Endogenous Opioids and Exercise Induced Cardioprotection

by

Lindsey Erin Miller

A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
May 6, 2012

Keywords: Myocardial infarction, Exercise, Opioids, Apoptosis, Necrosis, Cardioprotection

Approved by

John C. Quindry, Associate Professor Kinesiology
Bruce L. Gladden, Professor Kinesiology
David Pascoe, Professor Kinesiology
Rajesh Amin, Assistant Professor Pharmacal Sciences
Dean Schwartz, Associate Professor Anatomy, Physiology, and Pharmacology

Abstract

Acute exercise exposure dramatically decreases tissue injury and cellular death that results during the ischemia–reperfusion (IR) events of myocardial infarction, though the mechanisms aren't fully understood. Existing evidence indicates endogenous opioids are a critical component of exercise-induced cardioprotection, and may be linked to the cardioprotective peptide, calcitonin gene related peptide (CGRP). The specific opioid and/or receptor subtype responsible for these effects is unknown. Based upon pharmacological research, the delta opioid receptor appears to be the most likely opioid receptor involved in cardioprotection. The purpose of this study is to determine if the delta opioid receptor is involved in opioid-mediated exercise-induced cardioprotection. Seventy three rats were randomly assigned into an unstressed (n=32) or IR group (n=41). Animals in the unstressed group performed treadmill exercise, or remained sedentary. The effect of exercise on the mRNA and protein expression of the opioid compound leu-enkephalin, and delta opioid receptor, and CGRP were determined at 0min, 20min, and 120min following exercise. Leu-enkephalin mRNA expression was significantly increased at 0min and 120min following exercise ($p = 0.03$ and $p = 0.021$, respectively). However, no significant differences were found in tissue protein content. Animals exposed to IR were randomly divided into Sham, sedentary (S) or exercise (Ex) groups. A delta-opioid receptor antagonist, Naltrindole, was administered (5 mg/kg i.p injection) 15 minutes prior to exercise in a subset of animals (ExD), and at a corresponding time in a subset of sedentary animals (SD). Twenty-four hours following the final exercise bout, animals received

surgically-induced IR by left anterior descending (LAD) coronary artery ligation in vivo. Heart tissue was collected for determination of infarct area (necrotic tissue death) and apoptosis. Significant between group differences existed for tissue necrosis ($p < 0.0001$). Compared to Sham, S and ExD animals had significantly greater tissue necrosis ($p > 0.0001$, $p = 0.003$), while no difference existed compared to Ex groups. S had significantly increased tissue necrosis compared to Ex ($p = 0.003$), but was not different compared to SD. Significant between group differences existed for tissue apoptosis ($p = 0.013$). Compared to Sham, S had significantly greater level of apoptosis ($p = 0.016$), while no difference existed compared to Ex and ExD. Ex was significantly lower compared to S ($p = 0.035$), but not ExD. No difference existed between S and ExD or SD. These data provide evidence that the delta opioid receptor subtype is involved, at least in part, in exercise induced cardioprotection against tissue necrosis. Further research is needed to clarify the mechanisms involved in this observed protection.

Acknowledgments

I have had the opportunity to meet and work with many wonderful individuals who have had significant impact on me. Specifically, I would first like to thank my Doctoral Advisor, Dr. John C. Quindry. I have been working with Dr. Quindry since my early days as a graduate student, and he has shown dedication, commitment and genuine interest in helping me to realize and achieve my goals on both an academic and personal level. I'd also like to thank each of my committee members, Dr. Bruce Gladden, Dr. Dave Pascoe, Dr. Dean Schwartz, and Dr. Rajesh Amin. They have each been important educators in the classroom and laboratory setting, and have served as role models for my aspiration of future faculty member and mentor. A special thanks to Dr. Amin, also, for helping me learn rtPCR technique, and Gayani Nanayakkara who was also a huge help. I would also like to thank Dr. Thomas Denney, who kindly agreed to serve as my outside reader. My fellow lab members were Graham McGinnis, Bridget Peters, and Christopher Ballmann. Each of them helped with data collection, and also served the important purpose of general lab hilarity and keeping spirits high (even when things were not going as planned). I would also like to thank the Department of Kinesiology and Dr. Mary Rudisill for supporting my research and providing my assistantship. I would like to thank my family for their continued support and encouragement. Lastly, I thank my husband, Adam Miller, who has demonstrated true selflessness and has made many sacrifices in order to support me through the completion of my doctoral degree. I am very lucky to have him- Cheers to my best friend.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ACSM	American College of Sport Medicine
Akt	Serine/threonine kinase (protein kinase B)
ANOVA	Analysis of variance
Atg	Autophagy related homolog
ATP	Adenosine triphosphate
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X protein
Bcl-2	B cell leukemia/lymphoma-2
CAT	Catalase
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CuZnSOD	Copper-Zinc superoxide dismutase
CVD	Cardiovascular Disease
Cx43	Connexon 43
DNA	Deoxyribonucleic acid
DPDPE	[D-Pen^{2,5}] Enkephalin (delta opioid receptor agonist)
dUTP	deoxyuridine-triphosphate
GPx	Glutathione peroxidase

I	Ischemia
IL	interleukin
iNOS	inducible nitric oxide synthase
I.P	Intraperitoneal
IR	Ischemia-Reperfusion
LAD	Left anterior descending
MEAP	Methionine-enkephalin-arginine-phenylalanine
MI	Myocardial Infarction
MIPβ	Macrophage inflammatory Protein β
MIP-2	Macrophage inflammatory Protein-2
Mito K_{ATP}	Mitochondrial ATP-sensitive Potassium Channel
MnSOD	Manganese superoxide dismutase
MPTP	Mitochondrial permeability transition pore
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
NCX	Sodium-Calcium exchanger
PARP	Poly (ADP-ribose) polymerase
Pi	Inorganic phosphate
PKC	Protein kinase C
PLN	Phospholamban
PNS	Peripheral nervous system
POMC	Proopiomelanocortin
R	Reperfusion
RNA	Ribonucleic acid

ROS	Reactive oxygen species
rtPCR	reverse transcriptase polymerase chain reaction
Sarc K_{ATP}	Sarcolemmal ATP-sensitive potassium channel
SERCA	Sarcoendoplasmic reticulum calcium ATPase
Sdf1	Stromal cell-derived factor 1
SOD	Superoxide Dismutase
SR	Sarcoplasmic reticulum
TGF	Tumor growth factor
TNF	Tumor necrosis factor
TUNEL	Terminal deoxynucleotidal transferase dUTP nick end labeling
TTC	Triphenyl tetrazolium chloride
$\dot{V}O_{2max}$	Maximal oxygen uptake
5HD	5-hydroxydecanoate

Chapter 1

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in industrialized nations (72). Although CVD encompasses a variety of conditions, ischemic heart disease is the most prevalent form of CVD, often leading to myocardial infarction (MI), commonly known as a 'heart attack' (72). During ischemia, coronary blood flow is either severely inhibited or completely blocked, the result being ineffectual supply of oxygen and nutrients, and ultimately cardiac dysfunction and tissue death. Blood flow re-establishment following ischemia is termed reperfusion. Although critical for tissue survival, the majority of cellular damage occurs during reperfusion. The damage that occurs during ischemia and reperfusion (IR) is considered a collective pathology.

Severity of IR injury depends on the magnitude of tissue affected as well as the time of ischemic exposure (12, 65). Three established time-dependent phases of ischemic injury occur during IR. The first phase is approximately one to five minutes into ischemia producing arrhythmias without permanent injury or decrease in pump function (26). Phase two is termed 'myocardial stunning' and generally is characterized by decreased contractile function, occurring 5 to 20 minutes following the onset of ischemia. Myocardial stunning is completely reversible within 24-72 hours and does not result in myocardial cell death (12, 26, 27). The third phase results from ischemic exposure greater than 20 minutes and results in irreversible cardiomyocyte damage and death (12, 26). Tissue damage and cell death as a result of IR occurs due to multiple causes, most notably disruption of intracellular calcium homeostasis, impaired ion pump

function, mitochondrial dysfunction, inflammation, activation of proteases, alteration in membrane function and permeability, and accelerated oxidant production (12, 27, 65).

Cellular death resulting from IR injury is classified as necrotic or apoptotic (20, 89). Necrotic cell death is a result of inadequate nutrient delivery, while apoptosis is programmed cell death due to an attempt of the affected tissue to metabolically down regulate in order to enhance chances of tissue survival (20, 89). A third process, autophagy, translates literally to 'self-eating' and is characterized by cell shrinkage and removal of damaged or unnecessary cell components in the absence of an inflammatory response. Autophagy is a normal occurrence in healthy tissues, but may reach pathologic levels in IR stressed tissues, leading to the induction of apoptosis (89). Although an adaptive mechanism, widespread metabolic down regulation becomes maladaptive in that decreased cardiac function results in decreased nutrient delivery (89). Therefore, maintenance of cellular viability is critical in maintaining cardiac function; mitigating necrotic and apoptotic cellular death will greatly improve the likelihood of survival following an ischemic event (89).

An active lifestyle is linked to both decreased occurrence as well as increased survival rate associated with cardiovascular disease and myocardial infarction (72). Interestingly, brief exposure to exercise dramatically reduces tissue injury and cellular death that results during the IR cycle that occurs during myocardial infarction (15, 21, 23, 25, 29, 67, 69). The beneficial cellular adaptations allowing for increased cellular recovery that occur with exercise, referred to as exercise induced cardioprotection, are yet to be fully understood.

Mechanisms of exercise induced cardioprotection are multifaceted and are known to include up regulation of the antioxidant MnSOD (40), the preemptive opening of sarcolemmal and mitochondrial ATP-sensitive potassium channels within the myocardium, and up regulation

of endogenous opioids (15, 21, 25, 69). The mitochondrial ATP-sensitive potassium channels are essential for protection against cardiac dysfunction and arrhythmia generation following short term (20 minutes) ischemia, while the sarcolemmal ATP-sensitive potassium channels are essential for maintaining cell viability during prolonged exposure to ischemia in exercise induced cardioprotection (15, 69). Protection afforded by opening of sarcolemmal and mitochondrial ATP-sensitive potassium channels prior to an ischemic event may be due to minimization of calcium overload by the within the contracting myocardium (15, 69). The intracellular signaling cascade that occurs in response to exercise and results in channel opening is still being investigated.

Mechanisms of exercise preconditioning which originate from outside the cell, known as remote preconditioning, are a new area of investigation. To date, endogenous opioids are the only known mediators of exercise-induced remote preconditioning (25). Strong evidence indicates that endogenous opioids are a critical component of cardioprotection (25, 42, 76). Transient increases in the expression of opioid precursor molecules (proopiomelanocortin, prodynorphin, and proenkephalin) as well as in the three major receptor subtypes (delta, kappa, and mu), within the myocardium occur in response to an exercise stimulus (25). Furthermore, administration of Naltrexone, a non-specific opioid receptor antagonist, completely abolishes the cardioprotective effect of exercise (25). Although this research provides clear evidence for the role of endogenous opioids in cardioprotection, it is unclear which specific opioid and/or receptor is responsible for these effects. Based upon pharmacological research, the delta opioid receptor appears to be the most likely involved in cardioprotection, although it is likely that the effect is due to multiple receptors. Therefore, the purpose of this study is to examine the role of the delta opioid receptor in opioid mediated exercise induced cardioprotection.

CHAPTER II

REVIEW of LITERATURE

Despite scientific advances and a decline in the mortality rate due to cardiovascular disease (CVD) over the past ten years, CVD is still a leading cause of death in the United States (72). On average, every 25 seconds an individual suffers a coronary event, and every minute an individual dies of a heart attack (72). Of those deaths, 33% occur before the age of 75, well below the predicted life expectancy in industrialized nations (72). Healthcare costs associated with CVD are estimated at 286 billion for 2011, exceeding estimates for any other diagnostic group (72). Due to the high incidence and healthcare cost, solutions to cardiovascular disease are of utmost importance.

Cardiovascular Disease is a broad term used to describe all diseases of the circulatory system (72). Coronary heart disease is the most prevalent manifestation of CVD, typically resulting in myocardial infarction (MI), known commonly as a ‘heart attack’ (72). During MI, coronary blood flow is either severely inhibited or completely blocked, a condition known as ischemia (65). The lack of blood oxygen supply results in the formation of reactive oxygen species, accumulation of hydrogen ions, and impairs the functioning of ion pumps within the cell (27, 65). The production of free radicals, as well as increased cellular calcium concentration referred to as calcium overload, are large contributors to the compromised functionality observed with myocardial infarction (12). Decrements in myocardial function and tissue damage can be subdivided into two distinct types of injury; that which occurs during ischemia (I) and that which occurs upon reperfusion (R) of oxygenated blood to the ischemic area (65). Often the damaging

effects of MI are examined together due to the fact that reperfusion must occur for tissue survival. Therefore, although the damage is distinct and separable, ischemia-reperfusion (IR) damage is a collective pathology. The following sections review the mechanisms of IR injury, myocardial dysfunction and mechanisms of protection against IR injury.

Mechanisms of IR Injury

Insufficient oxygen levels during ischemia limit ATP production within the mitochondria, which is the fundamental event of IR pathology. Secondary to ATP deficits, ion dyshomeostasis is the major contributing factor to the observed cardiac arrhythmias. Production of reactive oxygen species and the resulting oxidative stress in combination with calcium overload are the central cellular events in IR injury.

Oxidative Stress

Oxidative stress is the term applied collectively to reactive oxygen, nitrogen, and carbon molecules that cause a shift in the cellular oxidation-reduction (redox) balance in favor of the oxidants, leading to cellular damage (45). The oxidative products of redox reactions are often free radicals, highly reactive molecules possessing an unpaired electron in their outer orbital. Non-radical oxygen-based molecules, such as H_2O_2 are also fundamental to redox balance (35). As such, 'reactive oxygen species' (ROS) is currently the favored terminology to collectively describe the molecules responsible for a shift in redox balance to oxidative stress. Over the past few decades the concept of redox balance and oxidative stress has been researched at length. Low levels of ROS are generated as normal and important mediators of metabolism, gene regulation and signal transduction, while high levels are involved with the pathology of IR (1, 2, 46). The major sources of ROS within the myocardium include the mitochondria, a variety of oxidases, nitric oxide and autoxidation of catecholamines. The production of these molecules is

typically compartmentalized within a particular cell or region within a cell due to the rapidity of these reactions (36). Therefore, oxidative stress during IR is viewed within the context of a given cellular environment and includes local inflammation, presence of transition metals and other redox sensitive constituents (17).

The mitochondria are among the primary sources of ROS during IR. Normally, as electrons are passed along the cytochromes affiliated with the inner mitochondrial membrane, each provide the energy to pump hydrogen ions into the inner membrane space, creating a proton gradient between the inner and outer mitochondrial spaces. At complex five this gradient, coupled with the presence of ADP, Pi, and an oxygen molecule, provides the energy to convert ADP to ATP, with oxygen acting as the final electron acceptor, forming a water molecule. However, electron leakage into the inner mitochondrial matrix may periodically occur, causing incomplete reduction of an oxygen atom, creating a potent reactive oxygen species, superoxide, within the mitochondrial matrix (28, 71). Superoxide production occurs at complexes I and III within the electron transport chain.

The majority of oxidative stress occurs during reperfusion, explained by the ‘start-stop-start’ interruption of oxidative phosphorylation that occurs during IR. Reestablishment of blood flow to the ischemic tissue results in a free radical ‘burst’ which damages the mitochondrion (88). ATP production is diminished, resulting in further ionic disturbances within the mitochondrial matrix and cytosol. Decreased ATP production results in an increased calcium level, which mediates activation of xanthine oxidase and NADPH oxidase, ultimately adding to ROS production during reperfusion. The resulting inflammation, phagocytosis, activation of cyclooxygenase-2 (COX-2) and the inducible form of nitric oxide (iNOS) also contribute to the production of ROS (9).

Oxidative damage to the myocardium involves nearly all cellular constituents of protein, lipid, and nucleic acid composition. Cellular function is critically impacted by altered membrane fluidity and permeability resulting from the oxidation of polyunsaturated fatty acids within organelles and the cell membrane. Oxidation of thiol groups on certain amino acids results in diminished function of enzymes, receptors, contractile elements and transporters, as well as loss of cell structural integrity (84, 91). Oxidative modification of DNA selectively targets guanine bases, affecting the genetic code, genome replication, and DNA repair function (36, 88). Damage occurs as cellular antioxidants are overwhelmed by the production of ROS (8, 36, 88). Collectively, the oxidative damage that occurs during IR is directly responsible for all forms of IR injury.

Calcium overload and IR injury

Normally, calcium levels are tightly controlled within the cell cytosol and mitochondria by ATPase fueled sequestration mechanisms. During IR, however, calcium overload occurs in both the mitochondrial matrix and cytosol (18, 78). Calcium overload is attributed largely to diminished oxygen supply and the resulting decreased ATP availability causing alterations in contractile function and pump activity of various ion channels. Calcium overload is exacerbated as cellular metabolism shifts from predominantly oxidative phosphorylation to glycolytic bioenergetics, leading to rapid pH disturbances. The sarcolemmal sodium-hydrogen exchanger activity increases in order to partially reconcile the increased proton load at the cost of elevated intracellular sodium concentrations. Contractile dysfunction results since the myocardial action potential is sodium dependent and increased intracellular sodium causes inability to repolarize. Rising intracellular sodium concentration further complicates ionic control as the sodium/potassium exchanger activity declines secondary to declining ATP availability. Calcium

dyshomeostasis is inevitable at this stage due the sodium calcium exchanger (NCX) antiport activity in attempt to maintain sodium gradient across the cell membrane necessary for action potential generation and cellular contraction (8, 78). Increased cellular calcium will be further increased by the calcium-induced calcium release mechanism. Calcium dyshomeostasis results in the impairment of contractile function and calcium-dependent promotion of oxidative stress. Increased production of reactive oxygen species cause oxidation to calcium handling proteins: phoshpolamban (PLN), sarcoendoplasmic reticulum calcium pump ATPase pump (SERCA2a), NCX, and the L-type calcium channels. Furthermore, increased calcium levels leads to the activation of the protease calpain- which is implicated in both necrotic and apoptotic cell death. The spread of calcium overload-induced damage from neighboring cells results in increased damaged tissue area. During ischemia, progressive dephosphorylation of connexon 43 (Cx43), the most common connexon gap junction in cardiomyocytes, results in increased conductance which is believed to have a role in injury propagation (16). Activated protein kinase C (PKC) epsilon phosphorylates Cx43, decreasing conductance between cardiomyocytes and the spread of injury via calcium overload (16). As with oxidative stress, calcium overload is responsible for all forms of IR injury.

Myocardial Injury

The presence of arrhythmias denotes three established phases of ischemia-reperfusion injury dependent upon the duration of ischemic exposure (5, 65). Estimated time courses in which each phases occurs have been established. The first phase is one to five minutes of ischemia followed by reperfusion, typically resulting in ventricular arrhythmias without significant injury (5, 65). The arrhythmic response to ischemia is due, in part, to the generation of ROS, and is attenuated by administration of antioxidant compounds (5).

The second phase is termed ‘myocardial stunning’ and is characterized by reperfusion following approximately five to 20 minutes of ischemia, resulting in reversible contractile dysfunction lasting 24-72 hours without cell death (6, 12, 27, 49). Myocardial stunning may occur as a result of a single episode of ischemia or multiple bouts of ischemia. The pathological processes involved in myocardial stunning include the generation of reactive oxygen species (ROS) as well as disruption in calcium homeostasis and sensitivity. The formation of ROS results in an oxidant stress, interferes with ion transport channels, and decreased calcium sensitivity of the myofilaments. Antioxidants alleviate stunning when administered prior to ischemia or just prior to reperfusion. However, antioxidants are not effective when administered one minute after reperfusion, indicating that the damaging ROS production occurs in the immediate moments following the re-establishment of blood flow (6, 49). Evidence that myocardial stunning is not completely abolished with antioxidant therapy underpins the notion that the phases of stunning are distinct between non-ROS mediated ischemic stunning and ROS-mediated reperfusion stunning (6, 49). Disruption in calcium homeostasis occurs as a result of calcium overload during the early phase of reperfusion, resulting in contractile dysfunction. Alteration of contractile proteins, (most likely the troponin regulatory complex, during reperfusion results in decreased calcium sensitivity and force generation (6, 49). That ROS production, calcium overload and impaired calcium sensitivity each occur during reperfusion indicates stunning is a manifestation of reperfusion injury and that ROS and calcium overload are not mutually exclusive pathological responses (6, 7, 49). The proposal of an underling multi-faceted pathology is supported by evidence that similar markers of damage are associated with both ROS production and calcium overload (7). Furthermore, ROS formation may alter calcium flux by altering sarcoplasmic reticulum (SR) function and may act directly on contractile

proteins to decrease calcium sensitivity (7). While many unknowns remain, it is clear that ROS, calcium overload, and calcium sensitivity are the main pathological facets of myocardial stunning (7).

The third level of IR injury results from ischemic durations greater than 20 minutes and results in both necrotic and apoptotic cell death (12). Necrotic cell death is characterized by swelling of cells and organelles and loss of plasma membrane integrity, and defined as unregulated death resulting from the lack of oxygen and metabolite supply as well as the ability to produce ATP (89). Increased Ca^{2+}, ROS production, cellular acidosis and low levels of ATP all contribute to necrotic cell death (55, 89). Necrotic and apoptotic cell death are linked with the opening of mitochondrial permeability transition pore (MPTP), which further limits ATP production by decreasing the inner mitochondrial membrane potential (89). In experimental studies, necrotic cell death following IR is quantified histologically via triphenyl tetrazolium chloride (TTC) staining of myocardial cross sections. TTC staining reacts with dehydrogenases to stain metabolically active tissue a deep red while necrotic tissue appears white, and are then analyzed colormetrically.

Apoptosis is an energy (ATP) dependent process and characterized by cell shrinkage, DNA fragmentation, nuclear condensation, and the formation of vesicles containing cellular constituents for phagocytosis (89). Apoptosis is a fundamental cellular process in growth and development and is also essential for tissue homeostasis (89). However, during IR, excessive apoptotic cell death is detrimental to tissue viability and overall cardiac function (89). The production of reactive oxygen species is linked to increased levels of myocardial apoptosis. Over accumulation of H_2O_2 causes decreased inner mitochondrial membrane potential and opening of the mitochondrial permeability transition pore (MPTP), resulting in increased cytochrome c

release and downstream caspase 3 activation (55). Agents that block MPTP opening and caspase activation have been demonstrated to decrease H_2O_2-driven apoptosis (10, 55). The concept that H_2O_2-induced myocardial apoptosis occurs in three phases of mitochondrial defects: priming, depolarization, and fragmentation has recently been proposed (10, 55). During the priming phase matrix calcium overload occurs while the inner mitochondrial membrane potential is unaltered. Depolarization occurs as the mitochondria undergo sudden loss of inner membrane potential, which is contingent upon MPTP opening. The fragmentation phase follows, in which the mitochondria undergo massive swelling and rupture, releasing cytochrome c and initiating apoptosis (55). The extent of apoptotic cell death is often assessed in experimental settings via terminal deoxynucleotidal transferase dUTP nick end labeling (TUNEL) based cytochemical technique to assess apoptosis-induced DNA strand breaks (10, 67). Measurement of protein expression for pro-apoptotic signaling molecules such as Bcl-2 associated X protein (Bax), Bcl-2 homologous antagonist/killer (Bak), caspases 3, 7, and 9, anti-apoptotic signals such as B cell leukemia/lymphoma-2 (Bcl-2), and markers of autophagy such as Autophagy related homolog (Atg) 5, 7, and 12 are determined via western blot to indicate the level of apoptosis and autophagy within a given tissue. Injury as a result of IR occurs as a result of multiple causes, most notably disruption of intracellular calcium homeostasis, mitochondrial dysfunction, inflammation, activation of proteases, alteration in membrane function and permeability, and accelerated oxidant production (7, 12).

Mechanisms of IR injury Protection

Many research efforts over the past few decades have focused on mechanisms that protect the heart from the damage incurred during IR, coining the term ‘cardioprotection’. Models of cardioprotection include pharmacologic preconditioning, ischemic preconditioning

and exercise preconditioning (6). Briefly, pharmacologic preconditioning involves exposure of the heart to pharmacologic agents prior to coronary occlusion (6), ischemic preconditioning involves bouts of brief surgically applied ischemia (59), and exercise preconditioning involves acute (1-3 days) of moderate exercise prior to an ischemic event to confer protection against cardiac damage (15, 23, 25, 40, 67). Elucidation of the mechanisms responsible for protection in each model may lead to development of therapies and prevention techniques against IR injury. However, both ischemic preconditioning and pharmacological preconditioning are unsustainable forms of protection. Repetitive exposure of the myocardium to ischemia eventually leads to cardiac dysfunction, while prolonged pharmacotherapy leads to tissue desensitization (77). Aerobic exercise therapy, by contrast, is pragmatic in that it is cost effective and sustainable for extended treatment. Therefore, exercise preconditioning is considered by some to be the only viable therapy that may provide long term protection against IR injury from a scientific perspective (13, 48, 65). A wealth of recent data supports a role of exercise-based research to better understand therapeutic solutions against IR injury (15, 23, 39, 66, 79)

Exercise induced cardioprotection against IR injury

The protective effect of exercise in cardiovascular disease was first documented scientifically more than forty years ago with the observation that men who had more active occupations suffered less from cardiovascular disease (83). Exercise is associated with decreased CVD risk, as well as cardiac vasculature and structural remodeling in ways that mitigate the damage caused during IR (66). The beneficial effects of exercise are observed in the presence of CVD, leading to important cardiac rehabilitation therapies for individuals with a variety of cardiac diseases (3, 19). Furthermore, exercise results in a myocardial phenotype that is resistant to IR injury, a phenomenon known as exercise preconditioning. This protective phenotype is

associated with myocardial specific biochemical adaptations in response to exercise independent of structural alterations, and is the least understood facet of exercise induced cardioprotection (23, 24, 66). As will be discussed in the following section, short term exercise (days) is as effective as long term exercise (weeks) for eliciting a cardioprotected phenotype (23, 66).

The exercised myocardium is protected against all three forms of IR injury; ventricular arrhythmias (40, 69), myocardial stunning (51-53, 79-82) and myocardial infarction (23, 24, 38, 39, 70). Furthermore, exercise is protective against both necrotic and apoptotic tissue death (29, 67, 70). Recent evidence indicates that apoptotic tissue death is prevented in the exercised myocardium by preservation of basal autophagy levels following IR (JQ 2011 in review). Exercise preconditioning is one of the most reproducible phenomena in medical science and is protective in both the male and female heart (15, 21, 29, 52, 70, 81), as well as in young and aged hearts (67, 68, 80).

Exercise duration and intensity

An important implication of exercise induced cardioprotection is the dose required to achieve the protected phenotype. Although many studies utilize an animal model, most of these studies incorporate the animal equivalent of human recommendations set forth by the American College of Sports Medicine (ACSM) for health and fitness. Initial studies demonstrated improved maintenance of ventricular pressure during IR following a 10 week protocol of treadmill exercise at approximately 70% $\dot{V}O_2$max (66). Subsequent research demonstrated equal levels of ventricular contractility maintenance with short duration (3 – 5 days) of similar exercise intensity (23). Furthermore, a single bout of exercise for 30 minutes protects against IR induced necrotic tissue death (90). These early investigations reveal that short duration exercise (days) is just as effective as long term exercise (weeks) in eliciting protection against IR injury.

The intensity threshold required to elicit exercise induced cardioprotection is relatively low compared to functional capacity. Exercise intensities of 55% and 75% of $\dot{V}O_2$max are equally protective against losses of ventricular contractility (51). While findings from one study indicate that an exercise intensity of 50% $\dot{V}O_2$max is not effective in eliciting an exercise induced cardioprotective response (81), it is likely that slight variation in exercise protocol intensity accounts for the difference in findings between these studies. Exercise protection against IR is maintained for at least 9 days following exercise cessation (52). These findings demonstrate multiple important facts in regard to the exercise induced cardioprotection stimulus. First, the intensity required is relatively low compared to intense training standards (51). Second, the stimulus required from a protected phenotype is threshold dependent (23, 51, 66, 81). Third, the apparent disconnect between the time period required for exercise induced cardioprotection versus structural alterations in vasculature as well as absence of alteration in coronary flow rates to the myocardium highlights the fact that the mechanisms of protection are not due to increased blood flow to the myocardium (51-53, 67). These facts support evidence that mechanisms of protection are largely a result of biochemical alterations within the myocardium.

Mechanisms of exercise induced cardioprotection

The mechanisms responsible for exercise cardioprotection are multifaceted and include the independent preservation of bioenergetics, calcium homeostasis (11) and prevention of oxidative stress (38, 40). Although the mechanisms of exercise induced cardioprotection are incompletely understood, several have been identified. Known mechanisms include enhanced endogenous antioxidants (40, 90), cellular components of calcium regulation (11, 29, 30), and alteration of cardioprotective proteins (15, 69) each involved in the regulation of apoptotic and necrotic cell death (10, 15).

Endogenous antioxidants

The exercise stimulus is paradoxical in that while it is associated with many health benefits, it also results in increased production of reactive oxygen species and oxidative stress which has been linked with the progression of various diseases. Despite this, the observed increase in ROS following exercise is beneficial in that it affects the tissue up-regulation of endogenous antioxidants (44, 45). One such antioxidant is superoxide dismutase (SOD), which converts superoxide into hydrogen peroxide through an enzymatic process (58). SOD exists in different forms specific to the cellular location in which it is located; within the cytosol it is associated with a copper-zinc cofactor (CuZnSOD) and within the mitochondrial matrix it is associated with a manganese cofactor (MnSOD) (36). A bi-phasic response of MnSOD to exercise has been demonstrated in exercised hearts, where increased activity is associated with decreased infarct size (90). Prevention of exercise-mediated over expression of MnSOD by use of anti-sense oligonucleotides completely abolished exercise induced protection (40). Further study confirmed that MnSOD was essential in preventing IR-induced ventricular arrhythmias (40) as well as necrotic and apoptotic tissue death (29, 37). In contrast, MnSOD is not essential in protection against contractile dysfunction during IR (53). MnSOD is the only antioxidant shown to be consistently increased in response to exercise and also essential to the protected phenotype (37, 40, 69). MnSOD converts superoxide into the ROS H_2O_2 (58). H_2O_2 is not technically a free radical, but it is thought to be more physiologically relevant compared to superoxide due to its relatively long half-life and ability to cross cell membranes (35). Several antioxidant defense systems against H_2O_2 exist within the myocardium, including catalase (CAT) and glutathione peroxidase (GPx), which converts H_2O_2 into the benign products oxygen and water. Some investigations report an increase in myocardial CAT (41, 51-53), while others do

not (38, 40). Similarly, GPx is reported to increase in response to exercise (33, 43, 56), while multiple studies have documented no change in activity following exercise (22, 23, 38, 39). Variation in results may be due to different study conditions imposed at the time of sampling. Still, it is well established that improved antioxidant fortifications are associated with exercise, and these antioxidant enzymes are essential to exercise induced cardioprotection (37). In summary, the removal of ROS by endogenous antioxidant systems is clearly important in conferring protection against IR induced injury. Findings from numerous studies demonstrate that short term exercise results in an increase in cardiac levels of one or several antioxidant defenses. To date, however, only MnSOD is scientifically verified as essential to exercise induced cardioprotection against IR injury.

Calcium homeostasis

Calcium overload within the cytosol and mitochondria is a cornerstone of IR injury. Exercise training improves ventricular function and calcium handling during IR by restoring calcium balance through increased calcium handling proteins and increased phosphorylation of phospholamban (11, 34, 73). Further study reveals that exercise is associated with decreased activation of calpain, a calcium activated protease, which has detrimental effects on calcium handling within the myocyte (30). Decreased calpain activation is associated with preservation of SERCA-2A ATPase activity during IR, leading to better calcium regulation (30). Exercise preserves calcium homeostasis by decreased oxidative modification of L-type calcium channels during IR (29). Calcium overload may also be inhibited by the opening of sarcolemmal ATP-sensitive potassium channels and mitochondrial potassium ATP-sensitive channels within the cardiac myocyte (15, 69). The preservation of calcium homeostasis extends to prevention of the calcium-dependent promotion of oxidative stress.

ATP sensitive potassium channels

The role of the inwardly rectifying ATP sensitive potassium (K_{ATP}) channels in protection against IR injury has been a popular area of cardioprotection research (47). ATP sensitive potassium channels are located at two cellular locations; within the sarcolemma (sarc K_{ATP}) and the inner mitochondrial membrane (mito K_{ATP}). These channels are well conserved metabolic sensing mechanisms located in a variety of tissues (50, 87). As such, the channels are closed in the presence of abundant ATP levels and open in response to diminished ATP, as occurs during metabolically stressful situations such as IR. Other stimuli may induce opening in the absence of ATP level disturbance such as protein kinase C (PKC) and other metabolically-linked signaling molecules. Opening of the K_{ATP} channels prior to IR robustly protects cardiac tissue against all forms of IR injury (32, 62). The mechanisms of this protection are not fully understood, however multiple explanations have been proposed. Opening of K_{ATP} channels is associated with improved calcium handling and a reduction in oxidant production (32). Opening of the sarcolemmal K_{ATP} channel prior to IR may result in decreased membrane potential by K^+ efflux, resulting in shortened action potential duration and decreased Ca^{2+} entry through the L-type Ca^{2+} channel (32, 60). Opening of the mitochondrial K_{ATP} channel prior to IR may result in lowering of the mitochondrial membrane potential, decreasing flux through the electron transport chain (60). Collectively, these effects result in the prevention of the free radical burst and oxidative stress by diminished supply-demand mismatch via more efficient electron transport in the mitochondria or a reduction in myocardial energy demand (32, 64).

The role of the K_{ATP} channel in the exercised heart is a relatively new topic of investigation in the realm of exercise-induced cardioprotection. The cardiac K_{ATP} channel is an essential component of normal cardiac function; genetic knockout animals have diminished

exercise capacity and are predisposed to ventricular arrhythmias in an unstressed heart (92). Due to the necessity of K_{ATP} channels for normal cardiac function and exercise capacity, knockout models are not suitable for exercise preconditioning research. Therefore, investigations of the mechanisms of K_{ATP} as a mediator of exercise induced protection employ pharmacological inhibitors specific to the mito K_{ATP} and sarc K_{ATP} channels. The mito K_{ATP} channel has been demonstrated to be essential in protection against IR induced ventricular arrhythmias, and protection corresponds to attenuation of oxidative stress within the left ventricle (69). Exercise mediated protection against IR-induced necrosis is mediated by the sarc K_{ATP} channel (14, 15, 21). Based on these results, it appears that the mito K_{ATP} and sarc K_{ATP} channels have variable functions in exercise cardioprotection. The mito K_{ATP} channel is essential to preservation of cardiac function, as evidenced by decreased arrhythmia occurrence, (69) while the sarc K_{ATP} channel is essential to preservation of tissue viability, as evidenced by a decrease in tissue death (14). Early investigations suggested that the sarc K_{ATP} channel was only protective in the female heart (14, 15, 21). More recent efforts, however, demonstrate that the sarc K_{ATP} channel is also protective in male hearts (Quindry in review AJP-Heart). Furthermore, both mito K_{ATP} and sarc K_{ATP} channels may contribute to cardioprotection by preservation of basal autophagy levels in the moments following IR (Quindry in review AJP-Heart). However, an effect of these channels on apoptosis is yet to be demonstrated. In conclusion, exercise cardioprotection against several forms of IR injury is mediated through preemptive opening of mito K_{ATP} and sarc K_{ATP} channels. The intracellular signaling cascade that occurs in response to exercise and results in channel opening is still being investigated. Interestingly, mechanisms of exercise preconditioning which originate from outside the cell, known as remote preconditioning, are also being investigated. To

date, endogenous opioids are the only mediators of exercise-induced remote preconditioning (25).

Endogenous Opioids and Cardiac Preconditioning

The role of opioid compounds in both pharmacological and ischemic preconditioning models of cardioprotection is well documented (25, 54, 76, 85, 86). Evidence that endogenous opioid compounds are released in response to exercise prompted recent investigation as to whether these molecules are involved in exercise induced cardioprotection (25). As expected, administration of a non-specific opioid receptor antagonist was demonstrated to abolish the cardioprotective effect of exercise (25). More research is required to determine the specific receptor and opioid compound involved, as well as the mechanisms of this observed protection.

Three major classes of opioid receptors have been identified, kappa (κ), mu (μ), and delta (δ), each consisting of multiple subtypes (31, 86). All three receptor types have structural homology and belong to the same superfamily of seven transmembrane receptor G-protein coupled receptors (31, 76). Endogenous opiates include three families of peptides, β-endorphins, enkephalins, and dynorphins; defined by their precursor molecules proopiomelamocortin, proenkephalin, and prodynorphin, respectively (31, 86).

Since the discovery of endogenous opioids, intense research has focused on the functions these peptides have, mainly within the central nervous system. Recent work indicates that both endogenous and pharmacologically administered opioids provide protection during cardiovascular system dysfunction (42, 57, 63, 76). Within the brain, receptors are the most concentrated in the brain stem, near the cardiovascular control center (85). Many attempts to clarify the exact effect of different opioids and receptor subtypes within the central nervous system (CNS) yield conflicting results (86). The observed effect is dependent upon the exact

location of opioid administration, opioid concentration, and species tested (85, 86). Generally, opioids at the cardiovascular control center have an inhibitory effect, resulting in decreased heart rate and blood pressure (85). This inhibitory effect is largely mediated via mu and delta receptors within the paraventricular nucleus of the hypothalamus, rostroventrolateral medulla, and the dorsal hippocampus via activation of potassium channels resulting in inhibitory hyperpolarization of nervous tissue (85). The action of opioid compounds within the CNS is separate from the action within the peripheral nervous system (PNS). The blood-brain barrier is not permeable to opioid molecules, and therefore, the concentrations within the CNS do not reflect the concentration in the peripheral circulation.

Within the peripheral nervous system, enkephalins are produced and released along with acetylcholine from neuronal terminals innervating cardiac tissue (85). Cardiomyocytes possess at least the three previously mentioned receptor types, (mu, kappa, and delta) within the sarcolemma (76, 85). Similarly, an opioid receptor agonist is released with norepinepherine from sympathetic nerve terminals innervating the heart. The co-release is thought to inhibit the beta-adrenergic effect (57). In fact, Leu-enkephalin inhibits the effect of norepinepherine via Beta1 receptors by suppressing the L-type calcium channel current and the Ca^{2+} transient within single myocytes (54). This mechanism may protect against “over-stimulation” from the sympathetic nervous system (54).

Under the circumstance of heart failure, the effect of enkephalin to decrease sympathetic responsiveness may be a protective mechanism to limit the demand placed upon the heart (86). In the heart failure model, increased sympathetic stimulation occurs to compensate for low cardiac output and subsequent drop in blood pressure by increasing heart rate, contractility, and vascular resistance (76). Although this response is compensatory, increases in both afterload and

preload result, ultimately over working the heart and causing greatly increased myocardial oxygen demand. Eventually these effects result in decreased cardiac output, leading to a 'vicious cycle' that characterizes decompensating heart failure. Therefore, enkephalins may reduce the down-cycling of cardiac function in this pathological setting. Interestingly, this effect does not translate to the beta 2 receptor (86).

Although neuronal release of enkephalins has an interesting role in cardiac function, the major source of production is within the myocardium (76, 86). The endogenous production of enkephalins within cardiac tissue allows the reversal of catecholamine effects in the absence of neuronal stimulation (86). The myocardial expression of the proenkephalin gene results in a 31 kDa polypeptide precursor, which either enters post-translational processing to yield penta-peptides Met and Leu-enkephalin, Methionine-enkephalin-arginine-phenylalanine (MEAP), and several other large peptides such as peptide B (86). Interestingly, preenkephalin mRNA levels are higher in cardiac tissue compared to the brain, suggesting higher levels of production within heart tissue; however, direct measures of enkephalin extracted from the heart are much lower than that extracted from the brain (86). A probable explanation for this occurrence includes the rapid degradation of peptides produced in the heart, the lack of peptide storage, and that most forms of enkephalins remain in precursor or large protein form (peptide B or MEAP) and are not included in the standard measurement (86). Although the function of these larger peptides is unclear, it may be that they serve as a reservoir for steady continuous enkephalin release (86). Storage of precursor peptides may allow for the ability to alter the type of enkephalin released in accordance to differing physiological stimuli (86). This reasoning supports the finding that the type of enkephalin in greatest circulating concentration is influenced by cardiac events (76, 86). For example, following ischemia, an increase in Leu-enkephalin is detected in the coronary sinus

(86). Taken together, these results support the idea that the heart itself is an endocrine organ, and that enkephalins have a role in autocrine and/or paracrine regulation of cardiac function. These novel revelations underpin recent interest in opiates as potential mediators of exercise induced cardioprotection.

Many factors mediate exercise induced cardioprotection, and strong evidence indicates endogenous opioids are a critical part of that mediation (25, 42, 76). Transient increases in myocardial expression of opioid peptide precursors, as well as in the delta, kappa, and mu receptor expression within the myocardium occur in response to exercise (25, 76). Furthermore, administration of Naltrexone, a non-specific opioid receptor antagonist, completely abolishes the protective effect of exercise treatment against tissue necrosis (25). Although preliminary evidence for the role of endogenous opioids in cardioprotection exists, it is still unclear if there is a specific opioid and/or receptor responsible for these effects. Therefore, in order to completely understand the mechanisms for therapeutic development, more research is needed.

Opioids in ischemic preconditioning

The delta opioid receptor is involved in protection against cardiac injury in an ischemic preconditioning model of cardioprotection (54). Administration of a delta receptor agonist attenuates apoptosis resulting from an ischemic insult (54, 57). The anti-apoptotic effects of the receptor agonist are reversed by a mitochondrial ATP sensitive potassium channel blocker (5HD), as well as a specific protein kinase C (PKC) inhibitor (54, 57). Ischemic preconditioning and delta opioid receptor agonist administration both result in activation of PKC, specifically the delta isoform (54). Stimulation of the delta opioid receptors results in opening of the mitochondrial ATP sensitive potassium channels, resulting in the generation of oxygen radicals within the mitochondria that activate the PKC delta isoform (54). The connection between

oxygen radicals and the activation of PKC delta is supported by the finding that antioxidant administration blocks the activation of PKC delta (54). Upon activation, PKC delta translocates to the membrane and exerts biological function (4, 54). However, another theory proposed is that delta opioid receptor binding results in PKC delta activation, which then translocates to the mitochondria, directly opening mitochondrial ATP sensitive potassium channels (54, 76). The opening of these channels results in the phosphorylation or increase in gene expression of rate limiting enzymes (54).

Although the exact pathway is uncertain, PKC has a role in cardioprotection (16, 54, 57, 76). Both the delta and epsilon isoforms are involved in cardioprotection, although they appear to have different effects (16). Administration of PKC epsilon prior to ischemia mimics the protection observed following ischemic preconditioning, while PKC delta protects against IR damage during the reperfusion phase (16). These findings are consistent across varying experimental set-ups; in cardiomyocyte culture, an isolated perfused heart preparation, and *in vivo* (16). As Lui et al. suggest, recent research indicates a central role of PKC in mitochondrial ATP sensitive potassium channel opening, as well as the involvement of reactive oxygen species (ROS) on PKC delta (16, 54, 76). Activated PKC epsilon also phosphorylates the mitochondrial ATP sensitive potassium channel, maintaining function and aiding the development of ROS production, which in turn increases the activation of PKC epsilon (16, 54). The mitochondrial permeability transition pore (MPTP) typically opens during reperfusion, resulting in mitochondrial swelling and protein release, uncoupling of oxidative phosphorylation, and eventually the reduction of the mitochondrial membrane potential, ultimately resulting in necrotic cell death (16). Activated PKC epsilon reduces the opening of the MPTP by direct

phosphorylation or via PKC epsilon effects on mitochondrial ATP sensitive potassium channel opening (16).

Opioids may have a cardioprotective effect via the neuropeptides, calcitonin gene related peptide (CGRP) and epinephrine (42). The left ventricular myocardium possesses the ability to release epinephrine in the absence of neural stimulation, and can produce and release CGRP, which is also stimulated by activation of the delta opioid receptor (42). In a cell culture ischemia-reperfusion model, application of delta opioid receptor agonist, [D-Pen^{2,5}] Enkephalin (DPDPE), reduced infarct size by 51%, increased phosphorylated Akt levels, and reduced myocyte apoptosis (42). Treatment of left ventricular cell culture with β_2-adrenergic and CGRP-receptor antagonists resulted in significantly greater myocyte death, and completely abolished the protective effect of DPDPE (42).

Calcitonin gene related peptide is present in sensory neurons, typically associated with the tachykinin, substance P, which interestingly is a potent vasodilator (61, 75). Within the myocardium, CGRP is in greatest concentration within the sinoatrial and atrioventricular nodes, and has a positive chronotropic and inotropic effects in high concentrations (75). Calcitonin gene related peptide increases following ischemic preconditioning; as measured in the coronary effluent (75). Cardioprotective effects of CGRP are mediated through inhibition of tumor necrosis factor alpha (TNF-α), but not potassium channels. Also, CGRP is linked to decreased reactive hyperemia, decreased creatine kinase release, reduction of infarct size, and improved left ventricular pressure recovery following ischemia (42, 75). Exogenously applied CGRP has the same effects as increased CGRP via ischemic preconditioning, and the cardioprotective effects in both exogenous/endogenous models are blocked by administration of CGRP receptor antagonist

(42, 75). To date, the effect of CGRP in an exercise preconditioning model has not been investigated.

Based on existing research, the delta opioid receptor is most likely to affect cardioprotection (42). However, the kappa receptor is also prominently expressed in the rat heart and contributes to cardioprotection in both ischemic preconditioning and pharmacologic research models (74). Administration of a kappa opioid receptor agonist attenuates mitochondrial swelling, dilation of the endoplasmic reticulum, and membrane damage of the cardiomyocyte following ischemia compared to control (74). Activation of the kappa opioid receptor may limit infarct size, creatine kinase and lactate dehydrogenase release form the myocardium, TUNEL-positive nuclei (a marker of apoptosis), and the Bax/Bcl-2 ratio (indicating reduction in apoptotic stimuli), inhibit genomic DNA fragmentation in the ischemic tissue, and lessen the alteration of caspase 3 and caspase 9 (74). As with the delta opioid receptor, the mechanisms of action are not completely understood. Protein kinase C dependent pathways are involved with kappa opioid receptor activation (74). Also, it is proposed that the decrease in infarct size is due to opening of the mitochondrial ATP sensitive potassium channel (74). Administration of 5HD, a mitochondrial KATP channel inhibitor, reverses the aforementioned protective effects of the kappa opioid receptor agonist (74).

Conclusion and Purpose

In summary, a strong rationale exists for the involvement of endogenous opioids in an exercise model of cardioprotection. In this model, the role of specific endogenous opioids has not been investigated. The purpose of this study is to determine the effect of specific endogenous opioids in exercise induced cardioprotection against IR induced cell death. The response of opioid precursor and receptor protein expression, tissue markers of oxidative stress and

antioxidant activity, and plasma CGRP and epinephrine will be determined to indicate the effect of exercise training on these cardioprotective variables. Furthermore, markers of apoptosis and autophagy as well as quantification of both necrotic and apoptotic tissue death following IR will be determined. The results from these studies will provide insight into the precise function of endogenous opioids in exercise induced cardioprotection.

CHAPTER III

JOURNAL MANUSCRIPT

ENDOGENOUS OPIOIDS AND EXERCISE CARDIOPROTECTION

ABSTRACT

Acute exercise exposure dramatically decreases tissue injury and cellular death that results during the ischemia–reperfusion (IR) events of myocardial infarction, though the mechanisms aren't fully understood. Existing evidence indicates endogenous opioids are a critical component of exercise-induced cardioprotection, and may be linked to the cardioprotective peptide, calcitonin gene related peptide (CGRP). The specific opioid and/or receptor subtype responsible for these effects is unknown. Based upon pharmacological research, the delta opioid receptor appears to be the most likely opioid receptor involved in cardioprotection. The purpose of this study is to determine if the delta opioid receptor is involved in opioid-mediated exercise-induced cardioprotection. Seventy three rats were randomly assigned into an unstressed (n=32) or IR group (n=41). Animals in the unstressed group performed treadmill exercise, or remained sedentary. The effect of exercise on the mRNA and protein expression of the opioid compound leu-enkephalin, and delta opioid receptor, and CGRP were determined at 0min, 20min, and 120min following exercise. Leu-enkephalin mRNA expression was significantly increased at 0min and 120min following exercise ($p = 0.03$ and $p = 0.021$, respectively). However, no significant differences were found in tissue protein content.

Animals exposed to IR were randomly divided into Sham, sedentary (S) or exercise (Ex) groups. A delta-opioid receptor antagonist, Naltrindole, was administered (5 mg/kg i.p injection) 15 minutes prior to exercise in a subset of animals (ExD), and at a corresponding time in a subset of sedentary animals (SD). Twenty-four hours following the final exercise bout, animals received surgically-induced IR by left anterior descending (LAD) coronary artery ligation *in vivo*. Heart tissue was collected for determination of infarct area (necrotic tissue death) and apoptosis. Significant between group differences existed for tissue necrosis ($p < 0.0001$). Compared to Sham, S and ExD animals had significantly greater tissue necrosis ($p > 0.0001$, $p = 0.003$), while no difference existed compared to Ex groups. S had significantly increased tissue necrosis compared to Ex ($p = 0.003$), but was not different compared to SD. Significant between group differences existed for tissue apoptosis ($p = 0.013$). Compared to Sham, S had significantly greater level of apoptosis ($p = 0.016$), while no difference existed compared to Ex and ExD. Ex was significantly lower compared to S ($p = 0.035$), but not ExD. No difference existed between S and ExD or SD. These data provide evidence that the delta opioid receptor subtype is involved, at least in part, in exercise induced cardioprotection against tissue necrosis. Further research is needed to clarify the mechanisms involved in this observed protection.

INTRODUCTION

Currently, cardiovascular disease (CVD) is the leading cause of death in industrialized nations (22). Within the variety of conditions encompassed in CVD, ischemic heart disease is the most prevalent, often resulting in myocardial infarction (MI), commonly known as a 'heart attack' (22). During ischemia, coronary blood flow is either severely inhibited or completely blocked, resulting in ineffectual supply of oxygen and nutrients, and ultimately cardiac dysfunction and tissue death. The re-establishment of blood flow following ischemia, termed reperfusion, is critical for tissue survival. Paradoxically, the majority of cellular damage occurs during reperfusion. An active lifestyle is linked with both decreased occurrence as well as increased survival rate associated with cardiovascular disease and MI (22). Interestingly, brief exposure to exercise dramatically reduces tissue injury and cellular death that results during the ischemia-reperfusion (IR) cycle that occurs during MI (2, 5-8, 20, 21). The beneficial cellular adaptations allowing for increased cellular recovery that occur with exercise, referred to as exercise induced preconditioning, are multifaceted and include the independent preservation of bioenergetics, calcium homeostasis (1) and prevention of oxidative stress (11, 12). Recent investigation reveals endogenous opioids have a role in exercise induced preconditioning against tissue death (7).

Circulating endogenous opioid compounds increase in response to exercise (16), and furthermore, transient increases in transcript expression of opioid precursor molecules and receptors within the myocardium occur in response to an exercise stimulus (7). Whether an acute exercise stimulus also results in increased protein expression of these molecules and receptors is currently unknown. Interestingly, administration of Naltrexone, a non-specific opioid receptor antagonist, abolishes the cardioprotective effect of exercise (7). However, the specific opioid

receptor subtype and endogenous opioid molecule involved in exercise mediated protection is yet to be identified. The role of opioid compounds in both pharmacologic and ischemic preconditioning models of cardioprotection against necrotic and apoptotic cell death is well documented (7, 14, 17, 18, 23-25). Based upon ischemic and pharmacologic models of preconditioning, the delta opioid receptor appears to be the most likely involved in cardioprotection (9, 17, 18). Cardioprotective effect of opioids may be linked to the neuropeptide calcitonin gene related peptide (CGRP) (14). The left ventricular myocardium possesses the ability to produce CGRP, and its release is stimulated by activation of the delta opioid receptor (14). Plasma CGRP levels are known to increase in response to exercise, indicating a potential role in exercise induced preconditioning (13).

Although preliminary evidence for the role of endogenous opioids in exercise preconditioning exists, it is still unclear if there is a specific opioid and/or receptor responsible for these effects. Therefore, the purpose of this study is to examine the effect of an exercise stimulus on opioid compounds and receptor transcript and protein expression within the left ventricle, and to determine the role of the delta opioid receptor in opioid-mediated exercise-induced preconditioning. The potential role of CGRP in an exercise preconditioning model has not yet been investigated. As an exploratory facet of this study we examined the effect of exercise on transcript and protein expression within the left ventricular tissue.

METHODS

Animals The experimental protocol was approved by the Auburn University Institutional Animal Care and Use Committee (IACUC PRN# 2011-1925, Appendix A). Seventy three Sprague Dawley rats were randomly assigned into two treatment groups; either 'Unstressed' or 'Ischemia-Reperfusion' (IR) group and housed in Auburn University's Biological Research Facility on a 12 hour light:dark schedule. All animals received unrestricted access to water and were fed a standard chow diet *ad libitum*. Rats either performed treadmill exercise for three consecutive days, proportional to that recommended for humans by the American College of Sports Medicine, or remained sedentary (sedentary-control and Sham).

Exercise Protocol The animal exercise protocol consisted of 3 days treadmill habituation beginning at 10 minutes at 30 m/minute 0% grade and increasing by 10 minutes each day to a final bout of 30 minutes on day three, shown in **Figure 1** with injection schedule for applicable groups described in the following sections. Following the last day of habituation, animals rested for one day and then performed 3 consecutive days for 30 minutes at 30 m/minute and 0% grade. Procedures specific to animal groups and protocols following exercise/sedentary-rest for each group are outlined in the following sections; a general outline is provided in **Figure 2**.

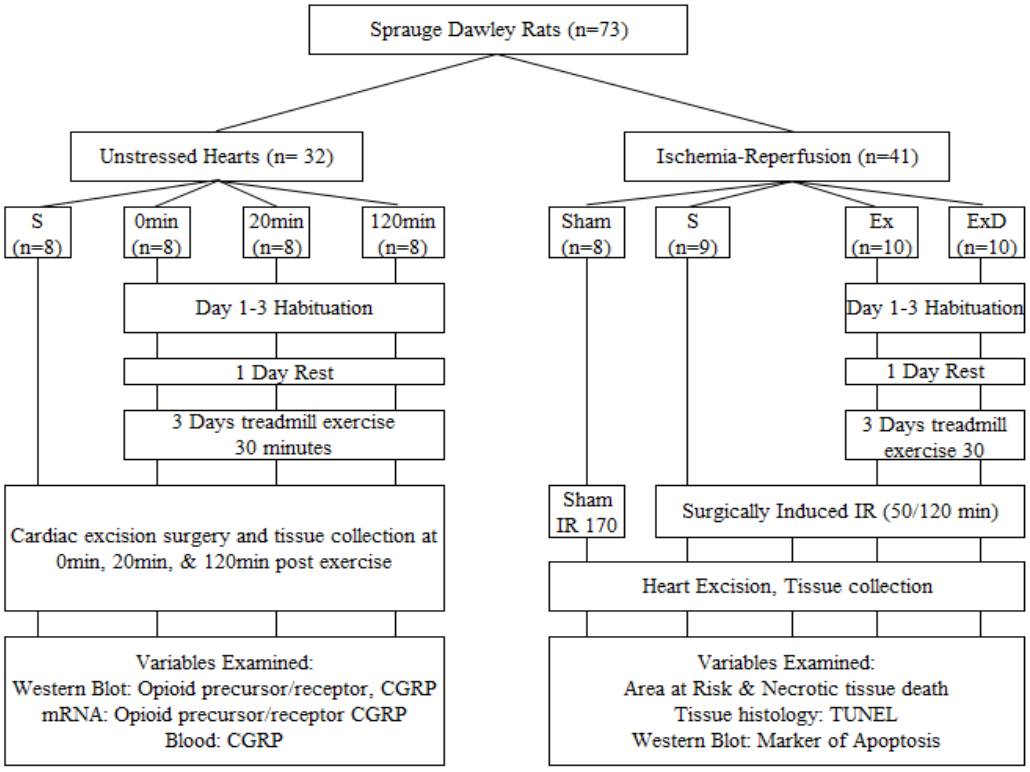
Figure 1. Exercise and injection protocol

Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
				Inject	Inject	Inject
10 min	20 min	30 min	0 min	30 min	30 min	30 min
Habituation Period			Rest	Exercise Stimulus		

Unstressed group Animals were anesthetized and sacrificed via cardiac excision immediately following exercise (0min), or at 20 minutes (20min) and 120 minutes (120min) following exercise; sedentary control animals (S) were sacrificed in the same manner. Serum and heart tissue were collected, immediately frozen in liquid nitrogen, and stored at -80˚C for future analysis.

IR group Animals were further divided into SHAM, Sedentary (S), Exercised (Ex), Exercised Delta specific opioid inhibitor (ExD), and Sedentary Delta specific opioid antagonist control (SD). Animals in the Ex and ExD groups performed treadmill exercise as described above. ExD animals received a selective peptide delta-opioid specific receptor inhibitor, Naltrindole at a dosage of 5 $mg \cdot kg^{-1}$ intraperitoneal (I.P) injection, 15 minutes prior to exercise on the three final exercise days. The SD group received injections of the delta opioid specific antagonist in the same time course as the ExD group (3 consecutive days, 3 $mg \cdot kg^{-1}$ I.P injection). Animals were anesthetized via I.P injection of sodium pentobarbital (80 $mg \cdot kg^{-1}$) and underwent surgically induced IR 24 hours following the final exercise bout as described in the following section.

Figure 2. Study Outline



Surgical IR Rats were anesthetized with 80 $mg \cdot kg^{-1}$ sodium pentobarbital intraperitoneally (I.P) and ventilated with room air through a tracheotomy tube. Blood pressure was continuously monitored via computer software connected with a pressure transducer attached to a saline filled catheter inserted into the right carotid artery (Biopac Student Lab Pro 2005). A catheter was placed in the right jugular vein for administration of sodium pentobarbital as needed throughout the experimental procedure to maintain a surgical plane of anesthesia. The left side of the heart was exposed by left thoracotomy. Rats were then exposed to an ischemia-reperfusion (IR) challenge *in vivo* consisting of 50 minutes ischemia followed by 120 minutes reperfusion, or Sham surgery (170 minutes no ischemia). Ischemia was induced by placing a ligature around the left anterior descending (LAD) coronary artery. Sedentary animals received the same surgical procedure; Sham animals received the same surgery minus the final tightening

of the ligature (non-ischemic surgical control). Reperfusion was initiated by loosening of the ligature in sedentary and exercised animals. Following 120 minutes of reperfusion, ligatures were re-tightened for all animals and Evans Blue dye was infused through the coronary artery catheter for visualization of the area at risk. Hearts were then immediately excised, weighed and imaged for quantification of perfused, area at risk, and infarct area.

Infarct Area Animals assigned to the Infarct group received 3-4ml 4% Evans Blue infusion as indicated above. Immediately following excision, hearts were cut into 1.5 mm cross sections and incubated at 37° C for 12 minutes in 1% triphenyltetrazolium chloride (TTC, Sigma T8877). Heart slices were then placed into 10mM PBS and imaged with a digital camera. Images were analyzed on the Kodak Gel Logic 2200 to determine the area at risk (ischemic area) and infarct area.

Apoptotic cell death/TUNEL Tissue slices designated for the TUNEL assay were coated in Richard Allen Scientific -50 embedding medium, frozen in 2-methyl butane chilled with liquid nitrogen, and stored at -80°C until sectioning. Hearts were sliced into 8μm cross sections and fixed in 10% Formalin, washed, and permeabilized (50 mg Sodium citrate, 50 μl Triton X-100, 50 ml deionized water) for 5 minutes, and blocked with 3% BSA and 20% Goat serum (vector S1000) for 30 minutes. Histological sections were incubated with rabbit anti-laminin (Sigma-Aldrich) followed by a secondary antibody conjugated to Texas Red fluorescent tag (Vector Laboratories, Burlingame, CA). The histological sections were then incubated with TUNEL label reaction mixture (Cell death detection kit from Roche). Slide covers were mounted with Dapi mounting medium (Vector Laboratories). Apoptotic nuclei were imaged via fluorescence microscopy (Nion Eclipse Ti-U).

Markers of Apoptosis and Precursor Protein Expression Tissues designated for western blot analysis were separated into area at risk (ischemic) and perfused regions, frozen in liquid nitrogen and stored at -80°C. Tissues were homogenized on ice 1:20 w/v in homogenization buffer, containing 19ml 100mM PBS with 10% EDTA and 1 ml protease inhibitor cocktail (Sigma 2714). Homogenized samples were centrifuged at 10,000 x g for 10minutes at 4°C and supernatant was collected and kept on ice. Homogenized samples were diluted to 2.6μg/μl protein concentration in SDS sample buffer and β-mercapta ethanol (βME), boiled for 5 minutes at 90°C and stored at -80°C. Forty μg protein was separated in precast gels (Lonza). Proteins were transferred to polyvinylidene flouride (PVDF) membranes. Following transfer, membranes were placed in blocking solution (ThermoScientific) for 2 hours at room temperature on a rocker. Antibodies for Apoptotic markers were purchased from Cell Signaling β-Actin: (#4970); Bax: (#2772); Bcl-2: (#2876). Opioid receptor antibodies were purchased from Abcam; delta opioid receptor: (#ab66318), Leu-enkephalin: (#ab8902), and CGRP: (#ab47027). Primary antibody (1°, Cell Signaling) were prepared 1:1000 in PBS-T 1%Super Block and added to each membrane to cover and incubated overnight at 4°C. Secondary antibody (2°, Cell Signaling #7074 and Abcam #6027) was diluted 1:2000 and 1:3000, respectively in PBS-T 1%Super Block and applied to each membrane to cover for 2 hours. Membranes were imaged in ECL (Peirce #32209) according to manufacturer instructions. Following image capture, antibodies were removed by 15 minute incubation with stripping solution (ThermoScientific #21059). Successful stripping was ensured by re-imaging of one membrane following the stripping step. The process was repeated for each primary antibody. The membrane-bound proteins were then stained with GelCode Blue (ThermoScientific #24590) and imaged.

mRNA extraction and analysis Primers for pPENK, DOR, CGRP and β-Actin mRNA were obtained from Integrated DNA Technologies (IDT), provided in **Table 1**. Primer specificity and Tm was verified by temperature gradient PCR (Appendix B). Left ventricular tissue was cut at -30°C and RNA was isolated using Qiagen RNeasy Mini kit, according to the Animal Tissue Protocol. RNA yield an purity was determined by Nano Drop (Thermo Scientific). One μg cDNA was synthesized from RNA using Verso cDNA kit (Thermo Scientific) following manufacturer instructions. qPCR was performed with a reaction solution prepared with SYBR green quantitative PCR kit (SA Biosciences) combined with 10μM of each primer and 50ng total RNA to a final volume of 25μl. The instrument cycles will be as follows: one cycle at 95°C for 2 minutes, 35 cycles of 95°C for 30 seconds followed by an elongation step of 62°C for 30seconds, and a third cycle of 72°C for 5 minute. Fluorescent signals were monitored sequentially for each cycle at the end of each elongation step. Specificity of RT-PCR products were confirmed by analysis of melting curves and by omission of the reverse transcriptase. All samples were run in duplicate and normalized to β-Actin mRNA using the $\Delta\Delta C_t$ method.

Statistical Analysis One-Way ANOVA was used to analyze between group differences for area at risk, infarct area, apoptotic tissue death (TUNEL), preopioid molecule, opioid receptor, and CGRP protein content. mRNA expression levels were analyzed using REST software (Qiagen). Two-Way repeated measures ANOVA was used to compare group differences between variables in perfused and non-perfused tissue samples. Significant Group differences were determined via a Tukey post hoc analysis. P was set *a priori* to ≤ 0.05.

Table 1. Primers for real-time PCR analysis

PENK	TCAGGAAAGATTGTCCCTGCTGGT
	TTGGAAAGAAGAATGCGCCTGTGG
DOR	TGAAGACGGCCACCAACATCTACA
	TTTCCATCAGGTACTTGGCGCTCT
CGRP	AGAAGAGATCCTGCAACACTGCCA
	TGGGCACAAAGTTGTCCTTCACCA

Forward and reverse primer sets: PENK, proenkephalin; DOR, delta opioid receptor; CGRP, calcitonin gene related peptide.

RESULTS

Animal Characteristics Animal Characteristic data is presented in **Table 2.** In the Unstressed group, no significant differences existed between groups for body weight. In the IR group, between group differences did not exist for either body or heart weight.

Table 2. Animal Characteristics

Unstressed	S	0min	20min	120min	
Body Weight (kg)	388.8±6.1	359.1±6.5	369.4±10.9	368.0±7.9	
IR	Sham	S	Ex	ExD	SD
Body Weight (kg)	348.6±5.8	351.0±10.7	373.8±5.3	375.2±6.9	341.0±5.9
Heart Weight (g)	0.97±0.03	1.05±0.03	1.07±0.02	1.04±0.03	1.00±0.05

Infarct Area Area at risk (AAR) and tissue infarct area are presented in **Figure 3**. and **Figure 4**. Significant between group differences existed for tissue necrosis ($p < 0.0001$). Compared to Sham, S and ExD animals had significantly greater tissue necrosis ($p > 0.0001$, $p = 0.003$). Tissue necrosis was not significantly different between Sham and Ex groups. Ex significantly decreased necrosis compared to S ($p = 0.003$). S was not different compared to SD.

Apoptosis Tissue levels of Apoptosis measured via the TUNEL assay are presented in **Figure 5.** Significant between group differences existed for tissue apoptosis ($p = 0.013$). S had significantly greater levels of TUNEL positive nuclei compared to Sham ($p = 0.016$), while Ex and ExD did not differ from Sham. Ex was significantly lower compared to S ($p = 0.035$), but not ExD. No difference existed between S and ExD or SD. The secondary measure of apoptosis, Bax/Bcl-2 ratio, measured by western blot was not significantly different between groups, or between ischemic and perfused tissue.

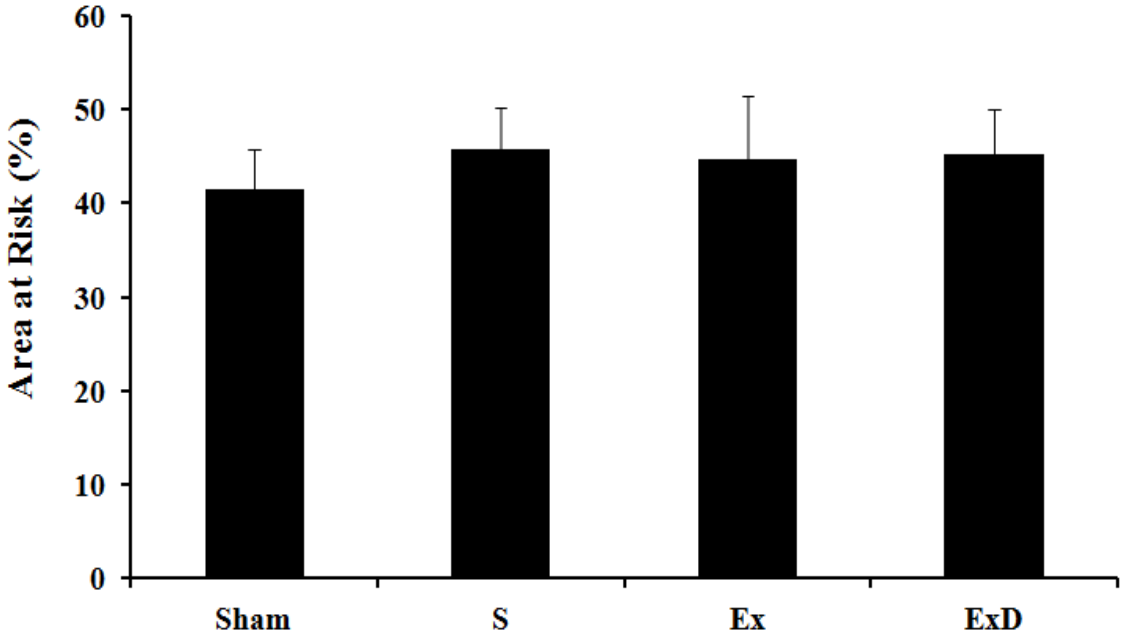


Figure 3. Area at risk, expressed as a percentage of ischemic area per total heart area. Data represents group means ± SEM. Group means: Sham (n-8), S (n=9), Ex (n=10), ExD (n=10)

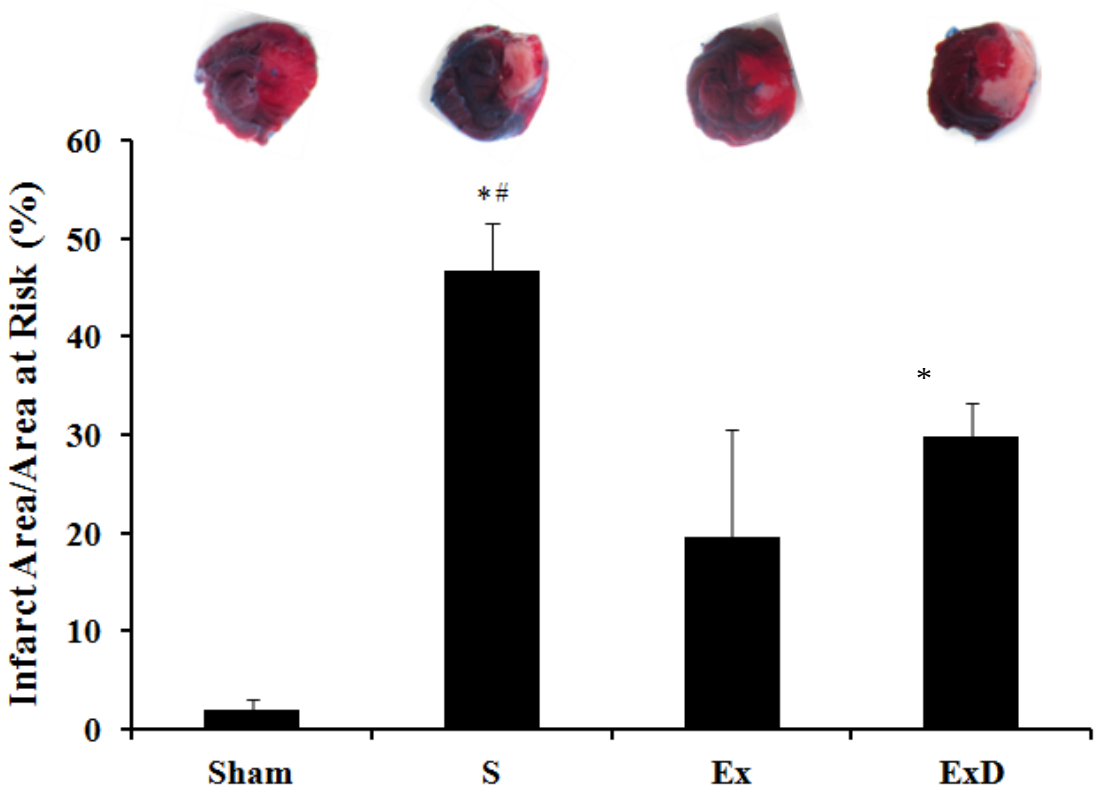


Figure 4. Infarct area expressed as a percentage of area at risk (ischemic area). Data represents group means ± SEM. * Different from Sham, $p \leq 0.05$; # Different from Ex, $p \leq 0.05$. Sham (n=8), S (n=9), Ex (n=10), ExD (n=10).

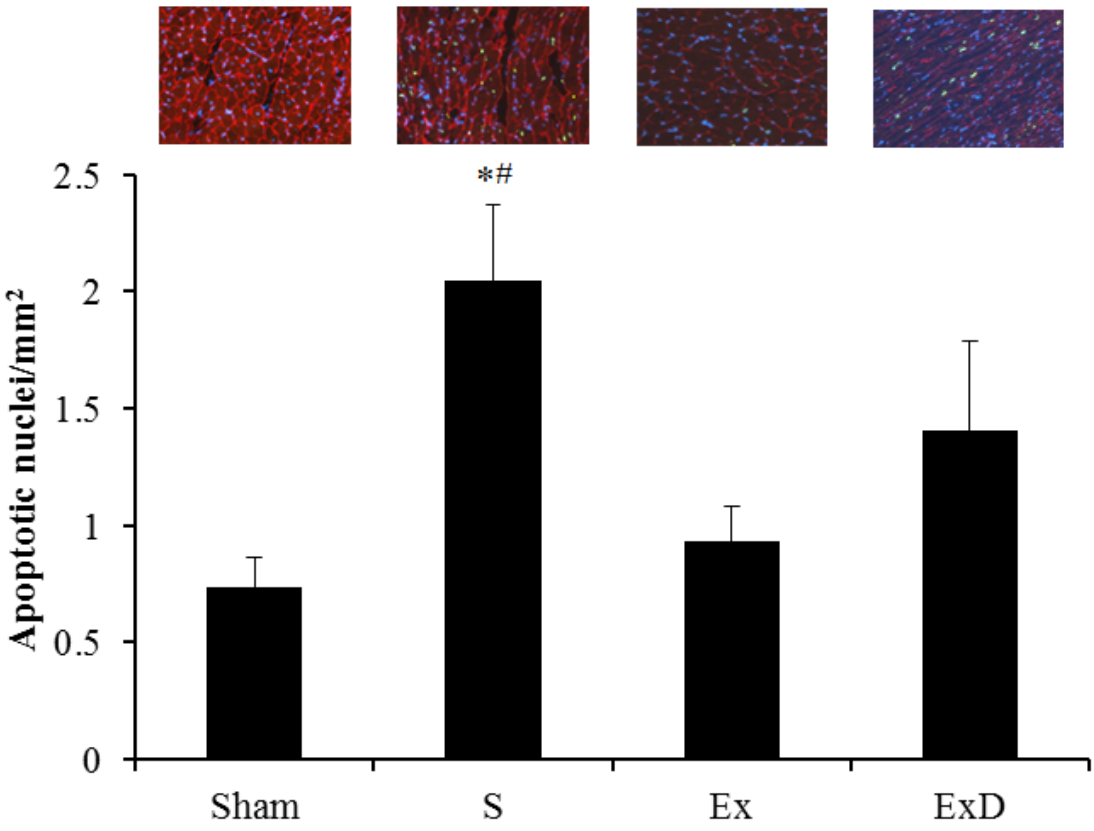


Figure 5. TUNEL assay, apoptotic nuclei per mm2 tissue area. Data represents group means ± SEM. * Different from Sham; # Different from Ex. Group means: Sham (n=8), S (n=9), Ex (n=10), ExD (n=10).

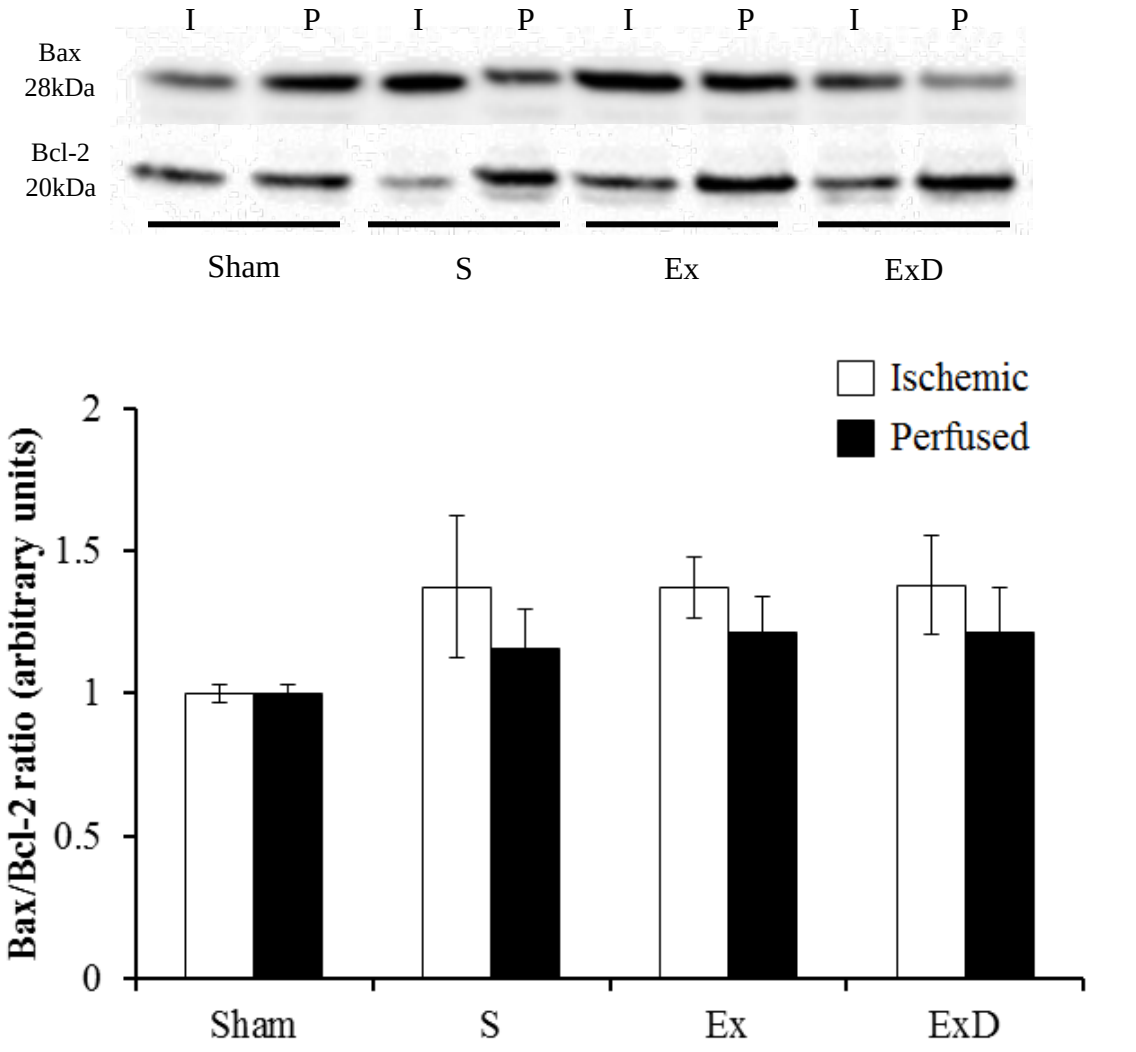


Figure 6. Bax/Bcl-2 ratio expressed relative to Sham values. Open bars represent ischemic tissue, closed bars represent perfused tissue. Data represents groups means ± SEM. Group means: Sham (n=8), S (n=9), Ex (n=10), ExD (n=10).

Real time rtPCR and Protein Expression mRNA levels within the left ventricular tissue for the delta opioid receptor, pre-enkephalin, and CGRP are presented in **Figure 7A**, **7B**, and **7C**, respectively. No significant differences existed between groups for DOR and CGRP mRNA levels. Pre-enkephalin mRNA was significantly elevated from sedentary control animals at 0min and 120min ($p = 0.03$ and $p = 0.021$, respectively). Levels of Leu-enkephalin, DOR, and CGRP were not significantly different between groups (data not shown). CGRP levels in the serum were not significantly different between groups (data not shown).

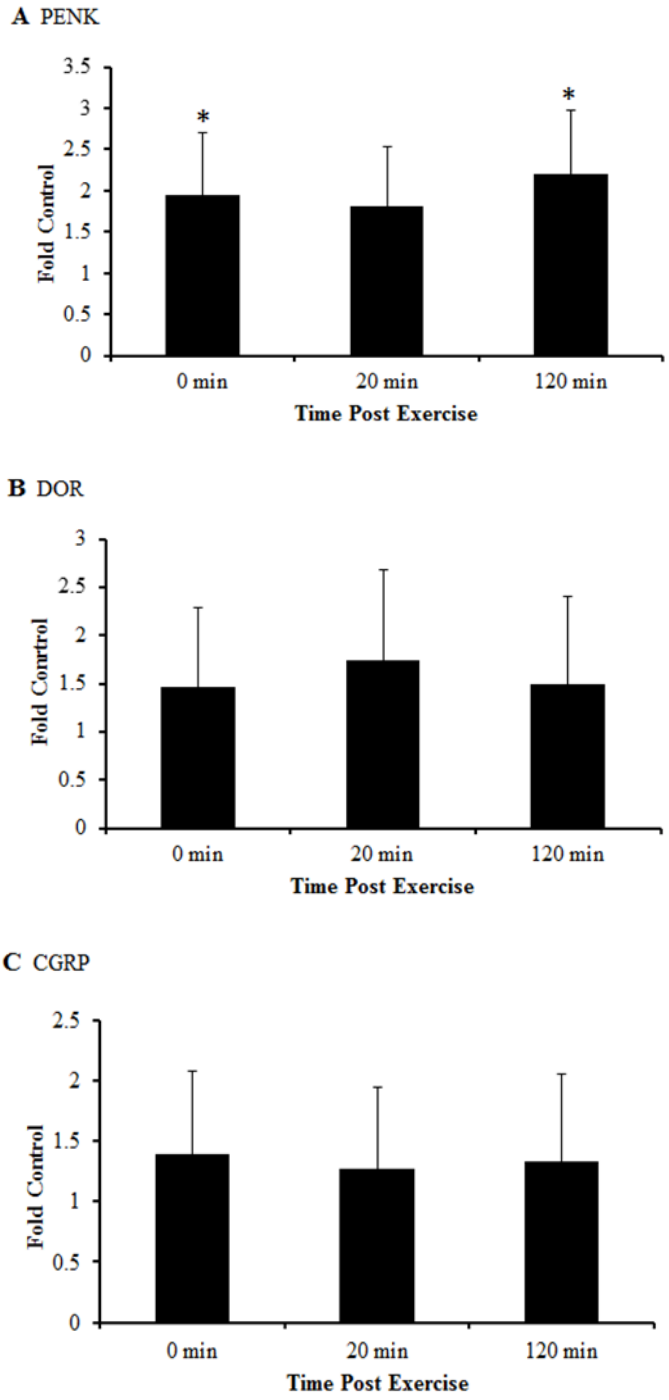


Figure 7. mRNA expression, expressed as fold control. **A**. PENK, proenkephalin; **B**. DOR, delta opioid receptor; **C**. CGRP, calcitonin gene related peptide. Data represents group means ± SEM.

DISCUSSION

Necrotic and Apoptotic tissue death Previous findings from our lab and others demonstrate exercise mediated protection against necrotic tissue death (7, 10). The observation that infarct protection was abolished by administration of a non-specific opioid receptor antagonist provides a strong rationale for the current investigation into the role of specific endogenous opioids in exercise mediated protection. Our results confirm previous findings that exercise protects against both necrotic and apoptotic tissue death (2, 10, 20). Furthermore, we present novel evidence that the delta opioid receptor is involved in protection against necrotic tissue death in short term exercised hearts.

While our results confirm exercise induced protection against apoptotic tissue death, involvement of the delta opioid receptor was not indicated. Conversely, the delta opioid receptor is involved in anti-apoptotic protection in ischemic and pharmacologic preconditioning models (14, 17, 18). Administration of a delta receptor agonist attenuates apoptosis resulting from an ischemic insult (17, 18). The anti-apoptotic effects of the receptor agonist are reversed by a mitochondrial ATP sensitive potassium channel blocker (5HD), as well as a specific protein kinase C delta (PKCδ) inhibitor (17, 18). Interestingly, the Mitochondrial ATP sensitive potassium channel is involved in exercise preconditioning (21), and PKCδ is known to increase following acute exercise (4). The seemingly conflicting data of this study compared to other models of preconditioning and known effects of exercise may be due to a variety of factors. For example, we were only able to observe apoptotic markers at one time point following reperfusion. While the delta opioid receptor does not appear to have a role in exercise mediated protection against apoptosis, it may be that observation of apoptotic markers in the hours and

days following IR may be an enlightening next step to confirm whether or not the delta opioid receptor has a role in exercise protection.

While it is tempting to draw conclusions based upon differing models of preconditioning, it is important to note that many mediators of ischemic preconditioning are not involved in exercise preconditioning. Currently, endogenous opioids appear to be one of the few common factors between the two models of preconditioning. Still, it may be that mediators involved in multiple forms of preconditioning may have differing mechanisms. For example, the mitochondrial K_{ATP} channel is implicated in both necrotic and apoptotic tissue death in the IPC model, while in an exercise model it is involved with protection against arrhythmia and not apoptotic tissue death (21). The exercise model of cardioprotection is sustainable, and not associated with inflammatory response. More research is needed to elucidate the mechanisms of exercise induced endogenous opioid mediated protection to uncover potential sustainable interventions.

mRNA transcripts and protein expression Exercise results in increased expression of both the delta opioid receptor and preproenkephalin in left ventricular tissue (7), while the effects on protein expression are unknown. Our results indicate increased expression of both the delta opioid receptor and proenkephalin following exercise, although only proenkephalin mRNA was significantly elevated at 0min and 120min. The precise factors that account for the discrepancy in our results and previous work are unclear; however it could be due to differences in exercise protocol, or quantification technique (i.e ΔCt vs $\Delta\Delta$Ct). We were unable to identify any differences in protein expression of leu-enkephalin, a common peptide cleavage product of proenkephalin. Detection of transcriptional change without associated translational change may be a result of multiple factors, which are undetermined currently. The myocardial expression of

proenkephalin gene results in a 31 kDa polypeptide precursor, which either enters post-translational processing to yield penta-peptides Met and Leu-enkephalin, hepa peptide MEAP, octa peptide Met-enekephalin-Arg-Gly-Leu, and several other large peptides (25). The disconnect between mRNA expression and protein levels may be explained by the rapid degradation of peptides produced in the heart, the lack of peptide storage, and/or that most forms of enkephalins remain in precursor or large protein form (peptide B or MEAP) and are not included in the measurement of our study or others (25). Although the function of these larger peptides is unclear, it may be that they serve as a reservoir for steady continuous enkephalin release (25). Storage of precursor peptides may allow for the ability to alter the type and amount of enkephalin released in accordance with differing physiological stimuli (25). More research is needed to further characterize the effect of exercise on the storage, release, and function of these opioid peptides.

Delta opioid receptor was detected via western blot, however no significant differences existed between groups ($p = 0.363$). Future research to determine potential effects of receptor binding activity and cellular location may provide useful insights into the mechanisms of delta opioid mediated cardioprotection.

CGRP An exploratory facet of this study was to examine whether CGRP levels were elevated in the minutes following exercise. Existing evidence indicates that the protective effect of endogenous opioids may be linked to CGRP. The left ventricular myocardium releases CGRP following activation of the delta opioid receptor (14). CGRP plasma concentrations are known to increase in response to exercise; however, the effect of CGRP in an exercise preconditioning model has not been examined. Therefore, we chose to examine the mRNA and protein content of CGRP in the left ventricular tissue, as well as plasma concentration in response to acute exercise.

The mRNA levels were not significantly elevated post exercise in any group, and our results do not indicate an effect of exercise on plasma CGRP concentration. Discrepancies between our data and those who have found elevated plasma CGRP following exercise may be due to differences in our exercise protocol, as well as species examined. For example, plasma CGRP is increased in humans following maximal, or near maximal exercise (13), but not following a less intense exercise protocol (15). Furthermore, we were unable to observe the release and/or rate of binding to the CGRP receptor. Although we did not detect any differences in CGRP mRNA or protein content, we cannot definitively rule out the possibility it may indeed have a role in the observed protection.

Further research is warranted to determine the downstream mechanisms of delta opioid receptor mediation against IR induced tissue necrosis. In an ischemic preconditioning model of cardioprotection, the protective effects of delta opioid receptor appear to be linked to the activation of PKC epsilon and opening of mitochondrial K_{ATP} channels (17). Activated PKC epsilon phosphorylates the mitochondrial ATP sensitive potassium channel, maintaining function and aiding the development of ROS production, which in turn increases the activation of PKC epsilon (3, 17). The mitochondrial permeability transition pore (MPTP) typically opens during reperfusion, resulting in mitochondrial swelling and protein release, uncoupling of oxidative phosphorylation, and eventually the reduction of the mitochondrial membrane potential, ultimately resulting in necrotic cell death (3). Activated PKC epsilon reduces the opening of MPTP by direct phosphorylation or via effects on mitochondrial ATP sensitive potassium channel opening (3). That these same mechanisms may be involved in the exercise model of delta opioid mediated cardioprotection is supported by evidence that both mitochondrial K_{ATP} channels and PKC are involved in exercise preconditioning (19, 21). Future research endeavors

to uncover if these mechanism are also involved in the exercise-delta opioid receptor mediated protection would provide insightful information regarding the mechanism of exercise preconditioning.

Conclusions We provide novel evidence for the role of the delta opioid receptor in exercise induced cardioprotection against necrotic tissue death. The known cardioprotective properties of opioid compounds, in combination with the sustainable protection afforded by exercise, make this area of research extremely important for the development of future therapies against IR injury. Furthermore, characterization of the exercise effect on opioid compounds and receptors may prove beneficial in regards to prescription and dosage of pharmacological opioid compounds.

References

1. Bowles DK, Starnes JW. Exercise training improves metabolic response after ischemia in isolated working rat heart. *J Appl Physiol*. 1994;76(4):1608-14.
2. Brown DA, Chicco AJ, Jew KN, Johnson MS, Lynch JM, Watson PA, et al. Cardioprotection afforded by chronic exercise is mediated by the sarcolemmal, and not the mitochondrial, isoform of the KATP channel in the rat. *J Physiol*. 2005;569(Pt 3):913-24. PMCID: 1464258.
3. Budas GR, Churchill EN, Mochly-Rosen D. Cardioprotective mechanisms of PKC isozyme-selective activators and inhibitors in the treatment of ischemia-reperfusion injury. *Pharmacol Res*. 2007;55(6):523-36.
4. Carson LD, Korzick DH. Dose-dependent effects of acute exercise on PKC levels in rat heart: is PKC the heart's prophylactic? *Acta Physiol Scand*. 2003;178(2):97-106.
5. Chicco AJ, Johnson MS, Armstrong CJ, Lynch JM, Gardner RT, Fasen GS, et al. Sex-specific and exercise-acquired cardioprotection is abolished by sarcolemmal KATP channel blockade in the rat heart. *Am J Physiol Heart Circ Physiol*. 2007;292(5):H2432-7.
6. Demirel HA, Powers SK, Zergeroglu MA, Shanely RA, Hamilton K, Coombes J, et al. Short-term exercise improves myocardial tolerance to in vivo ischemia-reperfusion in the rat. *J Appl Physiol*. 2001;91(5):2205-12.
7. Dickson EW, Hogrefe CP, Ludwig PS, Ackermann LW, Stoll LL, Denning GM. Exercise enhances myocardial ischemic tolerance via an opioid receptor-dependent mechanism. *Am J Physiol Heart Circ Physiol*. 2008;294(1):H402-8.
8. French JP, Hamilton KL, Quindry JC, Lee Y, Upchurch PA, Powers SK. Exercise-induced protection against myocardial apoptosis and necrosis: MnSOD, calcium-handling proteins, and calpain. *FASEB J*. 2008;22(8):2862-71. PMCID: 2493460.
9. Gross GJ, Peart JN. KATP channels and myocardial preconditioning: an update. *Am J Physiol Heart Circ Physiol*. 2003;285(3):H921-30.
10. Hamilton KL, Quindry J, French J, Lee Y, Powers SK. MnSOD antisense oligonucleotide treatment attenuates exercise induced protection against infarction and apoptosis following ischemia-reperfusion. *Faseb Journal*. 2005;19(4):A709-A.
11. Hamilton KL, Staib JL, Phillips T, Hess A, Lennon SL, Powers SK. Exercise, antioxidants, and HSP72: protection against myocardial ischemia/reperfusion. *Free Radic Biol Med*. 2003;34(7):800-9.
12. Hamilton KL, Quindry JC, French JP, Staib J, Hughes J, Mehta JL, et al. MnSOD antisense treatment and exercise-induced protection against arrhythmias. *Free Radic Biol Med*. 2004;37(9):1360-8.
13. Hasbak P, Lundby C, Olsen NV, Schifter S, Kanstrup IL. Calcitonin gene-related peptide and adrenomedullin release in humans: effects of exercise and hypoxia. *Regul Pept*. 2002;108(2-3):89-95.
14. Huang MH, Nguyen V, Wu Y, Rastogi S, Lui CY, Birnbaum Y, et al. Reducing ischaemia/reperfusion injury through delta-opioid-regulated intrinsic cardiac adrenergic cells: adrenopeptidergic co-signalling. *Cardiovasc Res*. 2009;84(3):452-60.
15. Kjaer M, Mohr T, Dela F, Secher N, Galbo H, Olesen H, et al. Leg uptake of calcitonin gene-related peptide during exercise in spinal cord injured humans. *Clin Physiol*. 2001;21(1):32-8.

16. Kraemer WJ, Noble B, Culver B, Lewis RV. Changes in plasma proenkephalin peptide F and catecholamine levels during graded exercise in men. *Proc Natl Acad Sci U S A*. 1985;82(18):6349-51. PMCID: 391051.
17. Liu H, Zhang HY, McPherson BC, Baman T, Roth S, Shao Z, et al. Role of opioid delta1 receptors, mitochondrial K(ATP) channels, and protein kinase C during cardiocyte apoptosis. *J Mol Cell Cardiol*. 2001;33(11):2007-14.
18. Maslov LN, Lishmanov YB, Oeltgen PR, Barzakh EI, Krylatov AV, Govindaswami M, et al. Activation of peripheral delta2 opioid receptors increases cardiac tolerance to ischemia/reperfusion injury Involvement of protein kinase C, NO-synthase, KATP channels and the autonomic nervous system. *Life Sci*. 2009;84(19-20):657-63.
19. Melling CW, Thorp DB, Milne KJ, Noble EG. Myocardial Hsp70 phosphorylation and PKC-mediated cardioprotection following exercise. *Cell Stress Chaperones*. 2009;14(2):141-50. PMCID: 2727986.
20. Quindry J, French J, Hamilton K, Lee Y, Mehta JL, Powers S. Exercise training provides cardioprotection against ischemia-reperfusion induced apoptosis in young and old animals. *Exp Gerontol*. 2005;40(5):416-25.
21. Quindry JC, Schreiber L, Hosick P, Wrieden J, Irwin JM, Hoyt E. Mitochondrial KATP channel inhibition blunts arrhythmia protection in ischemic exercised hearts. *Am J Physiol Heart Circ Physiol*. 2010;299(1):H175-83.
22. Roger VL, Go AS, Lloyd-Jones DM, Adams RJ, Berry JD, Brown TM, et al. Heart disease and stroke statistics--2011 update: a report from the American Heart Association. *Circulation*. 2011;123(4):e18-e209.
23. Schultz JE, Gross GJ. Opioids and cardioprotection. *Pharmacol Ther*. 2001;89(2):123-37.
24. Sun SY, Liu Z, Li P, Ingenito AJ. Central effects of opioid agonists and naloxone on blood pressure and heart rate in normotensive and hypertensive rats. *Gen Pharmacol*. 1996;27(7):1187-94.
25. van den Brink OW, Delbridge LM, Rosenfeldt FL, Penny D, Esmore DS, Quick D, et al. Endogenous cardiac opioids: enkephalins in adaptation and protection of the heart. *Heart Lung Circ*. 2003;12(3):178-87.

Cumulative References

References

1. Anderson EJ, Kypson AP, Rodriguez E, Anderson CA, Lehr EJ, Neufer PD. Substrate-specific derangements in mitochondrial metabolism and redox balance in the atrium of the type 2 diabetic human heart. *J Am Coll Cardiol*. 2009;54(20):1891-8. PMCID: 2800130.
2. Anderson EJ, Lustig ME, Boyle KE, Woodlief TL, Kane DA, Lin CT, et al. Mitochondrial H2O2 emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans. *J Clin Invest*. 2009;119(3):573-81. PMCID: 2648700.
3. Balady GJ, Williams MA, Ades PA, Bittner V, Comoss P, Foody JM, et al. Core components of cardiac rehabilitation/secondary prevention programs: 2007 update: a scientific statement from the American Heart Association Exercise, Cardiac Rehabilitation, and Prevention Committee, the Council on Clinical Cardiology; the Councils on Cardiovascular Nursing, Epidemiology and Prevention, and Nutrition, Physical Activity, and Metabolism; and the American Association of Cardiovascular and Pulmonary Rehabilitation. *Circulation*. 2007;115(20):2675-82.
4. Benson JD, Kraemer BB, Mackay B. Malignant melanoma of soft parts: an ultrastructural study of four cases. *Ultrastruct Pathol*. 1985;8(1):57-70.
5. Bernier M, Hearse DJ, Manning AS. Reperfusion-induced arrhythmias and oxygen-derived free radicals. Studies with "anti-free radical" interventions and a free radical-generating system in the isolated perfused rat heart. *Circ Res*. 1986;58(3):331-40.
6. Bolli R. Mechanism of myocardial "stunning". *Circulation*. 1990;82(3):723-38.
7. Bolli R, Marban E. Molecular and cellular mechanisms of myocardial stunning. *Physiol Rev*. 1999;79(2):609-34.
8. Bolli R, Bhatti ZA, Tang XL, Qiu Y, Zhang Q, Guo Y, et al. Evidence that late preconditioning against myocardial stunning in conscious rabbits is triggered by the generation of nitric oxide. *Circ Res*. 1997;81(1):42-52.
9. Bolli R, Shinmura K, Tang XL, Kodani E, Xuan YT, Guo Y, et al. Discovery of a new function of cyclooxygenase (COX)-2: COX-2 is a cardioprotective protein that alleviates ischemia/reperfusion injury and mediates the late phase of preconditioning. *Cardiovasc Res*. 2002;55(3):506-19.
10. Borst SE, Quindry JC, Yarrow JF, Conover CF, Powers SK. Testosterone administration induces protection against global myocardial ischemia. *Horm Metab Res*. 2010;42(2):122-9.
11. Bowles DK, Starnes JW. Exercise training improves metabolic response after ischemia in isolated working rat heart. *J Appl Physiol*. 1994;76(4):1608-14.
12. Braunwald E, Kloner RA. The stunned myocardium: prolonged, postischemic ventricular dysfunction. *Circulation*. 1982;66(6):1146-9.

13. Brown DA, Moore RL. Perspectives in innate and acquired cardioprotection: cardioprotection acquired through exercise. *J Appl Physiol*. 2007;103(5):1894-9.
14. Brown DA, Lynch JM, Armstrong CJ, Caruso NM, Ehlers LB, Johnson MS, et al. Susceptibility of the heart to ischaemia-reperfusion injury and exercise-induced cardioprotection are sex-dependent in the rat. *J Physiol*. 2005;564(Pt 2):619-30. PMCID: 1464442.
15. Brown DA, Chicco AJ, Jew KN, Johnson MS, Lynch JM, Watson PA, et al. Cardioprotection afforded by chronic exercise is mediated by the sarcolemmal, and not the mitochondrial, isoform of the KATP channel in the rat. *J Physiol*. 2005;569(Pt 3):913-24. PMCID: 1464258.
16. Budas GR, Churchill EN, Mochly-Rosen D. Cardioprotective mechanisms of PKC isozyme-selective activators and inhibitors in the treatment of ischemia-reperfusion injury. *Pharmacol Res*. 2007;55(6):523-36.
17. Buettner GR. The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate. *Arch Biochem Biophys*. 1993;300(2):535-43.
18. Carrozza JP, Jr., Bentivegna LA, Williams CP, Kuntz RE, Grossman W, Morgan JP. Decreased myofilament responsiveness in myocardial stunning follows transient calcium overload during ischemia and reperfusion. *Circ Res*. 1992;71(6):1334-40.
19. Chakravarthy MV, Joyner MJ, Booth FW. An obligation for primary care physicians to prescribe physical activity to sedentary patients to reduce the risk of chronic health conditions. *Mayo Clin Proc*. 2002;77(2):165-73.
20. Chen M, Won DJ, Krajewski S, Gottlieb RA. Calpain and mitochondria in ischemia/reperfusion injury. *J Biol Chem*. 2002;277(32):29181-6.
21. Chicco AJ, Johnson MS, Armstrong CJ, Lynch JM, Gardner RT, Fasen GS, et al. Sex-specific and exercise-acquired cardioprotection is abolished by sarcolemmal KATP channel blockade in the rat heart. *Am J Physiol Heart Circ Physiol*. 2007;292(5):H2432-7.
22. Criswell D, Powers S, Dodd S, Lawler J, Edwards W, Renshler K, et al. High intensity training-induced changes in skeletal muscle antioxidant enzyme activity. *Med Sci Sports Exerc*. 1993;25(10):1135-40.
23. Demirel HA, Powers SK, Zergeroglu MA, Shanely RA, Hamilton K, Coombes J, et al. Short-term exercise improves myocardial tolerance to in vivo ischemia-reperfusion in the rat. *J Appl Physiol*. 2001;91(5):2205-12.
24. Demirel HA, Powers SK, Caillaud C, Coombes JS, Naito H, Fletcher LA, et al. Exercise training reduces myocardial lipid peroxidation following short-term ischemia-reperfusion. *Med Sci Sports Exerc*. 1998;30(8):1211-6.
25. Dickson EW, Hogrefe CP, Ludwig PS, Ackermann LW, Stoll LL, Denning GM. Exercise enhances myocardial ischemic tolerance via an opioid receptor-dependent mechanism. *Am J Physiol Heart Circ Physiol*. 2008;294(1):H402-8.
26. Downey JM. Free radicals and their involvement during long-term myocardial ischemia and reperfusion. *Annu Rev Physiol*. 1990;52:487-504.
27. Farber NE, Vercellotti GM, Jacob HS, Pieper GM, Gross GJ. Evidence for a role of iron-catalyzed oxidants in functional and metabolic stunning in the canine heart. *Circ Res*. 1988;63(2):351-60.
28. Finaud J, Lac G, Filaire E. Oxidative stress : relationship with exercise and training. *Sports Med*. 2006;36(4):327-58.

29. French JP, Hamilton KL, Quindry JC, Lee Y, Upchurch PA, Powers SK. Exercise-induced protection against myocardial apoptosis and necrosis: MnSOD, calcium-handling proteins, and calpain. *FASEB J*. 2008;22(8):2862-71. PMCID: 2493460.
30. French JP, Quindry JC, Falk DJ, Staib JL, Lee Y, Wang KK, et al. Ischemia-reperfusion-induced calpain activation and SERCA2a degradation are attenuated by exercise training and calpain inhibition. *Am J Physiol Heart Circ Physiol*. 2006;290(1):H128-36.
31. Gianoulakis C. Endogenous opioids and addiction to alcohol and other drugs of abuse. *Curr Top Med Chem*. 2009;9(11):999-1015.
32. Gross GJ, Peart JN. KATP channels and myocardial preconditioning: an update. *Am J Physiol Heart Circ Physiol*. 2003;285(3):H921-30.
33. Gunduz F, Senturk UK, Kuru O, Aktekin B, Aktekin MR. The effect of one year's swimming exercise on oxidant stress and antioxidant capacity in aged rats. *Physiol Res*. 2004;53(2):171-6.
34. Ha KN, Traaseth NJ, Verardi R, Zamoon J, Cembran A, Karim CB, et al. Controlling the inhibition of the sarcoplasmic Ca2+-ATPase by tuning phospholamban structural dynamics. *J Biol Chem*. 2007;282(51):37205-14.
35. Halliwell B, Gutteridge JM. Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol*. 1990;186:1-85.
36. Halliwell B, Gutteridge JMC. *Free radicals in biology and medicine*. 3rd ed. Oxford
New York: Clarendon Press ;
Oxford University Press; 1999.
37. Hamilton KL, Quindry J, French J, Lee Y, Powers SK. MnSOD antisense oligonucleotide treatment attenuates exercise induced protection against infarction and apoptosis following ischemia-reperfusion. *Faseb Journal*. 2005;19(4):A709-A.
38. Hamilton KL, Staib JL, Phillips T, Hess A, Lennon SL, Powers SK. Exercise, antioxidants, and HSP72: protection against myocardial ischemia/reperfusion. *Free Radic Biol Med*. 2003;34(7):800-9.
39. Hamilton KL, Powers SK, Sugiura T, Kim S, Lennon S, Tumer N, et al. Short-term exercise training can improve myocardial tolerance to I/R without elevation in heat shock proteins. *Am J Physiol Heart Circ Physiol*. 2001;281(3):H1346-52.
40. Hamilton KL, Quindry JC, French JP, Staib J, Hughes J, Mehta JL, et al. MnSOD antisense treatment and exercise-induced protection against arrhythmias. *Free Radic Biol Med*. 2004;37(9):1360-8.
41. Harris MB, Starnes JW. Effects of body temperature during exercise training on myocardial adaptations. *Am J Physiol Heart Circ Physiol*. 2001;280(5):H2271-80.
42. Huang MH, Nguyen V, Wu Y, Rastogi S, Lui CY, Birnbaum Y, et al. Reducing ischaemia/reperfusion injury through delta-opioid-regulated intrinsic cardiac adrenergic cells: adrenopeptidergic co-signalling. *Cardiovasc Res*. 2009;84(3):452-60.
43. Husain K. Interaction of exercise training and chronic NOS inhibition on blood pressure, heart rate, NO and antioxidants in plasma of rats. *Pathophysiology*. 2003;10(1):47-56.
44. Jackson MJ, McArdle A, McArdle F. Antioxidant micronutrients and gene expression. *Proc Nutr Soc*. 1998;57(2):301-5.
45. Jones DP. Disruption of mitochondrial redox circuitry in oxidative stress. *Chem Biol Interact*. 2006;163(1-2):38-53.
46. Jones SP, Bolli R. The ubiquitous role of nitric oxide in cardioprotection. *J Mol Cell Cardiol*. 2006;40(1):16-23.

47. Kane GC, Liu XK, Yamada S, Olson TM, Terzic A. Cardiac KATP channels in health and disease. *J Mol Cell Cardiol*. 2005;38(6):937-43. PMCID: 2736958.
48. Kavazis AN. Exercise preconditioning of the myocardium. *Sports Med*. 2009;39(11):923-35.
49. Kloner RA, Bolli R, Marban E, Reinlib L, Braunwald E. Medical and cellular implications of stunning, hibernation, and preconditioning: an NHLBI workshop. *Circulation*. 1998;97(18):1848-67.
50. Kristiansen SB, Nielsen-Kudsk JE, Botker HE, Nielsen TT. Effects of KATP channel modulation on myocardial glycogen content, lactate, and amino acids in nonischemic and ischemic rat hearts. *J Cardiovasc Pharmacol*. 2005;45(5):456-61.
51. Lennon SL, Quindry JC, French JP, Kim S, Mehta JL, Powers SK. Exercise and myocardial tolerance to ischaemia-reperfusion. *Acta Physiol Scand*. 2004;182(2):161-9.
52. Lennon SL, Quindry J, Hamilton KL, French J, Staib J, Mehta JL, et al. Loss of exercise-induced cardioprotection after cessation of exercise. *J Appl Physiol*. 2004;96(4):1299-305.
53. Lennon SL, Quindry JC, Hamilton KL, French JP, Hughes J, Mehta JL, et al. Elevated MnSOD is not required for exercise-induced cardioprotection against myocardial stunning. *Am J Physiol Heart Circ Physiol*. 2004;287(2):H975-80.
54. Liu H, Zhang HY, McPherson BC, Baman T, Roth S, Shao Z, et al. Role of opioid delta1 receptors, mitochondrial K(ATP) channels, and protein kinase C during cardiocyte apoptosis. *J Mol Cell Cardiol*. 2001;33(11):2007-14.
55. Magal M, Dumke CL, Urbiztondo ZG, Cavill MJ, Triplett NT, Quindry JC, et al. Relationship between serum creatine kinase activity following exercise-induced muscle damage and muscle fibre composition. *J Sports Sci*. 2010;28(3):257-66.
56. Marini M, Lapalombella R, Margonato V, Ronchi R, Samaja M, Scapin C, et al. Mild exercise training, cardioprotection and stress genes profile. *Eur J Appl Physiol*. 2007;99(5):503-10.
57. Maslov LN, Lishmanov YB, Oeltgen PR, Barzakh EI, Krylatov AV, Govindaswami M, et al. Activation of peripheral delta2 opioid receptors increases cardiac tolerance to ischemia/reperfusion injury Involvement of protein kinase C, NO-synthase, KATP channels and the autonomic nervous system. *Life Sci*. 2009;84(19-20):657-63.
58. McCord JM. Oxygen-derived free radicals in postischemic tissue injury. *N Engl J Med*. 1985;312(3):159-63.
59. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation*. 1986;74(5):1124-36.
60. Nichols CG, Ripoll C, Lederer WJ. ATP-sensitive potassium channel modulation of the guinea pig ventricular action potential and contraction. *Circ Res*. 1991;68(1):280-7.
61. Nozdrachev AD, Kotel'nikov SA, Mazhara Iu P, Naumov KM. [A view on the cardiac rhythm control: intracardial regulation]. *Fiziol Cheloveka*. 2005;31(2):116-29.
62. O'Rourke B. Evidence for mitochondrial K+ channels and their role in cardioprotection. *Circ Res*. 2004;94(4):420-32. PMCID: 2712129.
63. Olson GA, Olson RD, Kastin AJ. Endogenous opiates: 1994. *Peptides*. 1995;16(8):1517-55.
64. Peart JN, Headrick JP. Sustained cardioprotection: exploring unconventional modalities. *Vascul Pharmacol*. 2008;49(2-3):63-70.
65. Powers SK, Quindry JC, Kavazis AN. Exercise-induced cardioprotection against myocardial ischemia-reperfusion injury. *Free Radic Biol Med*. 2008;44(2):193-201.

66. Powers SK, Demirel HA, Vincent HK, Coombes JS, Naito H, Hamilton KL, et al. Exercise training improves myocardial tolerance to in vivo ischemia-reperfusion in the rat. *Am J Physiol*. 1998;275(5 Pt 2):R1468-77.
67. Quindry J, French J, Hamilton K, Lee Y, Mehta JL, Powers S. Exercise training provides cardioprotection against ischemia-reperfusion induced apoptosis in young and old animals. *Exp Gerontol*. 2005;40(5):416-25.
68. Quindry JC, French J, Hamilton KL, Lee Y, Selsby J, Powers S. Exercise does not increase cyclooxygenase-2 myocardial levels in young or senescent hearts. *J Physiol Sci*. 2010;60(3):181-6. PMCID: 2854316.
69. Quindry JC, Schreiber L, Hosick P, Wrieden J, Irwin JM, Hoyt E. Mitochondrial KATP channel inhibition blunts arrhythmia protection in ischemic exercised hearts. *Am J Physiol Heart Circ Physiol*. 2010;299(1):H175-83. PMCID: 2904130.
70. Quindry JC, Hamilton KL, French JP, Lee Y, Murlasits Z, Tumer N, et al. Exercise-induced HSP-72 elevation and cardioprotection against infarct and apoptosis. *J Appl Physiol*. 2007;103(3):1056-62.
71. Reid MB, Shoji T, Moody MR, Entman ML. Reactive oxygen in skeletal muscle. II. Extracellular release of free radicals. *J Appl Physiol*. 1992;73(5):1805-9.
72. Roger VL, Go AS, Lloyd-Jones DM, Adams RJ, Berry JD, Brown TM, et al. Heart disease and stroke statistics--2011 update: a report from the American Heart Association. *Circulation*. 2011;123(4):e18-e209.
73. Rolim NP, Medeiros A, Rosa KT, Mattos KC, Irigoyen MC, Krieger EM, et al. Exercise training improves the net balance of cardiac Ca2+ handling protein expression in heart failure. *Physiol Genomics*. 2007;29(3):246-52.
74. Rong F, Peng Z, Ye MX, Zhang QY, Zhao Y, Zhang SM, et al. Myocardial apoptosis and infarction after ischemia/reperfusion are attenuated by kappa-opioid receptor agonist. *Arch Med Res*. 2009;40(4):227-34.
75. Schlier A, Schreckenberg R, Abdallah Y, Krasteva G, Piper HM, Pfeil U, et al. CGRP-alpha responsiveness of adult rat ventricular cardiomyocytes from normotensive and spontaneously hypertensive rats. *Eur J Cell Biol*. 2009;88(4):227-41.
76. Schultz JE, Gross GJ. Opioids and cardioprotection. *Pharmacol Ther*. 2001;89(2):123-37.
77. Sommerschild HT, Kirkeboen KA. Preconditioning - endogenous defence mechanisms of the heart. *Acta Anaesthesiol Scand*. 2002;46(2):123-37.
78. Sperelakis N. *Heart physiology and pathophysiology*. 4th ed. San Diego, Calif.: Academic Press; 2001.
79. Starnes JW, Bowles DK. Role of exercise in the cause and prevention of cardiac dysfunction. *Exerc Sport Sci Rev*. 1995;23:349-73.
80. Starnes JW, Taylor RP, Park Y. Exercise improves postischemic function in aging hearts. *Am J Physiol Heart Circ Physiol*. 2003;285(1):H347-51.
81. Starnes JW, Taylor RP, Ciccolo JT. Habitual low-intensity exercise does not protect against myocardial dysfunction after ischemia in rats. *Eur J Cardiovasc Prev Rehabil*. 2005;12(2):169-74.
82. Starnes JW, Barnes BD, Olsen ME. Exercise training decreases rat heart mitochondria free radical generation but does not prevent Ca2+-induced dysfunction. *J Appl Physiol*. 2007;102(5):1793-8.

83. Stevens JB, Olson WG, Kraemer R, Archambeau J. Serum selenium concentrations and glutathione peroxidase activities in cattle grazing forages of various selenium concentrations. *Am J Vet Res*. 1985;46(7):1556-60.
84. Sun J, Huang SH, Zhu YC, Whiteman M, Wang MJ, Tan BK, et al. Anti-oxidative stress effects of Herba leonuri on ischemic rat hearts. *Life Sci*. 2005;76(26):3043-56.
85. Sun SY, Liu Z, Li P, Ingenito AJ. Central effects of opioid agonists and naloxone on blood pressure and heart rate in normotensive and hypertensive rats. *Gen Pharmacol*. 1996;27(7):1187-94.
86. van den Brink OW, Delbridge LM, Rosenfeldt FL, Penny D, Esmore DS, Quick D, et al. Endogenous cardiac opioids: enkephalins in adaptation and protection of the heart. *Heart Lung Circ*. 2003;12(3):178-87.
87. Wang Y, Haider HK, Ahmad N, Ashraf M. Mechanisms by which K(ATP) channel openers produce acute and delayed cardioprotection. *Vascul Pharmacol*. 2005;42(5-6):253-64.
88. Werns SW, Fantone JC, Ventura A, Lucchesi BR. Myocardial glutathione depletion impairs recovery of isolated blood-perfused hearts after global ischaemia. *J Mol Cell Cardiol*. 1992;24(11):1215-20.
89. Whelan RS, Kaplinskiy V, Kitsis RN. Cell death in the pathogenesis of heart disease: mechanisms and significance. *Annu Rev Physiol*. 2010;72:19-44.
90. Yamashita N, Hoshida S, Otsu K, Asahi M, Kuzuya T, Hori M. Exercise provides direct biphasic cardioprotection via manganese superoxide dismutase activation. *J Exp Med*. 1999;189(11):1699-706. PMCID: 2193084.
91. Yu BP. Cellular defenses against damage from reactive oxygen species. *Physiol Rev*. 1994;74(1):139-62.
92. Zingman LV, Hodgson DM, Bast PH, Kane GC, Perez-Terzic C, Gumina RJ, et al. Kir6.2 is required for adaptation to stress. *Proc Natl Acad Sci U S A*. 2002;99(20):13278-83. PMCID: 130624.

APPENDIX 1

GENERAL INFORMATION AND INSTRUCTIONS

SUBMIT ORIGINAL AND 16 COPIES TO:

ANIMAL RESOURCES, 307 Samford Hall, MAIN CAMPUS

University policy requires that all research, teaching, production/maintenance, and demonstration activities involving vertebrate animals be approved by the Auburn University Institutional Animal Care and Use Committee (IACUC) prior to initiation of the project. The Auburn University IACUC policy is available from the Animal Resources office and website: www.auburn.edu/research/vpr/animals/documents/policy.pdf. This policy is in accordance with federal regulations and guidelines.

When submitting the original and 16 copies, the General Information and Instructions and the Additional Information sections should be omitted.

The IACUC meets the first and third Thursdays of each calendar month. Protocols received at least seven days prior to a scheduled meeting date (e.g. by 11:30 a.m. on Thursday of the week prior to a scheduled Thursday p.m. meeting) will be placed on the agenda. Approved protocols will be assigned a PRN (protocol review number). Approved Animal Subjects Review Forms will remain in the official files of the University for not less than three years beyond the completion of the project. If a project is not funded, please notify ANIMAL RESOURCES AS SOON AS POSSIBLE (844-5978).

Annual review of all protocols is required. An Annual Review Form will be sent to the Principal Investigator approximately 30 days prior to the anniversary date of the approved PRN. Please complete and return the form promptly to ANIMAL RESOURCES, 307 Samford Hall. This form is also available on the Animal Resources website http://www.auburn.edu/research/vpr/animals/forms.htm.

Animal users are encouraged to become familiar with all guidelines and regulations pertaining to the care and use of animals in research and teaching by visiting the Animal Welfare Information Center (AWIC) on the World Wide Web at http://www.nal.usda.gov/awic/

An Animal Subjects Review Form may be obtained by downloading it from the Animal Resources website http://www.auburn.edu/research/vpr/animals/forms.htm. Alternatively, this form may be

obtained at either of the following offices: Animal Resources, 307 Samford Hall, Main Campus or the Division of Laboratory Animal Health, 311 Greene Hall Annex, College of Veterinary Medicine.

Complete this form by providing **BOLD TYPED** answers to each item listed. If an item is not applicable, please indicate NA.

ANIMAL SUBJECTS REVIEW FORM

PRINCIPAL INVESTIGATOR: John Quindry
RANK/TITLE: **Associate Professor**
DEPARTMENT: **Kinesiology**
COLLEGE/SCHOOL: **College of Education**
CAMPUS ADDRESS: **2050 Memorial Coliseum** E-MAIL ADDRESS: **jcq0001@auburn.edu**
CAMPUS PHONE #: **4-1421** FAX #: **4-1467**
X Check if PI will serve as faculty advisor to the Lead Graduate Student or Resident associated with this activity.

LEAD GRADUATE STUDENT/RESIDENT: Lindsey Miller
RANK/TITLE: **Graduate Student**
DEPARTMENT: **Kinesiology** E-MAIL ADDRESS: **les0016@auburn.edu**
CAMPUS PHONE #: **4-1473** FAX #: **4-1467**

CO-INVESTIGATOR: Rajesh Amin
RANK/TITLE: **Assistant Professor**
DEPARTMENT: **Harrison School of Pharm** E-MAIL ADDRESS: **rha0003@auburn.edu**
CAMPUS PHONE #: **4-8295** FAX #:

PROJECT TITLE: Endogenous Opioids and Exercise Induced Cardioprotection in Remote Preconditioning

STARTING DATE: 5/2011 (Must not be prior to IACUC approval)
EXPIRATION DATE: 6/2014 (Must not exceed three years)

Is any part of the funding from a U.S. Public Health Service Agency: Yes ____ No _X_

REQUIRED SIGNATURES
The information contained on this form provides an accurate description of the animal care and use protocol which will be followed. I agree to abide by governmental regulations and university policies concerning the use of animals. I will allow veterinary oversight to be provided to animals showing evidence of pain or illness. If the information provided for this project concerning animal use should be revised, or procedures changed, I will so notify the committee of those changes in writing, and no proposed changes will be implemented until full IACUC approval has been granted.

_____
Principal Investigator **Date**

Medical care for animals will be available and provided as indicated by a qualified veterinarian. By accepting this responsibility, the veterinarian is providing assurance that any personal interest he/she might have in the project will not conflict with his/her responsibility for the provision of adequate veterinary care for the animals. Furthermore, the veterinarian provides assurance of review and consultation on the proper use of anesthetics and pain relieving medications for any painful procedures.

_____
Project Veterinarian **Date**

Dr Laura Tambrallo
Project Veterinarian (Please type or print)

_____
Departmental Chairperson **Date**

Lead Graduate Student/Resident **Date**

***IACUC Chair** **Date**

*IACUC Chair signs the protocol after IACUC approval has been granted.

PLEASE TYPE IN BOLD FONT AND COMPLETE THE FOLLOWING FORM IN FULL.

1. Will the animals be used in:

Teaching	
Research	**X**
Demonstration	
Production	

If Teaching, give the course number: **NA**

2.

Species Common Name	**Total Number**	**Source**	**Housing Location**
Sprague Dawley Rat	**150**	**Harlan Sprague Dawley**	**Biological Research Facility**

3. Will animals be maintained for a period of 12 or more consecutive hours in a location other than the housing location mentioned in Item 2? (*See Item 3 of Additional Information at the end of this form.*)

Yes _____ No **X**

If Yes, specify the location and reason.

4. PERSONNEL QUALIFICATIONS (*See Item 4 of Additional Information at the end of this form.*)

A. Indicate who will provide daily care and maintenance of the animal(s). Indicate name(s) or identify the particular unit staff.

Daily care and maintenance will be provided by the staff of the Biological Research Facility personnel under the direction of Dr Laura Tambrallo and the biological research facility staff. Additional routine care and exercise training will be provided by Dr. Quindry, Lindsey Miller, Graham McGinnis and Brian Kliszczewicz.

B. List the names of all individuals who will conduct procedures involving animals on this protocol. If all individuals are not currently known, please indicate as such.

Dr. John Quindry, P.I. for this project
Lindsey Miller, Lead Graduate Student for this project, trained by Dr. Quindry
Graham McGinnis, Graduate Student, trained by Dr. Quindry
Brian Kliszczewicz, Graduate Student, trained by Dr. Quindry

C. Principal Investigator Certifications

My signature on page 1 of this form certifies that:

1) Individuals performing animal procedures on this protocol are or will be qualified to perform their particular animal related duties through training and/or experience (individuals will be supervised until adequate training has occurred). Training and/or experience must encompass the following: *biology, handling, and care of the species; aseptic surgical methods and techniques (if applicable); the concept, availability, and use of research or testing methods that limit the use of animals or minimize distress; the proper use of anesthetics, analgesics, and tranquilizers (if applicable); and procedures for reporting animal welfare concerns. Informative links regarding training resources have been provided for assistance as needed at http://www.auburn.edu/research/vpr/animals.
2) All individuals working with animals, animal tissues, or animal products on this protocol will be informed of relevant *occupational health and safety issues prior to performing their duties. * Informative links have been provided for assistance in this and other areas as needed at http://www.auburn.edu/research/vpr/animals.

5. State how or why you selected the species to be used in this project.

Due to the invasive nature of this study design, an animal model is the only option. The Sprague Dawley model of exercise induced cardioprotection is chosen because of the similarity to human physiology, and has been well validated by the PI's group and others. Furthermore, the PI has extensive experience with this model.

6. STUDY/ACTIVITY JUSTIFICATION AND OBJECTIVES:

A. Justification:

Currently, cardiovascular disease (CVD) is the leading cause of death in industrialized nations. Although CVD encompasses a variety of conditions, ischemic heart disease is the most prevalent form of CVD, often leading to a myocardial infarction (MI), also known as a 'heart attack'. During ischemia coronary blood flow is either severely inhibited or completely blocked, resulting in ineffectual supply of oxygen and nutrients, and ultimately cardiac dysfunction and tissue death. The re-establishment of blood flow following ischemia is termed reperfusion. Although critical, during the reperfusion phase the majority of cellular damage occurs. The damaging effects of MI will be examined collectively due to the fact that reperfusion must occur in order to continue survival.

Due to the prevalence of ischemic heart disease, much research has been devoted to understand the causes of and responses to ischemia in order to develop potential therapies and prevention techniques.

Short duration exercise is a potent and viable mean of eliciting a protected phenotype against ischemia reperfusion injury- known as exercise induced cardioprotection. Exercise induced

cardioprotection against IR injury has been demonstrated by improved functional measures such as cardiac rhythm abnormalities (electrocardiogram) as well as decreased level of cellular damage and death. Although it is well established that exercise training provides cardioprotection against IR injury, the mechanisms behind this protection are not completely understood. Further research on this topic is critical for further development of treatment and therapies for the large number of individuals who suffer from ischemic heart disease.

B. Objectives:

The primary research aim of our laboratory is to examine mechanisms of exercise induced cardioprotection against ischemia-reperfusion (IR) injury. It has been shown by our lab and others that short term (3 days) of exercise training elicits a cardioprotective phenotype; however, the mechanisms of this protection are yet to be fully understood. Interestingly, recent work has shown endogenous opioids, which are elevated as a result of exercise, have a protective effect against ischemia-reperfusion injury. The specific opioid compound and receptor type involved in protection is yet to be elucidated. Based upon pharmacological research, the delta opioid receptor has an important role in ischemic tolerance via a calcitonin gene related peptide (CGRP) linked mechanism. It is proposed that elevated opioid compounds interact with their receptor to stimulate the release of CGRP, which then mediates the cellular effects affording less IR damage. Interestingly, although the delta opioid receptor is expressed on various tissues throughout the body, only one cell type within the heart known as the intrinsic cardiac adrenergic (ICA) cell, expresses the delta opioid receptor. Therefore, the purpose of our research three fold; First, we aim to determine if exercise induced cardioprotection is mediated via delta opioid and CGRP response and the delta opioid receptor. Secondly, we aim to determine if the protection is mediated via delta opioid receptors located on the heart tissue (as opposed to at another tissue where CGRP might be released to have an effect at the heart) and to characterize the response of plasma CGRP, pre-opioid compounds and opioid receptor expression to an acute exercise training stimulus. Thirdly, we aim to determine the specific role of both ICA cells and cardiac myocytes in exercise induced cardioprotection against ischemia reperfusion injury.

7. SUMMARY OF PROPOSED ACTIVITY: USE LAY TERMS to give a description of the proposed activity. *From reading this section it should be possible for a non-scientist to determine exactly how animals will be used in the context of the proposed activity.*

   This section should include a clear description of the experimental design (research protocols) or activities involving animals (teaching, demonstration, or production/maintenance protocols). This section should also include a brief description of each phase of activities involving animals and should make it possible to account for all animals requested in Item 2. Justification for animal numbers is required to assure that only the necessary number of animals is being used. (*See Item 7 of Additional Information at the end of this form for guidance in providing the appropriate information.)*

One hundred and fifty Sprague Dawley rats (two months old) will be randomly assigned into three treatment groups; either 'Unstressed', 'Unstressed- cell culture' or 'Ischemic-reperfusion' (IR) group (figure 1.) and housed in Auburn University's Biological Research Facility. All animals will receive free access to water and be fed a standard chow diet *ad*

***libidum*. Animals will be subjected to a 12 hour light:dark schedule. Rats will either perform treadmill exercise for three consecutive days, equivalent to that recommended for humans by the American College of Sports Medicine, or remain sedentary (sed-control and Sham). Specific animal exercise is as follows: 5 days treadmill habituation beginning at 10 min at 30 m/min and increasing by 10min each day to a final bout of 50 min on day five. Following the last day of habituation, animals will rest for two days and then perform 3 consecutive days for 60 min at 30 m/min. Breaks lasting at least two minutes will be given every 15 min. Animals will be exercised on a rodent treadmill within the Biological Research Facility. In the event that any rodent does not complete the exercise protocol; they will immediately be excluded from the study. Any injury to the animals will be immediately cared for by a veterinarian and the animal will be removed from the study until approval from veterinarian. Procedures specific to animal groups and protocols following exercise/sedentary-rest for each group are as follows:**

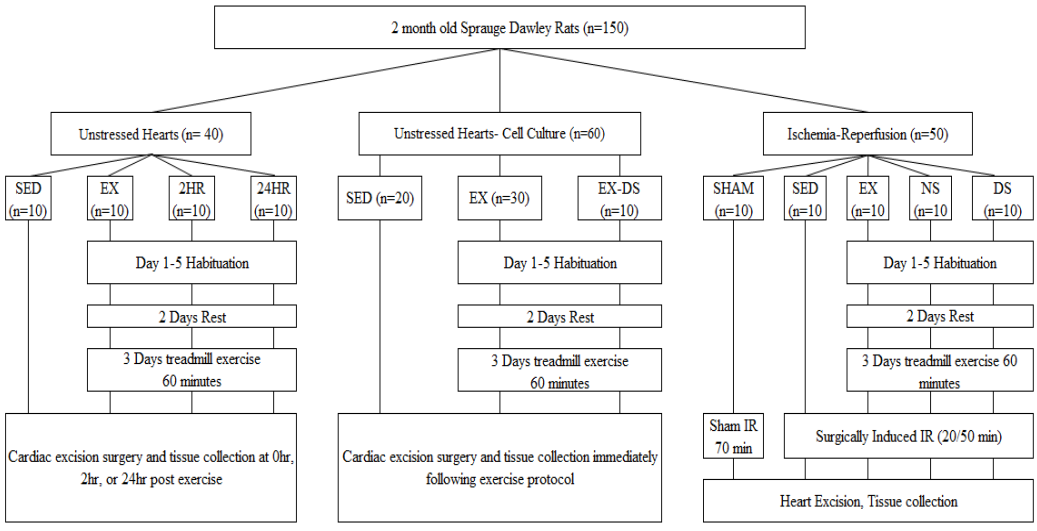
Animals in the 'Unstressed group' will be anesthetized and sacrificed via cardiac excision within the Cardioprotection Laboratory in the Beard Eaves Memorial Coliseum immediately following exercise, or at 2 and 24 hours following exercise; sedentary control animals will also be sacrificed in the same manner. Blood and heart tissue will be collected, immediately frozen in liquid nitrogen, transferred to the Cardioprotection Laboratory in the Department of Kinesiology and stored for future analysis. Measurements will include: opioid precursor molecules, receptors, calcitonin gene related peptide (CGRP -which is released in response to opioid receptor binding), and tissue staining. The humane endpoints for this group will be cardiac excision under surgical plane anesthesia.

Exercised animals in the 'Unstressed-cell culture' group will be further divided into sedentary (SED), exercised (EX) and exercise-delta opioid specific inhibitor (EX-DS). Animals in the EX-DS group will receive subcutaneous injections 15 minutes prior to exercise on the three 60 minute days of delta opioid receptor specific blocker, naltrindole (10mg/kg). Immediately following completion of the exercise protocol, animals will be transferred to the Harrison School of Pharmacy in order to collect and prepare cardiac tissue for cell culture studies. Once animals are brought to the Harrison School of Pharmacy, they will be immediately anesthetized and sacrificed via cardiac excision. Sedentary animals will also be transferred to the Harrison School of Pharmacy building and sacrificed in the same manner. Only animals in the 'Unstressed-cell culture' group will be transferred to the Harrison School of Pharmacy for subsequent cell culture studies. Animals will be euthanized immediately upon transition from Biological Research Facility to the Harrison School of Pharmacy, which is planned to be within one hour of exercise completion for all animals. Blood and heart tissue will be collected and prepared for cell culture studies. The humane endpoints for this group of animals will be by cardiac excision under surgical plane anesthesia.

The 'IR' group will be further divided into SHAM, SED, EX, non-specific opioid inhibitor (NS) and delta specific opioid inhibitor (DS). Animals in the EX, NS and DS groups will perform treadmill exercise described above. DS animals will receive a delta-opioid specific receptor inhibitor, naltrindole (10mg/kg) and NS animals will receive non-specifc opioid receptor inhibitor, naltrexone (10mg/kg) via subcutaneous injections 15 minutes prior to

exercise on the three 60 minute days. Following the final day of exercise, animals will be immediately transferred to the Cardioprotection Laboratory in the Kinesiology Department. Animals will then be anesthetized via IP injection of sodium pentobarbital (80mg/kg) and undergo surgically induced IR by ligation of the left anterior descending (LAD) coronary artery for 20 minutes. Reperfusion will be established by loosening of the ligature, lasting 50 minutes. Following the reperfusion phase, animals will be sacrificed by removal of the heart tissue for analysis. Sedentary animals will receive the same surgical procedure; Sham animals will receive the same surgery minus the final tightening of the ligature. The humane endpoints for this group will be by cardiac excision under surgical plane anesthesia.

Figure 1. Experimental Design



8. A. Select pain/distress category relevant to the use of animals in this study. (*See Item 8A of Additional Information at the end of this form.)*

C _____ D __X__ E _____

B. If category D or E was chosen in 8A, please complete the following. (*See Item 8B of Additional Information at the end of this form.)*

1) Database(s) searched or other sources consulted to determine the availability of alternatives.

Database Searched		Date of Search	Years Covered
Medline	**X**	**2-17-11**	**1950-2011**
Agricola			
CABA			
Altweb			
Other (describe)	**Pubmed**	**2-17-11**	**1950-2011**

2) Keywords and search strategy used when considering alternatives to the painful or distressful procedure(s).

Keywords: Ischemia, reperfusion, IR injury, opioids, cardioprotection, exercise and opioids, myocyte and opioid, cardiac excision

Began by searching 'ischemia reperfusion', then 'IR injury', and 'cardioprotection' to find alternative protocols for the control animals. Next I moved on to slightly more specific searches for my topic, using 'exercise and opioids', 'myocyte and opioid', 'opioid and cardioprotection', and 'cardiac excision'.

3) A succinct written narrative based on results of the database search, that will permit the IACUC to readily assess whether the search topics were appropriate and whether the search was sufficiently thorough. This narrative must address the following:

Reduction:

The number of animals per group has been chosen in order to minimize the number of animals used and still find significance between group means, with expected attrition rate of 15 percent, based upon previous work of the PI and others. Also, these animals will serve as sedentary and exercise controls for multiple groups, therefore limiting the total amount of animals used by our laboratory.

Replacement:

An invasive study design is required to examine the effects of exercise and opioids during ischemic heart disease at the cellular level to further understand how to treat and prevent

ischemic heart disease. Due to this invasive design, an animal model is essential. The Sprague Dawley rat model is ideal in that it will not require 'practice' since it has been utilized previously by our laboratory and it allows for comparison with other related research.

Refinement:

The PI has been conducting related research for 10 years and has the experience necessary to limit animal use.

4) If alternatives are available but will not be used, please provide a justification. **NA**

5) If pain/distress category E is to be employed, please provide a justification for withholding pain and/or distress relieving drugs. **NA**

9. SURGERY:

Will surgery be performed?
Yes X No ___
If yes, please address the following, as applicable:

A. Nonsurvival surgery - Describe all surgical procedures, including surgical preparation. Indicate where surgery will be performed (building and rooms). Identify the person(s) and describe their qualifications for performing the particular surgical procedure(s).

Ischemia-Reperfusion surgeries will be performed by Dr. John Quindry, with assistance from Lindsey Miller, Graham McGinnis and Brian Kliszczewitz. Dr. Quindry has 10 years experience in related animal surgical procedures. IR surgeries will be performed within the Department of Kinesiology Cardioprotection Laboratory at Auburn University. Animals will be transported to the Carioprotection Laboratory or Harrison School of Pharmacy building in a climate controlled vehicle and a covered box in order to minimize stress to the animals. The cardiac excision surgeries will be performed by Dr. John Quindry, Lindsey Miller, Graham McGinnis and Brian Kliszczewitz within the Cardioprotection Laboratory, or the Harrison School of Pharmacy building on campus at Auburn University. Animals will be anesthetized via IP injection of sodium pentobarbital (80mg/kg) to surgical plane anesthesia prior to both surgical procedures. All animals will be euthanized within 12 hours of removal from the Biological Research Facility.

Animals in both the Unstressed and Unstressed- cell culture group will be sacrificed via thoracotomy and cardiac excision following confirmation of surgical plane anesthesia. Animals in the Unstressed group will be sacrificed within the Biological Research Facility, and animals in the Unstressed- cell culture group will be sacrificed within the Harrison School of Pharmacy building. Animals in the Ischemia Reperfusion group will receive surgically induced IR, performed via left thoracotomy and ligation of the left anterior descending coronary artery.

The animals will be ventilated on room air and real time blood pressure and ECG will be recorded via carotid artery cannulation and paw electrodes, respectively. Animal body temperature will be maintained at 37˚ C using a Gaymar water bath and heating blanket. The Jugular vein will be cannulated with small tubing connected to a syringe of sodium pentobarbital, held in place with sutures placed around the vein, holding the tubing in place. Surgical plane of anesthesia will be maintained via periodic injections of sodium pentobarbital via the jugular vein cannulation. Ischemia will last 20 minutes, followed by loosening of the ligature to induce reperfusion, lasting 50 minutes. Following the reperfusion phase, Evans Blue dye will be injected into the heart via the jugular cannulation and the heart will immediately be removed for tissue analysis.

B. Survival surgery - Describe all surgical procedures, including surgical preparation and post-surgical care. Please indicate that aseptic technique will be followed if the procedure is a survival surgical procedure. Indicate where surgery will be performed and what postoperative care will be provided (building and rooms). Identify the person(s) and describe their qualifications for performing the particular surgical procedure(s).

NA

10. Administration of analgesics, anesthetics, tranquilizing drugs, and/or neuromuscular blocking agents (Indicate generic name, dose, route of administration and frequency; if by inhalation, method of scavenging waste anesthetic gases.)

Animals will be anesthetized prior to cardiac excision via i.p. injection of sodium pentobarbital at a dose of 80mg/kg. Additional sodium pentobarbital needed to reach surgical plane will be administered via i.p. injection at a concentration of 10mg/ml, in 1ml injection doses until surgical plane is reached. During IR surgery, additional sodium pentobarbital will be administered via jugular catheterization to maintain surgical plane anesthesia at a concentration of 10mg/ml.

11. Administration of reagents, cells, drugs (other than anesthetics or analgesics), infectious agents, carcinogens, recombinant DNA, etc. (Indicate generic name, dose, route of administration and frequency, anticipated side effects, monitoring protocol.)

Animals in the Unstressed-cell culture group will be further divided into three subgroups: sedentary (SED), exercise (EX), and exercise- delta opioid inhibited (EX-DS). Animals in the EX-DS group will receive a specific delta opioid receptor blocker, naltrindole (10mg/kg) 15 minutes prior to exercise on the last three consecutive days of the protocol. The IR group will be further divided to receive a specific delta opioid receptor blocker, naltrindole (10mg/kg) or non-specifc opioid receptor blocker naltrexone (10mg/kg) by subcutaneous injection 15 minutes prior to exercise during the three consecutive days of exercise treatment. No known side effects are expected with the given dose of these treatments; however, animals will be closely monitored for any signs of abnormal behavior before, during and after treadmill exercise. Furthermore, animals will be examined daily for normal grooming, food/water intake, and urinary/bowel movements, as evidenced by "normal" soiling of bedding.

12. ASSURANCES:

A. Provide a brief statement to confirm that proposed activities involving animals do not duplicate previous experiments unnecessarily.

An exercise induced increase in opioid receptor RNA and precursor molecules, as well as loss of cardioprotection by non-specific opioid receptor blockage has been demonstrated. However, it is essential to confirm these results in order to substantiate further findings which have not been previously demonstrated such as specific receptor protein expression levels, CGRP levels, and the specific receptor involved in exercise induced cardioprotection.

B. My signature on page 1 of this form certifies that exercise of caged dogs will be accomplished according to the Animal Welfare Act (AWA) or cage size provides adequate space for exercise to meet AWA requirements. Alternatively, explain why an exception should be approved by the IACUC.

C. Will wild caught or endangered animals be utilized?
Yes _____ No **X**

If Yes, the investigator is responsible for obtaining and maintaining valid permits (if required) for collecting, purchasing, transporting, and holding of these animals. List applicable federal and/or state permit numbers and expiration dates. **NA**

13. HAZARDOUS AGENTS

Use of hazardous agents in animals may require approval of the appropriate institutional committee. Contact the Department of Risk Management and Safety (844-4870) for specific information.

Hazardous Agent	**Yes**	**No**	**Agent**	**Date of Committee Approval and BUA #**
Radioisotopes		X		
Biological Agents		X		
Hazardous Chemicals or Drugs		X		
Recombinant DNA		X		

Describe the practices and procedures required for the safe handling and disposal of contaminated animals and material associated with this study. Also describe methods for removal of radioactive waste and, if applicable, the monitoring of radioactivity.

14. What will be the disposition of the animals at the termination of the project? If euthanasia is to be performed, what will be the method of carcass disposal?

All animals will be euthanized by sodium pentobarbital injection followed by cardiac excision as described previously. All carcasses will be incinerated at the College of Veterinary Medicine on the campus of Auburn University.

15 All protocols must include the method of euthanasia that will be used during the normal course of the protocol or in the event of unforeseen circumstances resulting from illness or injury. Please specify the method, agent, dose, and route of administration. The euthanasia method must be consistent with the AVMA Panel on Euthanasia or justification for deviation should be indicated. This document is available on the Animal Resources website, http://www.auburn.edu/research/vpr/animals/ resources/res_index.htm and in the Journal of the American Veterinary Medical Association (Vol. 218, No. 5, Pages 669-696, 2001).

All animals will be euthanized by IP injection of sodium pentobarbital (80 mg/kg) and cardiac excision.

ADDITIONAL INFORMATION

THIS PAGE NEED NOT BE INCLUDED WHEN SUBMITTING FORM FOR REVIEW

3. The IACUC is required to inspect animal housing areas and laboratories (at least twice per year) where animals are kept for 12 or more hours.

4. PERSONNEL QUALIFICATIONS:

Federal regulations require institutions to ensure that people caring for or using animals are qualified to do so through documented training or experience. This training is to include investigators, technical personnel, trainees, visiting investigators, and any other individuals who may perform animal husbandry, anesthesia, surgery, or other experimental manipulations involving animals.

7. Please use this procedure list for guidance in providing the necessary information. Please note that this is not meant to be an exhaustive list, but only a guide.

- $ **Body fluid sampling** (e.g. blood, cerebrospinal fluid, ascites, urine —describe method of collection, amount, frequency).
- $ **Antibody production** (indicate route of administration, volume administered per site, number of sites, adjuvant use and frequency, consideration of alternatives to Freund's adjuvant, anticipated side effects, monitoring protocol).
- $ **Ascites method for monoclonal antibody production**. Auburn University requires adherence to the Office for Protection from Research Risks (OPRR) policies concerning the production of monoclonal antibodies using the mouse ascites method. Please refer to the OPRR document http://oacu.od.nih.gov/ARAC/ascites.htm. Use of the ascites method requires justification as to why in vitro systems cannot be used.
- $ **Special diets** (describe any anticipated nutritional deficit or other health concerns).
- $ **Indwelling catheters or implants** (describe type, maintenance/monitoring protocol).
- $ **Restraint of an unanesthetized animal** other than that associated with brief routine procedures such as for the collection of blood (describe method, duration, frequency).
- $ **Tumor transplantation** (describe any anticipated functional deficit to the animal, monitoring protocol, endpoint).
- $ **Food or fluid restriction** (e.g. greater than that associated with pre-anesthetic procedures — describe, include justification and monitoring protocol.)
- $ **Special housing, equipment, animal care** (e.g. describe special caging, water, feed, waste disposal, etc.)
- $ **Experimental endpoint criteria** (list the criteria to be used to determine when euthanasia is to be performed. Death as an endpoint must always be scientifically justified.)

8A. USDA promulgated PAIN/DISTRESS CATEGORIES - Please use the following categories when categorizing the pain/distress level.

C Pain or Distress - None or Minor

These include studies that DO NOT involve surgery; induction of painful or stressful disease conditions, or pain or distress in excess of that associated with routine injections or blood collection. Included are induction or transplantation of tumors in animals (as long as the tumors do not cause pain and the animals are terminated prior to becoming ill), administration of mildly toxic substances or pathogenic agents that cause no significant disease or distress, polyclonal antibody production (antigen inoculations and blood collection) as long as significant disease does not result, mild food restriction, and, typically, the collection of animals from the wild or from experimental units (i.e. fish in earthen ponds) for minor procedures. NOTE: If blood is to be collected via the retro-orbital or intracardiac methods, then anesthesia is required and Pain/Distress D must be selected. Also, if *in vivo* monoclonal antibody production is to be performed, the pain category D must be selected.

D Pain or Distress Relieved by Appropriate Measures

A major concern of the reviewers of these protocols is the degree of pain and/or distress imposed on the animals in the studies, and the methods the investigators will use to prevent, relieve, or minimize such pain or distress.

Following is a partial list of procedures known to involve significant pain and/or distress:

1. Surgical procedures such as biopsies, gonadectomy, exposure of blood vessels, chronic catheter implementation, laparotomy, or laparoscopy
2. Administration of any chemical or organism that would be expected to produce pains or distress but which will be alleviated by analgesics
3. Intracardiac or retro-orbital blood collections
4. Monoclonal antibody production (ascites method)
5. Other procedures which would be painful or distressful to the animal if performed without the benefit of anesthesia, analgesic, and/or tranquilization (e.g., exsanguination).

E Pain or Distress without Anesthesia, Analgesia or Tranquilizers

If the nature of the study prohibits the use of pain and/or distress relieving drugs, or if unavoidable and unalleviable pain or distress will be produced, you must provide a written justification. (Include this in your response to Item 8, B, 5.) Such procedures include: direct stimulation of central nervous system pain tracts, nociceptor stimulation by physical or chemical means that cause severe pain (e.g., corneal abrasions), or any potentially painful procedure if performed without chemical relief of pain.

8B. The Animal Welfare Act (AWA) requires that the Principal Investigator (PI) consider alternatives and provide a written narrative of the sources consulted to determine whether or not alternatives exist to procedures which may cause pain or distress.

According to the Animal Welfare Information Center (AWIC) of the U.S. Department of Agriculture (USDA), an alternative to procedures that may cause more than momentary pain or distress to animals is any procedure which results in REDUCTION in number of animals used, REFINEMENT of techniques to alleviate such pain or distress, or REPLACEMENT of animals (e.g. with an insentient model such as might be accomplished through use of cell culture or computer simulation). For assistance in conducting database/network searches, as required by the AWA when procedures may cause more than momentary pain or distress to animals, investigators may contact the AU Library On-Line Services (844-1748). Alternatively, to explore a variety of resources for evaluating alternatives investigators may consult the following website: http://www.aaalac.org/alts.htm

PI Checklist for Animal Subjects Review Form

General:

- ❒ Did you use the newest version of the Animal Subjects Review Form?
- ❒ Did you spell out all acronyms the first time they were used?
- ❒ Did you verify the spelling of all drugs used?
- ❒ Did you include a copy of any referenced Standard Operating Procedures (SOPs) and/or existing protocols?
- ❒ Did you omit all irrelevant information when using a previous protocol file to create a new Animal Subjects Review Form?

All Protocols:

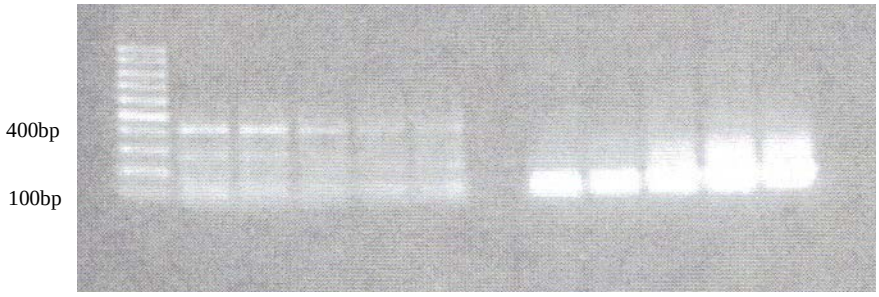
- ❒ Did you check yes or no to Public Health Service funding source?
- ❒ #2- Did you clarify animal numbers as "per year" or "project total"?
- ❒ #2 and #7- Did you make sure animal numbers in these two sections agree?
- ❒ #2- Did you name the commercial sources?
- ❒ #2- Did you provide the specific housing facility?
- ❒ #4 - Did you list all individuals involved in study by their names (if known)?
- ❒ #7- Did you address how the animal numbers were **determined** and/or **justify** these numbers?
- ❒ #7- Could the study design be presented more clearly using a table?
- ❒ #7 and #11- Did you, if applicable, include the route of administration and/or dosage for all drugs used?
- ❒ #7- Did you, if applicable, include the technique, location, and/or volume of blood drawn?
- ❒ #7- Did you, if applicable, provide the method of transportation and/ or the method of restraint?
- ❒ #8.B.3. - Did you specify reduction, replacement, and/or refinement as they pertain to this study?
- ❒ #10 and #11- Did you, if not applicable, put None or N/A?
- ❒ #12 – Did you provide, if applicable, permit numbers and expiration dates?
- ❒ #13 – Did you include, if applicable, the Biological Use Authorization (BUA) number and date of approval or indicate that it is pending?
- ❒ #14 - Did you address method of carcass disposal and/or the location in the event that euthanasia becomes necessary?
- ❒ #15 - Did you indicate the method of euthanasia should it become necessary?

Teaching Protocols:

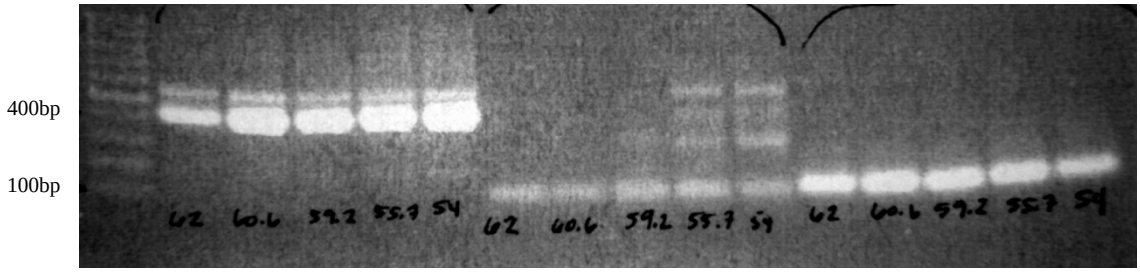
- ❒ #7- Did you include, if applicable, the number of students per animal, the number of animals per lab, and the number of labs per year?

Appendix 2

Temperature Gradient (°C) PCR Products



18S rRNA Delta Opioid Receptor



Beta Actin CGRP Proenkephanlin