Interleukin-6 and Exercise-Induced Cardioprotection

by

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Abstract

Ischemia-Reperfusion (IR) injury is the leading cause of death in the US and industrialized nations. Exercise preconditioning has emerged as a unique method to reduce the injury associated with IR, yet the mechanisms have not been fully elucidated. The purpose of this study was to examine the potential of the cytokine Interleukin-6 (IL-6) as an exercise-induced mediator of cardioprotection. Wild type (C57) and IL-6 knockout (IL-$6^{-/-}$) mice (n=32 for both groups) underwent treadmill habituation, and three days of cardioprotective exercise for 60 minutes at 18 $m*min^{-1}$ and 0% grade. Aim 1, animals were sacrificed PRE, POST, 30, and 60 minutes post-exercise on the third session, and serum, skeletal muscles and hearts were analyzed for indices of IL-6 signaling via PCR and western blotting. Aim 2, mice were separated into Sedentary (C57 SED and IL-$6^{-/-}$ SED), Exercised (C57 EX and IL-$6^{-/-}$ EX), and Sham (SH, C57 mice only) groups (C57 n=24; IL-$6^{-/-}$=16), and received a surgically induced *in vivo* IR surgery. Hearts were excised and incubated in TTC for myocardial necrosis. Ischemic and perfused tissues were collected and analyzed for indices of apoptosis and autophagy via PathScan analysis and conventional western blotting. Aim 1: Exercise increased IL-6 and sIL-6R in the blood, as well as IL-6R mRNA and protein in the gastrocnemius and myocardium. IL-6R protein was increased only in the C57 mice. P-STAT3 was similarly increased in gastrocnemius and heart in *both* C57 and IL-$6^{-/-}$ mice post exercise. Myocardial iNOS mRNA and protein were decreased by exercise, while COX-2 mRNA and protein were unchanged. Aim 2:

Exercise protected C57 EX mice against IR induced arrhythmias and necrosis, while C57 SED, IL-$6^{-/-}$ SED and IL-$6^{-/-}$ EX mice had significantly higher arrhythmia scores, and significantly greater % infarct area compared to SH. C57 EX mice had increased p44/42 MAPK and p38 MAPK phosphorylation compared to IL-$6^{-/-}$ EX mice, suggesting protection was dependent upon these pathways. This is the first study to show that exercise exerts cardioprotection via IL-6, and strongly implicates protective signaling originating from the exercised skeletal muscle.

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List of Abbreviations

AAR	Area At Risk
Akt	Serine/Threonine Protein Kinase B
ANOVA	Analysis of Variance
Atg	Autophagy Related
ATP	Adensoine Triphosphate
Bax	B-cell lymphoma 2-associated X protein
Bcl-2	B-cell lymphoma 2
BSA	Bovine Serum Albumin
BSF-2	B-cell Stimulatory Factor 2
CF	Cytosolic Fraction
COX-2	Cyclooxygenase 2
CVD	Cardiovascular Disease
DNA	Deoxyribonucleic Acid
ECG	Electrocardiogram
EDL	Extensor Digitorum Longus
Ex	Exercised
FasL/FasR	Fas Ligand/Fas Receptor
F'/R'	Forward/Reverse Primer Sequences
GIK	Glucose-Insulin-Potassium

Gp-130	Glycoprotein 130
HNE	Hydrogen Sodium Exchanger
HSP	Heat Shock Proteins
H_2O_2	Hydrogen Peroxide
IACUC	Institutional Animal Care and Use Committee
IF	Immunofluorescence
IHC	Immunohistochemistry
IL-6	Interleukin-6
IL-6R	Interleukin-6 Receptor
iNOS	Inducible Nitric Oxide Synthase
IPC	Ischemic Preconditioning
IP3	Inositol Triphosphate
IR	Ischemia-Reperfusion
Jak	Janus Kinase
LAD	Left Anterior Descending Coronary Artery
MAPK	Mitogen Activated Protein Kinase
MI	Myocardial Infarction
$Mito_{KATP}$	Mitochondrial ATP-sensitive Potassium Channel
MPTP	Mitochondrial Permeability Transition Pore
NADPH	Nicotinamide Adenine Dinucleotide Phosphate-Oxidase
NCX	Sodium Calcium Exchanger
NF	Nuclear Fraction
NFDM	Non Fat Dry Milk

NIH	National Institutes of Health
NO	Nitric Oxide
PARP	Poly (ADP-ribose) Polymerase
PBS	Phosphate Buffered Saline
PI3-K	Phosphoinositide 3-Kinase
PVDF	Polyvinylidene difluoride
ROS	Reactive Oxygen Species
RONS	Reactive Oxygen and Nitrogen Species
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Species
RT	Room Temperature
$Sarc_{KATP}$	Sarcolemmal ATP-sensitive Potassium Channel
Sed	Sedentary
SERCA	Sarco-Endoplasmic Reticulum Calcium ATPase
sIR	Simulated Ischemia-Reperfusion
SOCS	Suppressor of Cytokine Signaling
SOD1	Copper Zinc Superoxide Dismutase (CuZnSOD)
SOD2	Manganese Superoxide Dismutase (MnSOD)
STAT	Signal Transducer and Activator of Transcription
SWOP	Second Window of Protection
TBST	Tris Buffered Saline with 0.05% Tween
TNF	Tumor Necrosis Factor
TTC	2-3-5, Triphenyltetrazolium Chloride

qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
VO_2max	Maximum Volume of Oxygen Consumption
WT	Wild Type

CHAPTER I:

INTRODUCTION

Cardiovascular disease (CVD) and diseases of the heart are the leading causes of morbidity and mortality in developed nations, accounting for almost 1 in 3 deaths in the US (133). Mortality data indicate that over 2200 American deaths associated with CVD occur each day. Perhaps more shockingly stated, approximately 1 death every 40 seconds can be attributed to CVD (133). The most prevalent manifestation of CVD is the common heart attack, or, myocardial infarction (MI) (133). During MI, blood flow is reduced to a region of the heart for a period of time (ischemia) until blood flow is re-established (reperfusion). Collectively, the physiological stress and injury incurred is referred to as ischemia-reperfusion (IR) injury (23), and occurs by three pathologically different means, with increasing severity as a function of time (67, 105).

With increasing duration of ischemia, diagnostically different types of injury occur with increasing severity. Generally, during the first five minutes following the onset of ischemia, arrhythmias are the most prevalent cardiac dysfunction (104). Although acute ECG abnormalities are sometimes lethal during IR, these arrhythmias are usually not life threatening provided ventricular pump function is not unduly compromised. As such, ventricular arrhythmias associated with IR do not induce lasting pathology in and of themselves. With ischemia extending to twenty minutes, arrhythmias may persist and myocardial stunning, resulting in ventricular pump dysfunction, occurs. These contractile

deficits are generally non-lethal and recovery is expected in the hours and days following (13). Ischemic bouts persisting for greater than 20 minutes inflict irreversible injury to the cardiomyocytes. Myocardial tissue death occurs via two mechanistically and phenotypically distinct processes; Necrosis and Apoptosis. Necrosis occurs as the direct result of cellular injury or stress during IR, and creates a larger inflammatory response in neighboring cells. Alternatively, apoptosis is a form of cell-mediated cell death in which an ATP dependent pathway is activated resulting in apoptosome formation and systematic cellular breakdown. While apoptosis is adaptive in some acute scenarios during development, increased apoptosis post IR is pathologic. In contrast, necrosis is thought to be purely pathologic (95). More recently, attention reveals a third regulatory pathway, autophagy, which mediates the cellular outcomes during IR injury. Analogous to recycling, autophagy is a *compartmentalized* form of *organelle* death, in which low performing or damaged organelles are ubiquitin labeled for proteasomal degradation. Autophagy-driven digestion of cellular structures benefits the myocardium by lowering the cellular ATP demand during periods of ischemia and breaks down malfunctioning organelles that contribute to cellular injury (51, 58). The difference between pathologic and adaptive up regulation of autophagy is currently a topic of ongoing investigation. Evidence suggests that the acute preservation of basal levels of autophagy in the minutes and hours following IR injury benefits the cellular energetic state, and also limits cell death, while a prolonged or sustained up regulation of autophagy (i.e. days) is maladaptive (99).

Early reperfusion of the myocardium, thereby limiting the ischemic duration, is the most important intervention serving to improve post MI prognoses and reduce the severity of IR injury (67, 105). Paradoxically, the majority of myocardial damage occurs during

reperfusion. Thus, establishing robust and reproducible methods to protect the heart against the injury inflicted during both ischemia and reperfusion is of clinical importance. As such, understanding of myocardial "preconditioning" emphasizes three methodological approaches to protect the heart; Ischemic Preconditioning (IPC), Pharmacologic Preconditioning, and Exercise Preconditioning. IPC was initially observed in response to transient bouts of sub-lethal ischemia (108), and has been extensively investigated in a search for viable mechanisms to be exploited pharmacologically to reduce IR injury. Exercise is a unique form of preconditioning, and is demonstrated to be mechanistically different from IPC or pharmacologic PC (44, 126).

Exercise confers protection to the heart (cardioprotection) in four main areas; 1) modification and reduction of primary risk factors for CVD, 2) angiogenesis and structural remodeling of the myocardium, 3) post IR rehabilitation from IR injury, and 4) *exercise preconditioning*. Documentation as early as the 1800's revealed that physical activity reduces angina pectoris (66). Decades of research have been dedicated to understanding effective and sustainable means to activate myocardial preconditioning and thereby decrease the incidence and severity of IR injuries.

Several key mediators of cardiac preconditioning have emerged; these include Adenosine, Manganese Superoxide Dismutase (MnSOD), Heat shock proteins (HSP), and preemptive opening of the mitochondrial and sarcolemmal ATP sensitive potassium channels ($Mito_{KATP}$ and $Sarc_{KATP}$, respectively) (19, 129). Methods to activate these proposed mediators include pharmacologic agents, IPC and exercise. However, exercise is the only *sustainable* way to elicit a robust cardioprotected phenotype (20, 122, 127).

Previous research has focused on endogenous cardioprotective mediators, although recent findings indicate that remote factors induce cytoprotection through endocrine-like interactions. Recently, small glycoproteins, produced in and secreted from exercising skeletal muscle have been identified (93, 110, 113, 114, 150). Subsequently, these glycoproteins were termed "myokines," and skeletal muscle was re-classified as possessing endocrine-like properties (113). Among these myokines, interleukin 6 (IL-6) is most notably up regulated in response to exercise, with expression increasing as much as 100-fold (80, 114, 150). Though IL-6 is controversially discussed as both a pro- and anti-inflammatory cytokine in various physiological scenarios, as related to cardioprotective exercise, it appears that its functions are *anti*-inflammatory and metabolically regulatory (116, 117).

Mechanistic studies of IL-6 induced cardioprotection carried out both *in vivo* (32) and *in vitro* (144) lend support to the current study rationale. However, the aforementioned studies were investigating pharmacologic preconditioning or IPC, and are not in the context of exercise. Using an *in vivo* rodent IPC model, Dawn and Bolli found an essential role for IL-6 in preconditioning the heart against a 30 minute regional ischemia (32). The infarct sparing effect, elicited by 6, 4-min bouts of sub lethal ischemia, was abrogated in IL-$6^{-/-}$ mice exposed to an identical IPC stimulus and IR injury as WT mice. Tissue analyses revealed that the protection afforded to the IPC hearts was conferred via IL-6 signaling through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, resulting in the up regulation of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) (32). Smart et al. demonstrated a cardioprotective role for IL-6 against a simulated IR injury (sIR) *in vitro*. By pre-incubating cardiomyocytes with IL-

6, they observed a reduction in cellular damage, and preservation of cell viability and Ca^{2+} homeostasis following sIR (144). Similarly, the protection afforded to the cardiomyocytes was nitric oxide (NO) dependent, and was abolished by the addition of an iNOS inhibitor, aminoguanidine. Calcium homeostasis was found to be phosphotidyl-inositol 3-kinase (PI3-K)-dependent, and the protection was ameliorated by the addition of the PI3-K inhibitor, wortmannin (144).

While one of the hallmarks of exercise-induced preconditioning is its mechanistic differences from IPC, it is possible that both pre-conditioning stimuli invoke common mechanisms and/or pathways. Alternatively, given the marked differences in inflammatory versus non-inflammatory pathways at work in ischemic and exercise preconditioned hearts, respectively, further examination of the cellular pathways responsible for preconditioning are required. Collectively, a rationale exists that the exercise-induced increase in circulating IL-6, largely attributed to increased synthesis and release from exercising skeletal muscle, is sufficient to activate protective pathways in the myocardium. Therefore, the purpose of this study was to investigate the role of IL-6 in exercise induced myocardial preconditioning against IR injury. In that regard, it is hypothesized that exercise will increase IL-6 signaling in the heart and mitigate IR-induced apoptotic and necrotic tissue death in an IL-6 dependent fashion. Furthermore, exercise-induced increases in IL-6 will preserve autophagy immediately following IR.

CHAPTER II

REVIEW OF LITERATURE

Ischemia Reperfusion Injury

Cardiovascular disease is the leading cause of morbidity and mortality in the US and developed nations (133). The majority of CVD pathologies manifest as ischemic heart disease (133). Following an ischemic event, the most effective intervention to date is prompt reperfusion, clinically achieved via thrombolytic infusion (20). Paradoxically, cellular injury occurs during both ischemia and reperfusion, leading to a more encompassing term for the collective damage from both phases, ischemia reperfusion (IR) injury (23, 146). Decades of research delineate the pathophysiology and underpin theoretical interventions against IR injury. To better understand strategies to reduce myocardial injury, one must first understand a mechanistic progression of IR injury.

IR induced arrhythmias and myocardial stunning

Myocardial injury during IR is traditionally categorized as reversible (ventricular arrhythmias and myocardial stunning) or irreversible (necrotic and apoptotic tissue death). Secondary to cellular ATP deficiency due to reduced blood flow creating a supply-demand mismatch, all forms of injury are in the context of two underlying mechanisms during IR: Ca^{2+} overload and ROS production (during ischemia as well as the free radical "burst" immediately upon reperfusion) (122, 146, 163). During IR, accumulating atherosclerotic plaque or thrombi formation reduce blood flow to an area of the heart ventricle, making it

ischemic. Ischemic injury occurs in three clinically distinct phases, increasing in severity proportional to ischemic duration (67, 105). During ischemia, oxidative phosphorylation decreases in proportion to oxygenated blood delivery, creating a bioenergetic challenge to the effected myocardium. A subsequent increase in glycolytic metabolism occurs to maintain cellular ATP levels. This metabolic shift increases hydrogen ion production, resulting in acidosis (23, 34, 51, 146). As a result of low [ATP] and H^+ accumulation, the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA), Na^{2+}/K^+ ATPase and the sarcolemmal ATP sensitive K^+ channel ($Sarc_{KATP}$) become overwhelmed and an ionic imbalance of Na^{2+} and Ca^{2+} occurs (118, 146). Secondary to the accumulation of $[H^+]$, the activity of the hydrogen sodium exchanger (HNE) increases. Ionic disruption of Na^+ inside the cell, results in a directional reversal of Na^{2+}/Ca^{2+} exchanger (NCX), thereby increasing intracellular Ca^{2+} (51). Dysfunctions in ionic control during ischemia cause direct and indirect disruption of the contractile machinery, resulting in arrhythmias (23, 118, 130). Contractile deficits are the first clinically relevant manifestation of myocardial ischemia, which often appear within the first five minutes following coronary artery blockage. Provided reperfusion of the effected myocardium, arrhythmias are generally transient, disappearing within hours of reperfusion.

Extending beyond the first minutes of unremitting ischemic injury, myocardial stunning appears. Myocardial stunning was first identified by Heyndrickx in 1975 (68), and is described succinctly as persistence of contractile abnormalities during reperfusion in the absence of cellular death (11, 13, 81). Overproduction of ROS during ischemia, as well as the free radical burst upon reperfusion, both alter Ca^{2+} handling, and are contributory factors in myocardial stunning. The increased production of ROS leads to oxidation of the

sarcolemmal ion pumps and Ca^{2+} handling proteins on the sarcoplasmic reticulum. This interaction propagates ionic disruption, leading to Ca^{2+} induced stunning (13). In addition to the repercussions of ionic dysregulation, Ca^{2+} overload can activate calpains, a class of calcium sensitive proteases capable of digesting contractile proteins, contributing to stunning (46, 143), as well as initiating cell death pathways (109).

Oxidative stress plays an important role in the progression of IR injury (26, 85, 90, 121, 122). Specifically, the over production of reactive oxygen and nitrogen species (ROS and RNS, or RONS) promote the oxidation of cellular constituents, i.e. proteins, lipids and DNA, leading to a cascade of dysfunction and injury (85, 122). Cellular sources for RONS during IR include NADPH oxidases, xanthine oxidase, and the mitochondria (2). ROS also affect contractile proteins directly, creating contractile deficits that are reversed by antioxidant treatment (63, 142). Exposure to the superoxide anion attenuates contractile force in a calcium independent manner (94), demonstrating the dynamic interplay between Ca^{2+} homeostasis and oxidative stress.

Tissue death - Necrosis

During IR injury, cell death occurs by two mechanistically different outcomes; necrosis and apoptosis (95, 163). During necrosis, damaged cells swell due to intracellular hyperosmolarity and rupture releasing cellular contents (95). Thus, measuring levels of cardiac specific isoforms of creatine kinase and troponin in the blood are used clinically to diagnose cardiac injury or myocardial infarction. Infarct-induced necrosis is a passive cellular process that effects greater areas of cells subjected to bioenergetic stressors or mechanical injury. Necrosis generally elicits a greater inflammatory response, making it an important therapeutic target in the treatment of IR injury (40).

Disagreement exists in the literature in the use of term necrosis to describe a mechanism of cell death, since it refers to the degradation processes *following* cell death, which occur approximately 24 hours later (40, 95). For this reason, "oncosis" or "oncotic necrosis," can be used to describe the same pathology (40, 95). However, the term necrosis is commonly accepted to describe cell death in response to acute injury, where membrane integrity is compromised (59).

Tissue death - Apoptosis

Alternatively, cells undergoing an energetic stressor may enter an ATP-dependent programmed form of cell death called apoptosis. Apoptosis is characterized by a significant *decrease* in cell volume (15, 95). Initiation of apoptosis occurs by some combination of Intrinsic and Extrinsic pathways. Extrinsic initiation of apoptosis is mediated by activated membrane bound death receptors in complex with signaling peptides, such as FasL/FasR and TNFα/TNFR1 (40, 84). The Intrinsic initiation of apoptosis is mitochondrially driven, and is related to membrane permeability and release of mitochondrial proapoptotic proteins (40). Both pathways converge on the cleavage and activation of Caspase 3, which represents the committed step of apoptosis. Downstream processes include the fragmentation of DNA, apoptotic body formation, and phosphatidylserine translocation to the membrane to ensure non-inflammatory phagocytosis of vesicles (40, 131).

As related to IR injury in the hearts, Boehning et al. identified a Ca^{2+} induced amplification of apoptosis. Increased cytosolic [Ca^{2+}] triggers cytochrome *C* release from the mitochondria, which binds to inositol triphosphate (IP_3) receptors on the endoplasmic reticulum membrane (9). Receptor activation stimulates a large cytosolic flux of Ca^{2+} from the ER, and a concomitant rise in mitochondrial [Ca^{2+}], which dissipates the mitochondrial

potential and triggers the formation of the mitochondrial permeability transition pore (MPTP) (9, 101). Opening of the MPTP releases cytochrome *C,* dissipates mitochondrial membrane potential, and initiates cellular apoptosis.

Morphologically, differences exist between apoptosis and necrosis, which elicit different local outcomes. Necrotic cell death involves the rupture of cells and the release of cytosolic contents and promotion of subsequent inflammation that impacts nearby ventricular tissue (95). Conversely, apoptosis does not evoke an inflammatory response. Cellular contents and organelles are packaged in vesicles called apoptotic bodies, phagocytosed, and degraded within phagolysosomes (40, 138).

Autophagy

A third cellular outcome, autophagy, has been the focus of recent cardioprotection investigations. Quite literally translated, autophagy is loosely referred to as a "self-eating" and is a cellular process activated by bioenergetic/nutrient stress, ROS and cellular injury, where damaged or non-essential cellular constituents are selectively degraded (52). Through a process of ubiquitin-like labeling, targeted proteins are engulfed into an autophagosome and chaperoned to the lysosome. Merging of the lysosome and autophagosome produces an autophagolysosome, where proteins are digested thereby reducing the metabolic obligations of the cell (58). While basal autophagy levels mediate the normal turnover of cellular constituents, pathological increases in autophagic flux are associated with pathologic cardiac hypertrophy, heart failure, and detrimental outcomes post IR (58, 99). Importantly, the inhibition of autophagy in the $Atg5^{-/-}$ mouse magnifies infarct following IR (58, 62), suggesting that autophagic initiation rescues jeopardized cells from entering a cell death pathway. Gomes and Scorrano (2011) showed that, in response

to starvation, low functioning mitochondria can be selectively targeted for degradation based on morphology such that more efficient mitochondria are spared (48, 49). Targeting underperforming mitochondria may mitigate intrinsic activation of apoptosis. These findings from prior investigations support the current convention that autophagy, or manipulation of the activation of autophagy, is a potential therapeutic target for IR injury (124).

Myocardial Preconditioning

As heart disease is a leading cause of morbidity and mortality in the US, interventions to decrease infarct size and preserve the viability of ischemic cardiomyocytes are clinically important. Theoretically, the ability to harness preconditioning mechanisms prior to IR injury will improve post IR outcomes. To date, several scientific approaches to elicit preconditioning have been investigated; Ischemic Preconditioning (IPC), Pharmacologic Preconditioning, and Exercise-Induced Preconditioning.

The cornerstone paper in the field of preconditioning was published by Murry et al. (1986). The landmark discovery revealed that four discontinuous 5 minute ischemic periods 24 hours before an extended duration infarct drastically reduced cardiac tissue death (108). From this initial discovery thousands of follow-up investigations have been undertaken in order to reveal key mediators, and to discover pharmacological therapeutic agents that preempt the ischemic insult. From a phenomenological perspective, the protection afforded to the heart from IPC manifests in a biphasic response with an initial phase of preconditioning, referred to as classical preconditioning, and a delayed/late phase, or the second window of protection (SWOP) (6, 25, 166). Classical preconditioning is robust, yet brief, lasting less than 4 hours (25). The SWOP however is less fortifying, but is sustained

for up to 96 hours (6). In both cases, the threshold stimulus to elicit protection from IPC is at least a 1 x 5 minute coronary occlusion (6, 25). Candidate trigger mechanisms that initiate IPC and corresponding mediators are thoroughly outlined in prior reviews (166). Findings from several groups implicate two specific mediators, iNOS and COX-2, as central to late phase of IPC (32, 56, 76, 144, 164). Using immunohistochemical analysis of hearts harvested from ischemically preconditioned rats, Wang et al. (2002) found that iNOS was expressed by cardiac muscle during an IPC protocol, but by inflammatory cells during permanent occlusion, suggesting adaptive and maladaptive responses to sub-lethal and index ischemia, respectively (160). Xuan et al. (2001) demonstrated the iNOS mediated protection afforded to hearts by IPC was dependent on activation of the JAK/STAT pathway, specifically STAT3 (164). Similarly, Smith, et al. (2004) found STAT3 phosphorylation to be essential to both IPC and pharmacologic preconditioning in isolated cardiomyocytes and perfused hearts (145). Expanding upon those findings, Dawn, et al. (2004) showed that the STAT3 mediated up regulation of iNOS and COX-2 in IPC *in vivo* was mediated by IL-6. IPC was completely abrogated in the IL-$6^{-/-}$ mouse (32). Using an inducible cardiac specific $STAT3^{-/-}$ mouse, Bolli et al. (2011) demonstrated an obligatory role for STAT3 phosphorylation and activation in the up-regulation of protective mediators following IPC (14). Conversely, Zuurbier et al (2012) found that *reduced* IL-6 signaling through STAT3 was associated with attenuated cytoprotection following IPC (168). Furthermore, coronary effluent taken from preconditioned "donor" rats, when administered to non-preconditioned "acceptor" rats, mitigated IR injury (75). Analysis of the myocardial tissue of the "acceptor" rats showed increased levels of phospho-STAT3,

suggesting a humoral factor in the preconditioned effluent was capable of inducing STAT3 phosphorylation (75).

Taken together, these results present a strong rationale that transient increases in IL-6, resulting in IL-6 signaling in the heart, can activate protective mediators in a STAT3 dependent manner. While the previously presented evidence utilizes IPC induced increases in IL-6, or pharmacologically administered IL-6, the hypothesis that *exercise* triggers the same protective pathways in an IL-6 dependent fashion has never been tested.

Exercise-Induced Cardioprotection

While there are some obvious similarities between ischemic, pharmacologic preconditioning, and exercise preconditioning, both descriptive and mechanistic findings indicate the exercise stimulus is unique. A short course of exercise (3 days) produces a more sustainable window of protection, lasting between 9 and 18 days (87), while protection afforded by ischemic and pharmacologic means dissipates within 4-7 days. Perhaps more importantly from a clinical application, exercise provides robust protection in young and old animals, whereas non-exercise forms of preconditioning do not (125, 139). Characteristic of both IPC and pharmacologic preconditioning is a truncated window of protection (6, 7), and a physiological tolerance to the stimulus accumulated during a period of chronic treatment (30, 158). Importantly, neither pharmacologic nor ischemic preconditioning elicits cardioprotection in the aged myocardium (139). Of the many pharmacological preconditioning agents tested, none have been translated into clinical use. Of the possible pharmacological interventions, only adenosine and glucose-insulin-potassium (GIK) are effective as a means of post conditioning the myocardium when administered at reperfusion (12).

Distinct from these previously discussed methods to precondition the myocardium, exercise has emerged as a unique and interesting stimulus (20, 44, 78, 122). Dating back as early as the 1800's, Dr. William Heberden recorded in his *Commentaries on the History of Cures and Diseases*, "I knew one who set himself a task of sawing wood for half an hour every day, and was nearly cured [of angina pectoris] (66)," which has been heralded as one of the earliest records of the protective effects of exercise on the heart. More recent observation by Paffenbarger et al. (1970) indicated that men in more physically active occupations displayed less CVD (112). It is important to note that "exercise-induced cardioprotection" is a term encompassing 4 principle components; 1) Risk factor modification to reduce the incidence of CVD, 2) Structural remodeling of the myocardium to reduce IR induced damage, 3) Post conditioning through cardiac rehabilitation therapy, and 4) Exercise Preconditioning, which remains to be the least understood and was the focus of this research. Considering these preliminary observations, the concept of exercise-induced cardioprotection against IR injury has existed for some time. However, reductionist evaluation of the underlying mechanisms of exercise preconditioning has primarily materialized over the past decade.

Exercise Preconditioning refers to biochemical modulations to the cardiac phenotype that induce resistance to IR injury independent of structural adaptations (33, 119, 120). Cumulatively, existing data indicate that both chronic (weeks)(17, 18, 103) and acute (days) (35, 45, 63, 64, 88, 126, 128-130, 165) exercise protects the heart against all major classifications of IR injury. Following just a few (1-3) days of exercise, the heart is resistant to ventricular arrhythmias (63, 130), stunning (88), and both necrotic and apoptotic cell death (45, 128, 129). Individual mediators of exercise preconditioning have been studied

in pursuit of a "magic bullet" of cardioprotection, or an "exercise factor." As exercise is a multifaceted stimulus, the protection afforded to the myocardium is comprised of myriad mediators that improve myocardial tolerance to IR, either individually or in concert.

Oxidative Stress and Antioxidants

As oxidative stress is pivotal to the injury during IR, the role of enzymatic and non-enzymatic antioxidants, via supplementation or up regulation from exercise, are of interest in preconditioning the heart against oxidative stress during IR (26, 64, 122, 128). Superoxide dismutase (SOD) is a potent antioxidant enzyme responsible for catalyzing the conversion of superoxide (O_2^-) to hydrogen peroxide (H_2O_2). SOD exists in two forms; SOD1, or CuZnSOD (cytosolic) and SOD2, or MnSOD (mitochondrial). Antioxidant defense systems to buffer H_2O_2 to water include Catalase and Glutathione peroxidase (and the cycle of oxidized and reduced glutathione) (22). Of these antioxidants, SOD2 is the only one consistently found to be up regulated by exercise and contribute to exercise preconditioning (63, 64). Acute treatment with exercise, hyperthermia or TNF-α (delivered pharmacologically or from exercise) increased SOD2 activity in the left ventricle, yielded an equivalent magnitude infarct sparing effect in each treatment (74, 165). Exercise and antioxidant supplementation were also found to improve the left ventricular developed pressure (LVDP) during short duration IR and reduce infarct size during long duration IR when administered individually or in combination (64). Exercise preconditioning against apoptosis and necrosis is dependent on an increase in SOD2. Importantly, administering antisense oligonucleotides against SOD2 to counter exercise-induced increases in enzyme overexpression, abrogates the exercise induced increases in SOD2 as well as exercise preconditioning against arrhythmias (63) and infarct (45).

The interplay between oxidative stress and heat shock proteins (HSPs) was long held to be involved in exercise preconditioning (64). HSPs are a class of chaperone molecules that protect cells by regulating protein folding, repair, and degradation, and have been investigated as a potentially protective mechanism in exercise preconditioning. Key members of the HSP family include α-crystallin, HSP27, HSP72, and HSP90 (8, 96). Exercise consistently increases skeletal muscle levels of HSPs. The expression of *myocardial* HSP72 also increases in response to exercise (64, 120, 128, 156). Similarly, an increase in myocardial HSP72 is associated with attenuated IR injury. However, extensive investigation of these candidate mediators of cardioprotection has yet to identify an essential role for HSPs in myocardial preconditioning from exercise. In that regard, exercise, without the up regulation of HSPs, still imparts a robustly protected phenotype, thereby establishing that HSP72 is not essential to, exercise preconditioning (128, 156). However, recent findings implicate HSP72 in exercise-induced cardioprotection and blood flow regulation via smooth muscles in major coronary vessels and require further investigation to resolve whether an essential role in cardioprotection occurs (106).

ATP Sensitive Potassium Channels

Recent emphasis has been placed on the role of the ATP sensitive potassium channels (KATP) located in the sarcolemma ($Sarc_{KATP}$) and mitochondria ($Mito_{KATP}$) of cells and their role in the exercise induced cardioprotection. Pharmacologic studies indicate that $Mito_{KATP}$ and $Sarc_{KATP}$ opening are involved in preconditioning the heart against IR. Similarly, the roles of the Mito- and $Sarc_{KATP}$ channels have been investigated in the context of exercise preconditioning (19, 44, 129, 130). These channels function as metabolic sensors and are sensitive to intracellular ATP concentrations. These channels are

thought to shorten the cardiac action potential and preventing Ca^{2+} overload (55). Recent results from our lab and others indicate that the $Mito_{KATP}$ channel affords protection against arrhythmias during short duration IR, while the $Sarc_{KATP}$ channel mediates protection against IR induced necrotic tissue death (19, 129, 130).

Cytokines, Myokines and IL-6

Cytokines are a functionally distinct subset of glycoprotein signaling molecules with a molecular weight between 15 and 30 kD (36). Among these cytokines, interleukin 6 (IL-6) has received recent attention due to its pleiotropic ability to affect most tissues in the body, often with contradictory responses. IL-6 was initially identified by Hirano, et al. in 1986 for its interaction with B-cells, and was initially named B-cell stimulatory factor 2 (BSF-2) (71). IL-6 is approximately 21 kD, but reported sizes vary 21-28 kD, depending on the phosphorylation and glycosylation status (132). Comparative modeling and X-ray diffraction analysis reveal 4 alpha helical structures comprising the main structure of IL-6. Four cysteine residues are identified that play an integral role in the formation of two disulfide bonds, which are essential to the biological function of IL-6 (132). Studies across several animal models show that these residues are highly conserved among murine and human species, supporting their importance. The Cys45-Cys51 disulfide bond is essential for full biological function, while the disulfide bond at Cys74-Cys84 impacts the proper folding and preservation of binding capacity (132).

IL-6, Exercise, and Muscle

Acute exercise is a well-established physiologic stimulus to elicit an increase of the concentration of circulating IL-6 (37, 42, 61, 79, 80, 93, 148). Initially, it was thought that the increase in IL-6 following exercise was due to muscle fiber damage and infiltrating

immune cells (21, 115). However, subsequent investigation revealed that IL-6 was increased following both concentric and eccentric exercise (77), and displayed no association between IL-6 and markers for muscle damage post exercise (111). Fundamental to the evolution of the exercise/contraction induced IL-6 production, Ullum et al. conducted a study where subjects performed 60 minutes cycling exercise at 75% VO_2max (159). In this study, an increase in IL-6 was observed in the plasma, but not in the blood mononuclear cells, leading to the assertion that leukocytes do not account for the exercise induced increases in IL-6, and that other tissues must be responsible (159). In extension to these findings, the concentrations of IL-6 in arterial and venous blood perfusing an exercising skeletal muscle were compared, revealing an increase in IL-6 in venous blood. Steensberg et al. conclusively attributed the exercise induced increase in IL-6 to local production within, and release from, skeletal muscle in exercising humans (150). Importantly, Ono et al. established that mechanical contraction of the muscle is essential to the production of IL-6 via electrical stimulation of control and locally paralyzed skeletal muscle (110).

Interestingly, a recent study identified intramyofibrillar vesicles containing IL-6 *at rest* (83). Isolated muscle fibers from the tibialis anterior of mice subjected to *in situ* muscle contraction of the leg (contralateral limb used as a baseline/resting control) showed intracellular localization of IL-6 containing vesicles which could be depleted with 45 minutes of stimulation (1 train/sec, 500-ms train, 100 Hz, 0.1-ms pulse duration, 1-13 V). This study conclusively shows a contraction driven release of IL-6, but also raises interesting questions regarding the resting expression of IL-6 in muscle (83).

IL-6, IL-6R and "IL-6 Signaling"

IL-6 is biologically active in a signaling complex consisting of IL-6, IL-6 receptor (IL-6R), and a ubiquitously expressed receptor, gp-130. The IL-6/IL-6R complex associates with the gp-130 protein, causing dimerization and subsequent signal transduction (16, 135). Classical IL-6 signaling via the membrane bound IL-6R is restricted to certain tissues, which partially dictates the location and magnitude of IL-6 stimulation. However, the existence of a soluble receptor (sIL-6R) permits an alternative mechanism known as IL-6 *trans-signaling*, where a pre-formed complex of IL-6/sIL-6R circulating in the plasma binds to tissues expressing gp-130, allowing IL-6 to stimulate almost any tissue in the body (136). The two IL-6 signaling mechanisms are mechanistically different and play different physiologic and pathophysiologic roles (31, 136).

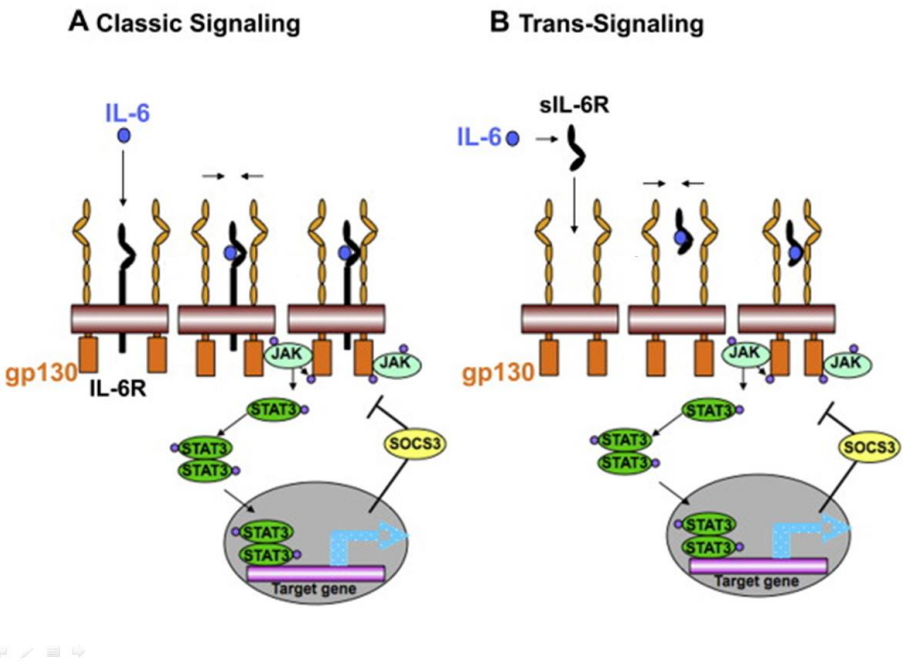

Figure 1. Classical and IL-6 Trans-Signaling. IL-6 binding to IL-6R causes dimerization of STAT3 and subsequent signal transduction through the JAK/STAT pathway leading to transcription of target genes. Also note that signal activation triggers a negative feedback inhibition through SOCS3 (38).

The sIL-6R is produced as the result of proteolytic cleavage of the membrane bound receptor, or the direct translation of mRNA coding for the IL-6R lacking the transmembrane portion (136, 137). Under normal conditions, the myocardium expresses little, if any, IL-6R. As mentioned previously, IL-6 signaling through gp-130 results in the activation of the JAK/STAT pathway, most notably via JAK1,2/STAT3. Phosphorylation of STAT3 at its tyrosine 705 residue by JAK2 initiates dimerization, nuclear translocation and DNA binding activity (89). Alternative regulation through phosphorylation of the serine 727 residue by mitogen activated protein kinase (MAPK) may also be involved in STAT3 activity (69). Although chronically elevated levels of IL-6 are implicated cardiac pathologies such as heart failure, dilated cardiomyopathy, ventricular hypertrophy, and MI, the elimination of IL-6 signaling in the heart via cardiac specific STAT3 deletion results in *reduced* resistance to IR and inflammation, reduced angiogenesis, and increased fibrosis (43, 69, 70). Global knockout of STAT3 is embryonically lethal, which emphasizes the importance of this signaling pathway (155). The duality and contradictory roles of STAT3 in the heart suggest necessity for tight regulation. As such, IL-6 signaling through the gp-130/STAT3 axis activates the suppressor of cytokine signaling (SOCS) proteins and the protein inhibitors of activated STATs (PIAS) in a negative feedback system (43, 147).

A rationale for IL-6 signaling and Exercise-Induced Cardioprotection

The mRNA and protein levels of IL-6 and IL-6R increase in response to sustained ischemia and IR in rat heart *in vivo* (28). Immunohistochemical (IHC) analysis indicates that the elevated IL-6 localized to the ischemic area, while the neighboring perfused tissue was unaffected (28). Although inflammatory by classification, other evidence supports the idea that IL-6 accumulation post IR benefits the heart. IL-6 has inotropic properties, reducing

contractility in a Ca^{2+} and NO dependent manner (144, 149, 151). Reducing contractility post IR could be beneficial for bioenergetic homeostasis and Ca^{2+} regulation. IL-6 also stimulates cardiac hypertrophy and angiogenesis (72, 97), and activates anti-apoptotic proteins (100, 140). Gwechenbarger et al. observed an increased presence of IL-6 in *viable* border zones of infarcted tissue. IL-6 expression was attributed to release from both cardiomyocytes (*in vitro* analysis of stimulated myocytes) and infiltrating immune cells (*in vivo* IR) (60). In regard to the up regulation of IL-6 in the post-ischemic myocardium, Gwechenbarger asserts, "[it] is interesting to speculate that these 3 responses, reduced contractility, positive protein balance (hypertrophy), and antiapoptosis, might favorably influence myocardial cells and allow them to survive in an area of jeopardy (60)." As such, the investigation of IL-6 as a cardioprotective mediator in the context of exercise represents a novel role for this protein.

To date, few studies have been conducted to investigate the effects of IL-6 as it pertains to myocardial preconditioning, none in the context of exercise. Of these studies, the application is restricted to IPC (32) or pharmacologically administering IL-6 *in vitro* (144). However, Matsushita et al (2005) established that IL-6 signaling through gp-130 reduced cardiomyocyte apoptosis during IR (100). Specifically, by infusing IL-6, IL-6R or the IL-6/IL-6R complex, Matsushita demonstrated that only the IL-6/IL-6R complex induced phosphorylation of gp-130 and was capable of reducing infarct size (100). Importantly, the IL-6/IL-6R combination group was the only treatment capable of inducing IL-6 signaling. Cardioprotection via IL-6 signaling is mediated by the phosphoinositide 3 kinase (PI-3k)/Akt pathway (41, 144), and the JAK/STAT pathway (32, 43). The activation of the PI-3k/Akt pathway is associated with cellular growth and survival (97), and confers protection

in the heart (41, 144). IL-6 signaling through the JAK/STAT pathway is protective against IR injury via the up regulation of iNOS and COX-2 (32). IL-6 also preconditions cardiomyocytes against IR injury *in vitro* in an Akt dependent fashion (144). Using a constitutively active Akt mutant mouse, Matsui et al. demonstrated that Akt activation reduced infarct size by 64% and attenuated cardiomyocyte apoptosis by 84% (98).

Though much is known of exercise-induced cardioprotection, several specific areas remain relatively untapped. A more comprehensive understanding of all contributing factors to the cardioprotected phenotype is essential to advance effective treatments or prevention of IR injury. Taken together, the exercise induce increase in IL-6 with the increase in either myocardial or soluble IL-6R could lead to increased IL-6 signaling in the heart, conferring protection against IR injury.

CHAPTER III

Manuscript for Journal Submission

Abstract:

Introduction: Interleukin-6 (IL-6) is a pleiotropic cytokine shown to protect the heart against ischemia-reperfusion (IR) injury *in vitro* and *in vivo* following pharmacologic and ischemic preconditioning (IPC). The purpose of this study was to characterize exercise induced IL-6 signaling in the myocardium (Aim 1) and to subsequently evaluate the potential to precondition the heart via exercise-induced IL-6 signaling (Aim 2). Methods: C57 and IL-6 knockout mice underwent 3 days of treadmill exercise for 60 min*day^{-1} at 18 m*min^{-1}. Aim 1: Serum, gastrocnemius and heart were collected from animals Pre, Post, 30, and 60 minutes following the final exercise session. Tissues were analyzed for indices of IL-6 signaling. Aim 2: Exercise Preconditioned and sedentary mice were subjected to IR injury (30 minutes I, 120 minutes R). Ischemic and perfused tissues were assayed for indices of necrosis, apoptosis and autophagy. Results: Aim 1: Exercise increased IL-6 and sIL-6R in the blood, as well as IL-6R mRNA and protein in the gastrocnemius and myocardium. IL-6R protein was increased only in the C57 mice. P-STAT3 was similarly increased in gastrocnemius and heart in *both* C57 and IL-6$^{-/-}$ mice post exercise. Myocardial iNOS mRNA and protein were decreased by exercise, while COX-2 mRNA and protein were unchanged. Aim 2: Exercise protected C57 EX mice against IR induced arrhythmias and necrosis, while C57 SED, IL-6$^{-/-}$ SED and IL-6$^{-/-}$ EX mice had

significantly higher arrhythmia scores, and significantly greater % infarct area compared to SH. C57 EX mice had increased Thr202/Tyr204 phosphorylation of p44/42 MAPK and Thr180/Tyr182 phosphorylation of p38 MAPK phosphorylation compared to IL-$6^{-/-}$ EX mice, suggesting protection likely operated through these pathways. This is the first study to show that exercise exerts cardioprotection via IL-6, and strongly implicates protective signaling originating from the exercised skeletal muscle.

Introduction:

Cardiovascular disease (CVD) and diseases of the heart are the leading causes of death, accounting for almost 1 death every 40 seconds in the US (133). The most prevalent manifestation of CVD is the myocardial infarction (MI) (133). Collectively, the physiological stresses incurred during MI are referred to as ischemia-reperfusion (IR) injury, which increase in severity as a function of time (23, 67, 105), ranging from transient and reversible arrhythmias and stunning to irreversible cell death by apoptosis and necrosis (24). Recently, a third regulatory pathway mediating cellular fates during IR injury, autophagy, has received notable attention (51, 57, 58). Though the difference between pathologic and adaptive autophagy is a topic of debate (99, 141), recent evidence suggests that the acute preservation of basal autophagy following IR injury is cardioprotective (129). Establishing robust and sustainable methods to protect or precondition the heart against IR injury is of tremendous clinical importance. Myocardial preconditioning has relied on three methodological approaches to protect the heart; Ischemic Preconditioning (IPC), Pharmacologic Preconditioning, and Exercise Preconditioning. IPC was initially observed in response to transient bouts of sub-lethal ischemia prior to an index ischemia (108), and has been extensively investigated in search of viable mechanisms to be exploited

pharmacologically. Endurance exercise is well demonstrated to be mechanistically different from IPC (44, 126), yet just as effective as an IPC stimulus. Specifically, exercise preconditioning provides a more sustained window of protection (87), and is capable of protecting the aged myocardium (125) whereas IPC does not (139).

Endogenous exercise-induced cardioprotection has been studied extensively (33, 44, 63, 64, 78, 88, 122, 129), yet remains incompletely understood. Several essential mediators have been described in detail including HSP's (64, 120, 128), antioxidants (45, 63, 64, 123), ATP-sensitive potassium channels (K_{ATP} channels) (19, 129, 130), opioids (Miller in submission) (35). However, less is known about the contribution of the exercising skeletal muscle as it pertains to exercise preconditioning through paracrine and endocrine activation. Cytokines, called myokines, produced in and secreted from skeletal muscle, have garnered recent attention and have been investigated in various physiological and pathophysiological scenarios including IR injury and cardioprotection (93, 110, 113, 114, 150). Among these myokines, interleukin 6 (IL-6) is most notably up regulated in response to exercise (80, 114, 150), and has been implicated as an essential mediator of IPC (32, 100, 144).

Mechanistic studies of IL-6 induced cardioprotection have been carried out both *in vivo* (32) and *in vitro* (144) with both pharmacologic and IPC. IPC induced signaling through the Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway reduced necrosis following 30 minute regional ischemia (32). The infarct sparing effects were abrogated in IL-$6^{-/-}$ mice, demonstrating an essential role for IL-6 in IPC (32). Bolli, et al. developed a sophisticated mouse model with a cardiac specific, inducible STAT3 deletion to demonstrate the importance of STAT3 signaling in IPC (14). Smart et al.

demonstrated IL-6 induced cardioprotection against IR injury *in vitro*. They observed a reduction in cellular damage, preservation of cell viability, and Ca^{2+} homeostasis following sIR (144). The protection afforded to the cardiomyocytes was nitric oxide (NO) and phosphotidyl-inositol 3-kinase (PI3-K) dependent, and was abolished by iNOS and PI3-K inhibitors (144).

Collectively, a rationale exists that the exercise induced increase in circulating IL-6, largely attributed to increased synthesis and release from exercising skeletal muscle, is sufficient to activate protective pathways in the myocardium. Therefore, the purpose of this study was to investigate the role of IL-6 in exercise induced cardioprotection against IR injury. In that regard, it is hypothesized that exercise will increase IL-6 signaling in the heart and decrease IR induced apoptotic and necrotic tissue death in an IL-6 dependent fashion. Furthermore, we hypothesized that exercise induced IL-6 signaling would preserve autophagy immediately following IR.

Methods:

Animals

Male mice (56 C57, C57BL/6J; 48 IL-$6^{-/-}$, B6;129S2-*Il6*^{tm1Kopf}/J) were used to complete the two aims of this study. All protocols utilized in the study received Auburn University IACUC approval. Animals were housed at the Auburn University Biological Research Facility (BRF) on a 12:12 *reversed* light:dark cycle with access to water and rodent chow *ad libitum*. In Aim 1, 64 mice (C57, n = 32 and IL-$6^{-/-}$, n = 32) were assigned to exercise or sedentary treatments to evaluate the acute myokine response to exercise in the blood, skeletal muscle and heart (**Figure 1**). Exercised mice were habituated to treadmill exercise for 10, 20, 30, and 40 minutes on consecutive days, followed by a rest day and then

performed 3 days of exercise for 60 min•day^{-1}, at 18 m*min^{-1} and 0% grade. Exercise was performed on a calibrated motorized rodent treadmill (Columbus Instruments, Columbus, OH) and sedentary mice, used as pre-exercise controls, spent a time matched duration on the treadmill at 0 m*min^{-1}. Under isofluorane anesthesia, tissue was collected from sedentary mice (PRE) and exercised mice immediately (POST), 30 minutes (30), or 60 minutes post exercise (60). Serum, gastrocnemius, soleus, extensor digitorum longus (EDL), and hearts were snap frozen for subsequent analysis. Mice in Aim 2 (C57 n = 24 and IL-6$^{-/-}$ n = 16) followed an identical habituation and preconditioning exercise protocol and received an *in vivo* ischemia reperfusion injury 24 hours following the final exercise session.

in vivo Ischemia Reperfusion Injury

Twenty-four hours following the final exercise session, mice received a surgically induced IR injury. Briefly, mice were anesthetized using sodium pentobarbital (50 mg/kg) and a tracheotomy and left thoracotomy were performed. Mice were supported with a pressure driven mechanical ventilator (Kent Scientific, Torrington, CT), and connected to limb lead electrodes integrated into a physiological data acquisition system (Biopac, Santa Barbara, CA) for the duration of the surgery. The left anterior descending (LAD) coronary artery was occluded with a sterile surgical suture passed through polyethylene tubing, creating a reversible ligature. Regional ischemia was administered for 30 minutes followed by 120 minutes reperfusion. At the conclusion of IR, the ligature was re-established and 4% Evan's Blue dye was injected via left ventricular cardiac puncture allowing for visualization of the area at risk (AAR). An additional group of C57 mice (n=8) received a sham operation.

Electrocardiogram data were collected and analyzed for arrhythmias including premature ventricular contractions (PVC's), episodes and duration of ventricular fibrillation (VF) and tachycardia (VT) using Biopac software and evaluated using the A Score method (104). To assess myocardial necrosis, hearts were excised, sectioned into 2 mm transverse cross sections and incubated with 1% triphenol-tetrazolium chloride (TTC) for 15 minutes at 37ºC. To assess apoptosis and autophagy, the ischemic and perfused areas of the myocardium were separated and snap frozen in liquid nitrogen for Western Blotting and PathScan analysis.

ELISA

Serum IL-6 was quantified using a commercially available ELISA (Invitrogen KMC0062) following manufacturer protocol. Briefly, blood was collected via direct cardiac puncture and allowed to clot. Samples were centrifuged for 10 minutes at 10,000 x g at 4ºC and stored at -80ºC until analysis. Serum samples were run in duplicate with the absorbance read at 450 nm and plotted against a standard curve of mouse IL-6 ($r^2 = 0.998$).

PCR

Approximately 20 mg of myocardial, gastrocnemius, soleus, and EDL tissue was used for RNA isolation using 500 µL Ribozol® RNA Extraction Reagent (Amresco N580), as per manufacturer instructions. RNA was precipitated with 500 µL Isopropanol with 0.5 µL Glycogen, washed with Ethanol, and reconstituted with DEPC H_2O. A 3% agarose gel was run to verify the purity of extracted RNA. Isolated RNA (1 µg) was converted to cDNA using a Verso cDNA kit (ThermoScientific AB-1453/B) with a reverse transcription cycle of 30 minutes at 42ºC. The resulting cDNA (50 ng/µL) was diluted to a final concentration of 5 ng/µL and stored at -20ºC. Primer efficiency curves were run for each primer set to

select optimum cDNA concentrations and ensure single amplification products. PerfeCTa® SYBR Green Super Mix (Quanta BioSciences 95054) was combined with forward and reverse primers (**Table 1**) and 25 ng cDNA. qPCR conditions were 95ºC for 2 minutes, followed by 35 cycles of denaturing at 95ºC for 30 seconds, re-annealing at 62ºC for 30 seconds, and extension at 72ºC for 5 minutes. mRNA expression was calculated using the $_{\Delta\Delta}$Ct method.

Western Blotting

Serum, gastrocnemius and heart (Aim 1) samples were homogenized in lysis buffer with phosphatase (GBiosciences Phosphatase Arrest II, 1:100) and protease (Sigma #P2714, 1:10) inhibitors to acquire a whole muscle homogenate. Cytosolic and nuclear fractions were obtained using a nuclear isolation kit (ThermoScientific, Waltham, MA) following manufacturer instructions. Ischemic and perfused regions of hearts (Aim 2) were homogenized in PathScan® Sandwich ELISA Lysis buffer containing protease and phosphatase inhibitors. Samples were normalized for protein concentration, diluted with Laemmli sample buffer with 2-mercaptoethanol, and heated at 95ºC for 5 minutes.

Proteins were separated on 6% (iNOS), 10% (IL-6R, COX-2, [P]-STAT3, [P]-Akt, Atg3, Atg5, Beclin 1) or 18% (LC3II/I) polyacrylamide gels and transferred onto methanol activated PVDF membranes. Membranes were exposed to primary antibodies (IL-6R, Santa Cruz #374259 1:1000 in 5% NFDM; P-STAT3, Cell Signaling #9145; STAT3, Cell Signaling #4904; P-Akt, Cell Signaling #9271; Akt, Cell Signaling #9272; iNOS, Cell Signaling #13120; COX-2, Cell Signaling #12282; Atg3, Cell Signaling #3415; Atg5, Cell Signaling #12994; Beclin 1, Cell Signaling #3495; LC3II/I, Cell Signaling #12741, 1:1000 in 5% BSA, and αTubulin, Developmental Studies Hybridoma Bank #12G10, 1:1000 in

5% NFDM) overnight at 4ºC. Membranes were incubated in mouse or rabbit targeted secondary antibodies (Cell Signaling #7076 and #7074, respectively, 1:2000 in BSA) for 1 hour at RT, and blots were imaged with Luminata Forte HRP substrate (Millipore). Images were captured with a ChemiDoc-It® Imager (UVP, Upland, CA) and analyzed using NIH ImageJ software.

PathScan

PathScan slides were assembled in gaskets and blocked for 15 minutes at RT. Ischemic and perfused homogenates, diluted to 1.0 $mg*mL^{-1}$, were added to wells and incubated overnight at 4ºC with rocking. Wells were incubated with a detection antibody followed by HRP-linked Streptavidin with four 5 minute washes separating each step. Slides were exposed with LumiGLO®/Peroxide substrate and quantified using UVP software, and normalized to the total intensity of each sample.

Statistical Analysis

All values are presented as means ± standard error (SEM). A 2 (group) x 4 (time) analysis of variance (ANOVA) was used to analyze effect of genotype and exercise on mRNA and protein expression of genes and proteins of interest, respectively, following exercise (Aim 1). Repeated measures ANOVA was used to analyze group differences in AAR, infarct size (necrosis), and apoptosis with Tukey's HSD post hoc analysis to evaluate significant differences when appropriate (Aim 2). Significance was set *a priori* at $p \leq 0.05$.

Results:

Aim 1:

Serum

Serum was collected from mice via cardiac puncture to quantify circulating levels of IL-6 after exercise. A post-exercise increase was seen in the blood, which reached significance at 30 minutes ($p < 0.05$) (**Figure 2**). Subsequently, western blotting was conducted to examine the expression of proteins associated with IL-6 signaling in response to the acute exercise stimulus.

A significant interaction effect ($p = 0.003$) was present for the IL-6R in the serum (sIL-6R). sIL-6R was increased in C57 mice 4.5 ± 0.4, 4.5 ± 0.8, and 5.0 ± 0.6 fold immediately post, 30 minutes post, and 60 minutes post exercise, respectively ($p < 0.05$). sIL-6R was significantly higher in the C57 mice compared to IL-$6^{-/-}$ mice at all sample times post exercise ($p < 0.05$), although no significant differences existed between C57 and IL-$6^{-/-}$ mice pre-exercise (**Figure 3**).

Gastrocnemius

Mixed gastrocnemius was assayed via conventional western blotting as an index of skeletal muscle IL-6 signaling (**Figure 4**). Exercise elicited an increase in gastrocnemius IL-6R expression. A significant strain main effect ($p < 0.001$) and time main effect ($p = 0.042$) were present. IL-6R expression was increased 2.2 ± 0.5 fold post, 2.4 ± 0.4 fold at 30 minutes, and 2.3 ± 0.3 fold 60 minutes post exercise compared to pre exercise in C57 mice ($p < 0.05$), and was significantly higher than IL-$6^{-/-}$ animals at all sample times post exercise ($p < 0.05$). The nuclear fraction of gastrocnemius homogenate was analyzed for P-STAT3 (Tyr705). A significant increase in nuclear P-STAT3 was present at 30 minutes post exercise compared to pre in *both* C57 and IL-$6^{-/-}$ mice ($p = 0.003$). Post hoc analysis showed an increase in P-STAT3 at 60 minutes post exercise in C57 mice only ($p < 0.05$).

Interestingly, there was not a significant main effect for strain ($p = 0.392$), demonstrating that exercise induced STAT3 phosphorylation in both C57 and IL-$6^{-/-}$ mice.

Proposed mediators of cardioprotection downstream of IL-6 signaling, iNOS and COX-2, were also measured in the gastrocnemius. iNOS demonstrated a modest, but significant increase in response to exercise (time main effect, $p = 0.001$), which showed an increase in iNOS at all sample times post exercise compared to pre exercise values in both C57 and IL-$6^{-/-}$ mice. Follow up t-tests revealed a 1.5 ± 0.09 fold increase POST ($p = 0.001$), 1.6 ± 0.09 fold increase at 30 ($p < 0.001$), and 1.4 ± 0.08 fold increase 60 minutes post ($p = 0.005$). Gastrocnemius COX-2 was significantly higher post exercise compared to 60 (time main effect; $p = 0.003$). No strain differences were seen in the expression of iNOS ($p = 0.838$) or COX-2 ($p = 0.587$).

Real Time qPCR results for Aim 1 gastrocnemius are presented in **Table 2**. Exercise did not elicit an increase in IL-6 gene expression in the gastrocnemius. The alternative IL-6 family cytokines CT-1 and LIF, similarly, were not increased in response to exercise in C57 or IL-$6^{-/-}$ mice. IL-6R mRNA expression was increased at 60 minutes post exercise in both C57 and IL-$6^{-/-}$ mice ($p < 0.01$). A significant interaction was noted in iNOS expression ($p = 0.004$). Subsequent post hoc analysis showed that the time and group effects were driven by significantly higher basal iNOS expression in IL-$6^{-/-}$ mice compared to all other groups ($p < 0.05$), whereas no differences existed between any other sample times. COX-2 mRNA expression was higher post exercise in the IL-$6^{-/-}$ mice compared to PRE, 30, and 60 ($p < 0.05$), and was also increased compared to C57 POST.

Myocardium

In the myocardial tissue (**Figure 5**), IL-6R expression followed the same pattern as seen in the serum and gastrocnemius. Significant time main effect ($p < 0.001$), main effect for strain ($p = 0.004$), and interaction effect ($p = 0.037$) were present. IL-6R expression was increased 2.6 ± 0.5 fold POST, 2.1 ± 0.4 fold at 30 minutes, and 2.6 ± 0.3 fold at 60 minutes post exercise in C57 animals only ($p < 0.05$), and was increased compared to IL-$6^{-/-}$ mice at each time point post exercise ($p < 0.05$). No difference in IL-6R was present between C57 and IL-$6^{-/-}$ mice PRE (**5A**). Serine 473 phosphorylation of Akt was measured in the myocardium as an index of IL-6 induced signaling through the PI3K/Akt pathway. Interestingly, in both groups, a significant reduction in P-Akt occurred POST, which returned to pre-exercise levels by 30 and 60 minutes post exercise (**5B**). Phosphorylated STAT3 at tyrosine 705 was increased post exercise in both groups at 30 minutes post exercise. Additionally, P-STAT3 in C57 mice was increased at 60 minutes compared to PRE and POST ($p < 0.05$) and was significantly higher than IL-$6^{-/-}$ mice ($p < 0.05$). IL-$6^{-/-}$ mice showed an earlier peak in P-STAT3 which increased immediately post exercise ($p < 0.05$), but was no longer elevated by 60 minutes post exercise. A significant interaction effect suggests the two groups were affected differently by exercise (**5C**).

No differences were present for myocardial COX-2. iNOS, however, was decreased at 60 minutes post exercise in both groups (time main effect; $p = 0.039$). No differences existed between groups for either COX-2 or iNOS (**5D** and **5E**, respectively).

mRNA expression in the heart is presented in **Table 2**. No increase in IL-6 mRNA expression was present post exercise. However, a post exercise increase in LIF mRNA existed at 60 minutes for both C57 and IL-$6^{-/-}$ mice. There was also higher myocardial CT-1 mRNA in the IL-$6^{-/-}$ mice at all sample times (group main effect; 0.044). Exercise elicited

an increase in myocardial IL-6R mRNA at 30 ($p < 0.001$) and 60 ($p < 0.001$ from PRE, $p = 0.048$ from POST) in both C57 and IL-$6^{-/-}$ mice. iNOS mRNA expression was significantly reduced POST ($p = 0.022$), 30 ($p = 0.001$), and 60 (0.002) minutes post exercise. No significant changes were seen in myocardial COX-2 mRNA expression. The mRNA expression of SOCS3, the negative feedback inhibitor for IL-6 signaling, was also unchanged by exercise.

Aim 2:

Animal Anthropometrics

Mice were weighed prior to IR, and the hearts were blotted dry and weighed following cardiac excision; the data are presented in **Table 3**. IL-$6^{-/-}$ mice had a significantly higher bodyweight (BW) than C57 mice (30.3 ± 0.9 g vs. 28.3 ± 0.5 g; $p = 0.026$), with no effect of exercise treatment ($p = 0.396$). Exercised IL-$6^{-/-}$ mice had a larger heart weight (HW) compared to sedentary mice, as well as C57 exercised mice ($p < 0.05$). Exercise increased the heart weight to bodyweight ratio (HW/BW) in both strains from C57 mice (4.2 ± 0.1 mg*g^{-1}) to IL-6-/- mice (4.6 ± 0.1 mg*g^{-1}) (exercise main effect; $p = 0.001$). Finally, exercised IL-$6^{-/-}$ mice had a higher HW/BW than sedentary IL-$6^{-/-}$ mice ($p < 0.05$).

Arrhythmia Scoring

ECG data were collected using a Biopac integration system, analyzed under blinded conditions for PVC's, VT and VF. Treatments were evaluated based on a standardized arrhythmia scoring system for clinical and research settings (A Score) (104). C57 SED mice had a significantly higher A Score than Sham animals ($p = 0.007$) indicating a higher incidence of ventricular arrhythmias. C57 EX mice were protected against arrhythmias, and had significantly lower A Scores than the C57 SED mice ($p = 0.007$), and were not

statistically different from SH. IL-6$^{-/-}$ SED mice had a higher A Score compared to SH mice ($p = 0.005$). The increase in arrhythmia score in IL-6$^{-/-}$ EX mice approached significance ($p = 0.077$), but was not statistically different from SH (**Figure 6**).

TTC Staining for Necrosis

At the conclusion of the IR injury, hearts were perfused with Evan's Blue dye to quantify the area at risk (AAR) and the percent infarct (**Figure 7A**). Digital images were analyzed with ImageJ (NIH). No differences were seen between groups in the AAR (in percent heart area; C57 SH = 34.6 ± 2.8, C57 SED = 35.6 ± 2.8, IL-6$^{-/-}$ SED = 28.6 ± 3.1, C57 EX = 40.2 ± 4.4, IL-6$^{-/-}$ EX = 38.9 ± 4.2) ($p = 0.208$). Tissues were incubated with 1% TTC to quantify necrosis (**Figure 7B**). In response to IR, there was a significant increase in necrosis in C57 SED (22.2 ± 4.0%, $p = 0.01$) and IL-6$^{-/-}$ SED (22.7 ± 4.4%, $p = 0.008$) in comparison to SH (2.8 ± 0.7%). In response to exercise, C57 EX mice (7.8 ± 1.8%) showed no increase in necrosis compared to SH ($p = 0.867$), while IL-6$^{-/-}$ EX (20.2 ± 4.9%) mice had a larger infarct than SH ($p = 0.02$). Furthermore, C57 SED mice had a significantly larger infarct compared to C57 EX ($p = 0.049$).

Autophagy

Western blotting for markers of autophagy Atg3, Atg5, LC3II/I, and Beclin 1 are presented in (**Figure 8**). There was a significant reduction in Atg3 in the ischemic tissue of C57 and IL-6$^{-/-}$ mice in both exercise and sedentary groups ($p < 0.001$). Additionally, there was a significant strain*treatment interaction such that IL-6$^{-/-}$ EX had increased Atg3 compared to IL-6$^{-/-}$ SED ($p < 0.05$). No significant differences existed for Atg5, Beclin 1, or LC3II/I.

Cell Stress and Apoptosis PathScan

The Cell Stress and Apoptosis PathScan array was used to analyze multiple targets in associated pathways. No significant differences were found between sham ischemic (SH I) and sham perfused (SH P) tissues for any assay target, thus the total SH average was used to calculate a fold change value for each target. Ischemia increased the cleavage of Caspase-3 ($p = 0.022$) and Caspase-7 ($p = 0.007$) in both C57 and IL-$6^{-/-}$ mice, with no difference between sedentary and exercised treatments. Interestingly, cleaved PARP at Asp214 showed no differences between sedentary C57 and IL-$6^{-/-}$ mice in the ischemic or perfused tissue. However, C57 EX mice had significantly reduced cleavage compared to IL-$6^{-/-}$ EX mice ($p < 0.05$). No differences were present between sedentary and exercised treatments for PARP. SAPK/JNK dual phosphorylation at Thr183/Tyr185 was increased in the ischemic tissue of both C57 and IL-$6^{-/-}$ mice in sedentary and exercised groups ($p = 0.011$).

Ischemia suppressed the dual phosphorylation of p44/42 MAPK at Thr202/Tyr204 across both strains and in sedentary and exercised mice ($p = 0.027$). C57 EX mice showed increased p44/42 MAPK phosphorylation compared to IL-$6^{-/-}$ EX mice ($p < 0.05$), while no differences between strain existed in sedentary mice. Similarly, a significant increase in p38 MAPK phosphorylation at Thr180/Tyr182 was seen in C57 EX compared to C57 SED and IL-$6^{-/-}$ EX ($p < 0.05$). No significant differences were present for Akt (Ser473), Bad (Ser136), HSP27 (Ser82), Smad2 (Ser465/467), p53 (Ser15), IκBα (total and Ser32/36), Chk1 (Ser345), Chk2 (Thr68), eIF2α (Ser51), TAK1 (Ser412), or Survivin (total). p44/42 MAPK, p38 MAPK and PARP are presented in **Figure 9**, all remaining data are presented in **Table 4**.

Discussion:

The current study was undertaken to characterize exercise induced IL-6 signaling in the heart (Aim 1), and to investigate the potential cardioprotective roles of IL-6 in exercise preconditioning (Aim 2). Current study findings reinforce that; 1) exercise increased circulating levels of IL-6 protein, and 2) exercise elicited an increase in sIL-6R and membrane bound IL-6R in the gastrocnemius. To our knowledge, this is the first study to demonstrate that exercise also increases myocardial IL-6R expression, and does so in an IL-6-dependent fashion. We have also demonstrated that exercise promoted nuclear translocation of P-STAT3 (Tyr-705), indicating induction of IL-6 signaling in skeletal muscle and heart, which was not eliminated in the IL-$6^{-/-}$ mouse.

Most importantly, this study is the first to show that exercise is cardioprotective against tissue necrosis following ischemia reperfusion injury, and that exercise induced protection was abrogated in IL-$6^{-/-}$ mice.

IL-6/IL-6R Regulation (gene and protein data)

The exercise induced increase in skeletal muscle IL-6 mRNA expression and circulating IL-6 protein is well documented in the literature (93, 110, 113, 114, 150). Interestingly, recent evidence suggests that *resting* skeletal muscle also contains significant quantities of IL-6 protein sequestered in storage vesicles which, in response to electrical stimulation, are stimulated to release IL-6 (83). Our data fall in line with these recent findings in that IL-6 mRNA expression was unchanged in the heart and gastrocnemius in response to exercise, yet circulating IL-6 protein levels were significantly elevated within minutes following an acute bout of exercise. Although IL-6 can be produced by other cells, previous research suggests that they do not contribute to the appearance of IL-6 in response to endurance exercise (107, 159). In agreement with our findings, Adser et al (1) found no increase in

IL-6 mRNA in the gastrocnemius of C57 mice following 1 hour of treadmill exercise comparable to the currently prescribed exercise protocol. Interestingly, using PCR primers targeting a segment of DNA in the IL-6 gene that is undisturbed by the neo cassette insertion used to generate the IL-$6^{-/-}$ mouse, Adser also showed a robust up regulation of IL-6 gene expression in IL-$6^{-/-}$ mice at rest, and in response to exercise, which also occurred in the current study. More careful primer design to flank Exon 2 of the IL-6 gene (the site of the neo cassette insertion) indicated no amplification of a gene product in the IL-$6^{-/-}$ mouse after 40 PCR cycles (data not shown).

To gain further perspective into IL-6 gene expression across different muscles, the post exercise mRNA expression of IL-6 was compared among heart, gastrocnemius, soleus, and EDL samples. β-actin expression did not differ between tissues, and thereby served as a normalizer gene. Compared to myocardium, IL-6 mRNA expression in the gastrocnemius was 2.3 ± 0.4 fold higher (n/s), while IL-6 mRNA expression in the soleus and EDL were 29.2 ± 4.8 fold higher ($p < 0.001$) and 61.5 ± 8.7 fold higher ($p < 0.001$), respectively. Given the fiber type distribution of the tissues analyzed, these data agree with previous findings by Hiscock et al (73), which indicated that type II fibers displayed greater IL-6 mRNA production in response to exercise. These data support the proposed notion that endogenous production of IL-6 within the myocardium is not the source of the observed increase in circulating IL-6, and that it was most likely the combined contribution of the exercised skeletal muscles.

Interleukin-6 signals through a hexameric structure with glycoprotein (gp)-130 and IL-6R. Though some discrepancies exist in the literature, IL-6R increases following exercise in skeletal muscle and in the blood (53, 54, 80, 86). In line with the current findings, Keller

et al showed that 1 hour of swimming exercise increased the IL-6R mRNA expression in both C57 and IL-$6^{-/-}$ mice, although they did not quantify IL-6R at the protein level. Keller attributes an IL-6-independent regulation of IL-6R mRNA expression, although there seems to be a significant IL-6 *dependent* component of IL-6R protein expression, as evidenced by the reduction in IL-6R in IL-$6^{-/-}$ mice.

Within the current study it was necessary to quantify sIL-6R and myocardial IL-6R levels post exercise to connect the rationale for myocardial IL-6 signaling since basal IL-6R expression in the heart is minimal (28). This study offers the novel findings that exercise increases myocardial IL-6R mRNA and protein. Furthermore, new evidence for a post-transcriptional regulation of cardiac and skeletal muscle IL-6R is also revealed. IL-6R mRNA was increased similarly in IL-$6^{-/-}$ and C57 mice post exercise, while IL-6R protein was only increased in the C57 mice. IL-6R is the direct target of several micro-RNAs (miRNAs) (50, 134) which offer attractive prospects for the discrepancy between strains. However, since these targets were beyond the scope of the current study, further investigation to support a rationale that one or more of these miRNAs would be differentially regulated in C57 or IL-$6^{-/-}$ mice, or by exercise, is warranted.

STAT3 and Akt pathway activation

Subsequent to the assembly of the IL-6-IL-6R-gp130 signaling complex, multiple signaling pathways including JAK2/STAT3, Akt, and MAPKs can be activated. Tyrosine 705 phosphorylation and nuclear translocation of STAT3 has been previously observed in response to exercise in skeletal muscle (157), but whether exercise elicits STAT3 signaling in the heart is unresolved. In the current study, it was hypothesized that IL-6 induced STAT-3 signaling mediates exercise induced cardioprotection. In order to gain more

comprehensive insight, P-STAT3 was measured in both skeletal muscle and the myocardium.

In agreement with previous findings in skeletal muscle, nuclear P-STAT3 was increased in the gastrocnemius following acute exercise. In extension to the current literature, P-STAT3 was similarly elevated in the heart post exercise. Surprisingly, we also observed a rapid induction of P-STAT3 in the gastrocnemius and heart of IL-$6^{-/-}$ mice. In both tissues, P-STAT3 returned to baseline values by 60 minutes post exercise, while P-STAT3 remained elevated in C57 mice. One possible alternative is that IL-6 family cytokines are responsible for the increase in P-STAT3 in the IL-$6^{-/-}$ mouse (43, 82). In support of that rationale, myocardial CT-1 mRNA expression was higher in IL-$6^{-/-}$ mice compared to C57 mice at all sample times, while myocardial LIF expression was similarly increased in both C57 and IL-$6^{-/-}$ mice 60 minutes post exercise. In a transaortic constriction pressure overload model of cardiac hypertrophy, STAT3 phosphorylation was the same in C57 and IL-$6^{-/-}$ mice, supporting IL-6 independent STAT3 activation (82). Although only partially resolved in the current data set, the possibility exists that IL-$6^{-/-}$ mice could be physiologically primed for STAT3 signaling via increased basal CT-1, and possibly other IL-6 family cytokines, which all operate through the common gp130 receptor and converge on STAT3.

Previous research employing IPC identified IL-6/STAT3 mediated protection which was dependent on the up regulation of iNOS and COX-2 (32), which was eliminated in the IL-$6^{-/-}$ mouse (32). The current findings differ from Dawn et al. (32) in that exercise induced STAT3 signaling was not completely inhibited in the IL-$6^{-/-}$ mice, and exercise induced a *decrease* in myocardial iNOS mRNA and protein, while COX-2 was unaffected. These data agree with previous findings by Quindry et al which show that exercise does not

increase myocardial iNOS and COX-2 in rats exposed to a proportional exercise protocol, and asserts that ischemic preconditioning relies on inflammatory mediated cardioprotection while exercise preconditioning does not (126).

While the major impetus of the current study was to investigate STAT3 primarily as a nuclear transcription factor, exciting new evidence suggests that STAT3 also plays an important role at the mitochondrial level. Measurable quantities of STAT3 have recently been found in the mitochondria (10, 153, 154, 161). STAT3 associates with mitochondrial ETC complexes and plays a role in cellular respiration and oxidation of fuels. STAT3 has also been shown to associate with cyclophilin D and prevent mPTP opening, reducing apoptosis (10). Perhaps an additive level of mitochondrial fortitude existed in the current study in the C57 mice through the combined functions of the IL-6-STAT3 axis and p38 MAPK (5).

Phosphoinositide 3-kinase/Akt/mTOR signaling has been extensively studied in cardiac and skeletal muscle, primarily in the context of muscle hypertrophy/atrophy in skeletal muscle and adaptive/maladaptive cardiac hypertrophy of the heart. The PI3K/Akt/mTOR pathway has been previously shown to be essential to IL-6 induced cytoprotection against simulated ischemia-reperfusion injury *in vitro* (144). Akt activation with IL-6 was essential for the increase in iNOS, and resultant protection from damage and preservation of viability. Ma et al found that chronic swim training was sufficient to elicit physiological hypertrophy via activation of the PI3K/Akt pathway over 8 weeks (92). Furthermore, exercise induced pro-survival pathways in the left ventricles of chronically trained STZ-induced diabetic rats, which was associated with the up regulation of PI3K/Akt signaling (29). Contrary to these findings, we saw a *decrease* in P-Akt (Ser473) immediately post

exercise in both C57 and IL-6$^{-/-}$ mice. Interestingly, Akt-induced hypertrophic signaling is down-regulated in cardiac myocytes by AMPK (27), which is tightly regulated by cellular energy status and bioenergetics stressors such as endurance exercise (65). These findings are supported in the current data set. Furthermore, in a subset of C57 mice in Aim 1, exercise increased P-AMPKα (Thr172) phosphorylation in the gastrocnemius and heart immediately post exercise (data not shown), which agrees with previous findings, and supports an AMPK-Akt molecular switch (4). We hypothesized a differential response to exercise in the two strains used based on recent findings by Adser et al., who showed that IL-6 regulates AMPK activity following exercise (1), and the findings that IL-6 activates Akt phosphorylation (152, 162). However, in the current study, no differences were identified in C57 and IL-6$^{-/-}$ mice.

Arrhythmia protection

This is the first study to implicate exercise induced IL-6 dependent protection against IR induced arrhythmias. We showed that IR increased the arrhythmia score in sedentary mice only, and that exercise confers cardioprotection against arrhythmias in the C57 mouse. A non-significant increase was seen in the arrhythmia score of IL-6$^{-/-}$ EX mice ($p = 0.077$) compared to SH, which makes it tempting to speculate that exercise exerted its protective effects in an IL-6-dependent manner, although these claims are not supported statistically in the current study. *In vitro* evidence strongly implicates that IL-6 has positive effects on contractility (102, 144). Smart showed that IL-6 improved calcium handling and cardiomyocyte contractility following simulated IR in culture (144). Similarly, recent findings by Maxeiner et al. show that cardiospheres cultured from hypertensive donor patients were high in IL-6 concentration and reduced contractility in acceptor rat hearts

(102). Perhaps most interestingly, a large cohort genome-wide association study (Cohorts for Heart and Aging Research in Genomic Epidemiology, or "CHARGE") found a single nucleotide polymorphism in the IL-6R gene that was associated with atrial fibrillation (91). Although more mechanistic studies need to be pursued, our findings agree with those in the CHARGE study that IL-6R expression could be important in arrhythmia prevention and/or protection.

p38 and p44/42 MAPK signaling and protection

In response to exercise and ischemia reperfusion injury, we showed that phosphorylation of p44/42 MAPK and p38 MAPK were both elevated in the ischemic and perfused tissue. Previous research has shown that IL-6 can induce p44/42 MAPK activation independently of STAT3 (41). Our findings dovetail nicely with those findings by Fahmi et al. and extend to an *in vivo* model. We demonstrate currently that exercise induced STAT3 signaling was not inhibited in IL-$6^{-/-}$ mice, but p44/42 and p38 phosphorylation, and protection against arrhythmias and infarction, was abolished. Interestingly, IL-6 alone could not induce STAT3 phosphorylation without the addition of the sIL-6R. Whether this is a function of low basal IL-6R expression, or a specific feature of the soluble vs membrane bound IL-6R is unclear, but the findings further support the current rationale for the necessity to up regulate either, or both forms of IL-6R (41, 152).

The fact that the MAPK response was not specific to the ischemic tissue, but was present in both, suggests that phosphorylation of p38 and p44/42 was most likely in response to preconditioning exercise, and not in response to IR. Phosphorylation of p38 in skeletal muscle plays an important role in mitochondrial regulation (3, 5, 47). Additionally, AMPK increases IL-6 expression in cardiomyocytes in a p38 MAPK-dependent manner (39),

which could lead to autocrine stimulation of IL-6 expression (39), and sensitization of cardiomyocytes to AMPK signaling. Interestingly, recent findings indicate that aldosterone preconditions Langendorff perfused rat hearts via p38 MAPK activation. Furthermore, they show that preconditioning increased phosphorylation of p38 MAPK, while IR severely reduced it, suggesting that acute increases in p38 MAPK phosphorylation, as seen in the current study, are protective (167).

Apoptosis and Necrosis

Exercise (45, 125, 128, 129) and IL-6 (98, 100, 140) both play an independent role in the reduction of IR injury, but no studies have investigated them together. In the current study, we found that hearts from exercised mice had increased p38 MAPK and p44/42 MAPK phosphorylation which coincided with a strain specific decrease in PARP in ischemic and perfused tissue. These findings suggest that IL-$6^{-/-}$ EX mice had higher levels of apoptosis compared to C57 EX mice and that IL-6 plays a role in the antiapoptotic response seen in PARP. Other markers for apoptosis including Caspase 3, Caspase 7 and JNK were increased in the ischemic tissues independent of IL-6 and exercise. Though the duration of ischemia was sufficient to induce myocardial injury, a more severe IR injury may have made treatment effects more discrepant for apoptosis-related outcomes. Previous studies by our lab using longer duration ischemic models showed that exercise conferred protection against apoptosis (45, 125, 128, 129). Extending this rationale to the current study, Matsushita et al. showed that infusion of IL-6/sIL-6R was capable of reducing IR induced apoptosis in rats (100).

We also confirm that exercise reduced IR induced infarction in the C57 mice, and report for the first time that the anti-infarct protection from exercise is dependent on IL-6, as the

protection granted by exercise was lost in IL-6$^{-/-}$ mice. Dawn and Bolli showed the infarct sparing effects of IPC were mediated by IL-6 (32), and Gwechenberger found that increased IL-6 levels were present in *viable* border zone surrounding infarcted tissue (60). The protective effects observed by Bolli were mediated by iNOS and COX-2; however, these mediators of IPC cardioprotection were not increased in the current study, and appear to be uninfluenced by exercise (126). That IL-6 family cytokines can activate p44/42 MAPK mediated protective pathways independent of STAT3 (41) and that these pathways were differentially mediated by exercise here, leads to the concept that the two pathways may operate synergistically to optimize the response to IR. In support, p38 MAPK has an inhibitory effect on P-STAT3 signaling (43), which in the context of IR could serve as an adaptive process to limit STAT3 signaling to an acute time frame post IR.

Conclusion:

In conclusion, this study expands the existing body of literature on the mechanisms of exercise induced cardioprotection against ischemia reperfusion injury in an *in vivo* model of IR. We have presented the novel findings that exercise increases serum, gastrocnemius, and myocardial IL-6R expression in an IL-6 dependent fashion, and that exercise induced STAT3 signaling in C57 and IL-6$^{-/-}$ mice. Furthermore, this paper is the first to show that exercise induced cardioprotection against myocardial necrosis is IL-6 dependent. This study offers compelling evidence to support the rationale that mediators of exercise cardioprotection originate from remote tissues outside of the heart, a topic which requires further elucidation.

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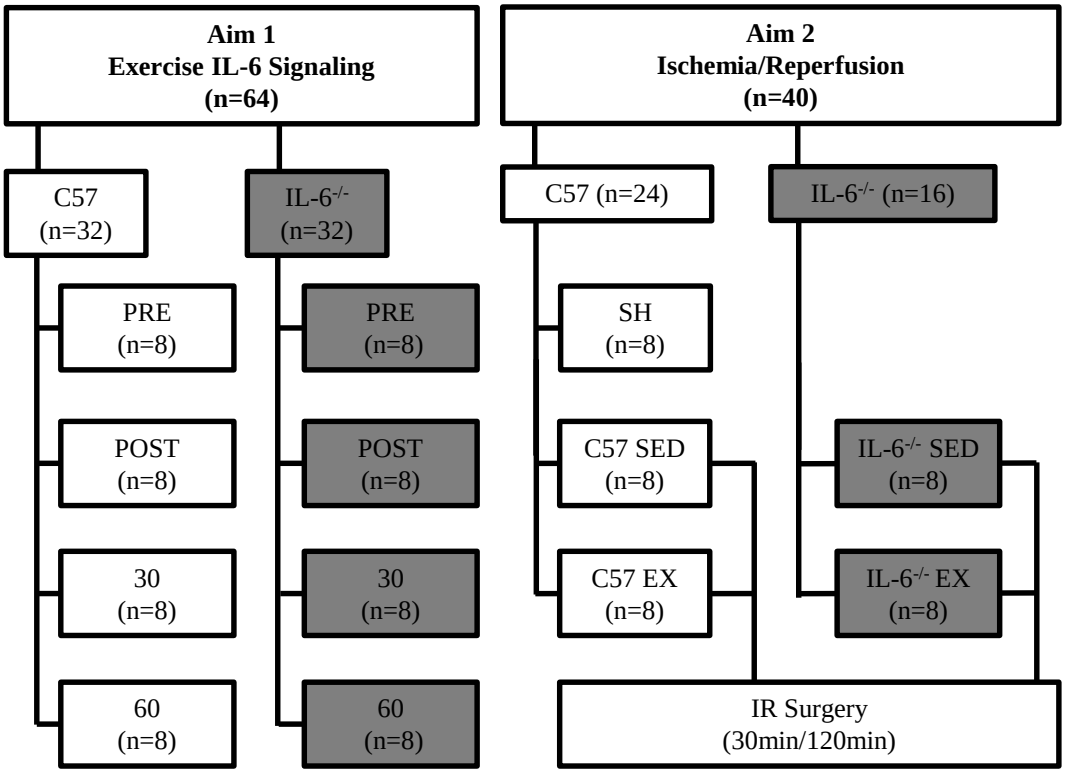
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Exercise IL-6 Signaling
(n=64)
Aim 2
Ischemia/Reperfusion
(n=40)
C57
(n=32)
IL-6-/-
(n=32)
C57 (n=24)
IL-6-/- (n=16)
PRE
(n=8)
PRE
(n=8)
SH
(n=8)
POST
(n=8)
POST
(n=8)
C57 SED
(n=8)
IL-6-/- SED
(n=8)
30
(n=8)
30
(n=8)
C57 EX
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IR Surgery
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Figure 1.

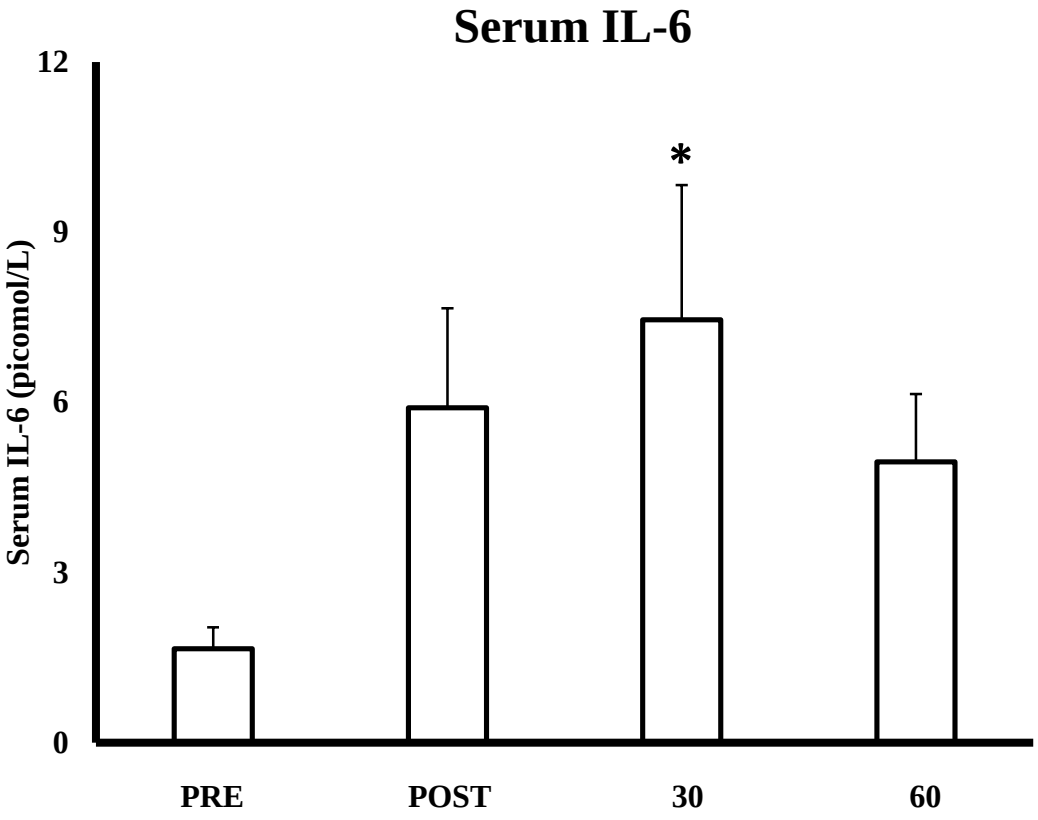

Figure 2.

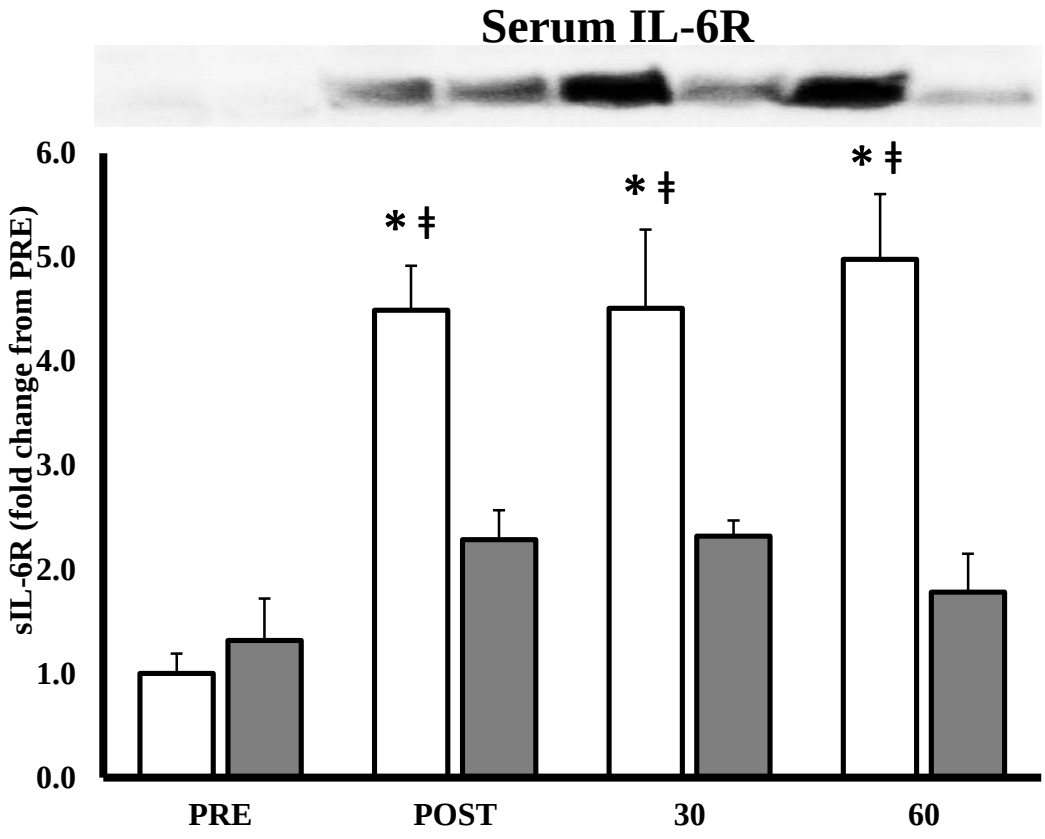
Serum IL-6R
sIL-6R (fold change from PRE)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
* ‡
* ‡
* ‡
PRE
POST
30
60

Figure 3.

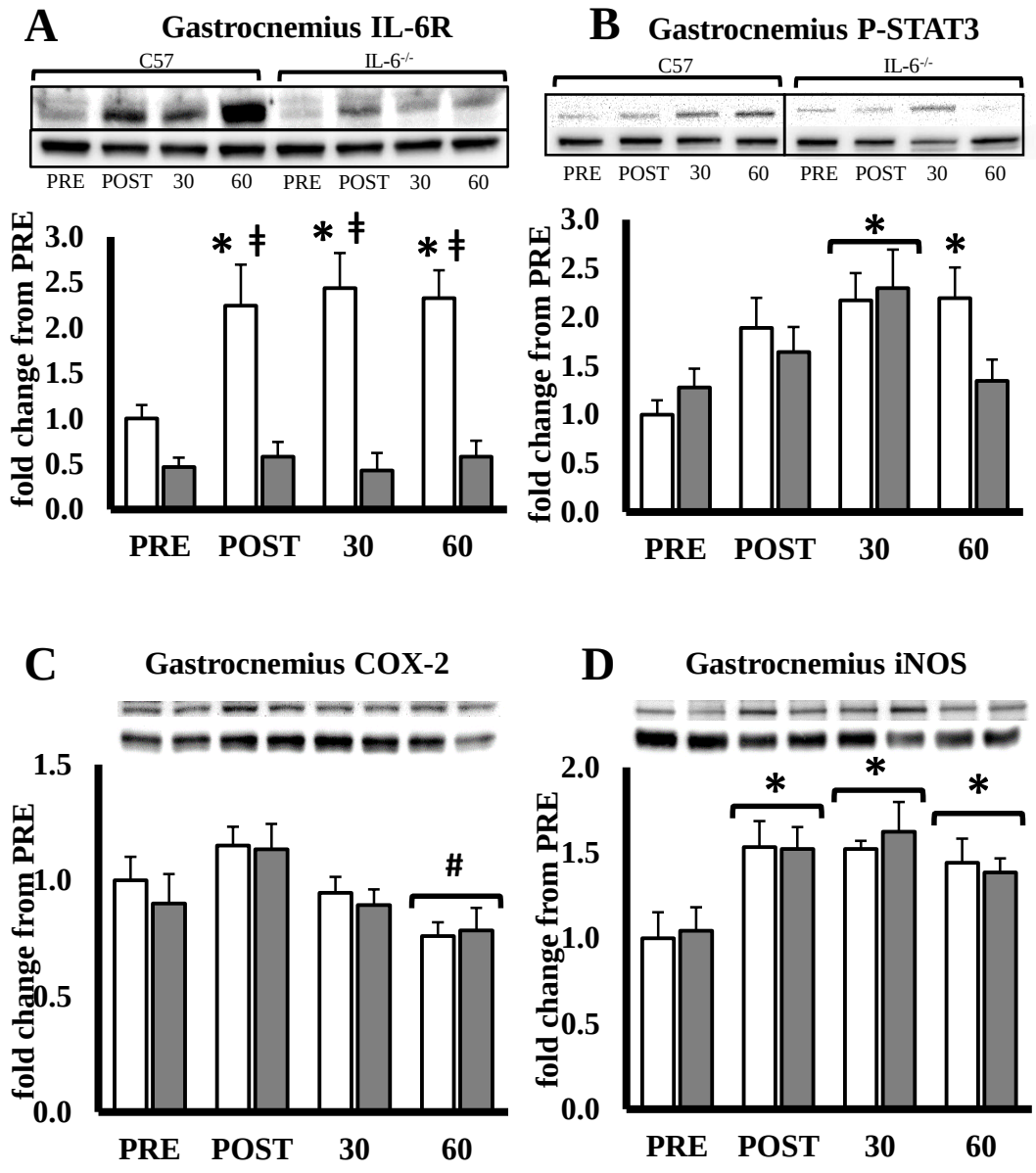
A
Gastrocnemius IL-6R
C57
IL-6-/-
PRE POST 30 60 PRE POST 30 60
fold change from PRE
3.0
2.5
2.0
1.5
1.0
0.5
0.0
* ‡
* ‡
* ‡
PRE POST 30 60
B
Gastrocnemius P-STAT3
C57
IL-6-/-
PRE POST 30 60 PRE POST 30 60
fold change from PRE
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
PRE POST 30 60
C
Gastrocnemius COX-2
fold change from PRE
1.5
1.0
0.5
0.0
#
PRE POST 30 60
D
Gastrocnemius iNOS
fold change from PRE
2.0
1.5
1.0
0.5
0.0
*
*
*
PRE POST 30 60

Figure 4.

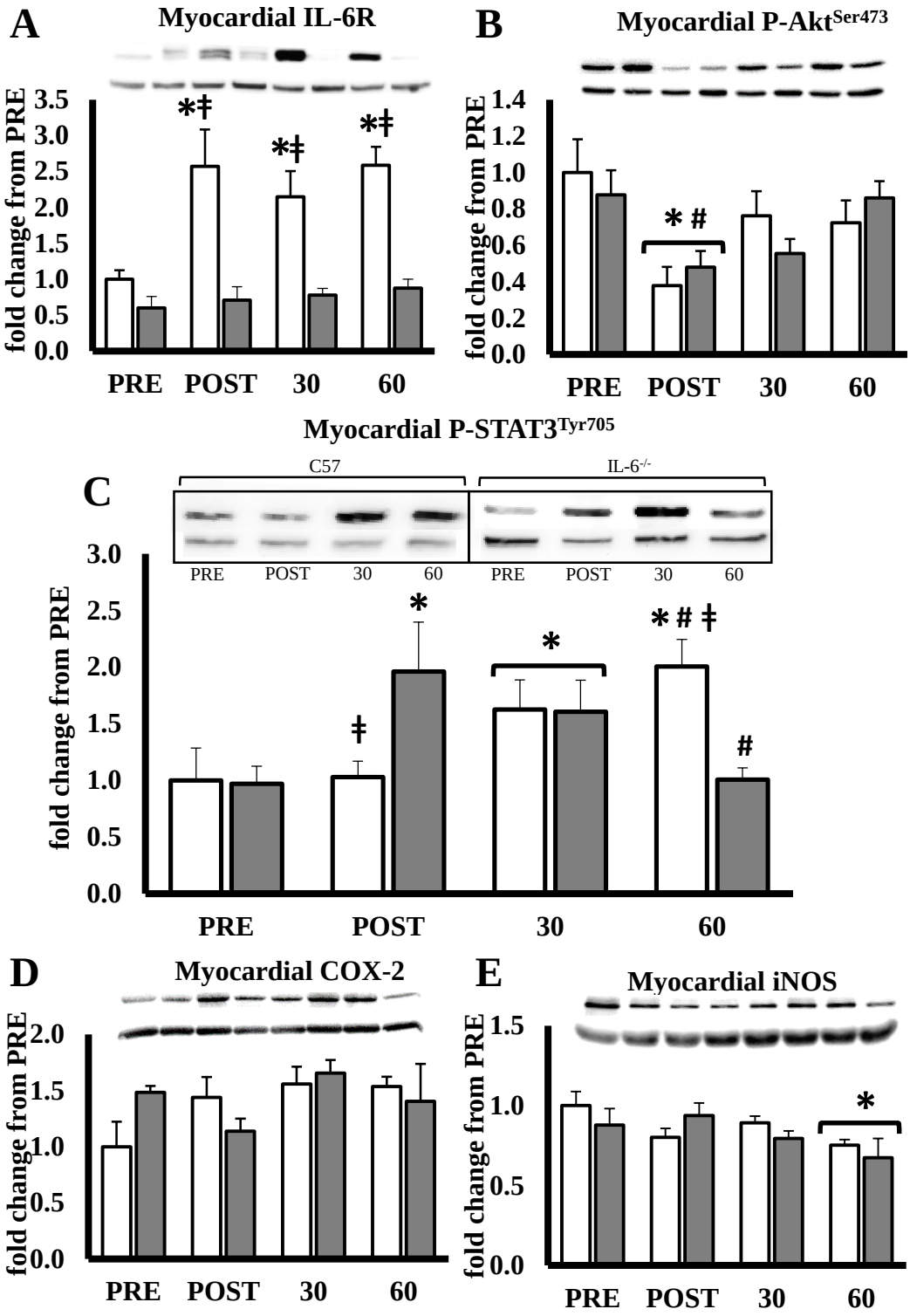
A
Myocardial IL-6R
fold change from PRE
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*‡
*‡
*‡
PRE
POST
30
60
B
Myocardial P-Akt Ser473
fold change from PRE
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
* #
PRE
POST
30
60
Myocardial P-STAT3 Tyr705
C
C57
IL-6-/-
PRE
POST
30
60
PRE
POST
30
60
fold change from PRE
3.0
2.5
2.0
1.5
1.0
0.5
0.0
‡
*
*
*
‡
#
PRE
POST
30
60
D
Myocardial COX-2
fold change from PRE
2.0
1.5
1.0
0.5
0.0
PRE
POST
30
60
E
Myocardial iNOS
fold change from PRE
1.5
1.0
0.5
0.0
*
PRE
POST
30
60

Figure 5.

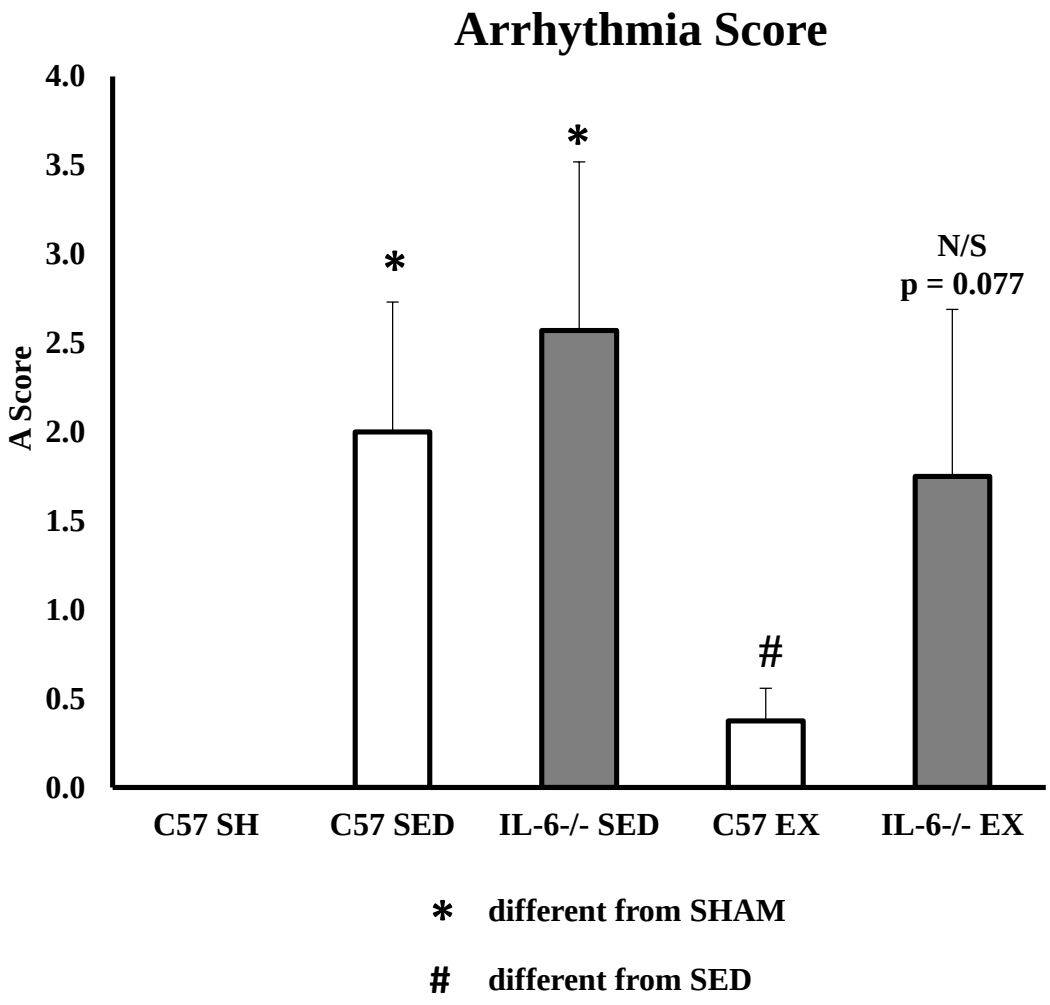
Arrhythmia Score
A Score
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
#
N/S
p = 0.077
C57 SH
C57 SED
IL-6-/- SED
C57 EX
IL-6-/- EX
* different from SHAM
# different from SED

Figure 6.

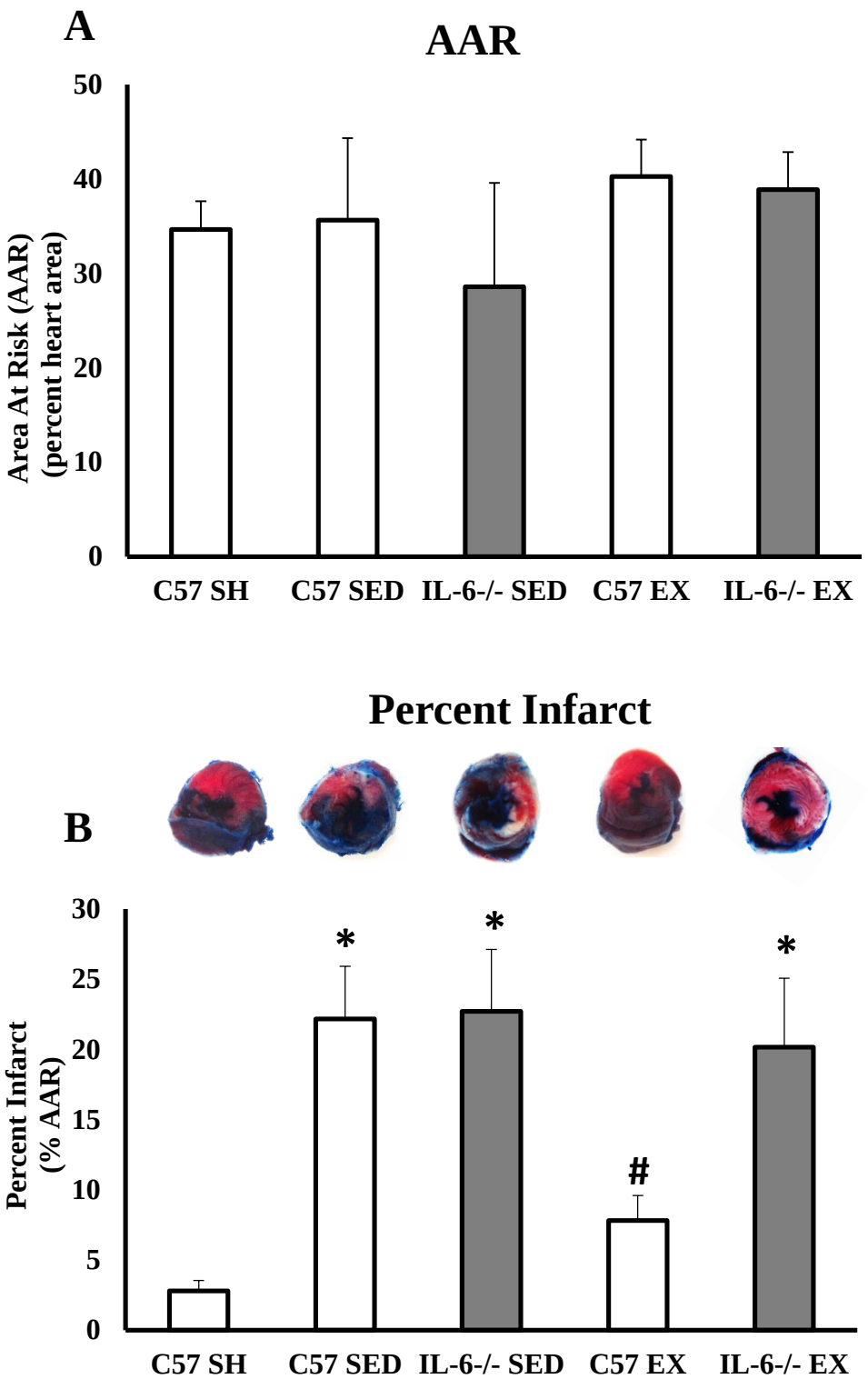
A
AAR
50
40
30
20
10
0
Area At Risk (AAR)
(percent heart area)
C57 SH
C57 SED
IL-6-/- SED
C57 EX
IL-6-/- EX
Percent Infarct
B
30
25
20
15
10
5
0
Percent Infarct
(% AAR)
*
*
#
*
C57 SH
C57 SED
IL-6-/- SED
C57 EX
IL-6-/- EX

Figure 7.

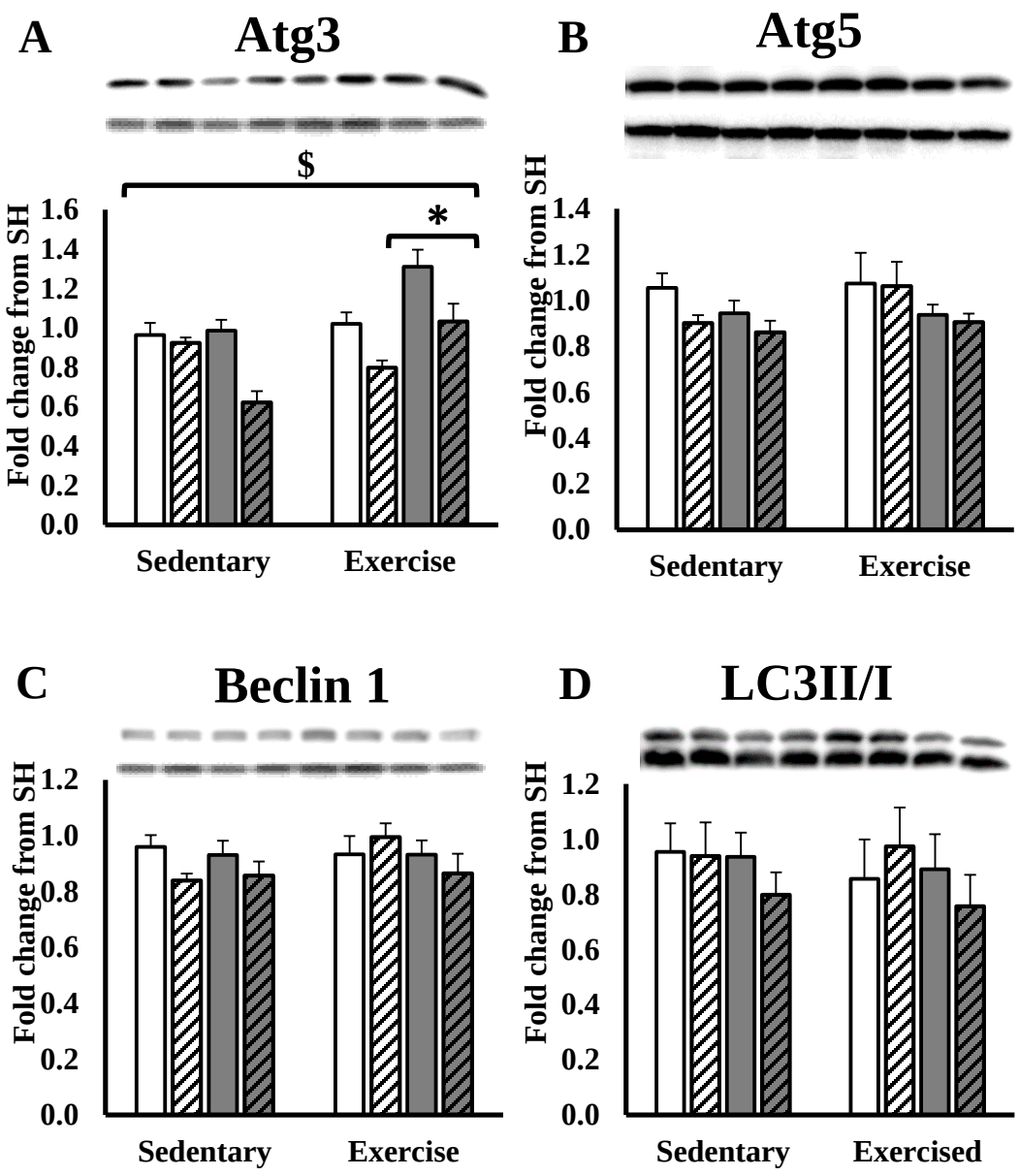
A
Atg3
$
*
Fold change from SH
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sedentary
Exercise
B
Atg5
Fold change from SH
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sedentary
Exercise
C
Beclin 1
Fold change from SH
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sedentary
Exercise
D
LC3II/I
Fold change from SH
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sedentary
Exercised

Figure 8.

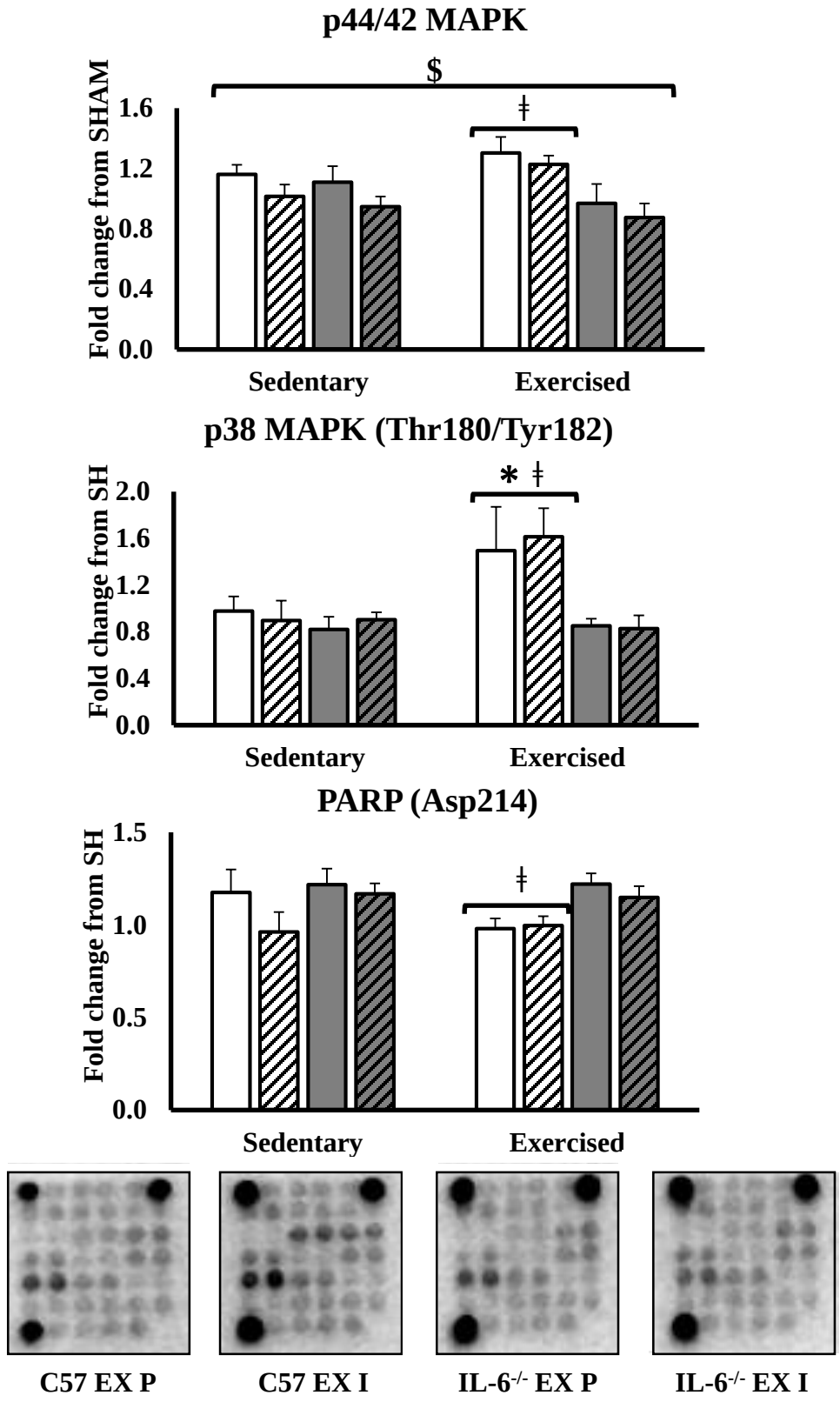
p44/42 MAPK
$
‡
Fold change from SHAM
1.6
1.2
0.8
0.4
0.0
Sedentary
Exercised
p38 MAPK (Thr180/Tyr182)
* ‡
Fold change from SH
2.0
1.6
1.2
0.8
0.4
0.0
Sedentary
Exercised
PARP (Asp214)
‡
Fold change from SH
1.5
1.0
0.5
0.0
Sedentary
Exercised
C57 EX P
C57 EX I
IL-6-/- EX P
IL-6-/- EX I

Figure 9.

Gene	Forward Primers (F')	Reverse Primers (R')
IL-6	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCCAGAGAAC
LIF (variant 1)	AGTAGCCGACTGCAGCTCTC	GACAGCTGTGCTGGATCAAA
Ctf1 (CT-1)	CCCATCTGTCTCCCTGTGAT	TCCTAAGGTGAATGGCCTTG
IL-6R	CTTGGTTCCGATTTCCTTCT	GAAATTGTCACTCGCGTAAAC
Ptgs2 (COX-2)	AGAAGGAAATGGCTGCAGAA	GCTCGGCTTCCAGTATTGAG
NOS2 (iNOS)	CACCTTGGAGTTCACCCAGT	ACCACTCGTACTTGGGATGC

Table 1.

		C57				IL-6$^{-/-}$			
Tissue	**Gene**	**PRE**	**POST**	**30**	**60**	**PRE**	**POST**	**30**	**60**
Heart	**IL-6**	1.0 ± 0.2	1.4 ± 0.1	1.2 ± 0.3	1.6 ± 0.6	-	-	-	-
	LIF	1.0 ± 0.3	1.5 ± 0.2	1.2 ± 0.2	2.9 ± 0.9 *	1.1 ± 0.1	1.6 ± 0.4	1.1 ± 0.1	2.2 ± 0.5*
	CT-1	1.0 ± 0.2 ǂ	1.1 ± 0.2 ǂ	1.4 ± 0.2 ǂ	1.2 ± 0.1 ǂ	1.5 ± 0.3	1.6 ± 0.2	1.5 ± 0.3	1.4 ± 0.2
	IL-6R	1.0 ± 0.1	1.3 ± 0.2	2.0 ± 0.3 *	1.8 ± 0.2 #*	0.8 ± 0.1	1.5 ± 0.1	1.6 ± 0.3 *	2.0 ± 0.2 #*
	iNOS	1.0 ± 0.2	0.6 ± 0.1 *	0.4 ± 0.1 *	0.5 ± 0.1 *	1.1 ± 0.2	0.7 ± 0.1 *	0.5 ± 0.1 *	0.5 ± 0.1 *
	COX-2	1.0 ± 0.1	1.5 ± 0.3	1.2 ± 0.2	1.2 ± 0.2	1.1 ± 0.2	1.5 ± 0.3	1.7 ± 0.3	1.5 ± 0.2
Gastroc	**IL-6**	1.0 ± 0.1	1.6 ± 0.5	2.0 ± 0.6	0.7 ± 0.1	-	-	-	-
	LIF	1.0 ± 0.2	2.0 ± 0.6	1.5 ± 0.3	1.1 ± 0.1	2.1 ± 0.3	2.6 ± 1.0	1.1 ± 0.2	1.4 ± 0.2
	CT-1	1.0 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
	IL-6R	1.0 ± 0.1	1.2 ± 0.2	2.0 ± 0.3	3.2 ± 0.8 #*	0.9 ± 0.1	1.9 ± 0.6	1.9 ± 0.3	4.0 ± 0.7 #*
	iNOS	1.0 ± 0.2 ǂ	1.4 ± 0.2	1.0 ± 0.3	0.6 ± 0.2	3.7 ± 0.8	1.3 ± 0.4 *	1.2 ± 0.2 *	1.4 ± 0.4 *
	COX-2	1.0 ± 0.1	2.2 ± 0.5 ǂ	1.9 ± 0.3	1.1 ± 0.2	1.9 ± 0.3	4.4 ± 1.0 *	1.2 ± 0.2	1.3 ± 0.3

Table 2.

	C57		IL-6$^{-/-}$	
	Sedentary	**Exercised**	**Sedentary**	**Exercised**
Body Weight (g)	28.6 ± 0.7 ǂ	27.6 ± 0.6 ǂ	30.6 ± 1.3	30.0 ± 1.2
Heart Weight (mg)	122.1 ± 2.6	126.0 ± 2.9 *ǂ	118.6 ± 6.4	136.8 ± 6.2 *
Heart Weight/ Body Weight	4.3 ± 0.1	4.6 ± 0.1	3.9 ± 0.2	4.6 ± 0.2 *

Table 3.

	C57 Sedentary		IL-6-/- Sedentary		C57 Exercised		IL-6-/- Exercised	
PathScan Target	**P**	**I**	**P**	**I**	**P**	**I**	**P**	**I**
Akt (Ser473)	0.75 ± 0.09	0.72 ± 0.07	0.77 ± 0.09	0.68 ± 0.07	0.70 ± 0.04	0.65 ± 0.05	0.59 ± 0.07	0.60 ± 0.09
Bad (Ser136)	1.14 ± 0.07	1.05 ± 0.08	1.13 ± 0.04	1.12 ± 0.07	1.08 ± 0.11	1.06 ± 0.06	1.06 ± 0.05	1.10 ± 0.09
HSP27 (Ser82)	0.72 ± 0.04	0.94 ± 0.05	0.85 ± 0.06	0.88 ± 0.08	0.84 ± 0.06	0.77 ± 0.06	0.91 ± 0.07	0.80 ± 0.07
Smad2 (ser465/467)	0.41 ± 0.03	0.45 ± 0.06	0.40 ± 0.07	0.49 ± 0.08	0.47 ± 0.06	0.46 ± 0.04	0.35 ± 0.03	0.37 ± 0.05
p53 (ser15)	0.19 ± 0.07	0.29 ± 0.05	0.29 ± 0.06	0.28 ± 0.04	0.24 ± 0.04	0.26 ± 0.05	0.22 ± 0.05	0.24 ± 0.06
JNK (Thr183/Tyr185)	1.48 ± 0.06	1.66 ± 0.10 $	1.51 ± 0.04	1.55 ± 0.09 $	1.40 ± 0.06	1.60 ± 0.08 $	1.61 ± 0.05	1.71 ± 0.10 $
Caspase3 (Asp175)	0.50 ± 0.05	0.54 ± 0.05 $	0.49 ± 0.08	0.53 ± 0.04 $	0.42 ± 0.07	0.48 ± 0.06 $	0.54 ± 0.07	0.58 ± 0.04 $
Caspase7 (Asp198)	1.28 ± 0.07	1.36 ± 0.09 $	1.24 ± 0.08	1.47 ± 0.05 $	1.24 ± 0.06	1.32 ± 0.03 $	1.27 ± 0.05	1.36 ± 0.09 $
IκBα (Total)	2.21 ± 0.29	2.17 ± 0.20	2.29 ± 0.18	2.11 ± 0.15	2.52 ± 0.20	2.43 ± 0.27	2.38 ± 0.22	1.98 ± 0.18
Chk1 (Ser345)	1.17 ± 0.09	1.22 ± 0.06	1.28 ± 0.07	1.32 ± 0.08	1.27 ± 0.09	1.26 ± 0.07	1.27 ± 0.06	1.26 ± 0.05
Chk2 (Thr 68)	0.64 ± 0.05	0.71 ± 0.08	0.60 ± 0.07	0.60 ± 0.07	0.59 ± 0.04	0.62 ± 0.04	0.61 ± 0.06	0.67 ± 0.06
IκBα (Ser 32/36)	0.52 ± 0.07	0.48 ± 0.10	0.46 ± 0.05	0.49 ± 0.10	0.54 ± 0.05	0.43 ± 0.07	0.32 ± 0.06	0.55 ± 0.09
elF2α (Ser51)	0.88 ± 0.07	0.85 ± 0.06	0.84 ± 0.08	0.97 ± 0.06	0.84 ± 0.07	0.81 ± 0.07	1.01 ± 0.05	0.98 ± 0.07
TAK1 (Ser 412)	0.92 ± 0.06	1.00 ± 0.05	0.93 ± 0.05	0.87 ± 0.06	0.90 ± 0.06	1.01 ± 0.08	1.21 ± 0.19	0.89 ± 0.06
Survivin (Total)	0.61 ± 0.06	0.66 ± 0.08	0.63 ± 0.03	0.81 ± 0.06	0.67 ± 0.07	0.67 ± 0.07	0.63 ± 0.07	0.63 ± 0.06

Table 4.

Figure Legends

Figure 1. Study Design. Aim 1: C57 and IL-$6^{-/-}$ mice were divided into sedentary and exercised groups and were sacrificed PRE, POST, 30, and 60 minutes post exercise by cardiac excision. Aim 2: Exercised and sedentary C57 and IL-$6^{-/-}$ mice received surgically induced IR injury with 30 minutes I and 120 minutes R. A group of C57 mice was used for a time matched sham operation.

Figure 2. Serum IL-6. Blood was drawn via cardiac puncture and assayed for IL-6 via commercially available ELISA. Exercise increased serum IL-6 at 30 minutes post exercise. * different from PRE $p < 0.05$

Figure 3. Soluble IL-6 Receptor. Serum was analyzed via western blotting for sIL-6R. Exercise increased sIL-6R at all time points in C57 mice only. C57 mice had greater sIL-6R expression compared to IL-$6^{-/-}$ mice POST, 30, and 60, but no differences were seen PRE. * different from PRE ǂ different from IL-$6^{-/-}$

Figure 4. Exercise induced signaling in gastrocnemius. Western blotting was conducted on gastrocnemius samples. Whole muscle homogenates were used for IL-6R, nuclear fractions were used for P-STAT3, and cytosolic fractions for iNOS and COX-2. Exercise elicited an increase in gastroc IL-6R in C57 mice only (A), while a post exercise increase in P-STAT3 was seen in both groups at 30, and in C57 mice at 60 (B). COX-2 was significantly higher POST compared to 60 (C), and iNOS was increased at POST, 30, and 60 (D). No differences were seen between C57 and IL-$6^{-/-}$ mice in COX-2 and iNOS. * different from PRE # different from POST ǂ different from IL-$6^{-/-}$.

Figure 5. Exercise induced signaling in the myocardium. Western blotting was conducted on myocardial samples. Whole muscle homogenates were used for IL-6R, nuclear fractions were used for P-STAT3, and cytosolic fractions for P-Akt, iNOS and COX-2. Exercise increase IL-6R expression in C57 animals only (A). P-Akt was decreased post exercise, and returned to baseline at 30 and 60 (B). Exercise increased P-STAT3 in C57 and IL-$6^{-/-}$ mice. COX-2 was unchanged, while iNOS was suppressed. * different from PRE # different from POST ǂ different from IL-$6^{-/-}$

Figure 6. Arrhythmia Score. ECG were analyzed under blinded conditions. C57 SED and IL-$6^{-/-}$ SED mice had significantly higher arrhythmia scores. C57 EX mice had a significantly lower score compared to C57 SED. A non-significant increase in arrhythmia score was seen in IL-$6^{-/-}$ EX. * different from SH # different from SED

Figure 7. Infarct Size. Evan's Blue dye allowed for the quantification of the AAR (A). Incubation in 1% TTC was used for quantifying the infarct size (B). No differences were seen in AAR. C57 SED, IL-$6^{-/-}$ SED and IL-$6^{-/-}$ EX had larger infarcts compared to SH. C57 EX had significantly smaller infarcts compared to C57 SED, and were not different from SH. * different from SH # different from SED

Figure 8. Autophagy western blotting. Ischemic and perfused myocardial samples were dissected and analyzed separately for autophagy markers. There was a decrease in Atg3 in ischemic tissue, with an increase in IL-$6^{-/-}$ EX mice compared to SED (A). No changes were seen in Atg5 (B), Beclin 1 (C), or LC3 (D). * different from SED $ different from perfused

Figure 9. PathScan data. Myocardial homogenates were analyzed with Cell Stress and Apoptosis PathScan. p44/42 MAPK was decreased in ischemic tissue compared to perfused tissue and was increased in C57 EX compared to IL-$6^{-/-}$ EX. p38 MAPK was increased by exercise in C57 mice only. PARP was higher in IL-$6^{-/-}$ EX mice compared to C57 EX. T different from IL-$6^{-/-}$ $ different from Perfused.

Table 1. PCR Primers. Primers were selected using Primer3Plus freeware and ordered from IDT Technologies.

Table 2. Aim 1 PCR results from gastrocnemius and myocardium. Note increase in myocardial and gastrocnemius IL-6R mRNA in both strains. Myocardial CT-1 was higher in IL-$6^{-/-}$. Myocardial iNOS and COX-2 were not increased following exercise. * different from PRE # different from POST ǂ different from IL-$6^{-/-}$

Table 3. Aim 2 Mouse Anthropometrics. IL-$6^{-/-}$ mice had slightly higher BW, but no differences exist between C57 and IL-$6^{-/-}$ HW/BW in either sedentary or exercise mice * different from PRE ǂ different from IL-$6^{-/-}$

Table 4. Supplementary PathScan Data. $ different from Perfused.

Appendix I

Extended Methodology

Animals

Male mice (56 C57, C57BL/6J; 48 IL-6$^{-/-}$, B6;129S2-*Il6*^{tm1Kopf}/J) were used to complete the two arms of this study. All protocols utilized in the study received Auburn University IACUC approval. Animals were housed at the Auburn University Biological Research Facility (BRF) on a 12:12 light:dark cycle with access to water and rodent chow *ad libitum*. In Branch 1 of the proposed study, 64 mice (C57, n = 32 and IL-6$^{-/-}$, n = 32) were randomly assigned to exercise or sedentary treatments to evaluate the acute myokine response to exercise in the blood, skeletal muscle and heart. In the second branch of the study, 40 mice (C57 n = 24 and IL-6$^{-/-}$ n = 16) were used to investigate the role of IL-6 in exercise induced preconditioning against IR injury.

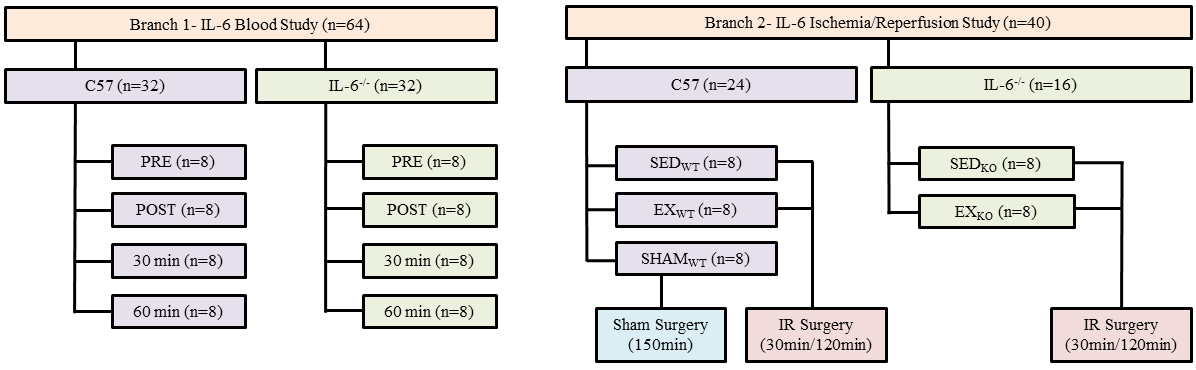

Figure 2. Animal groups for Branch 1 and Branch 2 study designs. A total of 104 mice were used including 56 C57 and 48 IL-6$^{-/-}$ mice.

DNA isolation and Genotyping

Tissues from animals of both strains, C57 and IL-$6^{-/-}$, were genotyped to verify strain specificity and effective knock-out of the *IL6* gene using Qiagen DNeasy Blood and Tissue Kit (Qiagen #69581) following manufacturer recommendations. Briefly, approximately 25 mg tissue gastrocnemius tissue was homogenized in 180 μL Buffer ATL. To the homogenate, 20 μL Proteinase K was added, and tubes were vortexed and incubated at 56ºC until complete lysis (overnight). Following lysis, samples were vortexed, and 200 μL Buffer AL and 200 μL 100% ethanol were added and the solution was mixed thoroughly. The mixture was then transferred into a spin column, housed in a 2 mL centrifuge tube, and centrifuged at 8000 rpm for 1 min. The spin column was placed in a fresh tube, and the flow through was discarded. To the spin column, 500 μL Buffer AW1 was added, and samples were centrifuged at 8000 rpm for 1 min. The spin column was placed in a fresh tube, and the flow through was discarded. To the spin column, 500 μL Buffer AW2 was added, and samples were centrifuged at 14,000 rpm for 3 min. Spin columns were transferred to a sterile 1.5 mL microcentrifuge tube and the previous tube and flow through discarded. To the spin column, 200 μL Buffer AE was added to elute DNA. Samples were incubated for 1 minute at room temperature (RT), and centrifuged at 8000 rpm for 1 min, collecting DNA in tube.

Endpoint PCR was conducted using 100 ng DNA combined with 10 μL 2X GoTaq Green Master Mix, 1.25 μL common forward primer (5'-TTCCATCCAGTTGCCTTCTTGG-3'), 1.25 μL wild type reverse primer (5'-TTCTCATTTCCACGATTTCCCAG-3'), 1.25 μL mutant reverse primer (5'-CCGGAGAACCTGCGTGCAATCC-3'), with sterile water to a final volume of 20 μL. PCR was conducted following manufacturer instructions (initializing- 3 min at 94°C, 35 cycles of 30 seconds denaturing at 94°C, 1 min annealing at 62°C, 1 min extension at 72°C, with a final

elongation for 2 min at 72°C) to amplify products of 174 and 380 bp, corresponding to wild type and mutant DNA, respectively (heterozygous animals would show yield products of both sizes). PCR products were separated on a 1% glycine gel (3 g tris glycine dissolved in 300 mL TAE buffer, heated with a microwave to a boil, 3 μL ethidium bromide added once cooled, and cast) at 80 V for 40 minutes and imaged with trans-UV light on a ChemiDoc-It imaging system.

Branch 1 - Exercise and IL-6 signaling

Mice were randomly assigned to sedentary or exercise treatments. Mice in the exercise group performed treadmill exercise on a rodent treadmill (Columbus Instruments, Columbus, OH). A mild electric shock was administered at the rear of the treadmill, and will serve as motivation. Mice were habituated to treadmill exercise over 4 days with exercise durations increasing from 10 minutes to 40 min over a four-day period. Following habituation and a one-day rest period, mice underwent 3 consecutive days of "preconditioning exercise" for 60 minutes/day at 18 m/min at 0% grade. Sedentary mice were given equal treadmill exposure time at 0 m/min. Under isofluorane anesthesia, a cardiac puncture was performed to collect blood. Mice were then sacrificed pre-exercise (PRE), immediately post-exercise (POST), 30 minutes post-exercise (30), or 60 minutes post-exercise (60) via decapitation. Sedentary mice were used for the PRE group. Blood was immediately centrifuged at 10,000 x g at 4ºC, and the serum aliquots were stored at -80ºC. Heart and skeletal muscle used for histological experiments was excised, coated in Neg50 embedding medium, frozen in 2-methylbutane cooled over liquid nitrogen, and stored at -80ºC until subsequent analysis. Contralateral skeletal muscles were snap frozen in liquid nitrogen and used for biochemical analysis.

Branch 2 – Exercise induced cardioprotection and IL-6 signaling

A second group of mice (C57 n=24 and IL-$6^{-/-}$ n=16) was used to investigate the role of IL-6 in exercise-induced cardioprotection. Mice were assigned to sedentary and exercise treatments, and exposed to an identical exercise stimulus as Branch 1. Such a short exercise stimulus is paramount to the design of the study in showing the biochemical mechanisms of preconditioning. Longer training regimen introduce the confounding possibility that myocardial and cardiovascular remodeling or reconstruction will impact the observed protection in exercised animals. Use of the preconditioning protocol previously described ensures that the biochemical interactions are being examined. Twenty-four hours following the final exercise session, animals received a surgically induced IR injury. A group of C57 mice (n=8), was further received a sham operation.

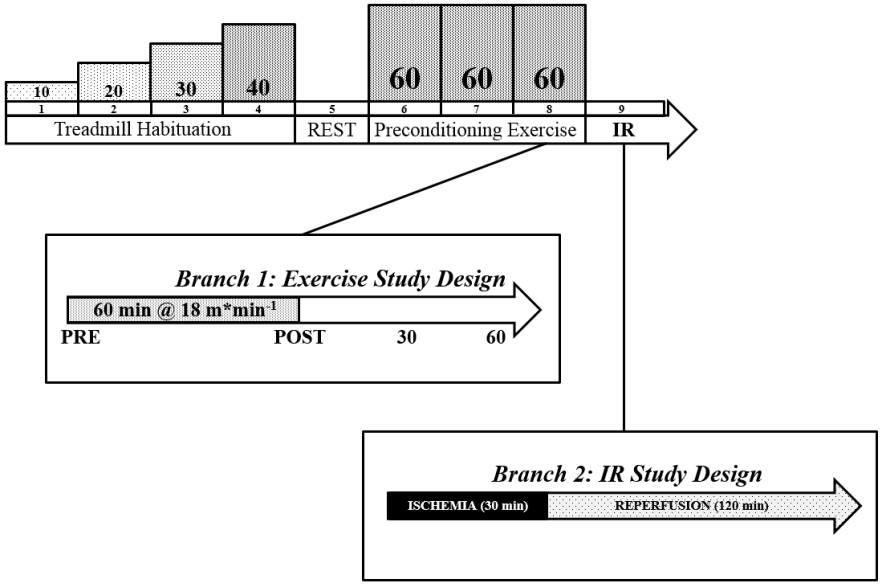

Figure 3. Summary of exercise habituation and preconditioning exercise protocols for Branch 1 and Branch 2 study designs. Branch 1) Animals will be sacrificed prior to or immediately following the final exercise session. Branch 2) Animals will undergo surgically induced IR (or sham operation) 24 hours following the final exercise session.

Surgical Preparation and in vivo IR

Twenty-four hours after the third preconditioning exercise bout, animals received a surgically induced, reversible *in vivo* regional IR injury. Mice were anesthetized using sodium pentobarbital (50 mg/kg) prior to surgical handling. Once a surgical plane of anesthesia was established, verified by the absence of paw pinch reflexes, animals were connected to limb lead electrodes integrated into a physiological data acquisition system (Biopac, Santa Barbara, CA). An incision was made at the anterior neck, and blunt dissection exposed the trachea. The trachea was penetrated with micro surgical scissors, and a tracheotomy, using a 23 gauge venous catheter, was inserted. Mice were supported with a pressure sensitive mechanical ventilator (Kent Scientific) for the duration of the surgery.

A reversible left anterior descending coronary artery ligation (LAD ligation) was performed to elicit IR injury. Briefly, the LAD was visualized via left thoracotomy and an 8-0 surgical suture (Ethicon J548G), looped through a small polyethylene tubing, was passed underneath the vessel and looped back through the tubing, creating a reversible ligature. To establish ischemia, the ligature was tightened manually and held in place with a surgical clip, producing regional ischemia for 30 minutes. Ischemia was verified by either visual confirmation of color change in the area at risk, as well as ECG rhythm changes. At the conclusion of the ischemic duration, the ligature was released to allow reperfusion for 120 minutes. Durations of ischemia and reperfusion have been verified by previous studies in our lab and others to produce quantifiable IR injury, resulting in cell death. Sham animals received an identical surgery, however, the ligature remained loose throughout an equivalent period (150 minutes). A surgical plane of anesthesia was monitored and maintained throughout the entire experiment and supplemental anesthetic was provided at the reappearance of any reflexes. Following reperfusion (or at the termination of sham surgery),

ligatures were briefly replaced and animals received an intraventricular injection of 4 % Evan's Blue dye (2 g dissolved in 50 mL 10 mM PBS). Dye circulated to the perfused areas of the heart allowing for location, quantification, and comparison of ischemic and perfused tissue. Promptly following dye injection, animals were sacrificed by cardiac excision.

Branch 2 tissue analysis for Infarct area/AAR vs. Perfused tissue

Following excision, the heart was stopped in ice cold saline (10 mM PBS) and cut into 2 mm coronal cross-sections using a mouse heart matrix (Zivic Instruments, Pittsburgh, PA). To measure tissue necrosis, sections were incubated in 1% triphenol-tetrazolium chloride (TTC; 100 mg TTC in 10 mL DI H_2O) for 15 minutes at 37°C. TTC reacts with dehydrogenases in viable tissue following IR injury, leaving non-reactive infarcted tissue white in appearance. TTC stained slices, incubated in ice-cold saline, were photographed for subsequent analysis of infarct size. Quantification of perfused tissue and ischemic tissue, or the area at risk (AAR), were used to ensure that a uniform IR injury occurred in all hearts. Within the AAR, quantification of necrotic tissue was used to evaluate the extent of tissue death as a result of ischemia. Perfused cardiac tissue, (blue) was dissected free of the ischemic AAR (pink/white) and analyzed separately.

Histological analysis of Arm 1 hearts

Hearts and skeletal muscles from Branch 1 animals were histologically assayed for IL-6R. Frozen tissues were cryosectioned at 10 µm thickness on a Microm HM-525 microtome (ThermoScientific) at -30°C. Samples were fixed in 10 % formalin (Sigma-Aldrich HT5014) for 20 minutes. After washing in 10 mM PBS, sections were permeabilized with Permeabilization Solution (50 mg Na^{2+} Citrate (Sigma-Aldrich S1804), 50 µL Triton X-100 (Sigma-Aldrich X-100) in 50 mL DI H_2O) for 5 minutes, washed, and dried with kimwipes. After drying, tissues were circled with a hydrophobic pap pen and blocked for 30 minutes in 3% BSA and 20% goat serum

(7.6 mL DI H_2O, 300 mg BSA (Sigma-Aldrich A7906), 158 mg trisma HCL (Sigma-Aldrich T3252), 2 mL 20% goat serum).

Primary antibody for IL-6R (Santa Cruz, sc-660), diluted 1:40 in blocking buffer, was applied to tissues overnight at 4°C with rocking. After washing, a fluorescent labeled secondary antibody (diluted 1:40) was applied for 40 minutes at RT in the dark, with rocking. Slides were mounted with Vecta Shield + DAPI and sealed with clear nail polish.

Slides were imaged at 40X magnification using a fluorescent microscope (Nikon Eclipse Ci), and images were captured with NIS Elements software using filters for DAPI (to visualize nuclei), and FITC (to visualize IL-6R). Signal intensity for IL-6R was measured in representative view fields (8 fields per sample), and compared to pre-exercise values.

Tissue Handling for Western Blotting

Cardiac and skeletal muscles were analyzed via Western Blotting for proteins involved in IL-6 signaling. Approximately 30 mg of muscle sample was cut from whole muscle samples (cardiac ventricles and skeletal muscles). The myocardium in Branch 2 was separated into ischemic and perfused tissue samples following TTC staining, and the two conditions were analyzed separately to allow for comparison. To obtain whole muscle homogenates, muscle samples were diluted 1:20 w/v in 10 mM PBS with protease inhibitors and homogenized glass-on-glass, kept on ice. Samples were centrifuged at 10,000 x g for 10 minutes at 4°C and the supernatant was transferred to a new tube.

Nuclear and Cytosolic fractions (NF and CF, respectively) were obtained with the aid of a NE-PER Nuclear Isolation Kit (ThermoScientific #78833), as per manufacturer instructions. Briefly, 200 μL CERI was combined with phosphatase (GBiosciences Phosphatase Arrest II, 1:100) and protease (Sigma #P2714, 1:10) inhibitors to make a homogenizing buffer. Approximately 20 mg

of sample was homogenized with 200 µL homogenizing buffer, vortexed vigorously for 15 seconds, and incubated on ice for 10 minutes. Following incubation, 11 µL CERII was added. Samples were vortexed vigorously for 15 seconds, incubated for 1 minute on ice and centrifuged at 25,000 x g for 5 minutes. The supernatant, containing the CF, was transferred to a fresh tube and stored at -80°C. The pellet, containing the NF, was resuspended with 500 µL PBS (0.1 M phosphate, 0.15 M NaCl; pH 7.2), and triturated to wash the sample, then re-spun at 25,000 x g for 5 minutes. The supernatant was discarded. To obtain the nuclear fraction, the nuclear lysis buffer (100 µL NERI with protease and phosphatase inhibitors) was added to the washed pellet and triturated. Samples were incubated for 40 minutes on ice, with a 15 second vortex every 10 minutes. Following incubation, samples were centrifuged for 10 minutes at 25,000 x g. The supernatant, containing the NF, was transferred to a fresh tube and stored at -80°C and the pellet was discarded. All centrifugation steps were at 4°C.

From each homogenate sample, 20 µL was diluted to a final concentration of 1:100 by adding 180 µL 10 mM PBS with 0.1 mM EDTA to determine protein concentration via Bradford Assay. Samples were compared to a standard curve of bovine serum albumin (BSA, Sigma-Aldrich) diluted in 10 mM PBS to a concentration of 1.25 mg/mL BSA, and serially diluted down to 0 mg/mL. In a 96 well plate, 280 µL Bradford Reagent (Sigma-Aldrich) and 20 µL diluted sample was added, mixed, and incubated 20 minutes in the dark. The absorbance was measured at 595 nm on a spectrophotometer. The protein concentration of each sample was normalized to 1.0 µg/µL for whole muscle homogenates, 6.0 µg/µL for CF samples, and 1.0 µg/µL for NF. Laemmli sample buffer (Biorad, 161-0737) with 5% v/v β-mercaptoethanol was added to each sample 1:1, resulting in a final sample concentration of 0.5, 3, and 0.5 µg/µL for whole muscle, CF, and NF samples,

respectively. Prepped sample were capped, heated at 90°C for 5 minutes and stored at -80°C until use.

Western Blotting

Resolving gels (10%) were made using 2.5 mL Bis-Acrylamide 40%, 50 µL APS 10%, 100 µL SDS 10%, 2.5 mL Resolving Gel Buffer (1.5 M Tris-HCl, pH = 8.8), and 15 µL TEMED. Stacking gels (4%) were made using 1 mL Bis-Acrylamide 40%, 50 µL APS 10%, 100 µL SDS 10%, 2.52 mL Stacking Gel Buffer (0.5 M Tris-HCl, pH = 6.8), and 10 µL TEMED. Western blot preps (~25 µg protein /well) were separated at 100 V for 2.5 hours (Biorad 165-8004), bathed in run buffer (Biorad 161-0772) and kept on ice. Proteins were transferred onto MeOH activated polyvinylidene difluoride (PVDF) membranes via standard wet transfer methodology in transfer buffer (Biorad 161-0771) at 40 V for 10 hours at 4°C. Membranes were washed 3 x 5 minutes with TBS, 0.05% Tween (TBS-T) and blocked with 5% NFDM/TBST for one hour, with rocking. Primary antibodies to analyze IL-6 signaling (IL-6Rα, (p-)STAT3, (p)-Akt), apoptosis (Cell Signaling Cell Stress and Apoptosis PathScan), autophagy (autophagy-related protein (Atg) 3, 5, Beclin 1, and LC3II/I), and protective mediators (COX-2, iNOS) were diluted 1:1,000 in 5% BSA/TBS-T and applied to membranes overnight at 4°C, with rocking. Anti-rabbit and anti-mouse IgG-horseradish peroxidase-conjugated secondary antibodies diluted in 5% BSA/TBS-T were applied to membranes for 2 hours at room temperature, with rocking. Membranes were washed 3 x 5 minutes in TBS-T before and after each antibody application. For detection, ~500 µL enhanced chemiluminscent reagent (ECL) (Millipore WBLUF0100) was applied to each membrane, sheathed with a plastic coverslip, and imaged in the dark with a ChemiDoc-It$^{®2}$ Imager (UVP, Upland, CA). Inverted images were analyzed using freely available image analysis software (ImageJ, NIH).

Cell Signaling PathScan Array

PathScan slides were assembled in gaskets as described in product manual and blocked for 15 minutes at RT. Ischemic and perfused homogenates, diluted to 1.0 mg*mL^{-1} in Array Diluent Buffer, were added to wells and incubated overnight at 4ºC with rocking. Following 4 x 5 minutes washes with 1X wash buffer (1 mL 20X wash buffer + 19 mL dH_2O), wells were incubated with a detection antibody (150 µL 10X Detection Antibody + 1350 µL Array Diluent Buffer, per slide, made immediately prior to use and kept on ice) for 1 hour at RT with rocking. Wells were then washed 4 x 5 minutes, and incubated with HRP-linked Streptavidin (150 µL 10X Streptavidin HRP + 1.35 mL Array Diluent Buffer, per slide) for 30 minutes at RT with rocking. Slides were exposed with LumiGLO®/Peroxide substrate (9 mL dH_2O + 500 µL 20X LumiGLO + 500 µL 20X Peroxide, made immediately prior to use) and quantified using UVP software, normalized to the total intensity of each sample.

mRNA Extraction

Approximately 20 mg of myocardial, gastrocnemius, soleus, and extensor digitorum longus (EDL) tissue was used for RNA isolation using Ribozol® RNA Extraction Reagent (Amresco N580), as per manufacturer directions. Briefly, tissue was homogenized in 500 µL Ribozol and incubated 5-10 minutes at room temperature (RT). After incubation, 200 µL chloroform was added, samples were shaken vigorously for 15 seconds, and incubated 2-3 minutes at RT. Following incubation, samples were centrifuged at 12,000 x g for 15 minutes at 4ºC. RNA, in the clear top aqueous phase, was transferred to a new sterile tube. RNA was precipitated with the addition of 250 µL Isopropanol, followed by 5-10 seconds vortex. For skeletal muscle samples, 0.5 µL Glycogen (Amresco #N632) was added to increase RNA yield. Following a 10 minute incubation at RT, samples were centrifuged at 12,000 x g for 10 minutes at 4ºC, resulting in a small, white, gel-like

pellet (RNA). The aqueous phase was poured off and the tube tapped dry on a clean kimwipe, making sure not to disrupt the pellet. To the pellet, 500 µL ethanol was added, and the pellet was lifted with gentle rocking. Samples were centrifuged at 7,500 x g for 5 minutes at 4ºC, the ethanol wash was poured off (leaving the RNA pellet in the tube), and samples were allowed to air dry for ~15 minutes or until ethanol was no longer visible. RNA was reconstituted in 40 µL DEPC treated, RNase Free H_2O, and RNA purity and yield was measured with a Nanodrop 2000c (ThermoScientific, Wilmington, DE).

cDNA Synthesis and qPCR

Isolated RNA was converted to cDNA using a Verso cDNA kit (ThermoScientific AB-1453/B) as per manufacturer directions. Briefly, 1 µg of RNA was converted, calculated from Nanodrop output. In 0.6 mL sterile thin walled PCR tubes, 20 µL cDNA synthesis reactions consisting of 4 µL 5x Buffer, 2 µL dNTP Mix, 1 µL RT Enhancer, 1 µL Verso Enzyme Mix, 0.5 µL Anchored Oligo-dT, 0.5 µL Random Hexamer, 1 µg RNA and RNase free H_2O were mixed and exposed to a reverse transcription cycle of 30 minutes at 42ºC (1 cycle). The resulting cDNA (50 ng/µL) was diluted to a final concentration of 5 ng/µL by adding 180 µL RNase free H_2O, and stored at -20ºC until later use.

A reaction solution (15 µL) containing PerfeCTa® SYBR Green Super Mix (Quanta BioSciences 95054), de-ionized H_2O, and forward (F') and reverse (R') primers (listed in **Table 1**), was added to each reaction tube in a PCR plate (Biorad HSP9601). To the reaction mixture, 5 µL (25 ng) total cDNA was added and triturated, avoiding bubbles, and the plate was sealed using microseals (Biorad MSB1001). After seals were places, plates were centrifuged for 1 minute at 100 x g at RT to ensure reactions were free of bubbles and were collected at the bottom of tubes. qPCR was

performed under the following conditions: 95ºC for 2 minutes, followed by 35 cycles of denaturing at 95ºC for 30 seconds, re-annealing at 62ºC for 30 seconds, and extension at 72ºC for 5 minutes.

Appendix II

IACUC Approval Form

APPROVED

ANIMAL SUBJECTS REVIEW FORM

2012-2116

PRINCIPAL INVESTIGATOR: John C. Quindry
RANK/TITLE: Associate Professor
DEPARTMENT: Kinesiology
COLLEGE/SCHOOL: Education
CAMPUS ADDRESS: 2050 Memorial Coliseum CAMPUS PHONE #: 4-1421
E-MAIL: Jcq0001@auburn.edu FAX #: 4-1467

☐ **Check if PI will serve as faculty advisor to the Lead Graduate Student or Resident associated with this activity.**

LEAD GRADUATE STUDENT/RESIDENT: Graham R. McGinnis
RANK/TITLE: Graduate Teaching/Research Assistant
DEPARTMENT: Kinesiology CAMPUS PHONE #: 4-1473
EMAIL: Grm0006@auburn.edu FAX #: 4-1467

CO-INVESTIGATOR: Thomas Denney
RANK/TITLE: Professor
DEPARTMENT: Engineering CAMPUS PHONE #: 4-1862
EMAIL: DENNETS@auburn.edu FAX #:

☒ **Check box if this protocol has more than one co-investigator. Additional co-investigators should be listed on page 2.**

PROJECT TITLE: Exercise and remote cardiac preconditioning by IL-6
STARTING DATE: ~~June 1, 2012~~ June 12, 2012 **EXPIRATION DATE:** ~~May 31 2015~~ June 11, 2015
(Must not be prior to IACUC approval) *(Must not exceed three years)*

Is any part of the funding from a U.S. PUBLIC HEALTH SERVICE AGENCY: YES ☐ NO ☒

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.

X _____ 5/7/12
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.

Dr. Patricia Rynders
Project Veterinarian Name (print or type)

X P. E. Rynders, DVM 5/17/12
Project Veterinarian Signature **Date**

Dr. Patricia Rynders
Unit Veterinarian Name (print or type)

X P. E. Rynders, DVM 5/17/12
Unit Veterinarian Signature **Date**

Dr. Mary Rudisill
Departmental Chairperson Name (print or type)

X Mary Rudisill 5/7/12
Departmental Chairperson Signature **Date**

X _____ 5/8/12
Lead Graduate Student/Resident signature **Date**

X James L. Bannon 6/12/2012
***IACUC Chair Signature** **Date**
*IACUC Chair signs the protocol after IACUC approval has been granted

Received
MAY 08 2012
Research Compliance

JUN 12 2012
Revised

CO-INVESTIGATOR: **Rajesh Amin**
RANK/TITLE: Assistant Professor
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EMAIL:
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CO-INVESTIGATOR:
RANK/TITLE:
DEPARTMENT:
CAMPUS PHONE #:
EMAIL:
FAX #:

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

IMPORTANT: Allow a minimum of 4-6 weeks for protocol approval.

1. Will the animals be used in:

A. Teaching [ ]
Research [x]
Demonstration [ ]
Production [ ]

If Teaching, give the course number(s): _____

B. If Teaching, complete the following chart:

Number of Students in the Class	Number of Students per animal	Number of Animals per Lab	Number of Labs per year

2. A.

Animal Common Name	Total Used[1]	Sex	Approximate Weight	Source[2]	Housing Location[3]
Mouse IL-6 KO (B6;129S2-*Il6*^{tm1Kopf}/J)	**78**	**M**	**30g**	**Jackson Laboratory**	**Biological Research Facility**
Mouse C57BL	**114**	**M**	**30g**	**Jackson Laboratory**	**Biological Research Facility**

[1]The number(s) listed in this column must match the total number of animals described in Question #7.

[2] If reusing animals from another protocol, please provide the protocol number and assurance statement that the animals' well-being has not been compromised by previous research and that the animals exhibit normal physiologic function. Please state how well-being and normal physiologic function was determined for these animals (i.e. physical exam prior to accepting animals for use in protocol).

[3] Please state the housing facility as well as the area (ft^2 or m^2) allocated per animal in cages, stanchions, floor pens, etc. and the reference used to determine the area i.e. *Ag Guide* (2010) or *Guide* (2011).

B. Select pain/distress category relevant to the use of animals in this study.
(*See Item 2B of Additional Information at the end of this form.)*

B [ ] C [ ] D [x] E [ ]

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:

N/A

If Yes, how will the animals be transported to that location, by whom, and was this vehicle inspected and approved by IACUC?

Animal transport is described below

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

Personnel Training Certification (PTC) and Occupational Health & Safety (OHS) Enrollment for all individuals listed in section 4.A and 4.B must be documented in the Office of Research Compliance prior to protocol approval.

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

Biological Research Facility staff

B. List the names of all individuals who will conduct procedures involving animals on this protocol. Any individual not identified by name prior to protocol review will not be approved to conduct procedures. To add personnel after IACUC review and approval of the protocol, a Modification Request Form (adding Personnel to the Protocol) including documentation of OHSP enrollment and PTC Training must be submitted and approved by the IACUC.
(Modification Request Form (adding personnel to protocol): https://fp.auburn.edu/vpr/compliance/animalresources/?Forms)

John Quindry, Graham McGinnis, Christopher Ballmann, Bridget Peters, Ron Beyers

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.

The invasive nature of a study examining ischemia-reperfusion (IR) injury requires the use of an animal model in order to gather the necessary information. Mice have been chosen for this study due to their cardiac/anatomical similarities to humans allowing translation of data. We are particularly interested in determining whether interleukin-6 (IL-6) over expression due to exercise is responsible for the cardioprotection observed in mammals that have experienced a few days of moderate intensity exercise. Within this experimental design, the wild type (C57BL) will serve as the control or reference animal model. The IL-6 knockout (IL-6 KO) mice do not produce IL-6 and will provide a means of comparison for investigating whether

IL-6 is involved in exercise induced cardioprotection against IR injury.

6. STUDY/ACTIVITY JUSTIFICATION AND OBJECTIVES:

A. Justify your animal use in one or two brief paragraphs:

Ischemic heart disease is the leading cause of morbidity and mortality in the US. While heart disease accounts for over 600,000 deaths annually, disease severity is variable. Ischemic insults can be mitigated by various methods of preparing or "preconditioning" the heart to withstand these pathological challenges. Understanding the mechanisms responsible for ischemic heart protection requires invasive experimental protocols that cannot be ethically performed in humans. The current animal use protocol in mice is designed to understand whether the muscle myokine interleukin-6 (IL-6) is responsible for exercise induced remote cardiac preconditioning against ischemic injury.

<u>Background information</u>: Our lab employs several days of moderate intensity treadmill exercise as a means of preconditioning the heart. Many animal based studies in our lab and others over the last decade confirm that exercise is protective against ischemia reperfusion (IR) injury. Acute bouts of exercise, few as 3 days, result in a cardiac phenotype that is largely resistant against IR injury. Within the context of experimental IR, this phenomenon is termed exercise-induced cardioprotection or exercise preconditioning. Our lab and others have undertaken this research line to understand the responsible mechanisms. To date several mechanisms have been identified, though it is clear that additional mechanisms of exercise induced cardioprotection remain undiscovered. Findings from our lab and others indicate that protection against IR injury is due to up-regulation of several protective mediators within the exercised myocardium. Recent research extends upon this understanding and indicates protective mechanisms also originate outside the heart. This alternative form of endogenous protection is termed "remote preconditioning". Our lab recently became the second research group to demonstrate remote preconditioning of the exercised heart against IR injury, demonstrating that the delta opioid receptor is essential to the exercise preconditioning response. Given the multifaceted nature of cardiac preconditioning, other mechanisms of remote preconditioning likely exist. A high potential candidate molecule for remote exercise-induced cardioprotection is interleukin 6 (IL-6). The IL-6 molecule is a member of a large family of glycoproteins. IL-6 is produced from many different tissues including skeletal muscle, which earned it a special classification amongst other cytokines, and is referred to as a *myokine*. Muscle derived IL-6, the form of interest currently, is released into the bloodstream in response to skeletal muscle contractions. Up-regulation of IL-6 and the IL-6 receptor (IL-6R) in response to exercise is established in the literature. The role of IL-6 as a mediator of cardiac preconditioning against ischemic injury in the exercised heart is currently unknown.

B. What are the main objectives of your study:

We will identify whether exercise induced cardioprotection is mediated by IL-6. Key dependent performance and outcome variables will yield 5 goals individually;

1) **Does IL-6 prevent apoptotic tissue death inflicted by IR injury?**
2) **Does IL-6 prevent necrotic tissue death inflicted by IR injury?**
3) **Does IL-6 prevent oxidative stress associated with IR injury?**
4) **Does IL-6 improve ventricular contractile performance during IR injury?**
5) **Does IL-6 prevent ventricular arrhythmias (as measured by ECG tracing)**

inflicted by IR injury?

7. A. 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. Tables may be helpful to show animal numbers. Justification for animal numbers is required to assure that only the necessary number of animals is being used. If applicable, include the technique, location and volume of blood drawn. If applicable, describe method of transportation and/or method of restraint. (*See Item 7 of Additional Information at the end of this form for guidance in providing the appropriate information.)*

Animal model and exercise: For this study, mice expressing the IL-6 chemical of interest (C57BL) will be examined as a point of comparison animal model. Genetically altered mice that do not express IL-6 (IL-6 knockout, IL-6 KO) will also be examined in order to understand the role of IL-6 as a potential chemical mediator of heart attack protection. Animals from both groups will be habituated for 5 days (10 min day 1, 10 min daily time increases resulting in 50 min exercise on day 5) to forced treadmill exercise (in a closed rodent treadmill with an electrified grid at the back of the treadmill) as a means of becoming familiar with exercise. Based on established use of the Columbus Instruments treadmill for exercise training mice, it is known that a typical impedance of a young adult mouse tail is ~500 Kohms which results in a peak grid current of ~0.3 milliamps. In perspective, electrical shock is barely perceptible in the ungloved human hand where impedance is ~900 Kohms (an impedance measure cannot be generated in the gloved hand). Because this knowledge is relative to our chosen commercially available treadmill model, which has been in use for more than a decade, electric shock will not be measured for the current study. Treadmill habituation will be followed by two days of rest. Mice will then complete 3 days, 60 min/day, 30 m/min, at an intensity (30m/min, 0% grade) which is of moderate to high intensity as compared to maximal aerobic capacity. Exercise will be performed on a Columbus Instruments rodent treadmill housed in the assigned second floor room within the Biological Research Facility (BRF) on the Auburn University campus. This exercise workload was chosen as a workload which is proportional to human exercise recommendations for physical activity from the American College of Sports Medicine.

IL-6 injections in sedentary C57BL and IL-6KO mice: A subset of sedentary animals will receive supplemental IL-6 injections as a cardioprotective control group. Subsets of sedentary mice from both C57BL and IL-6 KO animal pools will receive supplemental IL-6 (recombinant murine IL-6, $5x10^6$ units/mg in 200ul physiologic saline with 0.25% human serum albumin), via intraperioneal injection on 3 occasions designed to parallel the IL-6 spike elicited during 3 days of 60 minute treadmill exercise. Supplemental IL-6 injections are designed to mimic the potentially cardioprotective stimulus of IL-6 received during exercise. Animals receiving IL-6 injections will be exposed to both the IR protocol or plasma IL-6 experimental procedures as described below.

Animal transport: Separate subsets of mice will be moved to either the Memorial Coliseum Cardioprotection lab or Magnetic Resonance Imaging (MRI) research facility. 24 hours after the third day of exercise (after the conclusion of the exercise protocol) or sedentary treatments, animals will be transported to the memorial coliseum for non-

survival procedures in re-usable BRF cages contained within breathable cardboard boxes. The purpose of the cardboard box outer container is to prevent non-study and non-BRF individuals from recognizing the box contents. Mice will be delivered to the Cardioprotection Lab (2130 in the Memorial Coliseum) in an approved, climate controlled vehicle.

Animals will be transported to the AU MRI facility (see below) in an identical fashion to that described above.

Ischemia reperfusion injury: Mice will be anesthetized to a surgical plane using an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Once mice reflexes indicate a surgical plane of anesthesia (including paw pinch reflexes), animals will receive a surgically induced ischemia-reperfusion/heart attack surgery. Step one is to obtain measures of the heart rhythm using electrographic tracings. Electrocardiogram (ECG) data will be collected using a physiologic data acquisition system interfaced with a personal computer. ECG signals will be obtained from needle electrodes inserted into the paws. ECG will be evaluated through the entire duration of surgery and analyzed by trained technicians for subsequent identification and evaluation of aberrant heart beats based on clinically relevant understanding (heart muscle pump problems including premature ventricular contraction (PVC), ventricular tachycardia, and ventricular fibrillation).

Next animals will be mechanically ventilated (artificial breathing). An incision will be made in the mouse neck to access the trachea (breathing conduit) and a small piece of tubing (trachea tube) inserted. The trachea tube will be connected to the mechanical ventilator device via flexible tubing. Ventilators will be set to breath rates and pressures which are identical physical to normal physiologic values in conscious rodents.

Prior to the heart attack portion of the experiment, an indwelling catheter will be inserted into the jugular vein (a major neck vein) as a means of delivering additional anesthetic (sodium pentobarbital 10mg/ml) to maintain the surgical plane. Supplemental delivery of anesthetic will be gauged by physiologic data including ECG, blood pressure, and the potential appearance of reflexes which indicate the first signs of emergence from the anesthetic plane. Another indwelling catheter will be placed in the carotid artery (a large high pressure blood vessel of the neck region) in order to measure blood pressure during the experiment. The end of the catheter will be attached to a pressure transducer (pressure sensing device) also attached to a physiologic data acquisition system.

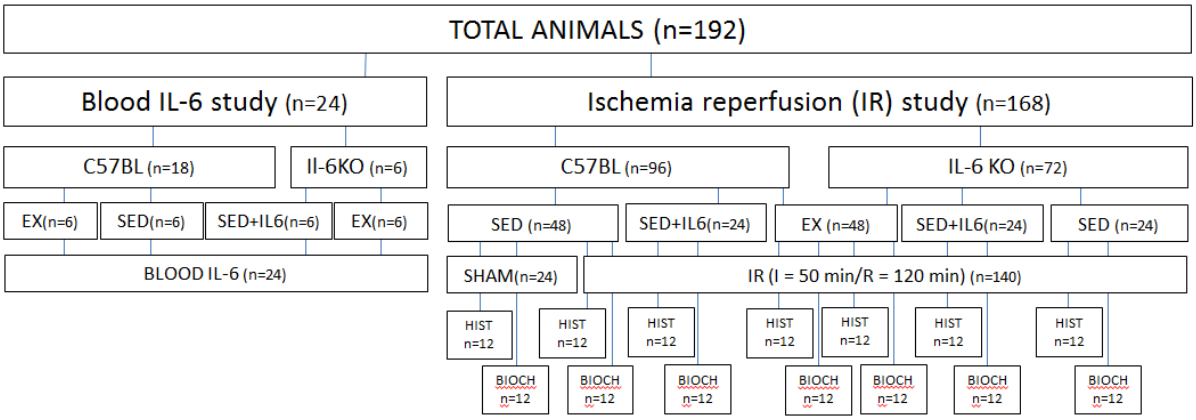
Heart attack will be performed by reversibly ligating (tying off of) a major coronary artery. The ligature is a surgical suture passed into the heart muscle and secured to the heart with a small piece of flexible tubing. The collective apparatus provides enough pressure to prevent blood flow to the downstream heart muscle. The blood flow to the heart muscle served by the coronary artery will be restricted for 50 minutes, a time we have determined in previous studies is adequate for production of heart tissue injury. The ligature apparatus will be loosened and blood flow restored for 120 minutes, which is the amount of time necessary to visualize cardiac death using post procedure chemical analyses. After the experimental procedure, the ligature will be re-affixed and ~1 ml of evan's blue dye contrast dye will be injected into the arterial circulation in the moments prior to cardiac excision. Dye infusion will permit differentiation between the tissue that receives blood flow and the ischemic tissue upon cardiac excision. Mice will be euthanized by heart excision while under a surgical plane of anesthesia. Cardiac and skeletal muscle will be saved for analysis of biomarkers related to cardioprotection. Plasma will be collected from the arterial catheter before and after the heart attack procedure will be saved for analysis of inflammatory biomarkers, myokines (IL-6), and

metabolic chemical profiles.

<u>Plasma IL-6 experiments</u>: A subset of animals will be sacrificed 20 minutes after completing either exercise (as described above) or sedentary cage treatments. Animals will be anesthetized with isoflurane (closed system with passive charcoal filtration) and euthanized via cardiac excision. Prior to cardiac excisions, 1 ml of blood will be sampled via cardiac puncture for the purpose of subsequent IL-6 myokine analysis. Because of time sensitive nature of these experiments (exercise-induced spikes in circulating IL-6 disappear within minutes to hours of exercise cessation), animals will be sacrificed in the BRF assigned procedure room on the first floor.

The figure below displays the study design for both Blood IL-6 and IR studies. Note that the eight treatments for the IR study are as follows:

Sham (C57BL) – surgery no IR, "sedentary" no exercise
Sed (C57BL) – IR, "sedentary" no exercise
Sed+IL-6 (C57BL) – IR, "sedentary no exercise, receives supplemental IL-6 injections
EX (C57BL) – IR, exercised
EX (IL-6 KO) – IR, exercised
Sed+IL-6 (IL-6 KO) – IR, "sedentary" no exercise, receives supplemental IL-6 Injections
Sed (IL-6 KO) – IR, "sedentary" no exercise



The requested animal numbers are based on previous investigations of exercise induced cardioprotection. As such, we generally need 8-10 animals/group to observe statistically different mean differences for key dependent outcome measures (% infarct, ECG arrhythmia scores, etc.) between Sham, Sed (C57BL), and Ex (C57BL). The requested animal numbers include a) an anticipated 15%-20% attrition rate due to complications during the IR surgery, and b) that several animals from many if not all of the IR groups will be investigated to generate preliminary data using cardiac MRI (described below). "Hist" denotes animals from which hearts will be used to gather histological variables. "Bioch" denotes animals from which hearts will be used to gather biochemical/molecular biology variables.

<u>Cardiac MRI</u>: A subset of mice (n= 2-3 for Sham, Sed (C57BL), Ex (C57BL), Ex (IL-6KO)) will be used to study cardiac function via MRI. These procedures will be undertaken at the Auburn University MRI Research facility in the AU Research Park. Mice will be transported to the MRI facility in a climate controlled vehicle. Animals will be covered from view as described above. Equipment needed to perform these experiments will be moved from the Cardioprotection Lab to the AU MRI animal

surgery suite. Mice will be anesthetized sodium pentobarbital (50 mg/kg) during the procedure and baseline measures taken before any surgical procedures. The IR procedure will be identical to that described previously (including the use of sodium pentobarbital as the anesthetic) save, for the fact that animals will be weaned from the ventilator following the ischemic period in order to obtain post IR cardiac function measures using MRI. To do this, a 'purse string' suture will be used to close the thoracotomy incision and lungs will be re-inflated by briefly providing back pressure on the small animal mechanical ventilator. Stated differently, simultaneous to closing of the thoracotomy, air will be prevented from escaping the ventilator for one ventilated breath thereby re-inflating the lungs and in the instant before the chest is closed. The PI traveled to Case Western Reserve University to learn this technique and successfully performed the procedure with animals surviving 6-8 weeks following. The PI will confirm this technique in the current study and report back to the IACUC after 5 IR experiments (on 5 animals, 1 experiment = 1 animal) have been performed where thoracotomies have been sutured closed and the lungs re-inflated. In the event that the PI is unsuccessful in performing this technique it will be discontinued, and under the direction of university veterinarians, alternative approaches will be proposed through subsequent IACUC amendments prior to any additional MRI experiments. As with the other IR experiments, mouse reflexes will be checked every few minutes and supplemental sodium pentobarbital delivered via jugular vein (sodium pentobarbital 10mg/ml). Once cardiac images are obtained, anesthetized mice will be euthanized by cardiac excision.

B. For experiments regarding food and/or fluid restriction:

1.) Describe animal health monitoring procedures and frequency (e.g. body weight, blood urea nitrogen, urine/fecal output, food/fluid consumed):

Not applicable

2.) Describe methods of ensuring adequate nutrition and hydration during the regulated period:

N/A

C. If this research involves production of genetically modified animals or is a pilot study and has the potential to result in unexpected outcomes, please address the following:

1.) New phenotypes or other unanticipated results which may affect animal health and well-being.

Not applicable

2.) Method for monitoring and managing unexpected outcomes to assure animal health and well-being.

N/A

3.) Procedure for reporting unexpected outcomes to the IACUC.

N/A

D. If exterior windows are present within the animal housing or procedure areas, describe how this may affect temperature and photoperiod control, as well as potential security risks.

Animals will be housed on a reverse dark (12 hour):light (12 hour) cycle. Animals will be housed briefly (a few hours max) in a dark room within the Cardioprotection Lab. Animals will be brought into the lighted lab facilities to be weighted and to receive

sodium pentobarbital in preparation for the IR surgery. The total time that an animal will remain conscious in the lighted room is expected to be less than 15-20 minutes maximum.

E. If this is a field study involving observation or use of a non-domesticated vertebrate species, please respond to the following:

1.) What is the potential impact on the wild population of the species to be studied?

Not applicable

2.) How might the study compromise health of either animals or persons e.g. zoonoses?

N/A

3.) Describe the final disposition of the animals being studied (i.e. return to wild population, preserve in museum collection, etc).:

N/A

F. Humane endpoints:

1.) If pain/distress category D/E or food/fluid restriction is applicable to this protocol, please define humane endpoints:

<u>Adverse effects during IR procedures</u>: Use of sodium pentobarbital and isoflurane will be used to prevent animal pain or distress during invasive procedures and MRI analysis. Because of the invasive nature of the proposed study, unanticipated outcomes during the IR surgeries or MRI analysis which eliminate a mouse for consideration use in the proposed study will necessitate the need to euthanize the animal immediately. Anesthetized mice will be euthanized following all of these procedures by cardiac excision. Concerns about animal conditions in the event of unanticipated outcomes will be reported to both animal care personnel and the project veterinarian.

<u>Secondary effects of IL-6 injections:</u> While previous work with IL-6 suggest that complications due to IL-6 use are unlikely, the PI and research team will carefully observe mice for signs of secondary effects due to IL-6 injections. Mice will be observed multiple times daily for unusual behavior and appearance which may suggest the animal is experiencing unanticipated effects of IL-6 injections. BRF animal care staff will be informed immediately of any animals suspected of experiencing IL-6 secondary effects. Mice that are believed to be experiencing untoward IL-6 secondary effects will be euthanized by CO_2 gas inhalation within the assigned first floor procedure room within the BRF.

<u>Treadmill exercise</u>: Due to the use of an electrified grid on the back of the motorized treadmill, there is a chance that animals will briefly experience pain or distress. To alleviate pain and distress we use a Columbus Instruments rodent treadmill which delivers a mild electrical shock. Because of the standardized use of this device in mice and rats for more than a decade, and adequate understanding of the amount of shock received by mice, shock will not be measured during this study. We have used this technique for more than 10 years, we use the mild electric shock sparingly to motivate rodents to run during the habituation period. The duration of the shock is dependent on the length of time the mouse remains on the electrical grid at the rear of the treadmill. In virtually all instances of mild electrical shock, less than one second

passes before running resumes. Importantly, the maximal current applied across the electrical grid of the treadmill in these experiments is based on the impedance of the animals' tail. A typical impedance of a young adult mouse tail is ~500 Kohms which results in a peak grid current of ~0.3 milliamps. In perspective, electrical shock is barely perceptible in the ungloved human hand where impedance is ~900 Kohms (note – that an impedance measure cannot be generated in the gloved hand). Once the animals resume running they are not subject to the mild electrical stimulus. In the event that animals are unable to avoid electrical shock for more than 3-5 seconds (e.g., undue fatigue, broken toenail, etc.), exercise will be discontinued. Based on previous experience fewer than 1:20 animals need to be removed from study for reasons almost exclusively limited to a broken toenail or repeated unwillingness to run on three consecutive days during the habituation period. Broken toenails sometimes prove to be an impediment to treadmill exercise because of discomfort associated with the "toe-off" of the normal mouse gait. In our experience, however, rats experience broken toenails more frequently than mice, suggesting that far fewer than 1:20 animals are likely to experience a broken toe nail in the proposed protocol. Animals deemed unsuitable for exercise training will be euthanized by CO_2 gas inhalation within the assigned first floor procedure room within the BRF.

2.) If research is novel and little or no information is available in the literature, state who you have collaborated with to define humane endpoints; please define humane endpoints resulting from your collaboration; state how you will periodically communicate with the IACUC to ensure the well-being of the animal(s) on this protocol:

Not applicable. The proposed procedures are well established in the PI's lab in addition to numerous labs worldwide.

3.) For category C and all other protocols where the potential exists for weight loss and/or other parameters that could be potentially harmful to the animals, state the humane end points:

G. Environmental Enrichment: *(See Bloomsmith et al. Lab Anim. Sci. 41:372-377); also the Ag Guide (2010) has a good discussion on this topic by species in Chapter 4*.

1.) Social enrichment: Please describe direct or indirect animal contact (visual, olfactory, auditory) with conspecifics or humans.

Mice will be housed 4-5/cage as a means of social enrichment.

2.) Occupational enrichment: Please describe any devices that provide animals with control or challenges (psychological enrichment); enrichment that encourages exercise.

We will use nestlets as a means of occupational enrichment for the mice. Nestlets provide an outlet for the species specific behavior of mice.

3.) Physical enrichment: Please describe alteration of the size or complexity of the animal's enclosure which may include objects, substrate or permanent structures (e.g. nestboxes, rocks and hiding places in an aquatic environment).

We will use nestlets as a means of physical enrichment for the mice. Nestlets provide mice with a physical task of nesting within the cage environment.

4.) Sensory enrichment: Please describe visual stimuli (television); auditory stimuli (music, vocalizations); olfactory, tactile, taste stimuli.

Within the cages we will use nylabones to enrich the sensory environment of the mice.

5.) Nutritional enrichment: Please describe presentation of varied or novel food types; changing the method of food delivery.

N/A

6.) Other types of enrichment: Please describe any other types of enrichment that do not fit the categories above.

Not applicable

7.) No Enrichment: Please justify the decision to provide no environmental enrichment if you have not responded to #7. G. 1-6 above.

N/A

8. If category D or E was chosen in Question 2B, please complete the following. (*See Item 8A of Additional Information at the end of this form.)*

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

Database Searched	X	Date of Search	Years Covered
Medline	X	**4/30/2012**	**1950-2012**
Agricola			
CABA			
Altweb			
Other (PubMed)	X	**4/30/2012**	**1950-2012**

B. Scientifically relevant terminology (e.g. keywords) and search strategy used when considering alternatives to the painful or distressful procedure(s):

Keywords: ischemia-reperfusion injury, exercise, cardioprotection, preconditioning, mice, IL-Strategy: started with a broad search on the general topic, ischemia reperfusion injury, and specified further to include other keywords like cardioprotection, IL-6, exercise, preconditioning and specifically in a rodent model using mice.

C. 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 minimum number of animals will be sacrificed in this study to observe statistically significant differences between the sham, sedentary, exercise, and IL-6 KO groups. Genetic knockout will be used to ensure the validity of the study as compared to inhibitor based research.

Replacement:

Due to the invasive nature of this experimental protocol and the aims to be accomplished, a mammalian model of the lowest order needs to be used to a) observe the

contribution of the intact and entire animal physiology, and b) translate the findings to the human heart. Though cellular models exist, the inability to accurately administer formal exercise in addition to the removal of extracellular and remote factors make those lower order models inappropriate for the aims of this study.

Refinement:

Over the past decade, the PI has refined the experimental procedure employed in this study design (left anterior descending coronary artery ligation) as well as the measurement techniques to identify ischemic and perfused myocardial tissue accurately. The volume of experience in this study design ensures the minimal number of animals used for the study.

D. If alternatives are available but will not be used, provide a justification.

N/A

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

N/A

9. SURGERY:

Will surgery be performed?

Yes [x] No [ ]

If yes, please address the following, as applicable:

A. Non-survival 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).

3 non-survival procedures will be performed: 1) animal sacrifice for the Blood IL-6 sub-study, 2) IR experiments for the main experiment, and 3) sub-study IR experiments performed between cardiac MRI procedures. The procedures are replicated here from previous sections of the protocol.

1) **<u>Animal sacrifice for the Blood IL-6 sub-study</u>: A subset of animals will be sacrificed 20 minutes after completing either exercise (as described above) or sedentary cage treatments. Animals will be anesthetized with isoflurane (closed system with passive charcoal filtration) and euthanized via cardiac excision. Prior to cardiac excisions, 1 ml of blood will be sampled via cardiac puncture for the purpose of subsequent IL-6 myokine analysis. Because of time sensitive nature of these experiments, animals will be sacrificed in the BRF. Procedures will be performed by Dr. Quindry, Graham McGinnis, Christopher Ballmann, and Bridget Peters.**
2) **<u>IR experiments</u>: IR experiments will be performed in both the Cardioproteection Lab (2134) Memorial Coliseum and the dedicated surgical facility of the AU MRI Research Facility. All IR procedures will be performed by Dr. Quindry and Graham McGinnis. Mice will be anesthetized to a surgical plane using an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Once the**

assessment of reflexes indicates a surgical plane of anesthesia (including paw pinch reflexes), animals will receive a surgically induced ischemia-reperfusion/heart attack surgery. Step one is to obtain measures of the heart rhythm using electrographic tracings. Electrocardiogram (ECG) data will be collected using a physiologic data acquisition system interfaced with a personal computer. ECG signals will be obtained from needle electrodes inserted into the paws. ECG will be evaluated through the entire duration of surgery and analyzed by trained technicians for subsequent identification and evaluation of aberrant heart beats based on clinically relevant understanding (heart muscle pump problems including premature ventricular contraction (PVC), ventricular tachycardia, and ventricular fibrillation). Detection of ECG arrhythmias is used to confirm successful ligation of the coronary artery and will be used as a key dependent variable/outcome for the study.

Next animals will be mechanically ventilated (artificial breathing). An incision will be made in the mouse neck to access the trachea (breathing conduit) and a small piece of tubing (trachea tube) inserted. The trachea tube will be connected to the mechanical ventilator device via flexible tubing. Ventilator settings will be set to breath rates and pressures which are identical physical to normal physiologic values in conscious rodents.

Prior to the heart attack portion of the experiment, an indwelling catheter will be inserted into the jugular vein (a major neck vein) as a means of delivering additional anesthetic (sodium pentobarbital 10mg/ml) to maintain the surgical plane. Supplemental delivery of anesthetic will be gauged by physiologic data including ECG, blood pressure, and the potential appearance of reflexes which indicate the first signs of emergence from the anesthetic plane. Another indwelling catheter will be placed in the carotid artery (a large high pressure blood vessel of the neck region) in order to measure blood pressure during the experiment. The end of the catheter will be attached to a pressure transducer (pressure sensing device) also attached to a physiologic data acquisition system.

Heart attack will be performed by reversibly ligating (tying off of) a major coronary artery. The ligature is a surgical suture passed into the heart muscle and secured to the heart with a small piece of flexible tubing. The collective apparatus provides enough pressure to prevent blood flow to the downstream heart muscle. The blood flow to the heart muscle served by the coronary artery will be restricted for 50 minutes, a time we have determined in previous studies is adequate for production of heart tissue injury. The ligature apparatus will be loosened and blood flow restored for 120 minutes, which is the amount of time necessary to visualize cardiac death using post procedure chemical analyses. After the experimental procedure, the ligature will be re-affixed and ~1 ml of evan's blue dye contrast dye will be injected into the arterial circulation in the moments prior to cardiac excision. Dye infusion will permit differentiation between the tissue that receives blood flow and the ischemic tissue upon cardiac excision. Mice will be euthanized by heart excision. Cardiac and skeletal muscle will be saved for analysis of biomarkers related to cardioprotection. Plasma will be collected from the arterial catheter before and after the heart attack procedure will be saved for analysis of inflammatory biomarkers, myokines (IL-6), and metabolic chemical profiles.

3) **<u>IR and cardiac MRI procedures</u>: Mice will be anesthetized sodium pentobarbital (50 mg/kg) during the procedure and baseline measures taken before any**

surgical procedures. The IR procedure will be identical to that described previously (including the use of sodium pentobarbital as the anesthetic) save, for the fact that animals will be weaned from the ventilator following the ischemic period in order to obtain post IR cardiac function measures using MRI. To do this, a 'purse string' suture will be used to close the thoracotomy incision and lungs will be re-inflated by briefly providing back pressure on the small animal mechanical ventilator. Stated differently, simultaneous to closing of the thoracotomy, air will be prevented from escaping the ventilator for one ventilated breath thereby re-inflating the lungs and in the instant before the chest is closed. The PI traveled to Case Western Reserve University to learn this technique and successfully performed the procedure with animals surviving 6-8 weeks following. As with the other IR experiments, mouse reflexes will be checked every few minutes and supplemental sodium pentobarbital delivered via jugular vein (sodium pentobarbital 10mg/ml). Once cardiac images are obtained, anesthetized mice will be euthanized by cardiac excision.

B. Survival surgery - Describe all surgical procedures, including SURGICAL PREPARATION. Indicate that aseptic technique will be followed if the procedure is a survival surgical procedure. Indicate WHERE surgery will be performed (building and rooms). Identify the person(s) and describe their qualifications for performing the particular surgical procedure(s).

Survival surgeries will not be performed.

C. Post-surgical Care - Describe POST-SURGICAL CARE including, who will be providing it (qualifications), what it will consist of, and where it will be provided (bldg., rooms).

Not applicable

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.)

Sodium pentobarbital will be administered via intraperitoneal injection (50 mg/kg) to induce a surgical plane of anesthesia for the IR main study and the IR-MRI sub-study. Supplemental sodium pentobarbital will be delivered via intravenous injection (10 mg/ml) as needed to maintain the surgical plane of anesthesia for both the IR main study and the IR-MRI sub-study.

Isoflurane will be administered to anesthetize mice for the blood IL-6 sub-study. An initial rate of 5% will be used to anesthetize mice, with supplemental doses of 2%-3% down to 1% used to maintain a surgical plane of anesthesia up to the point of cardiac excision. Isoflurane will be scavenged using an inline passive charcoal filtration system. The charcoal filters will be weighed before and after procedures as a means of documenting accumulation of isoflurane and discarded once the canister weight exceeds 50g of additional weight over the initial weight.

11. A. 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.)

Sub-sets of animals will receive supplemental IL-6 injections (recombinant murine IL-6, $5x10^6$ units/mg in 200ul physiologic saline with 0.25% human serum albumin) via the

intraperitoneal cavity.

If using cells, what is the source? Provide proper documentation to show that they are free from any infectious animal or human pathogens?

Not applicable

B. If a non-pharmaceutical grade compound or chemical is being used, the following criteria must be addressed:

1.) Provide a rationale for using less than pharmaceutical grade compounds. Cost savings alone do not adequately justify the use of non-pharmaceutical grade compounds in animals.

Non-pharmaceutical grade recombinant murine IL-6 will be administered as a means of spiking IL-6 levels for potential cardioprotection against IR injury. The use of this IL-6 is two-fold: 1) because pharmaceutical grade IL-6 is unavailable, and 2) because scientific continuity to previously published work within this research area is essential for successful completion of the proposed studies.

2.) Describe any expected side effects:

Given the fact that this dose and delivery of IL-6 has been well tolerated in previous research investigations, side effects are not expected. Animals receiving IL-6 injections will be observed daily in order to quickly identify any potential side effects. Indices of grooming, activity, and healthy appearance will be noted. Animal weights will be recorded throughout as an additional measure to assure wellbeing and to deliver a consistent weight dependent dose of IL-6. Mice will be weighed in a rodent cage placed on a scale. Mice found to be losing more than 10% of their body weight on 3 consecutive days will be noted to BRF animal care staff and euthanized by CO2 as deemed necessary by BRF animal care staff. Concerns about animal conditions in the event of unanticipated outcomes will be reported to both animal care personnel and the project veterinarian.

3.) Discuss the methods to be used to ensure sterility and storage of the drugs (e.g., sterile 0.22 micron filters, sterile diluent, storage in sterile vials, etc.):

Sterile saline and clean laboratory practices (clean sterile needles, swabbing bottle tops, etc.) will be used when preparing IL-6 injections (22µm filtered).

12. ASSURANCES:

A. Provide a brief statement to confirm that proposed activities involving animals do not duplicate previous experiments unnecessarily. If your protocol is a continuation of a previously approved project, include the PRN for the previous protocol and provide a brief statement summarizing previous work to justify study continuation.

The proposed animal use protocol has never been undertaken as evidenced by a literature search described above.

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.

Not applicable

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 including expiration dates and attach copies of the permits to the protocol. Copies of active collection permits must be provided prior to protocol approval.

N/A

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. Copies of an approval letter from the IBC along with the approved BUA must be provided prior to protocol approval.

Hazardous Agent	Yes	No	Agent	Date of Committee Approval & BUA #
Radioisotopes		x		
Biological Agents		x		
Hazardous Chemicals or Drugs		x		
Recombinant DNA		x		
Physical Agent (UV, Laser, Noise, Magnetic fields, etc.)		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 hazardous waste and, if applicable, the monitoring of hazardous waste.

Not applicable

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?

Animal carcasses will be returned to the BRF for incineration.

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 Guidelines on Euthanasia 2007 or justification for deviation should be indicated.
This document is available on the Office of Research Compliance website:
http://www.auburn.edu/research/vpr/animals/resources.htm

All animals will be euthanized by cardiac excision while under a surgical plane of

anesthesia. Cardiac excision is necessary for these experiments because many of the dependent variables to be examined are labile. Thus, cardiac tissues must be obtained in a physiologic state and frozen immediately. Alternative techniques including CO_2 euthanasia will not be performed for the main study because of the need for heart tissue to be physiologically viable at the time of freezing. The PI has published data demonstrating that key dependent biochemical and histological variables of IR injury, are labile to physiologic stressors such as hypoxia, and would be confounded by CO_2 euthanasia.

In contrast to animals used for the research study, mice that are deemed unsuitable for study (exercise intolerance or other complications) will be euthanized by CO_2 narcosis in accordance with the rules established by the 2007 American Veterinary Medical Association (AVMA) Guidelines on Euthanasia. The animals will be placed into the CO_2 chamber, followed by the addition of CO_2 gas from a compressed gas cylinder at a low flow rate. The chamber will not be pre-filled. The CO_2 flow rate will allow displacement of approximately 20% of the chamber volume per minute. Animals will be euthanized in this manner singly (not in groups of two or more). To ensure irreversibility of the euthanasia process, cervical dislocation will be performed following CO_2 euthanization. Dr. Quindry, Graham McGinnis, Christopher Ballmann, and Bridget are adequately trained to carry out this procedure. Following necropsy (for unexpected deaths), the carsasses will be incinerated at the incinerator located adjacent to the College of Veterinary Medicine and operated by Risk Safety Management.

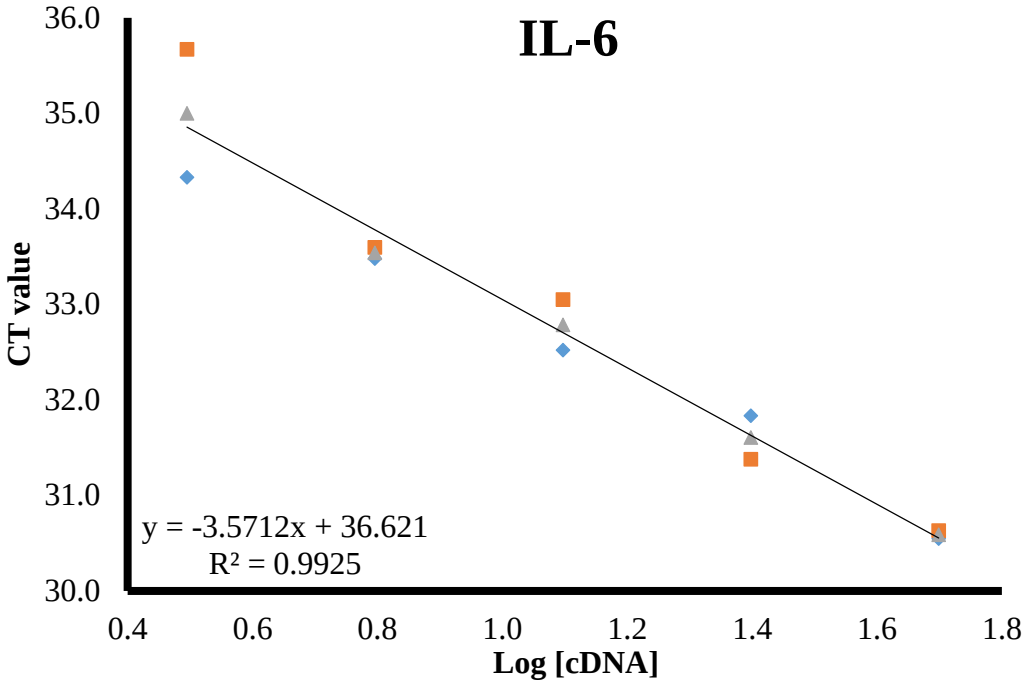
Appendix III

Arrhythmia Scoring Table

Scoring System A	ECG Criteria
0	0-49 PVC's
1	50-499 PVC's
2	>499 PVC's and/or 1 episode SVT VF
3	>1 episode VT or VF or both (<60 s combined)
4	VT or VF or both (60-119 s total combined duration)
5	VT or VF of both (>119 s total combined duration)
6	Fatal VF starting at >15 min after occlusion
7	Fatal VF starting between 4:00 and 14:59 after occlusion
8	Fatal VF starting between 1:00 and 3:59 after occlusion
9	Fatal VF starting <1 min after occlusion

Appendix IV

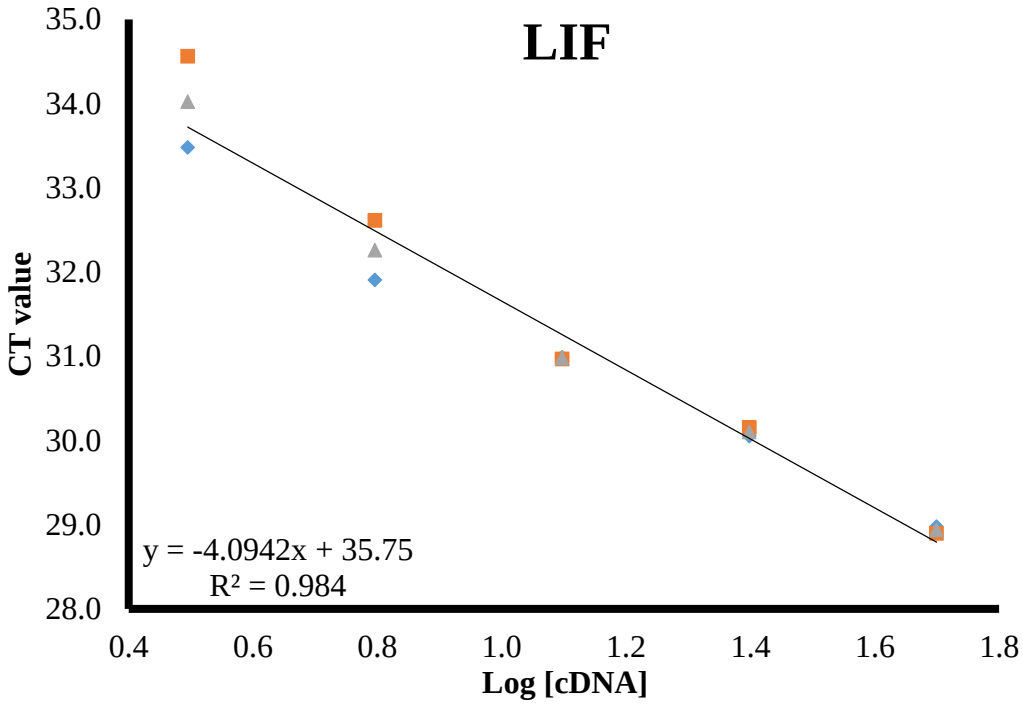
Primer Efficiency Analysis



[cDNA]	Log[cDNA]	Ct value1	Ct value 2	Avg
50	1.69897	30.55	30.63	30.589463
25	1.39794	31.83	31.38	31.606757
12.5	1.09691	32.52	33.05	32.784746
6.25	0.79588	33.48	33.60	33.537419
~~3.125~~	~~0.49485~~	~~34.33~~	~~35.67~~	~~34.999339~~
Blank	0	N/A	N/A	N/A

Efficiency = (10^(-1/slope)-1)*100

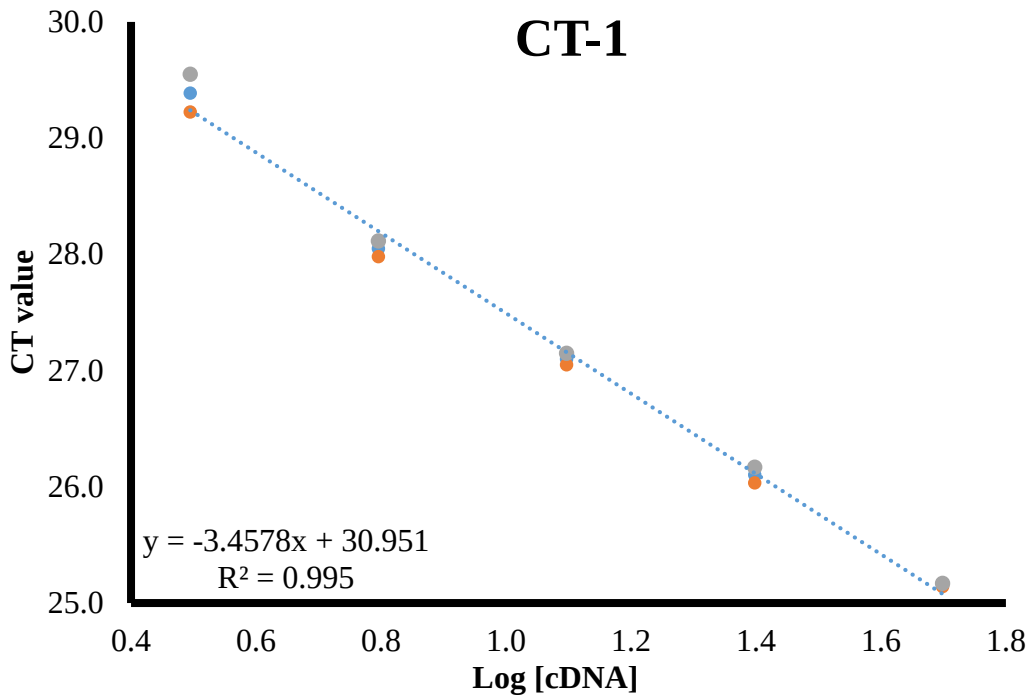
Slope	-3.3291879
Efficiency	99.697925



[cDNA]	Log[cDNA]	Ct value1	Ct value 2	Avg
50	1.69897	28.98	28.90	28.93646
25	1.39794	30.05	30.15	30.10183
12.5	1.09691	30.99	30.97	30.97667
6.25	0.79588	31.90	32.61	32.25883
~~3.125~~	~~0.49485~~	~~33.48~~	~~34.56~~	~~34.02036~~
Blank	0	N/A	N/A	N/A

Efficiency = (10^(-1/slope)-1)*100

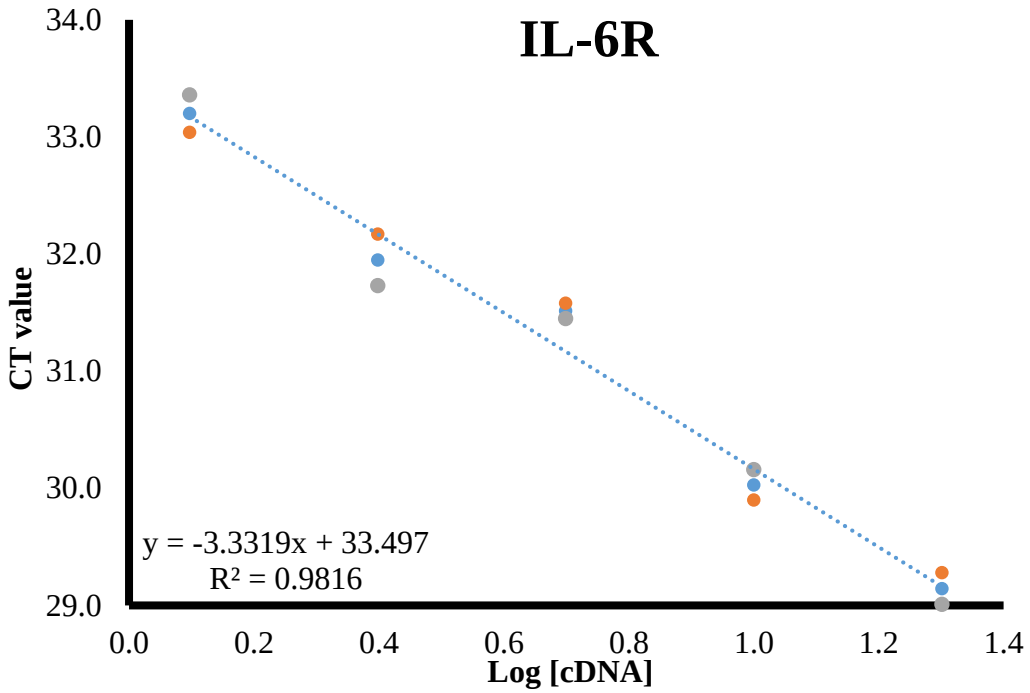
Slope	-3.60161
Efficiency	89.51923



[cDNA]	Log[cDNA]	Ct value1	Ct value 2	Avg
50	1.69897	25.14	25.17	25.15493
25	1.39794	26.03	26.17	26.10162
12.5	1.09691	27.05	27.15	27.09904
6.25	0.79588	27.98	28.12	28.04772
3.125	0.49485	29.22	29.55	29.38643
Blank	0	N/A	N/A	N/A

Efficiency = (10^(-1/slope)-1)*100

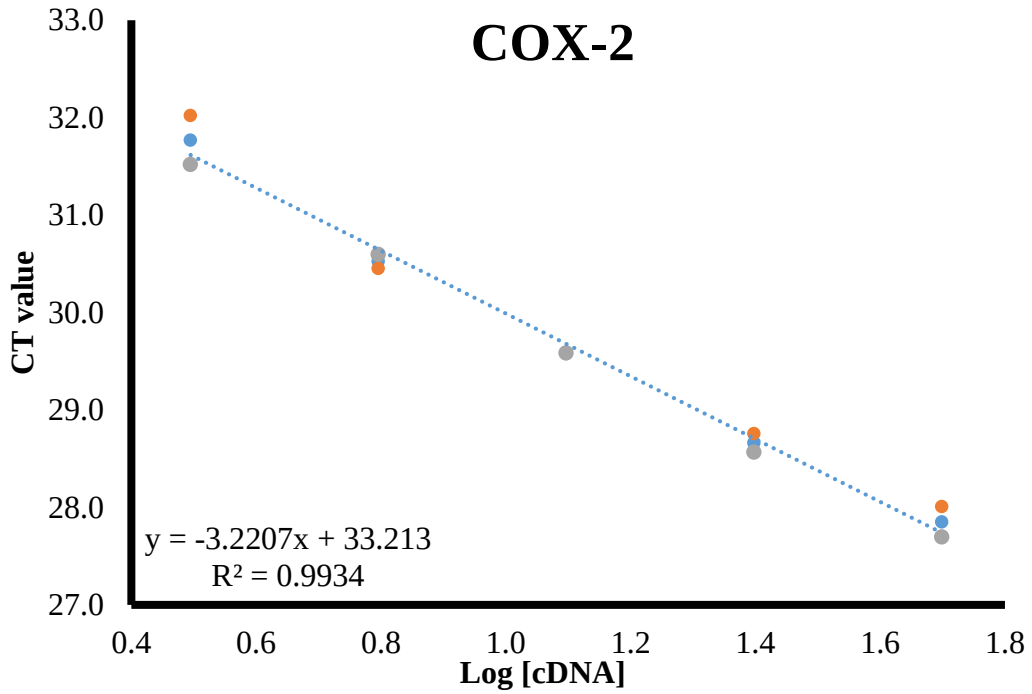
Slope	-3.45783
Efficiency	94.62504



[cDNA]	Log[cDNA]	Ct value1	Ct value 2	Avg
20	1.30103	29.28	29.01	29.145
10	1	29.9	30.16	30.03
5	0.69897	31.58	31.45	31.515
2.5	0.39794	32.17	31.73	31.95
1.25	0.09691	33.04	33.36	33.2
Blank	0	N/A	N/A	N/A

Efficiency = (10^(-1/slope)-1)*100

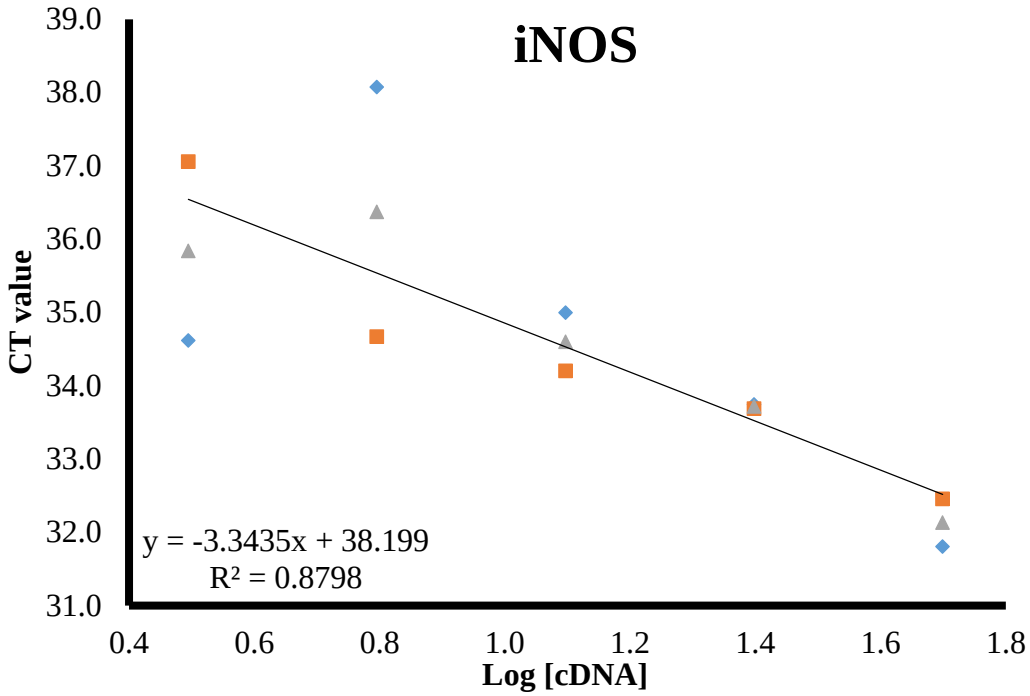
Slope	-3.3319
Efficiency	99.585532



[cDNA]	Log[cDNA]	Ct value1	Ct value 2	Avg
50	1.69897	28.01	27.70	27.85
25	1.39794	28.76	28.57	28.67
12.5	1.09691	29.58	29.59	29.58
6.25	0.79588	30.45	30.60	30.53
3.125	0.49485	32.02	31.52	31.77
Blank	0	N/A	N/A	N/A

Efficiency = (10^(-1/slope)-1)*100

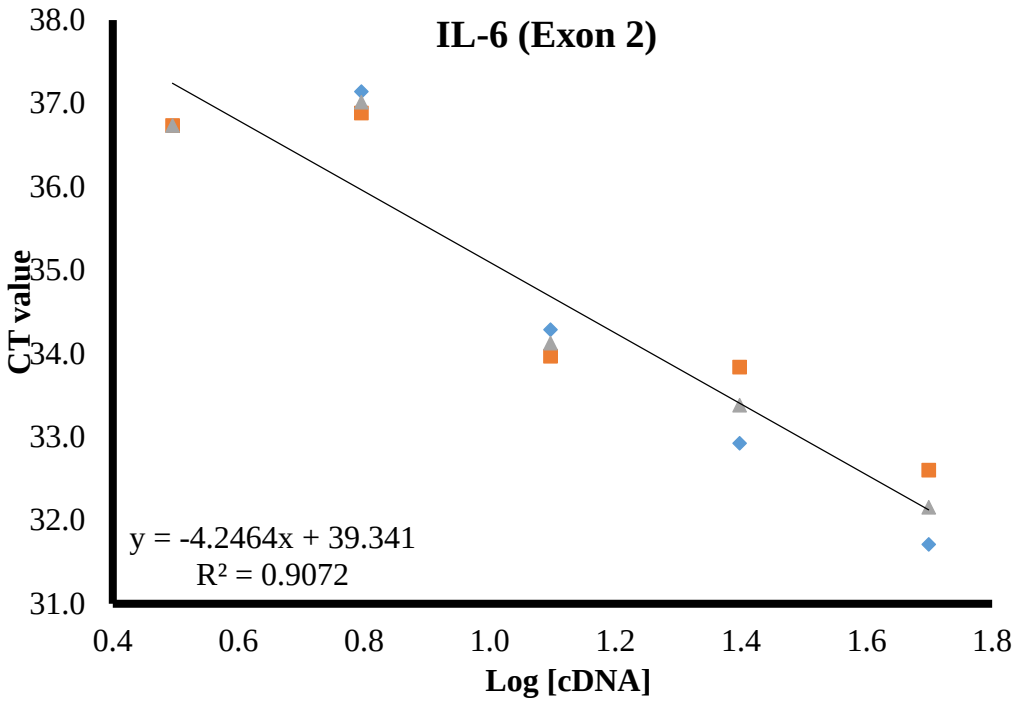
Slope	-3.22067
Efficiency	104.4062



[cDNA]	Log[cDNA]	Ct value1	Ct value 2	Avg
50	1.69897	31.81	32.46	32.1324
25	1.39794	33.75	33.69	33.71734
12.5	1.09691	35.00	34.20	34.59916
6.25	0.79588	38.07	34.67	36.37122
3.125	0.49485	34.62	37.06	35.83789
Blank	0	N/A	37.92	N/A

Efficiency = (10^(-1/slope)-1)*100

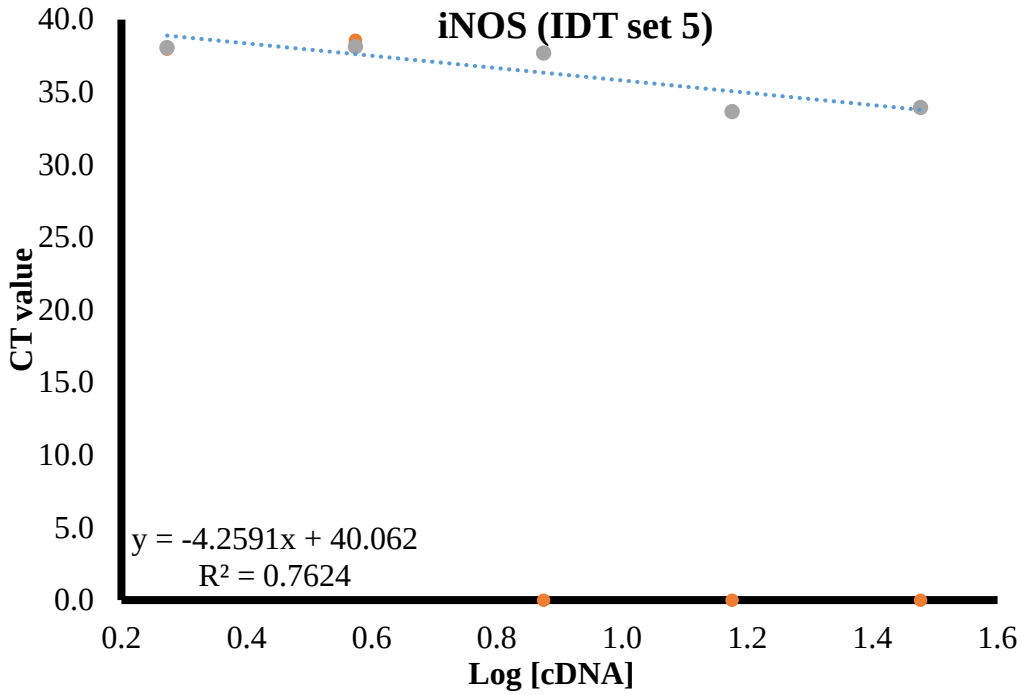
Slope	-3.34347
Efficiency	99.10861



[cDNA]	Log[cDNA]	Ct value1	Ct value 2	Avg
50	1.69897	31.71	32.60	32.15738
25	1.39794	32.93	33.84	33.38239
12.5	1.09691	34.29	33.97	34.128
6.25	0.79588	37.14	36.89	37.01293
3.125	0.49485		36.73	36.7336
BLANK	0	N/A	N/A	N/A

Efficiency = (10^(-1/slope)-1)*100

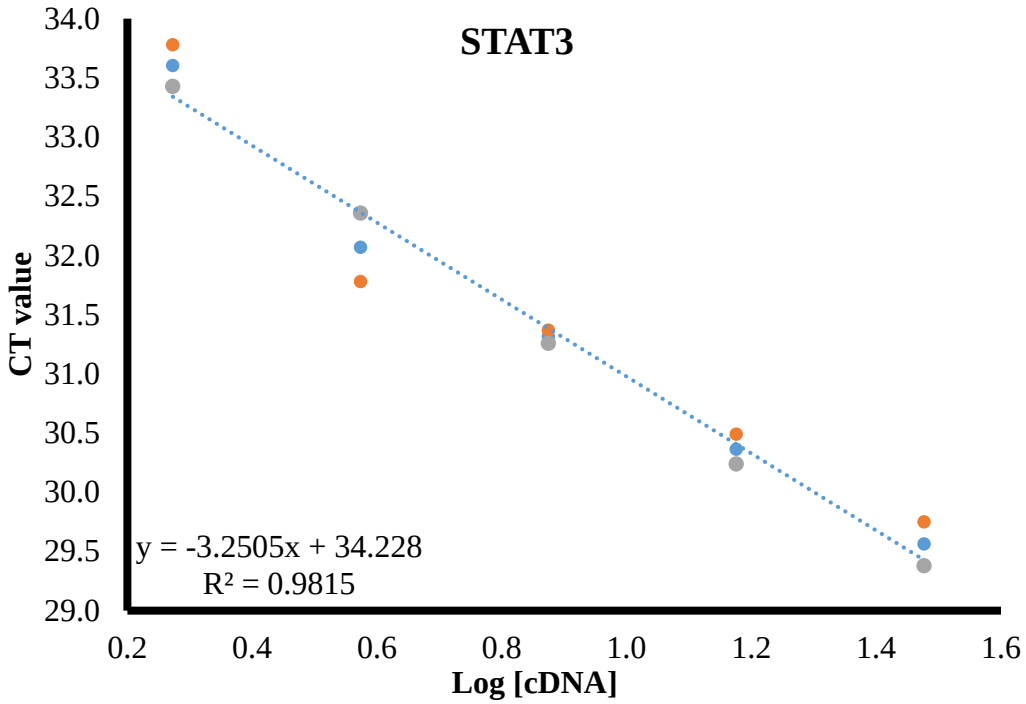
Slope	-4.24641
Efficiency	71.98588



[cDNA]	Log[cDNA]	Ct value1	Ct value 2	Avg
30	1.477121	33.9463875	N/A	33.94639
15	1.176091	33.6672282	N/A	33.66723
7.5	0.875061	37.6913942	N/A	37.69139
3.75	0.574031	38.1298947	38.58135	38.35562
1.875	0.273001	38.0632987	37.96238	38.01284
Blank	0	N/A	N/A	N/A

Efficiency = (10^(-1/slope)-1)*100

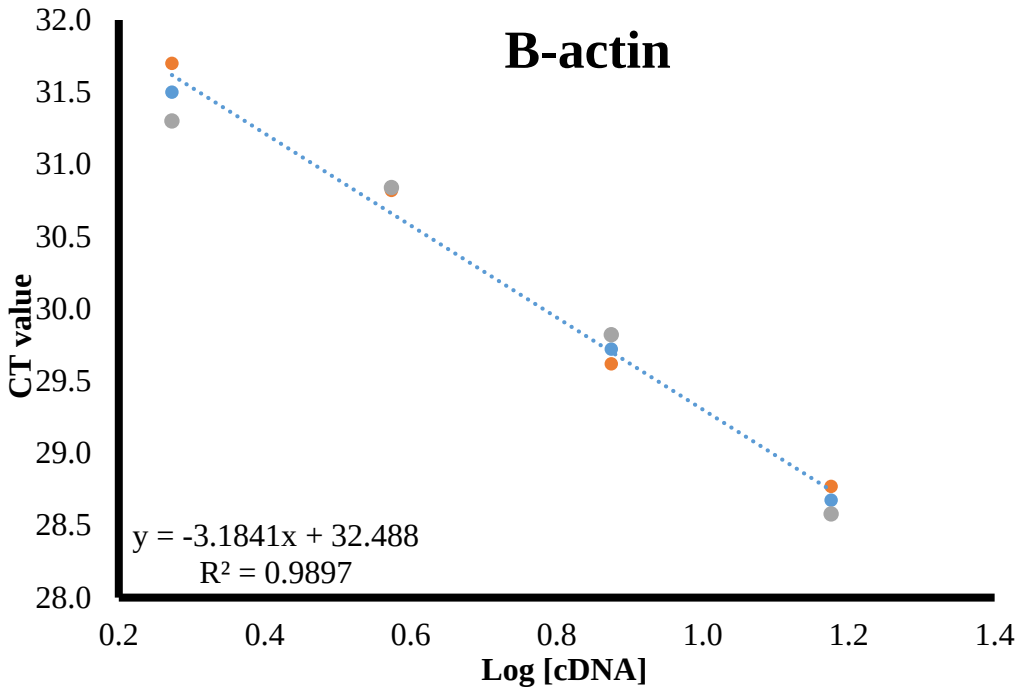
Slope	-4.2591
Efficiency	71.70834



[cDNA]	Log[cDNA]	Ct value1	Ct value 2	Avg
30	1.477121	29.75	29.38	29.565
15	1.176091	30.49	30.24	30.365
7.5	0.875061	31.37	31.26	31.315
3.75	0.574031	31.78	32.36	32.07
1.875	0.273001	33.78	33.43	33.605
Blank	0	N/A	N/A	N/A

Efficiency = (10^(-1/slope)-1)*100

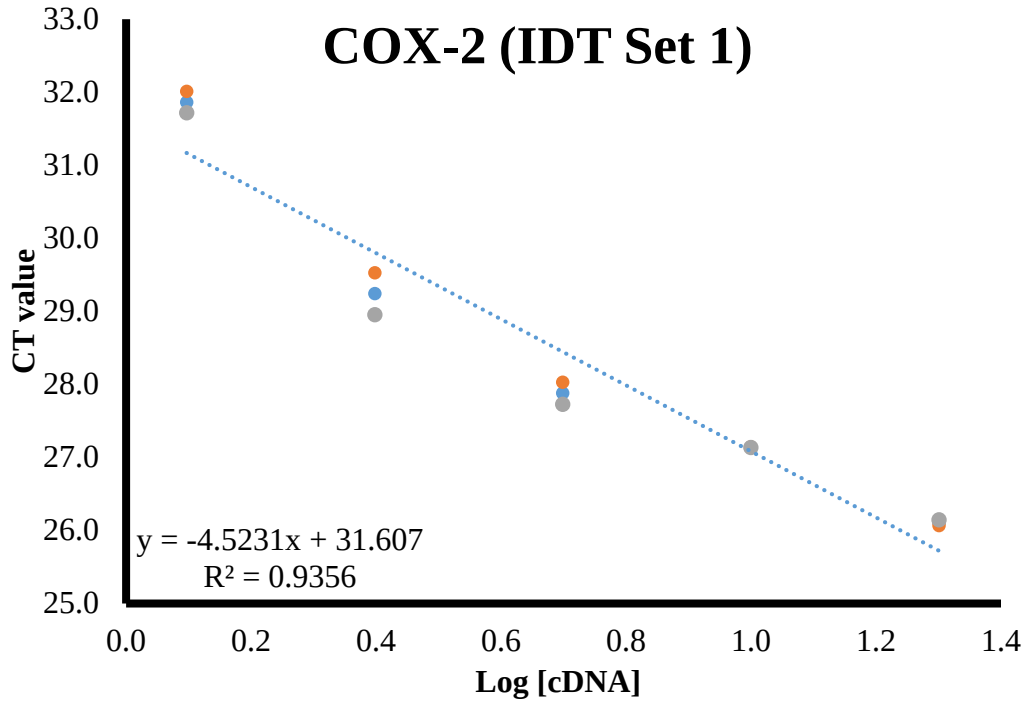
Slope	-3.2505
Efficiency	103.0696



[cDNA]	Log[cDNA]	Ct value1	Ct value 2	Avg
30	1.477121	-	-	-
15	1.176091	28.77	28.58	28.675
7.5	0.875061	29.62	29.82	29.72
3.75	0.574031	30.82	30.84	30.83
1.875	0.273001	31.7	31.3	31.5
Blank	~~0~~	~~37.57~~	~~36.9~~	~~37.235~~

Efficiency = (10^(-1/slope)-1)*100

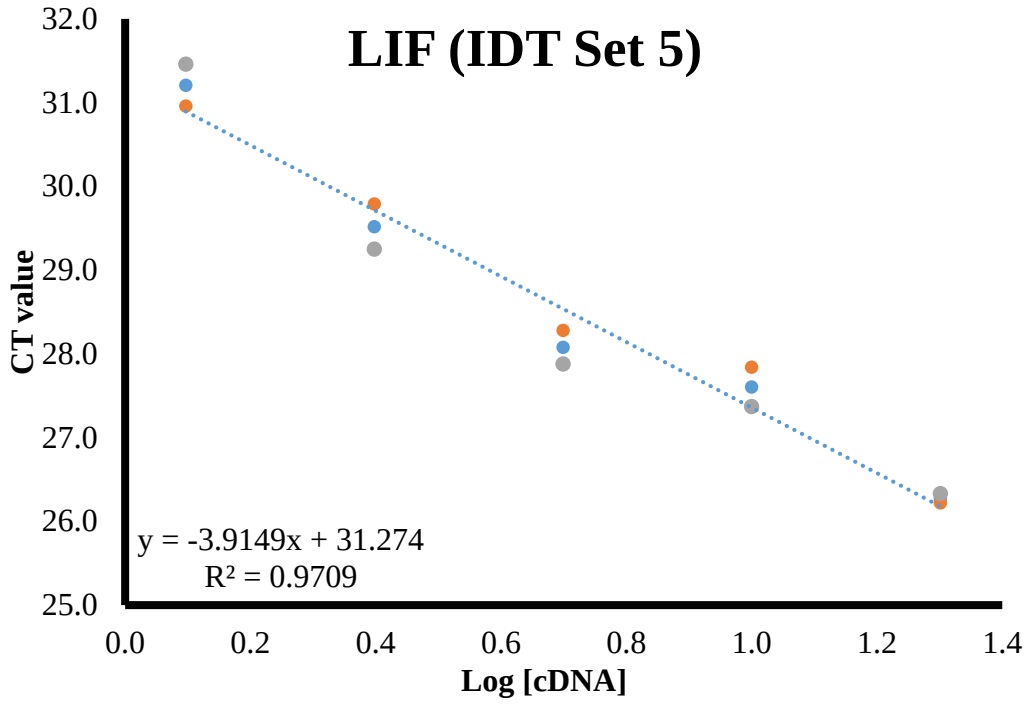
Slope	-3.1841
Efficiency	106.0917



[cDNA]	Log[cDNA]	Ct value1	Ct value 2	Avg
20	1.30103	26.06633	26.14371	26.10502
10	1	27.14371	27.13604	27.13988
5	0.69897	28.02707	27.72722	27.87715
2.5	0.39794	29.52713	28.95213	29.23963
1.25	0.09691	32.01068	31.71563	31.86316
Blank	0	N/A	N/A	N/A

Efficiency = (10^(-1/slope)-1)*100

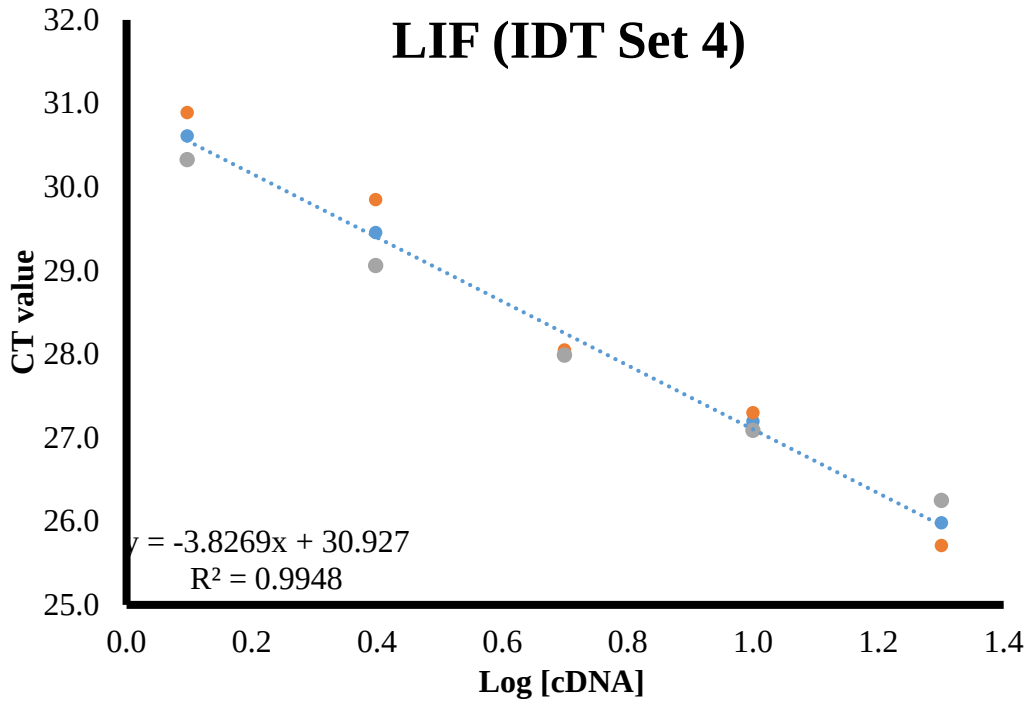
Slope	-4.52315
Efficiency	66.37385



[cDNA]	Log[cDNA]	Ct value1	Ct value 2	Avg
20	1.30103	26.22	26.33	26.275
10	1	27.84	27.37	27.605
5	0.69897	28.28	27.88	28.08
2.5	0.39794	29.79	29.25	29.52
1.25	0.09691	30.96	31.46	31.21
Blank	0	N/A	N/A	N/A

Efficiency = (10^(-1/slope)-1)*100

Slope	-3.91489
Efficiency	80.06731



[cDNA]	Log[cDNA]	Ct value1	Ct value 2	Avg
20	1.30103	25.71	26.25	25.98
10	1	27.3	27.09	27.195
5	0.69897	28.05	27.99	28.02
2.5	0.39794	29.85	29.06	29.455
1.25	0.09691	30.89	30.33	30.61
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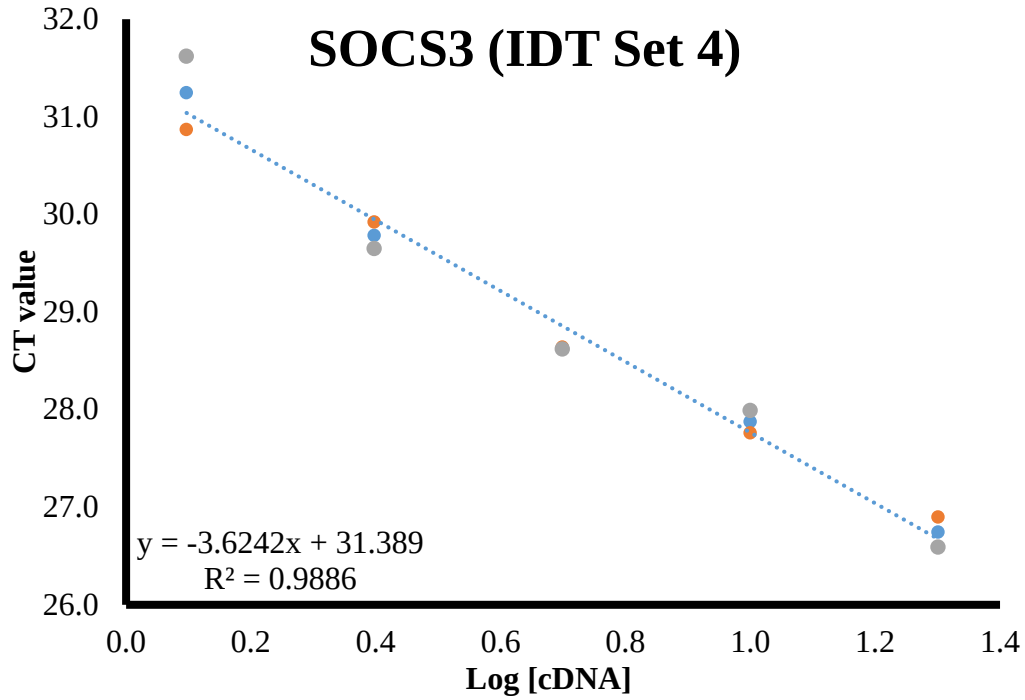
Efficiency = (10^(-1/slope)-1)*100

Slope	-3.8269
Efficiency	82.51901



[cDNA]	Log[cDNA]	Ct value1	Ct value 2	Avg
20	1.30103	26.90	26.59	26.745
10	1	27.76	27.99	27.875
5	0.69897	28.64	28.62	28.63
2.5	0.39794	29.92	29.65	29.785
1.25	0.09691	30.87	31.62	31.245
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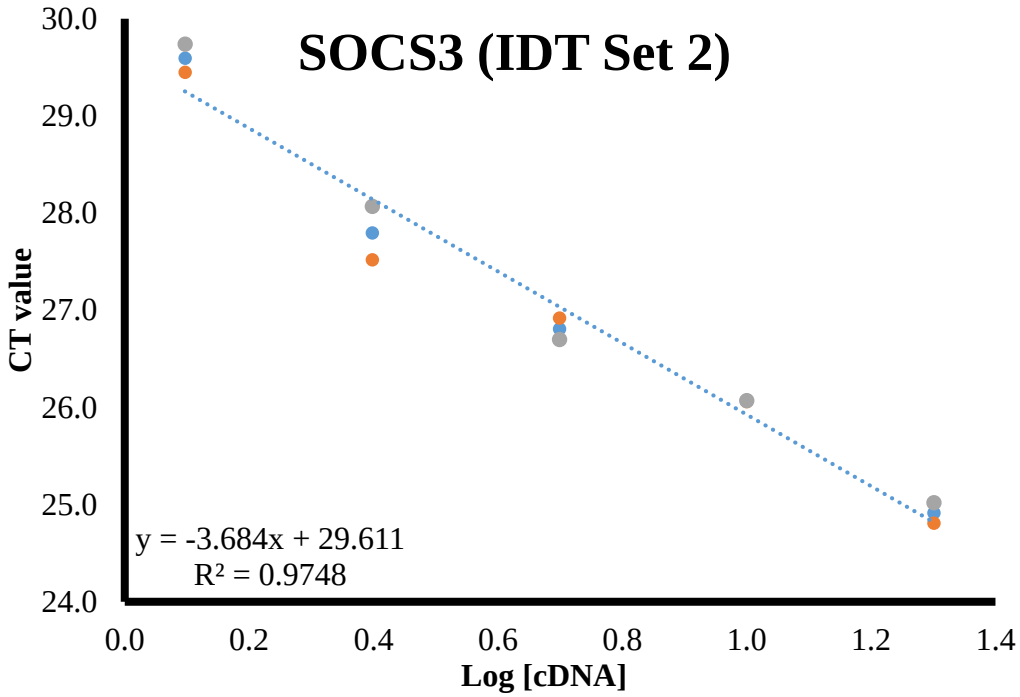
Efficiency = (10^(-1/slope)-1)*100

Slope	-3.62422
Efficiency	88.76487



[cDNA]	Log[cDNA]	Ct value1	Ct value 2	Avg
20	1.30103	24.81	25.02	24.915
10	1	26.06	26.07	26.065
5	0.69897	26.92	26.7	26.81
2.5	0.39794	27.52	28.07	27.795
1.25	0.09691	29.45	29.74	29.595
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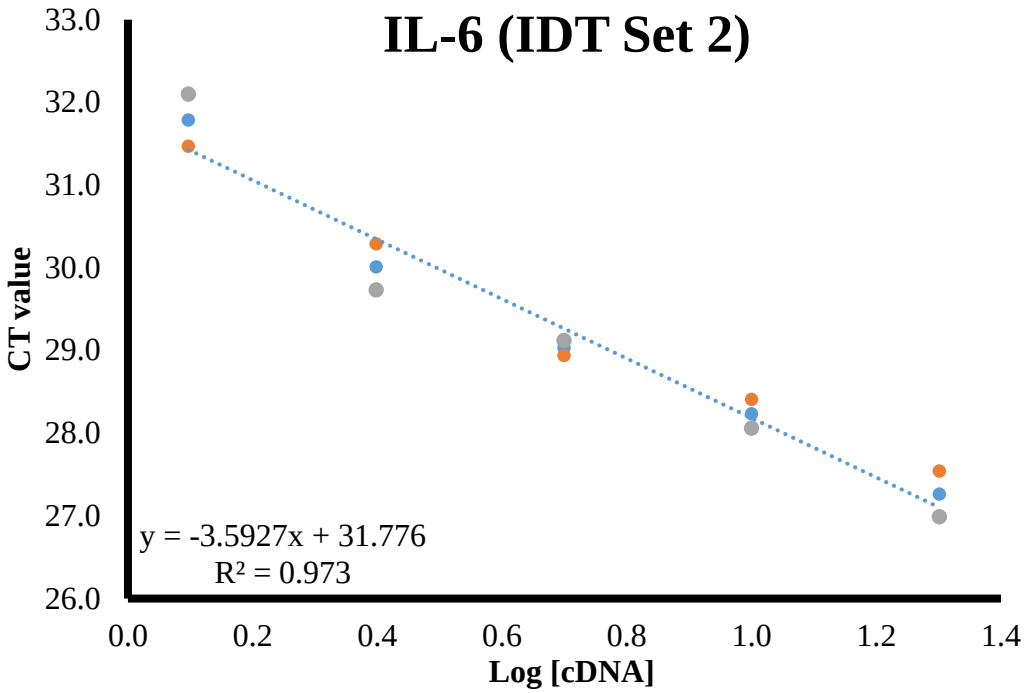
Efficiency = (10^(-1/slope)-1)*100

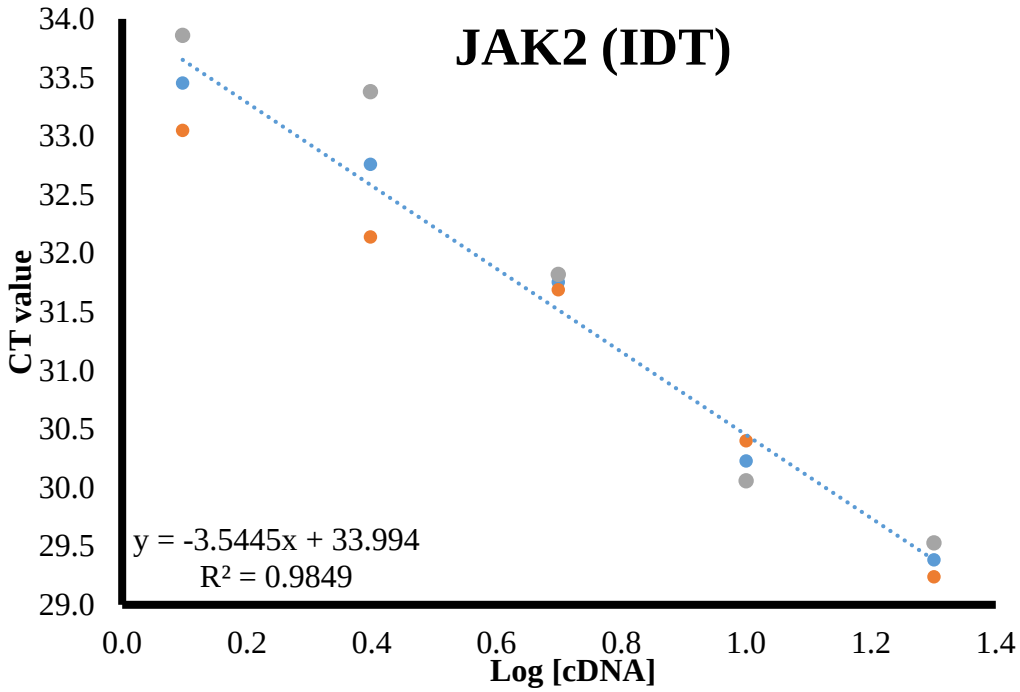
Slope	-3.684
Efficiency	86.82891



[cDNA]	Log[cDNA]	Ct value1	Ct value 2	Avg
20	1.30103	27.54	26.99	27.265
10	1	28.41	28.06	28.235
5	0.69897	28.94	29.12	29.03
2.5	0.39794	30.29	29.73	30.01
1.25	0.09691	31.47	32.1	31.785
Blank	0	N/A	N/A	N/A

Efficiency = (10^(-1/slope)-1)*100

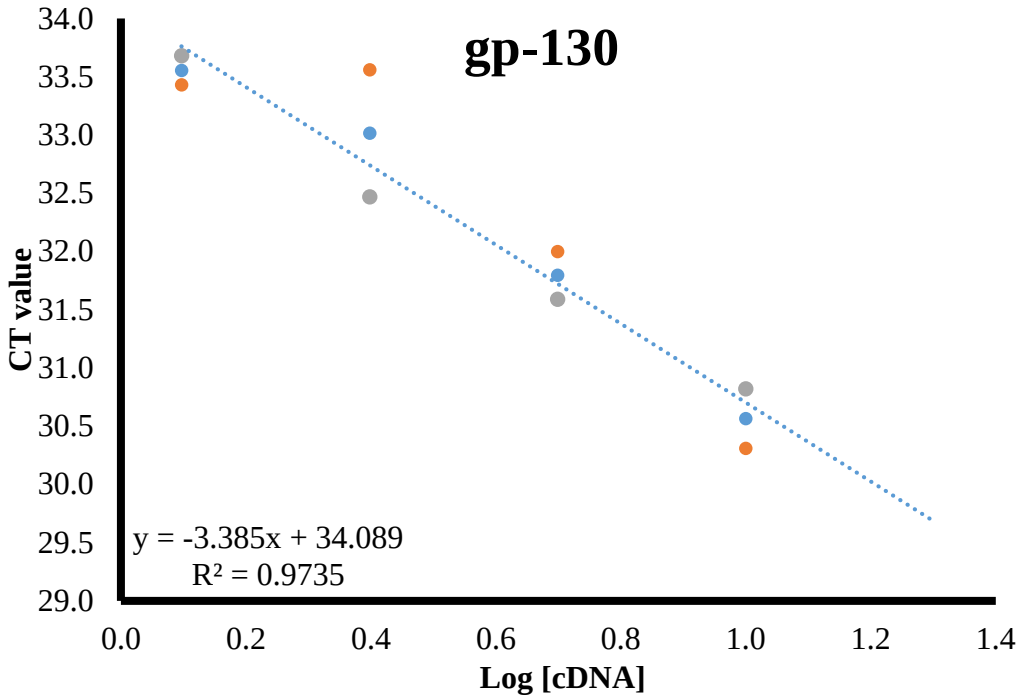
Slope	-3.5927
Efficiency	89.8201



[cDNA]	Log[cDNA]	Ct value1	Ct value 2	Avg
20	1.30103	29.24	29.53	29.385
10	1	30.4	30.06	30.23
5	0.69897	31.69	31.82	31.755
2.5	0.39794	32.14	33.38	32.76
1.25	0.09691	33.05	33.86	33.455
Blank	0	N/A	N/A	N/A

Efficiency = (10^(-1/slope)-1)*100

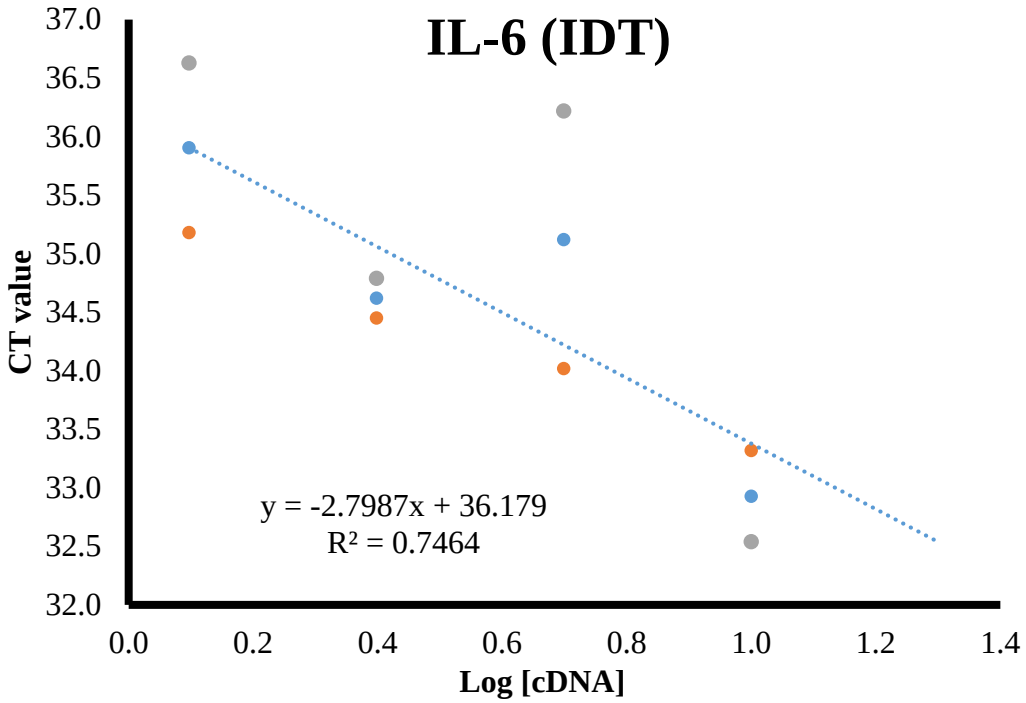
Slope	-3.5445
Efficiency	91.48168



[cDNA]	Log[cDNA]	Ct value1	Ct value 2	Avg
20	1.30103	-	-	-
10	1	30.31	30.82	30.565
5	0.69897	32	31.59	31.795
2.5	0.39794	33.56	32.47	33.015
1.25	0.09691	33.43	33.68	33.555
Blank	0	N/A	N/A	N/A

Efficiency = (10^(-1/slope)-1)*100

Slope	-3.385
Efficiency	97.43356



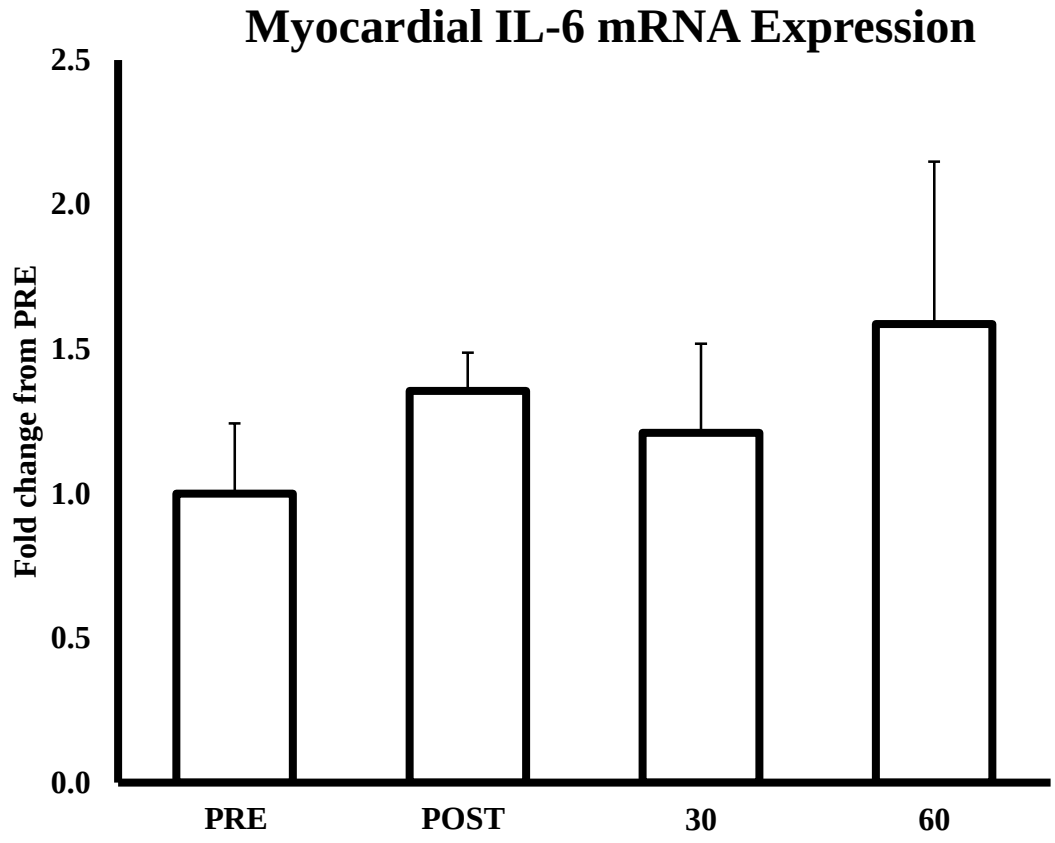
[cDNA]	Log[cDNA]	Ct value1	Ct value 2	Avg
20	1.30103	-	-	-
10	1	33.32	32.54	32.93
5	0.69897	34.02	36.22	35.12
2.5	0.39794	34.45	34.79	34.62
1.25	0.09691	35.18	36.63	35.905
Blank	0	N/A	N/A	N/A

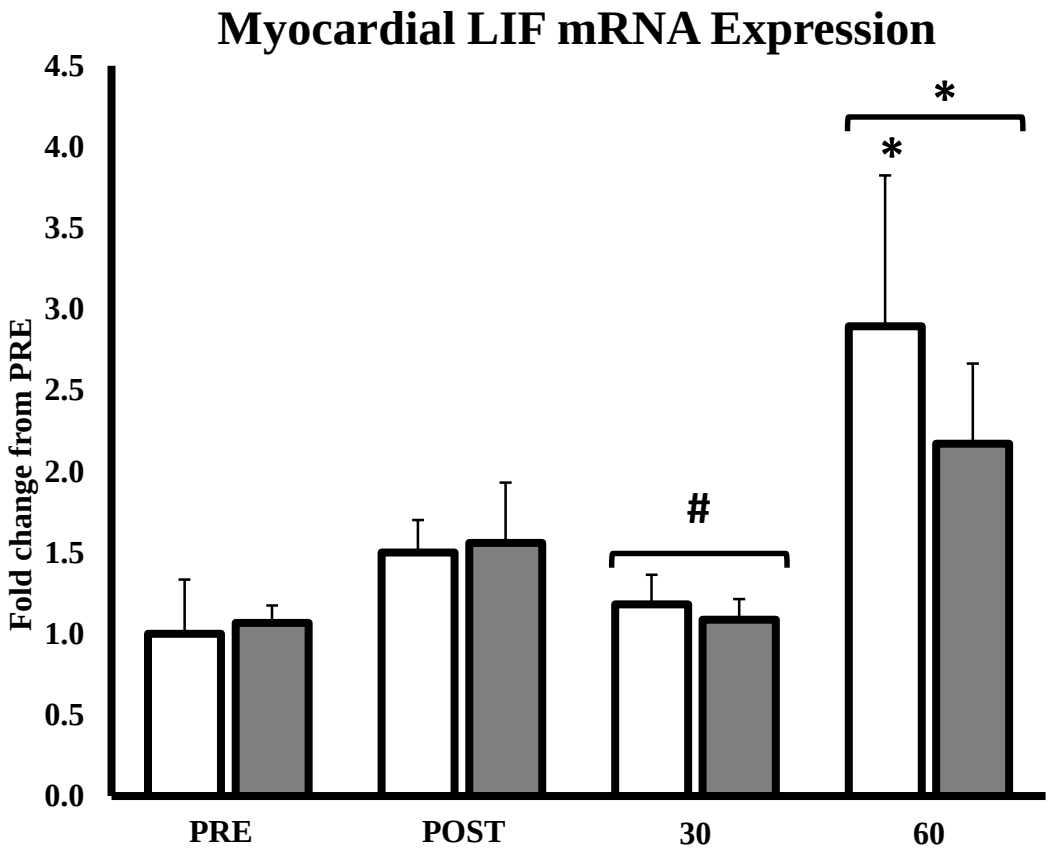
Efficiency = (10^(-1/slope)-1)*100

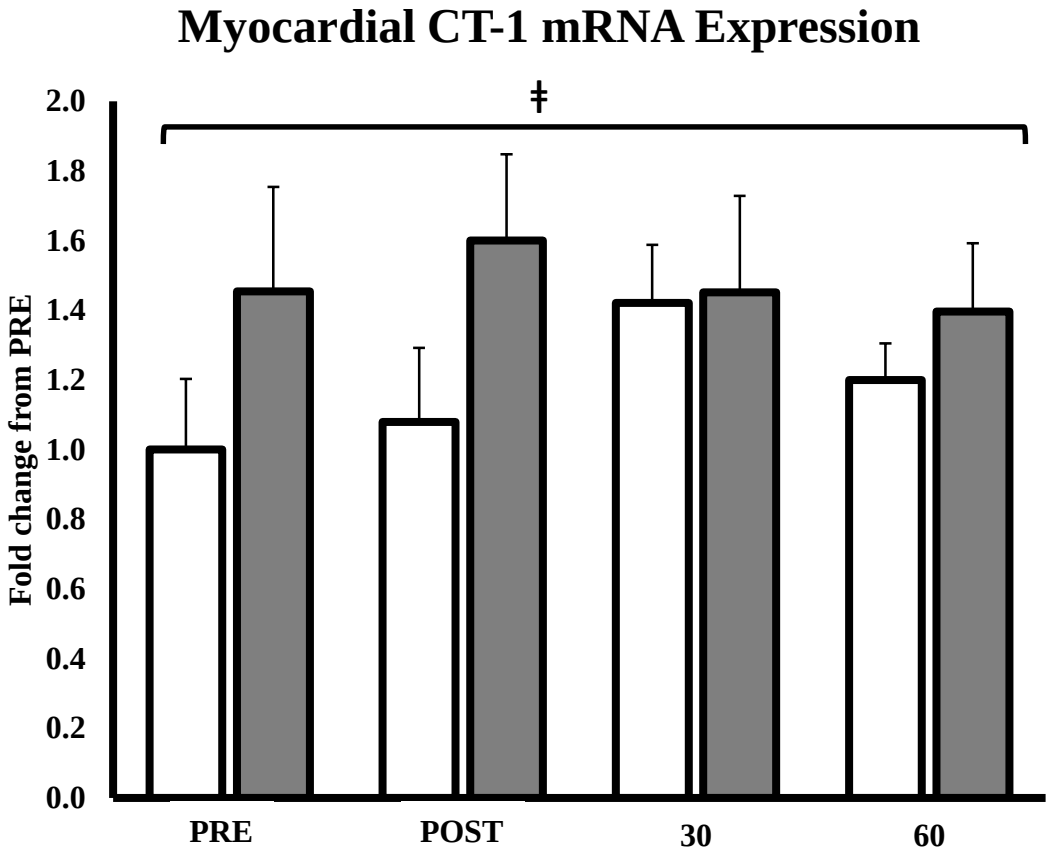
Slope	-2.7987
Efficiency	127.6715

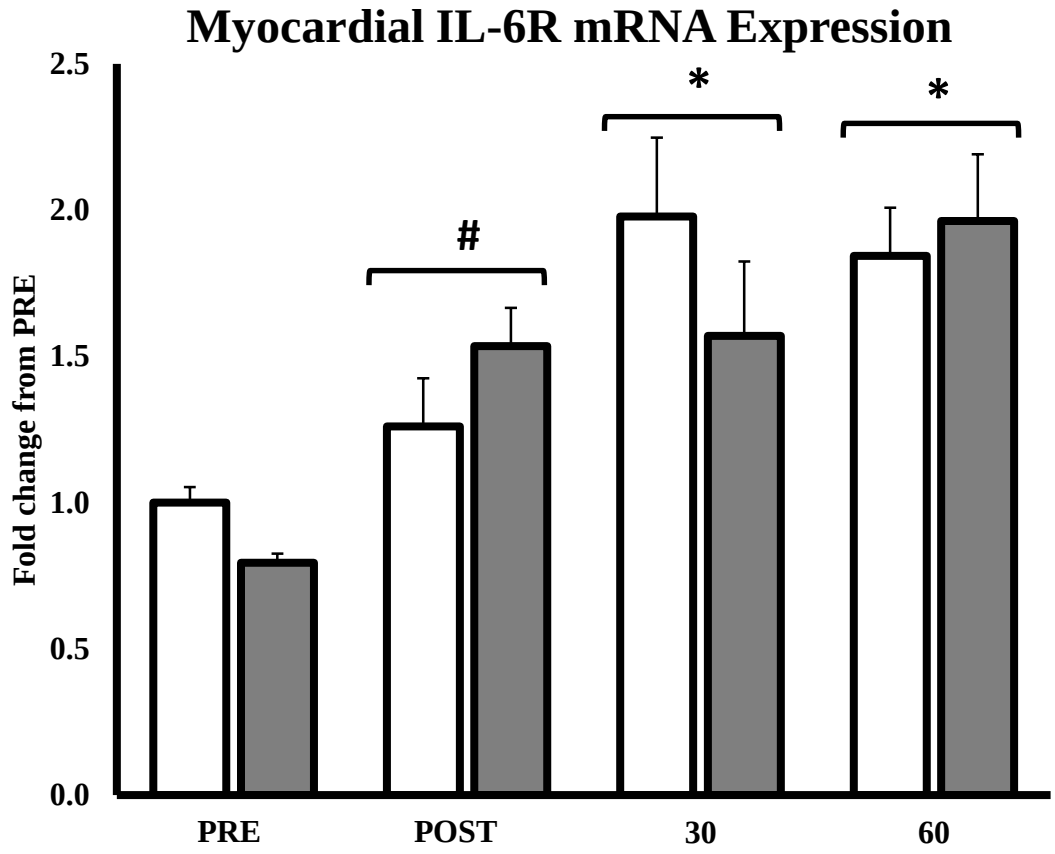
Appendix V

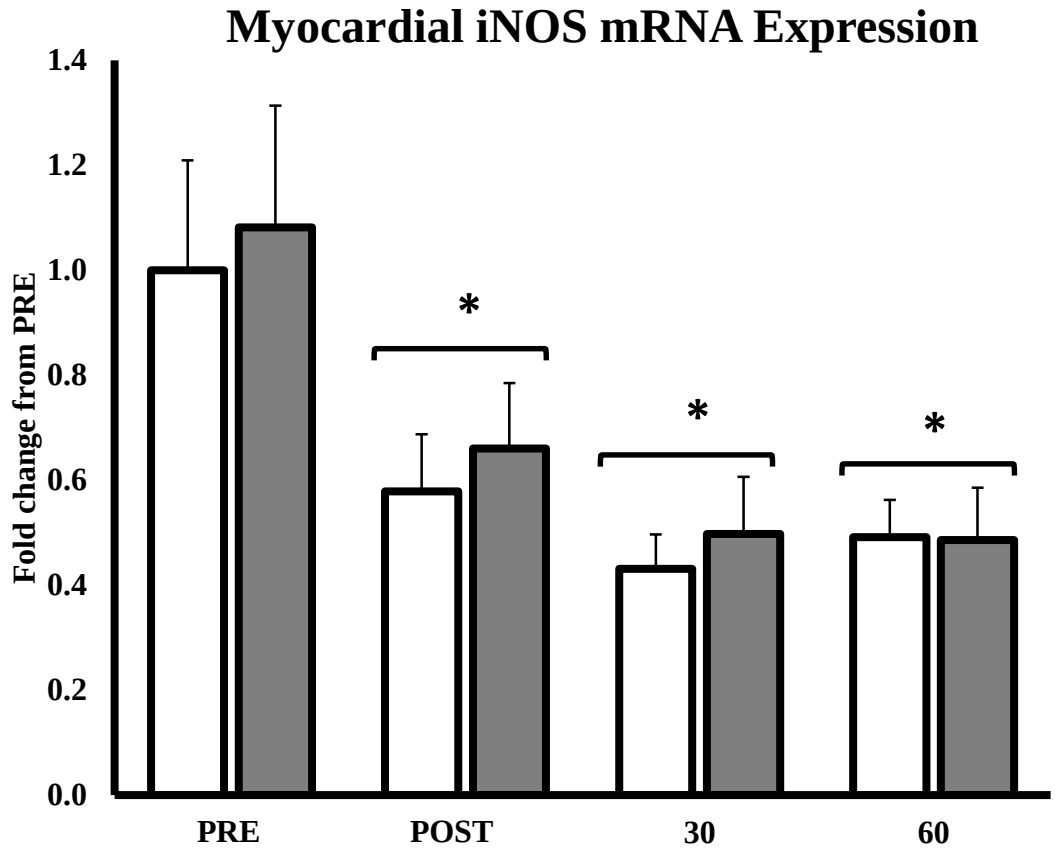
Supplementary Data Figures

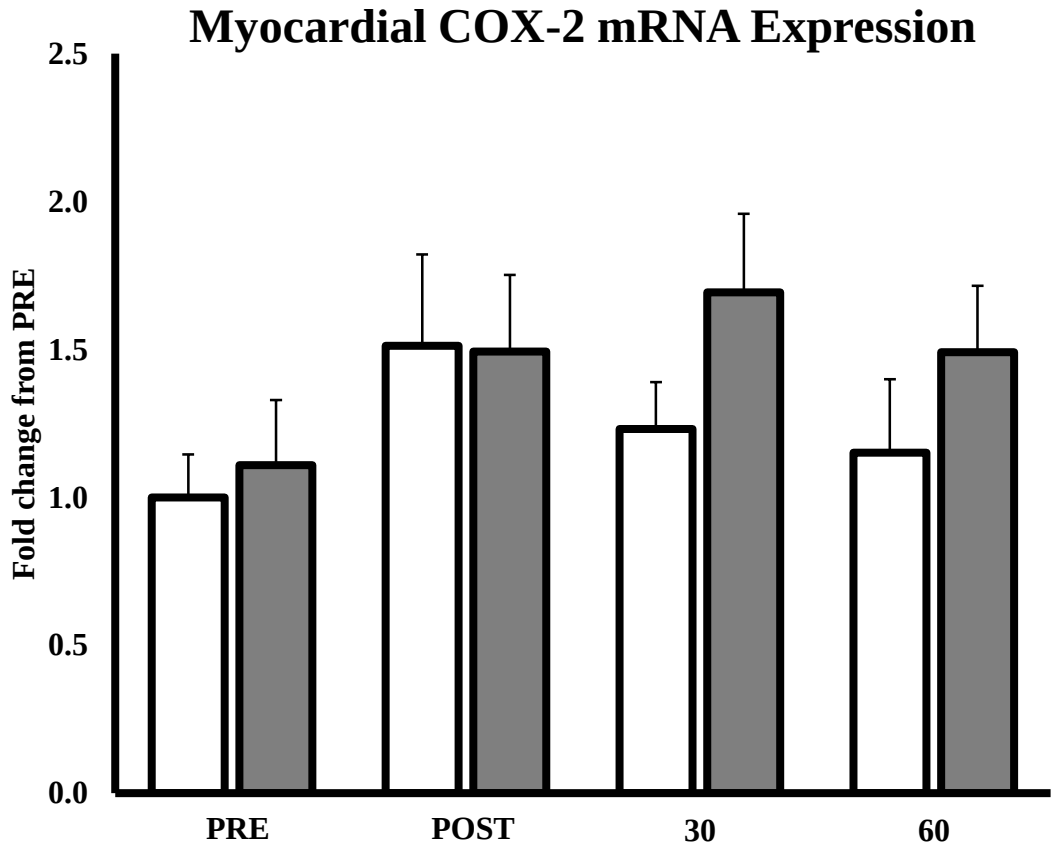

Myocardial IL-6 mRNA Expression
Fold change from PRE
2.5
2.0
1.5
1.0
0.5
0.0
PRE
POST
30
60

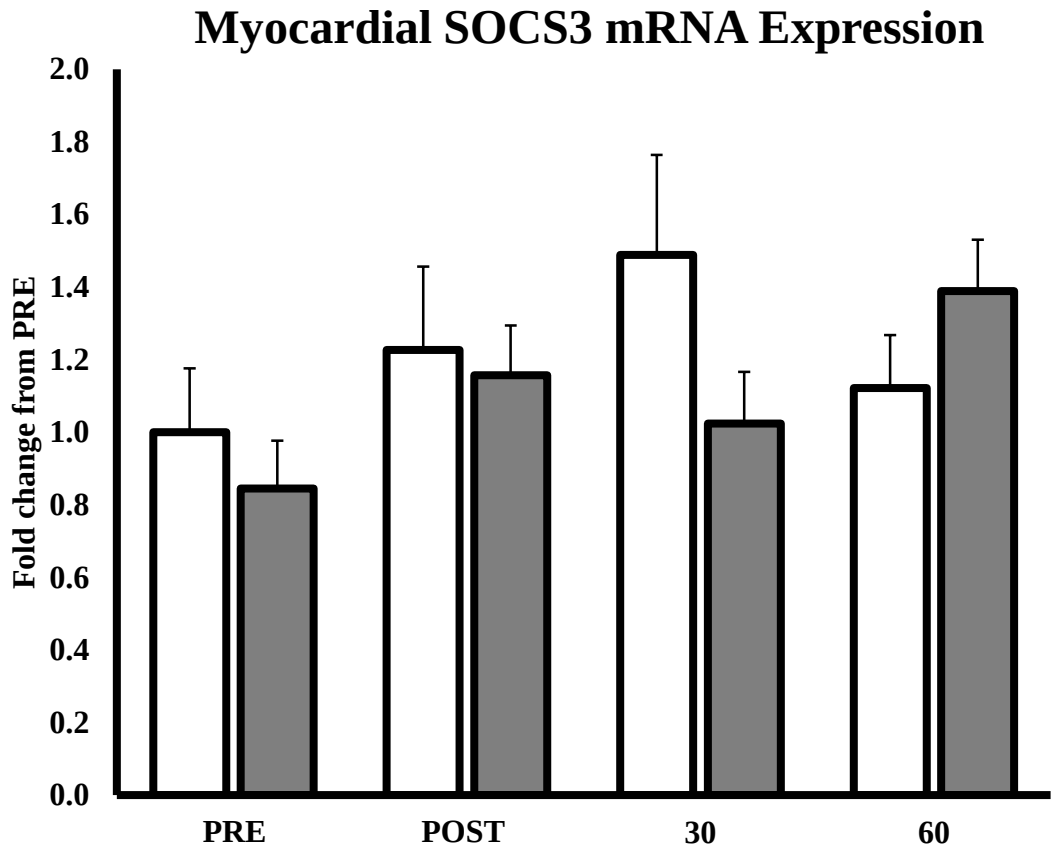

Myocardial LIF mRNA Expression
Fold change from PRE
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
#
PRE
POST
30
60

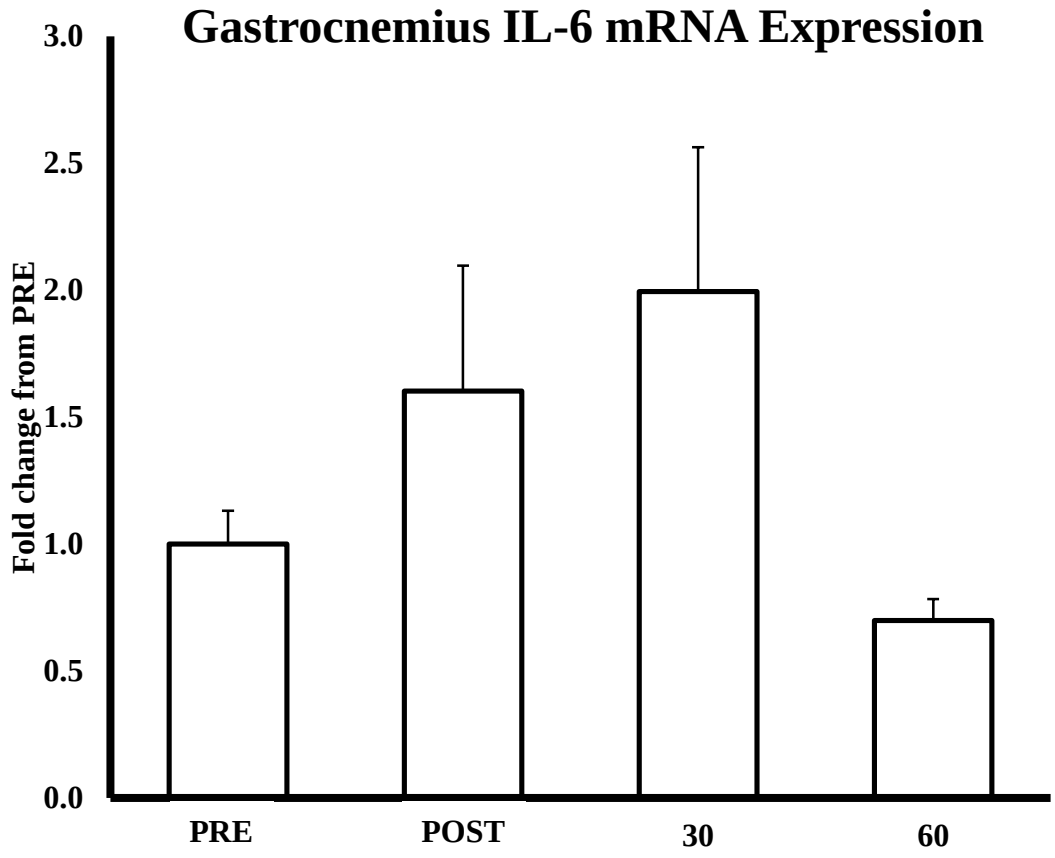

Myocardial CT-1 mRNA Expression
‡
Fold change from PRE
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
PRE
POST
30
60

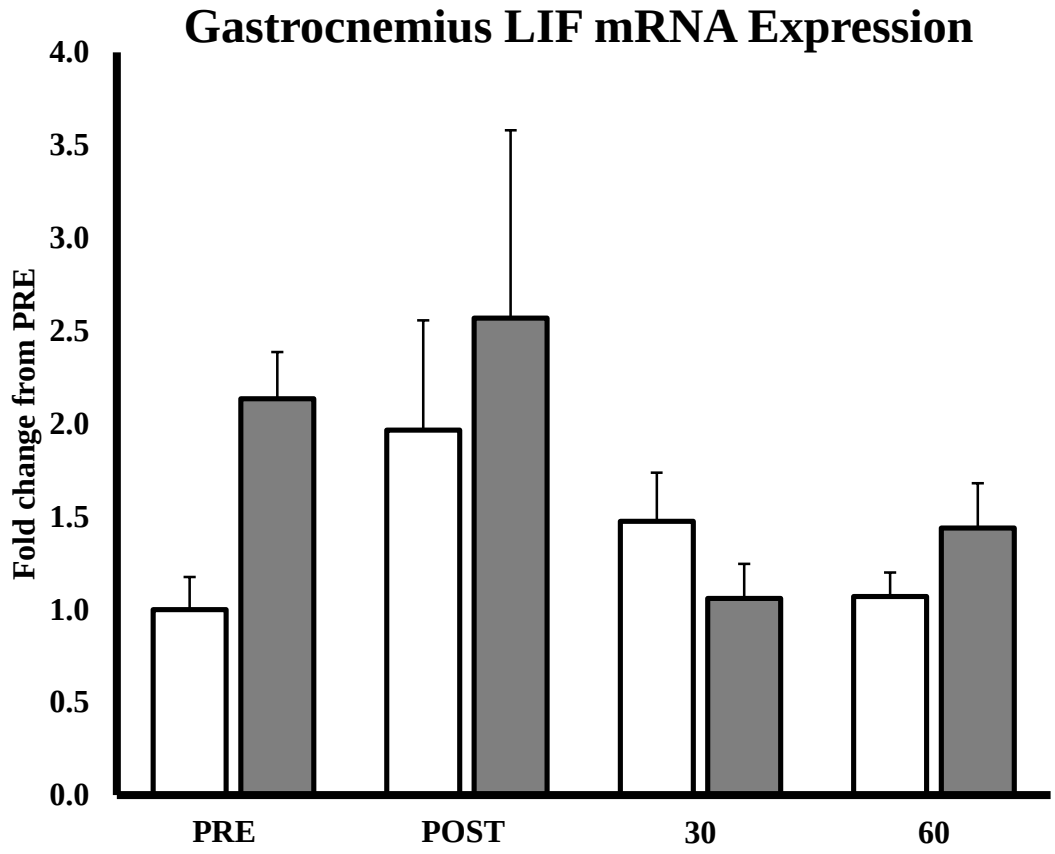

Myocardial IL-6R mRNA Expression
Fold change from PRE
2.5
2.0
1.5
1.0
0.5
0.0
#
*
*
PRE
POST
30
60

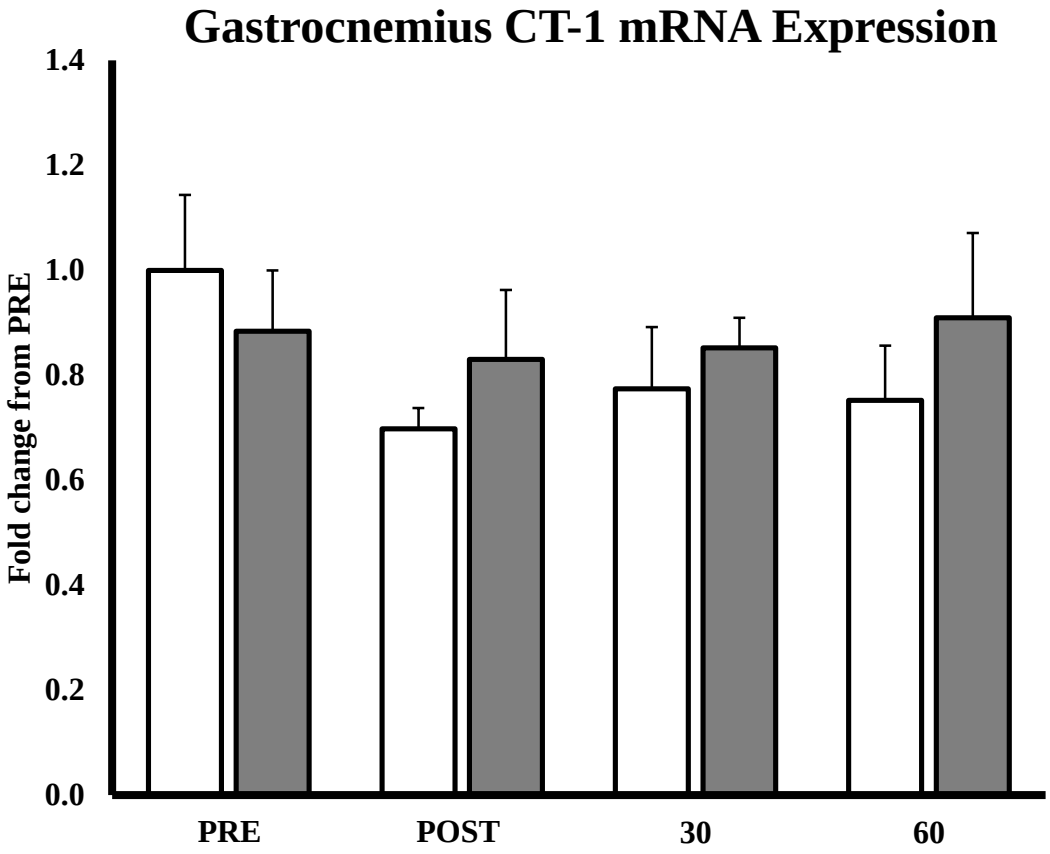

Myocardial iNOS mRNA Expression
Fold change from PRE
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
PRE
POST
30
60

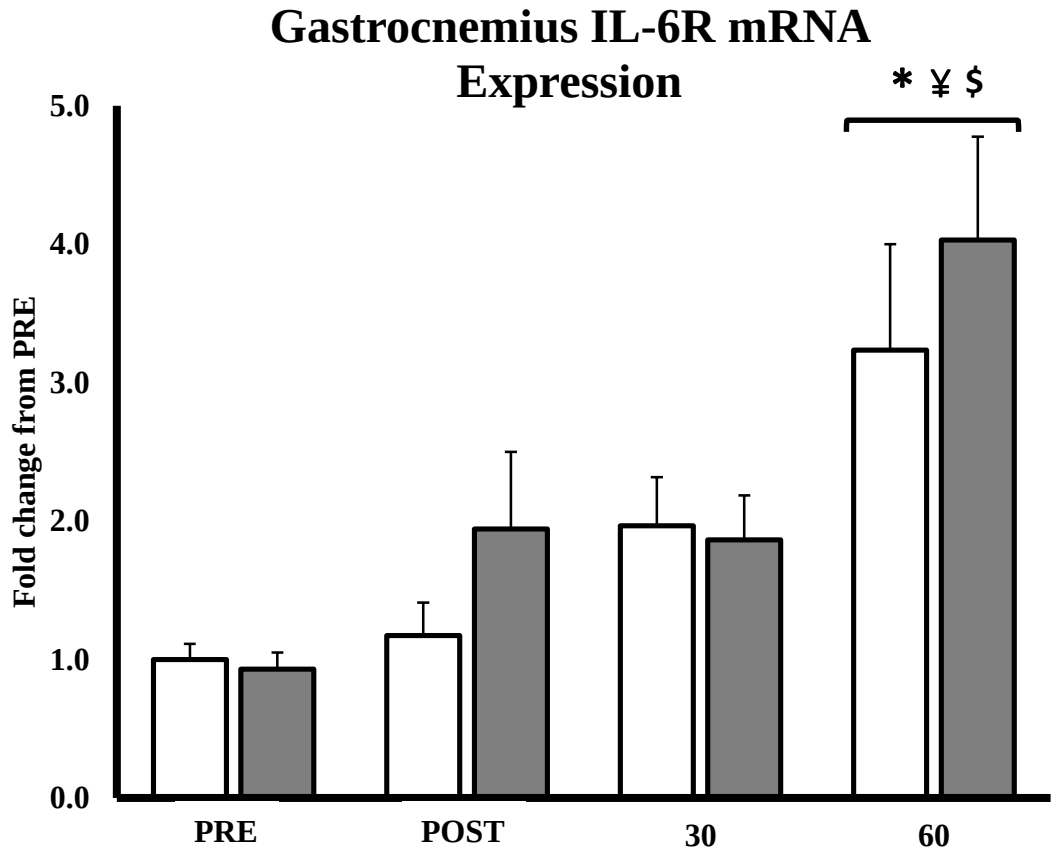

Myocardial COX-2 mRNA Expression
Fold change from PRE
2.5
2.0
1.5
1.0
0.5
0.0
PRE
POST
30
60

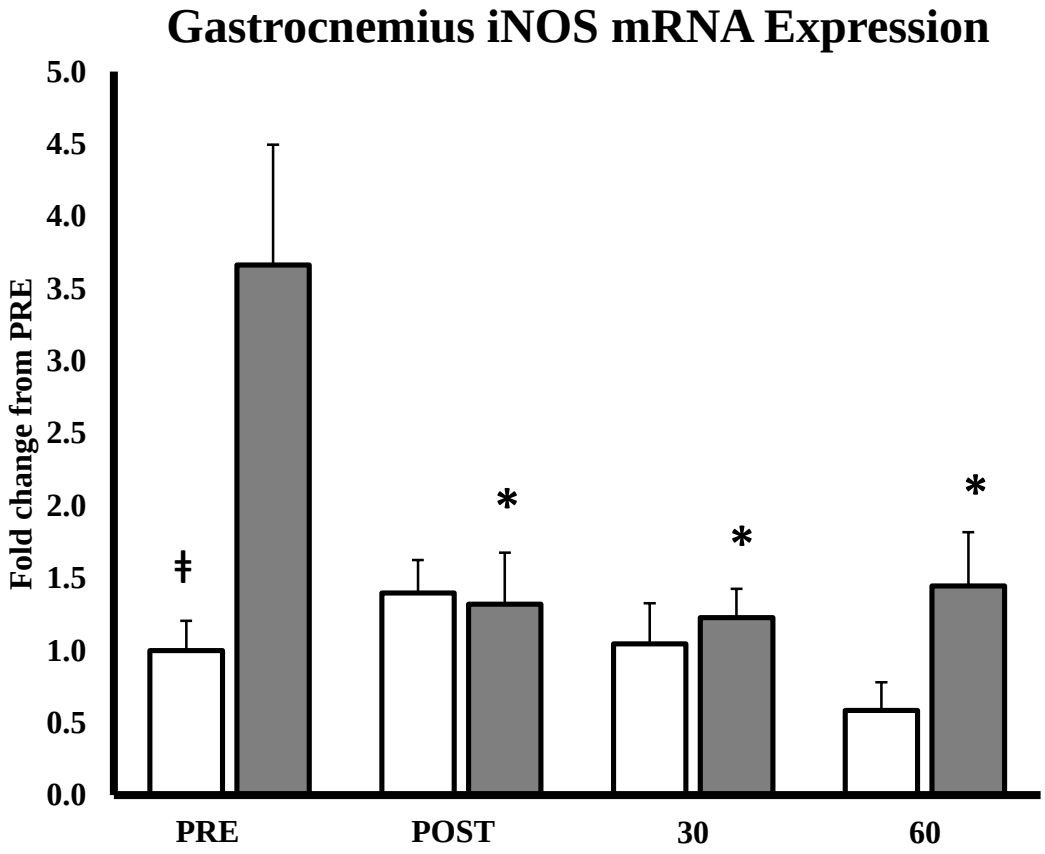

Myocardial SOCS3 mRNA Expression
Fold change from PRE
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
PRE
POST
30
60

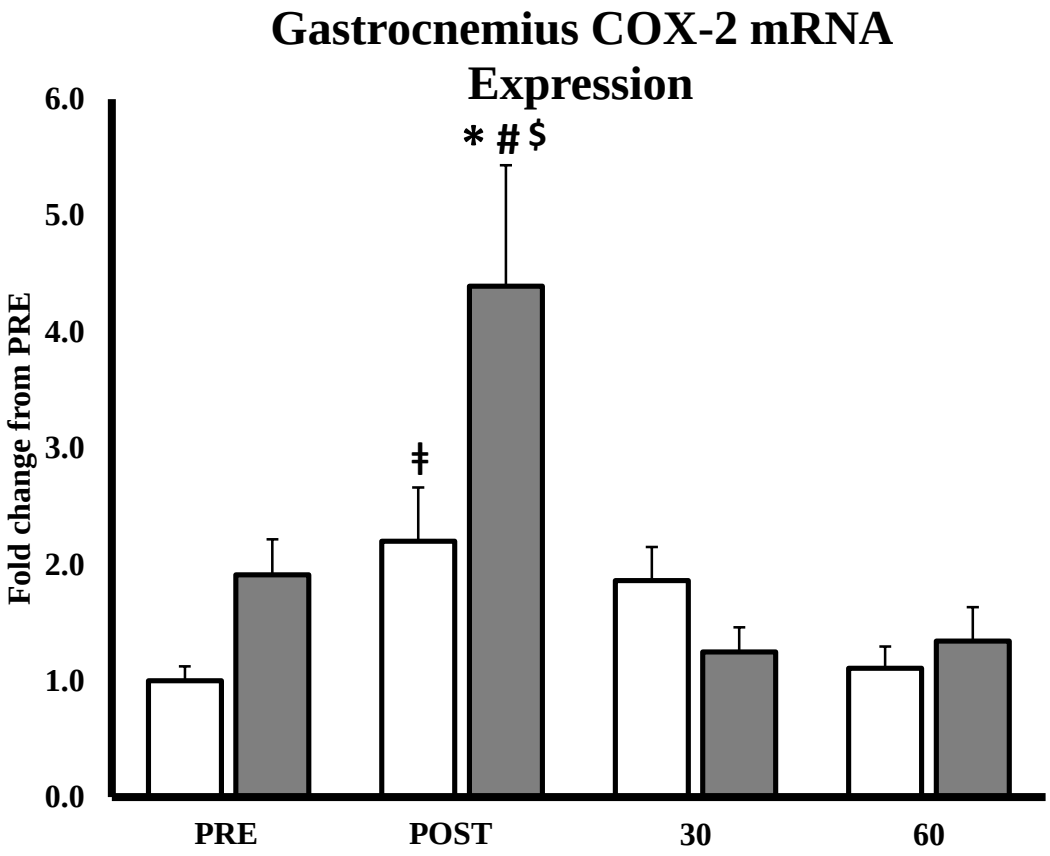

Gastrocnemius IL-6 mRNA Expression
Fold change from PRE
3.0
2.5
2.0
1.5
1.0
0.5
0.0
PRE
POST
30
60

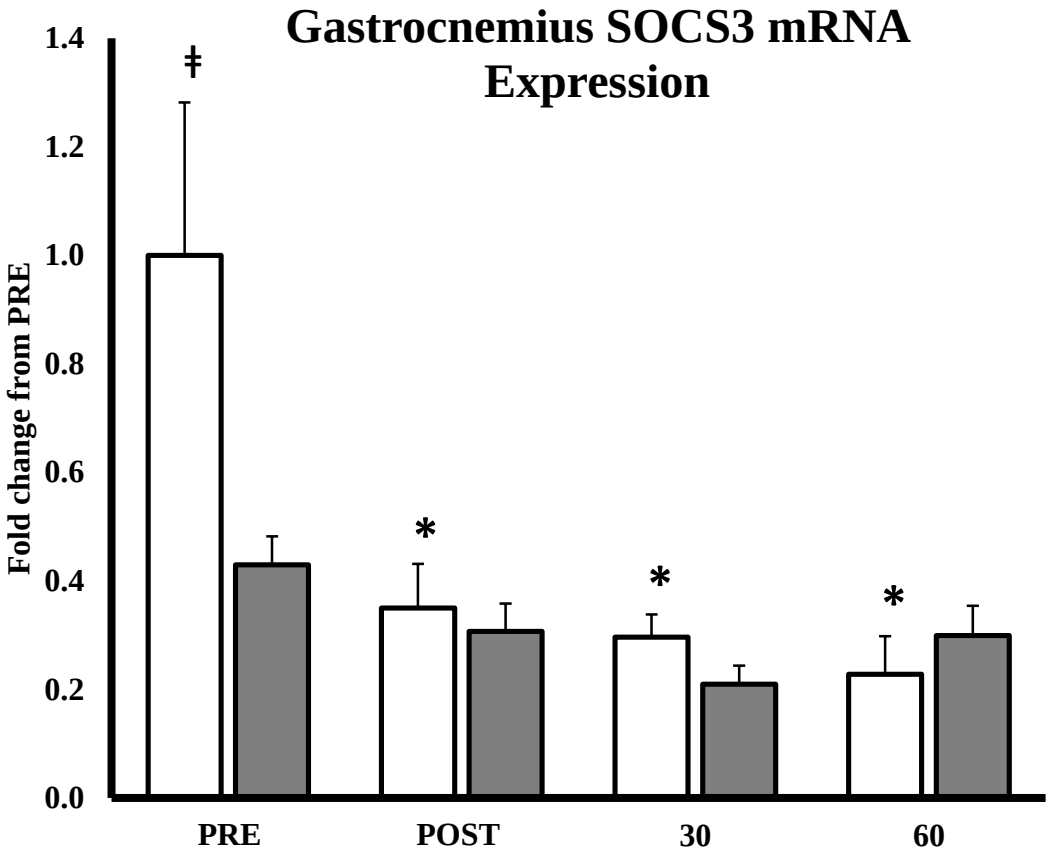

Gastrocnemius LIF mRNA Expression
Fold change from PRE
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
PRE
POST
30
60


Gastrocnemius CT-1 mRNA Expression
Fold change from PRE
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
PRE
POST
30
60

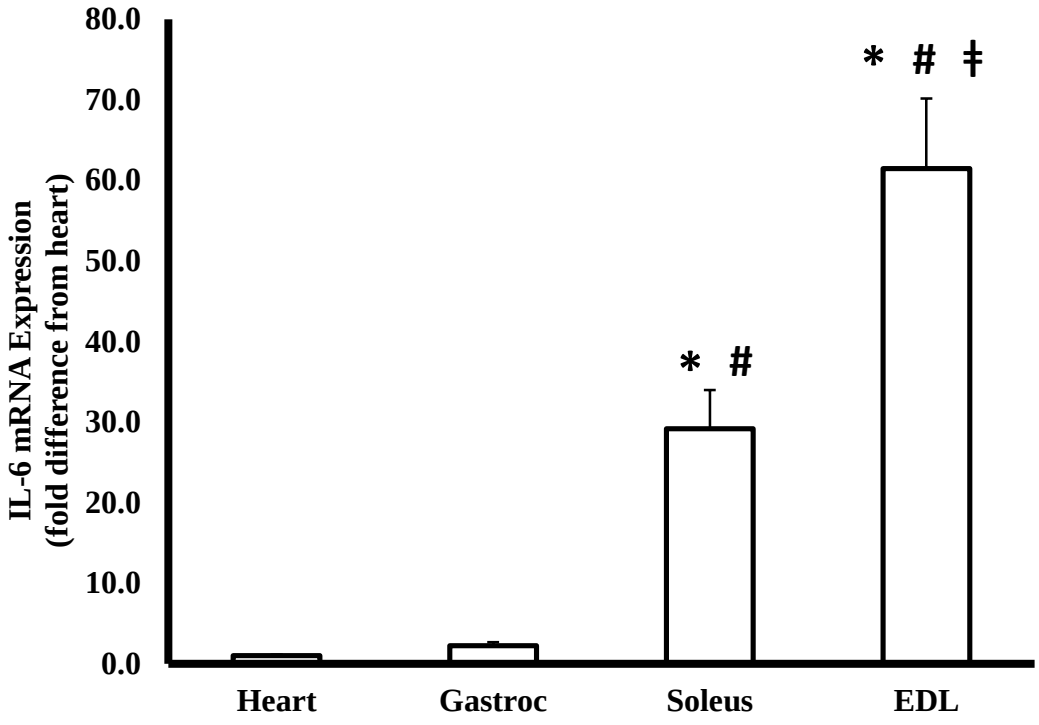

Gastrocnemius IL-6R mRNA Expression
* ¥ $
Fold change from PRE
5.0
4.0
3.0
2.0
1.0
0.0
PRE
POST
30
60


Gastrocnemius iNOS mRNA Expression
Fold change from PRE
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
‡
*
*
*
PRE
POST
30
60


Gastrocnemius COX-2 mRNA Expression
* # $
‡
Fold change from PRE
6.0
5.0
4.0
3.0
2.0
1.0
0.0
PRE
POST
30
60


Gastrocnemius SOCS3 mRNA Expression
Fold change from PRE
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
‡
*
*
*
PRE
POST
30
60

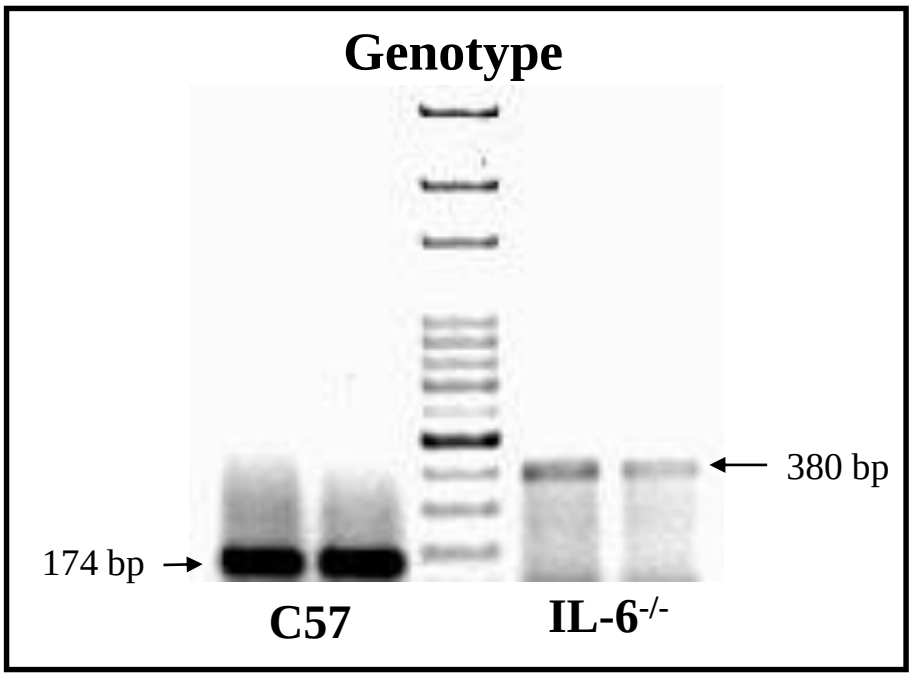
IL-6 mRNA Expression in Exercised Mice

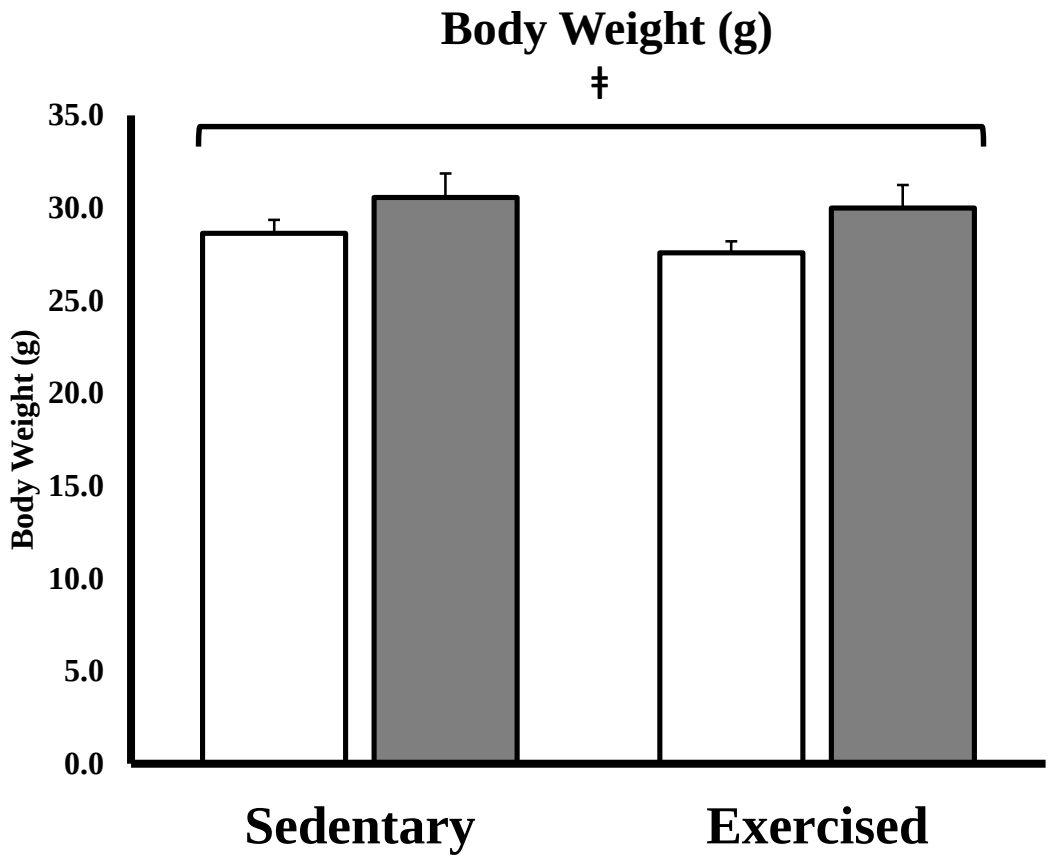


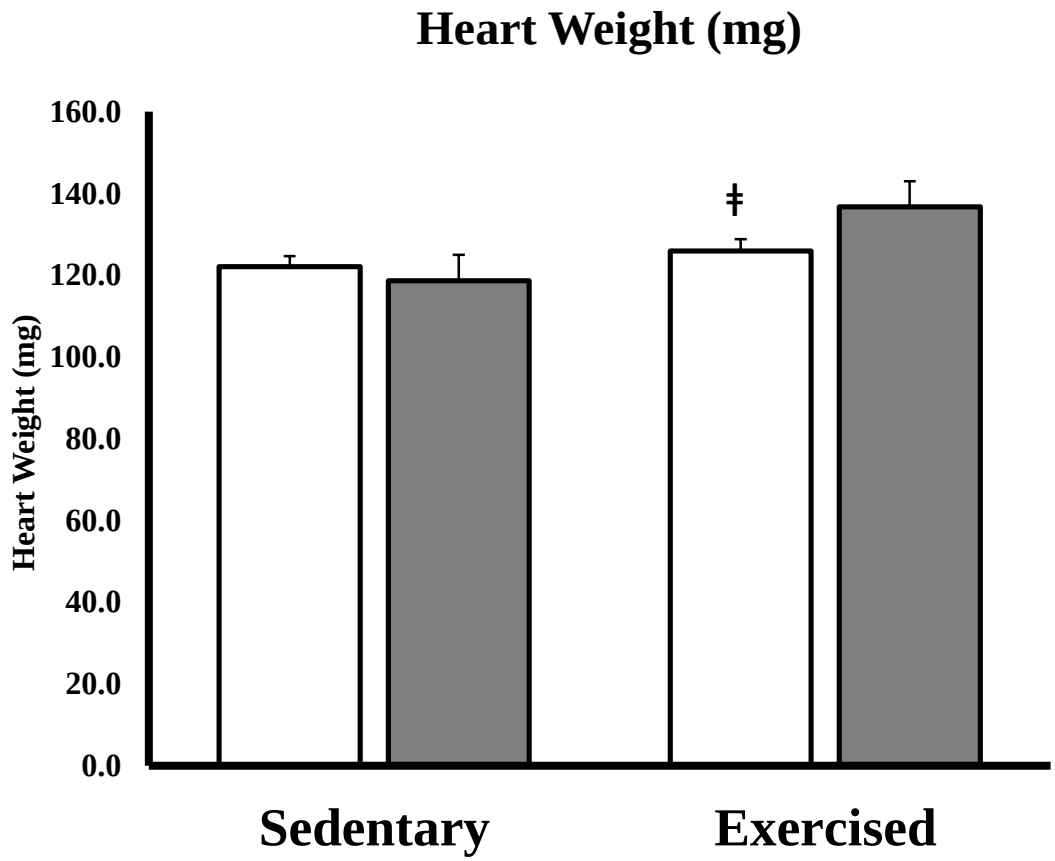
***** **Different from Heart**

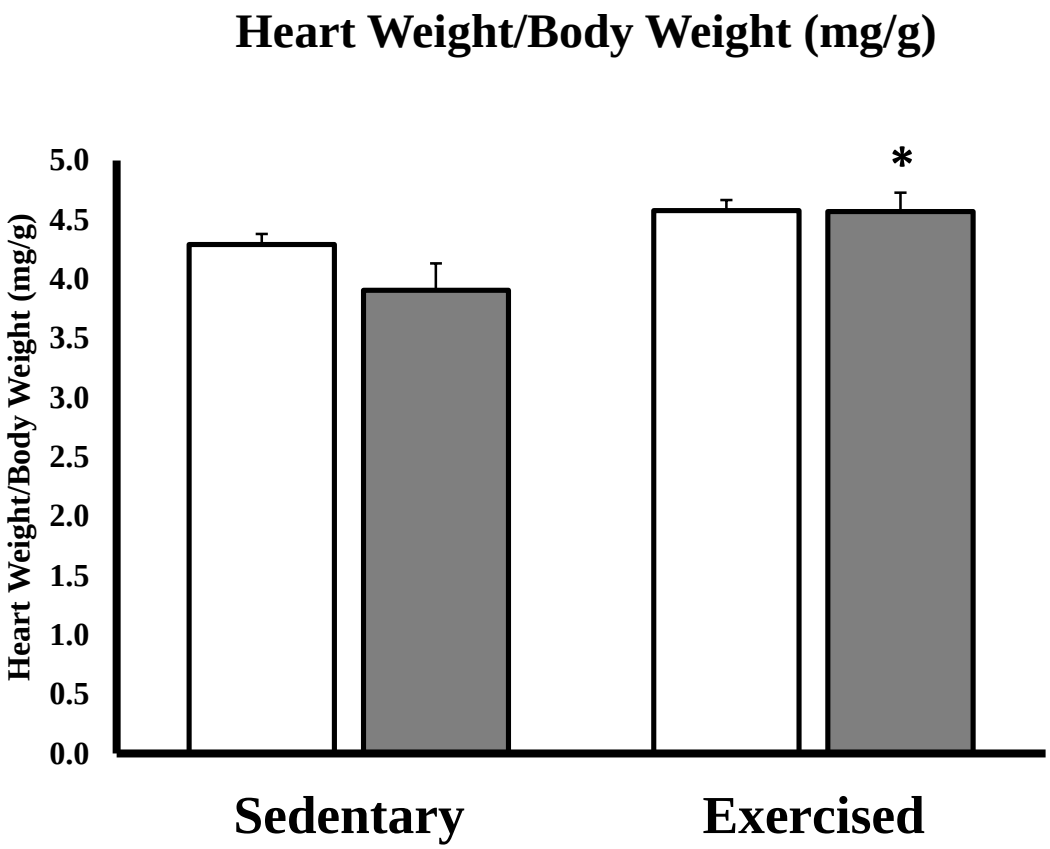
**Different from Gastrocnemius**

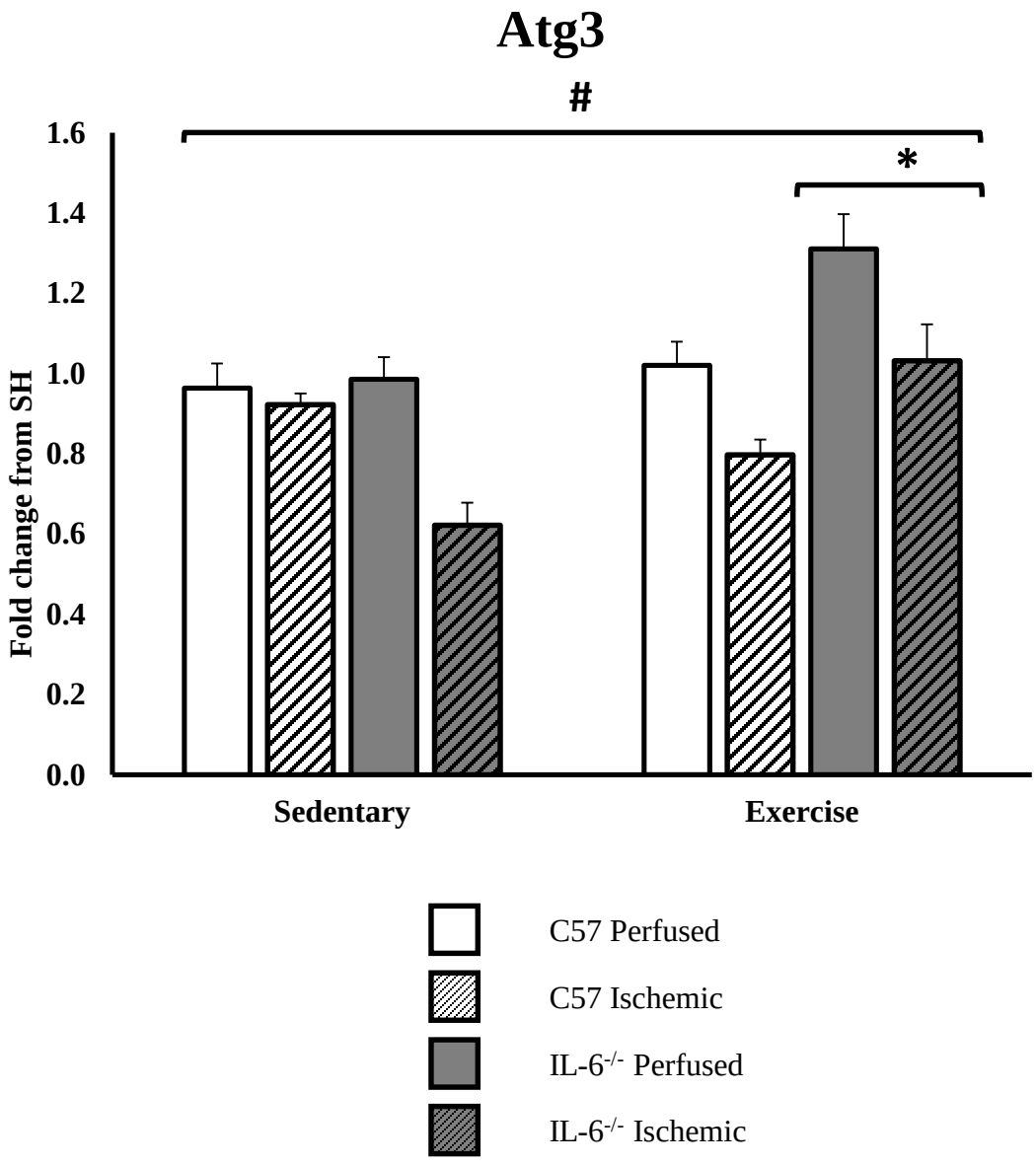
‡ **Different from Soleus**

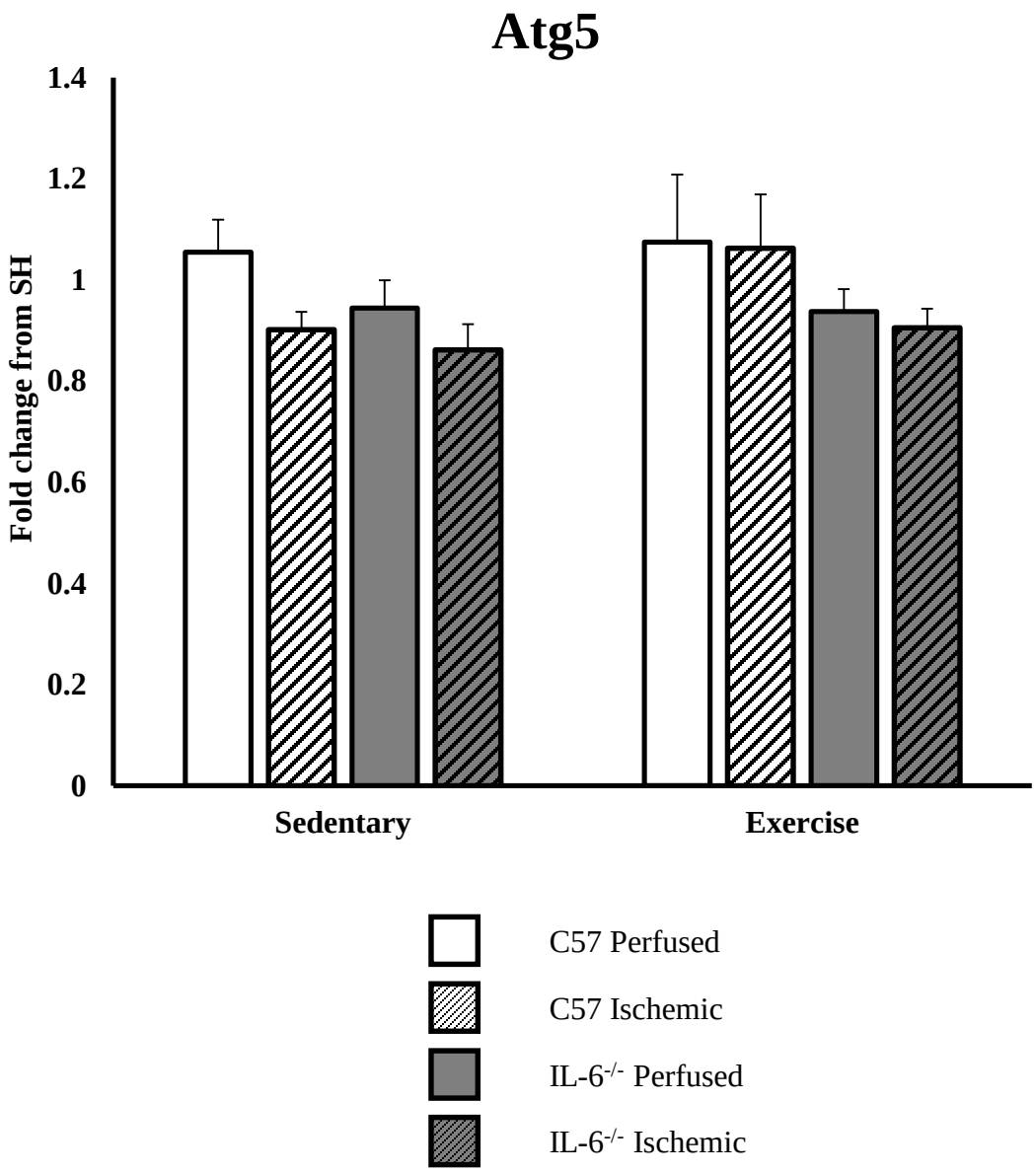

Genotype
380 bp
174 bp
C57
IL-6-/-

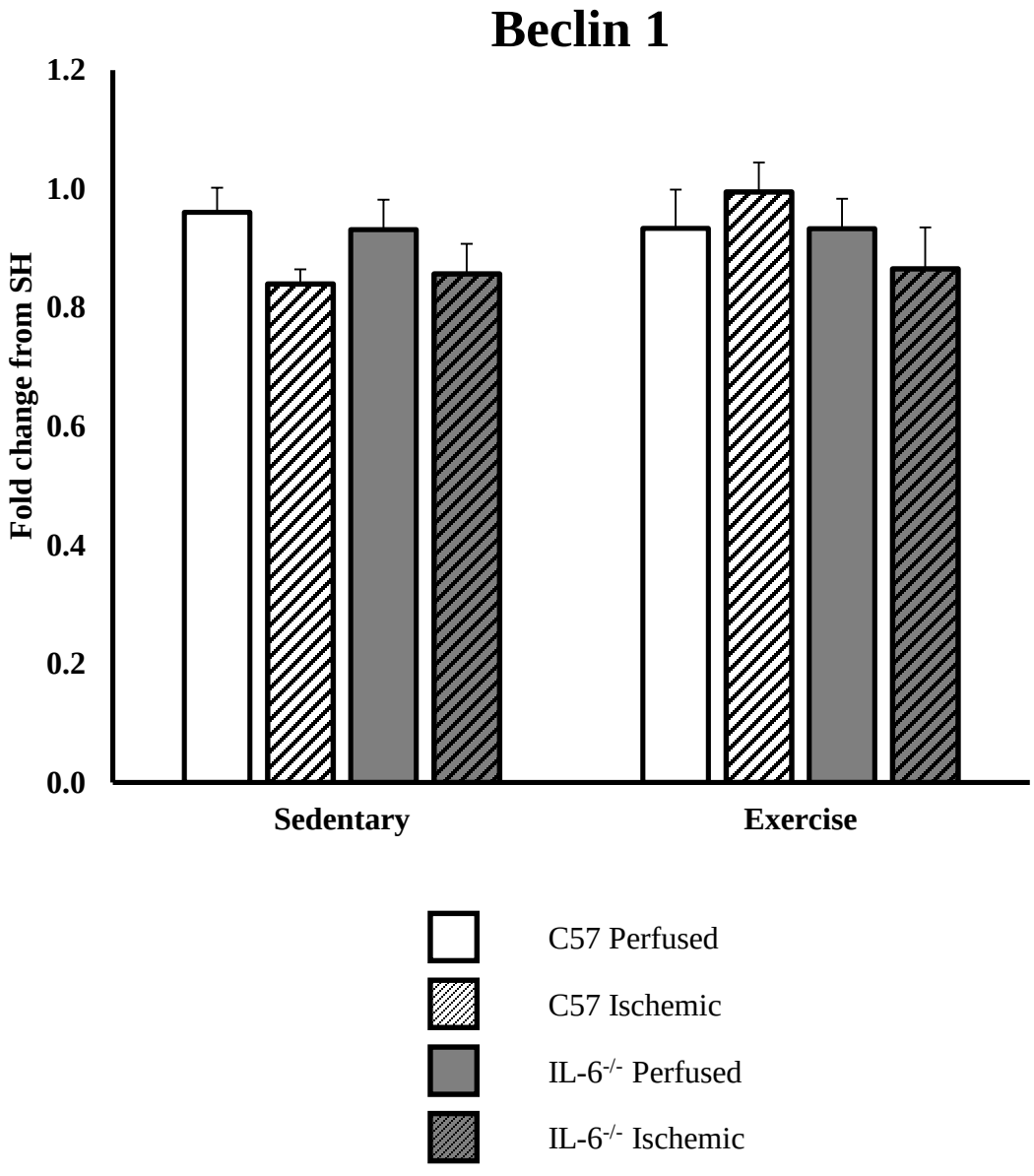

Body Weight (g)
‡
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Body Weight (g)
Sedentary
Exercised

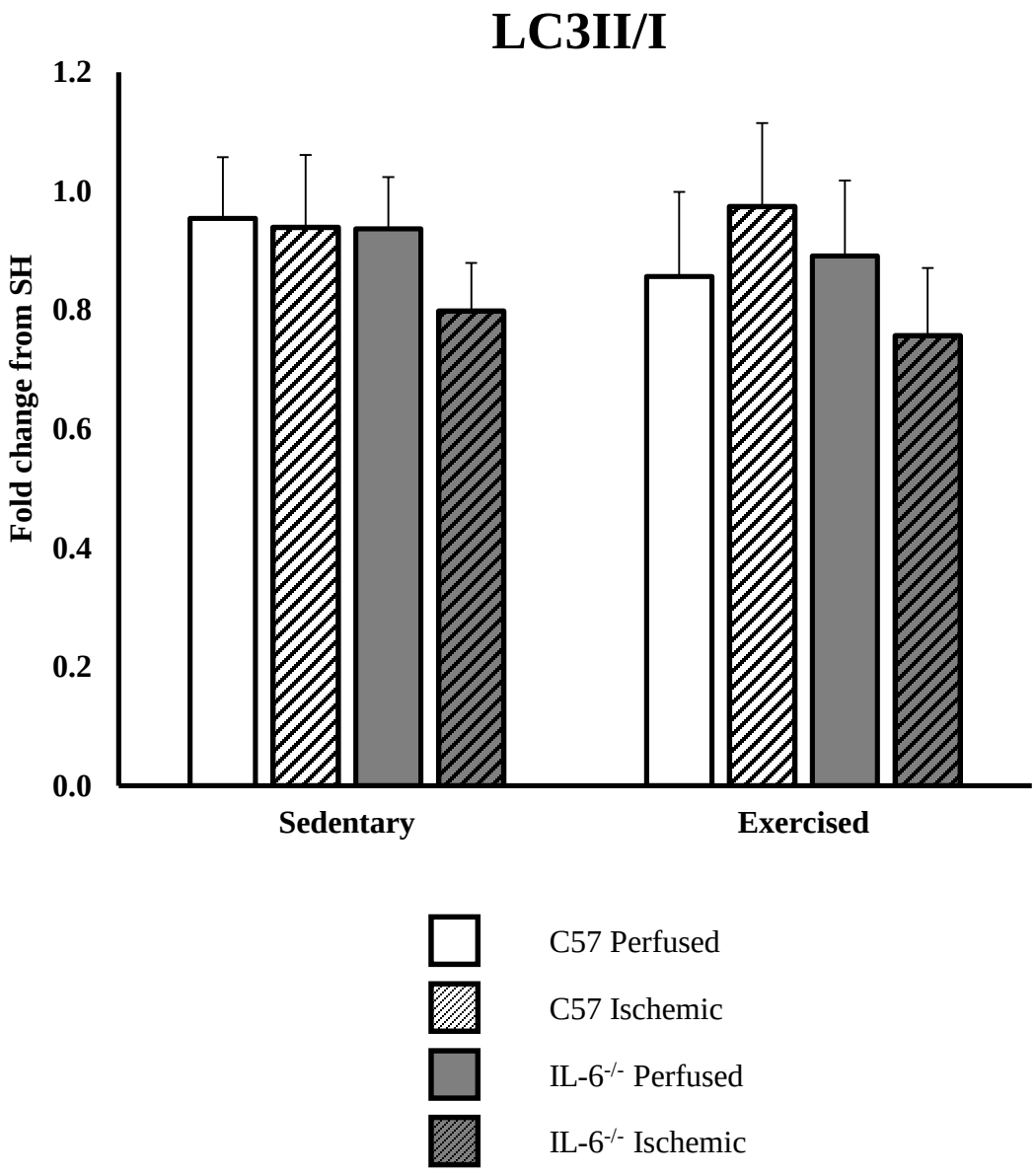

Heart Weight (mg)
Heart Weight (mg)
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
‡
Sedentary
Exercised

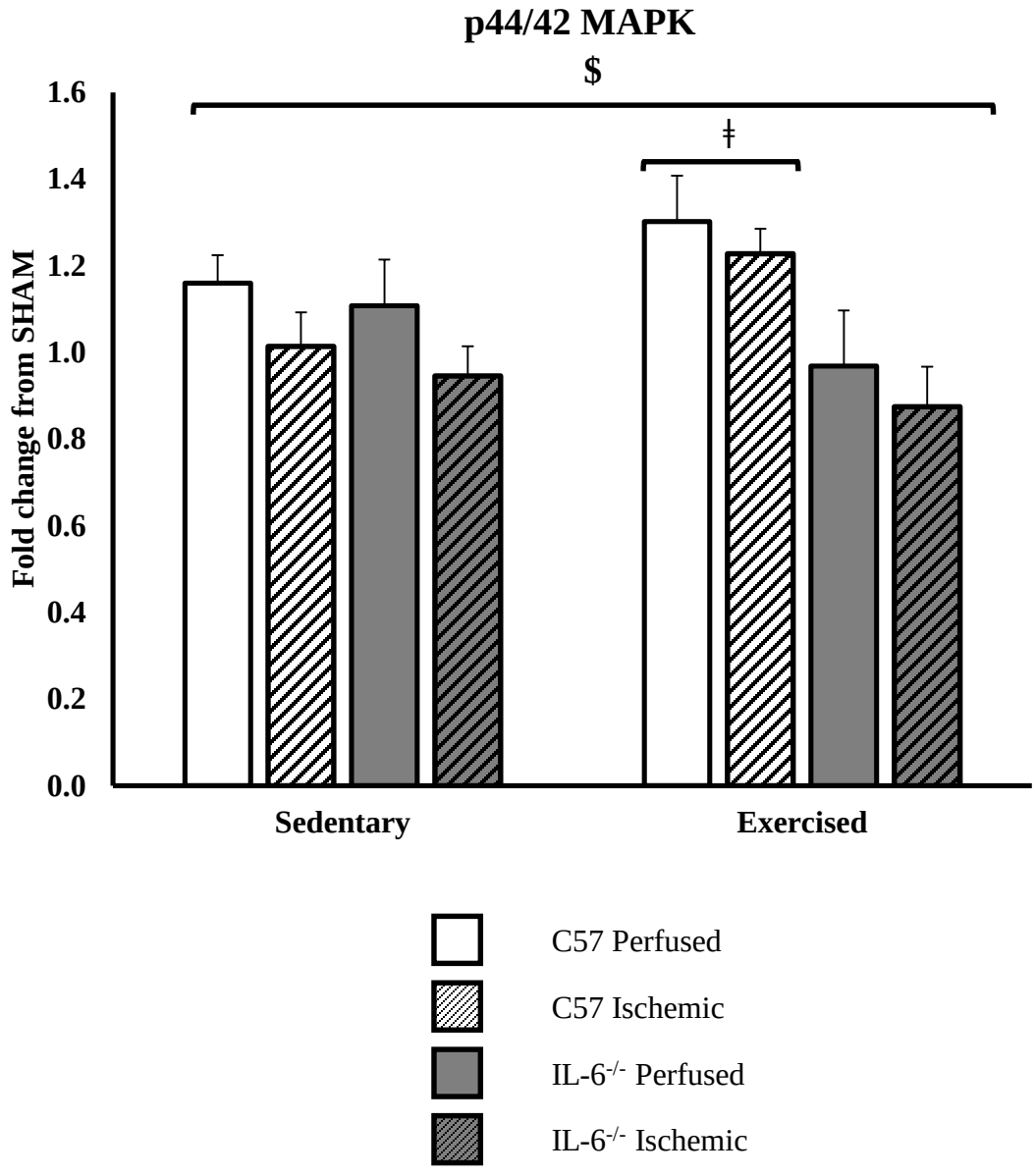

Heart Weight/Body Weight (mg/g)
Heart Weight/Body Weight (mg/g)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Sedentary
Exercised

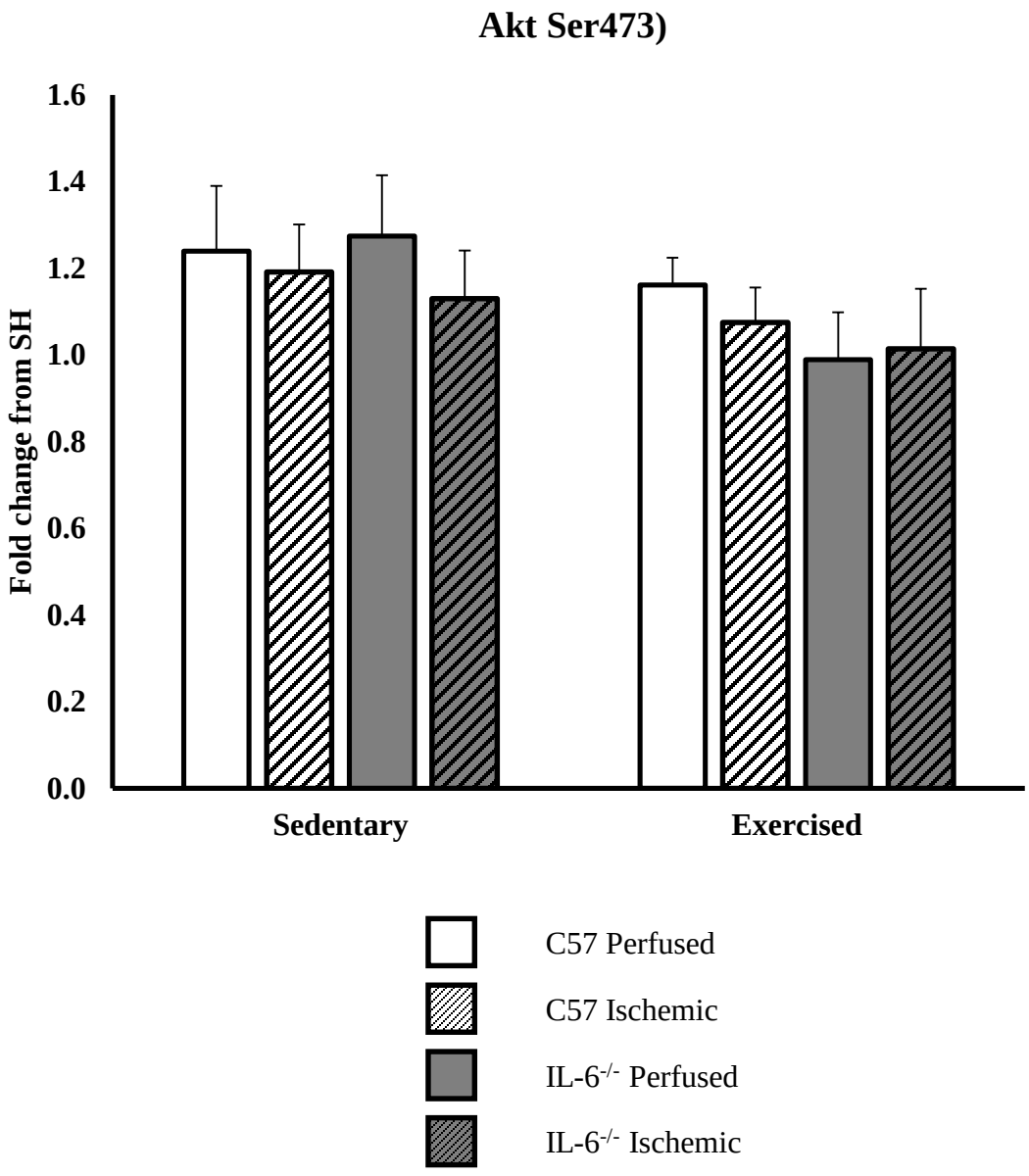

Atg3
#
*
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Fold change from SH
Sedentary
Exercise
C57 Perfused
C57 Ischemic
IL-6-/- Perfused
IL-6-/- Ischemic

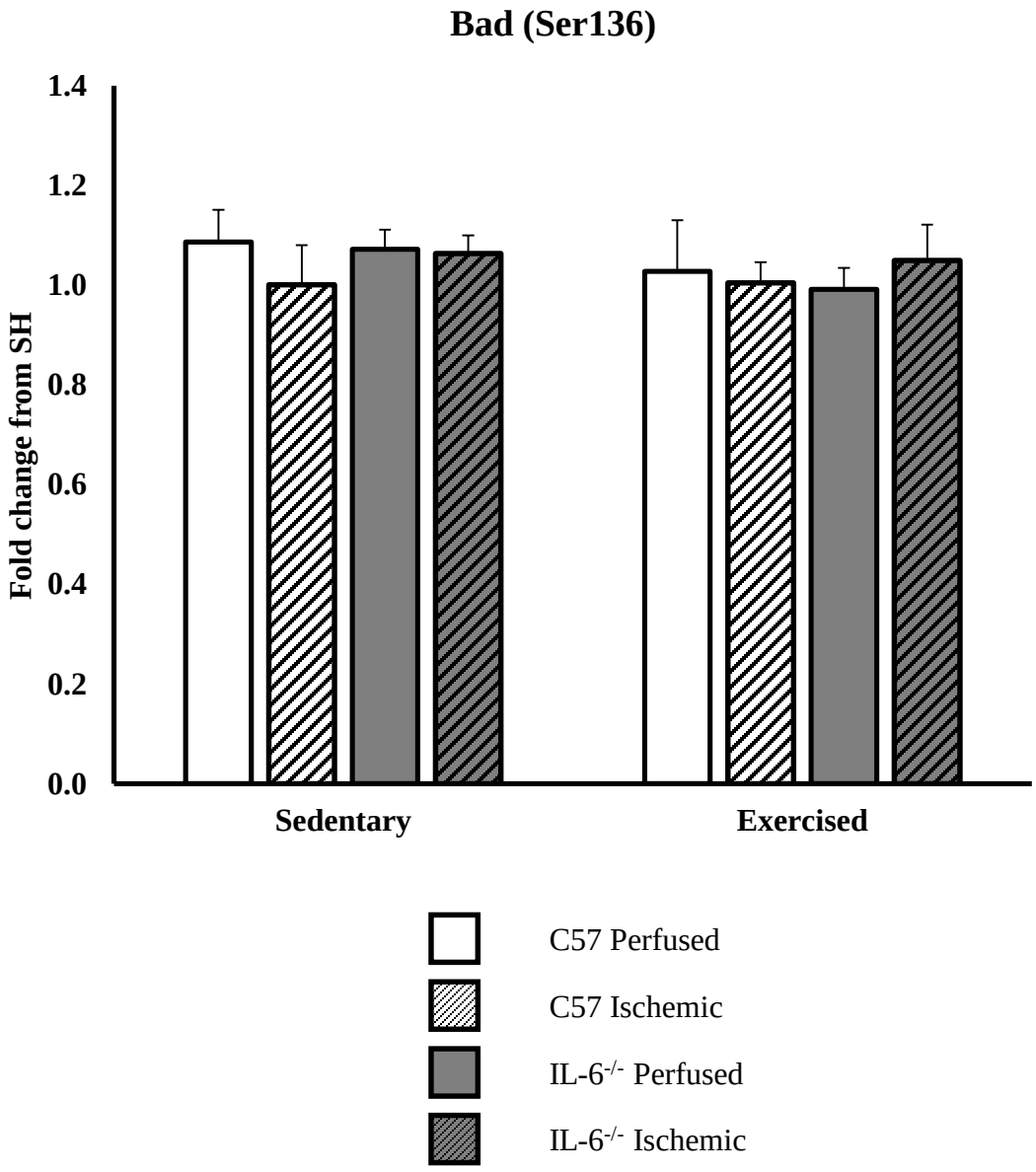

Atg5
Fold change from SH
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Sedentary
Exercise
C57 Perfused
C57 Ischemic
IL-6-/- Perfused
IL-6-/- Ischemic

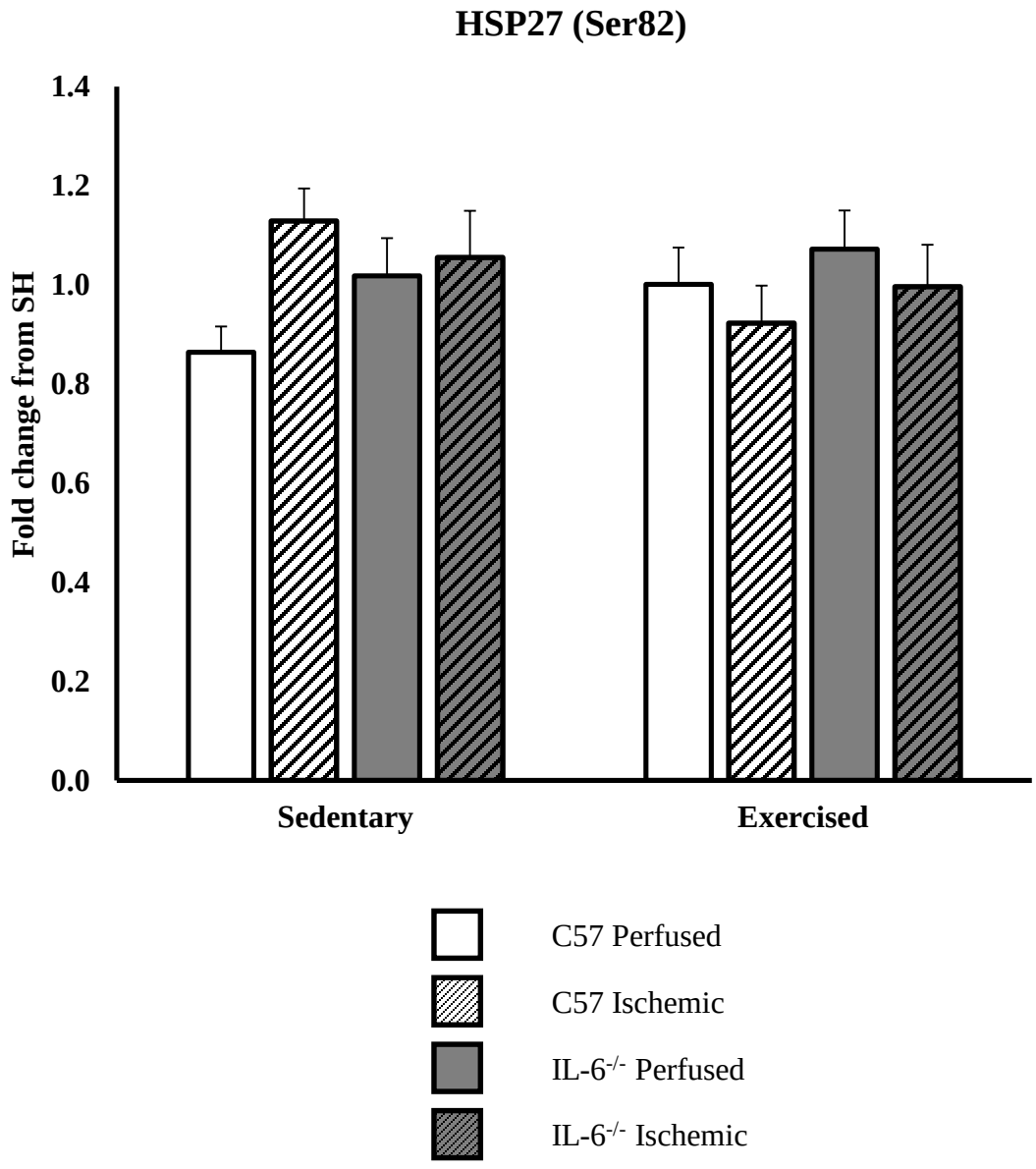

Beclin 1
Fold change from SH
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sedentary
Exercise
C57 Perfused
C57 Ischemic
IL-6-/- Perfused
IL-6-/- Ischemic

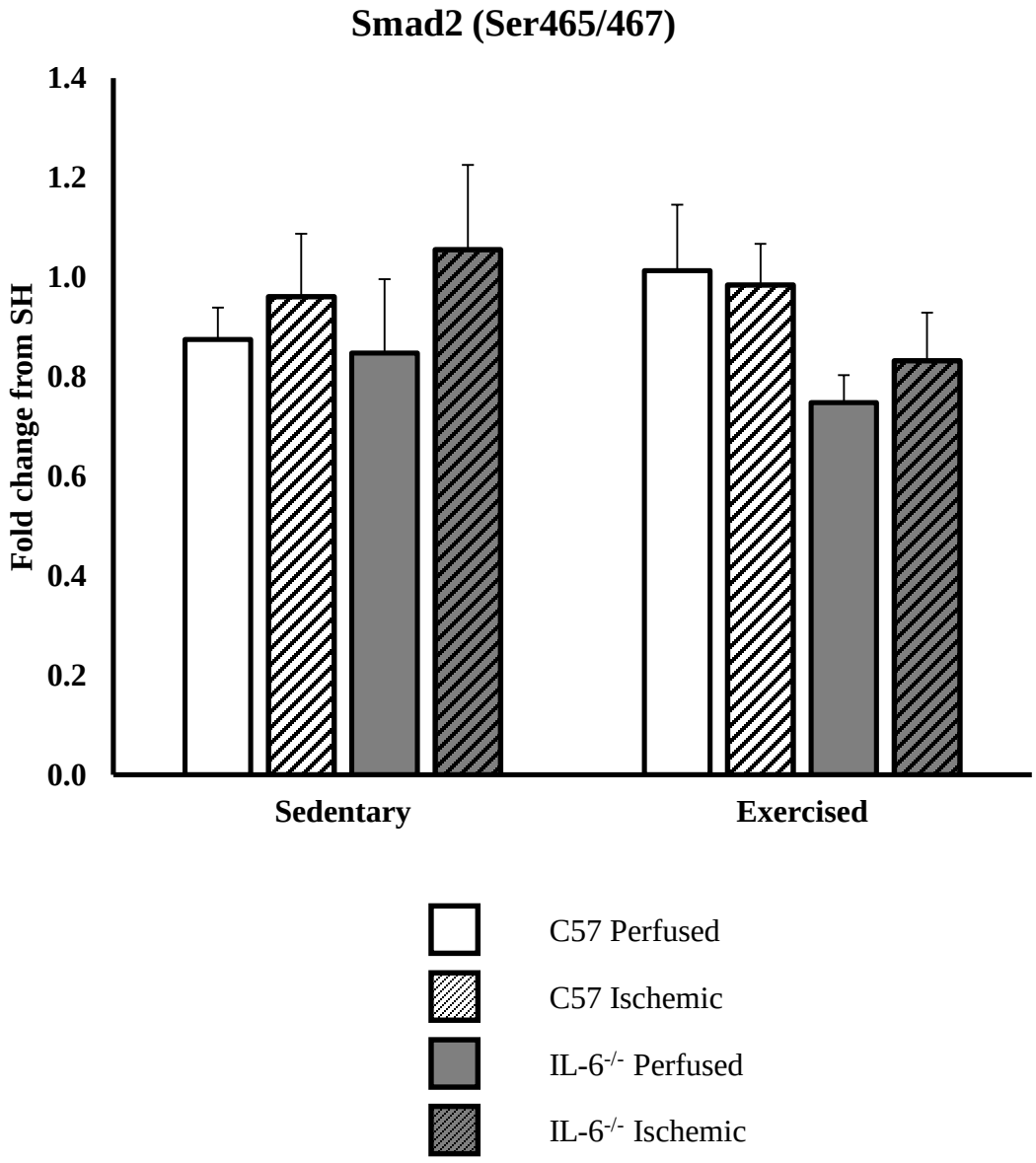

LC3II/I
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Fold change from SH
Sedentary
Exercised
C57 Perfused
C57 Ischemic
IL-6-/- Perfused
IL-6-/- Ischemic

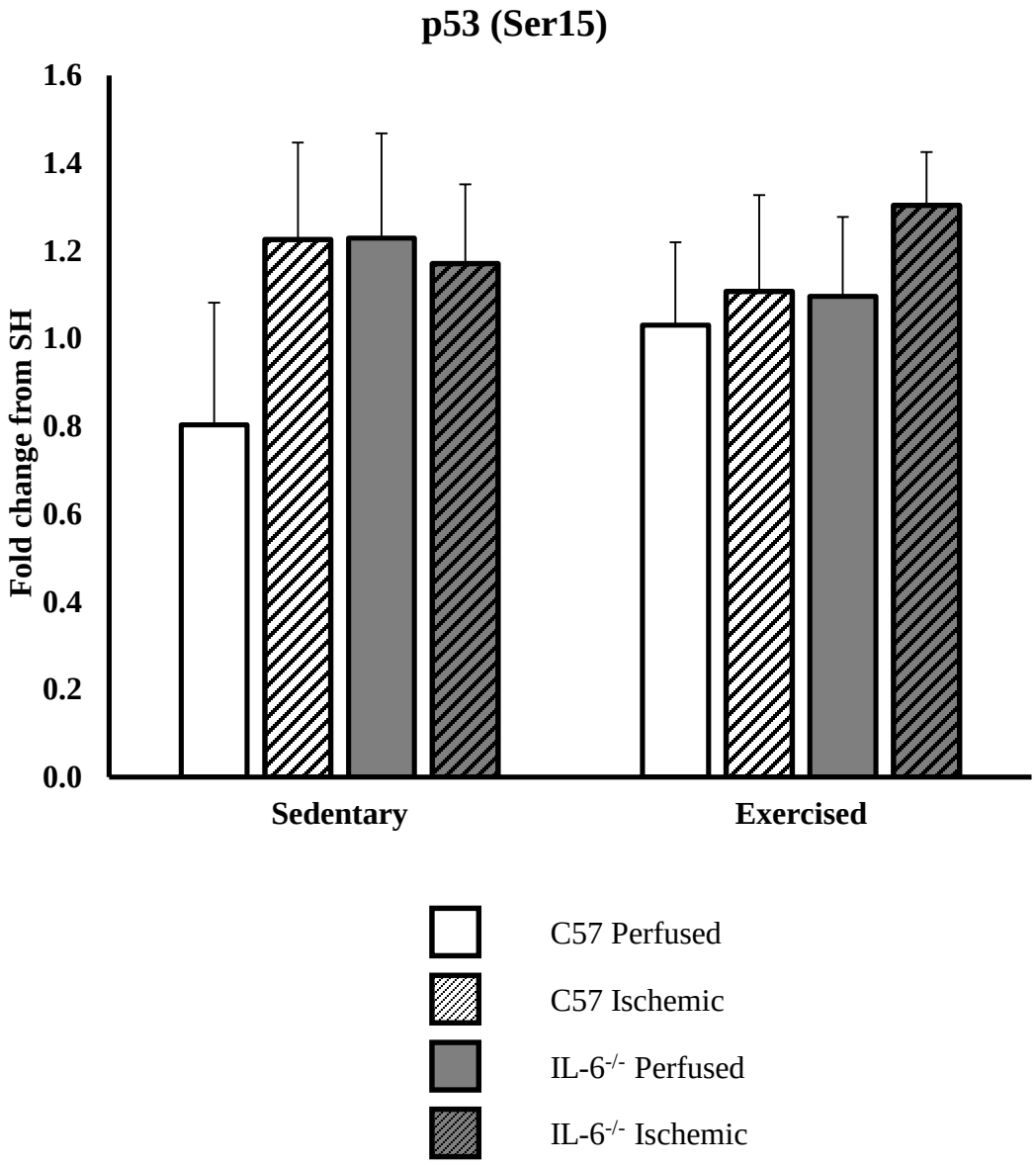

p44/42 MAPK
$
‡
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Fold change from SHAM
Sedentary
Exercised
C57 Perfused
C57 Ischemic
IL-6-/- Perfused
IL-6-/- Ischemic

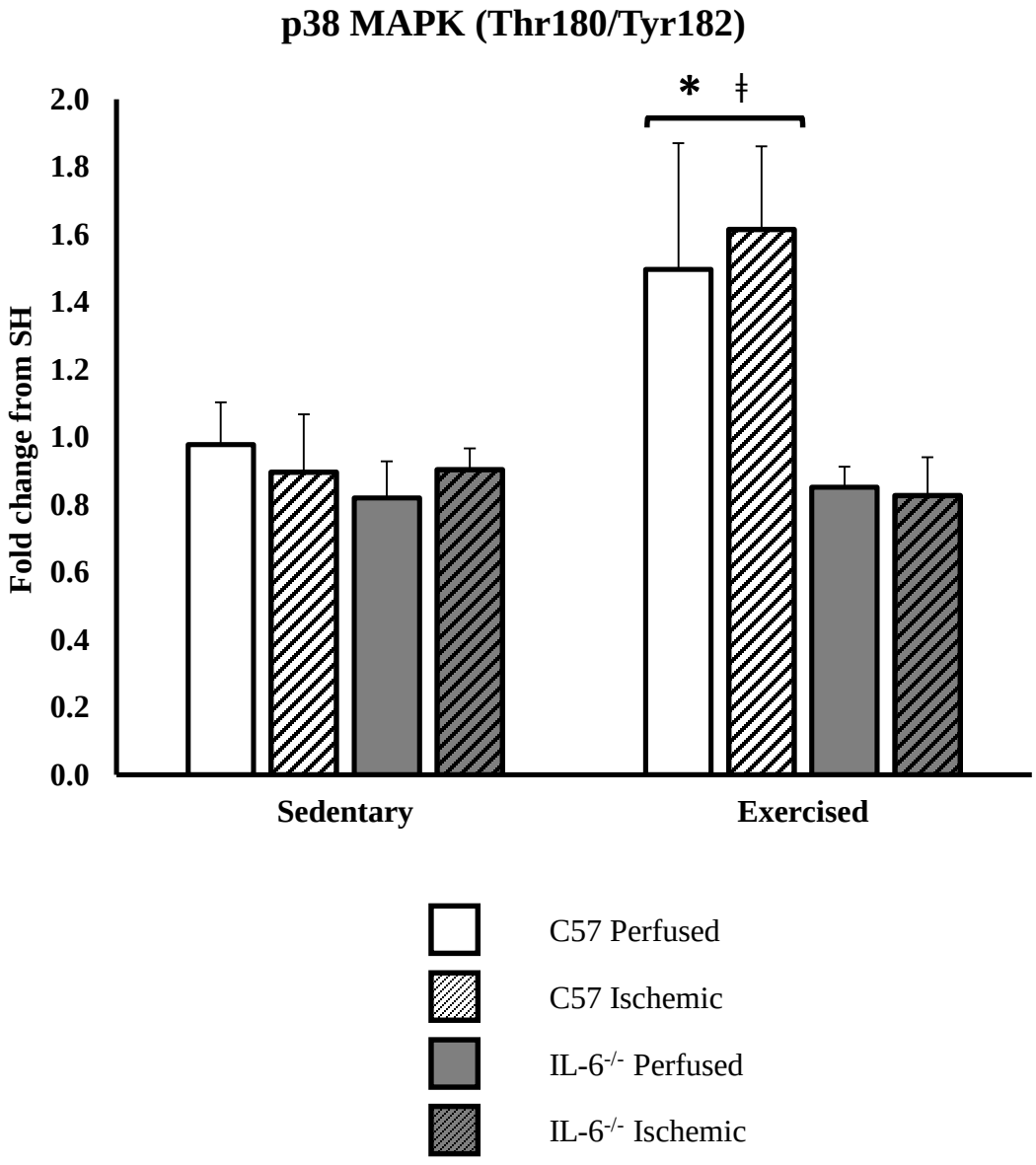

Akt Ser473)
Fold change from SH
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sedentary
Exercised
C57 Perfused
C57 Ischemic
IL-6-/- Perfused
IL-6-/- Ischemic

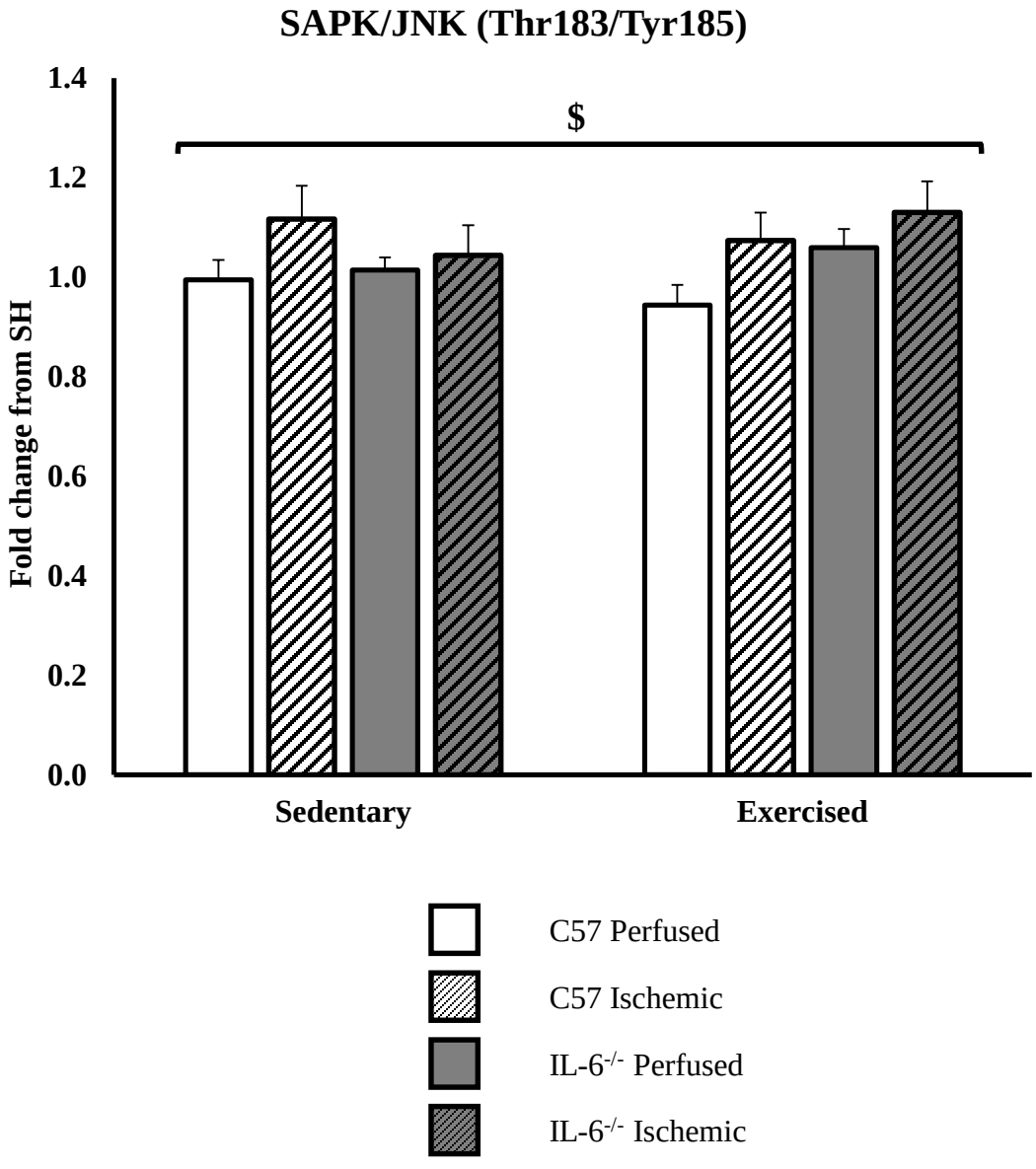

Bad (Ser136)
Fold change from SH
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sedentary
Exercised
C57 Perfused
C57 Ischemic
IL-6-/- Perfused
IL-6-/- Ischemic

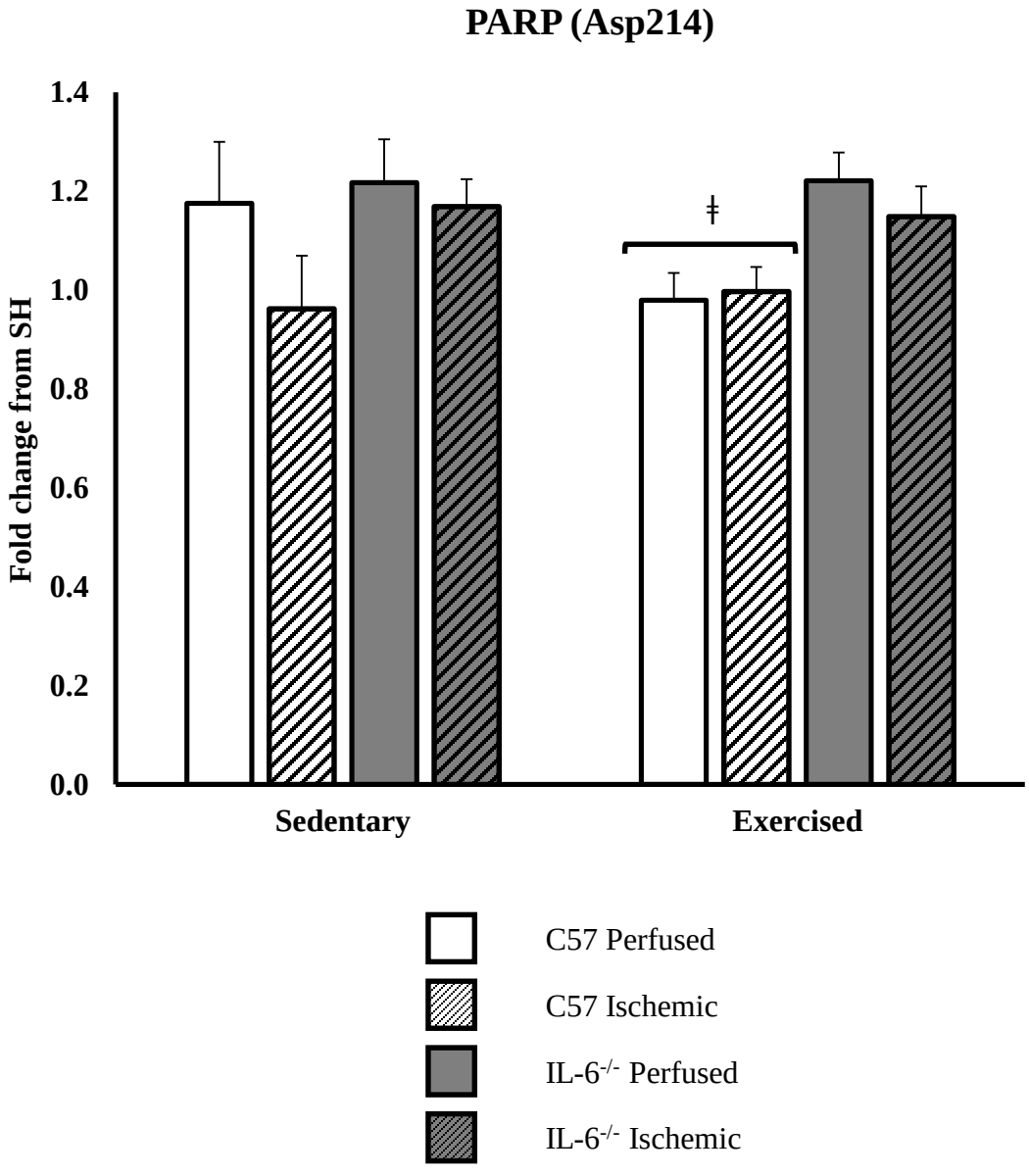

HSP27 (Ser82)
Fold change from SH
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sedentary
Exercised
C57 Perfused
C57 Ischemic
IL-6-/- Perfused
IL-6-/- Ischemic

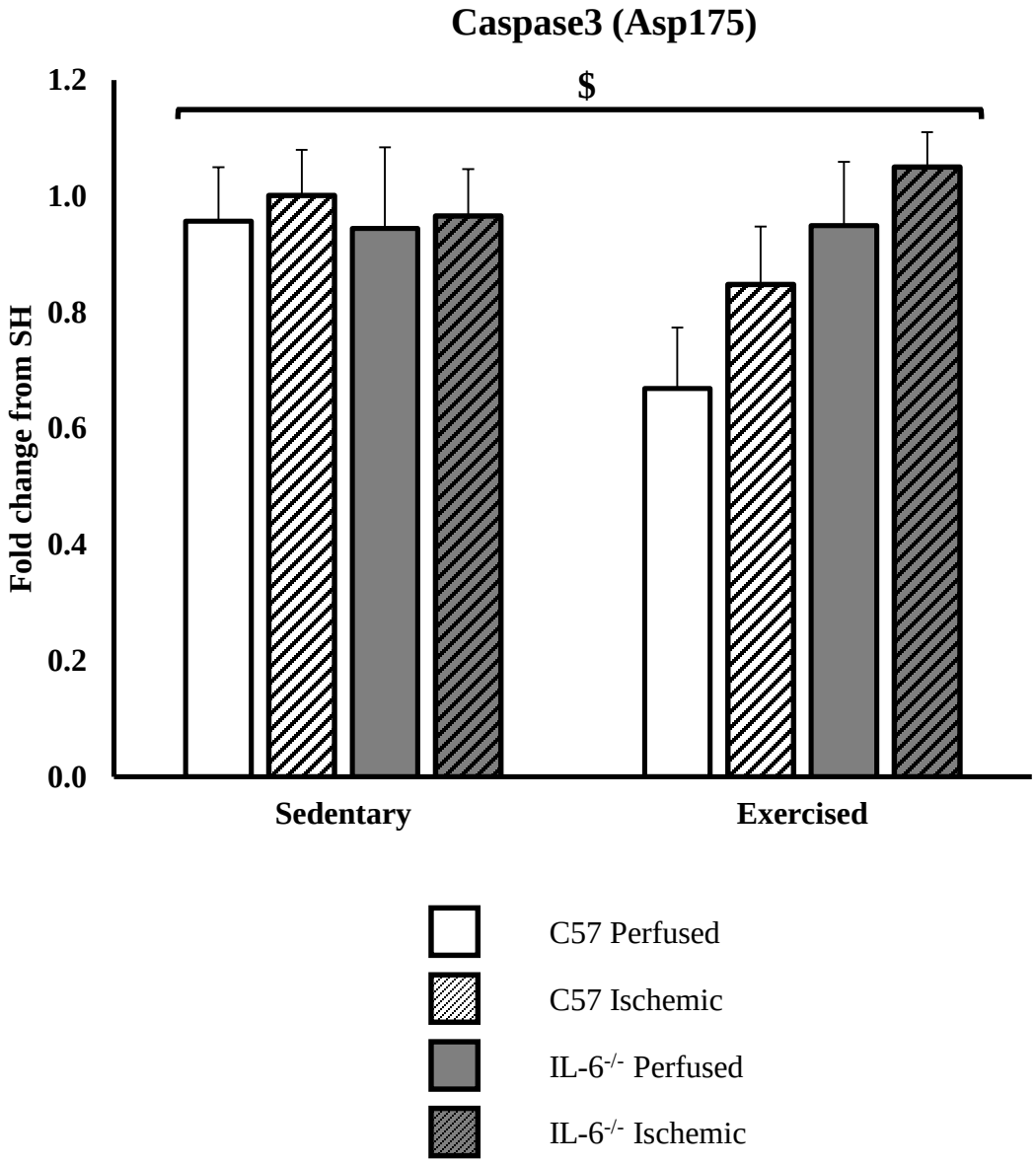

Smad2 (Ser465/467)
Fold change from SH
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sedentary
Exercised
C57 Perfused
C57 Ischemic
IL-6-/- Perfused
IL-6-/- Ischemic

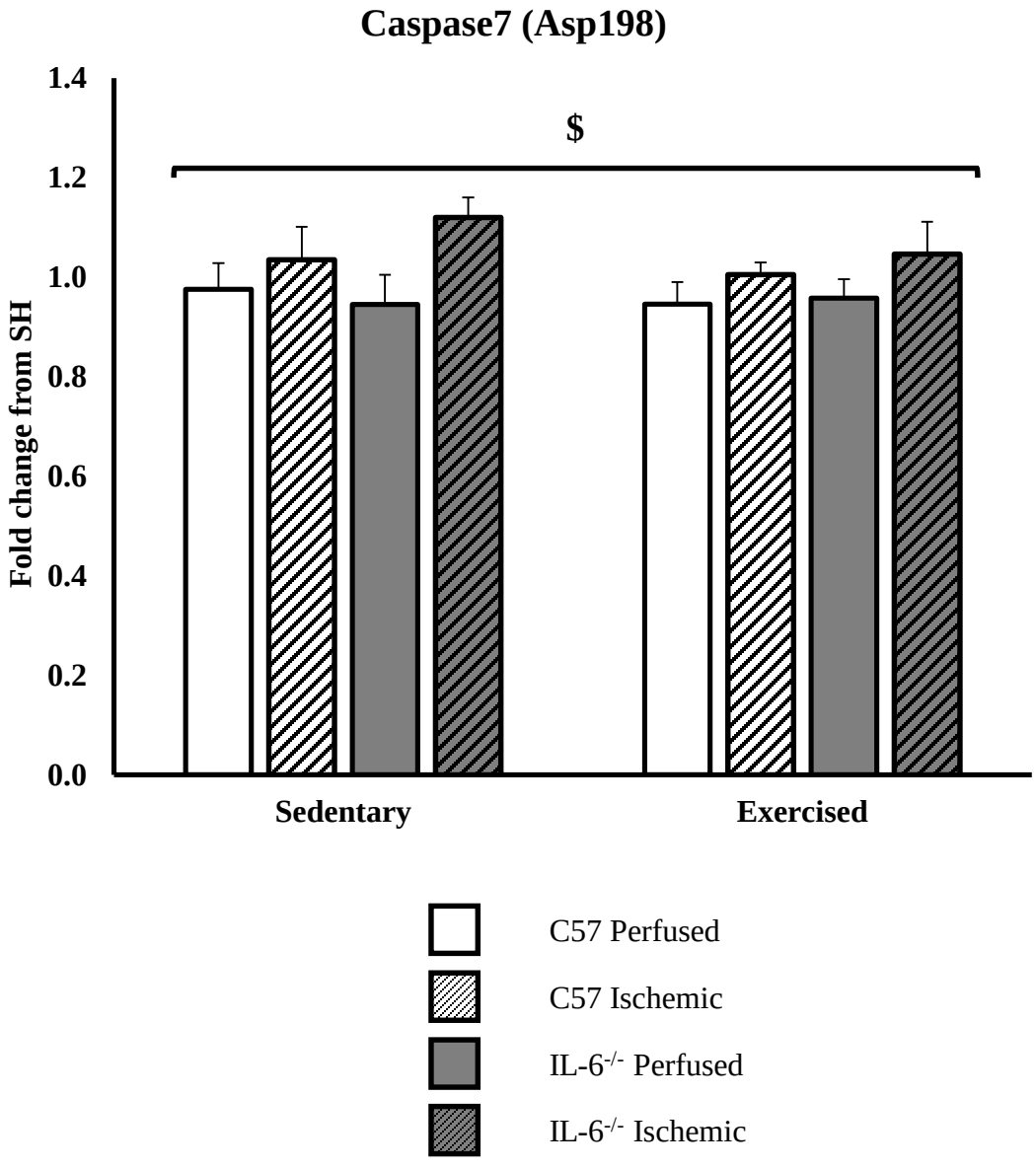

p53 (Ser15)
Fold change from SH
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sedentary
Exercised
C57 Perfused
C57 Ischemic
IL-6-/- Perfused
IL-6-/- Ischemic

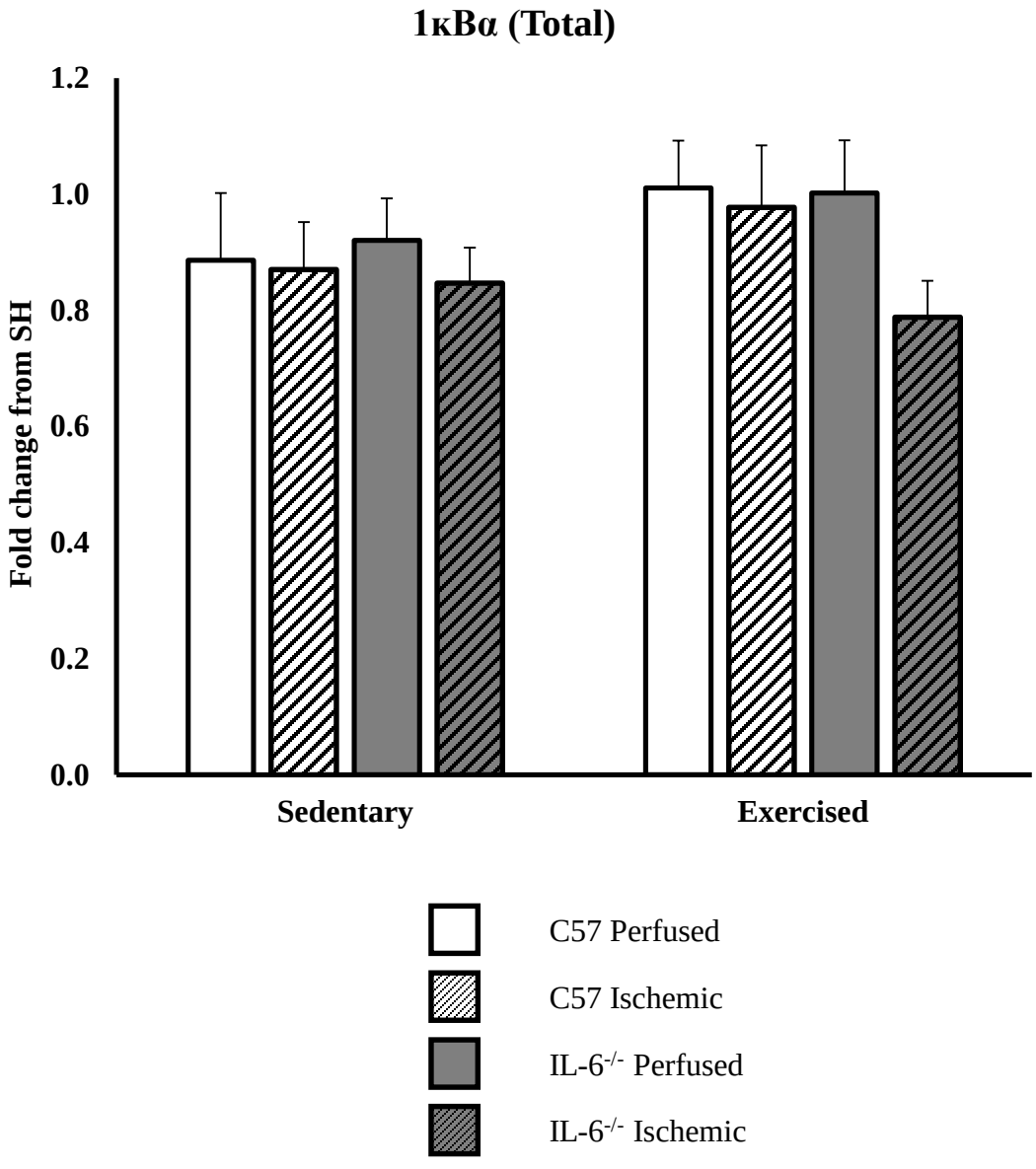

p38 MAPK (Thr180/Tyr182)
* ‡
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Fold change from SH
Sedentary
Exercised
C57 Perfused
C57 Ischemic
IL-6-/- Perfused
IL-6-/- Ischemic

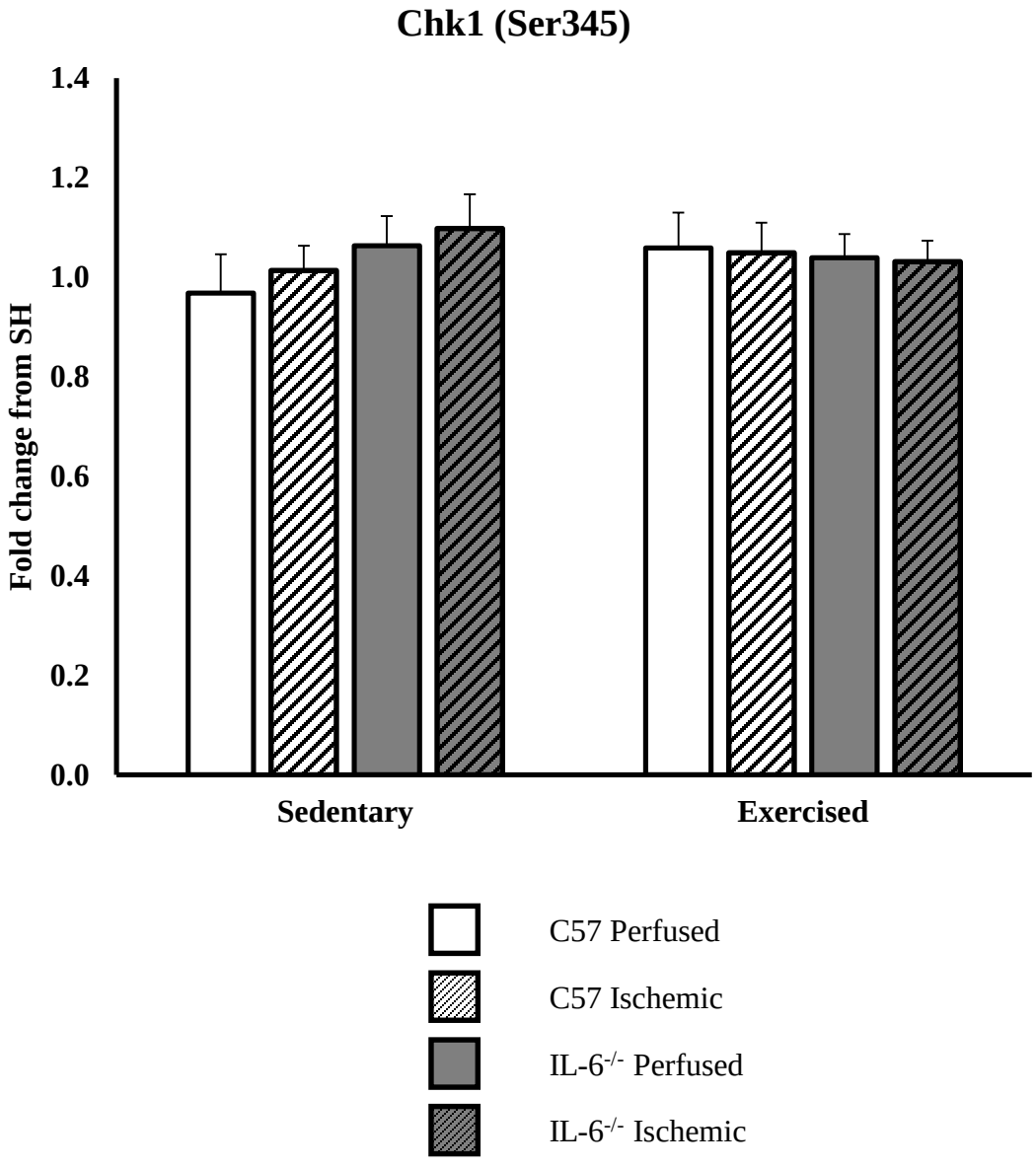

SAPK/JNK (Thr183/Tyr185)
Fold change from SH
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
$
Sedentary
Exercised
C57 Perfused
C57 Ischemic
IL-6-/- Perfused
IL-6-/- Ischemic

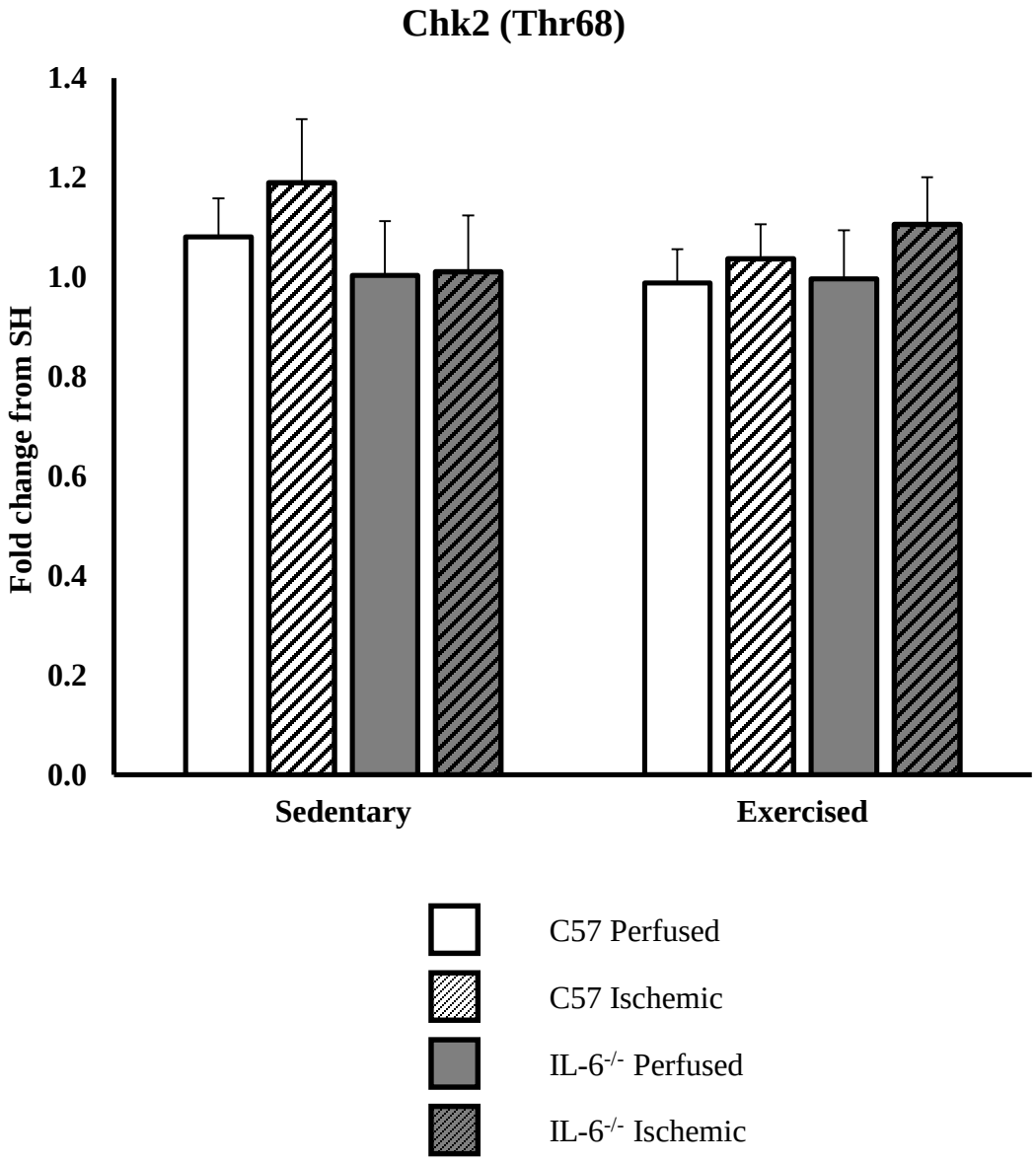

PARP (Asp214)
Fold change from SH
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
‡
Sedentary
Exercised
C57 Perfused
C57 Ischemic
IL-6-/- Perfused
IL-6-/- Ischemic

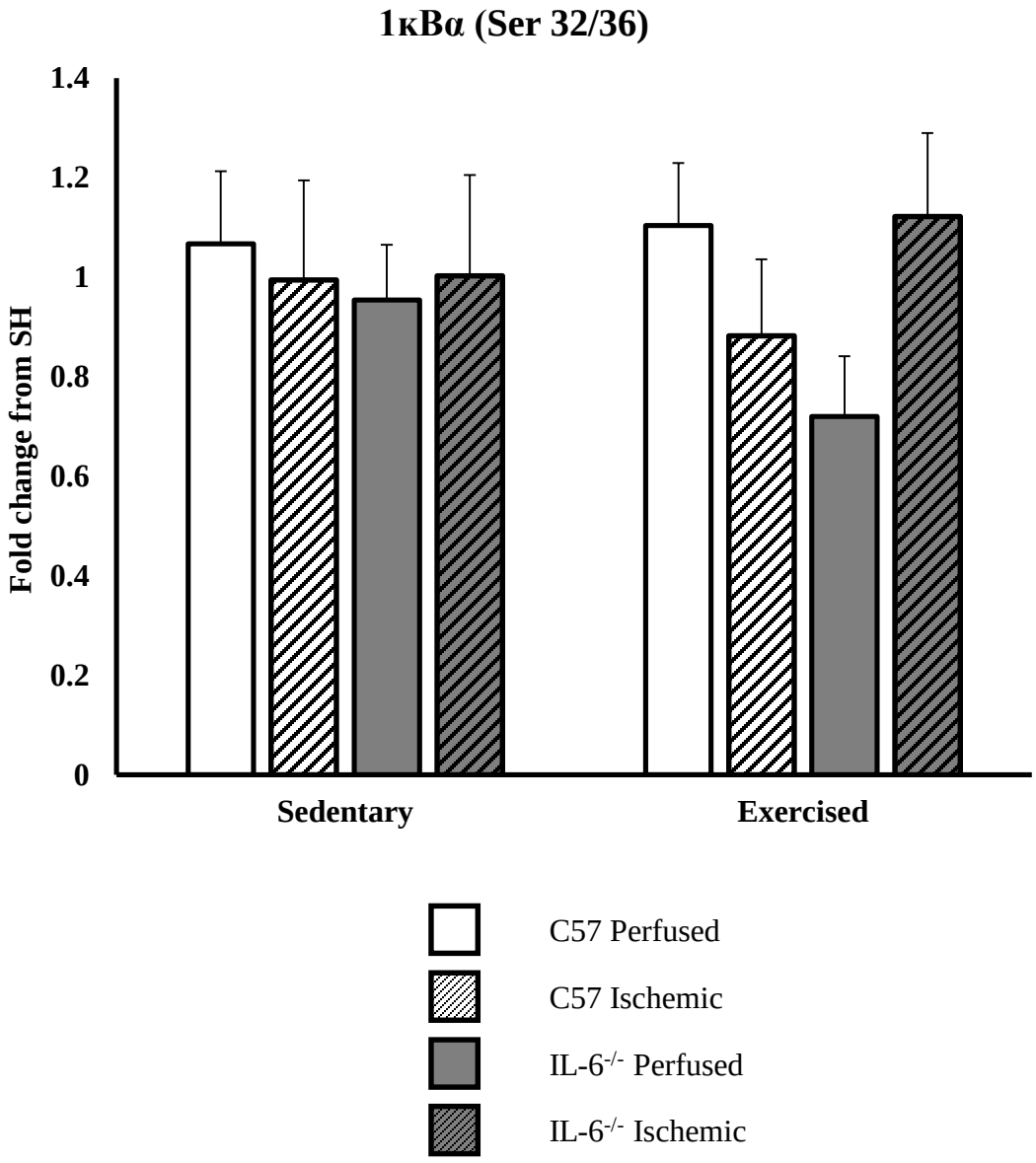

Caspase3 (Asp175)
$
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Fold change from SH
Sedentary
Exercised
C57 Perfused
C57 Ischemic
IL-6-/- Perfused
IL-6-/- Ischemic

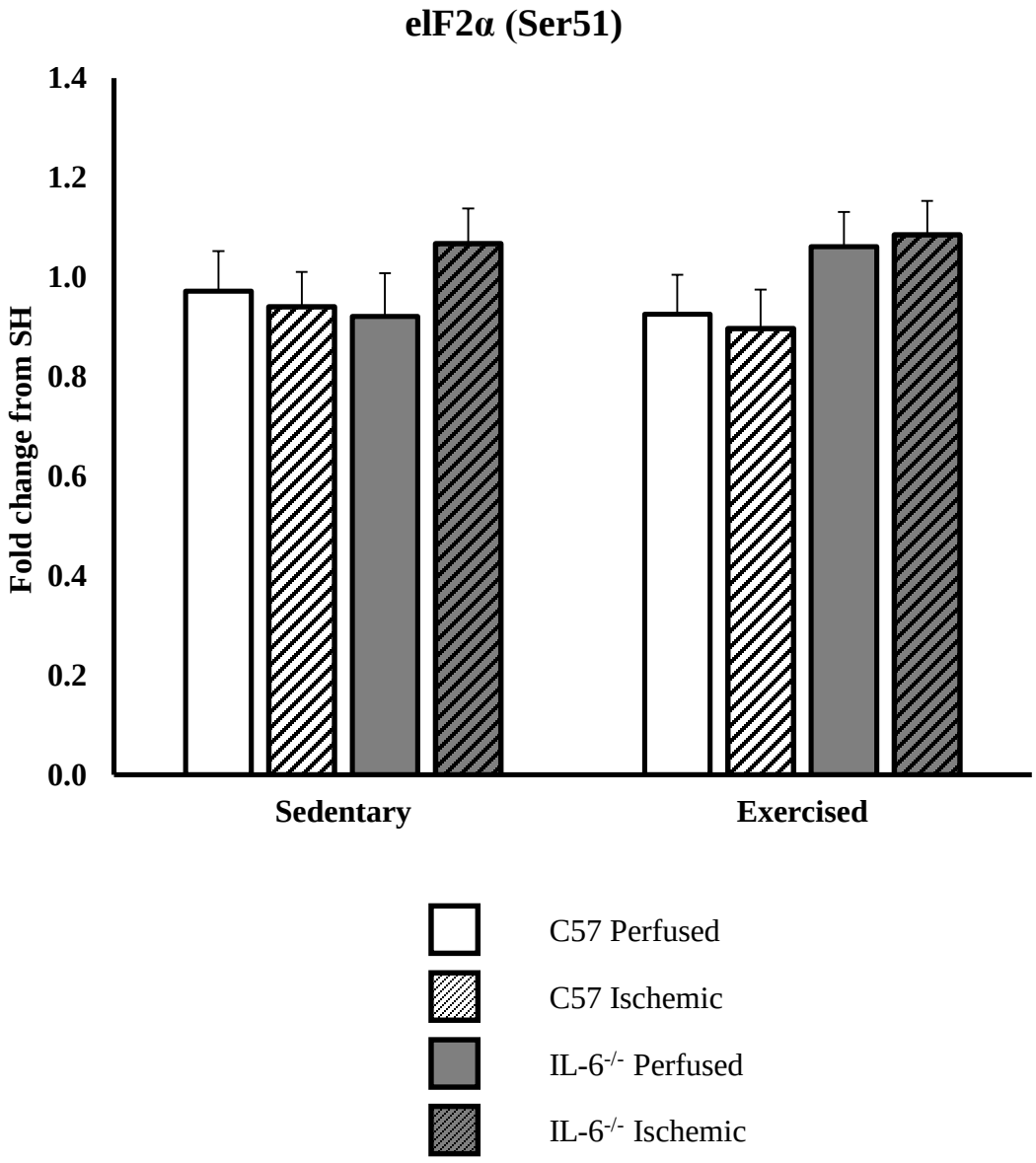

Caspase7 (Asp198)
$
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Fold change from SH
Sedentary
Exercised
C57 Perfused
C57 Ischemic
IL-6-/- Perfused
IL-6-/- Ischemic

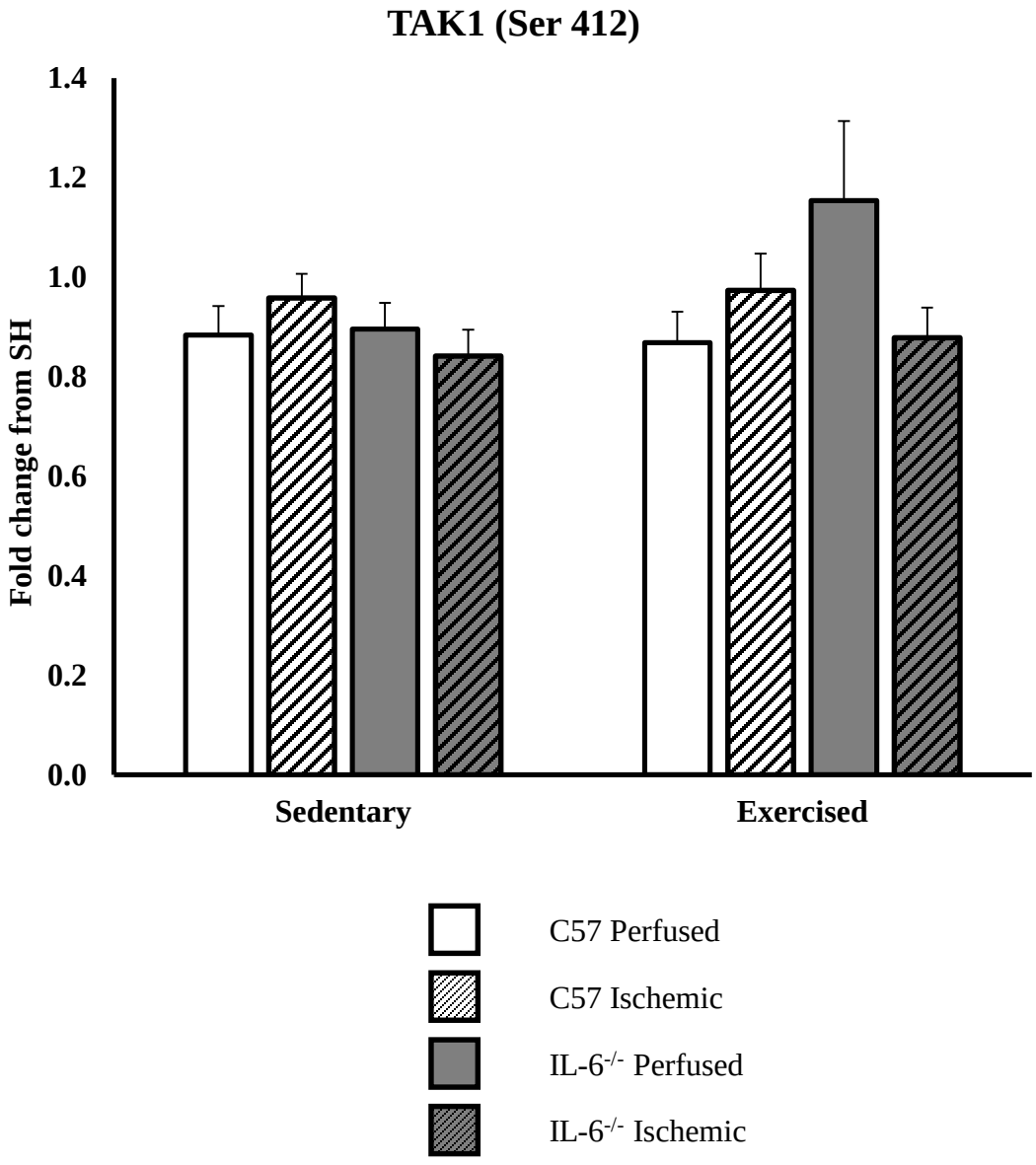

1κBα (Total)
Fold change from SH
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sedentary
Exercised
C57 Perfused
C57 Ischemic
IL-6-/- Perfused
IL-6-/- Ischemic

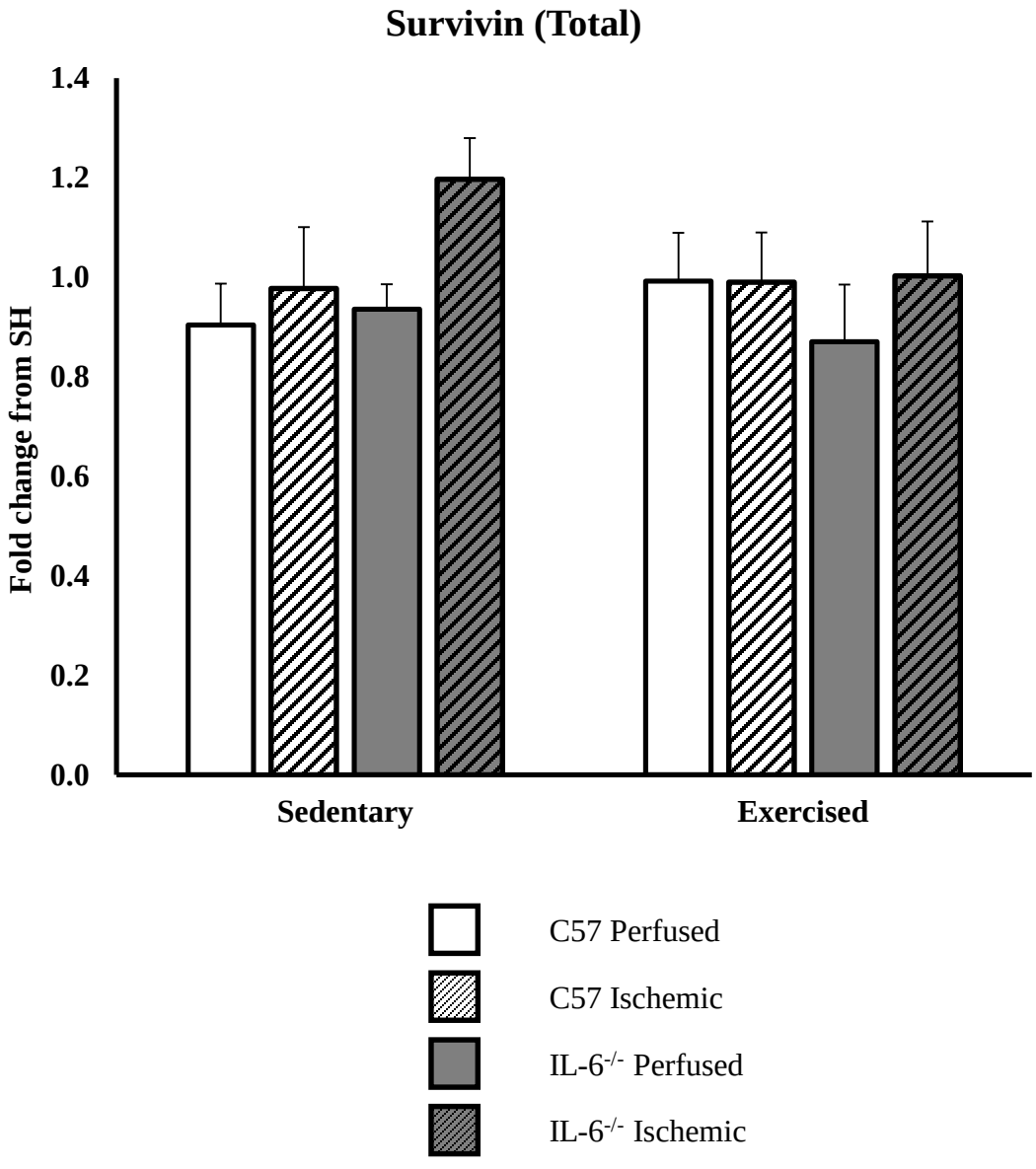

Chk1 (Ser345)
Fold change from SH
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sedentary
Exercised
C57 Perfused
C57 Ischemic
IL-6-/- Perfused
IL-6-/- Ischemic

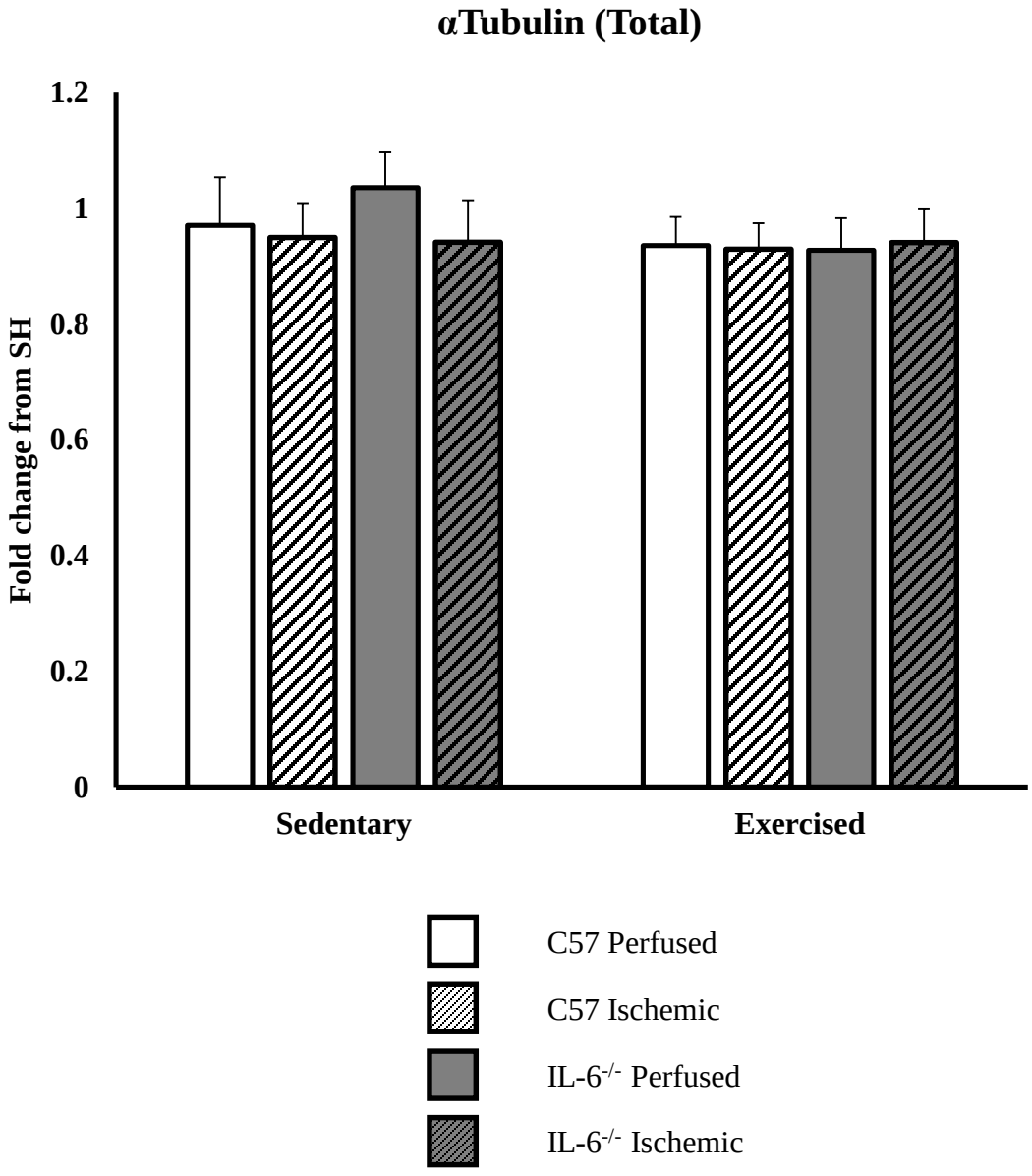

Chk2 (Thr68)
Fold change from SH
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sedentary
Exercised
C57 Perfused
C57 Ischemic
IL-6-/- Perfused
IL-6-/- Ischemic

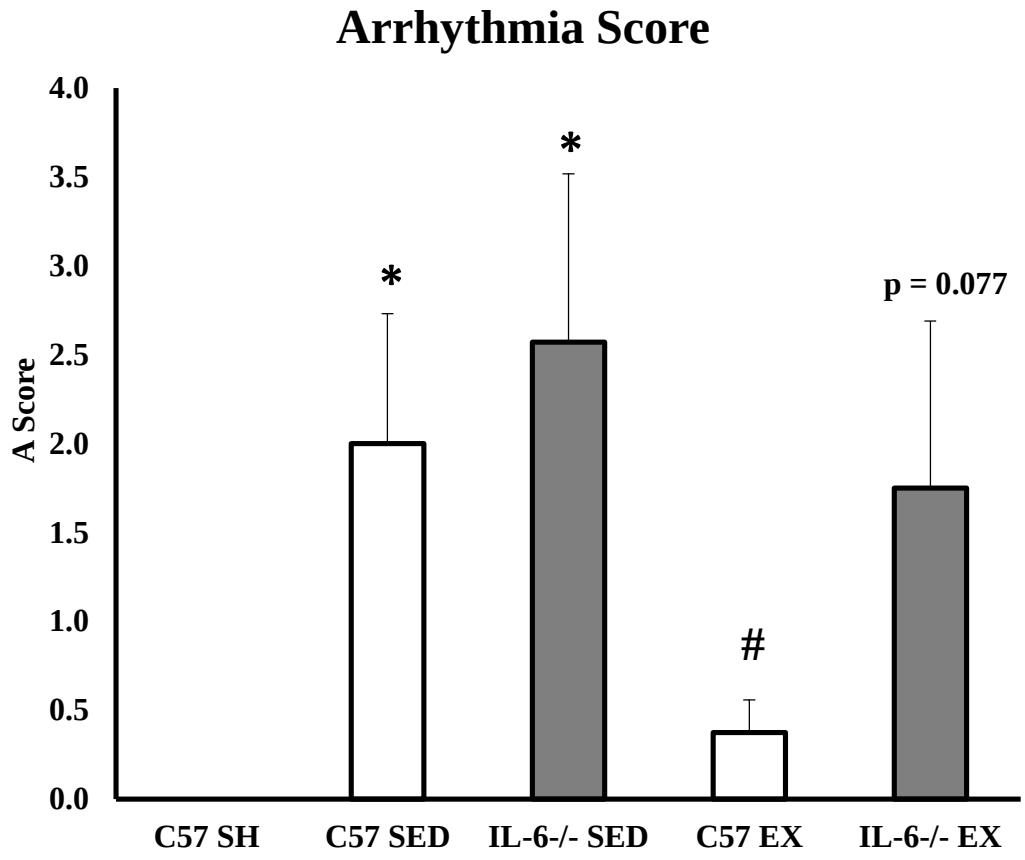

1κBα (Ser 32/36)
Fold change from SH
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Sedentary
Exercised
C57 Perfused
C57 Ischemic
IL-6-/- Perfused
IL-6-/- Ischemic


elF2α (Ser51)
Fold change from SH
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sedentary
Exercised
C57 Perfused
C57 Ischemic
IL-6-/- Perfused
IL-6-/- Ischemic


TAK1 (Ser 412)
Fold change from SH
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sedentary
Exercised
C57 Perfused
C57 Ischemic
IL-6-/- Perfused
IL-6-/- Ischemic

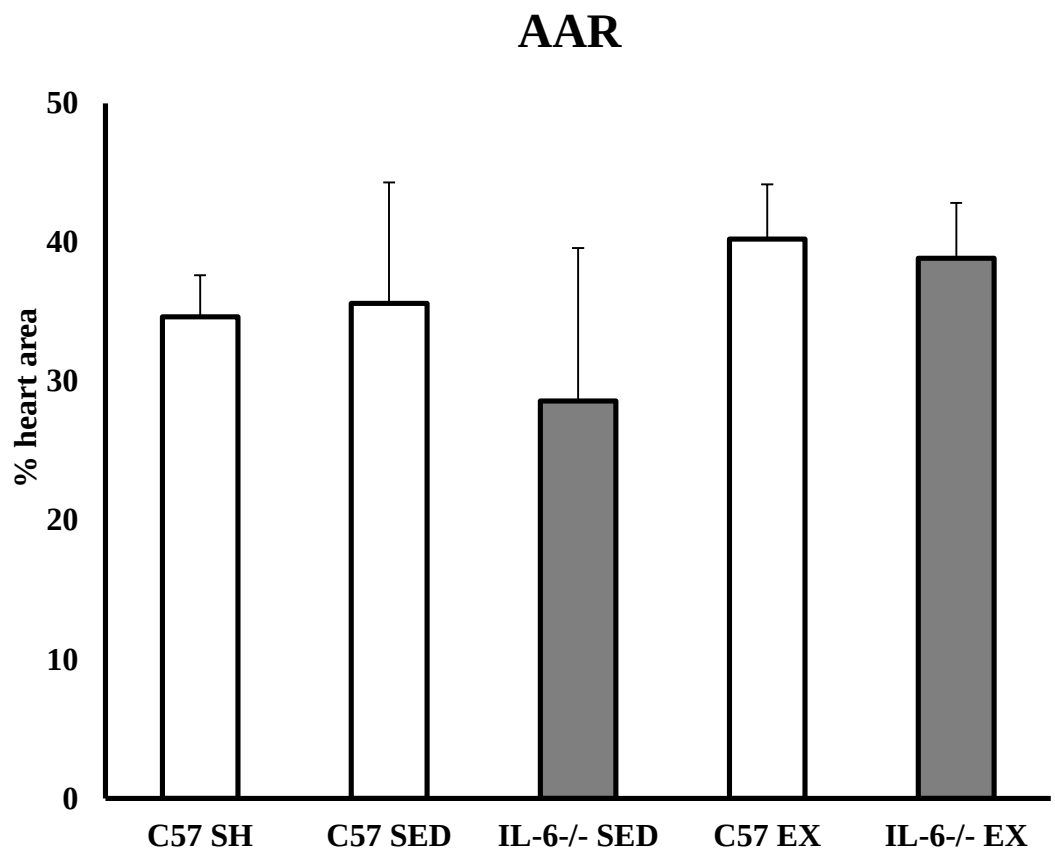

Survivin (Total)
Fold change from SH
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sedentary
Exercised
C57 Perfused
C57 Ischemic
IL-6-/- Perfused
IL-6-/- Ischemic

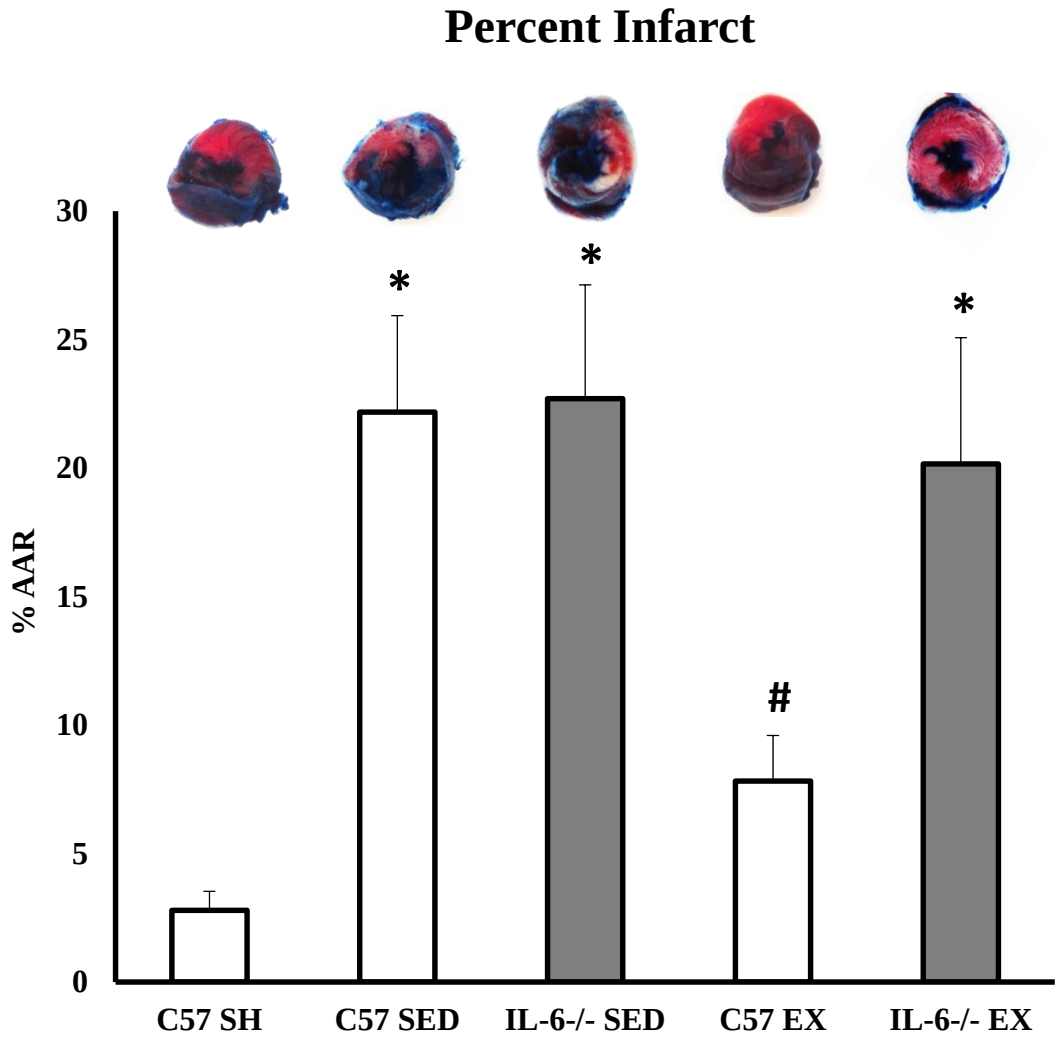

αTubulin (Total)
Fold change from SH
1.2
1
0.8
0.6
0.4
0.2
0
Sedentary
Exercised
C57 Perfused
C57 Ischemic
IL-6-/- Perfused
IL-6-/- Ischemic



***** **different from SHAM**

**different from SED**


AAR
% heart area
50
40
30
20
10
0
C57 SH
C57 SED
IL-6-/- SED
C57 EX
IL-6-/- EX



∗ different from SHAM

different from SED