail (42). Additionally, H_2O_2 stimulation increases phosphorylation of ERK1/2 in a time- and concentration-dependent manner that corresponds to H_2O_2-mediated NHE1 activation (185,210); therefore, ERK1/2 are implicated in NHE1's role in H_2O_2-induced damage. Inhibition of ERK1/2 using the inhibitor U0126 attenuated the typical H_2O_2-induced increase in NHE1 activity in isolated cardiomyocytes (169). Activation of ERK1/2 has been reported to be increased for at least 30 min following a single 30 min exercise bout in previously sedentary animals (97). These authors also reported that after 12 weeks of aerobic exercise training for 1 hr/day at the same speed as the acute exercise bout, ERK1/2 phosphorylation was no longer increased immediately after the same 30 min exercise bout that produced activation in the sedentary rats and that resting ERK1/2 phosphorylation levels were not different between sedentary and exercise trained animals. The authors concluded that acute exercise-induced activation of ERK1/2 gradually declines with development of exercise training-induced cardiac hypertrophy (97); however, they did not take into account that the exercise bout was less stressful after chronic training than the acute bout. Thus, whether ERK1/2 may become less sensitive to stress activation following exercise training is still in doubt. Neither H_2O_2-stimulated

ERK1/2 phosphorylation nor ERK1/2 regulation of NHE1 has been examined following aerobic exercise training. Therefore, the present study exercise trained adult rats for 5 weeks and used isolated ventricular myocytes to examine the effect of exercise on NHE1 content, intrinsic NHE1 activity, H_2O_2-mediated activation of NHE1, and H_2O_2-mediated phosphorylation of ERK1/2.

Methods.

Animals. Female, 5-mo old, Sprague-Dawley rats were purchased and housed in the campus animal facility in an isolated room maintained at 22°C with a 12-hour light-dark cycle and fed ad libitum with Harlan 2018 Teklad Global 18% Protein Rodent Diet. Animals were randomly divided into either a sedentary control (S) (n=14) or exercise trained (E) (n=11) group.

Exercise treatment. Exercise was carried out on a motor-driven treadmill using a protocol previously found to result in cardiac hypertrophy and a decrease in NHE1 content in the whole heart (67). Animals were habituated for 1 week by running for 10 min/day at 10 m/min up a 6˚ incline. During this week the time and speed was gradually increased, so that at the end of the week animals were running for 30 min at 25 m/min (70-75% VO_2max (31)). Following habituation animals began running in the cold (8˚C) to prevent an excessive increase in core temperature and running time was gradually increased over the course of one week until the animals were running for 1 hr/day at 25 m/min. Speed was then gradually increased until it reached 30 m/min and maintained at that speed for the final weeks. Exercising rats rested for 24 hrs after the last exercise bout to remove acute training effects and then the heart was isolated and perfused as described

below. This investigation was approved by the University's Animal Care and Use Committee and conforms to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85–23, Revised 1996).

Preparation of isolated myocytes. Myocytes were isolated as described by Xu et al. (215). Briefly, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg body wt). Hearts were removed following a transverse thoracotomy and rapidly mounted on a Langendorff apparatus. The heart was perfused at a perfusion pressure of 61 mmHg in a non-recirculating mode with Krebs-Henseleit buffer (37°C) containing (in mM) 118 NaCl, 4.7 KCl, 1.25 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 24.7 $NaHCO_3$ and 10 glucose for 5 min to wash-out blood. The buffer was bubbled with 95% O_2/5% CO_2. Then the heart was perfused with Ca-free buffer that contained all of the above components except $CaCl_2$. After 5 min of perfusion, collagenase (type II) was added to the buffer (0.075%) with 25 μM $CaCl_2$ and the heart was perfused in a recirculating mode for ~25 min. The heart was removed from the apparatus and the ventricles placed into a beaker containing the Ca-free buffer. The ventricles were agitated in a shaking bath (37°C) for 10 min at a rate of 50 cycles/min, which allowed individual cells to be released. Additional trituration using a wide-tipped transfer pipette assisted in cell dispersion. The released cells were suspended and washed three times in an incubation buffer containing all the components of the Ca-free buffer, 1% BSA, 30mM HEPES, 60mM taurine, 20mM creatine, and amino acid supplements at 37°C. Cells for NHE1 activity were filtered through nylon mesh and plated onto laminin-coated

coverslips in 35-mm dishes. Cells for ERK1/2 activation were filtered and resuspended in Tyrode's solution containing (in mM) 135 NaCl, 5.9 KCl, 1.2 MgCl, 11.5 Glucose, and 11.6 HEPES, pH 7.4. Aliquots of cell suspensions were stored in Tyrode's at -80°C for later measurements of activities of antioxidant enzymes and cytochrome *c* oxidase. Experiments were performed on isolated cells within 4 hr of isolation.

Preparation of cell lysates for detection of ERK1/2 phosphorylation. Myocytes in suspension were incubated in Tyrode's containing H_2O_2 at concentrations of 0 (Con), 50, 100, and 200 μM for 5 min followed by a 10-min washout. Concentrations >200 μM were not used since maximum ERK1/2 phosphorylation has been shown to occur at H_2O_2 concentrations below 200 μM and then to decrease as H_2O_2 concentration rises (210). The 5-min H_2O_2 perturbation followed by the 10-min washout simulates the time course of free radical insult upon reperfusion following ischemia. The 5-min exposure was used since peak ERK1/2 phosphorylation in response to 100 μM has been shown to occur at or before 6 min (173,185). The 10-min washout was used since ERK1/2 phosphorylation continues to increase following exposure (210). The ERK1/2 kinase inhibitors PD98059 (10 μM (185)) and U0126 (5 μM (210)) were present with a 100 μM H_2O_2 exposure to determine the extent of H_2O_2 regulation on ERK1/2. Inhibitor or DMSO was present 10 min before H_2O_2 exposure. More than one pharmacological inhibitor was used to display ERK1/2's involvement, because all studies utilizing pharmacological inhibitors are subject to potential nonspecific effects. Following perturbation, cells were lysed in ice-cold buffer containing (in mM) 50 NaCl, 50 NaF, 50 sodium pyrophosphate, 5 EDTA, 5 EGTA, 2 Na_3VO_4, 0.5 phenylmethylsulfonyl fluoride, 10 HEPES, pH 7.4, 0.1% Triton

X-100, and 10 μg/mL leupeptin. Lysed cells were stored at -80°C. Lysates were thawed, sonicated, and centrifuged at 10,000 x *g* for 30 min at 4°C. Protein concentrations were determined on supernatants using the Lowry method and samples were processed for immunoblotting.

Measurement of NHE1 activity. The acetoxy methyl ester of 2'-7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM) was used to measure intracellular pH on a Varian Cary Eclipse fluorescence spectrophotometer. BCECF-AM (1.25 μM (173)) was used to incubate cells for 15 min at room temp in the dark in Tyrode's buffer supplemented with 0.02% pluronic acid. A coverslip with >80% rod-shaped myocytes was inserted into a 4.5-mL optical methacrylate cuvette at a 30° angle to the light beam. The solution bathing the cells (pH 7.4) was changed by perfusing fresh solution into the bottom of the cuvette while aspirating continuously from just above the coverslip. The perfusion rate (7.5 mL/min) was kept constant using a peristaltic pump. At the perfusion rate used, the half-time of mixing in the cuvette was ~20 sec. The cells were excited alternately at 490- and 440-nm light every 0.02 sec using a rotating excitation filter wheel, and average fluorescence intensity ratios (490/440 nm) were recorded at 1.0-sec intervals. This technique allowed measurement of pH_i that is independent of cell concentration and loading. Emission was at 535 nm. At the end of each experiment, the fluorescence ratio values were converted to pH_i using the nigericin high-K^+ technique (197). This clamps the H^+ gradient and sets pH_i equal to external pH. Cells were perfused with high-K^+ solution containing (in mM) 5 NaCl, 135 KCl, and 50 HEPES at varying pH levels (6.8 to 7.8) in the presence of nigericin (4 mg/L). There was a linear

relationship between fluorescence intensity ratios and pH over this range ($r^2 = 0.982 \pm 0.002$, n = 35).

Tyrode's with ammonium chloride (15 mM) was used for 2 min to transiently generate an acid load (185). To recover, the cuvette was perfused with a normal Tyrode's buffer (pH 7.4). The cells were then acidified with a second ammonium chloride pulse to assess the response to H_2O_2; the first pulse acted as an internal control. 100 μM H_2O_2 was used as this concentration is a physiologically relevant ROS concentration (83) and was also used to determine ERK1/2 phosphorylation (described above). Additionally, the 10-min H_2O_2 exposure for NHE1 activity has been previously used (185) and was shown to elicit an increase in NHE1 activity in preliminary experiments (data not shown). Cells were maintained in HCO_3^- free medium throughout each experiment, so that the buffering capacity could be used as the indicator of NHE1 activity and HCO_3^-/H^+ exchanger activity did not interfere. The NHE1 protein activity was determined by measuring the slope of the first linear 20 sec of the recovery period and was expressed as ΔpH/min. The change in rate of recovery during the second pulse was normalized to the rate of recovery during the first pulse (125). When studying the effects of ERK1/2 on NHE1 activity, the second pulse was performed in the presence of PD98059 (10 μM). The inhibitor was present 10 min before the start of exposure to H_2O_2; DMSO was present during the first pulse.

Determination of intrinsic buffering capacity. Intracellular intrinsic buffering capacity (*B*, mmol/liter/pH unit) was determined for each group by stepwise removal of extracellular NH_4Cl and observing the change of pH_i produced by this load. Cells were

exposed to Tyrode solution (pH 7.4) containing 20 mM NH_4Cl for 90 s, followed by a stepwise reduction of extracellular NH_4^+ to 10, 5, 2.5, and 1 mM. At each step calculated changes in $[NH_4^+]_i$ and measured changes in pH_i were used to estimate *B*, from the equation $B = \Delta NH_4 + i/\Delta pH_i$ (NH_4Cl_o x $10(pKa - pH_i)$) / $[1 + 10(pKa - pH_o)]$. The Henderson-Hasselbach relation using a pKa for NH_4^+ of 9.21 determined the equilibrium between NH_4^+, NH_3 and pH in extracellular medium.

A volume correction was used to compare proton efflux (J_{H+}) in E and S heart cells. The volume factor was determined after fixing cells in phosphate buffered glutaraldehyde (1% final concentration) and observing the length and width of 100 cells per group (from 3 hearts per group) under a light microscope. The equation V = l*w*0.00759 was used to determine cell volume according to Satoh et al. (176), and a correction for J_{H+} (CJ_{H+}) was made using the equation CJ_{H+} = d(B*pH*V)/dt.

Western blotting. Cells used for detecting ERK1/2 were processed for immunoblotting as described above (*Preparation of cell lysates to determine ERK1/2 activation*). Laemmli's sample buffer was added to prepare samples for electrophoresis; sample volume was based on protein concentration of the sample. Protein concentrations of the homogenates were determined by the Lowry method and used to ensure that each sample was loaded with the same amount of protein. All samples were subjected to SDS-PAGE electrophoresis on a resolving gel until the desired molecular weight bands were separated. Thereafter, proteins were transferred onto a PVDF membrane, incubated at room temp in 5% evaporated milk in Tris buffered saline. The membranes were immunoblotted overnight at 4°C with a primary antibody: Anti-NHE1 (Millipore,

AB3081), β-Actin (Cell Signaling, 4967), Phospho-p44/42 (Erk1/2, Thr202/Tyr204) (Cell Signaling, 9101), or p44/42 MAPK (Erk1/2) (Cell Signaling, 9102). Membranes were incubated for 1-2 hr at room temp with a HRP-linked secondary antibody: Anti-rabbit IgG (Cell Signaling, 7074). Proteins were detected with SuperSignal West Pico chemiluminescent substrate in a BioRad Chemidoc Station and densities were measured using Quantity One software. The membrane was stripped with a buffer containing (in mM) 13.3 Glycine, 3.5 SDS, and 1% Tween-20, pH 2.2. The membrane was reprobed for another protein. β-Actin and total ERK1/2 served as loading controls for NHE1 and phospho-ERK1/2, respectively. Densities revealed changes in protein expression between groups.

Catalase and glutathione peroxidase (GPx) activity. Because H_2O_2 was used as the stimulus for the aforementioned experiments and crossed the cell membrane into the cytosol, the two primary antioxidants that quench H_2O_2 were examined. Briefly, the cells were homogenized on ice in approximately one volume of 50 mM KH_2PO_4, 0.1 mM EDTA, 0.1% (v/v) Triton X-100, pH 7.4. They were then centrifuged at 10,000 x *g* for 5 min and the supernatant kept on ice for measurement of the maximum activities of catalase, GPx, and cytochrome *c* oxidase (described later) within 1 hr. GPx activity was measured by the method described by Flohe and Gunzler (74). This assay was performed at 37°C in a Varian Cary 50 Bio UV-spectrophotometer at a wavelength of 340 nm and done in triplicate. Catalase activity was measured polarographically with a Clark-type O_2 electrode at 37°C as described by Abei et al. (2) and done in duplicate.

Training status. Whole plantaris muscle and cardiomyocyte suspensions were homogenized in ratios of 1:4 w/v or ~1:1 v/v, respectively, in 50 mM KH_2PO_4, 0.1 mM EDTA, 0.1% (v/v) Triton X-100, pH 7.4, centrifuged at 10,000 x *g* for 5 minutes, and the supernatants analyzed. Cytochrome *c* oxidase (cytox) activity, a marker of mitochondria content, was determined polarographically using a Clark-type O_2 electrode as described previously (171) and done in duplicate. Heart weight:body weight ratio was also used as a measure of training. Protein content for all enzyme assays and Western blotting was determined by the Lowry method using BSA as the standard.

Statistical analysis. Factorial ANOVAs or independent t-tests were used to uncover main effects. All significance values were set at the $\alpha = 0.05$ level and, if necessary, a Tukey's HSD was implemented to dissect main effects. For NHE1 protein expression and intrinsic NHE1 activity characteristics, an unpaired t-test was used to detect differences between groups S and E. For ERK1/2 phosphorylation, a 2x6 factorial ANOVA was used; the 2 levels were S and E, while the 6 treatments were H_2O_2 concentrations of 0 μM (Con), 50, 100, 200, 100+PD98059, and 100+U0126. For H_2O_2-stimulated NHE1 activity, a 2x3 factorial ANOVA was used; the 2 groups were S and E, while the 3 treatments were control, 100 μM H_2O_2, and 100 μM H_2O_2+PD98059. Unpaired t-tests were used to compare cell volume measurements, catalase activity, GPx activity, and cytochrome oxidase activity between S and E.

Results.

Exercise training adaptations. The animal characteristics of Table 1 display the differences between sedentary and exercise trained rats. The exercise training protocol

increased both heart weight and body weight in the E group by 11.8% ($P < 0.001$) and 5.7% ($P < 0.01$), respectively. The greater increase in heart weight explains the significant increase in heart weight:body weight ratio of 6.8% ($P < 0.01$). An increase in skeletal muscle mitochondrial content, as assessed by cytochrome *c* oxidase (cytox) activity, is a hallmark adaptation of exercise training and plantaris cytox activity was 1.9-fold higher in E animals ($P < 0.001$). No exercise-induced change was observed for heart cytox ($P>0.05$), which is typical for this extremely aerobic muscle. Overall, the presence of cardiac hypertrophy and the almost 2-fold increase in skeletal muscle mitochondria indicate a high state of endurance training.

NHE1 protein expression. To verify that exercise training does not increase NHE1 protein content, as shown previously (67), protein density was assessed via immunoblotting from isolated adult ventricular myocytes from six animals in both groups. No significant difference was detected between S and E (1.87 ± 0.83 vs. 1.47 ± 0.37 Arbitrary Units, $P = 0.25$).

Estimation of cell volume. The cell volume characteristics of sedentary and exercise trained myocytes are presented in Table 2. Cell volume was 55% greater in E myocytes ($P < 0.01$), a result of both greater cell length and width. The estimated volumes of sedentary adult myocytes are comparable to previously reported values (93). The respective cell volume data for S and E were used to calculate the corrected rates of proton efflux in the two groups.

Intrinsic buffering capacity. As depicted in Figure 1, a marked difference existed between the intrinsic buffering capacity of S and E myocytes. The best-fit line equations

developed following NH_4Cl titration were -27.976x + 229.18 and 15.747x - 70.395 for S and E, respectively, indicating a 48% greater buffering capacity in exercise-trained myocytes at pH_i during recovery (~7.16). As pH_i decreases, the difference in buffering capacity becomes less and less until at pH_i 6.8, *B* is virtually identical between groups.

NHE1 activity. As shown in Table 3, there was no between-group difference regarding the rate of pH change and the minimum pH reached following the NH_4Cl pulse. The E group had a 48% greater buffering capacity at these pH values compared to S ($P < 0.001$). Buffering capacity at at the pH_i in the middle of recovery was used to determine rate of proton efflux (J_{H+}). Because all experiments were carried out in the absence of HCO_3^-, J_{H+} could be used as a direct index of sarcolemmal NHE1 activity. The J_{H+} was significantly greater in E compared to S ($P < 0.05$). Taking into account cell volume changes, the final difference, CJ_{H+}, was observed to be a 161% increase in NHE1 activity in rat cardiomyocytes from E hearts ($P < 0.001$). These results suggest that the increase in NHE1 activity observed in E myocytes is a factor of their greater buffering capacity and cell volume.

Influence of H_2O_2 on NHE1 activity. Figure 2 displays the response of NHE1 activity to 100 μM H_2O_2 stimulation. Both S and E NHE1 activity were significantly increased upon H_2O_2 stimulation relative to control values ($P < 0.05$, $n \geq 7$ experiments, with cells from ≥ 7 hearts); however, the increase in E myocytes was significantly lower than S myocytes ($P < 0.05$). In both groups, the response to 100 μM H_2O_2 was abolished in the presence of the ERK kinase inhibitor PD98059, supporting ERKs role as a mediator of the H_2O_2-induced increase in NHE1 activity.

Effect of H_2O_2 on ERK phosphorylation. Total ERK1/2 and amount of phosphorylated ERK1/2 are displayed in Figure 3. Experiments using a range of H_2O_2 concentrations from 50 to 200 μM verified that maximum phosphorylation was achieved with 100 μM H_2O_2 in both groups. The expression of total ERK1/2 was significantly reduced in E by 30% for ERK1 and 43% for ERK2 compared to S ($P < 0.05$, $n = 30$, each H_2O_2 treatment from 5 different hearts). However, the amount of phosphorylated ERK1/2 both in the basal state (control) and following stimulation by H_2O_2 was not significantly different between groups. The observations that total ERK1/2 are lower in E than S, yet activated ERK1/2 are similar in both groups, indicate that a greater proportion of total ERK1/2 is phosphorylated in E compared to S. Both inhibitors (U0126 and PD98059) reduced phosphorylation to values not different compared to control values.

Effect of exercise training on antioxidant activity. The results for catalase and GPx are displayed in Table 4. No significant difference was observed between S and E groups for either catalase ($P = 0.09$) or glutathione peroxidase ($P = 0.10$). This suggests that both S and E cells received the same intracellular H_2O_2 perturbation.

Discussion.

The present study is the first to determine the impact of aerobic exercise training on myocardial NHE1 activity. There were two major findings. First, we observed that the exercise program used herein resulted in a significant increase in the intrinsic capacity for proton efflux from cardiomyocytes via NHE1 at physiological pH_i compared to those from sedentary animals. Second, the exercise program led to a significant decrease in the

sensitivity of NHE1 activity changes in response to ROS signaling pathways. These findings will now be discussed.

Exercise increases intrinsic NHE1 activity. NHE1 activity was measured in intact, isolated cardiomyocytes so that intracellular vs extracellular pH differences could be set to produce maximum activity, at pH levels where NHE1 activity typically is quiescent (205), and the rate of proton efflux across the sarcolemma, i.e., activity, could be directly determined. Because our experiments were performed in HCO_3^--free medium, any recovery from NH_4Cl-induced intracellular acidosis is from the contribution of NHE1 (93). E myocytes had a 161% greater CJ_{H+} compared to S myocytes (Table 3). This difference can be at least partially explained by the increased buffering capacity (Fig. 1) and cell volume (Table 2) detected in E cells. The enhanced buffering capacity is likely a reflection of the change in intracellular protein composition in response to training (for example, myofilaments), as intracellular buffers are believed to include H^+-titratable groups (16,19). When accounting for the larger cell volume of E myocytes, proton efflux increased by another ~96% relative to S myocytes.

Importantly, the increase in cellular NHE1 activity is not due to an increase in the expression of the protein compared to total myocyte proteins. We previously observed that the exercise training protocol used for this study resulted in a decrease in NHE1 content in whole heart (67). In the present study we observed a trend for a decrease in NHE1 in isolated ventricular myocytes, but it was not significant at the 0.05 level. Data using whole heart tissue sometimes can be difficult to generalize to the heart cell, because a heterogeneous population of cells, such as fibroblasts, smooth muscle and endothelial

cells, may contribute to the expression. In fact, NHE1 is the predominant pH_i regulator in fibroblasts (84), smooth muscle and endothelial cells (17). The fact that no change in NHE1 protein content was observed at the myocyte level suggests that any between-group difference in pH_i regulation is a direct result of NHE1 activity changes.

Most exercise-induced adaptations typically inherently present an understandable physiological reason for why the alteration occurred. What is the purpose of this increased activity? Wisloff et al. (214) exercise trained adult rats on the treadmill for 4 weeks and examined pH_i changes in response to increasing stimulation frequencies between 2 and 10 Hz. They observed that at $\geq$5 Hz both groups acquired acidotic pH_i; however, exercised cells had significantly less acidosis compared to sedentary cells. The increased NHE1 activity observed in our E cells can largely explain the attenuated acidosis observed with increasing stimulation frequency. This mechanism also could help explain why the trained myocytes displayed enhanced contractility and shorter systolic relative cell length compared to untrained myocytes at the same frequencies (214); the increased NHE1 activity would result in less H^+s being present to interfere with Ca^{2+} binding to Troponin C (14). Although NHE1 is the predominant H^+ extruding transporter, Na^+-coupled Cl^-/HCO_3^- exchange and Na^+-HCO_3^- cotransport warrant investigation to more fully understand how the exercise trained heart improves its ability to regulate pH_i during faster pacing (i.e. exercise) (153).

The NHE1 activity measured herein was carried out at or near physiological pH_i, since the extracellular pH was constant at pH 7.4 and the lowest pH_i reached was ~7.05. Using only a physiological pH range is a limitation of this study because pH_i can reach

considerably lower levels during pathological situations. However, we can speculate on exercise-induced NHE1 activity changes outside this pH range using the buffering capacity equations presented above. At pH_i 6.3, a level possible during 15 min of no-flow ischemia (149), E myocytes would have 45% lower *B* capacity. Following, the CJ_{H+} would be ~10% lower in E myocytes compared to S myocytes, which hypothetically would be protective against downstream cytosolic Ca^{2+} overload. Moreover, slower recovery from pHi would result in lower pHi values, which have been shown to be protective against mitochondria permeability transition pore opening (87). Further investigation examining NHE1 activity in exercise-trained myocytes at levels more comparable to those reached during ischemia are warranted and would provide more detailed insight into the role NHE1 plays in the exercise-induced cardioprotective phenotype. Also, investigating how exercise-trained myocyte buffering capacity decreases with decreasing pH_i, possibly by other H^+-extruding mechanism, is needed.

Exercise decreases sensitivity of NHE1 activity to ROS signaling pathways. H_2O_2 has been implicated previously in increasing NHE1 activity in both neonate (173) and adult (185) heart cells. In many pathological situations, including ischemia-reperfusion, NHE1 activity is upregulated by ROS generated from the stress. Thus, we examined changes in NHE1 activity in response to H_2O_2, which is a major by-product of reperfusion following an ischemic insult. We observed that addition of 100 μM H_2O_2 provided 10 min before recovery from the second NH_4Cl pulse increased NHE1 activity in both sedentary and exercise trained cells, as anticipated based on previous results (185); however, exercised myocytes had an attenuated response compared to sedentary.

Lower NHE1 activity in response to H_2O_2 during an ischemic insult (and possibly acidosis, as noted above), would be protective because it would result in less cytosolic Na^+ accumulation, which would result in less cytosolic Ca^{2+} overload (195). In addition, it would slow pH_i recovery, which would inhibit mitochondria permeability transition pore opening (87). Interestingly, if the increase in CJ_{H+} in response to H_2O_2 stimulation is viewed in light of the intrinsic CJ_{H+} of each respective group, then activity appears to be equal at ~0.92 fmol/min. This then would suggest that NHE1 may not be an exercise-induced protective mechanism by which cytosolic Ca^{2+} overload is slowed at physiological pH_i under H_2O_2 stimulation. The decreased response by the E group could be a result of a lower NHE1 protein content, a decreased H_2O_2 concentration observed by the cell due to increased antioxidant capacity, or altered signaling responsible for mediating the H_2O_2 induction of NHE1 activity. As noted earlier, there was no change in cardiocyte NHE1 protein content, meaning it likely is not a contributor to the altered response. Therefore, the other two possibilities will now be discussed.

H_2O_2 can be quenched by catalase and glutathione peroxidase. Catalase reduces H_2O_2 to H_2O and O_2, while GPx reduces H_2O_2 to H_2O. The literature is inconsistent regarding whether or not these antioxidants change following exercise training. We observed both catalase and GPx were not different between exercise and sedentary animals at the 0.05 level. These results are similar to other studies that did not detect changes in these antioxidants (22,56,57,90,103,164,189). Therefore, it appears that the attenuated response of NHE1 activity to H_2O_2 is not a result of lower perceived H_2O_2 concentration.

ERK1/2 are major regulators of NHE1 activity since they can phosphorylate NHE1 on its cytosolic tail (42). Iemitsu et al. (97) suggested that ERK1/2 may become less sensitive to stress activation following exercise training based on their findings that endurance trained rats had less ERK1/2 activation following an exercise bout compared to previously sedentary rats subjected to the same exercise bout. However, when we provided identical H_2O_2 stresses to cells from S and E we observed similar amounts of phosphorylated ERK1/2. Since there was lower total ERK1/2 in E, there was actually greater activation in E by the H_2O_2 stimulation. This suggests that ERK1/2 is more sensitive to stress activation by exercise training. This is in direct contrast to the conclusions of Iemitsu et al. (97). Their results are likely due to the fact that the trained animals experienced less stress during the exercise bout because of training-induced adaptations.

Previous studies focusing on the relationship of ERK1/2 activation and NHE1 activation in rat cardiomyocytes used sedentary animals (185,210). They reported that H_2O_2 stimulation increases phosphorylation of ERK1/2 in a manner that corresponds to H_2O_2-induced NHE1 activation. An important finding in the present study is that the signaling between ERK1/2 and NHE1 is dampened by exercise training. Although we observed that a 100 μM H_2O_2 stress resulted similar amounts of phosphorylated ERK1/2 in S and E (Fig. 3), there was less NHE1 activation in E (Fig. 2). Thus, NHE1 activation becomes less responsive to activated ERK1/2 following endurance training. Although we do not have an explanation for this dampening effect due to exercise, it clearly deserves

further investigation (i.e. phosphorylation of NHE1 cytosolic tail) because of the implications to the effect of enhanced cardioprotection.

Summary. This was the first study to examine the characteristics of sarcolemmal NHE1 activity and signaling in exercise-trained adult rat ventricular cardiomyocytes under basal and H_2O_2-stimulated conditions. We observed that NHE1 activity is increased in E myocytes compared to S myocytes at near physiological pH_i. This increase in activity is a result of enhanced buffering capacity and increased cell volume in E myocytes. Additionally, 100 μM H_2O_2 stimulation increased NHE1 activity in both groups; however, to a significantly lower activity level in E cells. The smaller increase in NHE1 activity was attributed to altered signaling between ER1/2 and NHE1. These results shed light on pH_i regulation during exercise and create a starting point for examining NHE1 as part of the exercise-induced cardioprotective phenotype.

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Contributions. BF and JW designed experiments. BF performed experiments, analyzed data, and wrote the paper.

Table 4.1. Animal characteristics.

Group	Body Wt (g)	Heart Wt (mg)	Heart Wt: Body Wt (mg/g)	Plantaris Cytox (nmol/mg)	Heart Cytox (nmol/mg)
S	246.5 ± 2.2 (15)	860 ± 14 (15)	3.49 ± 0.05 (15)	322.0 ± 17.5 (8)	1767 ± 139 (9)
E	261.4 ± 3.3* (11)	975 ± 22† (11)	3.73 ± 0.08* (11)	608.8 ± 41.3† (8)	1457 ± 104 (9)

Values are means ± SEM (n). S, sedentary group; E, exercise trained group; Cytox, cytochrome *c* oxidase (nmol O_2 consumed/min/mg pro). *P<0.01, †P<0.001 vs. S animals.

Table 4.2. Cell volume characteristics.

Group	Length (μm)	Width (μm)	Volume (pL)
S	109.6 ± 1.8	26.7 ± 0.6	22.2 ± 0.6
E	131.5 ± 2.4*	34.5 ± 0.9*	34.3 ± 1.1*

Volume = width x length x 0.00759 pL/μm^2 (176). An n = 100 cells from 3 hearts was used for each group. The estimated S volumes compare to previously reported values (93). Values are represented as mean ± SEM. *P<0.01 vs. S animals.

Table 4.3. NHE1 activity changes.

	S (n=16)	E (n=15)
CJ_{H+}(fmol/min)	0.57 ± 0.10	1.49 ± 0.18*
J_{H+}(mM/min)	0.026 ± 0.004	0.043 ± 0.005**
B (mM/pH unit)	28.79 ± 0.72	42.65 ± 0.79*
dpH/min	0.0533 ± 0.0088	0.0607 ± 0.0072
min pH	7.10 ± 0.01	7.07 ± 0.02

Pooled data from the first NH_4Cl pulse between control and H_2O_2 treatments to examine intrinsic characteristics of NHE1 between the two groups. S, sedentary group; E, exercise trained group. *B*, buffering capacity; dpH/min, rate of pH_i recovery. Values are represented as mean ± SEM. **P<0.05, *P<0.001 vs. S animals.

Table 4.4. Antioxidant levels.

Group	Catalase (Unit/mg)	GSH Peroxidase (nmol/min/mg)
S (n=10)	16.19 ± 0.92	25.77 ± 3.64
E (n=9)	19.41 ± 1.56	33.88 ± 2.83

Values are means ± SEM. S, sedentary group; E, exercise trained group; Catalase (Unit/mg protein, where 1 Unit = 1 umol H_2O_2 consumed/min); GSH Peroxidase, glutathione peroxidase (nmol NADH consumed/min/mg protein).

Figure 4.1. Intrinsic buffering capacity.

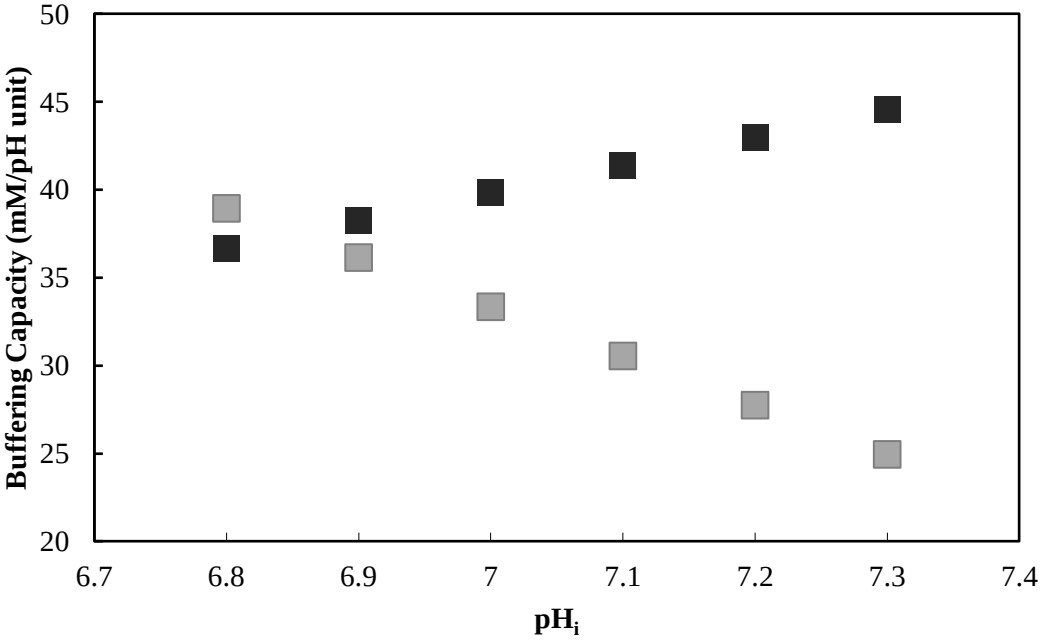

Figure 4.1. Dependency of intrinsic buffering capacity on pH_i in sedentary (light boxes) and exercise trained (dark boxes) ventricular myocytes based on the calculated equations following titrations with NH_4Cl. Sedentary: y = -27.98(x) + 229.18; Exercise trained: y = 15.75(x) + -70.40.

Figure 4.2. H_2O_2-mediated NHE1 activity.

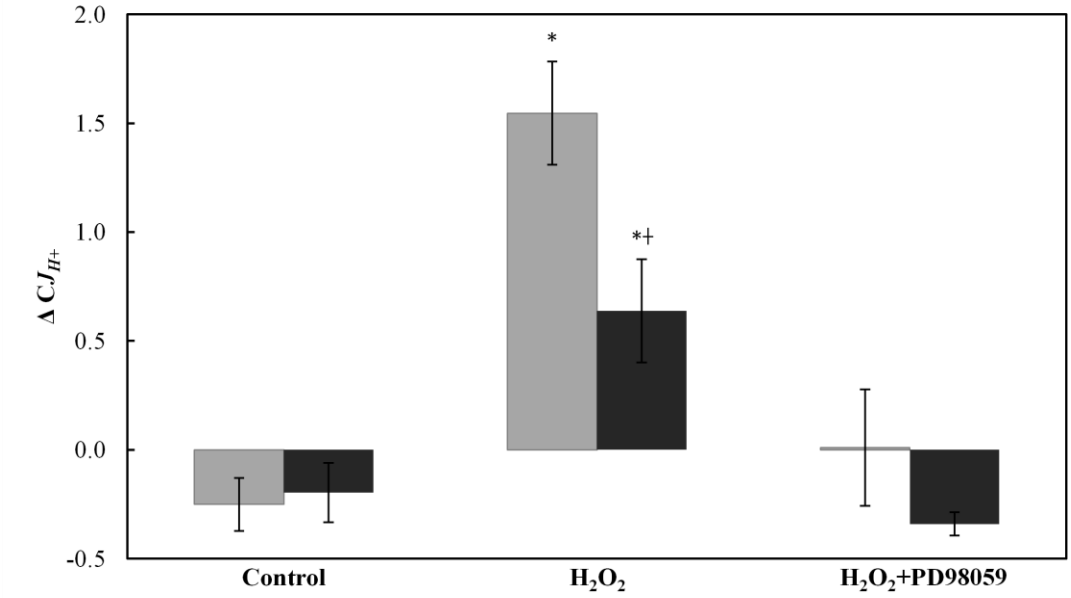

Figure 4.2. Effects of 100 μM H_2O_2 on NHE1 activity and the effects of ERK1/2 inhibition on H_2O_2-induced increase in NHE1 activity. Cells were subjected to two consecutive acid pulses. The change in proton efflux (ΔCJ_{H+}) during the second acid pulse relative to the first is shown in cells exposed to vehicle or 100 μM H_2O_2 from 10 min before the second acid pulse, in the absence or presence of PD98059 (10 μM) pre-treatment. Sedentary is light grey; Exercise trained is dark grey. *P<0.05 versus control, $^{+}$P<0.05 versus S H_2O_2 (≥7 experiments, with cells from ≥7 hearts).

Figure 4.3. H_2O_2-mediated ERK1/2 phosphorylation.

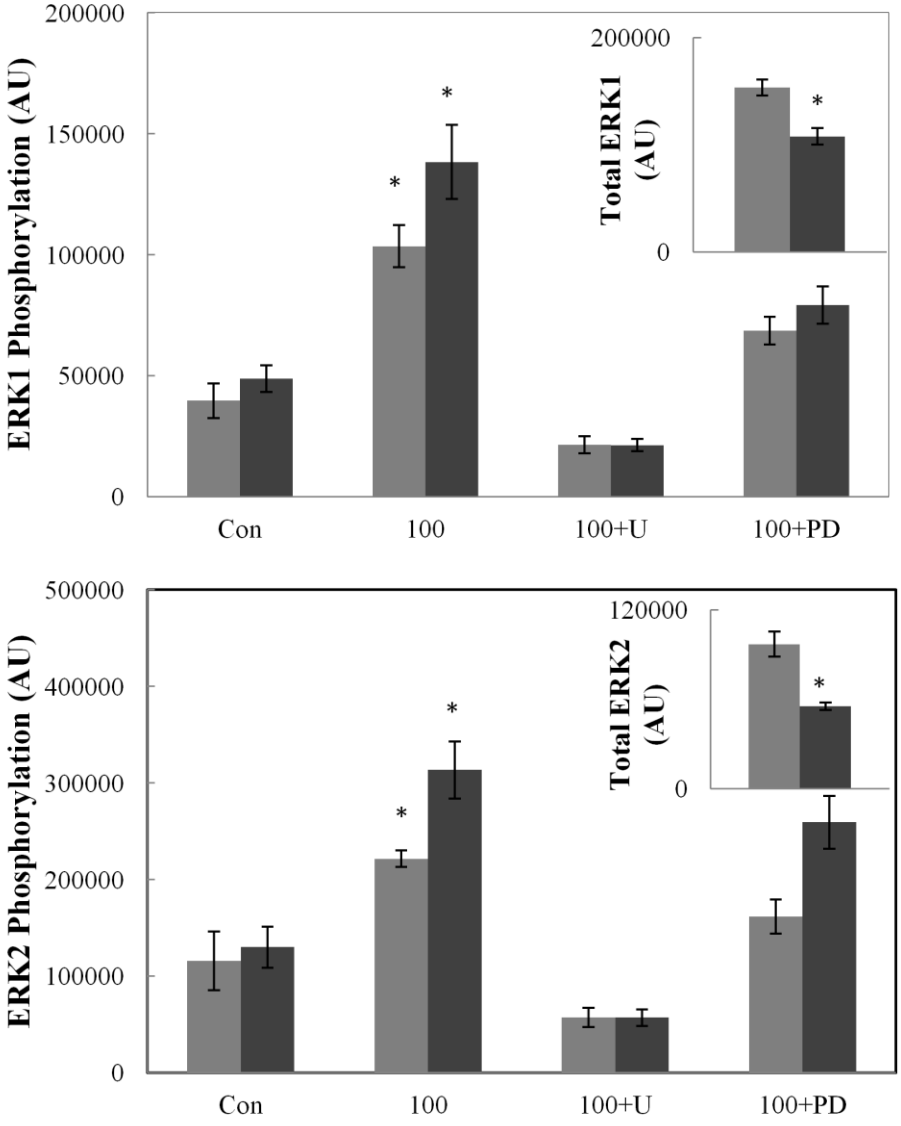

Figure 4.3. ERK1/2 phosphorylation was increased following exposure to H_2O_2; however, a concentration-dependent increase was not observed. Inhibition with ERK kinase inhibitors attenuated ERK1/2 phosphorylation to levels not different from controls for both groups. S is light grey columns and E is dark grey columns. Representative bands are displayed below. Con, control; U, U0126; PD, PD98059. *P < 0.05 vs. S control; $^{+}$P < 0.05 vs. E control. The inset displays total ERK1/2 density differences between S and E. E total ERK2 was significantly lower than S. AU, Arbitrary Units. *P < 0.05 vs. S (n = 5 hearts from each group).

CHAPTER V

GENERAL DISCUSSION

The primary purposes of this dissertation were to examine the effect of exercise training on myocardial NHE1 content, activity, and regulation, and the implication of changes to these characteristics. We initially observed that NHE1 content was decreased by 38% in the whole heart following exercise training (Chapter III). Because previous literature shows that suppression of NHE1 content and/or activity is protective against many stresses, especially ischemia-reperfusion and ROS, we hypothesized that this exercise-induced decrease in NHE1 content would present as a major mechanism of the exercise-induced cardioprotective phenotype. However, to gain a clearer picture of NHE1's potential in the exercise-induced phenotype, NHE1 activity and its regulation needed to be studied in response to exercise training. Chapter IV is the first study to determine the impact of aerobic exercise training on myocardial NHE1 activity and its regulation in the presence of ROS. The two major findings include: 1) the exercise program resulted in a significant increase in the intrinsic capacity for proton efflux from cardiomyocytes via NHE1 at physiological pH_i compared to those from sedentary animals, and 2) the exercise program led to a significant decrease in the sensitivity of NHE1 activity changes in response to ROS signaling pathways. The increased NHE1 activity was a result of enhanced buffering capacity and greater cell volume in the exercise-trained myocytes. Although, we observed ROS signaling to be not different

between groups, there was diminished NHE1 activation in the exercise-trained group following ROS stimulation. We do not have an explanation for this dampening effect due to exercise, but it clearly deserves further investigation because of the potential implications of the effect to enhanced cardioprotection.

The implications of altered NHE1 activity include increased or decreased intracellular Ca^{2+} and caspase-3 activation. After observing an exercise-induced decrease in NHE1 content, we hypothesized that exercise-trained myocytes would have less Ca^{2+} overload and caspase-3 activation compared to sedentary myocytes. This hypothesis was based on the fact that inhibition of NHE1 activity or knockout of its content provides protection against ischemia-reperfusion injury. We found that neither Ca^{2+} nor caspase-3 activity were altered in S and E following H_2O_2 stimulation and there was no between-group difference ($P > 0.05$), as depicted in Figure 1 and Figure 2, respectively. These results make sense since there was no significant decrease in NHE1 content in the isolated cells as discussed in Chapter IV; however, the diminished response of NHE1 activity to H_2O_2 stimulation suggests that NHE1 still may be protective. To that end, the equal Ca^{2+} and caspase-3 activity response may be due to the increased intrinsic NHE1 activity offsetting the decrease in response to ERK1/2-mediated H_2O_2 stimulation. A decrease in NHE1 activity would decrease Ca^{2+} overload and caspase-3 activity in a manner similar to a decrease in NHE1 content. Extrapolating the buffering capacity results in Chapter IV to pH_i values typically reached during ischemia-reperfusion suggests that NHE1 activity would be decreased at those pH_i values and, thus, protective. Therefore, further investigation of Ca^{2+} levels and caspase-3 activity at pH_i values

observed during ischemia-reperfusion are warranted and would provide more detailed insight of NHE1 as an exercise-induced cardioprotective mechanism. Due to high variability in caspase-3 activity measurements on the luminometer, an inability to detect caspase-3 cleavage via Western blotting, and various misfortunes using the microscope, the story is incomplete.

Future directions of this project include observing NHE1 activity in myocytes at pH_i more representative of that obtained during ischemia-reperfusion, examining other pH_i-regulating mechanisms of the heart cell following exercise, and using NMR in combination with an isolated, working-heart model to assess real time Na^+, H^+, Ca^{2+} changes following an ischemic insult. Using a true ischemia-reperfusion model on the whole heart or other IR-like models on isolated cells will extend the protective characteristics of NHE1. Additionally, examining a dose response between H_2O_2 and ERK1/2 phosphorylation at lower H_2O_2 concentrations than those used herein will help further characterize any exercise-induced effect on ERK1/2 signaling. Further investigation of NHE1 in the exercised heart is warranted, because mechanisms that regulate pH_i in the myocyte are not well studied and the cardioprotective potential of NHE1 is so well documented.

Figure 5.1. Cytosolic Ca^{2+}.

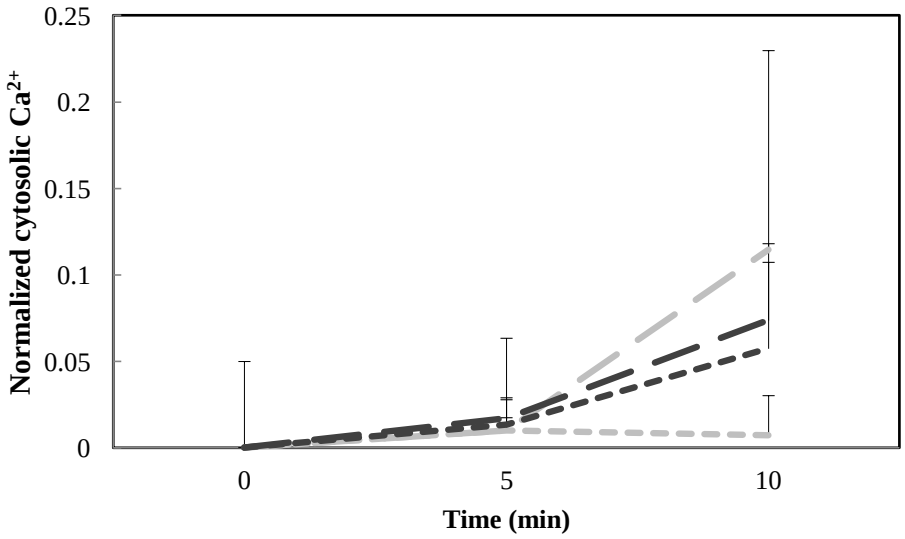

Figure 5.1. The effect of exercise-training induced changes to NHE1 on cytosolic Ca^{2+} content in the presence or absence of 100 μM H_2O_2. S control (light, short dash); S H_2O_2 (light, long dash); E control (dark, short dash); E H_2O_2 (dark, short dash). There were no between-group differences.

Figure 5.2. Caspase-3 activity.

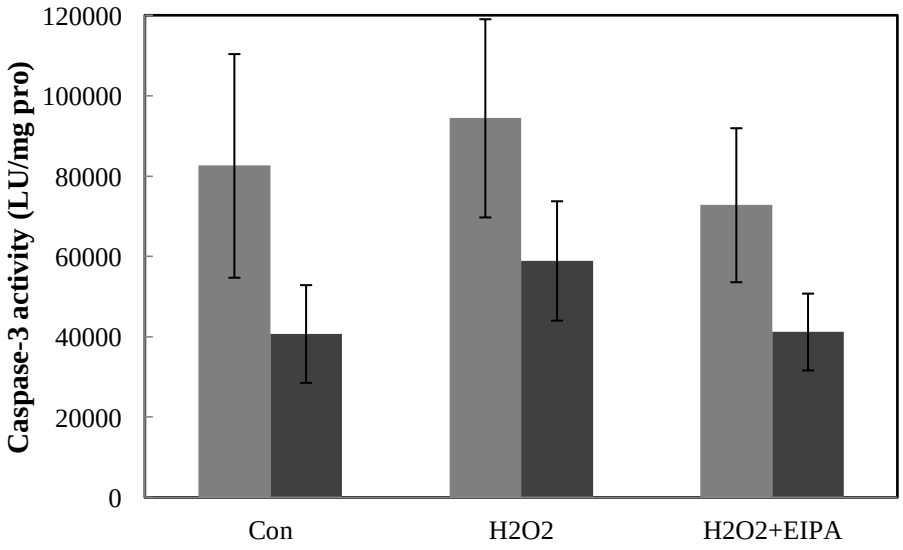

Figure 5.2. The effect of exercise-training induced changes to NHE1 on caspase-3 activity in the presence or absence of 100 μM H_2O_2. S is light grey and E is dark grey. There were no between-group differences.

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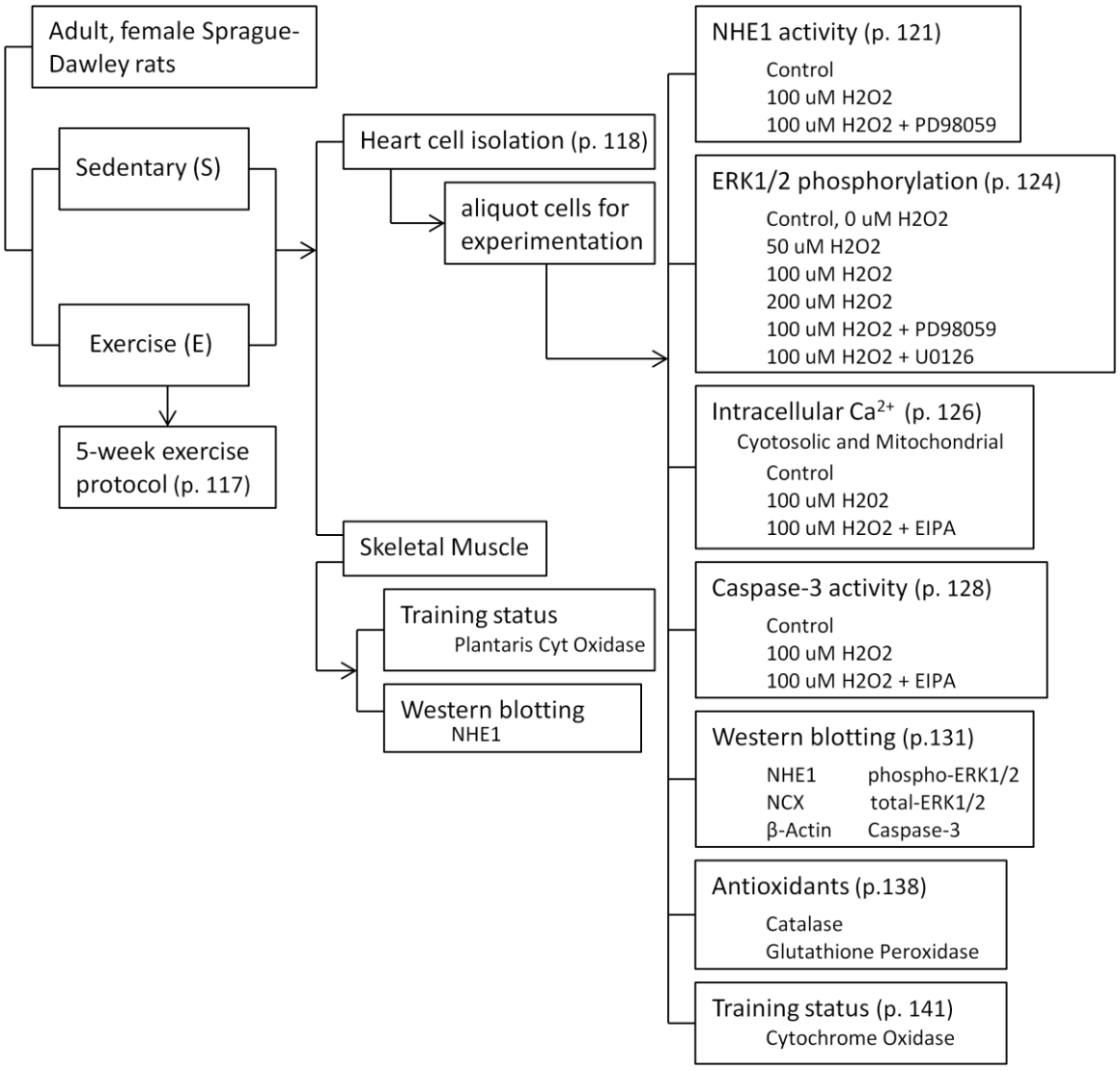
APPENDIX A

OUTLINE FOR STUDY PROCEDURE



APPENDIX B

ANIMAL USE

Description of use. During the duration of this project, approximately 30 animals were utilized. Animals were purchased at 4 months of age and evaluated approximately 3-7 weeks after arrival. Exercise trained rats were run on a motorized treadmill for 5 weeks in the temperature controlled, all-purpose room of Stone. Rats were run in the 8C room for the final 4 weeks of the protocol. On the day of experiment, rats were transported from their housing room in Stone 126 to the laboratory of Dr. Joseph W. Starnes in Eberhart 215. Experiments were 24 hours following the last exercise bout. In Dr. Starnes' lab the animals were anesthetized with an intraperitoneal injection of sodium pentobarbital at 40 mg/kg body weight, which prevented any conscious response during surgery. The heart was then excised from the anesthetized animal, the heart cells were isolated, and all experiments carried out on the isolated heart cells.

Justification of use. Exercise consistently provides protection to the heart following a bout of ischemia-reperfusion. This exercise-induced beneficial adaptation may be at least partially responsible for the increased survivability of heart attacks reported in physically active humans and the decreased risk of having a heart attack during physical activity in people who regularly exercise. Many intrinsic mechanisms have been studied; however, the Na+/H+ exchanger-1 (NHE1) has not been examined. NHE1 is a sarcolemmal protein implicated in intracellular pH regulation. Increases in this protein's content or activity result in greater damage to the heart and heart cell following ischemia-reperfusion; however, inhibition of NHE1 activity or removal of its content consistently provides protection against heart attack injury. Thus, suppression of NHE1 seems cardioprotective. We have previously used the same strain, gender, age of rat to show that 5 weeks of exercise treadmill training results in a 38% decrease in NHE1 content. However, how this exercise-induced decreased affects heart attack injury or how it plays a role in exercise-induced cardioprotection is not known. Therefore, the overall specific aim of this research project was to determine the role NHE1 in the attenuation of H_2O_2-induced injury. Finding ways to protect the heart during ischemic episodes is important as the occurrence of these episodes may lead to myocardial infarction, which is the leading cause of death in the United States. Human subjects are inappropriate because the end-point of the experiments requires that the heart be damaged. Non-animal species are inappropriate because they do not have a heart that is similar to that of a human. The rat is deemed most appropriate because of the prior studies carried out on this specie, ease of handling, and expense.

Information on veterinary care. Animals were housed in a central animal care facility under the direction of veterinarian specializing in laboratory animals. The University of North Carolina at Greensboro has an animal welfare assurance letter on file with NIH (#A 3706).

Limited injury and use of anesthetic drugs. There is modest risk of injury while exercising on the rodent treadmill. This risk was minimized by first acclimatizing the rats to the treadmill at sub-maximum intensities so that stress and fatigue was not a factor. If a rat refused to run, it was removed from the study. The only injury experienced by a rat was a cut toenail, and the animal was removed from the treadmill and antibiotic cream was administered to the toe area. The rats were anesthetized with sodium pentobarbital at a dosage of 40 mg/kg body weight, administered intraperitoneally by Bryan Feger, who has been trained in this procedure by Dr. Joseph Starnes, who has been routinely carrying out this procedure since 1976.

Method of euthanasia. Removing the heart from the anesthetized animal produced a euthanizing effect. This method is consistent with the recommendations of the Panel on

Euthanasia of the American Veterinary Medical Association. Dead animals were placed in the morgue freezer located in the Animal Care Facility in Eberhart.

APPENDIX C

EXERCISE PROTOCOL

Week 1 – at room temperature
Day 1 – 15 min at 15 m/min at 6% grade
Day 2 – 25 min at 20 m/min
Day 3 – 25 min at 20 m/min; 5 min at 25 m/min
Day 4 – 20 min at 20 m/min; 10 min at 25 m/min
Day 5 – 15 min at 20 m/min; 15 min at 25 m/min
Week 2 – at 8°C, refrigerated room
Day 6 – 20 min at 20 m/min; 10 min at 25 m/min
Day 7 – 30 min at 25 m/min
Day 8 – 35 min at 25 m/min
Day 9 – 40 min at 25 m/min
Day 10 – 45 min at 25 m/min
Week 3 – at 8°C, refrigerated room
Day 11 – 50 min at 25 m/min
Day 12 – 55 min at 25 m/min
Day 13 – 60 min at 25 m/min
Day 14 – 60 min at 26 m/min
Day 15 – 60 min at 27 m/min
Week 4 – at 8°C, refrigerated room
Day 16 – 60 min at 28 m/min
Day 17 – 60 min at 29 m/min
Day 18 – 60 min at 30 m/min
Day 19 – 60 min at 30 m/min
Day 20 – 60 min at 30 m/min
Week 5 – at 8°C, refrigerated room
Day 21 – 60 min at 30 m/min
Day 22 – 60 min at 30 m/min
Day 23 – 60 min at 30 m/min
Day 24 – 60 min at 30 m/min
Day 25 – 60 min at 30 m/min

APPENDIX D

HEART CELL ISOLATION

Solutions:

Krebs-Heinslet buffer (KHB), stock (10x), 1L

KH_2PO_4	1.62 g
KCl	3.54 g
NaCl	69.24 g

Dissolve in 1 L dH_2O
Store at RT

KHB, working solution, 400 mL

KHB Stock	40 mL
ddH_2O	360 mL
$MgSO_4$ (0.48 M)	1 mL
Glucose	0.72 g
$NaHCO_3$	0.83 g
Taurine	1 g

Make fresh daily

Incubation buffer, 1 L

KHB Stock		100 mL
BME amino acids		20 mL
MEM non-essential amino acids		10 mL
BME vitamin solution		10 mL
$MgSO_4$ (0.48 M)	2.5 mL	
HEPES	7.15 g	
Taurine	7.5 g	
Creatine	2.62 g	
$NaHCO_3$	2.08 g	
Glucose	1.8 g	

Dissolve in 1 L dH_2O
Aliquot into 50 mL conical tubes
Store at -20°C

500 mM $CaCl_2$
250 mM $CaCl_2$
1 and 2 N HCl

Procedure:

1. Turn on circulating water through perfusion system at 37°C, pH meter, and shaking water bath at 37°C.
2. Thaw 1 tube of incubation buffer in shaking water bath.
3. Prepare KHB working solution; add $NaHCO_3$ first.
4. Put spin bar in cylinder and stir on stir plate until solutes are dissolved. Be careful to make sure all $NaHCO_3$ is dissolved. Remember to bring to 400 mL.
5. Measure 0.5 g BSA in 50 mL Erlenmeyer flask.
6. Measure 100 mg collagenase type II and place in 20 mL beaker.

7. Put 1 more 50 mL Erlenmeyer and 1 more 20 mL beaker at work station.
8. Filter KHB solution using vaccum filter and 0.2 um, 47 mm circular filters.
9. Pour KHB solution into 400 mL Erlenmeyer flask.
10. Pour 150 mL into a graduated cylinder and oxygenate.
11. Add 375 uL of 500 mM $CaCl_2$ (1.25 mM) to graduated cylinder.
12. Add Ca-free KHB to left reservoir and Ca-containing to right reservoir.
13. Place bubblers in both reservoirs, and the pH electrode in the Ca-free.
14. Fill Langendorff tubing with necessary solutions. Be careful to make sure there are no air bubbles in the lines, and set-up bubble trap.
15. Anesthetize rat with intraperitoneal injection of ~0.3 mL of 50 mg/mL Na-pentobarbitol.
16. Make sure there is cold saline in small cup for heart isolation.
17. When asleep, weigh rat on balance and record weight.
18. Cut rat transversally below diaphragm and inject inferior vena cava with 0.1 mL heparin. Get saline in cup to tare.
19. Cut up both sides of the rib cage and cut the aorta to excise the heart.
20. Blot outside of heart with tissue and place in tared saline to get the weight of the heart. Record the weight.
21. With a slow drip, quickly mount the heart onto the canula and clip with gator clip, and quickly open flow to the heart with Ca-containing solution.
22. Tie aorta to canula with thread, cut pulmonary artery, and move drip reservoir to surround heart.
23. After 5 min of Ca-containing solution, switch flow to Ca-free solution.
24. Dissolve 100 mg collagenase type II in 10 mL Ca-free solution.
25. When the Ca-free buffer falls to the 100 mL line, add the 10 mL collagenase solution to the reservoir with the 12 or 20 mL syringe and 0.45 mm filter, and reduce amount of bubbling to attenuate foam buildup.
26. After 1.5 min, close drip reservoir to start recirculating collagenase solution. During recirculation, the collagenase reservoir must constantly be titrated with 2N HCl to keep the pH at ~7.4. Add drop of HCl on top of bubbles, so that it will disperse more evenly.
27. Dissolve BSA in incubation buffer; pour 50 mL of buffer into Erlenmeyer and place Erlenmeyer into water bath.
28. Watch for the heart to swell and become very soft. After ~20-30 min, the flow from the heart will increase markedly. When this happens, allow ~10 mL to drip into a 100 mL beaker and pH to 7.4.
29. Remove left atrium, cut ventricles from heart and place in the 100 mL beaker with collagenase (from step 27). Stop flow through canula to save buffer.
30. Mince ventricles into 6 pieces and place beaker in shaking water bath. Allow to shake at 50 rpm.
31. Collect ~50 mL of collagenase and pH to 7.4 with 1 N HCl. Add ~35-40 mL of this solution to the beaker containing the ventricles.
32. Add the pH electrode and stick bubbler to the 100 mL beaker with ventricles. Be careful to keep pH at ~7.4, but only use 1N HCl; usually 1 drop at pH 7.6 will work.
33. After 5 min, gently swirl and shake the beaker with ventricles. Use wide-tipped plastic transfer pipette to agitate the tissue and disperse the cells. Triturate the tissue 10 times. After 5 more min, repeat. Then, perform a 7-10 min trituration. Be gentle! Remember to keep watch of pH changes following trituration. If there is still enough tissue, continue to triturate it in incubation buffer, while the already dispersed cells settle in the digestion buffer.

34. Filter cells through mosquito netting into a 50 mL conical tube.
35. Allow cells to settle for 5 min.
36. Check pH of incubation buffer, and then remove supernatant from filtered cells, and add ~20 mL of incubation buffer.
37. Gently rock tube to re-suspend cells, and allow to settle for ~5 min.
38. Remove supernatant, add ~15 mL of incubation buffer.
39. Gently rock tube to re-suspend cells, and allow to settle for ~5 min.
40. Remove supernatant, add 10 mL of incubation buffer if adding calcium back to cells. If not, then add needed amount of Ca-free Tyrodes to cells, filter, and use.
41. With 10 mL of cells, place these cells in a 50 mL Erlenmeyer and place in water bath. To these cells add 250mL $CaCl_2$ every 5 min to gradually restore Ca^{2+} concentration to 1 mM: 4, 4, 4, 8, 10, 10 uL.

42. After adding last calcium addition, add 7 mL of 1 mM Ca incubation buffer and filter cells. Ca-containing cells then can be settled, supernatant removed, and resuspended in 1 mM Tyrodes (Appendix G – Intracellular Ca^{2+}).
43. Ca-free cells can be plated onto laminin-coated coverslips (Appendix E – NHE1 Activity) or left in suspension.

APPENDIX E

NHE1 ACTIVITY

Solutions:

Tyrodes stocks

NaCl	4.5 M	1000 mL
KCl	1.54 M	500 mL
HEPES, pH 7.5	0.386 M	500 mL
Glucose	1.15 M	500 mL
$MgCl_2$	0.240 M	500 mL

Ca-free Tyrodes, 1L

NaCl	30 mL	135 mM
KCl	3.83 mL	5.9 mM
HEPES	30 mL	11.6 mM
Glucose	10 mL	11.5 mM
$MgCl_2$	5 mL	1.2 mM

Bring to 1 L with dH2O
Check for pH 7.4

Laminin stock, 50 ug/mL

Laminin, 1 mg (Sigma, L2020)
add 1 mg to 20 mL of Tyrodes
Store aliquots at -20°C; keep usable in 4°C

BCECF-AM stock, 5.625 mM

BCECF-AM, 1 mg (Invitrogen, B1150)
220 uL of DMSO into 1 mg vial
Store aliquots at -20°C

Pluronic F127 stock

Pluronic F127 (Invitrogen P6866)

NH4Cl stock, 500 mM

2.675 g NH4Cl into 100 mL ddH2O
Store at 4°C

PD98059 stock, 20 mM

PD98059, 5 mg (Sigma, P215)
900 uL of DMSO into 5 mg vial
Store aliquots at -20°C

DMSO stock

DMSO (Calbiochem, 317275)

H_2O_2 stock, 10 mM

1.13 mL from 882 mM stock (in 4°C) into 100 mL
Store at 4°C

Nigericin stock, 2 mg/mL

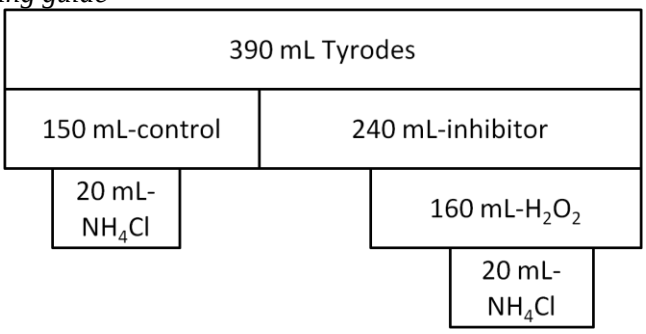
Nigericin, 10 mg, (Invitrogen, N1495)
Dissolve 10 mg in 5 mL of ethanol
Store at -20°C

Standards, 100 mL

NaCl	5 mM

KCl 130 mM
HEPES 50 mM
pH 6.8, 7.3, 7.8

Solution-making guide



The inhibitor is the most complex; the others simply need to make sure an aliquot is used for NH_4Cl pulse.

Procedure:

Before isolation

1. Place 2 coverslips (Thomas Scientific, 6663-F10, 11x22, #1) side-by-side in a 35-mm culture dish (Corning, 430588, non-treated).
2. Add 80 uL of Laminin stock to each coverslip; use pipette tip to spread.
3. Set out all solutions so they can warm to room temperature.

Post isolation

4. Transfer 3 mL of cells to each culture dish.
5. Allow 30 min for cells to settle.
6. Using transfer pipette, slowly remove supernatant with floating cells and add back 2 mL of normal Tyrodes.
7. Allow 30 min for cells to equilibrate.
8. Add 1.1 uL of BCECF-AM stock and 1.0 uL of Pluronic F127 to 5 mL of Tyrodes in a 15-mL conical tube.
9. Decant Tyrodes from dish, and add 2.5 mL of BCECF-AM Tyrodes; place remaining 2.5 mL back in drawer to keep in dark.
10. Put dish on shaker underneath box top (to keep dark), and rock at tilt 2 for 15 min.
11. While cells are incubating, turn on computer and fluorometer. Set-up software as described under Software Setup.
12. Load perfusion line (into fluorometer) with appropriate solution; fill three cuvettes each with 2.5 mL of one standard; put cuvette with 2.5 mL of Tyrodes and cuvette holder by workstation.
13. After 15 min of loading, decant BCECF-AM Tyrodes and add fresh Tyrodes. Allow 15 min in dark (under box top) for probe to de-esterify.
14. Grab coverslip with adhered cells using skinny tweezers and place into cuvette holder; have cells facing the excitation light beam.
15. Place cuvette into single-cell holder of fluorometer with cells facing excitation light beam, and slowly insert perfusion system into cuvette; the in-flow tip should be on the side of the coverslip with no cells.
16. Start Ratio software program.

17. Turn on in-flow pump; only flip toggle because the flow rate is set. Turn on out-flow pump at a speed higher than in-flow.
18. When the run is complete, add 5 uL of Nigericin to each standard, pause the software, place the coverslip into a standard and read for ~30 seconds. Repeat for remaining standards.

Software Setup

Click on **Setup** button

Cary tab

Instrument setup:	Data mode → Fluorescence	
Wavelength setup:	Collection type → Excitation fast filter	
	Em. Wavelength (nm) → 535	
Collect timing:	Simple collect → filled	
	Ave time (s) → 1	
	Stop (min) → 55	

Options tab

Emmission filter → Auto

Accessories tab

→ Fast Filter

Fast filter:	Result 1 → Check (Filter 3/Filter 1)	
Filter 1:	Name → BCECF	Wavelength → 440
Filter 3:	Name → BCECF	Wavelength → 490

Analyze tab

Nothing

Reports tab

X-Y Pairs table → Check

Auto-store tab

Storage:	On; prompt at end → filled
Auto-convert:	ASCII (csv) → filled

Click **OK** for instrument to set itself

APPENDIX F

ERK1/2 PHOSPHORYLATION

Solutions:

Tyrodes stocks

NaCl	4.5 M	1000 mL
KCl	1.54 M	500 mL
HEPES, pH 7.5	0.386 M	500 mL
Glucose	1.15 M	500 mL
$MgCl_2$	0.240 M	500 mL

Ca-free Tyrodes, 1L

NaCl	30 mL	135 mM
KCl	3.83 mL	5.9 mM
HEPES	30 mL	11.6 mM
Glucose	10 mL	11.5 mM
$MgCl_2$	5 mL	1.2 mM

Bring to 1 L with dH2O; Check for pH 7.4

U0126 stock, 10 mM

U0126, 1 mg (Sigma, U120-1MG)
234 uL of DMSO into 1 mg vial
Store at 4°C

PD98059 stock, 20 mM

PD98059, 5 mg (Sigma, P215)
900 uL of DMSO into 5 mg vial
Store aliquots at -20°C

H_2O_2 stock, 10 mM

1.13 mL from 882 mM stock into 100 mL
Store at 4°C

DMSO stock

DMSO (Calbiochem, 317275)

Lysis Buffer

NaCl	50 mM
NaF	50 mM
Na pyrophosphate	50 mM
EDTA	5 mM
EGTA	5 mM
Na_3VO_4	3 mM
phenylmethylsulfonyl Fl	0.5 mM
HEPES	10 mM
Triton X-100	0.1 %
Leupeptin	10 ug/mL

Store at 4°C

Procedure:

1. Aliquot 3 mL of Ca-free cells into each treatment conical tube.
2. Add 1.5 uL of DMSO, PD98059, or U0126 to cells; in doing multiple it is necessary to add with 1-2 min between each addition.
3. Keep tube on side for 10 min.
4. Add 30 uL of H_2O_2 stock to the 3 mL of cells, if necessary.
5. Put tube upright so cells can settle during 5 min perturbation.
6. Decant supernatant and add fresh, H_2O_2-free Tyrodes.
7. Keep tube on side for 5 min; turn tube upright for final 5 min so cells can settle.
8. Decant supernatant, add 100 uL of cold Lysis Buffer, and put in -80°C freezer.
9. Upon thawing for Western blotting (Appendix G), vortex for 10 seconds, spin at 10,000 *xg* at 4°C for 30 min, and keep supernatant for protein determination (via Lowry, Appendix I).

APPENDIX G

INTRACELLULAR Ca^{2+}

Solutions:

Tyrodes stocks

NaCl	4.5 M	1000 mL
$KCl/CaCl_2$	1.18/0.3 M	500 mL
HEPES, pH 7.5	0.386 M	500 mL
Glucose	1.15 M	500 mL
$MgCl_2$	0.240 M	500 mL

Ca-containing Tyrodes, 1L

NaCl	30 mL	135 mM
$KCl/CaCl_2$	5 mL	5.9/1.5 mM
HEPES	30 mL	11.6 mM
Glucose	10 mL	11.5 mM
$MgCl_2$	5 mL	1.2 mM

Bring to 1 L with dH2O
Check for pH 7.4

Fluo 4-AM stock, 4 mM

Add 11.4 uL of DMSO to 50 ug
Store at -20°C

Rhod 2-AM stock, 4 mM

Add 11.1 uL of DMSO to 50 ug
Store at -20°C

EIPA stock, 10 mM

EIPA, 25 mg (Sigma, A3085)
8.3 mL of DMSO into 25 mg vial
Store at 4°C

Pluronic F127 stock

Pluronic F127 (Invitrogen P6866)

H_2O_2 stock, 10 mM

1.13 mL from 882 mM stock into 100 mL
Store at 4°C

DMSO stock

DMSO (Calbiochem, 317275)

Procedures:

Before microscopy

1. To 3 mL of Ca-containing Tyrodes, add 1.5 uL of Fluo 4-AM stock (for cytosolic), 3 uL of Rhod 2-AM (for mitochondrial), and 1.5 uL of Pluronic F127. Add this to 3 mL of cells.
2. Let cells incubate for 30 min in dark at room temperature on rocker; have cells settle in remaining 5 min of incubation period.
3. Remove supernatant and add 2 mL of fresh Tyrodes.
4. Plate 200 uL of cells to the coverslip bottom of the culture dish.
5. Allow 30 min for cells to settle (or drive to JSN).

6. Add 2 mL of Tyrodes to dish and subsequently remove to wash off any floating cells; then add 2 mL of Tyrodes back to dish.

Microscopy of cells

7. Turn on computer and microscope using the numbers and associated power buttons.
8. Open the AxioVision software.
9. Place dish onto microscope stage and make sure it is secure; add tape to back of dish.
10. In *Acquisition*, turn on FITC and RHOD (widefield view) to observe cells and find
11. Once cells are found, turn on FITC SD and RHOD SD (spinning disk) and *Measure* for exposure time. Find the best plane for fastest exposure time (i.e. milliseconds).
12. Set time and number of exposures.
13. Add EIPA or DMSO and begin recording.
14. Add H_2O_2 when necessary.
15. To remove the H_2O_2-treated media and add fresh Tyrodes, quickly push back lens head and use transfer pipettes to change media. Be very careful not to move the dish or add media back so harshly that cells wash off. Then, quickly and carefully re-set lens head for next image to be taken.
16. Save images so data can be analyzed later with Image J.

APPENDIX H

CASPASE-3 ACTIVITY

Solutions:

Tyrodes stocks

NaCl	4.5 M	1000 mL
$KCl/CaCl_2$	1.18/0.3M	500 mL
HEPES, pH 7.5	0.386 M	500 mL
Glucose	1.15 M	500 mL
$MgCl_2$	0.240 M	500 mL

Ca-containing Tyrodes, 1L

NaCl	30 mL	135 mM
$KCl/CaCl_2$	5 mL	5.9/1.5 mM
HEPES	30 mL	11.6 mM
Glucose	10 mL	11.5 mM
$MgCl_2$	5 mL	1.2 mM

Bring to 1 L with dH_2O; Check for pH 7.4

EIPA stock, 10 mM

EIPA, 25 mg (Sigma, A3085)
8.3 mL of DMSO into 25 mg vial
Store at 4°C

H_2O_2 stock, 10 mM

1.13 mL from 882 mM stock into 100 mL
Store at 4°C

DMSO stock

DMSO (Calbiochem, 317275)

Lysis Buffer

NaCl	50 mM
NaF	50 mM
Na pyrophosphate	50 mM
EDTA	5 mM
EGTA	5 mM
Na_3VO_4	3 mM
phenylmethylsulfonyl Fl	0.5 mM
HEPES	10 mM
Triton X-100	0.1 %
Leupeptin	10 ug/mL

Store at 4°C

Procedures:

1. Aliquot 3 mL of Ca-containing cells into 15-mL conical tube.
2. Add 1.5 uL of DMSO or EIPA to cells; in doing multiple it is necessary to add with 1-2 min between each addition.
3. Keep tube on side for 10 min.
4. Add 30 uL of H_2O_2 stock to the 3 mL of cells, if necessary.
5. Put tube upright so cells can settle during 5 min perturbation.

6. Decant supernatant and add 500 uL of fresh, H_2O_2-free Tyrodes (w/DMSO or EIPA).
7. Keep tube on side for 10 min.
8. Add reagents from Promega Caspase-3/7 Glo Assay in equal portions – 90 uL.
9. Aliquot 90 uL, 3X from one tube into a 96-well plate. And follow directions from manufacturer.
10. Put conical tube upright so remaining cells can settle.
11. Decant supernatant, add 50 uL of cold Lysis Buffer, and put in -80°C freezer.
12. Upon thawing for Western blotting (Appendix G), vortex for 10 seconds, spin at 10,000 *xg* at 4°C for 30 min, and keep supernatant for protein determination (via Lowry, Appendix I).

APPENDIX I

LOWRY'S METHOD FOR DETERMINING PROTEIN CONCENTRATION

Solutions:

Protein Standard

For 10 mL of 1 mg/mL stock:

10 mg Bovine Serum Albumin (Sigma, A-4378) in dH_2O

Solution A, 2 L

40 g Na_2CO_3, 8 g NaOH, and 0.4 g NaK tartrate in dH_2O

Store in dark

Solution B, 1 L

5 g $CuSO_4$, $5H_2O$ in dH_2O

Store in dark

Solution C

50 parts Solution A to 1 part Solution B

Prepare fresh

Folin Ciocalteau's Phenol Reagent (Sigma, F-9252)

Dilute 5 parts of reagent with 6 parts dH_2O.

Procedure:

1. Standard Curve

   Prepare duplicate samples in the following manner:

[mg/mL]	1 mg/mL BSA stock (µl)	dH_2O (µl)	Final Volume (µl)
0.00	0	500	500
0.03	15	485	500
0.06	30	470	500
0.09	45	455	500
0.12	60	440	500
0.15	75	425	500
0.18	90	410	500
0.21	105	395	500

   * note concentrations at 0.18 and 0.21 mg/mL are probably out of range make sure std curve does not begin to level off if using these concentrations.

2. Experimental Samples, Each sample is done in duplicate. Add 490 µl of dH_2O, 10 µl of sample, and 2.5 mL of Solution C.
3. Initiation of reaction and reading, add 0.5 mL of diluted Folin Ciocalteau's Phenol Reagent to each sample and vortex. Incubate at room temperature for 30 minutes, with timing starting upon the first addition of the reagent. Read O.D. at 660 nm.

APPENDIX J

WESTERN BLOTTING – SDS-PAGE

Solutions:

30% Acrylamide/Bis Solution, 29:1
Stock solution (Bio-Rad, 161-0156)

1.5 M Trizma
45.42 g Trizma Base in 250 mL dH_2O, pH 8.8

1.0 M Trizma
30.29 g Trizma Base in 250 mL dH_2O, pH 6.8

20% SDS (sodium dodecyl sulfate)
Stock solution (Bio-Rad, 161-0418)

TEMED
Stock solution of N, N, N', N' - tetramethylethylenediamine (Sigma, 87689)

Ammonium Persulfate (APS)
For 1 mL of 10%
100 mg APS in dH_2O
Prepare fresh daily

10X Running Buffer, 1 L

Trizma	30.28 g
Glycine	144.2 g

in dH_2O

1X Running Buffer, 1 L

10X Running Buffer	100 mL
20 % SDS	5 mL

in dH_2O

β-Mercaptoethanol
Stock from Fisher (BP176-100)

Dithiothreitol (DTT)
For 10 mL of 2 M
3.086 g in dH_2O

4X Sample Buffer

	4X	Final 1X Concentration
1.25 M Trizma, pH 6.8	10 mL	62.5 mM
Glycerol	20 mL	10.0%
20% SDS	10 mL	1.0%
bromophenol blue	0.008 g	0.004%
ddH_2O	5 mL	

To use sample buffer, add 100 µl of β-Mercaptoethanol (or 2 M DTT) to 900 µL of 4X sample buffer.
Then use 1:3 with sample (i.e. 80 uL sample with 20 uL buffer).

Resolving Gel, 2 gels

	10 %	12 %	8 %
30% Acrylamide/Bis	3.3 Ml	4.0	2.7
1.5 M Tris, pH 8.8	2.5 mL	2.5	2.5
ddH_2O	4.0 mL	3.3	4.6
20 % SDS	100 uL	100	100
10 % APS**	100 uL	100	100
TEMED**	4.0 uL	4.0	6.0

** immediately prior to casting

Stacking Gel, 2 gels

	5%
30 % Acrylamide/Bis	1.7 mL
1.0 M Tris, pH 6.8	1.25 mL
ddH_2O	6.8 mL
20 % SDS	100 uL
10 % APS**	100 uL
TEMED**	10 uL

**immediately prior to casting

Procedure:

1. Assemble gel apparatus according to manufacturer's instructions in casting stand. Prepare resolving gel solution for number of gels to be cast in 15 mL conical tube.
2. Gently swirl solution and then use a Pasteur transfer pipette to fill caster 3/4 full – up to black mark. Overlay resolving gel with 200 µl of butanol and allow gel to polymerize.
3. Pour butanol off the resolving gel and rinse with dH_2O.
4. Prepare stacking gel solution for the number of gels cast, gently swirl solution, pour on top of the resolving gel and insert Teflon comb while avoiding bubbles. Allow 1 hour for polymerization.
5. While the stacking gel is polymerizing prepare samples. Samples that were homogenized 1:10 in homogenization buffer are further diluted 1:3 with 4X sample buffer, vortexed for 10 seconds, and allowed to incubate 20 min at room temperature. (Samples that were prepared through affinity chromatography will have been prepared in 1X sample buffer.) Place samples in boiling water for 5 min prior to loading to help SDS coat proteins.
6. Following polymerization of the stacking gel, assemble the gel apparatus for electrophoresis and place in the electrophoresis chamber. Fill the inner chamber with running buffer and bring the outer chamber to 1/4 full with running buffer.
7. Use yellow loading guide to load protein samples based on Lowry-determined protein concentrations and appropriate amounts of standard. Electrophorese at a constant 50 mA per 2 gels and a maximum of 200 V and 200 W until the tracking dye runs off the gel.
8. Following electrophoresis, remove clamping frame from mini tank and pour off Running Buffer. Remove gel cassette sandwiches from clamping frame. Separate glass plates using green wedge. Discard stacking gels. Carefully mark an edge of the resolving gel for orientation.
9. Carefully place resolving gels into Transfer Buffer.

APPENDIX K

WESTERN BLOTTING – WET TRANSFER

Solutions:

Transfer Buffer, 1 L

Trizma	3.03 g
Glycine	14.4 g
Methanol	200 mL
20 % SDS	2 mL

in dH_2O

*SDS for proteins that are hard to elute from gel, like larger proteins; but SDS not needed for smaller proteins that elute easily

10X TBS (Tris buffered saline), 1 L

Tris	24.23 g
NaCl	80.06 g

pH 7.4 in dH_2O

1X TBS (TBS), 1 L

10X TBS	100 mL

in dH_2O

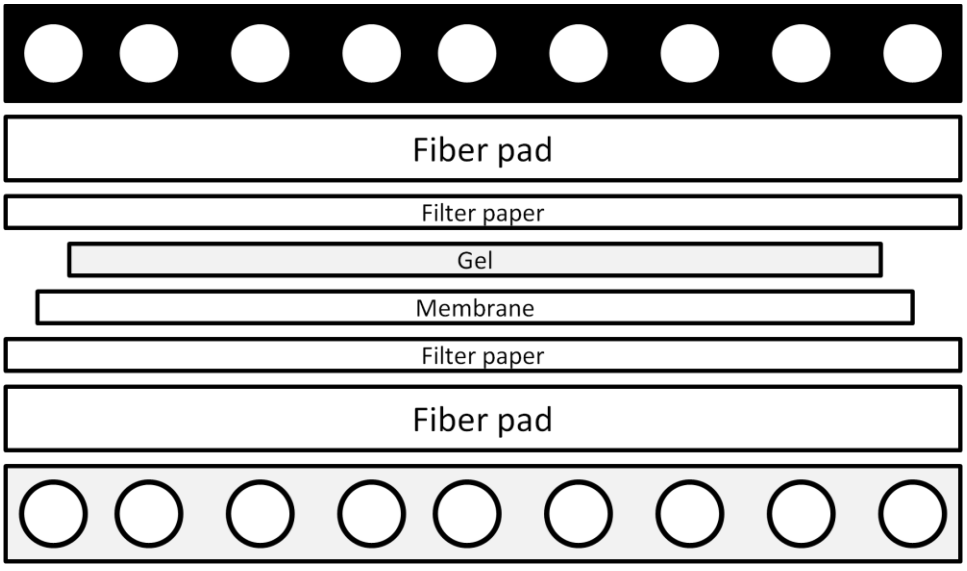
Blocking Solution, 100 mL

Dry Milk	5 g
TBS	100 mL

stir, filter with cheesecloth, 50 mL/membrane

Procedure:

1. Soak in Transfer Buffer for 20 min: 2 gels, 4 fiber pads, 4 filter papers, 2 PVDF membranes.
2. Assemble transfer cassettes in the following manner:



3. Place gel cassettes in electrode module (align black side of cassette with black side of module).
4. Place ice pack and stir bar in mini tank with electrode module.
5. Fill mini tank with remaining Transfer Buffer and put on greed lid (with red to red and black to black).
6. Place mini tank on stir plate and begin spinning and transfer: 350 mA (constant), 100 V, 200 W, 1 hr.
7. Following transfer, place membranes in blocking solution for 1 hr at room temperature on shaker.
8. Optional, check the gel to see if proteins were eluted and the membrane to see if proteins were transferred. Use coomasie blue to stain gel for the proteins. Wash gel 3x5 min with water, then 45 min with coomasie blue, then wash 3x5 min with water. The gel can be stored in water. Use Ponceau Red to stain membrane. The stain can be stripped with 2% v/v acetic acid and water, and does not interfere with immunoblotting.

APPENDIX L

WESTERN BLOTTING – IMMUNOBLOTTING

Solutions:

10X TBS (Tris buffered saline), 1 L

Tris 24.23 g
NaCl 80.06 g
pH 7.4 in dH_2O

1X TBS-0.1% Tween (TBST), 1 L

10X TBS 100 mL
Tween 20 1 mL
in dH_2O

Primary antibody (1° Ab)

Dilute 1° Ab to optimal conc. (Appendix J) in TBST with 5% BSA (10 mL per gel).

Secondary antibody (2° Ab)

Dilute appropriate HRP-labeled 2° Ab to optimized concentration (Appendix J) in TBST in 5% milk (10 mL per gel); dissolve milk in TBST and then filter through cheesecloth before adding 2° Ab.

SuperSignal solutions

West Pico Chemiluminescent Substrates:
Luminol/Enhancer (Thermo Scientific, 1859675)
Peroxide Buffer (Thermo Scientific, 1859674)

Stripping buffer, 1 L

Glycine 15 g
SDS 1 % (50 mL of 20 %)
Tween 20 10 mL
pH 2.2 in dH_2O (w/ conc. HCl)

Procedure:

1. Block PVDF membranes for 1 hr at room temperature 1X TBS with 5% milk; dissolve milk in TBS and then filter through cheesecloth before adding membrane.
2. Rinse the membranes with two quick washes of TBST.
3. Incubate membranes with 1° Ab overnight at 4°C on shaker.
4. Wash 3X5 minutes in TBST.
5. Incubate membranes with 2° Ab for 1-3 hrs at room temperature on shaker.
6. Wash 5X5 minutes with TBST.
7. Prepare the membranes for autoradiography using PIERCE SuperSignal solutions (Pierce Chemical, Rockford, IL) according to manufacturer's instructions; mix 3 mL of each solution and pour on membrane; incubate for 5 min.
8. Turn on computer, Chemidoc, camera.
9. Open QuantityOne software.
10. Open scanner from Volumes Quick Guide
11. Place membranes directly onto surface of Chemidoc, and situate them so that they can be seen by the camera using Live Focus.
12. Freeze the frame and close the door.
13. Use Live Acquire to obtain image; save images that are desirable.

14. When finished, place membranes in TBST for stripping, if necessary (step 18).
15. Use Volume Rectangular Tool or Volume Contour Tool to obtain density of bands.
16. Data can be exported to comma delimited format, and then copy/pasted into Excel for statistical analysis.
17. TURN OFF camera and Chemidoc station!

Stripping of membrane

18. Wash membrane in TBST for 5 min.
19. Incubate membrane in Stripping buffer for 20 min.
20. Wash membrane in TBST for 10 min.
21. Block membrane in TBS with 5% milk, and begin immunoblotting again.

APPENDIX M

BLOTTING OPTIMIZATION

	NHE1	**NCX**	**β-Actin**	**phospho-ERK1/2**	**total ERK1/2**	**Caspase3**
Molecular Weight:	110 kDa	67 kDa	45 kDa	42, 44 kDa	42, 44 kDa	17,35kDa
Lysis Buffer:	Appendix	Appendix	Appendix	Appendix	Appendix	Appendix
Total Protein Load:	100 ug	100 ug		30-50 ug		100 ug
Resolving Gel:	10%	10%		10%		10%
Primary Antibody:	Anti-NHE1 pAb, Rabbit (Millipore, AB3081)	Anti-NCX1 affinity purified pAb, Rabbit (Millipore, AB3516P)	β-Actin pAb, Rabbit (Cell Signaling, 4967)	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) pAb, Rabbit (Cell Signaling, 9101)	p44/42 MAPK (Erk1/2) pAb, Rabbit (Cell Signaling, 9102)	Caspase-3 pAb, Rabbit (Cell Signaling, 9662)
Primary Dilution:	1:360 in 5% BSA-TBST	1:400 in 5% BSA-TBST	1:1000 in 5% BSA-TBST	1:1000 in 5% BSA-TBST	1:500 in 5% BSA-TBST	
Secondary Antibody:	Anti-rabbit IgG, HRP-linked Ab (Cell Signaling, 7074)	Anti-rabbit IgG, HRP-linked Ab (Cell Signaling, 7074)	Anti-rabbit IgG, HRP-linked Ab (Cell Signaling, 7074)	Anti-rabbit IgG, HRP-linked Ab (Cell Signaling, 7074)	Anti-rabbit IgG, HRP-linked Ab (Cell Signaling, 7074)	Anti-rabbit IgG, HRP-linked Ab (Cell Signaling, 7074)
Secondary Dilution:	1:1000 in 5% milk-TBST	1:1000 in 5% milk-TBST	1:1000 in 5% milk-TBST	1:1000 in 5% milk-TBST	1:1000 in 5% milk-TBST	1:1000 in 5% milk-TBST
Exposure time:	3-5 min	3-5 min	4-6 min	4-6 min	3-5 min	
Comments:	Heat sample in block at 55°C; 2 hr 2°Ab incubation	2 hr for 2°Ab incubation; strips fairly well		strips really well; do before total ERK1/2		

APPENDIX N

CATALASE ACTIVITY

Sample Preparation:

1. Pipette 1 mL of tissue homogenate into centrifuge tube.
2. Centrifuge 1,500 x g for 10 minutes at 4°C, retain supernatant for analysis.

Solutions:

Homogenization Buffer

K2HPO4	50 mM
EDTA	0.1 mM
Triton X-100	0.1 %

pH 7.4

H_2O_2 – Hydrogen Peroxide

1 M H_2O_2 stock solution

Procedure:

1. Equilibrate the incubation buffer with N_2 gas in order to decrease O_2 concentration to a very low amount so that the O_2 produced from the catalase reaction will remain in solution during the assay.
2. Add 1.5 mL nitrogen-equilibrated buffer to the oxygen electrode cuvette.
3. Add 15 µL of supernatant and obtain baseline oxygen production rate.
4. Add 15 µL of 1 M H_2O_2 to initiate the reaction and obtain oxygen production rate. (Final concentration of H_2O_2 = 9.8 mM).

Calculations:

Catalase activity in Units/mg heart wet weight.

Where 1 Unit = 1 µmol H_2O_2/min

Net O_2 production rate (Step 4 - Step 3) X 2 (as 2 hydrogen peroxides are consumed for each oxygen produced) divided by amount of tissue in 15 µL.

APPENDIX O

GLUTATHIONE PEROXIDASE (GPx) ACTIVITY

Solutions:

Potassium phosphate buffer	50 mM
with DPTA	0.5 mM
pH 7.0	
NADPH	8 mM
Reduced Glutathione (GSH)	0.1 M
Glutathione Reductase (GSR)	50 U/mL

t-Butyl hydroperoxide (t-BOOH), 30 mM

Procedure:

1. Turn on UV-spectrophotometer to allow UV lamp to warm for ~15 min, and the water bath recirculator at 37°C for 5 min.
2. Set up spectrophotometer to read for 3 min at 340 nm.
3. Add the following to a 1.5 mL cuvette.
    a. 100 uL potassium phosphate buffer
    b. 20.0 uL GSH
    c. 2.0 uL GSR
    d. 26.0 uL NADPH
4. Add 20 uL of sample homogenate supernatant to cuvette.
5. Blank the spectrophotometer.
6. Insert cuvette into cell of spectrophotometer.
7. Add 20 uL t-BOOH to cuvette to begin reaction.
8. Mix contents of cuvette by re-pipetting.
9. Run spectrophotometer.
10. Record absorbance (OD) change for 3 minutes at 340 nm.

Calculations:

GPx/mg Protein – (OD/minute * total volume * 2) / (6.22 * volume of sample * mg protein in cuvette), where:
mg protein in cuvette is calculated by Lowry (Appendix I)

This is designated as the total rate of NADPH consumption. The non-enzyme dependent consumption of NADPH is also measured as above except that the 5 uL of sample is replaced by 5 uL of assay buffer. The rate of enzyme-dependent NADPH consumption is obtained by subtracting the non-enzyme-dependent NADPH consumption rate from the total NADPH consumption rate. GSH peroxidase activity is calculated using the extinction coefficient of 6.22 mM^{-1} cm^{-1}, and expressed as nmoles of NADPH consumed/min/mg of cellular protein.

APPENDIX P

HOMOGENIZATION PROTOCOL FOR TISSUE

Solutions:

Homogenization Buffer

K_2HPO_4	50 mM
EDTA	0.1 mM
Triton X-100	0.1 %
pH 7.4	

Procedure:

1. Trim approximately 150 mg of left ventricle from frozen heart.
2. Weigh tissue, and place remaining tissue in -20°C freezer.
3. Calculate buffer volume as: mass of tissue in grams X 19 = volume in mL of homogenization buffer.
4. Cut tissue into small pieces and place in homogenization tube.
5. Add volume of buffer from step 3 to homogenization tube, and place on ice.
6. Insert Teflon wand and start rotation of wand.
7. Homogenize by making approximately 5 passes of the wand into the tube.
8. Remove wand and inspect for non-homogenized pieces of tissue to put back in tube.
9. Repeat passes 2 more times, keeping tube on ice during and between each step.
10. Strain homogenate through one layer of cheese cloth.
11. Remove number of aliquots necessary for completion of assays and place remaining homogenate in -80°C.
12. Put tissue in -20°C back into -80°C for later.

APPENDIX Q

CYTOCHROME *C* OXIDASE (CYTOX) ACTIVITY

Solutions:

Assay medium

K_2HPO_4	50 mM
EDTA	0.1 mM

pH 7.4
Store at 4°C

Ascorbate solution

TMPD	62 mM
Ascorbate	20 mM

Store at -20°C, in dark bottle (light sensitive)
If solution is not clear, then remake

Ascorbate

Ascorbate	1.25 M

pH 5.6
Store at -20°C in small aliquots

Cytochrome c solution

cytochrome *c*	3.92 mM
Tris	50 mM

pH 7.2
0.243 g cytochrome c to 5 mL Tris
Store at -20°C

Procedure:

1. Before beginning assay, warm needed amount of Assay medium to 37°C.
2. Assemble stirred, closed cuvette with a Clark-type oxygen electrode and connect to a strip chart recorder in a 25C chamber.
3. Open oxygen electrode cuvette and add:
    a. 1.45 mL Assay medium
    b. 15.0 uL of Ascorbate solution
    c. 15.0 uL of 1.25 M Ascorbate
    d. 15.0 uL of Cytochrome c solution
4. Close cuvette and record changes in oxygen tension.
5. Open cuvette and add 30 uL of homogenate supernatant.
6. Close cuvette and record total rate of oxygen consumption.

APPENDIX R

GLOSSARY

Adenosine triphosphate (ATP) – The energy molecule of the cell; its phosphate bonds hold chemical potential energy that drives the processes of the human body.

Antioxidant – A substance that can reduce or destroy oxidants.

Aortic Flow (AF) – The volume of blood flowing through the aorta per minute, normalized for the size of the heart in milliliters per minute per gram of heart wet weight.

Apoptosis – Programmed cell death; the controlled degradation and death of the cell through the use of ATP and specific intracellular signaling.

Calcium overload – a compartmentalized increase in Ca^{2+} concentration above homeostatic levels that leads to cellular damage. The compartmentalization is typically seen in either the cytosol or the mitochondria.

Calpains – A group of Ca^{2+}-dependent proteins that degrade regulatory proteins within the cell.

Cardiac Function (COxSP) – An index of external work that an isolated working heart is performing during perfusion. Calculated as the product of the cardiac output and peak systolic pressure.

Cardiac Output (CO) – The volume of blood the left ventricle of the heart ejects per minute, normalized for the size of the heart. Calculated as the sum of the coronary flow and the aortic flow in milliliters per minute per gram of heart wet weight.

Cardioprotection – Improved intrinsic tolerance of the myocardium to an imposed stress.

Caspase-3 – An enzyme that degrades proteins, which leads to cell death, and is the effector protein of the mitochondrial-mediated cell death pathway.

Catalase (CAT) – The antioxidant enzyme that catalyzes the conversion of hydrogen peroxide to H_2O and O_2.

Coronary Flow (CF) – The volume of blood flowing through the coronary vasculature per minute, normalized for the size of the heart in milliliters per minute per gram of heart wet weight.

Dihydropyridine receptor – A membrane-bound channel that allows Ca^{2+} to enter the cell upon depoloarization.

Efficiency – The quotient of cardiac function (COxSP) and oxygen consumption.

Exercise-induced cardioprotection – The intrinsic protection that acute and/or chronic aerobic exercise confers to the heart.

Free Radicals – A highly reactive class of molecules, categorized by the presence of an unpaired electron in the outer orbital.

Glutathione (GSH) – A tri-peptide with a reduced sulfhydryl group, which is capable of being oxidized by glutathione peroxidase during the conversion of hydrogen peroxide to water.

Glutathione Peroxidase (GPX) – The antioxidant enzyme that catalyzes the conversion of hydrogen peroxide to H_2O.

Heart Rate (HR) - The rate at which the heart contracts, in beats per minute.

Heat Shock Protein 72 (HSP72) – The 72 kilo-Dalton inducible form of a protein in a class of proteins called stress proteins, implicated in the facilitation of cardioprotective mechanisms. The abbreviation HSP70 represents both the constitutive and inducible form of the protein

Hypertension – A chronic state of high blood pressure that causes damage and remodeling of the heart due to the increased workload this situation places on the heart.

Hyperthermia – An increase in body temperature above normal.

Hypertrophy – An increase in the size of the heart. Pathological hypertrophy can result from a chronic load to the heart (i.e. hypertension). Physiological hypertrophy is a result of normal development or exercise, when intermittent increases in workload are presented to the heart.

Ischemia – Period of no-flow in the myocardium during which the delivery of substrates and the removal of metabolic byproducts is prevented.

Ischemic preconditioning – Protection from heart attack injury that is afforded to the cell after receiving short, intermittent bouts of ischemia.

Isolated Heart Perfusion – Procedure for measuring the function of the heart *ex vivo* by providing artificial blood flow to the heart and allowing the heart to pump independently from the organism.

Krebs-Henseleit Buffer – Solution used to perfuse the hearts containing essential ions and substrates at an osmolarity and pH similar to that observed *in vivo*.

Lactate Dehydrogenase (LDH) – The enzyme which catalyzes the reversible reaction from Lactate to Pyruvate. It is normally located in the cytosol of all cells and its presence in the coronary effluent indicates sarcolemma damage, which may lead to cellular necrosis.

Langendorff Perfusion – Isolated heart perfusion in which the heart does not perform any external work, as the heart only contracts against a retrograde perfusion pressure. It is also used to perfuse hearts in order to isolate heart cells.

Lω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) – A competitive inhibitor of all three isoforms of nitric oxide synthase.

Mitochondrial calcium uniporter (MCU) – A mitochondrial-membrane-bound protein that is the only way cytosolic Ca^{2+} can enter into the mitochondrial matrix in normal, physiological situations.

N-(2-Mercaptopropionyl)glycine (MPG) – Pharmacological, potent free radical scavenger.

Myocardium - The heart muscle.

Myocardial infarction (MI) – Dead heart tissue resulting from a heart attack.

Nitric Oxide (NO) – A reactive nitrogen species produced by the action of nitric oxide synthase, which is responsible for signaling vasodilatation and initiating other cell-signaling events.

Nitric Oxide Synthase (NOS) – The enzyme that produces nitric oxide during the enzymatic conversion of L-arginine to L-citrulline. The three predominant isoforms include: eNOS, the endothelial isoform; iNOS, the inducible isoform; and nNOS the neuronal isoform.

Oxidative Stress – A stress in an organism caused by increased production of free radicals or reactive oxygen species, or a decrease in the antioxidant defense system.

Phospholamban – A protein that inhibits the activity of SERCA.

Preconditioning – Any treatment capable of triggering a cardioprotective response in the myocardium that can protect against subsequent stresses. Preconditioning falls into two general categories, the early and late phase. The early phase appears minutes following a treatment, and is generally characterized by post-translational modification of existing proteins. The late phase occurs anywhere from hours to days following treatment, and is generally characterized by de-novo synthesis of proteins.

Potassium-sensitive ATPase (KATP) – A channel in both the plasma and mitochondrial membranes that allows for K^+ to move through it in response to changes in ATP concentration.

Rate pressure product (RPP) – The product of heart rate and systolic blood pressure; one measure of the amount of work a heart is performing.

Reactive Oxygen Species (ROS) – A class of highly reactive molecules, including free radicals, which can cause oxidative stress.

Reperfusion – The period following ischemia, after the occlusion is removed, when oxygen is provided back to the cell and metabolic by-products are removed. Here is when most of the damage to the cell/tissue occurs.

Sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) – A protein on the membrane of the sarco-endoplasmic reticulum that uses ATP to move Ca^{2+} from the cytosol into the organelle.

Sodium-calcium exchanger (NCX) – A membrane-bound protein that exchanges one extracellular Na^{+} for one intracellular Ca^{2+}, which is the forward mode. This protein can act in reverse-mode, which can lead to calcium overload.

Sodium-hydrogen exchanger (NHE) – A membrane-bound protein that exchanges one extracellular Na^{+} for one intracellular H^{+}. The NHE-1 isoform is in the heart.

Sodium-potassium ATPase (NK ATPase) – A membrane-bound protein that exchanges 2 extracellular K^{+} for 3 intracellular Na^{+}. This protein is the major way the cell re-establishes the membrane potential.

Superoxide Dismutase (SOD) – The antioxidant enzyme that catalyzes the conversion of superoxide to hydrogen peroxide. The predominant isoforms include: MnSOD, located in the mitochondria; and CuZnSOD, located in the cytosol of the cell.

Systolic Pressure (SP) – The maximum pressure in the aorta during the contraction of the left ventricle, measured via the placement of a pressure transducer at the level of the aortic valve.

Tachycardia – A rapid increase in heart rate above normal.

Transgenic mice – A genetically altered line of mice; in most cases one gene is altered so that the effect of that gene at the organ or system level can be determined.

Tyrode's Buffer – Solution used to bath heart cells containing essential ions and substrates at an osmolarity and pH similar to that observed *in vivo*.

Unfolded protein response (UPR) – A cellular stress response related to the endoplasmic reticulum; the endoplasmic reticulum typically responds by upregulating protective proteins.

Wild-type mice – The non-genetically altered counterparts of transgenic mice; there is no change in any gene in this mice strains.

Working Heart Perfusion – Isolated heart perfusion in which the heart functions in a physiological, recirculating manner while producing measurable external work.