IMPACT OF INTRINSIC AEROBIC EXERCISE CAPACITY ON CARDIOVASCULAR OUTCOMES FOLLOWING VARIOUS FORMS OF CARDIOVASCULAR STRESS

by

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Cardiovascular diseases (CVD) remain the major leading cause of death in the western world. Therefore, several approaches to achieve cardioprotection against any cardiovascular insult have been explored and to date, the only practical and justifiable countermeasure capable of providing cardioprotection is regular sessions of endurance exercise. However, active exercise programs, while consistently beneficial, still generate a very diverse response, suggesting the potential for critical contribution from the genetic composition determining intrinsic aerobic exercise capacity. It has been estimated that up to 60-70% of the variation in exercise capacity is due to the genetic component. This heterogeneous response elaborates the importance of investigating the influence of intrinsic exercise capacity alone (independently from any other external factors such as exercise training) on the vascular, metabolic and myocyte adaptive responses following acute, chronic, or even to non-cardiovascular stress. Intrinsic capacity can be studied using high (HCR) and low (LCR) aerobic running capacity rat strains. The phenotypes differ by more than 5-fold in sedentary average running distance and time. The selection process used to develop these animals has generated a shift in metabolic and cardiovascular risk factors

between the two strains. LCR rats have accumulated cardiovascular risk factors, such as a large gain in visceral adiposity, hypertension, dyslipidemia, impaired glucose tolerance, endothelial dysfunction, hyperglycemia, hypertriglyceridemia, insulin resistance and elevated plasma free fatty acids. LCR phenotype scored high on cardiovascular risk factors and the HCR score high for health factors. Therefore, we tested the hypothesis that LCR phenotype will greater alterations in vascular, metabolic, and cardiac adaptive responses to various forms of cardiovascular stress, including doxorubin-induced cardiotoxicity, and ischemia reperfusion injury *in vivo* and *ex vivo*. We found that phenotypic vascular reactivity differences presented in males but not females and females had higher responses to vasoconstriction (endothelin 1) and vasodilation agents (Ach) in both strains. Aged HCR and LCR animals treated with Doxorubicin did not differ in resting cardiac function. However, HCRs decreased ejection fraction (lost contractility) and increased heart rate (reflex tachycardia) 10 days after Dox, but LCRs did neither. Moreover, LCRs had significantly higher mitochondrial respiration after Dox while HCRs appeared to have been physiologically primed and therefore did not exhibit an adaptive compensatory response. With 30 min coronary artery ligation followed by 2 hr. reperfusion, there was no differences in infarct size between HCRs and LCRs, but males in both phenotypes showed larger infractions than females. However, with 15 min coronary artery ligation followed by 2 hr. reperfusion, HCRs showed smaller myocardial infarct size comparing to LCR counterparts. The sex differences were maintained in both strains with different time frames of coronary ligation. In acute ischemic reperfusion (IR) study, cardiac mitochondria from HCR were significantly higher in control conditions with each substrate tested. However, after IR insult, the cardiac mitochondrial respiratory rates were similar between phenotypes, as was infarct size. In these post IR fibroblast/myofibroblasts, LCRs showed significantly higher gene expression in collagen I, lower

collagen III and higher collagen I/collagen III ratio. Furthermore, LCRs showed lower expression of extracellular matrix regulatory genes including MMP2, TIMP1, and SMAD5 compared to HCRs counterparts. BMPR2, Agtr1a, also were both decreased, and the anti-apoptotic gene, Bag3, was significantly higher in LCRs. Moreover, The Angiotensin receptor pathway is responsive only in post-ischemic LCR cardiac fibroblasts, and the BMP receptor pathway is responsive only in post-ischemic HCR cardiac fibroblasts.

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by

Musaad Bedah Alsahly

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DEDICATION

This dissertation is lovingly dedicated to my great parents, Mr. Bedah Alsahly & Ms. Norah Alajlan; my lovely wife, Ghadeer, and my brothers and sisters. Without their support, prayers, care and love this work would not have been possible.

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LIST OF SYMBOLS OR ABBREVIATIONS

α-SMA: Alpha-smooth muscle actin

5-HT: Serotonin

AAR: Area at risk

ACH: Acetylcholine

ADP: Adenosine diphosphate

AngII: Angiotensin II

ATP: Adenosine triphosphate

BAG3: Bcl-2–associated athanogene 3

BMPs: Bone morphogenetic proteins

BSA: Bovine serum albumin

CF: Coronary flow

CFBs: Cardiac fibroblasts

CK: Creatine kinase

Col I: Collagen I

Col III: Collagen III

Cr: Creatine

CSA: Citrate synthase activity

CVS: Cardiovascular diseases

DOX: Doxorubicin

ET-1: Endothelin-1

FAO: Fatty acid oxidation

FCCP: Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone

FFA: Free fatty acid

FSK: Forskolin

Glut: Glutamate

H&E: Hematoxylin and eosin

HA1077: Inhibitor of Rho-kinase

HCR: High capacity runner

IA: Infarcted area

IR: Ischemic Reperfusion

KHB: Krebs-Henseleit buffer

LCR: Low capacity runner

LV: Left ventricle

MI: Myocardial infarction

MITO: Mitochondria

MMPs: Matrix metalloproteinases

MOPS: 2; 3-[N-morpholino]-propane sulfonic acid

mPTP: Mitochondrial permeability transition pore

NE: Norepinephrine

NIH: National Institutes of Health

NO: nitric oxide

Oct: Octanyl carnitine

P/M: Pyruvate/Malate

Pcr: Phosphocreatine

PE: Phenylephrine

PMN: Polymorphonuclear leukocytes

PSS: Physiological Salt Solution

RISK: Reperfusion injury salvage kinase

ROS: Reactive oxygen species

SNP: Sodium nitroprusside

Succ: Succinate

TA: Total area

TBS-T: Tris buffered saline containing 0.1% Tween

TGFβ: Transforming growth factor beta

TIMPs: Tissue inhibitor of metalloproteinases

TNF-α: Tumor necrosis factor alpha

TTC: Triphenyltetrazolium chloride

CHAPTER 1: INTRODUCTION TO IMPACT OF INTRINCIC AEROBIC EXERCISE CAPACITY ON CARDIOVASCULAR OUTCOMES FOLLOWING VARIOUS FORM OF CARDIOVASCULAR STRESS

Problem and Approach:

Cardiovascular diseases (CVD) remain the major leading cause of death in the western world. Based on report from American Heart Association, the prevalence of cardiovascular disease is projected to increase by 9.9%, and the prevalence of heart failure and stroke are expected to increase by almost 25% by 2030 (1). Given the current and the expected worldwide prevalence and the higher mortality and morbidity of cardiovascular disease, developing a strategy to provide cardio protection CVD is very important and inevitable. Multifactorial basis of cardiovascular injury demands the use of multiple pharmacological agents, inhibiting several pathways of cardiac injury. Nevertheless, the use of these medications in their therapeutic doses, away from their therapeutic role in pathological events, may also interrupt physiological processes leading to several side-effects. Therefore, several approaches to achieve cardioprotection against any cardiovascular insult have been explored and to date, the only practical and justifiable countermeasure capable of providing cardioprotection is regular sessions of endurance exercise. Several studies have wonderfully established the beneficial effects of exercise including cardiac effects and systemic factors on skeletal muscle, metabolism, and vascular dysfunction. However, active exercise programs, while consistently beneficial, still generate a very diverse response, suggesting the potential for critical contribution from the genetic composition determining intrinsic aerobic exercise capacity. It has been estimated that up to 60-70% of the variation in exercise capacity is due to the genetic component (2,3). The genetic component appears to have two parts, the genes that regulate adaptive responses to exercise training and the

genes that determine intrinsic exercise capacity. However, it is not clear whether the genetic component for enhanced exercise capacity alone can result in protection from CVD or whether the training stimulus is necessary to produce the positive results. Therefore, it is very reasonable to investigate the influence of intrinsic exercise capacity alone on the vascular, metabolic and myocyte adaptive responses to acute, chronic, or even to non-cardiovascular stress. This dissertation sets to test the potential impact of intrinsic aerobic exercise to response to various form of cardiovascular stress including vascular reactivity to different vasoconstriction and vasodilation agents, metabolic and cardiac response to cardiotoxicity, For this reason, we are going to use rats with inherently different running capacity that are artificially selected over generations to produce strains with genetically determined high and low intrinsic capacity (HCR and LCR).

Exercise Physiology

Exercise or physical activity is planned, structured and repetitive bodily movement produced by skeletal muscles that result in energy expenditure above resting (basal) levels for the purpose of improving health and maintaining fitness. Exercise physiology is the study of how the body reacts to physical activity that includes the study of the acute responses and chronic adaptations to exercise. Acute response is direct response to exercise which may last for the duration of the exercise or training session. This could include changes in the cardiovascular, respiratory and muscular systems depending on the intensity and period of exercise. Chronic adaptation refers to long term changes, over several weeks or months, that occur during exercise and may include improved cardiovascular adaptation, respiratory adaptation, muscle tissue adaptation and metabolic adaptations to prolonged aerobic training. Exercise physiology is becoming a hot area of study in both the medical and fitness fields. Exercise physiology emphases on how physical activity can improve health and wellness. People do exercise for two main reasons

which include improve their fitness and protect or recover from several health insults or injuries (4). Generally, when it comes to leading a healthy lifestyle, exercise ranks high on the list of importance and the great health benefits of exercise are undeniable.

General Exercise and Diseases

The relationship between regular exercise and overall health has been well established. Several studies have revealed that physical activity and exercise are critically important for health promotion and wellbeing of people of all ages. Beyond that, there is undisputable evidence demonstrating the beneficial effects of regular exercise both to prevent and to treat several diseases. Furthermore, there is an inverse relationship between physical activity and all-cause mortality. Thus, it is widely accepted among scientists to consider exercises as a drug (5). A previous review study has comprehensively showed the effective role of regular exercise in prevention of many diseases and in the promotion of healthy longevity and increasing of the life expectancy for males or females (5). In healthy people, physical activities have been reported to reduce the risk of death by about 20%–35% (6,7). Physically inactive middle-aged women face a 52% increase in all-cause mortality, including cardiovascular and cancer related mortality in comparison with physically active ones (8). Several observational and clinical randomized trials have shown that regular physical activity contributes effectively to the treatment of several chronic diseases. For example, There is evidence for prescribing exercise in the primary and secondary prevention of pulmonary and cardiovascular diseases such as coronary heart disease, chronic obstructive pulmonary disease, hypertension, intermittent claudication; metabolic disorders which include type 2 diabetes, dyslipidemia, obesity, and insulin resistance; musculoskeletal diseases such as osteoporosis, rheumatoid arthritis, chronic fatigue syndrome, and fibromyalgia; cancer; and depression (9,10). All these studies showed that exercise could be considered as treatment of

established diseases. Furthermore, these studies suggest the importance of investigating the molecular signaling mechanisms in the adaptation to exercise for both physiological and pathological conditions. Like any other drug, a proper dose of exercise is critical to achieve the highest level of benefit. Moreover, special considerations should be taken when prescribing exercise for people with special needs such as elderly, children, pregnant women, overweight or obese patients.

Cardiovascular Diseases and Exercise

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality across the United States and worldwide (11). In the United States, more than 250000 deaths are caused by cardiovascular disease resulting from sedentary lifestyle. Moreover, physical inactivity is estimated to cause 30% of ischemic heart disease (12). In 1996, the relationship between physical inactivity and CV disease gained a footing in the medical community, when the American Heart Association released evidence supporting the effective role of aerobic exercise in improving hemodynamic, metabolic, neurological, endocrinal and respiratory function (13). As the association between physical inactivity and the increased risk of CV morbidity settled, additional data and studies supported the advantages of exercise as one of the most effective approaches to help prevent cardiovascular (CV) disease and to promote CV health. CVD involves a wide range of conditions that affect the heart and vasculature including arrhythmias, dilated, hypertrophic, or idiopathic cardiomyopathies, heart failure and atherosclerosis (14). These conditions can lead to potentially lethal cardiac events such as myocardial infarction, stroke ,or cardiac arrest (11). Thus, determining various therapeutic tools to prevent or reduce the incidence of CVD is vital. Thus, lifestyle interventions that aim to increase physical activity could be favorable therapeutic method

to cure most of CVD such as hypertension, ischemic infarction, atherosclerosis, and heart failure and most risk factors of CVD such as type 2 diabetes.

Diabetes and Exercise. Diabetes mellitus is one of the most prevalent metabolic disorders and a major health problem characterized by increased glucose level in the blood as a result of insulin deficiency or insulin resistance or both. The main feature of diabetes mellitus is insulin resistance which is also an independent risk factor for fatal cardiovascular disease such as heart failure (15). Excessive body weight and lacking exercise are the most risk factor of diabetes mellitus (16). Exercise training programs have emerged as a useful therapeutic regimen for treatment of type 2 diabetes mellitus as it has effective beneficial role on physiological parameters and decreases the metabolic risk factors in insulin resistance diabetes mellitus. Aerobic exercise session have shown to maintain the blood pressure in patient with diabetic neuropathy(15). Numerous of randomized trial studies showed that high intensity of aerobic exercise can result in weight loss with substantial improvement in insulin sensitivity (17–19). Moreover, aerobic exercise can improve glycemic control, fasting blood-glucose profile and lipid level. Furthermore, it can improve the endothelial function and reduces the arterial stiffness which is the positive denominator for emerging cardiovascular complications in of type 2 diabetes mellitus (19,20). Importantly, exercise therapy could also restore the defects of insulin by increasing glucose uptake into skeletal muscle through the glucose transporter, GLUT4, from an intracellular to the cell-surface (21).

Hypertension and Exercise. Hypertension is the leading risk factor for morbidity and mortality in the worldwide causing an estimated 9.4 million deaths in 2010 (22–24). Left ventricle hypertrophy is the major characteristic feature of the hypertensive heart which could act as an independent predictor of contrary cardiovascular events. Lifestyle factors, including physical inactivity, are vital adaptable risk factors in the development of hypertension. Therefore, Exercise

training is a key element of lifestyle therapy for the prevention and treatment of hypertension (25). Several epidemiologic studies consistently report the positive effects of aerobic exercise on hypertension with reductions in both systolic and diastolic blood pressure (25–27). Regular exercise protects against life-threatening hypertension exist, some of which involve the autonomic nervous system (28). In hypertensive patient, vagal tone might be improved with exercise training because blood vessels in barosensitive areas of the carotid arteries become more compliant than in their pre-training state causing arteries to be distend more in response to an increase in blood pressure (29). The improving in blood pressure with physical activity is thought to be due to reduction in peripheral vascular resistance, which may be due to neurohormonal and structural responses with reductions in sympathetic activity, stimulation of parasympathetic activity and increase in arterial lumen diameters, respectively (30).

Myocardial Ischemia and Exercise. Cardiovascular disease remains the major cause of mortality worldwide (1). Coronary artery disease is defined as a reduction or an interruption in coronary blood flow to the cardiac muscle, which result in a myocardial ischemia, the main element of cardiovascular mortality (31). Previously, exercises or physical activity was considered as contraindication after a cardiovascular event; nowadays, there is an agreement that exercise training should be part of cardiac rehabilitation programs. A great bulk of evidence supports the concept that regular exercise training can decrease the incidence of coronary events and increase survival chances after myocardial infarction (32,33). It is well established that exercise also confers cardioprotection against ischemia reperfusion (IR) injury through a direct effect on the myocardium (34,35). In previous study, McElroy and his colleagues found that physical exercise for 5 weeks after irreversible occlusion of the left coronary artery results in a 30% reduction of the infarcted area in trained mice in comparison to the non-trained mice (32).

In another study, it has been previously confirmed that performing a short term of aerobic exercise prior to an IR injury is adequate to increase cardiac output and improve cardiac function during and after a cardiac insult (36). There is increasing evidence demonstrating that exercise training, when appropriately prescribed and supervised, after myocardial infarction can protect from future complications and improve the quality of life and longevity of infarcted patients (37). Regular exercise is known to decrease arrhythmia (38,39), reduce myocardial stunning (40), and improve coronary vascular reactivity in hearts exposed to I/R injury. Moreover, physical exercise can decrease vascular resistance and enhance structural adaptations in the coronary artery such as increased number of capillaries and number and size of arteries and arterioles , thus improving the blood flow capacity (41). It is well demonstrated that physical training can improve endothelial functions and endothelial-dependent vasodilatation in addition to increase production of endothelial NO synthase(eNOS) (42,43). Furthermore, exercise can alter the cellular-molecular control of intracellular Ca2+ in both endothelial and vascular smooth muscle cells of coronary arteries in exercise-trained animals (41). Lastly, it has recently been demonstrated that physical exercise can also mimic the “Ischemic Preconditioning” (IP) phenomenon, which refers to the capacity of short episodes of ischemia to render the myocardium more resistant to subsequent ischemic insult and to limit infarct size during prolonged ischemia (44,45). These findings further support the potential positive effect of exercise on coronary disease.

Exercise and Longevity

Age is considered as one of the most reliable causes of death. As the number of older populations continuous to grow up, it is vital to develop interventions that can be simply applied and contribute to “successful aging” (46). Many factors can influence the physiological process of aging, some of which are modifiable. Among many factors that affect the aging process, physical

activity state is one of the major modifiable risk factors. In addition to a healthy diet and good psychosocial status, the advantages of exercise training on mortality, and the protection and management of chronic disease impacting both life span expectancy and quality of life are undoubted. Regular exercise can partially contrary the effects of the aging actions on physiological functions and preserve functional reserve in old people. Several studies have revealed that maintaining an adequate exercise sessions could decrease the risk of death, protect against numerous age-related conditions including cardiovascular diseases, certain cancers, metabolic abnormalities, dementia, depression, osteoporosis and increases longevity (47). Furthermore, Meta-analyses prove a clear positive dose-response association with respect to physical activity and longevity (48). In aged individuals, exercise training programs should be aimed to improve cardiorespiratory fitness, muscle function, cognitive function, as well as flexibility and balance. Determining the appropriate dose of exercise is critical to gain the maximum health benefits. Thus, further research is required in the elderly, in order to develop appropriate effective dose of exercise in this growing population (47).

Active Exercise and Genetic Predisposition

As mentioned previously, regular exercise has several health benefits including prevention and treatment of various chronic diseases such as cardiovascular disease, diabetes, cancer, neuro-cognitive disorders, all-cause mortality rates, quality of life, and longevity. However, physiological responses to physical exercises between individuals are variable and not consistent (49,50). That is, some people respond well to exercise, while others respond weakly or have no response at all to exercise, even when controlling other factors such as age, sex, and ethnic origin (49). Thus, scientists try to understand why health benefits of regular exercise programs vary from person to person. This inter-individual diversity in response to exercise training has mainly been

characterized by aerobic capacity (VO_{2max}) for endurance training (50,51)and by muscle mass and strength measurements for resistance training (52). In addition to these measures, genetic background has been strongly contributed to inter-individual variation. However, the existing knowledge regarding relationships between genetics and inter-individual variation in response to exercise training is limited. Indeed, genetic factor was found to be responsible for 9%-80% of physical activity depending on the intensity of the physical training, suggesting the important impact of genes in the physical activity (53–55).

Unlike exercise training, which is relatively easy to characterize and qualify, the genetic elements that define intrinsic endurance exercise capacity are more subtle. The genetic component appears to have two parts, the genes that regulate adaptive responses to exercise training and the genes that determine intrinsic exercise capacity (56,57). Studies on animals and humans have revealed that genes could be a driving force for someone being physiologically active. In animals, rodent strains showed to play a role in the divergence of spontaneous physical activity levels (58,59). Moreover, selective breeding studies of mice and rats result in developing generations of high and low active animals that vary in their predisposition for voluntary physical activity as well as identifying multiple quantitative trait loci (QTL) specific for running distance, speed, and duration (59–62). In humans, previous studies found that physically active parents tend to raise more active children than inactive parents, even after controlling the environmental factors such as exercise training (63). Other studies have revealed that individuals with the same genotypes respond equally to exercises training comparing to those with different genotypes, suggesting that genetic background plays a significant role in determination of individual differences in response to exercise training or physical activities(64). Furthermore, several genes have been discovered to be linked with exercise performance and response to physical activity (65). These genes influence

the physiological outcomes of exercise or physical activity which include: body size or mass, leanness, lipidemia, glucosemia, and hemodynamics (65). Understanding the influence of genetic background on the aerobic endurance will allow us to illuminate the standards of physical activities for individuals. Moreover, this knowledge would help to identify people who are expected to respond well or poorly to exercise training, thus structuring more efficient and safer training programs (allowing accurate estimation of the training outcomes including weight loss and improved health and wellness, prevention of potential overload, injuries, cardiomyopathies, and sudden death). However, in studies involving human subjects it is often difficult to test the effect of aerobic capacity on different health outcomes due to many external factors, such as training. To overcome this limitation, an animal model is required that allows in-depth analyses of aerobic exercise capacity.

Rat Model and Low and High Capacity Running Rats

As previously described, aerobic capacity is determined by several complex traits that include both environmental (extrinsic) and genetic (intrinsic) factors. It has been estimated that up to 60-70% of the variation in exercise capacity is due to the genetic component (2,3,57). However, the environment or active exercise is maximally accounted for 10-20% of influences on the aerobic capacity. This suggests the significant impact of the intrinsic factor on the overall aerobic capacity, as well as its dominant influences in the diseases outcomes that can be prevented or managed by physical exercise, such as in cardiovascular diseases. It is not clear whether the genetic component for enhanced exercise capacity alone can result in protection and treatment of cardiovascular diseases or whether the training stimulus is necessary to produce the positive results. However, it has been difficult to assess the effect of aerobic capacity on various health outcomes due to many external factors, such as training.

The development of a novel rat model, by Drs. Koch and Britton that emphasizes the differences in intrinsic aerobic exercise capacity has advanced the ability to differentiate the genetic components from the environmental effects, such as active exercise, that also influence the aerobic capacity (66). This novel rat model was developed by using a genetically heterogeneous outbred stock of N:NIH rats, two rat strains, low endurance running capacity (LCR) rats and high endurance running capacity (HCR) rats were created by artificial selection, using an endurance treadmill running test (66,67). Artificial selection for intrinsic aerobic endurance capacity was initiated with a founder population of 80 male and 88 female rats. Genetic variance among the population has been maximized by not selecting among brother and sisters. At ten weeks of age, rats undergo a protocol to estimate aerobic running capacity (run time for five days to exhaustion on a graded treadmill exercise test). The thirteen rats with the highest running capacity (HCR) and the thirteen rats with the lowest capacity were selected from each sex and randomly paired for mating. The treadmill protocol and the selection of the high and low capacity groups were repeated over subsequent generations. Over several generations, genetic heritability of the phenotype for aerobic capacity was clearly established by a difference in running capacity of 347% was present between groups at 11th generation (67,68). The treadmill running capacity difference can be translated to the LCR rats on average, as a decreased by an average of 16 meters for the LCRs and increased by an average of 41 meters in the HCRs in response to the selection. The divergence has further increased to a difference of 461% by the 21st generation (67,68). This animal model is very valuable in studying the interaction between aerobic capacity and chronic diseases such as cardiovascular diseases because the differences observed in aerobic capacity between these strains is evident under sedentary conditions, which eliminates the conflicting effects of training adaptation due to exercise training (68,69).

Previous work has demonstrated that these rat strains have different risk profiles for cardiovascular disease. Wisloff et al. utilized rats from the 10th and 11th generations to measure differences in cardiovascular disease risk factors between the two phenotypes (68). LCR rats were found to be more insulin resistant, with higher random glucose, fasting glucose, insulin level, visceral adiposity to body weight ratio, free fatty acids, and blood pressure when compared to HCR rats(68). Moreover, LCR rats expressed decreased levels of proteins involved in mitochondrial function in skeletal muscle, supporting the notion that impaired regulation of oxidative pathways in mitochondria may be a linkage between aerobic capacity and cardiovascular disease (68). In addition, LCR rats have been reported to decrease stroke volume, reduce systolic and diastolic cardiac function, and decrease oxygen supply extraction ratio and tissue diffusion capacity in skeletal muscle as compared to HCR rats (70). In another study, aged animals from both phenotypes has been compared to test the hypothesis that an intrinsic capacity for aerobic metabolism sets an initial divide for longevity and a compression of age-related morbidity. This study showed the first evidence of the "aerobic hypothesis of longevity" which is an important discovery in order to study the mechanisms of longevity and aging biology, especially the role of the myocardium and the circulatory system (71).

Our lab has been working with this model for about ten years. Noland et al. (98) in our laboratory utilized a cohort of the LCR and HCR rats at generation 13 rats for the purposes of assessing specific differences in fatty acid disposal by skeletal muscle as a function of intrinsic oxidative capacity under normal and high fat diet conditions. In addition to confirming basic characteristics of relative insulin resistance in the LCRs as reported previously by Wisloff et al.(68), the study demonstrated the phenotypic differences in adaptive responses to the stress of a high fat diet between HCR and LCR rats. These results suggest potential differences in adaptive

responses between phenotypes in to other environmental or chronic stresses other than high fat feeding.

Additional work from our lab utilized this model to investigate whether differences in intrinsic aerobic exercise capacity offered protection against acute ischemic reperfusion injury (AIRI). Results from this study revealed that there were phenotypic differences in infarct size to AIRI when ischemic challenge is 15 minutes, but these phenotypic differences disappear when ischemic challenge increases to 30 minutes. This data suggests the potential for intrinsic exercise capacity to influence the response of tissue to ischemic challenge, but if the challenge is too severe, intrinsic protective pathways in the HCR disappear. HCR and LCR also were tested in our lab to determine the vascular and metabolic adaptive response to acute peripheral artery ligation. This study showed that LCR vascular and metabolic responses deteriorated after acute ischemic injury and that HCR was more resistant to the ischemic injury.

From these studies we developed an interest in assessing the potential impact of intrinsic aerobic exercise to response to various form of cardiovascular stress including vascular reactivity to different vasoconstriction and vasodilation agents, metabolic and cardiac response to cardiotoxicity, For this reason, we are going to use rats with inherently different running capacity that are artificially selected over generations to produce strains with genetically determined high and low intrinsic capacity (HCR and LCR).

Thesis Overview:

The purpose of this project is to gain a better understanding about the deferential changes in vascular, metabolic and cardiac adaptations in response to different form of cardiovascular stress. Our previous studies and most other studies using LCR and HCR animals found that LCR rats score high on disease risks associated to the metabolic syndrome, which is defined as collection

of symptoms that may predispose for cardiovascular disease, and HCR rats score high for health factors related to maximal oxygen consumption. LCR rats were more insulin resistant, had higher mean blood pressures, decreased nitric oxide mediated vascular relaxation, increased susceptibility to tumor growth, impaired wound healing, reduced longevity, and displayed higher arrhythmogenicity following short term myocardial ischemia and reperfusion (68,72). LCR animals were predisposed to weight gain and increased blood free fatty acid (FFA) levels compared to HCR counterparts challenged with a high fat diet (70). These results suggest potential differences in adaptive responses between phenotypes in to other environmental or chronic stresses other than high fat feeding. Therefore, we will test the hypothesis that the low aerobic endurance running capacity (LCR) phenotype will show altered vascular, metabolic, and cardiac adaptive responses to various forms of cardiovascular stress than the high aerobic endurance running capacity (HCR) phenotype. In this thesis, we have evaluated the impact of intrinsic aerobic exercise capacity on adaptation responses following different cardiovascular insults. In chapter two, we describe how intrinsic aerobic exercise capacity, gender differences, and age influence aortic reactivity in response to both dilator and constrictor stimuli. In chapter three, we evaluate cardiac and mitochondrial adaptations in response to aging and Doxorubicin in rats bred for divergent aerobic capacities. In chapter four, five, and six, we investigate the impact of intrinsic aerobic capacity on cardiac myocyte, metabolic and fibroblast responses following different forms of acute ischemic-reperfusion injury. Finally, last chapter summarizes and discusses the findings of this thesis and what may teach us cumulatively about the relationship between intrinsic aerobic exercise capacity and CVD in addition to a glimpse of future direction.

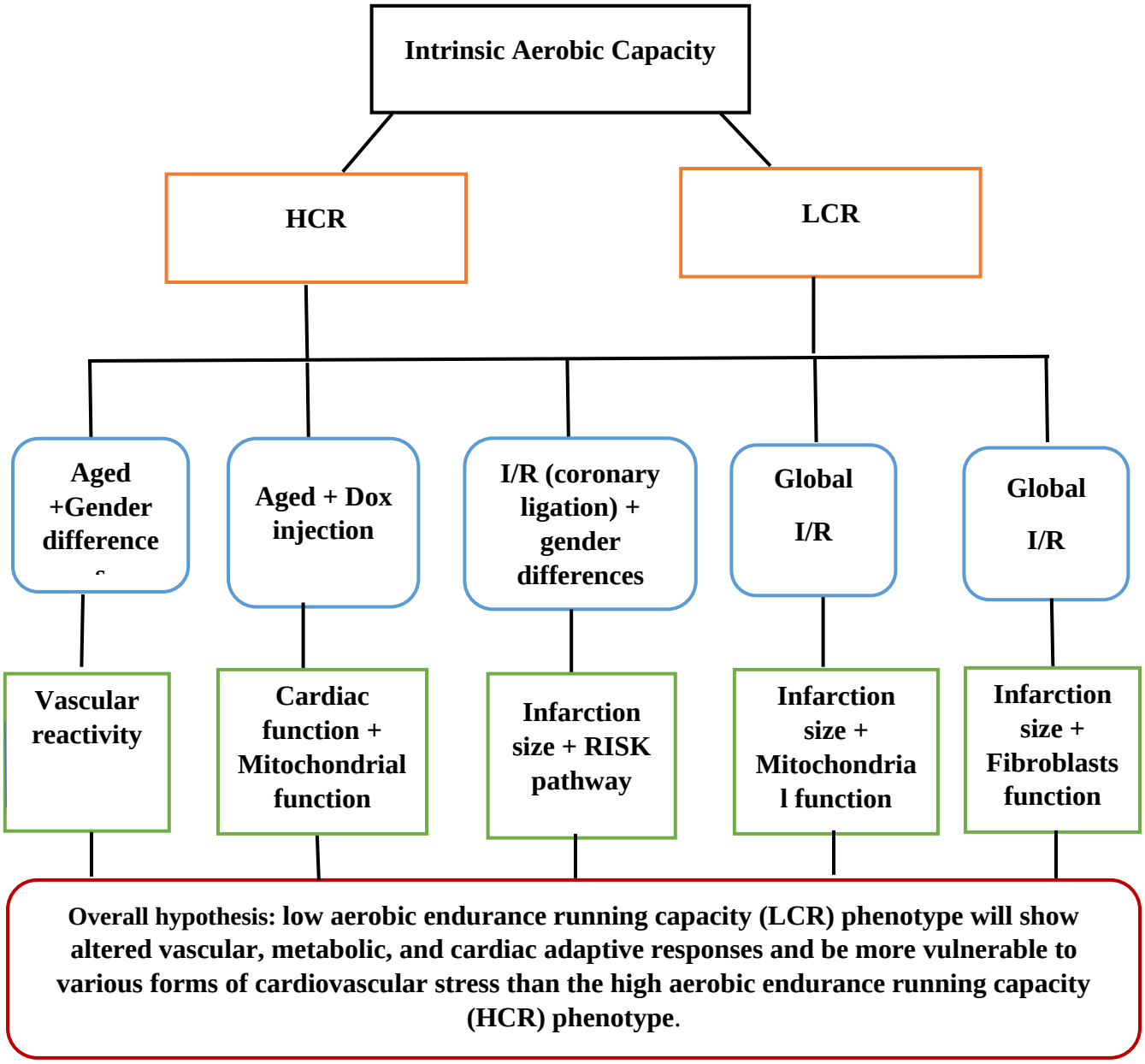


Figure 1.1: Schematic of overall project studies and general hypothesis

CHAPTER 2: SELECTION FOR INHERENT RUNNING CAPACITY ALTERS AORTIC REACTIVITY IN A STRAIN-DEPENDENT FASHION WHILE PRESERVING GENDER DIFFERENCES

ABSTRACT:

Purpose: The selection for high- or low-capacity (HCR/LCR) endurance running in rats revealed a divergent expression of cardiovascular risk factors. Physical activity is reported to be a positive modulator for endothelial function. While more is known about the impact of dynamic exercise on the vascular function, the effects of intrinsic aerobic capacity on the overall vascular reactivity are largely unknown. We tested the hypothesis that sedentary rats selectively bred for a high capacity to run (HCR) would be predisposed to robust endothelium-dependent and independent vascular responses to both dilator and constrictor stimuli compared to low capacity running (LCR) rats.

Methods: Using HCR/LCR generations 11 and 18 of both sexes, cumulative relaxation and contraction concentration-response curves were generated from thoracic aortic rings to the dilators; sodium nitroprusside (SNP), forskolin (FSK), HA1077 and acetylcholine (ACH), or constrictors; phenylephrine (PE), (5-HT), endothelin-1 (ET-1), norepinephrine (NE) and K^+.

Results: In both strains, relaxations of rings, to SNP, FSK, HA1077 and ACH were smaller in magnitude in males than females. The LCR male aortas showed the lowest relaxations and the highest EC_{50} values to all dilator agents. The maximal constrictions elicited by PE, ET-1, NE, 5-HT and K^+ were greater in females of either strain. Additionally, there was a significantly larger contractile response to K^+ in LCR males that was endothelium dependent. Strain differences in NO signaling were seen with higher levels of nitrate/nitrite production and eNOS protein

expression in HCRs as compared to LCRs, although no significant gender specific differences were observed.

Conclusions: The results indicated that LCR male aortas had impaired vascular and endothelium reactivity, linked to eNOS-NO signaling. The selection for/against aerobic running capacity generally preserved gender differences in aortic vascular responses, but within strains, the phenotypic selection exaggerated an impaired vascular function in a gender specific manner.

INTRODUCTION

Gender is recognized as an important determinant in the responses of the cardiovascular system (73), and has been shown to influence the susceptibility to ischemia-reperfusion injury (74) and endothelial function (75). Female sex hormones have been reported to enhance/preserve endothelial function, reduce oxidative stress, and protect against atherosclerosis (76–78). In the rat, aortas from females have reported greater basal NO production when compared to aortas from males, but the relaxation responses to protein kinase G activation were greater in aortas from males (79).

The health benefits of active aerobic exercise have been well established. Epidemiologic studies have shown that aerobic exercise reduces cardiovascular morbidity and mortality in the general population (80,81). Moreover, it has been shown, in both hypertensive patients and animal models of hypertension, that exercise training improves endothelial function (81–85). In animal studies, exercise training showed to be positive modulator in decreasing risk of heart failure, reducing arrhythmia, lowering myocardial stunning and improving coronary vascular reactivity in hearts exposed to ischemia reperfusion (86,87) . However, the specific mechanisms underlying the cardiovascular protective effects of exercise remain unclear (88).

Unlike exercise training, which is relatively easy to characterize and qualify, the genetic elements that define intrinsic endurance exercise capacity are more subtle. The genetic component appears to have two parts, the genes that regulate adaptive responses to exercise training and the genes that determine intrinsic exercise capacity (2,3,57). Although aerobic exercise training has beneficial effects on several of cardiovascular diseases, the variability in the physiological response to exercise training suggests potential impact of the genetic composition. It has been estimated that up to 60%-70% of the variation in exercise capacity is due to the genetic component (3,57,89,90). It is not clear whether the genetic component for enhanced exercise capacity alone can result in protection from cardiovascular diseases or whether the training stimulus is necessary to produce the positive results.

The development of a novel rat model, by Drs. Koch and Britton (91–94), that emphasizes the differences in intrinsic aerobic exercise capacity has advanced the ability to differentiate the genetic components from the environmental effects, such as active exercise, that also influence the aerobic capacity (92). Rat genetic models of intrinsic (i.e., untrained) low-capacity runners (LCR) and high-capacity runners (HCR) have been developed by artificial selective breeding using treadmill running as the discriminating phenotype. This selection process has generated a shift in metabolic and cardiovascular risk factors between the two strains (94). In previous study, LCR were found to be insulin resistant and predisposed to obesity, while the HCR animals are protected from high fat diet induced obesity and insulin resistance (95). Moreover, compared to the male HCRs, the male LCR rats have a 13% higher blood pressure, a significantly lower endothelial-dependent vascular relaxation as induced by acetylcholine, and an impaired regulation of oxidative pathways in mitochondria (94).

The HCR and LCR strains provide a unique model with inherent differences in aerobic capacity that allows for the testing of this supposition without the confounding effects of a training stimulus. Given that dynamic physical activity is reported to be a positive modulator for endothelial function and decreased blood pressure, and since the HCR rats have at least the intrinsic potential for increased physical activity, we tested the hypothesis that sedentary HCR animals would be predisposed to robust endothelium-dependent and independent vascular responses to both dilator and constrictor stimuli.

MATERIALS AND METHODS

Chemicals and drugs. The reagents listed below were purchased from Sigma-Aldrich, St. Louis, MO, USA: Phenylephrine (PE), norepinephrine (NE), serotonin (5-HT), endothelin-1 (ET-1), sodium nitroprusside (SNP), acetylcholine (ACH), the Rho kinase inhibitor (HA1077), and forskolin (FSK). All the salts were purchased from Fisher Chemicals, New Jersey, USA. Physiological Salt Solution (PSS) composition was, in mM: NaCl, 140.0; KCl, 5.0; $CaCl_2$, 1.6; $MgSO_4$, 1.2; 3-[N-morpholino]-propane sulfonic acid (MOPS), 1.2; EDTA, 0.1; d-glucose, 5.6; pH adjusted to 7.4 at 37 °C. K^+-PSS is a substituted PSS solution in which K^+ has been substituted for Na^+ in an equal molar fashion.

Animal strains. The HCR and LCR rats used in this study were provided by Drs. Koch and Britton (92) from strains they derived using artificial selective breeding for maximal treadmill running capacity, starting with a heterogeneous rat population from the N:NIH stock (National Institutes of Health, USA). Response to selection produced rat strains differing significantly in intrinsic aerobic capacity (92). In brief, endurance running capacity was assessed in the founder population at 11 weeks of age using distance to exhaustion on a treadmill running test (15-degree incline; initial velocity 10 M/minute and increased 1 M/minute every 2 minutes) as a phenotypic

endpoint. The top 20 % performers of each sex were used to establish the HCR (high capacity runner) strain, while the bottom 20% performers were mated similarly to produce the LCR (low capacity runner) strain. At each subsequent generation, offspring from 13 families each of LCR and HCR were phenotyped and bred with precaution being taken to minimize inbreeding (<1% per generation). See Table 2.1 for run characteristics of animals at 11 weeks used in these experiments.

In this study, males from generation 11 and females from generation 18 were used for the purpose of this investigation and were housed in a temperature-controlled environment with a 12:12 hour light: dark cycle. Rodents arrived at East Carolina University at 4 months of age and were quarantined for at least 12 weeks to satisfy institutional IACUC requirements. Once cleared by the veterinary staff for study, the animals were transferred to general housing and were provided standard rat chow and water *ad libitum* throughout. Animals were sacrificed in HCR: LCR pairs and studied between 50 and 52 weeks of age. (See Table 2.1 for body weights at time of arrival at ECU and at time of sacrifice). On experimental days, rats were anaesthetized using 0.1 mL/100 g body weight of a mixture containing 90 mg/mL ketamine and 10 mg/mL xylazine, and tissues were harvested. All procedures conformed to the Animal Care and Use Committee standards approved at East Carolina University and in compliance with NIH standards.

Preparation of isolated aortic rings. Under anesthesia, a thoracotomy was performed and the aorta from arch to diaphragm was gently excised and rinsed of blood in ice-cold PSS. The thoracic aortas were cleaned of connective tissue, fat, and blood with care to insure minimal damage to the smooth muscle and endothelial lining. Aortic rings, approximately 4 mm in length, were cut from the cleaned vessel. In some experiments, when denuded rings were required, the endothelium was removed mechanically.

Isometric force measurement. Individual aorta rings were mounted in organ bath chambers of the DMT 610 myograph system (DMT International, Atlanta, GA) and bathed with PSS continuously bubbled with air (37ºC, pH 7.4). Isometric tension was recorded with the Powerlab data acquisition system (ADI Instruments Pty Ltd, Castle Hill, NSW, Australia). Aortic rings were loaded with an initial resting tension of 20 mN. During an equilibration period, tissues were stretched to 40 mN every 7 minutes, until 40 mN of force was maintained. Tissue length then was shortened rapidly to achieve a passive force of approximately 20 mN, and then allowed to stabilize for 7 minutes before beginning any experiments.

After rings were equilibrated and passive force set, the responsiveness of each individual ring was checked using a 109 mM K^+-PSS stimulation for 10 minutes. Rings were then relaxed with successive washes of PSS at 10-minute intervals. The integrity of the vascular endothelium was tested pharmacologically by application of 1 μM acetylcholine (ACH) to 1 μM phenylephrine (PE) pre-contracted rings. The endothelial relaxation response was considered adequate when the force was reduced by greater than 50 % of the PE stimulated force. The endothelial-denuded rings were verified by ACH (1 μM) relaxation response of less than 10 % against PE (1 μM)-induced contraction.

Protocol for aortic relaxation dose-response experiment. The vasodilator responses to SNP (0.001-10 μM), FSK (0.001-100 μM) and HA1077 (0.001-100 μM) were conducted in endothelium-denuded aortas, while those to ACH (0.001-10 μM) were conducted in endothelium-intact aortas. All concentration responses were assessed in aorta rings pre-contracted with PE (1 or 3 μM) for 10 minutes to normalize for starting force. The vasodilators were cumulatively added to generate a concentration-dependent response profile. The relaxant responses were normalized to the stable PE force, or expressed as actual force per square area of rings (mN/mm^2).

Protocol for aortic contractile dose-response curve. In aortic rings, either with or without endothelium, the contractile response to cumulative addition of PE (0.01-30 μM), ET-1 (0.0001-1 μM), 5-HT (0.01-30 μM) and NE (0.001-10 μM) was evaluated. The contractile responses were expressed as force per square area of rings (mN/mm^2) or normalized to the maximal contractile response.

RNA isolation, reverse transcriptase-PCR, and real-time PCR. Total RNA was extracted from harvested aortas using TriReagent (Sigma-Aldrich, USA) and cDNA was generated using the High Capacity cDNA kit from Applied Biosystems (Foster City, CA) following the manufacturer's protocol. Real-time PCR was performed using TaqMan Primers and probes mixes from Applied Biosystems following the manufacturer's protocol and using GAPDH as the internal standard. The Real-Time PCR detection system used was the iCycler iQ (Bio-Rad Laboratories, USA).

Nitric oxide measurement. Nitrates plus nitrites were measured in aorta homogenates using a colorimetric assay kit (Cayman Chemicals; # 780001) following the manufacturer's protocol. Absorbance was measured at 542 nm with a plate reader (Synergy HT, Bio-Tek Instruments, Winooski, Vermont, USA) and the concentration of total nitrites and nitrates in each sample was calculated using a standard curve and expressed per mg protein.

Immunobloting. Aortas were homogenized in a lysis buffer (25 mM Tris-HCl, 150 mM NaCl, 1% NP-401% Sodium deoxycholate, 0.1% SDS, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 0.5 mM phenylmethylsulfonyl, pH 7.4) and the homogenate was centrifuged (12,000 *g* at 4º C for 15 minutes). Proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad), which were then incubated overnight in a blocking buffer composed of Tris buffered saline containing 0.1% Tween (TBS-T) and 4% bovine serum albumin, followed by incubation for 2 hours with the indicated antibodies in the blocking buffer. After washing 3 times for 10 minutes

each with TBS-T, the membrane was incubated with alkaline phosphatase-conjugated anti-mouse IgG (Sigma Aldrich) antibodies followed by washing with TBS-T. Immunoreactive bands were visualized with ECF substrate (GE Healthcare Buckinghamshire, UK). The image signals were quantified by densitometric analysis of digitized films using UnScanIt (Silk Scientific, Orem, UT, USA).

Statistics. Results are expressed as mean ± SEM. Non-linear regression analysis for all concentration response curves were performed using a Hill algorithm in Sigma Plot (Systat Software Inc, Richmond, CA) allowing for calculation of individual 'EC_{50}'s, the effective concentration to cause 50 % of a maximal response to be calculated. Mean data were analyzed by t-test or ANOVA. When the ANOVA manifested a statistical difference, the Holm-Sidak method was applied (Sigma Stat, Ver 3.1.1). Significance was accepted in all tests when $p < 0.05$ and statistical significance is identified as * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Data were organized into two evaluation groups: 1) examined general gender differences, with all data grouped into male and female pools without regard for strain; 2) examined strain differences, with data grouped into pools with regard for both gender and strain.

RESULTS

1. Selection process for aerobic phenotype preserves gender differences

1. a. Female rats demonstrate an enhanced vascular reactivity in both strains. When comparing the grouped male and female responses to different relaxation agonists, regardless of their strains, the females showed a significantly higher sensitivity to a NO donor (SNP), to a stimulator of cyclic AMP production (FSK), and to an inhibitor of Rho-kinase (HA1077) (Table 2.2). Although the difference of EC_{50} values between the male and female responses to acetylcholine did not reach significance, a trend towards higher sensitivity in females was observed

(Table 2.2). With regard to the grouped gender responses to the constrictors, only the ET-1 EC_{50} from the denuded aorta rings was statistically different (Table 2.2). Sensitivity to ET-1 and 5-hydroxytryptamine based on the calculated EC_{50} values was minimally greater for males compared to females in either intact or denuded aortic rings. Responses to the adrenergic agonists PE and NE revealed slightly greater sensitivity from the females as they had a lower calculated EC_{50} values than the males.

In denuded aortic rings from male rats, the concentration-dependent relaxation response to SNP (0.001-10 µM, pre-contracted with 1 µM PE) was shifted to the right in both HCR and LCR animals (Figure 2.1A) from the female responses. The same trend was observed in the concentration-dependant relaxation response to FSK (0.001-100 µM, pre-contracted with 1 µM PE) (Figure 2.2B). However, the relaxations of the LCR-males to both ACH (0.001-100 µM, pre-contracted with 1 µM PE; Figure 2.1B) and HA1077 (0.001-100 µM, pre-contracted with 3 µM PE; Figure 2.2A) were the only group to have a right shifted concentration-response profile.

The contraction response of denuded aortas to phenylephrine (PE) (0.01-10 µM, Figure 2.3A), ET-1 (0.0001-1 µM, Figure 2.3C), 5-HT (0.01-10 µM, Figure 2.4A) and K^+PSS (109 mM, Figure 2.4C) were significantly stronger in females than males in both HCR and LCR strains. The same trend was observed in responses of the intact aortas to PE (Figure 2.3B) and NE (0.001-10 µM, Figure 2.4B) but not for responses to ET-1 (Figure 2.3D) nor K^+PSS which displayed a significant gender difference only in HCRs (Figure 2.4D).

1. b. The gender difference is greater in the LCR Rats. When comparing male and female responses to different relaxation agonists within the same strain, it appears that the difference between male and female LCR animals was more pronounced than the difference between male and female HCRs. The calculated EC_{50} values (Table 2.3) for LCR males were higher than those

of females with SNP (80.9 ± 33.9 nM compared to 13.2 ± 6.2 nM), FSK (11.6 ± 2.8 μM compared to 3.2 ± 1.1 μM) and, HA1077 (14.4 ± 3.5 μM compared to 8.8 ± 0.9 μM). This difference was even more pronounced in aortas relaxed with acetylcholine (109.7 ± 56.3 nM compared to 32.2 ± 16.0 nM) (Figure 2.1B).

These results, in aggregate, illustrate that the gender difference was maintained with the breading selection for running capacity. The gender difference favors greater relaxations in aortas from the females, which may be linked to differences in the endothelial, NO, cAMP as well as Rho-kinase signaling cascades.

2. The intrinsic exercise capacity impacts the endothelial function most in male rats

2. a. The least relaxation was seen in LCR male aortas. In both denuded and intact aortas, SNP (0.001-10 μM, Figure 2.1A) and ACH (0.001-10 μM, Figure 2.1B) produced greater relaxations in aortas from HCR males than in aortas from LCR males. EC_{50} values (Table 2.3) were significantly higher for LCR over HCR males with both SNP (80.9 ± 33.9 nM vs. 23.2 ± 8.0 nM) and acetylcholine (109.7 ± 56.3 nM vs.14.3 ± 1.7 nM). The same trend was observed in the LCR males with HA1077 and FSK, as they showed the smallest relaxations and a consistent trend for the highest EC_{50} values (Table 2.3).

2. b. Contractile responses in HCR and LCR male aortas. Endothelin-1 (Figure 2.3C) and 5-HT (Figure 2.4A) produced the weakest contractions in aortas of LCR males, while PE (Figure 3A, 3B), NE (Figure 2.4B) and K^+PSS (Figure 2.4C, 2.4D) generated the smallest contractile responses in aortas of HCR males, without any significant change in the calculated EC_{50} between the groups (Table 2.3). The LCR males showed both strain and gender differences, suggesting that there were vascular dilation impairments in the aortas from these animals which involved the responses to NO, cAMP and Rho kinase signaling pathways.

3. The eNOS-NO system

Nitric oxide production can be used as an indicator of eNOS activity. We measured the nitrites and nitrates produced in aorta homogenates and the results revealed that, while there were no significant gender differences, the HCR strain was capable of producing more nitric oxide as compared to the LCR (Figure 2.5A). To further investigate this apparent strain difference, we measured the eNOS mRNA (Figure 2.5B) as well as the protein expression levels (Figure 2.5C). Figure 2.5B shows that HCR males and females had a higher eNOS level of expression compared to the LCR counterparts. Western blotting revealed a higher level of eNOS protein expression in the HCR strain as compared to the LCRs, and a tendency for higher levels of protein expression in the females as compared to the males in either strain (Figure 2.5C and 2.5D). These data were consistent with the functional vascular reactivity assessments (Figures 2.1-4), that also indicated a general difference between HCR and LCR in the behavior to the NO response and confirm that the difference was most evident in the LCR males.

DISCUSSION

The aim of this study was to elucidate the impact of differences in intrinsic aerobic running capacity on vascular reactivity and endothelial function and to test whether gender-based differences in vascular reactivity were influenced by the artificial selection process for the endurance running phenotype. Our data showed that intrinsic selection for endurance running capacity did not eliminate, and in some cases, may have exaggerated the gender effect on vascular reactivity. We observed that the aortic responses from all female rats were larger in magnitude to both constrictor and vasodilator agents in a strain-independent way. Our findings are consistent with the literature since several other reports have shown gender differences in the reactivity of blood vessels and suggest that the female vascular reactivity was greater than those of the male

(96,97). Indeed, female hormones such as estrogens and other sex steroids affect vascular reactivity both indirectly, by modulating the release of endothelium-derived vasoactive factors, and directly, by modulating intracellular calcium levels in vascular smooth muscle cells (98). However, the most important finding was that the differences in the aortic responses between male and female was more pronounced in LCRs than in the HCRs.

Epidemiological studies report that daily physical activity at a moderate intensity is effective in reducing the incidence of coronary heart diseases (99–101) and experimental data indicates that regular physical activity has beneficial effects on many risk factors for diabetes and cardiovascular diseases (102–104). The risk factors improved by moderate physical activity include insulin sensitivity, blood pressure, metabolic syndrome, dyslipidemia and endothelial dysfunction (105–108) which is an important early predictor of adverse coronary outcomes (109). Gender has been recognized as an important factor in determining the risk for cardiovascular diseases (73) and cardioprotective effects of sex hormones have been reported in both experimental and clinical studies (110,111). The impacts of sex hormones on cardiovascular system hemostasis and vascular reactivity have been well documented (112,113). The data generally suggest that female sex hormones, especially estrogens have vascular protective effects and that their influence is mainly on the regulation of the vascular tone (114–116). Thus, most studies reporting gender differences in vascular tone, highlight NO production (117) and release as being greater in females as compared to males and suggest that the NO/eNOS system is responsible for the greater vascular reactivity in females (96,118–120). This gender difference in endothelium-dependent relaxation also has been reported in isolated aortas of spontaneously hypertensive rats (121,122).

In this study, we investigated the NO/eNOS system and the results revealed that there was a distinct difference between the two strains with the HCR rats presenting higher expression of

eNOS and higher NO production than LCR. The difference between HCR and LCR has been initially reported by Wisloff et *al.* using female rats (16 to 24 weeks old) from the 11^{th} generation (54). They reported that ACH mediated vascular relaxation in ring segments from carotid arteries was 48% higher in the HCR compared to the LCR rats and that the EC_{50} for ACH was 7.8-fold greater in LCR than HCR rats. In this study, we report that in addition to the strain difference, the gender differences were more pronounced in the LCR animals than in the HCRs just as we observed with vascular responses. The successive breeding out to generation 18 provided, in addition to increasing the differences in aerobic capacity between the strains (Table 1), an increased strain difference in vascular reactivity.

There are three possible explanations for the observed changes in these results. First, the difference between males and females NO/eNOS system was narrowed via selection for the higher intrinsic aerobic capacity; second and alternately, the gender differences were exacerbated as a result of selection in the LCR and third, there was both a narrowing in the HCR and an expansion in the LCR strains that occurred concurrently. Although the NO/eNOS system has been reported to be one factor accounting for gender differences in vascular reactivity, vascular smooth muscle cells (VSMC) signaling pathways play a role as well (116). Studies in isolated VSMC have shown that the resting cell length is longer and the basal intracellular Ca^{2+} level is lower in female compared to male rats, suggesting gender differences in the mechanisms controlling Ca^{2+} that were estrogen related (123). In ovariectomized females, the resting cell length was significantly shorter and the basal [Ca2+]i was significantly greater than that in intact female, but there was no different between ovariectomized females with estrogen implants and intact females nor between castrated and intact males (123). KCl-induced VSMC contraction also has been reported to be gender dependent. In fact, Ca^{2+} influx, intracellular Ca^{2+} levels and VSMC contractions following KCl

depolarization were all greater in males compared to females (123–125). Moreover, KCl-induced contraction was enhanced in ovariectomized females, which further suggest an interaction between estrogen and the entry mechanisms of Ca^{2+} (123,124,126). Contractile responses to endothelin also have been shown to display gender specific differences. Arteries from female pigs developed greater contractile force in response to ET-1 than arteries from male pigs (127) and active exercise training increased this response in male but not in female arteries (127). Our data also showed a female specific increase in contractile responses to constricting agents (hydroxytryptamine, norepinephrine and KCl) but in contrast to the active exercise response, in sedentary animals, it was the female HCRs that demonstrated larger constrictor responses. For the KCl-induced contractile responses, there was a gender difference in the endothelium-intact aortas of HCRs but not of LCRs. However, in the denuded aortas, both strains showed a gender difference in their KCl-induced contraction responses. The persistence of the gender differences in HCRs and the existence of this differences in LCRs with denuded aorta in response to KCl-induced contraction suggest that the gender difference in both strains is also beyond the endothelial cell influence. Other possible mechanisms that can be endothelial dependent that were not investigated in this study were the roles of prostaglandin signaling and hyperpolarizing factor which may counteract the vasoconstriction response, and their impact is abolished by endothelial removing.

The current investigation demonstrated that in adult HCR/LCR rats, the gender difference was maintained in the artificial selection for intrinsic aerobic endurance running capacity. However, one confounding element to the vascular responsiveness might be a differential in aging between LCR and HCR rats. Aging is associated with changes in blood vessel tone which is determined by the balance between vasoconstrictors and vasodilators. Several reports indicate that with aging, there is an impairment of vasodilator responsiveness and enhancement of the

constrictor responses with different rates in males and females (128–131). Our data collected at 52 weeks of age showed significant gender differences for both the vasodilatation and vasoconstriction responses in the LCRs while in the HCRs, the differences were greater for vasodilatation responses and the K^+ depolarization. This finding suggests that selection for higher aerobic capacity may provide protection within the vasculature with aging. The differences seen in this study must be viewed in the light that the reposes were observed in a conduit vessel, the thoracic aorta. Generally, if differences are seen in this preparation, there are usually more robust changes in other segments of the vascular tree which may be more related to regulating perfusion characteristics to match metabolic demand with nutrient/oxygen supply.

CONCLUSION

The HCR and LCR strains provide an animal model with inherent differences in aerobic capacity that allows for the testing of impact of inherited elements on vascular responses without the confounding effects of a training stimulus. Given that dynamic physical activity is reported to be a positive modulator for endothelial function, we anticipated that the rats bred for high aerobic capacity would be predisposed to robust endothelium-dependent and independent vascular responses to both dilator and constrictor stimuli.

In conclusion, we provide evidence that selective breeding for intrinsic aerobic capacity, in the absence of exercise training, modulates vasomotor function and vascular phenotype in the aorta of mature female and male rats. We found that the gender difference was maintained with the breading selection for running capacity. However, the differences in the aortic responses between male and female was more pronounced in LCRs than in the HCRs. The LCR males had the poorest aortic responses, while the females, in both strains, retained the best overall dynamic responses. The gender difference favors greater relaxations in aortas from the females, which may

be linked to differences in the endothelial, NO, cAMP as well as Rho-kinase signaling cascades. Other physiological mechanism contributing to the differences between strains may involve differences in eNOS and NO production but also appear to include the response to direct cyclic nucleotide activators as well. Future experiments are required to investigate changes in calcium handling, contractile elements sensitivity or other protein modification between LCR and HCR phenotype.

Table 2.1. Body weight and running performance characteristics of HCR/LCR strains

	HCR		LCR	
	Male	**Female**	**Male**	**Female**
Characteristic	**n = 20**	**n = 20**	**n = 20**	**n = 20**
Arrival Body Weight (g)	244.4 ± 4.8 *	161.9 ± 2.5 *	336.5 ± 6.3	210.6 ± 3.9 *
Run time (min)	61.4 ± 0.6 *	64.9 ± 0.3 *	16.8 ± 0.5	23.1 ± 0.2 *
Run distance (m)	1526.5 ± 25.0 *	1670.7 ± 13.3 *	231.7 ± 7.7	353.8 ± 4.3 *
Body weight at sacrifice(g)	421.8 ± 14.6 * n = 12	232.9 ± 9.8 * n = 12	565.1 ± 20.1 n = 12	340.3 ± 16.6 * n = 12

Data are expressed as mean ± SEM. *P<0.05, *vs* LCR-Male, one-way ANOVA followed by Holm-Sidak test.

Table 2.2. Effects of gender on calculated EC_{50} values of relaxant and constrictor responses. Relaxant include, sodium nitroprusside (SNP), forskolin (FSK), HA1077 and acetylcholine (ACH) in phenylephrine preconstricted rings and constrictor include phenylephrine (PE), endothelin-1 (ET-1), 5-hydroxytryptamine (5-HT) and norepinephrine (NE) in endothelium-denuded (-) or endothelium-intact (+) rat aorta rings.

Treatment	Female		Male	
	EC_{50}	n	EC_{50}	n
Relaxation agents				
SNP (-)	11.9 ± 3.8 nM *	11	36.2 ± 8.7 nM	13
FSK (-)	2.9 ± 0.7 µM **	11	9.5 ± 1.8 µM	13
HA1077 (-)	5.7 ± 1.2 µM *	8	11.4 ± 1.9 µM	11
ACH (+)	35.1 ± 8.8 nM	13	70.0 ± 34.8 nM	12
Constrictor agents				
PE (-)	0.40 ± 0.04 µM	10	0.49 ± 0.04 µM	12
PE (+)	0.40 ± 0.03 µM	13	0.42 ± 0.04 µM	12
ET-1 (-)	0.19 ± 0.03 µM *	9	0.10 ± 0.02 µM	10
ET-1 (+)	0.11 ± 0.02 µM	11	0.07 ± 0.01 µM	12
5-HT (-)	8.77 ± 1.75 µM	10	6.23 ± 0.92 µM	12
NE (+)	0.13 ± 0.06 µM	13	0.15 ± 0.07 µM	12

Data are expressed as mean ± SEM. *P<0.05, **P<0.01 t-test, *vs* male.

Table 2.3. Effects of strain and gender on EC_{50} values of relaxant and constrictor responses induced by sodium nitroprusside (SNP), forskolin (FSK), HA1077 and acetylcholine (ACH) or phenylephrine (PE), endothelin-1 (ET-1), 5-hydroxytryptamine (5-HT) and norepinephrine (NE) in endothelium-denuded (-) or endothelium-intact (+) rat aorta rings pre-contracted by 1-3 µM phenylephrine (PE).

	EC_{50}			
Treatment	**HCR-Female**	**HCR-Male**	**LCR-Female**	**LCR-Male**
SNP (-)	10.3 ± 4.4 nM	23.2 ± 8.0 nM	13.2 ± 6.2 nM	80.9 ± 33.9 nM
n	5	6	6	7
FSK (-)	2.6 ± 0.9 µM	7.0 ± 2.1 µM	3.2 ± 1.1 µM *	11.6 ± 2.8 µM
n	5	6	6	7
HA1077 (-)	2.7 ± 0.7 µM	8.9 ± 1.8 µM	8.82 ± 0.95 µM	14.4 ± 3.5 µM
n	4	6	4	5
ACH (+)	38.4 ± 6.3 nM	14.3 ± 1.7 nM	32.22 ± 16.02 nM	109.7 ± 56.3 nM
n	6	5	7	7
NE (+)	120 ± 90 nM	126 ± 66 nM	135 ± 90 nM	166 ± 50 nM
n	6	5	6	6
PE (+)	356 ± 38 nM	388 ± 40 nM	446 ± 50 nM	439 ± 55 nM
n	6	5	6	6
ET-1 (+)	89 ± 18 nM	52 ± 11 nM	126 ± 30 nM	84 ± 17 nM
n	6	5	6	6
5-HT (-)	6.6 ± 0.5 µM	5.1 ± 0.6 µM	10.9 ± 3.3 µM	7.0 ± 1.5 µM
n	6	5	6	6

Data are expressed as mean ± SEM. *P<0.05, *vs* LCR-Male, one-way ANOVA followed by Holm-Sidak method.

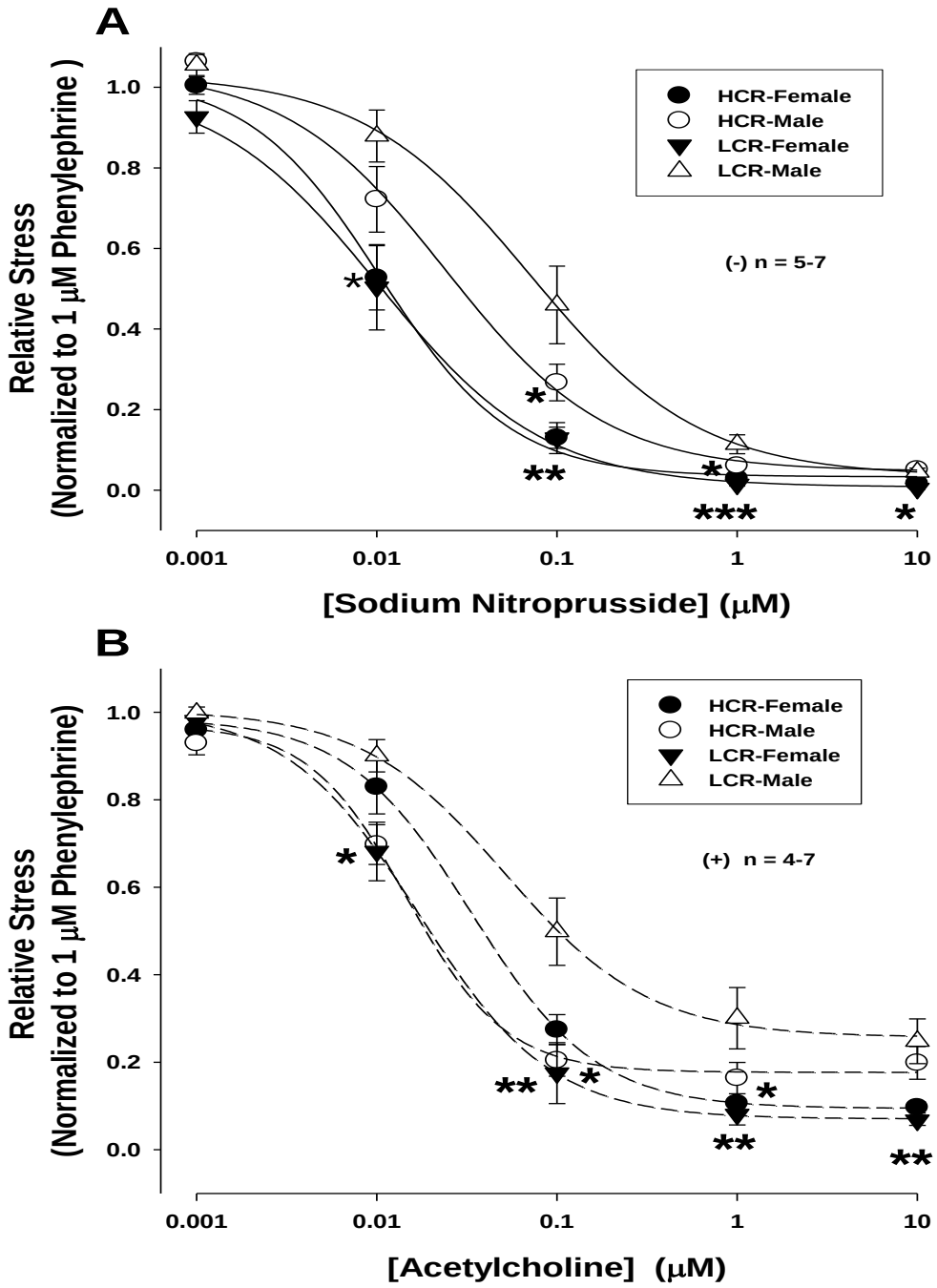


Figure 2.1. Strain and gender effect on sodium nitroprusside (SNP, Panel A) and acetylcholine (ACH, Panel B) induced relaxant responses in endothelium-denuded (-) isolated aortic rings (pre-contracted with 1μM phenylephrine) from HCR and LCR rats. Each point represents mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, *vs* LCR-Male; one-way ANOVA followed by Holm-Sidak method.

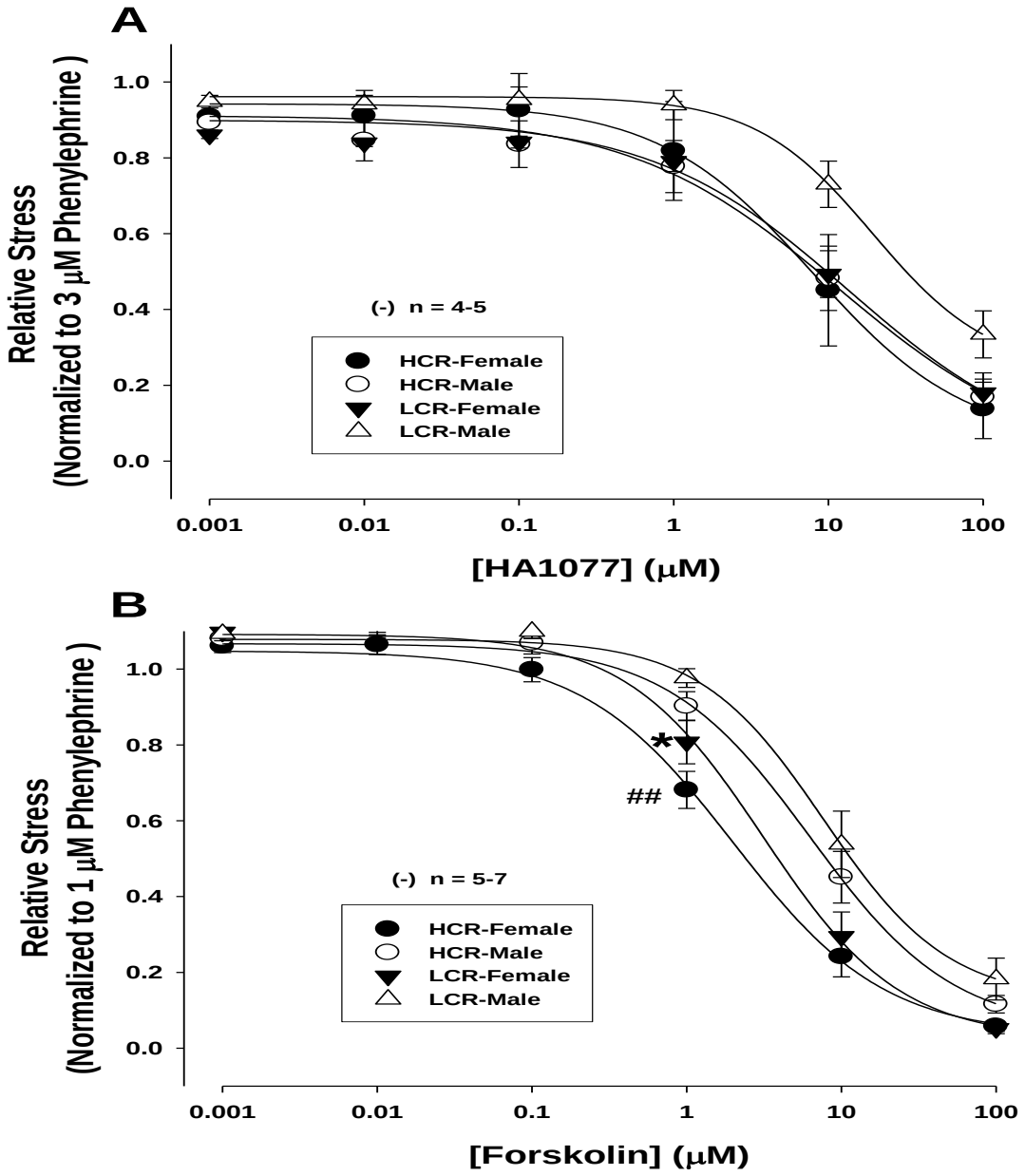


Figure 2.2. Strain and gender effect on Rho kinase inhibitor HA1077 (Panel A) and forskolin (FSK, Panel B) induced relaxant responses in endothelium-denuded (-) or endothelium-intact (+) isolated aortic rings (pre-contracted with 1-3 μM phenylephrine) from HCR and LCR rats. Each point represents mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, *vs* LCR-Male; ##P<0.01, *vs* HCR-Male; one-way ANOVA followed by Holm-Sidak method.

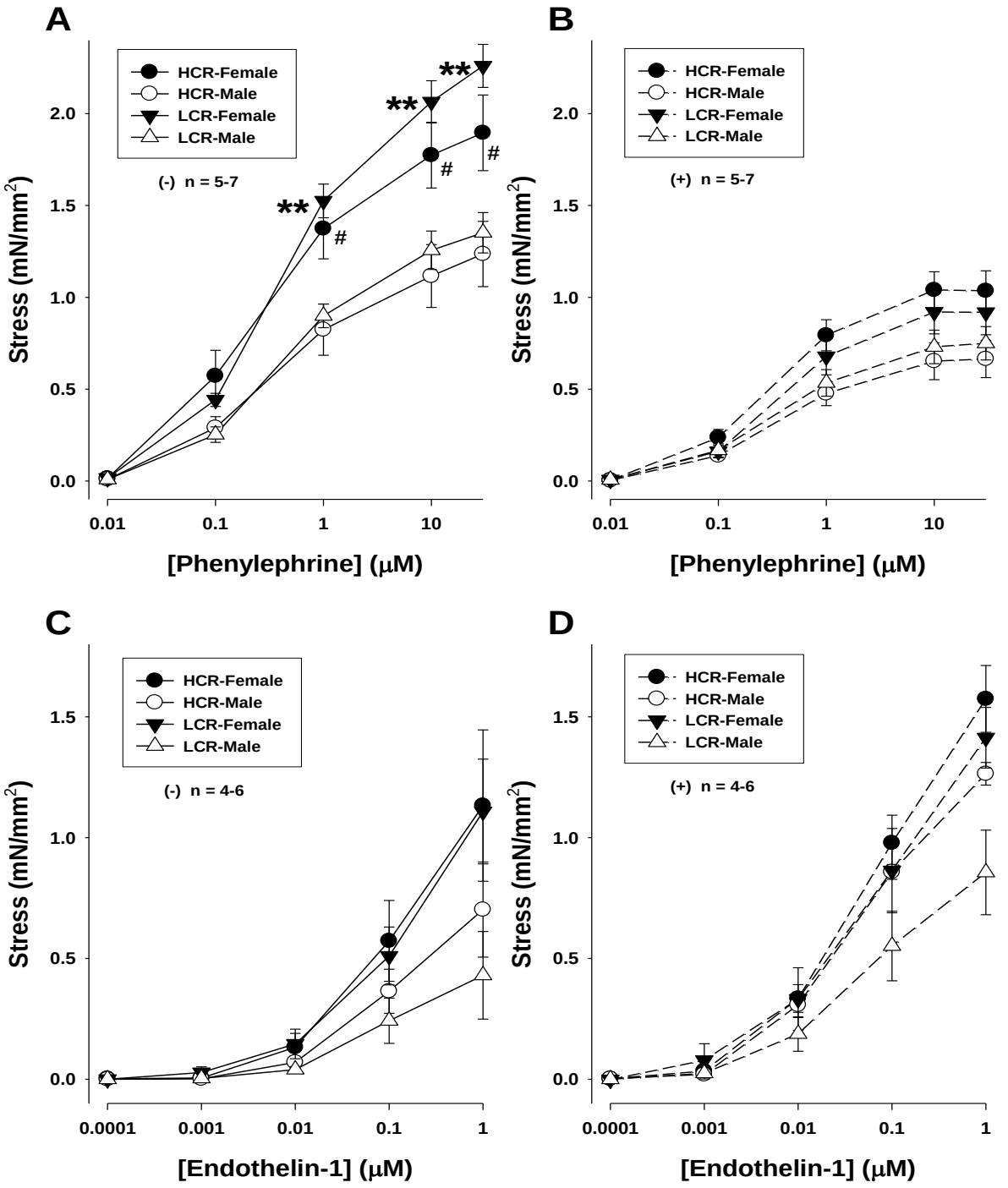


Figure 2.3. Strain and gender effect on phenylephrine (PE, Panel A and B), endothelin-1 (ET-1, Panel C and D) produced contraction in endothelium-denuded (-) and endothelium-intact (+) isolated aortic from HCR and LCR rats. Each point represents mean ± SEM. **P<0.01, *vs* LCR-Male; #P<0.05, *vs* HCR-Male; one-way ANOVA followed by Holm-Sidak method.

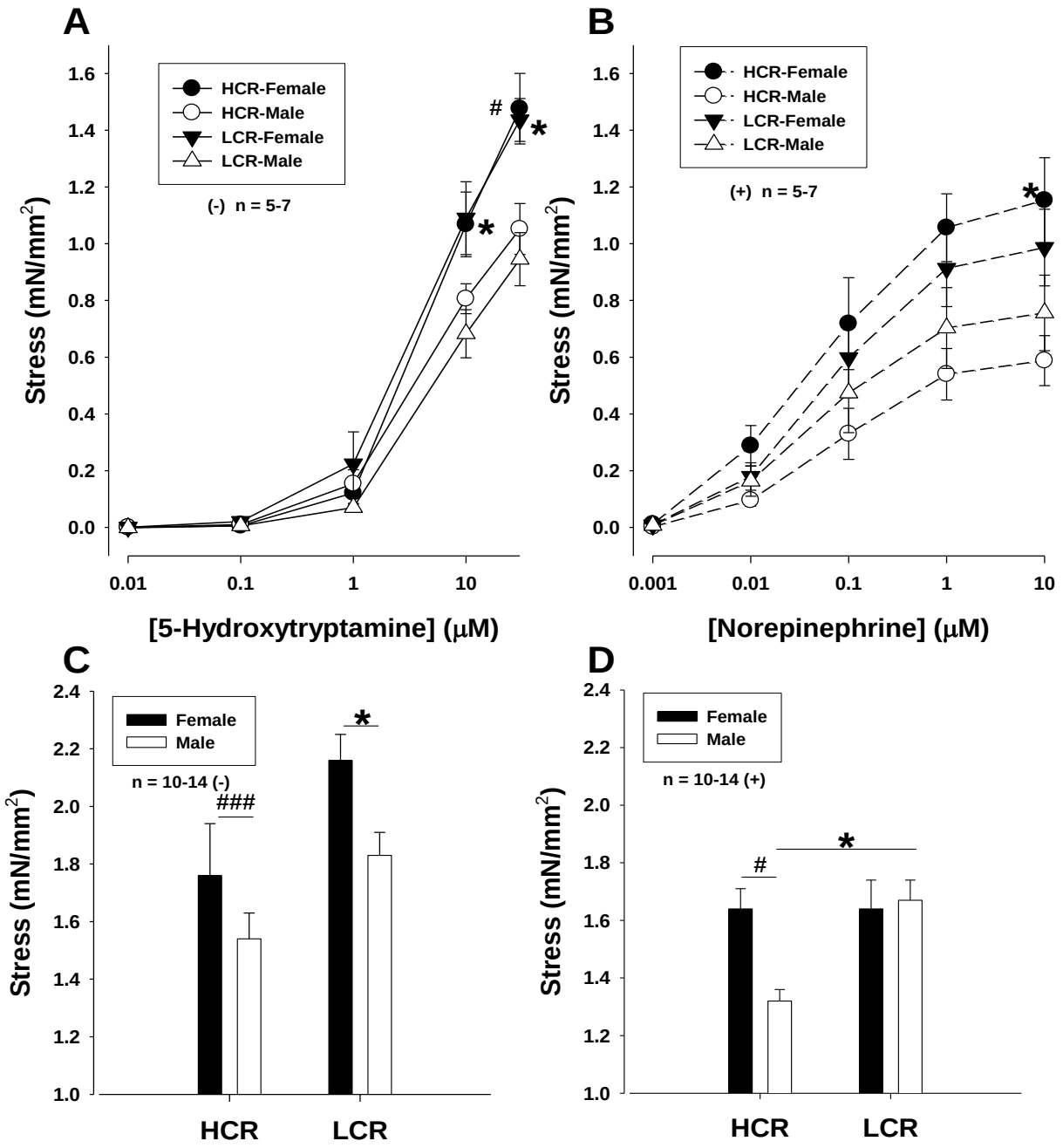


Figure 2.4. Strain and gender effect on contraction induced by 5-hydroxytryptamine (5-HT, Panel A), norepinephrine (NE, Panel B) and KPSS (109 mM KCl, Panel C and D) in endothelium-denuded (-) or endothelium-intact (+) aorta rings from HCR and LCR rats. Each point represents mean ± SEM. *P<0.05, *vs* LCR-Male; #P<0.05, ###P<0.001, *vs* HCR-Male; one-way ANOVA followed by Holm-Sidak method.

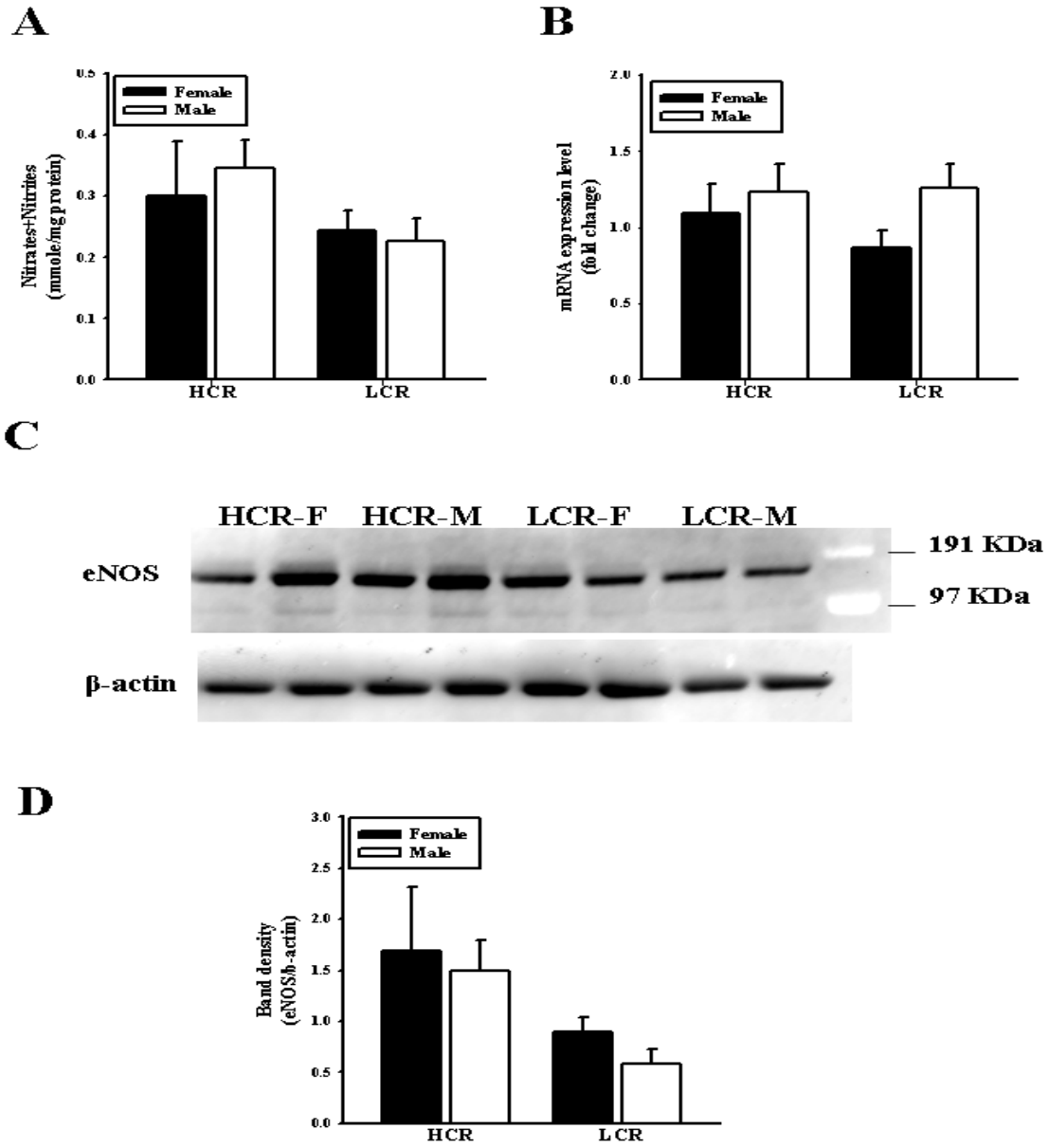


Figure 2.5. Strain and gender effect on total nitrites amount determined by the Griess method as described in the Methods (A), eNOS mRNA levels (B), and protein levels (D) in the aorta of HCR and LCR rats. (C), shows a representative blot of eNOS expression. Each point represents mean ± SEM of 5-7 animals. The data was analyzed by a two-way ANOVA followed by Holm-Sidak method.

CHAPTER 3: CARDIAC AND MITOCHONDRIAL ADAPTATIONS IN RESPONSE TO AGING AND DOXORUBICIN IN RATS BRED FOR DIVERGENT AEROBIC CAPACITIES

ABSTRACT

Purpose: Doxorubicin (DOX) remains as one of the most widely prescribed and effective anticancer agents. A major limitation of the therapeutic effectiveness of the drug is the occurrence of irreversible, progressive, dose-dependent cardiotoxicity. Active aerobic running capacity has been shown to protect against DOX-induced cardiac dysfunction, but little is known of the protective effects of intrinsic non-trained aerobic capacity. We hypothesized that a low aerobic capacity running (LCR) phenotype will be more susceptible for cardiac mitochondrial dysfunction and decreased cardiac performance in response to doxorubicin stress, when compared to the high aerobic running capacity (HCR) animals.

Methods: To test this hypothesis, cardiac function was assessed in rats specifically selected over 26 generations for their low (LCR) and high (HCR) intrinsic aerobic running capacity. HCR/LCR rats received a single doxorubicin (7.5mg/kg of body weight) intraperitoneal injection and cardiac performance was studied longitudinally through echocardiography. On the tenth day, the animal was sacrificed, cardiac mitochondria were isolated and mitochondrial function was assessed through respirometry studies.

Results: Our results indicated that animals with low inherent aerobic capacity were susceptible to doxorubicin insult as evidenced by an adaptive mitochondrial response, while the high aerobic capacity animals appeared to have been physiologically primed and therefore did not exhibit an adaptive compensatory response.

INTRODUCTION

Cancer is one of the greatest public health concerns in the United States (132). It is a prevalent cause of mortality and morbidity in the United States and suggest that current therapies may not be effective at combating this illness. Cancer accounts for a significant proportion of overall health care costs (133). Current cancer treatment options include surgical intervention, radiation and antitumor chemotherapy. Despite their relative antitumor efficacy, a common patient survival value/benefit-versus-cost adjustment to chemotherapy treatment is attributed to the non-selective cytotoxicity side effects, such as myelosuppression, nausea and vomiting, mouth ulcers and alopecia (134). Serious adverse side effects force patients who may otherwise benefit from continued administration of a drug to withdraw from chemotherapy and switch to an alternative agent, which may be less effective (135). Doxorubicin (DOX) remains as one of the most widely prescribed and effective anticancer agents. Toxic side effect limitations to chemotherapy is especially true for the anticancer agent doxorubicin (DOX, adriamycin), in this case due to its notorious cardiotoxic side effects (136). DOX-induced toxicity have been well recognized and vary from vasodilation, and hypotension to left ventricular dysfunction and congestive heart failure (137). Patients with cardiomyopathy due to DOX-induced toxicity have an especially poor prognosis, as their survival chances are worse than patients with ischemic cardiomyopathy (138). As the number of long-term cancer survivors continues to increase, chronic cardiotoxicity remains a clinically significant problem as the cardiac damage acquired after DOX-infusion is irreversible and progressively worsens (139). Several studies have attempted to explain the predilection of heart tissue to DOX toxicity. To begin with, the drug seems to be retained in cardiomyocytes more than in other cell types (140). The exact pathogenesis of DOX-induced cardiotoxicity remains unclear although it is hypothesized that the drug exerts its antineoplastic and cardiotoxic action by

distinct mechanisms: the anticancer response has been associated with lipid peroxidation, DNA intercalation, and inhibition of protein synthesis enzymes such as topoisomerase II (136,141–145). All of these effects result in cell cycle arrest that culminates in pro-apoptotic machinery leading to the death of cancer cells and tumor growth arrest (146). There is increasing evidence that oxidative and pro-apoptotic stressors are the primary causal mechanisms responsible for the cardiotoxic activity (147–150). Moreover, evidence suggests that the chemical structure of doxorubicin is prone to the generation of free radicals, leading to an increase in toxic reactive oxygen species (ROS) produced by the mitochondria, which trigger DNA damage and induces intrinsic mitochondria-dependent apoptotic pathways in cardiomyocytes (151–153). Interestingly, this oxidative stress pathway has been found to be distinct from DOX-induced apoptosis induced in tumor cells (145). This suggests that oxidative stress is the principal cause of cardiac damage induced by the drug with the myocardial mitochondria.

As there is no effective treatment for Dox-induced cardiotoxicity, prevention remains the best option. The aim of prevention is not only to prevent the toxicity, but also to increase the antitumor efficacy (146). To circumvent the adverse side effects of doxorubicin, aerobic capacity and exercise have been suggested as one of the few interventions to improve acute and chronic cardiotoxicity (154,155). Studies have shown that exercise capacity is a strong predictor of early morbidity and mortality and that low exercise capacity is a stronger predictor of death than other established risk factors, such as hypertension, diabetes, or smoking (156–158). The benefits of aerobic exercise training are well established as regular exercise has been shown to reduce the risk of heart disease, control hypertension and protect the heart against oxidative stress and apoptosis (159,160). Moreover, exercise has been shown to reduce arrhythmia, decrease myocardial stunning, and improve vascular reactivity in hearts exposed to ischemia reperfusion (161). In

regards to doxorubicin-induced toxicity, there are various exercise training regimens - acute vs. chronic (repeated) - that have shown that exercise preserved cardiac function in mice receiving the drug (162). More specifically, exercise alleviates doxorubicin toxicity by improving antioxidant status, attenuating apoptotic pathways, and preserving contractile function expression (163). It is theorized that exercise training induces increases in catalase and glutathione peroxidase activities which are beneficial under elevated ROS conditions, such as when induced by DOX (164,165).

Unlike exercise training, which is relatively easy to characterize and qualify, the genetic elements that define intrinsic endurance exercise capacity are more subtle. The genetic component appears to have two parts, the genes that regulate adaptive responses to exercise training and the genes that determine intrinsic exercise capacity (2,166,167). Although aerobic exercise training has beneficial effects on several of cardiovascular diseases, the variability in the physiological response to exercise training suggests potential impact of the genetic composition. It has been estimated that up to 60%-70% of the variation in exercise capacity is due to the genetic component (35-37,172). It is not clear whether the genetic component for enhanced exercise capacity alone can result in protection from cardiovascular diseases or whether the training stimulus is necessary to produce the positive results.

The development of a novel rat model, by Drs. Koch and Britton (91–94)that emphasizes the differences in intrinsic aerobic exercise capacity has advanced the ability to differentiate the genetic components from the environmental effects, such as active exercise, that also influence the aerobic capacity. Rat genetic models of intrinsic (i.e., untrained) low-capacity runners (LCR) and high-capacity runners (HCR) have been developed by artificial selective breeding using treadmill running as the discriminating phenotype. This selection process has generated a shift in metabolic and cardiovascular risk factors between the two strains (94). Current results suggest that HCR/LCR

rats can serve as genetic models that contrast for disease risks and indirectly support a mechanistic role for oxygen metabolism (168). Thus far, many studies have observed divergent characteristics between these model organisms. LCR rats have accumulated cardiovascular risk factors, such as a large gain in visceral adiposity, hypertension, dyslipidemia, impaired glucose tolerance, endothelial dysfunction, hyperglycemia, hypertriglyceridemia, insulin resistance and elevated plasma free fatty acids (94,157). In addition, LCR rats have decreased stroke volume, reduced systolic and diastolic cardiac function, as well as impaired oxygen supply extraction ratio and tissue diffusion capacity in skeletal muscle as compared to HCR rats (70). Moreover, LCR rats expressed decreased levels of proteins involved in mitochondrial function in skeletal muscle, supporting the notion that impaired regulation of oxidative pathways in mitochondria may be a linkage between aerobic capacity and cardiovascular disease (42). In summary, LCR rats score high on disease risks associated to the metabolic syndrome, which is defined as collection of symptoms that may predispose for cardiovascular disease, and HCR rats score high for health factors related to maximal oxygen consumption (67).

LCR rats also respond more negatively to environmental health risks, such as high fat diets (95). Therefore, it would be worthwhile to examine how this aerobic rat models respond differentially to doxorubicin induced cardiotoxicity. Thus, the aim of this study was to investigate the influence of intrinsic aerobic exercise capacity on metabolic and cardiac adaptive responses to doxorubicin-induced toxicity, and tests the overall hypothesis that the low aerobic endurance running capacity (LCR) phenotype will show altered metabolic and cardiac responses to doxorubicin when compared to the high aerobic endurance running capacity (HCR) phenotype. Moreover, we expect that the HCR phenotype confers cardioprotection against doxorubicin-induced cardiotoxicity as compared to the LCR phenotype.

MATERIALS AND METHODS

Animal Strains. Female HCR/LCR rats were obtained from Drs. Lauren Koch and Steven Britton at the University of Michigan (aged approximately 25 months, generation 26). The creation of the HCR/LCR rat model has been previously described in detail (92,168,169). All animals were housed 2 per cage in a temperature-controlled 12/12-hour light/dark cycle facility, where standard rat chow and water were provided ad libitum. Rats were randomly assigned into four groups: LCR injected with saline, (LCR + SAL) (n=6), LCR injected with DOX (LCR + DOX) (n=9), HCR injected with saline (HCR + SAL) (n=3) and HCR injected with DOX (HCR+DOX) (n=5). The number of rats in the LCR + DOX and HCR + DOX groups was greater to accommodate the potential mortality following DOX. All protocols were approved by the Brody School of Medicine at East Carolina University Institutional Animal Care and Use Committee and were in compliance with Animal Welfare Act guidelines.

Doxorubicin Administration. The LCR + DOX and HCR + DOX groups received a single intraperitoneal DOX injection (7.5mg/kg of body weight), while the control groups received an injection of 0.9% sterile saline at equivalent volumes.

Assessment of Cardiac Function. Transthoracic echocardiography was conducted on sedated rats using a commercially available echocardiographic system (Vevo 2100, Visual Sonics Inc.,Toronto, Ontario, Canada) with a 13-24 MHz linear array transducer (MS250). Echocardiogram and body weight measurements were made prior to injection (baseline) and 1, 4, 7- and 10-days post-injection. Rats were anesthetized by isoflurane (2-2.5%) delivered through a nose cone and the echocardiography was completed within 15 minutes after the administration of the sedative. The hair on the thoracic area was removed by applying a depilatory. Ultrasound transonic blue gel was placed on the thorax to optimize visibility. Two-dimensional images of the

left ventricle were obtained in the parasternal long-axis and short-axis views. B and M-mode images were obtained at the midventricular level in both views, from which internal dimensions of the left ventricle were obtained at end diastole and end systole (Figure 3.1). All images were analyzed using Vevo 2100 1.3.0 software (Visual Sonics Inc.).

Isolated Rat Heart. Rats were anesthetized using 90mg/mL ketamine and 10mg/mL xylazine, dosed at 0.1 mL/100g body weight. Once animal sensation reflexes, including eye blink, pedal and tail pinch reflexes were absent, the heart was rapidly harvested by midline thoracotomy and placed in ice-cold mitochondrial isolation medium (MIM) buffer for myocardial mitochondrial isolation.

Myocardial Mitochondrial Isolation. Mitochondria were isolated from the left ventricle (LV) using a modified protocol (170). From the excised heart, LV was removed, minced, and digested in 10mLs of MIM buffer (300 mmol/L sucrose, 10mmol/L Na-HEPES, and 0.2mmol/L EDTA, pH 7.2) containing 125mg/mL trypsin for 2 minutes and then diluted with trypsin inhibitor medium (10mL of MIM, pH 7.4, 1mg/mL BSA, and 165mg/mL trypsin inhibitor). The partially digested muscle was suspended in 10mL of MIM containing 1mg/mL BSA and homogenized briefly using a Potter-Elvehjem homogenizer. By differential centrifugation (once at 600 g (4°C) for 10 minutes and twice at 8000 g (4°C) for 30 minutes, a mitochondrial fraction was obtained as a protein pellet. The final mitochondrial pellet was suspended in 200uLs of MIM and protein quantification was determined using a Pierce BCA kit.

Mitochondrial Respiration. The respiratory rates of isolated cardiac mitochondria (100 ug) were measured at 25°C in an Oroboros oxygraph in mitochondrial respiration medium (MiR05) (Oroboros Oxygraph-2K, Oroboros Instruments Corp., Innsbruck, Austria). To prevent oxygen limitation, the respiration chambers were hyperoxygenated up to ~400 umol/l O2. Once

oxygen concentration flux stabilized, substrates were added according to protocol A and B. Protocol A consisted of the following substrate additions: (i) 5 mM glutamate (complex I substrate) + 2 mM malate (complex I substrate), (ii) 2 mM ADP (state 3 condition), (iii) 5 mM succinate (complex II substrate), (iv) 10μM rotenone (inhibitor of complex I), and (v) 0.75 μM carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP, a protonophoric uncoupler). Protocol B consisted of (i) 25 mM palmitoyl carnitine (fatty acid substrate) + 2 μM malate, (ii) 2 mM ADP, (iii) 5 mM glutamate, (iv) 5 mM succinate, and (v) 0.75 μM FCCP. Specific substrate additions allowed for measurement of state 4 (substrate only, no ADP added), state 3 (ADP), and chemically uncoupled (FCCP) respiration rates. Protocol B observed respiration supported exclusively by lipid (PC) under state 4 (PCM4) and state 3 (PCM3). The stable portion of the oxygen concentration slope was determined for each addition in both protocols and normalized as in previous respiration studies (171), (172). The rate of mitochondrial O2 consumption was expressed as picomoles per second per mg of protein. The respiratory control ratio (RCR) was set as the ratio of oxygen consumption at state 3 over oxygen consumption at state 4.

Statistics. Statistical analyses were performed using commercial software (Prism Software, Irvine, CA) on raw or log-transformed data. For HCR/LCR animal characteristics, t-tests were performed. Analysis of variance with repeated measures was used to compare changes in any echocardiographic parameter over time. Similarly, analysis of variance was used to compare substrate responses in respirometry protocols. Analysis of variance also was used to compare differences between groups at any given time. Specifically, the following comparisons were considered: LCR control vs. HCR Control, LCR Control vs. LCR + DOX, HCR Control vs. DOX, and LCR + DOX vs. HCR + DOX. The following comparisons were considered biologically irrelevant and were excluded from statistical comparison; LCR Control vs. HCR + DOX, HCR

Control vs. LCR + DOX. In each case, data are presented as the mean ± S.E.M. Statistical significance was accepted when $p < 0.05$.

RESULTS

1. Phenotype Characteristics

1. a. Body weight. HCRs had significantly lower body weights (211 ± 3 vs. 158 ± 2 grams, mean ± SEM, LCR vs. HCR, $p < 0.0000$) (Figure 3.2, a)

1. b. Best run time. HCR animals also ran more than 400% longer (78 ± 1 vs. 19 ± 0 minutes, mean ± SEM, HCR vs. LCR, $p < 0.0000$) (Figure 3.2, b)

1. c. Best run distance. HCR animals ran more than 8 times farther (2276 ± 43 vs. 274 ± 7 meters, mean ± SEM, HCR vs. LCR, $p < 0.0000$) (Figure 3.2, c)

2. Echocardiographic Assessment

2. a. Control Comparisons. In general, there were no statistical differences overall under control conditions between HCR and LCR animals. While not statistically different in absolute terms, HCR animals trend to having higher end diastolic volumes (Figure 3.3, a), cardiac outputs (Figure 3.3, b) and heart rates (Figure 3.3, c) in the aging animals. There were no meaningful differences in ejection fraction between phenotypes in untreated, aged animals (Figure 3.3, d), and therefore, a significant difference in intrinsic cardiac contractility likely does not explain the overall difference in performance.

2. b. Phenotypic responses to Doxorubicin. Doxorubicin treatment had no real effect on end-diastolic volume in the HCR animals, but it increased significantly in the LCR animals (Figure 3.4, a & b). The data are consistent with a mild loss of function in the LCR animals in the first 4-7 days.

The HCR animals tended to have increased heart rates in response to doxorubicin treatment, while the LCRS tended to show decreasing rate patterns. The increased heart rates in the HCRs were significantly increased throughout the treatment period, while the decrease in heart rate in the LCRs abated within 7 days after treatment began (Figure 3.4, c & d). Cardiac output is the product of heart rate and stroke volume. The aggregate effects of doxorubicin treatment on the HCRs were an increase in total output that was significant by Day 7 after treatment. In LCR animals, doxorubicin treatment tended to increase output early after treatment, but only weakly so, and cardiac output was consistently reduced compared to the HCR animals (Figure 3.4, e & f). Ejection fraction decreased at all times in the HCR animals following treatment with doxorubicin, consistent with a decrease in contractility, but did not change at any time after treatment in the LCRs. The differences in response to doxorubicin treatment remained significant throughout the first week, but had normalized by ten days (Figure 3.4, g & h)

3. **Mitochondrial Respiratory Capacity**

3. a. Control Comparisons. Basal and maximal oxygen consumption rates were not different between LCR and HCR phenotypes, but there were significant differences in ADP stimulated respiration, suggesting higher State III oxygen consumption in the LCRs in the glutamate malate protocol (Figure 3.5, a). Interestingly, when ADP stimulation is preceded by malate and Palmitoyl carnitine instead of glutamate/malate, the phenotype difference in ADP stimulated oxygen consumption is not seen

(Figure 3.5, b).

3. b. Phenotypic responses to Doxorubicin Doxorubicin treatment caused a significant increase in the oxygen consumption of cardiac mitochondria isolated from LCR animals stimulated with glutamate malate, ADP, and succinate, but not in mitochondria isolated from HCR animals

(Figure 3.6, a). In contrast, doxorubicin treatment did not produce phenotype specific differences in mitochondrial oxygen consumption when stimulated with the fatty acid-based protocol (Figure 3.6, c).

When compared to the control responses, doxorubicin increased mitochondrial respiratory capacity 2-5-fold in LCRs in the glutamate protocol (figure 3.6 b), but HCR isolates did not increase respiratory capacity in response doxorubicin treatment in any substrate protocol (figure 3.6, b & d). The doxorubicin induced increase in respiratory capacity may have been a compensation for a decrease in total mitochondrial number in the LCR animals, as mitochondrial protein was approximately 30% higher ($p = 0.061$) in the HCR animals under all conditions tested (Figure 3.7). Doxorubicin did not cause a decrease in mitochondrial protein content, but the baseline differences between LCRs and HCRs may have required an inducible adaptation in the LCRs at the mitochondrial level to respond to the increased stress placed on the system by doxorubicin treatment.

DISCUSSION

In this study, it was hypothesized that impairment of mitochondrial function and cardiac performance may link reduced fitness from doxorubicin-induced cardiotoxicity. The results indicate that our hypothesis was oversimplified, as the mitochondrial function and cardiac performance responses to Doxorubicin varied with the divergent phenotypes. The control animals that were not treated with doxorubicin did not perform significantly different in either test. This was a somewhat surprising result. Increased cardiac performance has been observed in young adults, suggesting perhaps that some of the cardiac associated aspects of intrinsic aerobic capacity are not durable throughout age. A possible explanation to the demise of this benefit is that oxidative damage to the mitochondrial DNA and electron transport chain accumulates with age (173,174).

This reduces mitochondrial energetic capacity, further stimulating oxygen free radical production. The resulting mitochondrial DNA damage inhibits mitochondrial biogenesis and increases replication errors and mitochondrial DNA deletions, thus creating a vicious circle (173).

Doxorubicin treatment in conjunction with an aged phenotype is, therefore, an ideal situation for these events to occur, as oxygen free radicals are produced, and mitochondrial DNA deletion takes place. Cardiac failure as a result of Dox administration is attributed to increased reactive oxygen species (ROS) production by the mitochondria (175). Specifically, it has been proposed that Dox stimulates ROS through mitochondrial NADH dehydrogenase, leading to the generation of a free radical cascade with a potent oxidizing potential (176,177). However, one study reported lower oxidative DNA mutations despite greater reactive oxygen species in skeletal muscles from HCR rats (178). Therefore, the phenotypic benefit of a high aerobic capacity may have disappeared due to an altered mitochondrial biogenesis from the preexisting excessive amounts of oxidative damage due to aging (179,180). Furthermore, it is expected that if the dosage and consequently, the concentration of the doxorubicin was higher, the inhibition of both fatty acids and glucose oxidation would have been seen (181).

In this study, doxorubicin treatment caused elevated levels of mitochondrial respiration in the LCR animals, especially in the glutamate-based protocol. Also, the LCR animals responded by increasing their cardiac volumes but failed in increasing their cardiac output, while the HCRs increased their heart rate and cardiac output, albeit in the total absence of exercise training. Contrary to our expectations, doxorubicin treatment consistently impaired contraction in the HCRs but not the LCRs, suggesting that the increased aerobic capacity phenotype did not confer protection against the cardiotoxic effects of the chemotherapeutic. However, a significant difference in intrinsic cardiac contractility likely does not explain the overall difference in

performance. Though, it is possible that the present results simply represent the residual vestige effects of a larger difference that may have been present at earlier ages. Taken together, these data demonstrate that intrinsic aerobic capacity influences the adaptive responses following DOX injury and perhaps the progression of DOX-induced cardiotoxicity.

Similarly to the effects of endurance exercise, the possibility exists that HCRs have improvements in innate myocardial antioxidant capacity which contribute protection against the doxorubicin-induced damage (177,182). Inversely, LCR rat hearts may be susceptible to doxorubicin cardiotoxicity due to limited antioxidant mechanisms that could protect them from oxidative stress (183). However, antioxidants are not cardiac specific and therefore reduce oxidative stress nonspecifically (184). Thus, the results from the present investigation provide novel insight into potential mechanisms associated with cardiovascular failure in a system that allows for each strain to serve as a control for unknown environmental changes (185).

There are several explanations for the differences exhibited in the LCR and HCR responses to doxorubicin treatment. The first explanation is that because of their genetic endowment, the HCR rats have less need to adapt to the doxorubicin injury than the LCR rats. The HCRs may have been intrinsically prepared to handle the doxorubicin insult. Thus, the lack of a mitochondrial respiration response by the HCR animals to the drug may be because the stress was not as impactful for that phenotype in comparison to the LCRs. Supporting evidence for such a postulate can be seen in our oxygen consumption data; the LCR rats increased their oxygen consumption after doxorubicin treatment while the HCR rats exhibited relatively stable rates. Moreover, after doxorubicin treatment, the HCRs responded by increasing their heart rate, while the LCRs increased their volumes. This supports the explanation that this same experimental drug treatment induced a different injury within the phenotypes due to the genetic endowment. Although both

phenotypes were given similar dosages, it is likely that the HCRs are endowed with inherent advantages that make them better equipped to tolerate the doxorubicin cardiac insult and therefore did not need to recover like the LCRs. One innate advantage of the HCRs may be a higher mitochondrial density.

As previously reported, doxorubicin is associated with several signs of cardiomyopathy: LV hypertrophy, changes in ventricle diameter, cardiomyocytes hypertrophy and loss, fibrosis and collagen deposition (186–188). These findings are consistent with our observation that DOX-treated LCR hearts have increased geometric dimensions and stroke volume, but a depressed cardiac output, which may reflect a compensatory remodeling response to the doxorubicin toxicity in the heart. Our investigation supports the thesis that in aged populations, depressed aerobic capacity is the antecedent of cardiac dysfunction.

Other characteristics of the animal model may have played a role in the divergent response to doxorubicin. The LCR animals' characteristic large adiposity could have been a factor in doxorubicin's cardiotoxicity as it is recognized that the drug does not achieve high concentrations in fat tissue and that obesity has been shown to slow the metabolism of the drug (189,190). Therefore, it is hypothesized that since anthracyclines do not distribute into fat then equivalent doses based on body surface area may lead to higher concentrations of doxorubicin in the hearts of LCRs than HCRs (191). Moreover, HCR rats have been shown to have higher markers of mitochondrial content in their locomotor muscles (178). Increased body weight, decreased fatty acid oxidation, and reduced insulin sensitivity has been associated with the reduced mitochondrial content in the skeletal muscle of LCR rats (185). A determinant of aerobic capacity performance is local oxidative capacity, namely, mitochondrial density. To achieve a given rate of oxygen uptake, greater mitochondrial density will require a lesser degree of activation per mitochondrion.

Subsequently, smaller increases in controllers of respiration, such as ADP (192). That is also supported by our results which show significant differences in ADP stimulated respiration, suggesting higher State III oxygen consumption in the LCRs. However, HCRs have about 30% more mitochondrial protein/μg LV protein. One possibility is that LCRs increase cardiac mitochondrial respiratory rates as an alternative to decreased mitochondrial content. Taken together, there is a strong argument that the differences between the phenotypes in their adaptive responses to doxorubicin is determined by the inherent differences that exist due to the genetic endowment.

A second possible explanation for the differences in response to DOX between the phenotypes is that the two groups adapt similarly, but the LCR response is attenuated due to poor intrinsic aerobic capacity. In the cardiac performance data, we see that the LCR animals' cardiac output is initially decreased, but by the fourteenth day, the LCRs demonstrated increases in this value to similar levels as the HCRs. These data support the explanation that the adaptive responses employed by the HCR and LCR animals are similar, but the timing of the response is the component that is influenced by inherent aerobic exercise capacity.

Our study results may have also varied if we sacrificed the animals earlier (such as day 4 or day 7) and examined mitochondrial function sooner. We may have seen a more pronounced difference in the cardiac mitochondrial function between the phenotypes. Based on our results, we most likely examined acute doxorubicin cardiotoxicity, which are nonlife threatening events that are resolved within a week. Acute cardiotoxicity damage resolves promptly to the cessation of doxorubicin infusion and rarely precludes further continuation of treatment. However, the types of chronic toxicity are irreversible and clinically significant, substantially affecting overall morbidity and mortality and requiring long-term therapy (193,194). Studies have shown that the genetic

makeup of patients may modulate the individual risk to develop cardiotoxicity (195–197). Thus, in experimental toxicity induced by doxorubicin, the dose of DOX used is crucial as it appears to be an important determinant not only of the severity of the cardiotoxicity that eventually ensues, but notably of the responsiveness of the HCR and LCR animals.

A third possible explanation is that the animal phenotypes utilize different mechanisms to adapt to the doxorubicin treatment. This conclusion is supported by the cardiac performance and mitochondria respiration results that show that the HCR rats adapted to a mild dose of DOX by increasing heart rate which transpired into an increased cardiac output with no change in mitochondrial respiration while LCR rats respond to DOX by increasing the volumes and the glutamate-based oxygen consumption of the mitochondria. In contrast, it is theorized that a more severe dosage results in an accumulation of doxorubicin in mitochondria that subsequently leads to heart failure (198). Thus, these results suggest that the beneficial effects of a high aerobic capacity phenotype encompass unique protective mechanisms to cardiotoxicity, guiding to an improved mitochondrial and cardiac function, and thereby, to a higher fitness.

CONCLUSION

This study provides new insight into the effects of doxorubicin treatment on mitochondrial and cardiac function in rats with divergent aerobic capacities. DOX injection caused an impairment of cardiac output in animals bred for low aerobic capacity which was associated with elevated cardiac volume dimensions and elevated mitochondrial respiration, particularly in the glutamate-based protocol. Furthermore, selection for high aerobic capacity, in the absence of exercise training, endows increased mitochondrial density, heart rate and cardiac output after doxorubicin treatment. These data provide evidence that differences in the mitochondrial function and cardiac performance may have a role in the divergence in aerobic capacity in the LCR/HCR model.

Although several mechanisms have been postulated to explain the pathogenesis of cardiotoxicity and its associated clinical manifestations, the precise details of the cellular and subcellular alterations remain to be elucidated. There has been a recent surge in experimental studies suggesting mitochondria may be intimately involved in the cardiotoxicity. Further investigation of whether there are differences in markers of cardiac mitochondrial capacity and density between phenotypes should be determined. Also, it has been suggested that differences in individual antioxidant defenses may hold the key to understanding doxorubicin susceptibility therefore, direct measurements of oxidative stress should be also taken in the consideration for future studies. It is vital that advances to effectively protect against doxorubicin's cardiotoxicity are developed to prevent cancer survivors from forming chronic life-threatening conditions, such as heart failure secondary to this therapy. Aerobic capacity is an integrated, polygenic phenomenon, however, through this study it is becoming clear that selection for high aerobic capacity, in the absence of exercise training may contribute in the improvement of DOX-induced cardiotoxity.

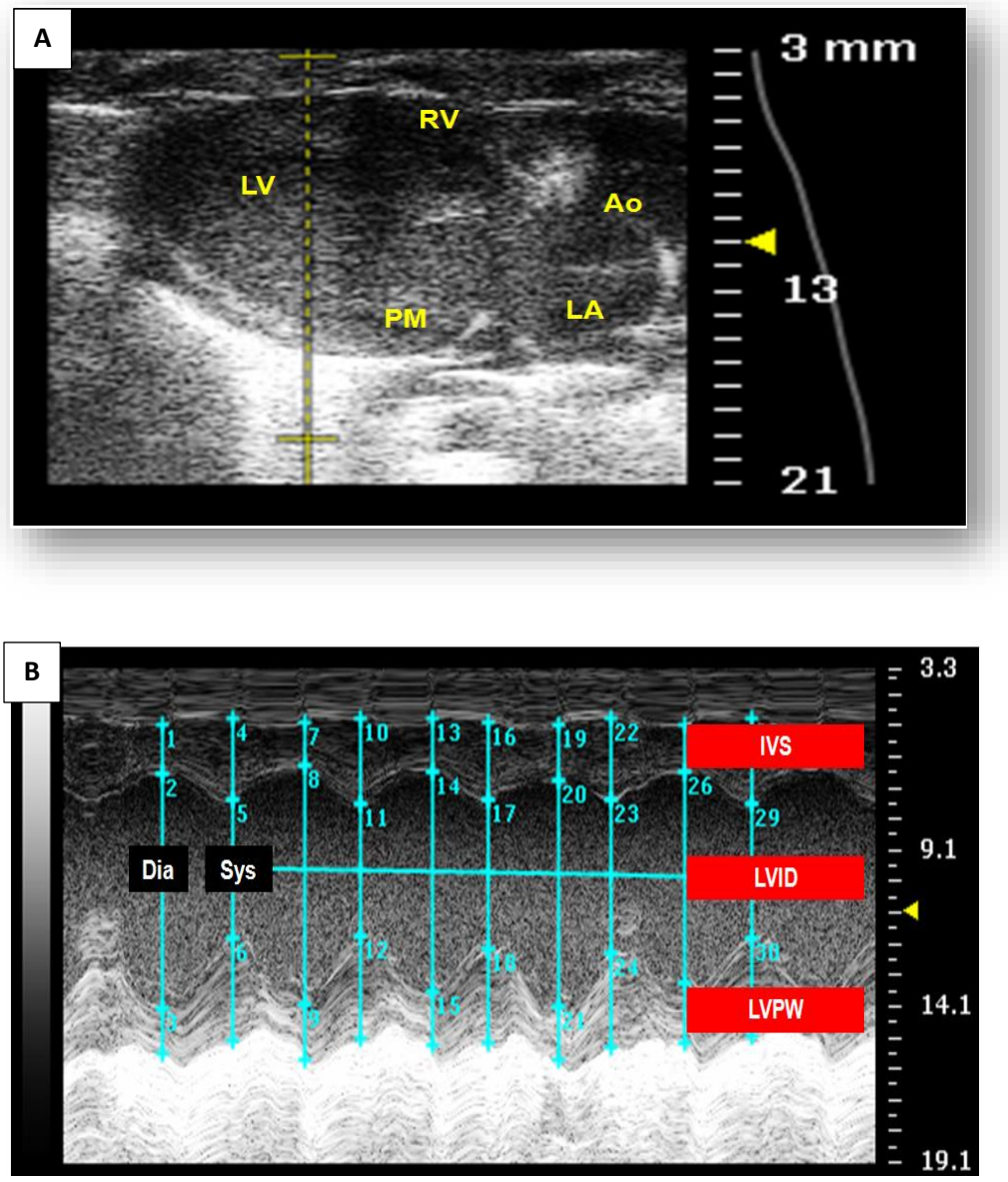


Figure 3.1. Echocardiography parasternal long-axis image of the rat heart at level of the papillary muscle. A, B-Mod. B, M-mode. Images were acquired using Vevo 2100 ultrasound system (Visualsonics). Dia, Diastole: IVS, inter ventricular septum; LVID, left ventricular internal diameter: LVPW, left ventricular posterior wall; Sys, Systole.

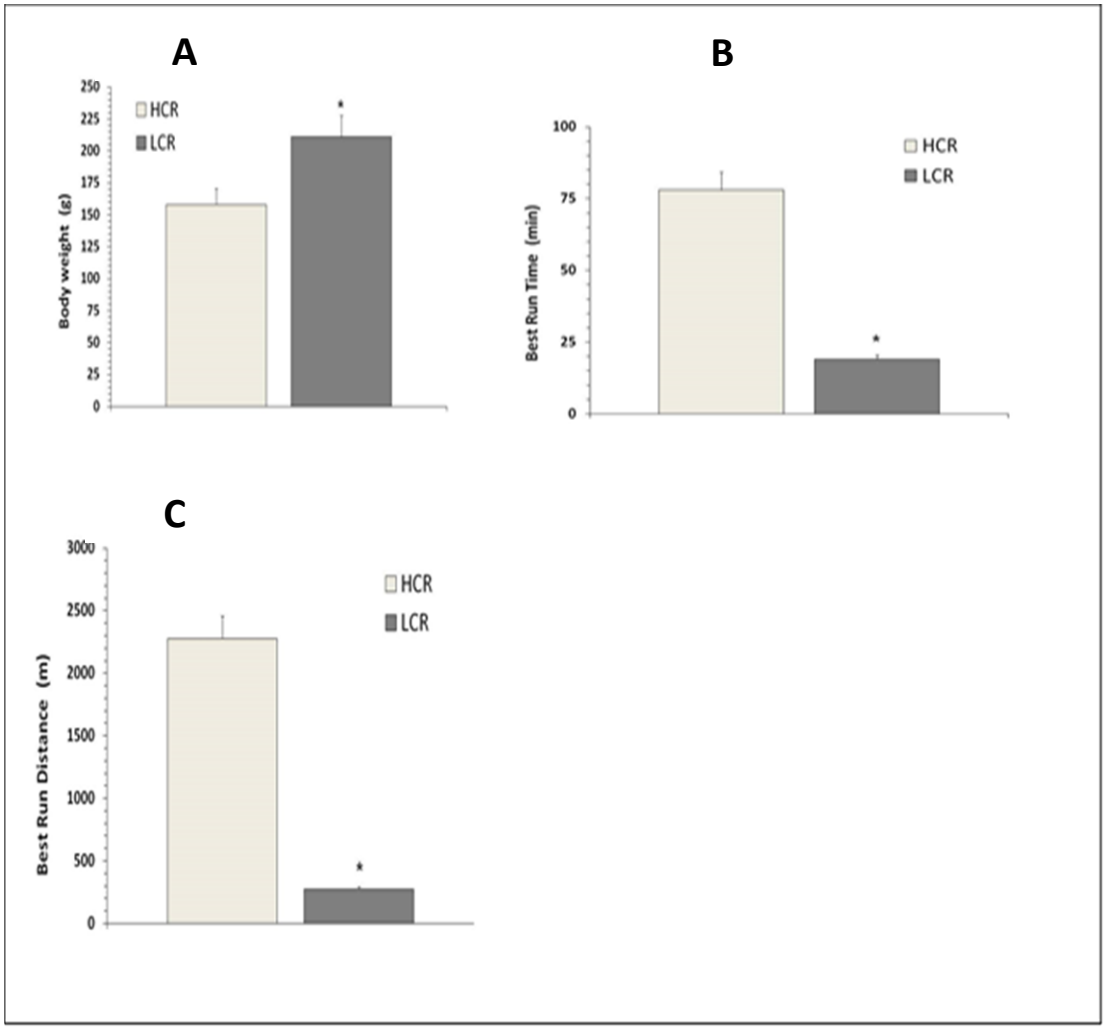


Figure 3.2. Summary of body weight differences; a, best run time; b, and best run distance; c between HCR and LCR cohorts at the time of phenotyping (12 weeks of age). (mean ± SEM, * indicates $p < 0.05$ vs. HCR)

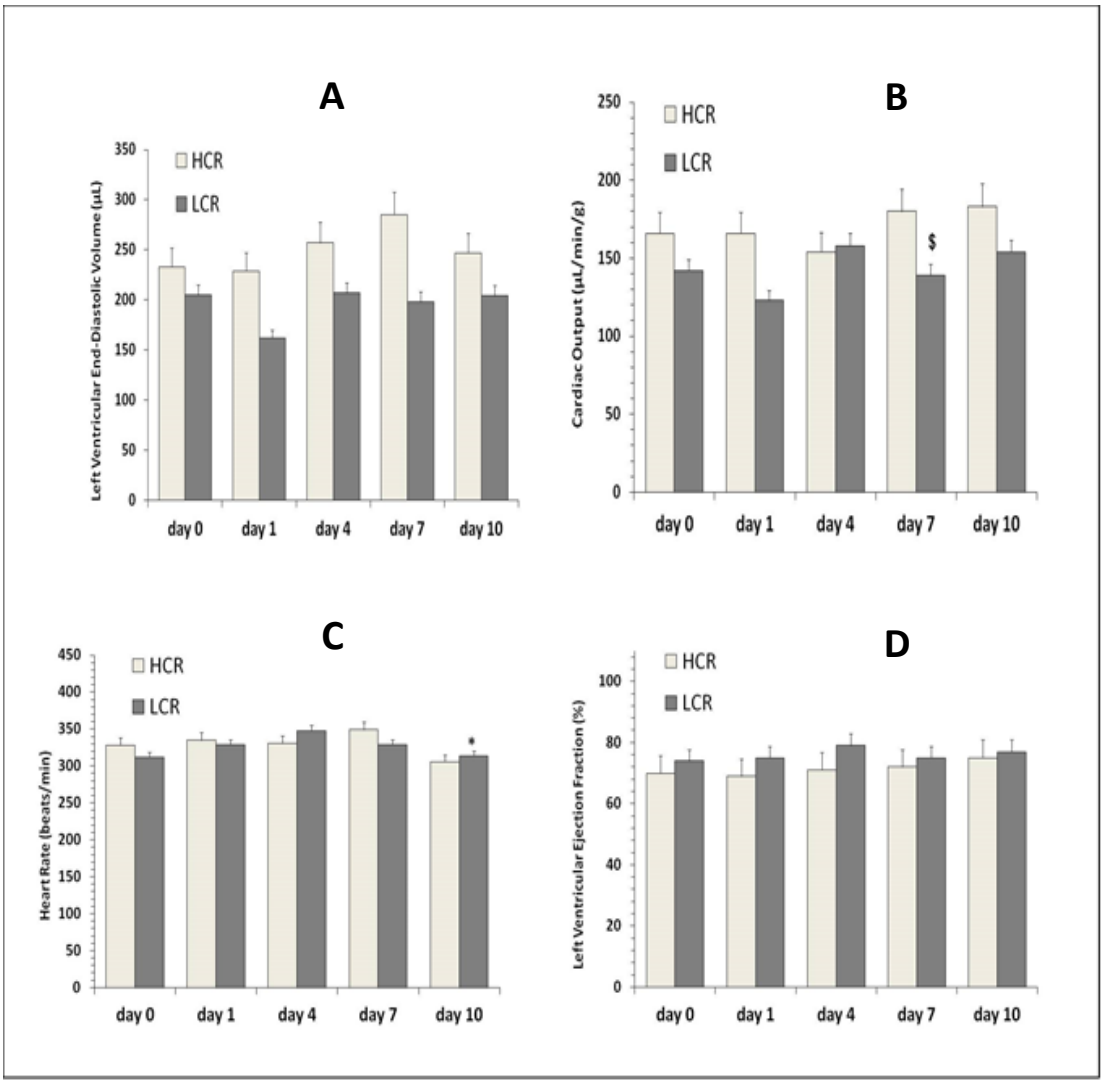


Figure 3.3. Summary of diastolic ventricular chamber size; a, cardiac output; b, heart rate; c, and ejection fraction; d between control HCR and LCR cohorts. (mean ± SEM,* indicates $p < 0.05$. \$ $p < 0.05$ vs. HCR phenotype at the same time point). LCR-Ctrl (n=6), HCR-Ctrl (n=3)

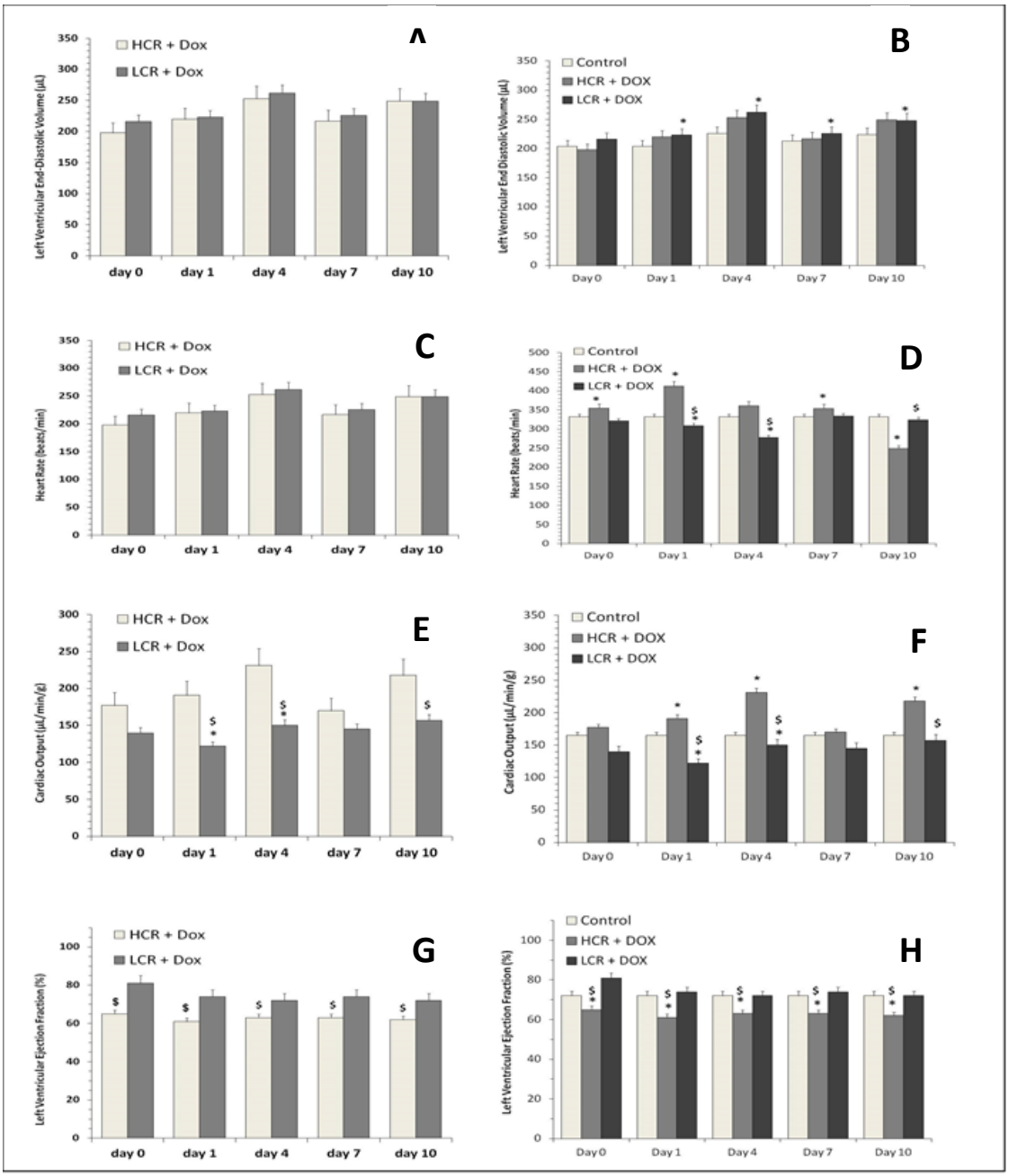


Figure 3.4. Summary of doxorubicin effects on diastolic ventricular chamber size; a & b, heart rate; c & d, cardiac output; e & f, and ejection fraction; g & h between HCR and LCR cohorts. (mean ± SEM, * indicates $p< 0.05$. \$ $p < 0.05$ vs. HCR phenotype at the same time point). LCR-Ctrl (n=6), HCR-Ctrl (n=3), LCR + DOX (n=9), HCR+DOX (n=5).

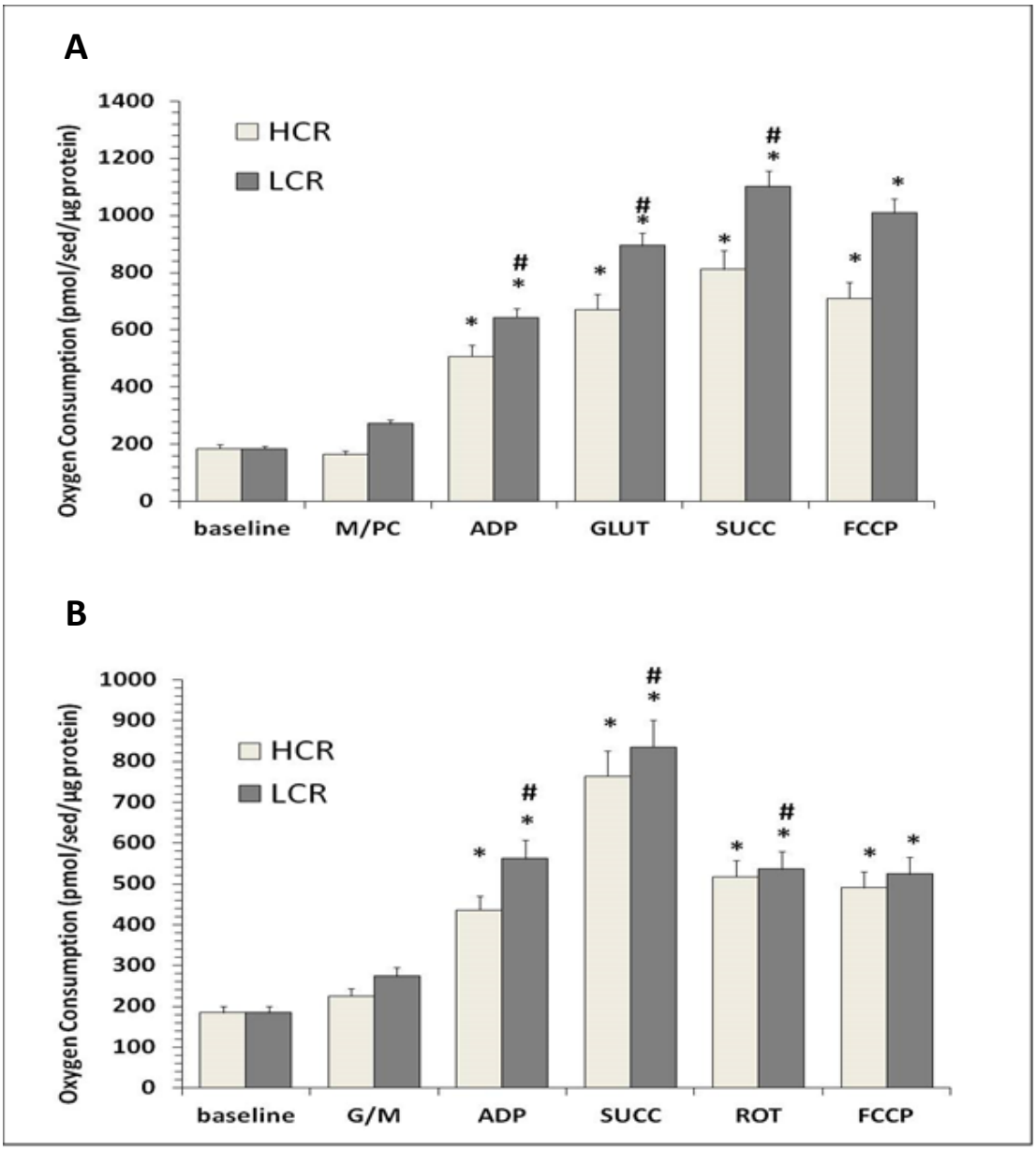


Figure 3.5. Control animal mitochondrial function. Baseline value for the comparison of phenotype effects on aging dependent changes in cardiac mitochondrial function, measured as oxygen consumption, under different substrate conditions in a glutamate-based protocol, a and a fatty acid-based protocol, b. (mean ± SEM, * indicates $p<0.05$ vs. baseline value for the phenotype; # indicates $p< 0.05$ vs. previous treatment condition for the phenotype. LCR-Ctrl (n=6), HCR-Ctrl (n=3), LCR + DOX (n=9), HCR+DOX (n=5).

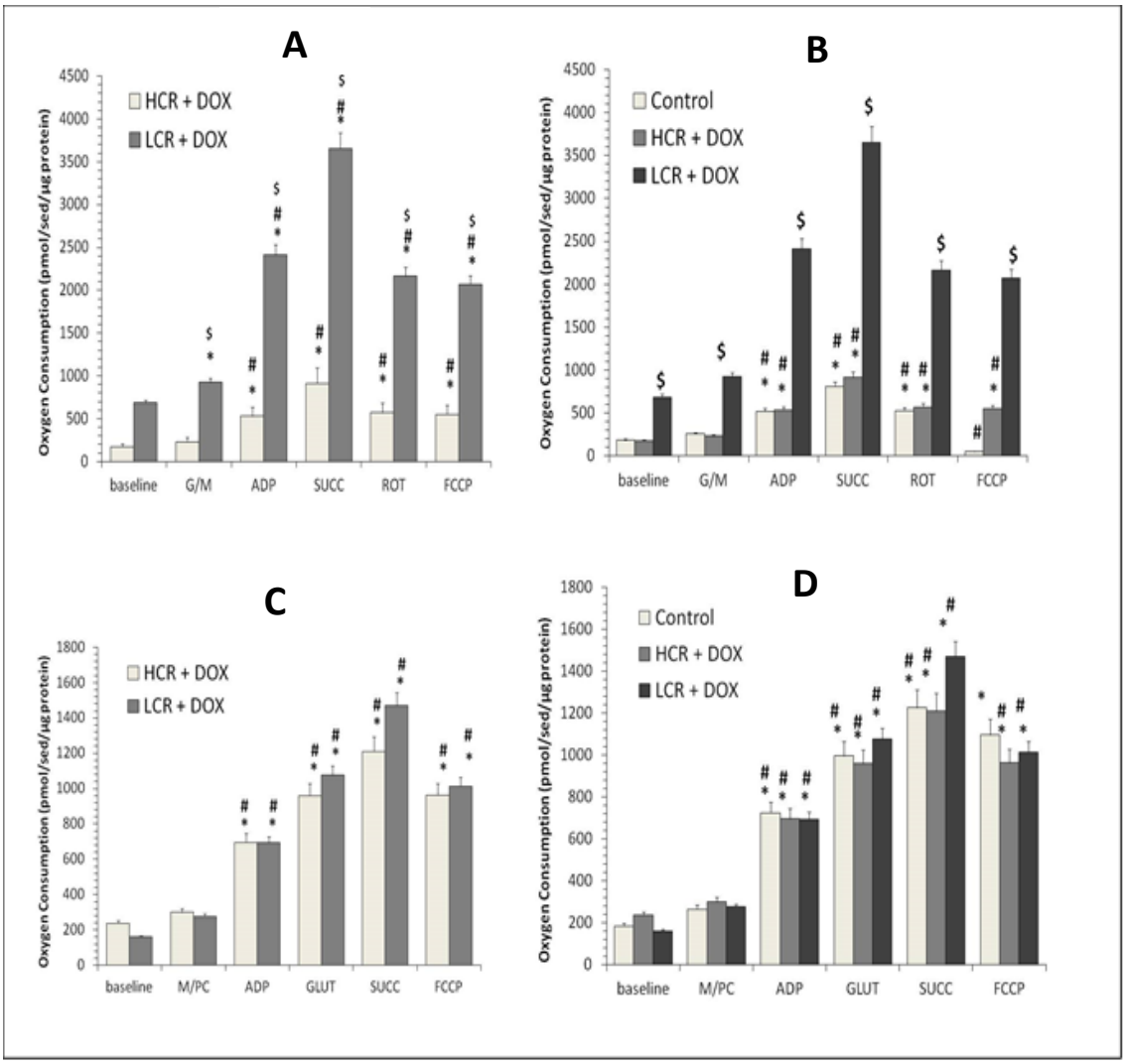


Figure 3.6. DOX-treated animal mitochondrial function. Comparison of phenotype influence on doxorubicin dependent changes in cardiac mitochondrial function, measured as oxygen consumption, under different substrate conditions in a glutamate based protocol, a & b and a fatty acid based protocol, c & d. * indicates $p<0.05$ vs. baseline value for the phenotype; # indicates $p< 0.05$ vs. previous treatment condition for the phenotype. LCR-Ctrl (n=6), HCR-Ctrl (n=3), LCR + DOX (n=9), HCR+DOX (n=5).

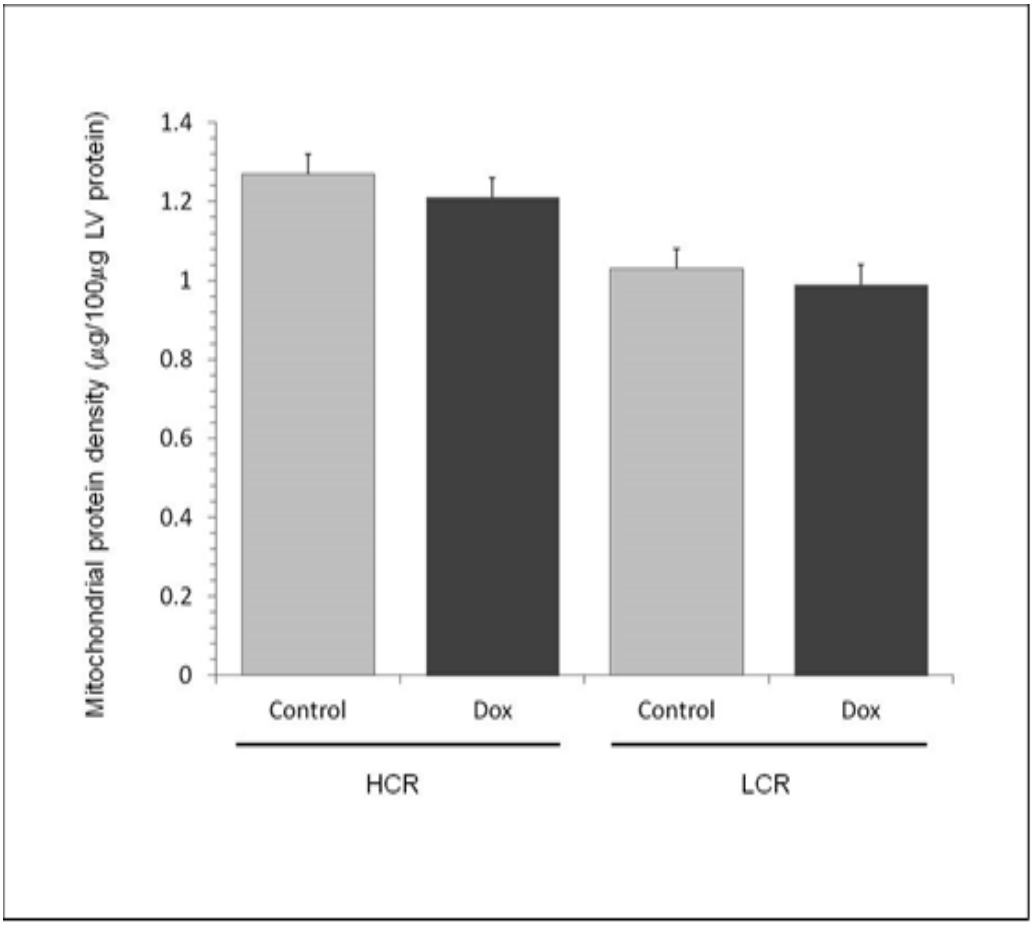


Figure 3.7. Mitochondrial protein amounts. Comparison of mitochondrial protein amounts in control and doxorubicin treated animals. Doxorubicin did not cause a loss in mitochondrial protein in either phenotype, but HCRs consistently demonstrated 30-35% higher mitochondrial protein content per unit LV mass than the LCRs. LCR-Ctrl (n=6), HCR-Ctrl (n=3), LCR + DOX (n=9), HCR+DOX (n=5).

CHAPTER 4: IMPACT OF INTRINSIC AEROBIC EXERCISE CAPACITY ON MYOCARDIAL RESPONSE FOLLOWING ACUTE ISCHEMIA-REPERFUSION INJURY (AIRI)

ABSTRACT

Purpose: Previous reports have suggested that the LCR phenotype appears to be at risk for cardiovascular diseases relative to the HCR phenotype, both in the susceptibility to ischemia induced arrhythmias and to diet induced insulin resistance. Whether these effects extend to injury limitations in an overt infarction or whether the effects are gender specific is not known. One pathway commonly associated with myocardial protection is the reperfusion injury salvage kinase (RISK), which utilizes several kinases including AKT and PKCε. Therefore, this study was designed to determine whether HCR/LCR phenotype differences would be evident in responses to acute myocardial ischemia-reperfusion injury (AIRI), measured as infarct size and to determine whether sex differences in infarction size were preserved with phenotypic selection.

Methods: Myocardial AIRI was induced in intubated, mechanically ventilated, anesthetized rats by 15- and 30-min ligation of the left anterior descending coronary artery, followed by 2 hr of reperfusion. The infarct size was determined by 2, 3, 5 - triphenyltetrazolium chloride staining, and normalized to area at risk. Portions of the tissue were snap frozen for subsequent protein isolations and western blot analysis or were paraffin embedded for H&E staining and histology analysis.

Results: Phenotype dependent differences in infarct size were seen in short time ligation but not in longer time ligation. not evident, but sex differences in infarct size were present regardless of phenotype. (male: HCR (n = 7), 42. 70 + 7. 05; LCR (n = 7), 44. 66 + 8. 95, p = n. s., female: HCR (n = 5), 32. 62 + 4. 76; LCR (n = 8), 31. 89 + 7. 20, p = n. s.). Infarct size could

not be correlated significantly with either body weight or run distance at selection. Western blots revealed that enzymes associated with the cardioprotective RISK pathway (AKT, P-AKT, PKC) and xanthine oxidase, an intrinsic antioxidant enzyme, were not different between the phenotypes. Histological analysis indicated that there were no differences in neutrophil infiltration between any of the groups.

Conclusions: The protection of high inherent aerobic capacity against AIRI seems to be time-dependent protection. However, with long time ligation, the phenotypic potential to exercise did not alter myocardial injury induced by AIRI, and selection based on endurance running capacity preserved sex differences. The induction of the RISK pathway was similar in both sexes and did not affect by phenotype. Phenotypic cardioprotection associated with arrhythmias and metabolic syndrome did not extend to injury reduction in an acute onset infarction.

INTRODUCTION

Enhanced aerobic capacity has been associated with diminished morbidity, improved quality of life, and decrease risk for cardiovascular diseases (156,199,200). Coronary artery disease is currently the leading cause of mortality and morbidity in the western world. The most common fatal consequence of coronary artery disease is an acute myocardial infarction, (201) and death due to coronary artery disease appears to decrease with treatments that include an aerobic exercise component (199,200). It remains unclear whether the benefits of exercise derive primarily from altered risk profiles (lipid, obesity, etc.) or directly from intrinsic myocardial resistance to injury.

Aerobic exercise capacity has genetic and environmental components. The genetic component define the intrinsic endurance exercise capacity and appears to have two parts, the genes that regulate adaptive responses to exercise training and the genes that determine intrinsic

exercise capacity (2,3,57). Although aerobic exercise training has beneficial effects on several of cardiovascular diseases, the variability in the physiological response to exercise training suggests potential impact of the genetic composition. It has been estimated that up to 60%-70% of the variation in exercise capacity is due to the genetic component (2,57,90). It is not clear if the genetic component for enhanced exercise capacity alone can result in protection from cardiovascular diseases or whether the training stimulus is necessary to produce the positive results, or a combination of both. The development of a novel rat model by Drs. Koch and Britton that emphasizes the differences in intrinsic aerobic exercise capacity has advanced the ability to differentiate the genetic components from the environmental effects, such as exercise training, that also influence the aerobic capacity (68,92,202). This novel rat model contrasts intrinsic aerobic capacity as a phenotype, low-capacity runners (LCR) and high-capacity runners (HCR), and developed by artificial selective breeding for endurance running provides a mechanism to address the genetic component of exercise and its contribution to cardiovascular disease (92). The LCR/ HCR models provide a means to test if phenotypic selection for aerobic capacity also selects for enhanced inductors of protective ischemia and reperfusion intracellular pathways. Untrained low endurance running capacity (LCR) rats were found to have a higher incidence of risk factors associated with cardiovascular disease than their untrained high endurance running capacity (HCR) counterparts (68,95). The LCR rats were more insulin resistant, had higher mean blood pressures, decreased nitric oxide mediated vascular relaxation, and had lower expression of proteins critical to skeletal muscle fatty acid oxidation (68,95). LCR animals were predisposed to weight gain and increased blood free fatty acid (FFA) levels compared to HCR counterparts when challenged with a high fat diet (95). Moreover, compared to the HCRs, the LCRs displayed higher arrythmogenicity following short term myocardial ischemia and reperfusion (72).

Early studies in this model also demonstrated differences in several skeletal muscle metabolic and vascular endpoints. In generation 7, HCR rats demonstrated increased VO2 max that was attributed to an increased O2 capacity and/ or increased capillary density in skeletal muscle under both normoxic and hypoxic exercise conditions (203). In generation 15, HCR rats had increased VO2 max while LCRs appeared to have decreased VO2 max values. The HCRs still exhibited an increases in skeletal muscle capillary density, and increased oxidative enzymes but in addition, they also demonstrated higher maximum cardiac stroke volume (SV) values in comparison to their LCR counterparts (204). In addition to differences in SV, the HCR's demonstrated a larger exercise induced peripheral vasodilation response. HCRs and LCRs also were tested in our lab to determine the vascular and metabolic adaptive response to acute peripheral artery ligation. This study showed that LCR vascular and metabolic responses deteriorated after acute ischemic injury and that HCR was more resistant to the ischemic injury. Another recent study from our lab have investigated the impact of intrinsic aerobic capacity on the vascular adaptive response to active exercise following lower limb ischemia. The results demonstrated that HCR rats showed 26% better preservation of muscle fiber area in the post-ischemic limb, but LCR rats showed at least a 2-fold increase in fiber area in response to active exercise. Moreover, HCR samples showed a greater increase (80%) in capillary density and perfusion exchange ratio (40%) in response to exercise. These data suggested that HCRs have improved collateral circulation and improved vascular vasodilation in skeletal muscle during maximal exercise and these differences are the factors that contribute to the phenotypic differences in this model.

Another study utilized a high fat diet to produce increased weight gain and fat mass within the LCR rats which exacerbate their insulin resistant condition (95). Noland et al. demonstrated that the LCR phenotype did not tolerate a high fat diet as well as their HCR counterparts and that

the artificial selection for aerobic capacity that produces the HCR phenotype has also isolated protective measures against high fat diet induced obesity and insulin resistance. The aggregate results of study to date have suggested that artificial selection for endurance running capacity also selects for intracellular pathways that provide protection against metabolic and cardiovascular stresses. At the cellular level, it has been demonstrated that HCRs show larger amplitude of calcium transients and higher efficiency in energy production (205), along with faster sarcomeric shortening and relaxation (68,206). Previous reports also have suggested a role for several kinases in the ability of the myocardium to withstand ischemic and reperfusion injury, including phosphorylated AKT, and PKCε (207,208). In addition, intrinsic antioxidant capacity provided by enzymes such as xanthine oxidase also can be cardioprotective following ischemic injury. Yellon et al. has demonstrated that the reperfusion injury salvage kinase (RISK) pathway is one intracellular cascade within myocardium that appears to mediate cardioprotective effects and utilizes the kinases AKT and PKC (209–211). In examining diabetic models, Yellon et al. has established that these models appear to have suppressed RISK pathways and attributed the inability of diabetic myocardium to respond to ischemic preconditioning to their inability to activate the RISK pathway (210).

Subjecting animals of the HCR/ LCR phenotype to conditions of acute ischemia and reperfusion permits an examination of whether the capacity for exercise itself is cardioprotective at rest, or whether selection against running capacity is associated with impaired expression and induction of RISK pathways as the trend to insulin resistance in these animals might predict.

To test this hypothesis, the following study was performed to test whether the LCR phenotype have limited protection to myocardium following acute ischemia – reperfusion injury (AIRI).

MATERIALS AND METHODS

Animal Strain. The development of LCR and HCR rats has been described previously in detail (92). Rats were selected from a heterogeneous rat population in the N: NIH stock (National Institutes of Health, USA) based on inherent running capacity. Endurance running capacity was assessed at 11 weeks of age using run time and distance to exhaustion on a treadmill (15 degrees incline; initial velocity 10m/ min and increased 1m/ min every 2 mins) as parameters. The highest 20 % in running capacity of each gender were randomly inbred to produce the HCR strain and the lowest 20 % in running capacity were inbred to produce the LCR strain. Subsequent generations were assessed and bred in a similar fashion with precautions taken to minimize inbreeding (< 1 % per generation).

Both female and male HRC and LCR rats, 16-18 months of age, from generation 17 were used in this investigation. They were housed in a temperature-controlled environment with a 12: 12h light:dark cycle throughout their lifespan. There were provided standard rat chow and water ad libitum. All animal procedures followed in this study were approved by the East Carolina University Committee on the Use and Care of Laboratory animals and conformed to the standards in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Acute I/R Injury. AIRI was induced in four experimental groups and two-time frames (15 & 30 min). AIRI was induced using procedures essentially as described previously (212,213). Briefly, all animals were anesthetized (pentobarbitol 65mg/ ml) and mechanically ventilated with room air. The heart was exposed through a thoracotomy performed in the left fifth intercostal space. The pericardium was gently separated, and a ligation of the left anterior descending coronary artery was performed using 6.0 silk suture and a reversible snare. Occlusion was

confirmed by cyanosis of the distal myocardial tissue. Following 15- or 30-min occlusion, the snare was released, and the tissue was under reperfusion for 2 hr.

Determination and quantification of infarct size. At the end of the 2 hr reperfusion period, animals were euthanized, and the hearts were excised. The heart was infused retrogradedly through the aorta with 1.0% 2,3,5-triphenyltetrazolium chloride (TTC) solution to delineate the infarcted area. The ligature tightened to re-occlude the artery and 1.0% methylene blue dye was retrogradely infused through the aorta to delineate the area at risk. Multiple transverse sections were made of the hearts and photographed using a digital camera. From these photographs, the LV area, area at risk, and area of infarction in each image were determined using NIH image software (ImageJ, version 1.34s). Values from each section were averaged to obtain a value for each animal. The area at risk was expressed as a percentage of the LV area and the area of the infracted zone was expressed as a percentage of the area at risk. Average values for each animal were used for statistical summary and comparisons of groups.

Western Blot. Tissue was homogenized, and centrifuged at 15,000 g for 25 min at 4°C. The supernatant was removed, and protein content was determined (Invitrogen EZQ kit). Following the protein quantitation, aliquots of protein were solubilized and subjected to Tris-HCL gel electrophoresis and transferred to an immobilon-p transfer membrane. After blocking, membranes were incubated with 1° antibody overnight (anti-AKT, anti P-AKT, anti-xanthine oxidase, or anti- PKCε, each at 1:1000). After washing and incubating with HRP conjugated 2º antibody, the blot was incubated in ECL solution, exposed to X-ray film for autoradiography, and quantified.

Histology. Histology was performed on the same tissue sections used for infarct measurements. Following TTC staining and digital photography, one of the myocardial sections

was fixed in 4% parafamaldhyde, embedded in paraffin wax, and cut at 5-um sections. Sections were stained with hematoxylin and eosin (H&E) and examined by light microscopy under high magnification (X60). The number of infiltrated neutrophils in each high - power field was counted and normalized to the area of the field, based on the microscope specifications. Neutrophil counts were made on two sections per rat heart and on five fields per section.

Statistics. Data are expressed as means + SE. Differences between groups were determined by ANOVA and Tukey ' s post hoc test with significance determined when $p < 0.05$.

RESULTS

Risk areas were not different between phenotypes subjected to acute I/R injury

The LV area at risk after AIRI was normalized to total LV area in each rat. There was no significant difference in the area at risk across all groups (HCR males (n = 6) 57.3 ± 3.0%, LCR males (n=6) 63.0 ± 2.7%, HCR females (n=5), 52.8 ± 2.2%, LCR females (n=6), 56.7% ± 2. 6) and both different time frames, 15 or 30 min, (Figure 4.1) indicating that the placement of the ligature was consistent across all groups.

Phenotypic differences in infarct size exist with 15 minutes, but not 30 minutes ischemia

With short time ligation (15 minutes), myocardial infarct size following AIRI was significantly different between HCRs and LCRs. Significantly less myocardial infarct size was observed in the HCRs vs. LCRs hearts (13% relative reduction) following 15 minutes of ischemia and 2 hours of reperfusion. With longer time ligation (30 minutes), myocardial infarct size following AIRI was not altered by phenotype in this model of endurance exercise capacity, but female rats in both the HCR and LCR groups exhibited smaller infarct size compared to their male counterparts (HCR males, 42.7 ± 1.7%, LCR males, 44.7 ± 2.5%, HCR females, 32.6 ± 1.5%, LCR females, 31.9 ± 1.8%) (figure 4.2). Thus, the AIRI in the model of endurance exercise capacity

demonstrates the gender difference and phenotypic differences in the short time ligation and gender differences but not a phenotypic difference with longer time ligation.

RISK pathway protein expression markers were not different between phenotypes after IR

Representative western blots demonstrated that in both phenotypes the basal expression of RISK pathway enzymes did not differ. Following AIRI, the phenotypes all demonstrated the ability to increases total AKT, PKCE and Xanthine Oxidase levels in comparison to resting conditions (Figure 4.3). The increase in these RISK pathway enzymes did not differ between the phenotypes.

Neutrophilic infiltration after IR was similar in both phenotypes

Histology results indicated that myocardial neutrophil infiltrate following AIRI increased in all groups. There was no differ with infiltrate according to phenotype or gender (Figure 4.4) (Table 4.1).

DISCUSSION

In this study, using *in vivo* ischemia reperfusion model incorporating with the application of different durations of ischemia and reperfusion in the same disease, the major findings demonstrated that LCRs heart are more vulnerable to AIRI at its early stage. However, with longer time ligation, the phenotypic difference in myocardia infarct size was disappeared but the gender differences were preserved. The two-time frames were used to just test the infarct size between the two phenotypes, while other evaluation has been done in 30 min ligation rats only.

The phenotypic differences observed in myocardial infarction size with shorter ischemic times but not with longer time ligation suggest that the increased aerobic capacity phenotype may not be able to resist the severity of oxidative stress and reduction of endogenous antioxidant

capacity and the impairment of protective signaling pathway related to the activation of Akt, the major mechanisms for the increased myocardial injury after prolonged ischemic insult (214).

After 30 min ligation, the ideal time for AIRI model, the RISK pathway that has been associated with cardioprotection in ischemia and reperfusion injuries was intact and inducible in both phenotypes but did not differ between the phenotypes or the sexes. Myocardial neutrophil infiltrate following AIRI did not differ according to phenotype or gender.

Gender has been recognized as an important factor in determining the risk for cardiovascular diseases (73) and cardioprotective effects of sex hormones have been reported in both experimental and clinical studies (111). Moreover, gender differences have been well established in models of ischemia reperfusion injury (214). Bae and Zhang observed significantly less myocardial injury in female vs. male hearts following 25 minutes of ischemia and 2 hours of reperfusion in Langendorff-perfused rat hearts (358). In the present study, the gender differences have been preserved in both strain which is consistent with other previous studies.

In humans there is a strong association with fitness and cardiovascular protection. Regular physical exercise is obviously effective in the secondary prevention of cardiovascular disease and is effective in attenuating the risk of premature death among men and women. The HCR and LCR phenotypes where developed as a model to assess the genetic components of aerobic capacity. In other rat models, it has been demonstrated that acute exercise training can induce cardioprotection that results in reduced infarctions following ischemia - reperfusion injuries (215–217). The HCR/ LCR model has inherited differences in aerobic capacity without prior training. In addition, Wisloff et al. found that the LCR phenotype scored high on cardiovascular risk factors and the HCR score high for health factors (68) while Lujan et al. reported that the LCR phenotype demonstrated increased ischemia-reperfusion–mediated ventricular tachyarrhythmias and the

HCR phenotype decreased susceptibility to tachyarrhythmias (72). This led to the hypothesis that the LCR phenotype would have limited protection to myocardium following AIRI. The LCR phenotype did not, however, demonstrate a limitation with the AIRI. This leads to a few possible explanations that might account for these unexpected observations. The injury of an AIRI may be too severe to pick up the differences within this model. Yaoita et al, and Yoshinari et al. have demonstrated that a myocardial infarction injury was unable to demonstrated differences in exercise training and high cholesterol dieting (218,219). These studies utilized a less severe injury of partial occlusion that was able to demonstrate differences in myocardial tissue.

Powers (34,285,289) has demonstrated that exercise training produces cardioprotection which may be a greater role in cardioprotection than the genetic component. However, our results suggest that high intrinsic aerobic capacity phenotype did not confer protection against AIRI injury, several studies indicate the protective effect of exercise preconditioning against diverse cardiovascular diseases (220,221). Regarding myocardial infarction (MI), Morris et al. reported greater survival rate in fit compared with inactive subjects after MI (217). Other experimental animal studies suggested that exercise training preconditions the heart and protects it against ischemia-reperfusion injury (221–223) and promotes healing of the infarcted area (223).

This model has demonstrated consistently that peripheral tissues have phenotypic differences. It appears that the phenotypic differences maybe more pronounced in peripheral tissues and not in cardiac. Noland et al. found changes in metabolic FFA utilization was affected by phenotype and was more pronounced in skeletal muscle while cardiac tissue did not demonstrate these differences even with high fat feeding (95). Recent work from our lab has also suggest that HCR rats showed 26% better preservation of muscle fiber area in the post-ischemic limb.

The AIRI injury may produce the same end result in infarction size but achieve this through different mechanisms. One of the potential explanations for the absence of detectable protection in the present study is that the phenotypic changes developed through generation in rats selected for low aerobic capacity may have triggered mechanisms that, to some extent, may precondition the heart against ischemic damage. These mechanisms were not investigated in the current study, but previous studies with using this model have shown that the immune response is exaggerated in LCR (214), and particularly TNF-α (224) as well as interleukin-10 (225), which have been shown to have preconditioning influences on ischemia-reperfusion injury and is significantly upregulated in the LCR rats (157).

Ischemia-reperfusion activates the anti-apoptotic pro-survival kinase signaling cascades which include: phosphatidylinositol-3- OH kinase (PI3K)-Akt and p42/p44 extra-cellular signal-regulated kinases (Erk 1/ 2) (207,209,210). Activating these pro - survival kinase cascades at the time of reperfusion has been demonstrated to confer protection against reperfusion - induced injury (207,209,210). Tsang et al. demonstrated that in a diabetic rat model the RISK signaling cascade was functionally limited with AIRI. This lead to the diabetic model to be less responsive to ischemia preconditioning (210). Data suggest that the LCR phenotype is more insulin resistant (72),(68) and when pushed with high fat feeding this insulin resistance is exacerbated (95). The insulin resistance does not appear to be severe enough to depress the inducible RISK pathway in this phenotype following AIRI.

AIRI is marked by an inflammatory reaction with the infiltration of polymorphonuclear leukocytes (PMN), predominantly neutrophils (226,227). Myocardial neutrophil infiltration is commonly linked to myocardial damage due to massive release of elastases, lipases and proteases as well as reactive oxygen species generated by NADPH oxidase in neutrophil membranes

(212,226). The myocardial neutrophil infiltration did not differ between the phenotypes nor the genders. The increased infarction size between the genders does not appear to be linked to differences in neutrophil accumulation. Sex based differences are present which indicates phenotypic selection based on aerobic capacity did not alter sex-based patterns of response to AIRI. These sex differences do not appear to be related to the RISK pathway or neutrophil infiltration but are present and having a pronounced effect on infarction size.

CONCLUSION

In conclusion, this study suggests that selection for intrinsic endurance capacity has allowed the opportunity to evaluate the independent role of genetics and training on cardioprotection. It could appear that the selection process provides more robust protection to metabolic stressors in comparison to ischemic stressors. Phenotypic selection for inducible exercise capacity maybe more indicative for cardioprotection pathways. Although selection for higher aerobic capacity may provide protection in the early stage of AIRI, this protection benefit is completely disappeared with a longer duration of ligation. Thus, our data do not clearly support a cardioprotective effect of higher inborn aerobic capacity. The cardioprotective role associated with high aerobic capacity seems, therefore, not to depend on inborn characteristics, but rather on acquired aerobic capacity and cardiac preconditioning. Aerobic capacity is an integrated, polygenic phenomenon, and it is becoming clear that simply possessing the intrinsic capacity for aerobic exercise does not confer the same outcomes as has been reported with active exercise.

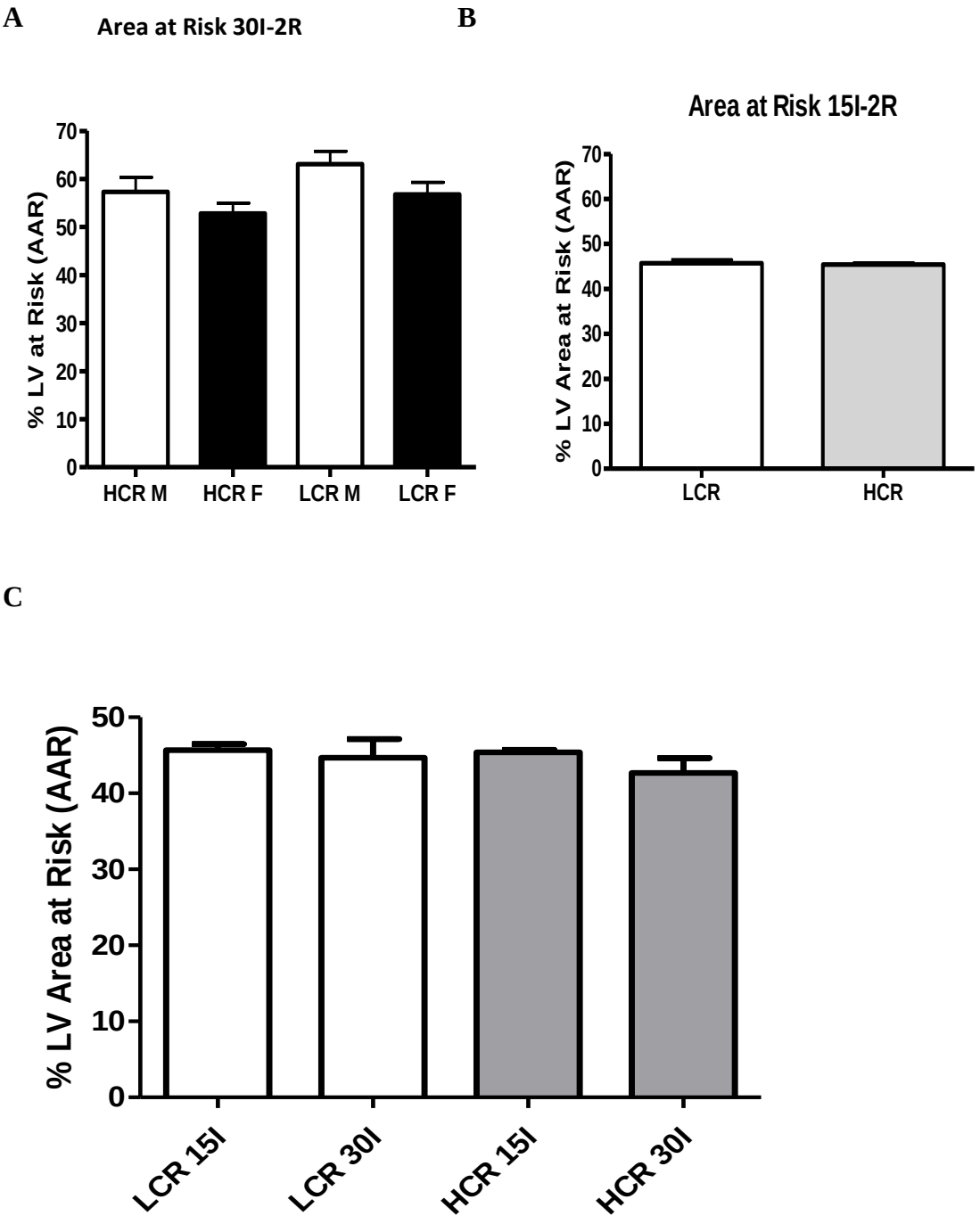


Figure 4.1. A, Comparison of the percentage of the LV at risk for ischemia (AAR) HCR male/female and LCR male/female rats after 30 min ligation. B, Comparison of the percentage of the LV at risk for ischemia (AAR) between HCRs/LCRs after 15 min ligation. C, Comparison of the Average Infarction Area (%AAR) between HCRs/LCRs within two-time frames (30 min and 15 min). (mean ± SEM, * indicates $p < 0.05$). HCR-M (n=7), LCR-M (n=7), HCR-F (n=5), LCR-F (n=8).

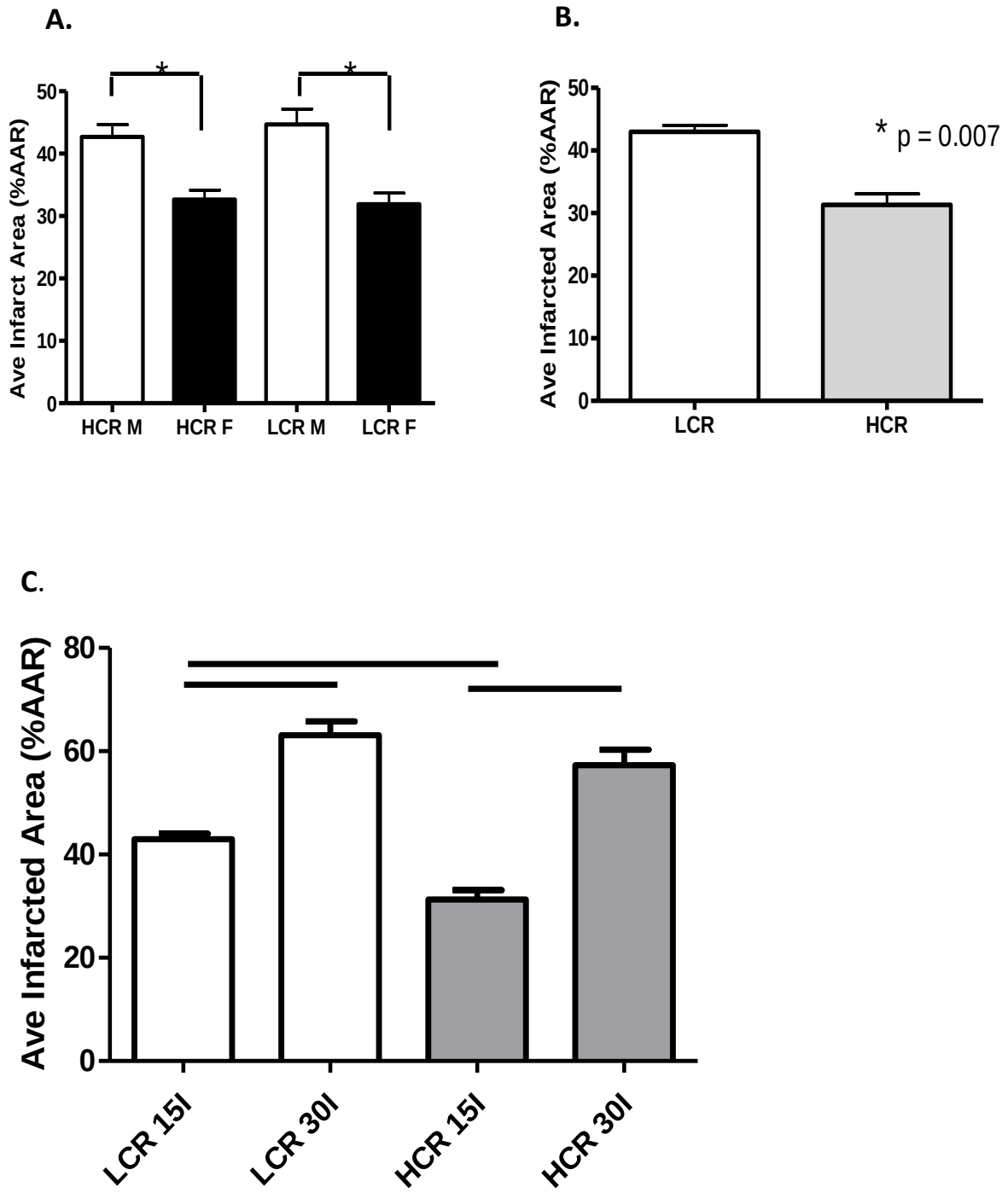


Figure 4.2. A, Comparison of the Average Infarction Area (%AAR) between HCR male/female and LCR male/female rats after 30 min ligation. B, Comparison of the Average Infarction Area (%AAR) between HCRs/LCRs after 15 min ligation. C, Comparison of the Average Infarction Area (%AAR) between HCRs/LCRs within two-time frames (30 min and 15 min). (mean ± SEM, * indicates $p < 0.05$). HCR-M (n=7), LCR-M (n=7), HCR-F (n=5), LCR-F (n=8).

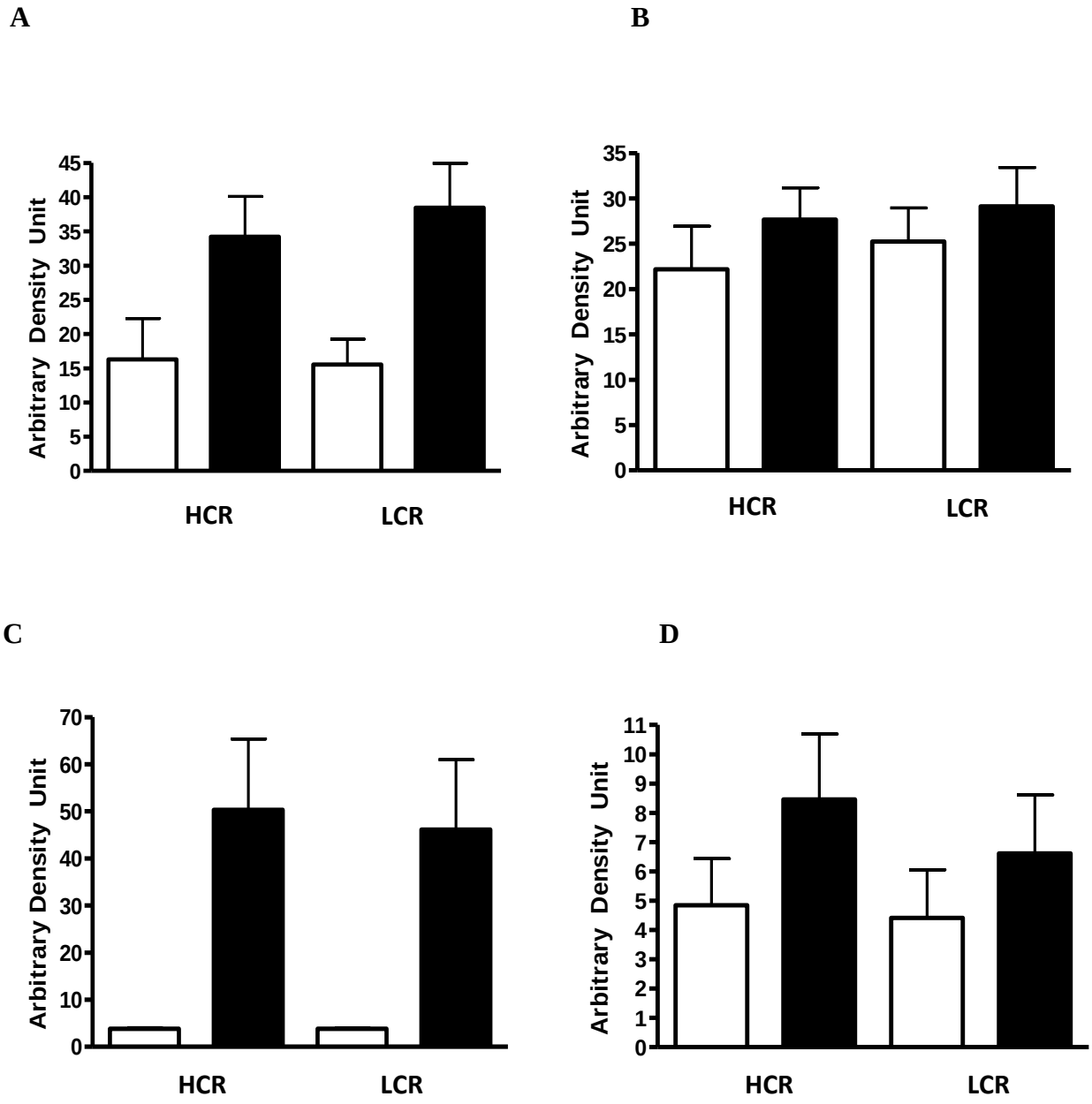


Figure 4.3 Densitometric summary of protein levels between HCR and LCR without (control, white bars) or with (IR, black bars) IR injury. A, AKT. B, Phospo-AKT. C, PKCε. D, Xanthine Oxidase. (mean ± SEM, * indicates $p < 0.05$). HCR-M (n=7), LCR-M (n=7), HCR-F (n=5), LCR-F (n=8).

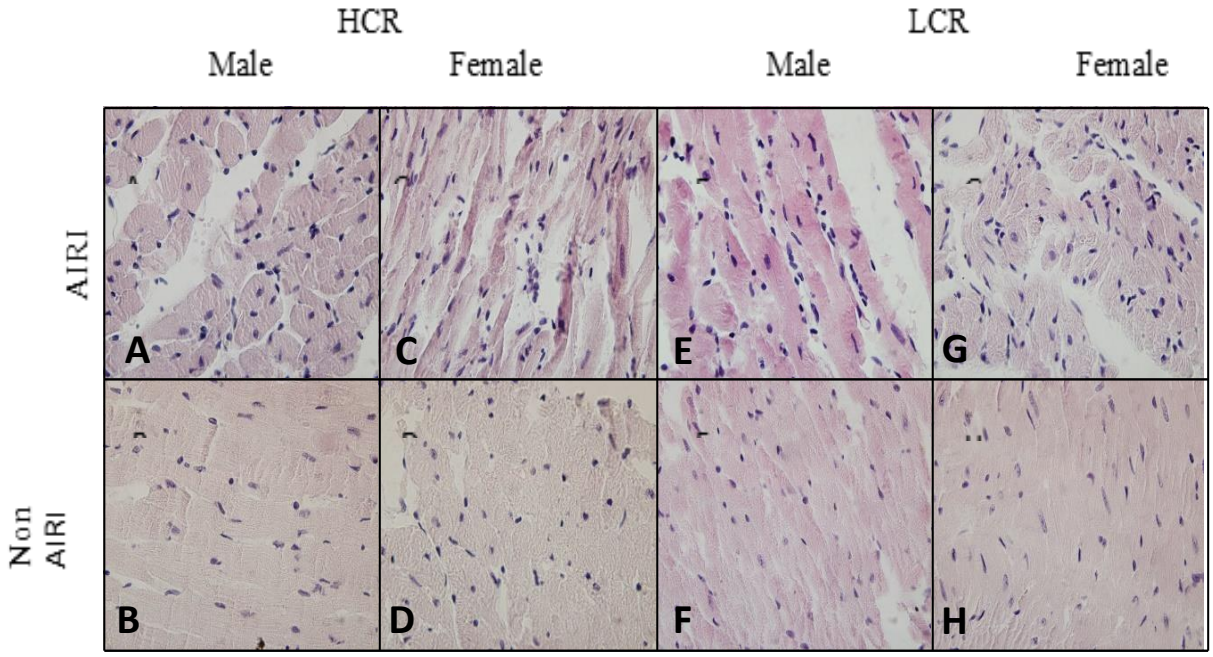


Figure 4.4. Myocardial neutrophil infiltration in HCR male/female and LCR male/female with and without IR injury using H&E stain: A= HCR Male AIRI; B = HCR Male non AIRI; C = HCR Female AIRI; D = HCR Female non AIRI; E = LCR Male AIRI; F = LCR Male non AIRI; G = LCR Female AIRI; H = LCR Female non AIRI. (all images shown at X60 magnification). (mean ± SEM, * indicates $p < 0.05$). HCR-M (n=7), LCR-M (n=7), HCR-F (n=5), LCR-F (n=8).

Table 2.1. Average Cell Counts

	HCR		LCR	
	Males	Females	Males	Females
AIRI	105.5 ± 4.5	110.1 ± 5.6	100.3 ± 3.9	105.4 ± 6.8
Non-AIRI	82.6 ± 1.0	81.3 ± 7.5	81.6± 3.5	81.8 ± 3.2

CHAPTER 5: AUGMENTED CARDIAC MITOCHONDRIAL CAPACITY IN HIGH AEROBIC CAPACITY "DISEASE RESISTANT" PHENOTYPE AT REST IS LOST FOLLOWING ISCHEMIA REPERFUSION

ABSTRACT

Rationale: Ischemic heart disease is a major cause of morbidity. Regular active exercise is therapeutic, but up to 70% of individual exercise capacity is due to an intrinsic genetic component. Intrinsic capacity can be studied using high (HCR) and low (LCR) aerobic running capacity rat strains. The phenotypes differ by more than 5-fold in sedentary average running distance and time. The HCR rats have been characterized as "disease resistant", while the LCRs are characterized as "disease prone". A consistent characteristic of the LCR is reduced metabolic capacity in several tissues, but metabolic capacity in cardiac tissue is not well studied in these phenotypes, particularly following the metabolic stress of ischemia and reperfusion.

Methods: 30 HCR and LCR rats were obtained from the parent colony at the University of Toledo, housed under sedentary conditions, and fed normal chow. LCR and HCR animals were randomly assigned to either control (C, n = 6 each) or ischemia reperfusion (IR, n = 9 each). On each study day, one HCR/LCR pair was anesthetized, and hearts were rapidly excised. In IR animals, the hearts were immediately flushed with iced hyperkalemic, hyperosmotic, cardioplegia solution, and subjected to cold global ischemic arrest (80 min). Following arrest, the hearts underwent warm reperfusion (120 min) using a Langendorff style perfusion system. Following reperfusion, the heart was weighed, and the LV was isolated. A mid ventricular ring was obtained to estimate infarction size (TTC), and part of the remaining tissue (~150 mg) was transferred to homogenation buffer on ice. Isolated mitochondria (MITO) samples were prepared and used to determine respiratory capacity under different metabolic conditions (OROBOROS). MITO from

control animals were obtained in prepared in similar fashion, but immediately following anesthesia and heart removal, and without IR. Citrate synthase activity was measured in each sample.

Results: In the control rats, HCR MITO showed respiratory rates 32% higher at rest and more than 40% higher under maximally stimulated conditions, compared to LCR MITO (both $p < 0.05$). After IR, resting MITO respiratory rates were decreased to about 10% in both strains, and the augmented capacity in HCRs was absent. Maximally stimulated rates also were decreased more than 50% from control and were no longer different between phenotypes. Ca^{++} retention capacity and infarct size were not significantly different between HCR and LCR (49.2% ± 5.6 vs. 53.7% ± 4.9), nor was average coronary flow during reperfusion.

Conclusion: Cardiac mitochondria from HCR were significantly higher in control conditions with each substrate tested. After IR insult, the cardiac mitochondrial respiratory rates were similar between phenotypes, as was Ca^{++} retention capacity and infract size. Relatively, the loss of respiratory capacity was actually greater in HCR than LCR. Together, these data could suggest limits in the extent to which the HCR phenotype might be "protective" against acute tissue stressors. The extent to which any of these deficits could be "rescued" by an active exercise component is unknown.

INTRODUCTION

Cardiovascular disease (CVD), including ischemia reperfusion (IR) injury, remains the leading cause of morbidity and mortality in developed countries. Of the most common forms of CVD, ischemic heart disease is the most prevalent counting more than one million deaths in the United States alone every year (228). Moreover, CVD is the most widespread and costly health problems in the world with a price tag of $555 billion U.S. dollars in 2016 (229). Ischemic heart disease occurs when the heart muscle is being deprived of the oxygen-rich blood as a result of

narrowed or blocked coronary arteries. Ischemic heart disease may lead to critical cardiac complications such as ventricular arrhythmias and congestive heart failure. Over recent decades, scientists and researchers reveal that ischemic pathology includes injurious events experienced during both ischemia and reperfusion which together described as ischemia-reperfusion (IR) injury (230,231).

Myocardial ischemia reperfusion injury results in detrimental cardiovascular outcomes after myocardial ischemia, cardiac surgery or circulatory arrest. Mostly, depriving the heart from blood flow due to coronary occlusion causes an imbalance between oxygen demand and supply, named *ischemia,* which in turn leads to cardiac tissue damage or dysfunction (232). Intuitively, early and fast restoration of blood flow has been considered the treatment of preference to protect against more tissue injury. Unfortunately, returning blood flow to the ischemic myocardium, called reperfusion, while necessary, can also aggravate or even induce an irreversible injury and be more deleterious than proceeding ischemia. This situation was therefore named *myocardial ischemia reperfusion injury* (232).

Continuing research is aimed to explore therapeutic interventions against IR injury. Although numerous pharmacological and preconditioning approaches to cardioprotection have been explored, regular exercise participation is recognized as an important, cost effective, safer lifestyle intervention in the prevention and treatment of IR injury (228). Redundant protective effects are evident in the exercised heart including increased levels of heat shock proteins, altered nitric oxide (NO) signaling, enhanced Ca^{2+} handling proteins, improved ATP-sensitive potassium channels, and enhanced endogenous antioxidant (233).

As an important site for ATP production via oxidative phosphorylation, mitochondria are critical in regulating normal cardiac metabolism and play a key role in the susceptibility of the

heart to IR injury (234,235). The heart is an organ with high metabolic demand; therefore, providing the myocardium with enough oxygen through the coronary arteries to support the physiological cardiac function is crucial. Blocking one or both coronary arteries leads to ischemia in areas distal to the blockage, whereby cardiac myocyte metabolism is compromised. Mitochondrial respiratory rate and enzyme activity are the major elements that drive the oxidative phosphorylation process and structural integrity of the mitochondria. Myocardial IR can cause severe effects on mitochondrial homeostasis which dramatically affects mitochondrial function and survival (236). In addition to the detrimental effects of impaired mitochondrial energy production, mitochondrial ionic imbalance and cell stress signaling can cause mitochondrial mediated cell death (236–238).

Myocardial IR injury is an important cause of impaired heart function in the early postoperative period subsequent to cardiac surgery and in acute myocardial ischemia. Growing evidence has become available supporting a crucial role of mitochondrial dysfunction in myocardial IR injury. Mitochondrial dysfunction during ischemia is a major mechanism that contributes to cardiomyocytes damage during IR (239). Increased reactive oxygen species (ROS) generation, defects in electron transport chain (ETC) activity and OXPHOS process, impaired respiratory chain complexes activity, opening of the mitochondrial permeability transition pore (MPTP) and release of cytochrome C are considered contributing factors in mitochondrial dysfunction associated with heart IR (240–244).

During ischemia, like many cells, when deprived of oxygen (anoxia), cardiac cells can produce ATP via anaerobic glycolysis to meet at least the most basic energy demand of cardiomyocytes (245). Anaerobic glycolysis increases intracellular H^+ ion concentration inducing acidosis(246). However, with severe and prolonged ischemia, the affected area of the heart

develops a condition called *contracture-rigor* in which anaerobic glycolysis is inhibited and no more glycolytic ATP is formed and then totally depleted (247). Ischemia can damage cardiac cells irreversibly. On reperfusion, mitochondrial oxidative phosphorylation returns to pre-ischemic levels within seconds and the cells typically undergo further contraction (hypercontracture). This phenomenon is termed *myocardial stunning* (248,249). Reperfusion can cause membrane damage, followed by cell death.

Stunned myocardium has relative excess oxygen consumption for a specified rate of contractile work, and therefore has a declined mechanical efficiency. This may be due to a rapid recovery of the intracellular pH during reperfusion (250). Once perfusion is restored, the intracellular increased accumulation of H^+ during ischemia is transported into the extracellular space in order to normalize the pH in exchange for Na^+ via Na^+/H^+ exchanger, while ATP depletion inactivates Na^+/K^+ ATPase. The combined effect results in increase intracellular Na^+ which in turn activates the sarcolemmal $2Na^+/Ca^{2+}$ exchanger, resulting in exchange of intracellular Na^+ with extracellular Ca^{2+}. A high rate of $2Na^+/Ca^{2+}$ exchange can finally lead to Ca^{2+} overload which in turn induce arrhythmogenesis, myocardial stunning, contracture, and ultimately apoptotic or autophagic cell death (251–253). Fluctuations in Ca^{2+} from the sarcoplasmic reticulum during reperfusion stimulate opening of the mPTP (254),(255). Opening of the mPTP leads to rapid dissipation of the membrane potential gradient which is essential for synthesis of ATP, water enters through the open pore causing mitochondrial swelling and lysis triggering apoptosis and cell death (256).

During myocardial reperfusion there is an increased fatty acid oxidation, impaired pyruvate oxidation and accelerated anaerobic glycolysis. High rates of fatty acid beta-oxidation inhibit glucose oxidation which lead to imbalance between glycolysis and glucose oxidation. Pyruvate

oxidation is likely further inhibited by the high plasma free fatty acid concentration observed with acute myocardial infarction. This 'uncoupling' between glycolysis and glucose oxidation is the main source of H^+ production in the heart. Therefore, if glycolysis is coupled to glucose oxidation, there will be no more H^+ production from glycolysis. However, if glycolysis is uncoupled from glucose oxidation, and pyruvate resulting from glycolysis is converted to lactate, there is a net production of 2 H^+ from each glucose molecule (257,258).

The proposed mechanisms underlying cardiac protection of exercise after IR include expression of selected mitochondrial proteins resulting in a mitochondrial phenotype that is resistant to IR-induced injury (34,259). This conclusion is based on believe that mitochondria play an essential role in cardiac injury after IR (260). Numerous reports suggest that mitochondria isolated from the hearts of exercise-trained animals are protected against a variety of *in vitro* challenges including anoxia/reoxygenation (261–263). In vivo, a study showed that exercise training protects the heart against IR-induced contractile dysfunction, defends against IR-induced impairment of oxidative phosphorylation, protects against IR-induced release of proapoptotic proteins from the mitochondria, prevents the IR-induced increase in mitochondrial H_2O_2 emission, protects cardiac mitochondria from IR-induced oxidative damage and promotes increased mitochondrial antioxidant enzymes in the heart (264).

Although regular exercise training is recognized as an important lifestyle intervention in the prevention and treatment of CVD and IR injury, not all individuals experience the same benefits from participating in exercise. It has been estimated that up to 70% of the variation in exercise capacity is due to the intrinsic genetic component (2). Some people have many risk factors, don't exercise, and don't get CVD, while some people have no risk factors, exercise regularly, and still experience diseases. Thus, studying the differential impacts of intrinsic aerobic

exercise capacity after cardiac ischemic reperfusion injury using intrinsic aerobic phenotype rats bred for low and high aerobic running capacity would provide a better platform for understanding the influences of intrinsic aerobic capacity on cardiac metabolic capacity and mitochondrial adaptive response pre and post ischemic reperfusion injury in these phenotypes.

Using a rat model to determine how intrinsic aerobic exercise capacity responds and adapts to several diseases has been developed by Koch and Britton (92). The HCR animals generally are characterized as "disease resistant", while the LCR animals are characterized as disease prone. The low aerobic capacity rats are a genetic model for metabolic syndrome that contribute to an increased risk of diabetes and cardiovascular disease. A consistent characteristic of the LCR is reduced metabolic capacity in several tissues, but metabolic capacity in cardiac tissue is not well studied in these phenotypes, particularly following the metabolic stress of ischemia and reperfusion.

We are interested in understanding differential changes in mitochondrial adaptive response pre and post cardiac ischemic reperfusion injury between HCR and LCR. Our hypothesis is that LCR rat's mitochondrial adaptive response is deteriorated by IR injury due decreased mitochondrial respiratory rat and impaired mitochondrial calcium retention capacity compared to the HCR rats. Our model utilizes cold cardioplegia as global ischemic arrest (80 min) and warm reperfusion (120 min) using a Langendorff style perfusion system.

MATERIALS AND METHODS

Animal Strains. 34 HCR and LCR, female rats, 40 weeks, generation 32, housed under sedentary conditions, and fed normal chow. LCR and HCR animals were randomly assigned to either control (C, n = 8 each) or ischemia reperfusion (IR, n = 9 each). HCR and LCR rats were obtained from the parent colony at the University of Toledo. This novel animal model has been

developed by Drs. Lauren Koch and Steven Britton at the University of Michigan. They have previously described the artificial selection used for the generation of the HCR and LCR strains (92). Briefly, each sex was selected from the founder population (N: NIH stock). Two-way artificial selective breeding was used to create low capacity runner (LCR) and high capacity runner (HCR) strains that were divergent for treadmill running capacity (run time until exhaustion on a graded treadmill exercise test). The 13 lowest and 13 highest running capacity rats of each sex were randomly paired for mating. This pattern was repeated over subsequent generations to produce the divergent strains using a rotational breeding scheme. In the present study animals from generation 32 were used. All the animals were exposed to treadmill exercise for the 5 days at 11 weeks of age when the animals were phenotyped for treadmill running capacity. Upon verifying the phenotypes of the animals, animals were prepared for shipping at 14 weeks of age, or soon after as weather conditions (airport tarmac temperatures < 85°F) permitted. Once received by the Department of Comparative Medicine at ECU, the animals were maintained under mandatory quarantine for 10 weeks before they were released for study. Rats were provided standard rat chow and water ad libitum and were kept on a 12 h light/ 12 h dark time schedule until sacrifice. Animal procedures were conducted in accordance with American Physiological Society guidelines for the humane and safe use of animals, and all protocols involving animals used for these experiments were approved by the East Carolina University Animal Care and Use Committee (Figures 2.1 & 2.2).

Cardiac ischemic-reprefusion injury. Rats were anesthetized with an intraperitoneal injection of ketamine (80mg/kg; Cenvet Australia, Kings Park, NSW, Australia) and xylazine (10mg/kg; Provet, Eastern Creek NSW, Australia).The heart was harvested after being arrested with 1,000 mL of iced St. Thomas' cardioplegic solution (NaCl 110.0 mM, NaHCO3 10.0 mM,

KCl 16.0 mM, MgCl2 16.0 mM, CaCl2 1.2 mM, pH 7.8) at 4°C, infused at a pressure of 60 mm Hg to 65 mm Hg into the aortic root, and the hearts were then stored in this solution at the same temperature for 80 min. After 80 min of cold global ischemic arrest, the heart was cannulated and immediately perfused retrogradely on a Langendorff perfusion apparatus with Krebs-Henseleit buffer (KHB) for 120 mins at 37°C (composition (mM): NaCl 118; KCl 4.7; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25; $CaCl_2$ 1.4; glucose 11; pH 7.3–7.4) and the a hydrostatic pressure maintained at 90 mm Hg (range, 80 to 95 mm Hg) by adjustment of the pump speed. Coronary flow (CF) was measured manually at the baseline and every 30 min of reprefusion.

Tissue isolation and infarct size quantification. Following reperfusion, the heart was taken off the canula and weighed and trimmed at the AV groove and LV was then isolated. A mid ventricular ring was obtained for infarct size quantification and the rest of LV was sliced into 3 approximately equal sections for mitochondrial experiments. For infarct size quantification**,** A mid ventricular ring was then placed in a 0.1 % triphenyltetrazolium chloride (TTC) solution and incubated at 37 °C for 10 minutes in a shaking water bath. Following incubation both sides of the slice were photographed with a digital camera attached to a dissecting microscope. Images were quantified using Image J software where total area (TA), lumen area and infarcted area (IA) were measured. The area at risk (AAR) was considered to be the entire left ventricle and was calculated by taking the TA and subtracting the lumen area. The infarcted area was determined by measuring the white appearing tissue in the AAR. Areas were converted to weights by multiplying the mean of the AAR and IA of both sides of the slice by the weight of each slice. The total weight of the IA from all sections was then divided by the total weight of the AAR from all sections to obtain a percentage of the heart that was infarcted.

Mitochondria Isolation. Left ventricular cardiac mitochondria were isolated as described in Wellman *et al.* (265). Briefly, one slice of isolated LV was immediately placed in ice-cold Buffer A (phosphate buffered saline (pH = 7.4), supplemented with EDTA (10mM)). All tissues were minced and resuspended in Buffer C (MOPS (50mM; pH = 7.1), KCl (100mM), EGTA (1mM), MgSO4 (5mM) supplemented with bovine serum albumin (BSA; 2g/L)) and then homogenized via a Teflon pestle and boroscilicate glass vessel. Tissue homogenates were centrifuged at 500 × g for 10-minutes at 4°C. Supernatant from each tissue was then filtered through thin layers of gauze and subjected to an additional centrifugation at 10,000 × G for 10-minutes at 4°C. Mitochondrial pellets were washed in 1.4ml of Buffer B (MOPS (50mM; pH = 7.1), KCl (100mM), EGTA (1mM), MgSO4 (5mM)), transferred to microcentrifuge tubes and centrifuged at 10,000 × g for 10-minutes at 4°C. Buffer B was aspirated from each tube and final mitochondrial pellets were suspended in 100–200 μL of Buffer B. Protein content was determined via the Pierce BCA protein assay. Functional assays involving isolated mitochondria were carried out in the following buffers; Buffer D – Potassium-MES (105mM; pH = 7.2), KCl (30mM), KH_2PO_4 (10mM), $MgCl_2$ (5mM), EGTA (1mM), BSA (2.5g/L); Buffer E – HEPES (20mM; pH = 8.0), KCl (100mM), KH_2PO_4 (2.5mM), $MgCl_2$ (2.5mM), Glycerol (1%).

Mitochondrial respiratory control (JO2). High-resolution O2 consumption measurements were conducted at 37C in 2 mL of assay buffer using the Oroboros Oxygraph-2K (Oroboros Instruments), as previously described (265). Briefly, isolated mitochondria (0.025 mg/mL) were added to assay buffer, supplemented creatine (Cr; 5 mM), phosphocreatine (PCr; 1 mM) and creatine kinase (CK; 20 U/mL), followed by the addition of respiratory substrates then ATP (5 mM). The following substrate conditions were tested: [Pyruvate/Malate – (P/M; 5/2.5 mM), 20U/mL CK (10uL) + 1mM PCR (5uLof 0.2M stock) + 5mM ATP (10uL), 5mM PCR (5uL) ~

total = 6mM, 9mM PCR (9uL) ~ total = 15mM, 6mM PCR (6uL) ~ total = 21mM, 0.02uM FCCP and 4uL of Oct + 5uL of Glut + 10uL of Succ. Respiratory control was assessed through sequential additions of PCr to final concentrations of 6 mM, 11 mM, 16 mM and 21 mM before additions of 5 µM FCCP.

Citrate synthase activity assay. Protein content of mitochondrial homogenates were assessed using a BCA assay. Citrate synthase activity was determined using a commercially available citrate synthase assay kit (#CS0720, Millipore-Sigma). Citrate synthase activity was determined using the manufacturer's instructions.

Ca2+ Retention Capacity. Calcium retention protocols were modified from Sloan et al. (266) where 0.5 mg mitochondria were suspended in an assay buffer containing: 125 mM KCl, 5 mM HEPES, 2 mM KH2PO4, 1 mM MgCl2 and (25 °C, pH = 7.3). The fluorescent Ca^{2+} indicator, calcium green 5 N salt, was utilized to track changes in extra-mitochondrial calcium levels. Extramitochondrial calcium fluorescence was measured using a fluorescence spectrophotometer (Photon Technology International, Birmingham, NJ, USA), with excitation and emission wavelengths set to 506/532 nm, respectively. Calcium induced mPTP opening experiments were performed under state 2 respiration conditions (5 mM glutamate/5 mM malate). Mitochondrial PTP opening was induced by subjecting mitochondria to sequential 50 nmol CaCl2 pulses every 3 minutes, which causes repeated decreases in the fluorescent signal as Ca^{2+} is taken up by the mitochondria. Induction of mPTP was denoted by a sharp increase in extra mitochondrial Ca^{2+} fluorescence, representing the release of the accumulated Ca^{2+} from the mitochondrial matrix. Calcium retention capacity was quantified as the amount of calcium needed to induce PTP opening (nmol CaCl2/mg mitochondria). This experiment was done to just IR LCR and HCR animals.

Statistics. Statistical analysis was performed using data analysis software in Microsoft Excel worksheet (Windows 10). Data is expressed as means ± SEM, and a p value <0.05 was considered statistically significant. Differences in infarct size between groups, coronary flow, mitochondrial respiratory rate, Ca^{++} retention capacity using single factor ANOFVA and paired t-test.

RESULTS

Myocardial infarct size

After 80 min of cold global ischemic arrest and 120 mins of warm reperfusion, myocardial infarct size was not significantly different between HCRs and LCRs. HCR and LCR animals were variable in overall infarct size, and in phenotypic differences on any given experimental day, but overall, both have relatively similar size of myocardial infarction. There was high variability in infarct size between the animals in both phenotypes. (Figure 5.3; $P > 0.05$).

Coronary Artery Flow

Coronary artery flow, was sampled manually at the beginning, after 30 min, and after 60 minutes of reperfusion. Values from each measurement were averaged to obtain a value for each animal. Although the HCRs showed a trend to have higher coronary artery flow than LCRs, the differences were not significantly different in all time points between HCRs and LCRs following IR injury. (Figure 5.4; $P > 0.05$).

Mitochondrial Respiratory Capacity

***Pre IR injury Comparisons*.** Baseline assessment of cardiac mitochondria respiratory rates revealed that HCR MITO show respiratory rates 32% higher at rest and more than 40% higher under maximally stimulated conditions, compared to LCR MITO (both $p < 0.05$). Generally, HCR

MITO showed significantly higher of mitochondrial respiration with all substrates than LCR MITO. Addition of the uncoupling agent, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) (1μM), at the end of the respirometry experiments rescued absolute rates of JO_2 in oligomycin. In both strain and ventricles, complex I-linked substrates (pyruvate/malate) showed markedly increasing in mitochondrial respiration. Collective analysis of all respirometry data suggest that Low aerobic capacity impairs cardiac muscle bioenergetics via impinging on the respiratory complexes. (Figures 5.5, 5,7; $P < 0.05$)

Post IR injury comparisons. There were no significant differences of cardiac mitochondria respiratory rates between HCR MITO and LCR MITO after ischemia as we have seen before ischemia. Regardless of the phenotype differences, the mitochondrial respiratory rate of the different substrates has been compromised post-ischemia comparing to pre-ischemia in both strains. Surprisingly, HCR MITO showed more deterioration of respiratory rate comparing to LCR MITO after IR insult. Interestingly, the pyruvate/malate substrate, which involve complex I, shows the most markedly decreased which is in consistence with the previous studies which have revealed that complex I is a major site of damage to the respiratory chain in ischemia (Figures 5.6, 5,7; $P > 0.05$).

Citrate synthase activity

Pre IR injury Comparisons. Citrate synthase activity was not affected by the differences of innate aerobic capacity, suggesting mitochondrial content per mitochondrial isolation was similar between HCR and LCR animals. Although the HCRs trend to have higher citrate synthase activity than LCRs, the differences were not significant (Figure 5.8; $P > 0.05$).

Post IR injury Comparisons. Generally, citrate synthase activity was decreased in both HCR (%40) and LCR (%45) animals after IR injury. Although the HCRs trend to have higher CSA

than LCR, the differences were not significant (Figure 5.8; $P > 0.05$). These data suggest losing relatively the same amount of mitochondrial content in both HCR and LCR animals after IR insult.

Ca2+ Retention Capacity

After 80 min of cold global ischemic arrest and 120 mins of warm reperfusion, Ca2+ retention capacity was not significantly different between HCRs and LCRs. Although HCR has relatively higher Ca2+ retention capacity than LCR animals, it is not considered to be statistically different (Figure 5.9; $P > 0.05$). Our data suggest that mitochondria isolated from the left ventricle of HCR and LCR rats required same amount of Ca2+ to induce mPTP as measured by sustained Ca2+ fluorescence.

DISCUSSION

The increase in the incidence and prevalence of CVD is concerning. Sedentary lifestyle and physical inactivity are potentially at high risk for the morbidity and mortality of cardiovascular disease. The benefits of aerobic exercise training are well established as regular exercise has been shown to reduce the risk of heart disease, control hypertension and protect the heart against oxidative stress and apoptosis (259). Exercise has been shown to reduce arrhythmia, decrease myocardial stunning, and improve vascular reactivity in hearts exposed to ischemia reperfusion (267,268). It is well known that aerobic exercise capacity has genetic and environmental components (2,166). The genetic component defines the intrinsic endurance exercise capacity and appears to have two parts, the genes that regulate adaptive responses to exercise training and the genes that determine intrinsic exercise capacity (166).

Although aerobic exercise training has beneficial effects on several of cardiovascular diseases, the variability in the physiological response to exercise training suggests potential impact of the genetic composition. It has been estimated that up to 60%-70% of the variation in exercise

capacity is due to the genetic component (2). It is not clear if the genetic component for enhanced exercise capacity alone can result in protection from cardiovascular diseases or whether the training stimulus is necessary to produce the positive results, or a combination of both.

The development of a novel rat model, by Drs. Koch and Britton (92) that emphasizes the differences in intrinsic aerobic exercise capacity has advanced the ability to differentiate the genetic components from the environmental effects, such as active exercise, that also influence the aerobic capacity. Rat genetic models of intrinsic (i.e., untrained) low-capacity runners (LCR) and high-capacity runners (HCR) have been developed by artificial selective breeding using treadmill running as the discriminating phenotype. This selection process has generated a shift in metabolic and cardiovascular risk factors between the two strains (68). In summary, LCR rats score high on disease risks associated to the metabolic syndrome, which is defined as collection of symptoms that may predispose for cardiovascular disease, and HCR rats score high for health factors related to maximal oxygen consumption (67). LCR rats also respond more negatively to environmental health risks, such as high fat diets. A consistent characteristic of the LCR is reduced metabolic capacity in several tissues, but metabolic capacity in cardiac tissue is not well studied in these phenotypes, particularly following the metabolic stress of ischemia and reperfusion. Therefore, the goal of this study was to determine how the metabolic adaptive response between rats bred for low intrinsic aerobic capacity and high intrinsic aerobic capacity are different after acute ischemic reperfusion injury. We hypothesized that a low aerobic capacity running (LCR) phenotype will be more susceptible to cardiac mitochondrial dysfunction after IR injury by impairing the respiratory rate and promoting the opening of the mPTP.

The results of this study showed that the left ventricle of rats selectively bred for high endurance running capacity (HCR) and low endurance running capacity (LCR) After 80 min of

cold global ischemic arrest and 120 mins of warm reperfusion have a relatively equal myocardial infarct size. Moreover, the results showed that coronary artery flow was not significantly different between HCR and LCR animals at all selected time points of reperfusion. These results were somewhat unexpected as the dynamic exercise showed that exercise training preserves coronary flow and reduces infarct size after ischemia-reperfusion in rat heart and HCR rats have at least the intrinsic potential for increased physical activity. One previous study evaluated the impacts of swim training on myocardial infarct size after irreversible coronary artery ligation in the rat (32). Their results showed a decrease in infarct size after training and proposed that the cardioprotective effect of exercise was facilitated by increased myocardial vascularity which might act to minimize the area of myocardium at risk during coronary artery obstruction (32). Another study confirmed that a 5-day program of exercise training regimen was adequate to confer infarct sparing in hearts subjected to a transient regional ischemia and followed by reperfusion (269). They hypothesized that the improved tolerance to ischemia-reperfusion insult was caused by exercise-induced increases in myocardial antioxidant defenses (269). In 2003, David A. Brown and his colleagues have demonstrated that prolonged endurance training confers a cardioprotective effect against infarction in myocardium subjected to severe ischemia and subsequent reperfusion (270). In addition, they observed that during severe ischemia, coronary flow to regions of the myocardium outside the ischemic area at risk was better maintained in hearts isolated from endurance-trained rats. Furthermore, on reperfusion of the area at risk, the increase in flow to the previously ischemic region of the heart was markedly higher in hearts isolated from trained rats (270). Together, all these results suggest the importance of exercise to maintain a good coronary artery flow in order to reduce the infarct size after IR insult. Our results showed that all coronary flow has impaired at same level in both HCR and LCR animals.

One explanation is that injury was too massive to maintain the cardioprotective impact of HCR phenotype. This concept is supported by the results of a previous study (not published) from our lab which showed that the phenotypic differences exists in myocardial infarction size with short time (15 min) but with not longer time (30 min) ligation which suggest that the increased aerobic capacity phenotype may not be able to resist the severity of oxidative stress and reduction of endogenous antioxidant capacity and the impairment of protective signaling pathway related to the activation of STAT3 and Akt, the major mechanisms for the increased myocardial injury after prolonged ischemic insult.

Regarding mitochondria respiration, the baseline results showed that HCR have a better respiratory rate with all substrates than LCR rats, however, after IR injury, the respiratory rat has been markedly compromised in both strains and the phenotypic differences has been abolished. The better baseline respiratory rate of HCR animals is in agreement with the broadly notion says that selection process has generated a shift in metabolic and cardiovascular risk factors between the two strains. In addition to the previous studies which reported that LCR phenotype is characterized by reduced metabolic capacity in several tissues such as skeletal muscle and liver, we demonstrated here the same baseline phenotypic differences in cardiac tissue. Initial studies in the HCR/LCR model established clear metabolic phenotypic differences between the strains. Wisloff and his colleagues reported that skeletal muscle of the HCR rats showed higher mitochondrial content and oxidative capacity than the LCR, matching the well-established muscle mitochondrial phenotypes described by several studies in exercise trained vs. untrained humans (68). Other studies using this animal model demonstrated that these animals display significant differences in hepatic mitochondrial phenotypes where LCR rats display lower hepatic

mitochondrial oxidative capacity as defined by reduced hepatic fatty acid oxidation (FAO), enzyme activity, and mitochondrial respiratory capacity compared with HCR rats (271,272).

Unlike the metabolic responses of peripheral tissues (skeletal muscle, liver) which have been previously investigated after subjected to different insults and showed phenotypic differences, the left ventricle mitochondria of rats selectively bred for high endurance running capacity (HCR) did not tolerant the insult of IR as the mitochondrial respiratory rate and Ca++ retention capacity have been compromised to the same level of low endurance running capacity (LCR) or even worst. However, our results suggest that high intrinsic aerobic capacity phenotype did not confer protection against IR injury, several studies indicate the protective effect of exercise preconditioning against diverse cardiovascular diseases (220,221). Regarding myocardial infarction (MI), Morris et al. reported greater survival rate in fit compared with inactive subjects after MI (217). Other experimental animal studies suggested that exercise training preconditions the heart and protects it against ischemia-reperfusion injury (221–223) and promotes healing of the infarcted area (223). In human study, biopsies performed in older men showed that even with aging, exercise increases mitochondrial DNA and mitochondrial respiratory chain activity which is likely related to increases in mitochondria biogenesis.

The IR injury may produce the same end-result in metabolic response but achieve this through different mechanisms. One of the potential explanations for the absence of demonstrable protection in the present study is that the phenotypic changes developed through generation in rats selected for low aerobic capacity (including characteristics comparable to the metabolic condition) may have triggered mechanisms that may precondition the heart against ischemic injury. These mechanisms were not investigated in the present study, but previous studies with this model have shown that the immune response is exaggerated in LCR (214), and particularly TNF-α (224) as

well as interleukin-10 (225), which have been shown to have preconditioning influences on ischemia-reperfusion injury and is significantly upregulated in the LCR rats (157).

Another explanation is that injury of IR is too severe to pick up the differences within this model. Therefore, the detectable protection of HCR phenotype might be limited to some lower level of injury but not tolerate this type of insult as 80 min of ischemia and 120 min of reperfusion. Surprisingly, HCR animals showed a more severe drop in mitochondrial respiratory rates with all substrates compared to LCR counterparts. The respiratory rate data showed that HCR is more deteriorated than LCR after IR injury, which, in some extend, makes the cardio-protection of HCR phenotype conditional on the present of oxygen and to a certain limit and make exceeding that limit causing deterioration of the situation.

Citrate synthase activity (CSA) was measured before and after IR injury in both strains. Our results showed that there were no phenotypic differences in CSA either pre or post IR injury. Generally, citrate synthase activity was decreased in both HCR (%40) and LCR (%45) animals after IR injury. Although the HCRs trend to have higher CSA than LCRS, the differences were not significant. These data suggest that HCR MITO and LCR MITO have the same mitochondrial content before IR and they have relatively lost the same amount of mitochondrial content after IR insult. Therefore, any phenotypic different detected in mitochondrial respiratory rates between HCR and LCR is not due to the differences in CSA.

Our results revealed that the pyruvate/malate substrate, which involve complex I, shows the most markedly decreased after IR insult in both strains which is in consistence with the earlier studies which have reported that complex I is considered the main site of damage to the respiratory chain in ischemia and activity of complex I is significantly reduced after 20 min to 30 min of ischemia due to the influences of low pH on the enzyme, while downstream electron transport

chains are relatively resistant to IR injury (273). Furthermore, Veitch and his colleagues (274) found a major declining in complex I activity in perfused rat hearts subjected to 20 min of global ischemia, and Cairns and his group (275) demonstrated that this damage was exacerbated by reperfusion.

The cause of complex I retrogression is unidentified. According to one previous study, Paradies et al. linked the decline in complex I activity under conditions of oxidative stress with the mitochondrial cardiolipin damage by ROS (276). Other studies have proposed that NO could be possible cause via peroxynitrite intermediates (277,278), since inhibitors of iNOS prevent the inactivation. This observation is supported by Jekabsone et al. (279), who indicated that complex I activity decreases in isolated mitochondria exposed to NO, and that this decrease is prevented by SOD. Therefore, either ROS or NO or both at the concentrations reachable during ischaemia and reperfusion could inhibit the enzyme directly, or through an influence on cardiolipin.

Mitochondria comprise about one-third of the mass of the heart and are critical for the normal mechanical and electrophysiological function of the cardiomyocyte, playing roles that extend beyond bioenergetics and metabolism. Proper function is required to meet the high energetic demand of the cardiomyocyte, as well as playing an essential role in controlling oxidative stress and Ca^{2+} handling (280). Ischemia-reperfusion injury increases production of reactive oxygen species (ROS) and induces calcium overload into mitochondria (281) which can interact together to induce opening of the mitochondria permeability transition pore (mtPTP) and, therefore, triggering apoptosis by promoting the release of proapoptotic proteins (i.e., cytochrome *c*) and subsequent activation of programmed cell (282,283).

Endurance exercise training has been proved to protect cardiac myocytes against ischemia-reperfusion-induced oxidative stress (284,285). Exercise has also been shown to increase

mitochondrial biogenesis and to improve mitochondrial function (286). Andreas et al (287), has reported that endurance exercise training reduces reactive oxygen species-induced cytochrome *c* release from heart mitochondria. These changes are associated with a lower maximal rate of mitochondrial permeability transition pore opening (V_{max}) and prolonged time to V_{max} in both subsarcolemmal and intermyofibrilla cardiac mitochondria (287). Moreover, it has been demonstrated that acute exercise protects against cardiac mitochondrial dysfunction, preserving mitochondrial phosphorylation capacity and protects against calcium-induced cardiac mitochondrial permeability transition pore opening in doxorubicin-treated rats (288).

This study evaluated, as the first time, the impact of intrinsic aerobic capacity on cardiac mitochondrial Ca^{2+} handling, and how subsequent alterations in tolerance to Ca^{2+} loading may contribute to cardiovascular dysfunction and injury through mitochondrial function. Our results revealed that high intrinsic aerobic capacity (HCR) phenotype did not confer protection against IR injury as the number of Ca^{2+} pulses required to induce opening of the mPTP is relatively the same number required to induce opening of the mPTP in low intrinsic aerobic capacity (LCR) phenotype. Although dynamic aerobic exercise has a lot of positive effects on the cardiac mitochondria after IR injury, selection for high aerobic capacity did not show the same benefit as the exercise training. Powers has demonstrated that exercise training produces cardioprotection which maybe a greater role in cardioprotection than the genetic component (289). Also, it is possible that the IR insult was too massive, so the benefit of high intrinsic aerobic capacity phenotype has abolished.

CONCLUSION

Sedentary lifestyle and physical inactivity have been strongly associated with increasing prevalence, incidence, and severity of CVD. Although regular endurance exercise training has

been reported as a non-invasive, cost effective, and safe treatment for many of CVD and IR, not all individuals experience the same benefits from participating in exercise. It has been estimated that up to 70% of the variation in exercise capacity is due to the intrinsic genetic component. We investigated the impact of intrinsic (genetic) aerobic capacity (as represented by genetic models of intrinsic (i.e., untrained) low-capacity runners (LCR) and high-capacity runners (HCR) on the LV infarct size and metabolic response after subjected to IR insult. Although HCR may provide better baseline respiratory rate than LCR phenotype, this advantage of this phenotype has been abolished after IR insult. Furthermore, we observed no phenotypic differences in overall infarct size, calcium retention capacity, as well as citrate synthase activity after IR injury. These findings suggest that injury might be too severe to pick up the differences within this model. Once a certain threshold is passed, the HCRs actually show proportionally greater loss of respiratory capacity compared to LCR. Thus, our data do not clearly support a cardioprotective effect of higher inborn aerobic capacity and if there is an effect, it can be overwhelmed. The cardioprotective role associated with high aerobic capacity seems, therefore, not to depend on inborn characteristics, but rather on acquired aerobic capacity and cardiac precondition.

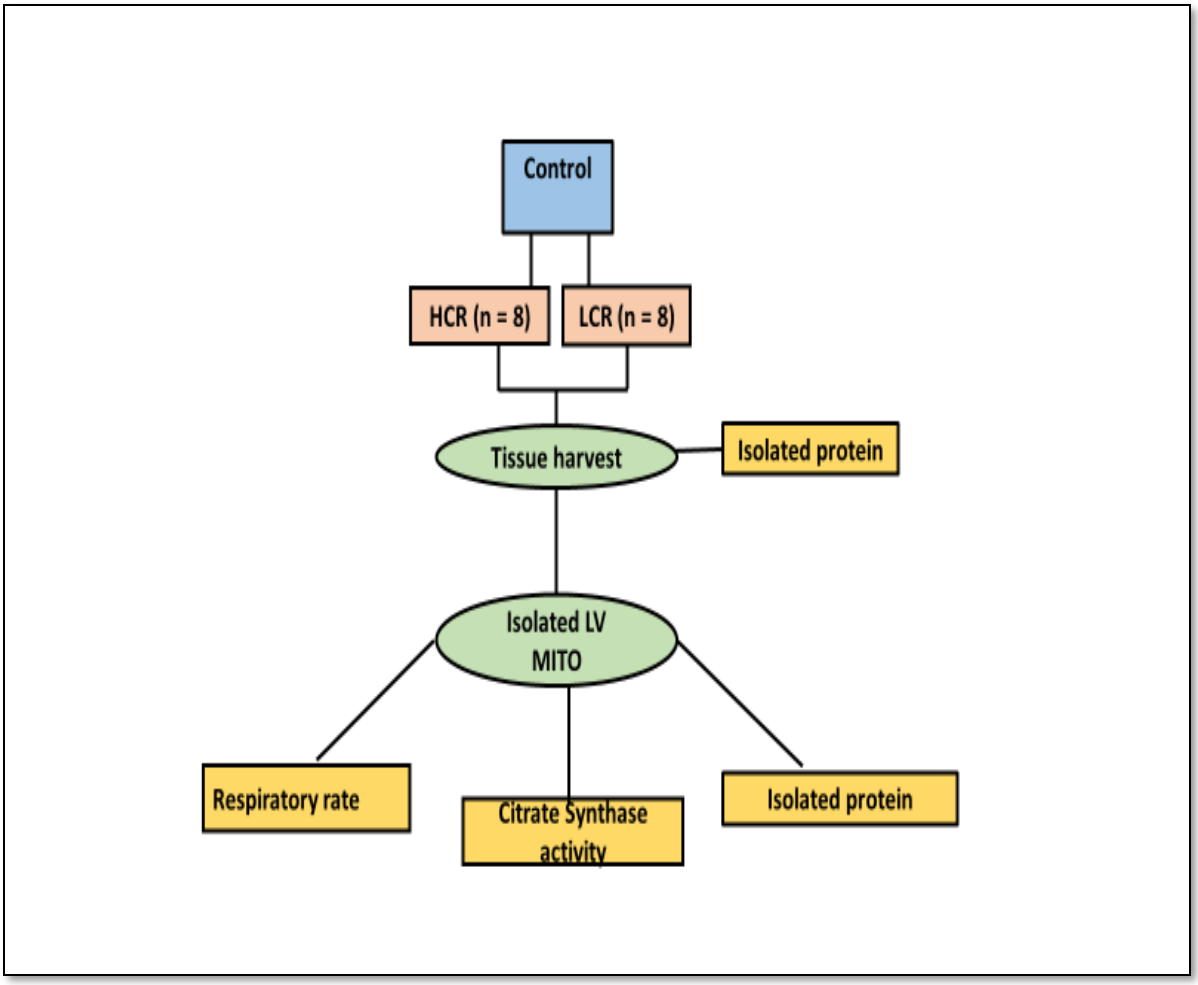


Figure 5.1. Schematic of mitochondrial study design for control HCR and LCR rats.

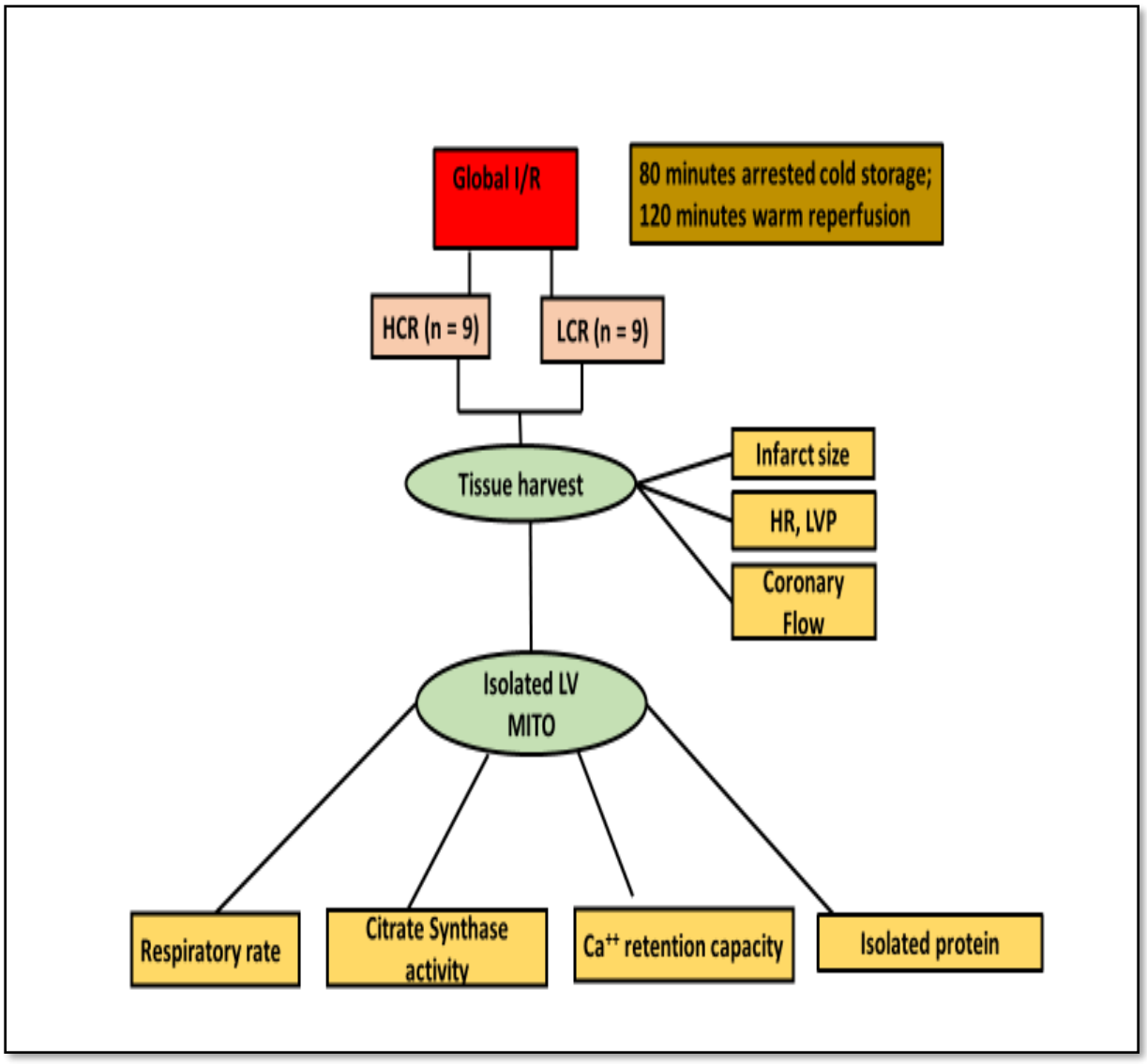


Figure 5.2. 2chematic of mitochondrial study design for I/R HCR and I/R LCR rats.

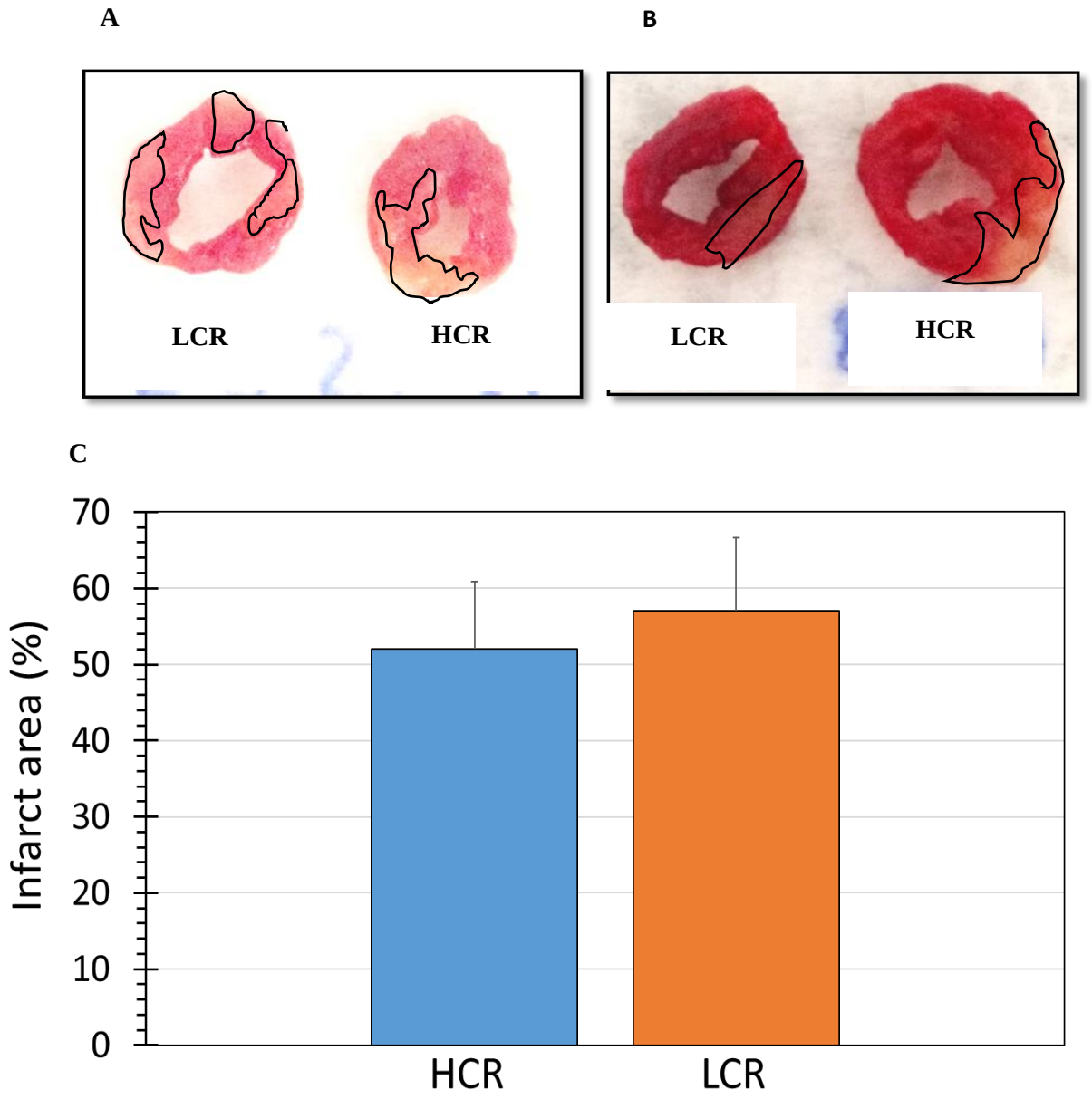


Figure 5.3. Comparsion of infarct size after IR injury between HCR (blue) and LCR (orange) phenotypes. A, B representive image the variation of left ventricle infarct size between HCR and LCR animals after IR injury. C, represent the differences of LV infarct size after 80 min of cold global ischemic arrest and 120 mins of warm reperfusion. Paired t-test was used to analyze the data, * indicates $p < 0.05$. The error bars represent standard error. HCR (n=9), LCR (n=9).

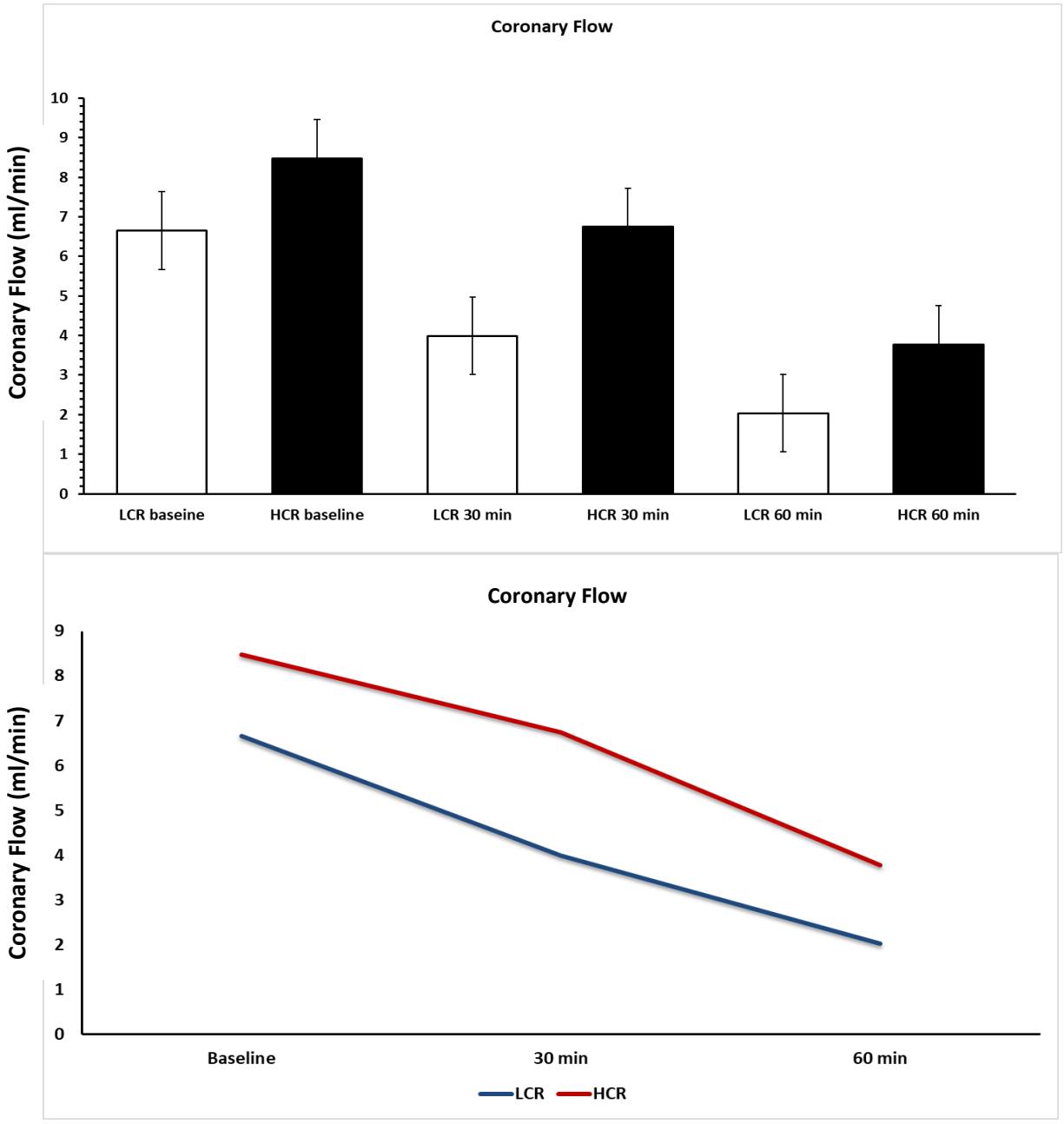
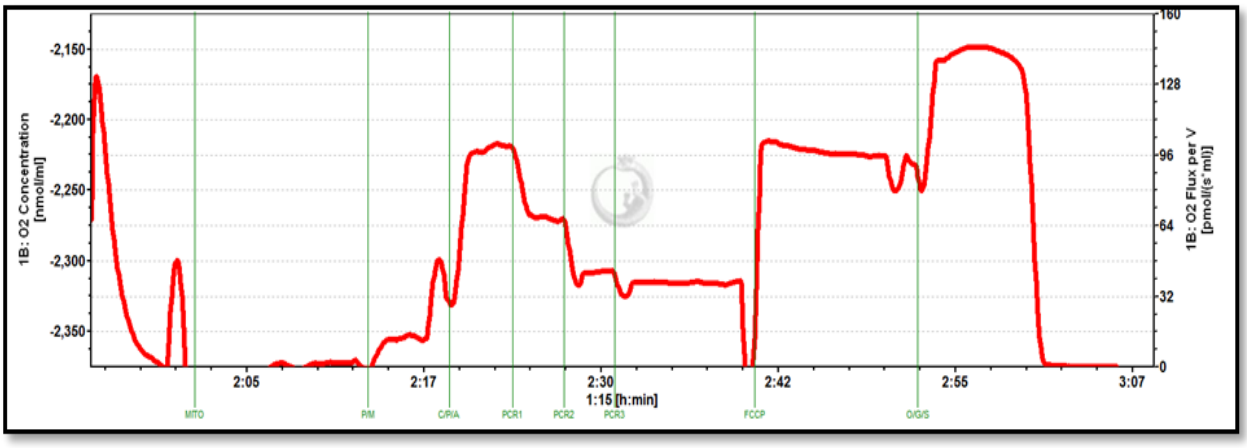


Figure 5.4. Comparison of coronary artery flow between HCR and LCR phenotypes. The coronary artery flow has been taken manually in different time frames of prefusion (baseline, and every 30 min of prefusion). Single factor ANOVA test was used to analyze the data, * indicates $p < 0.05$. The error bars represent standard error. HCR (n=9), LCR (n=9).



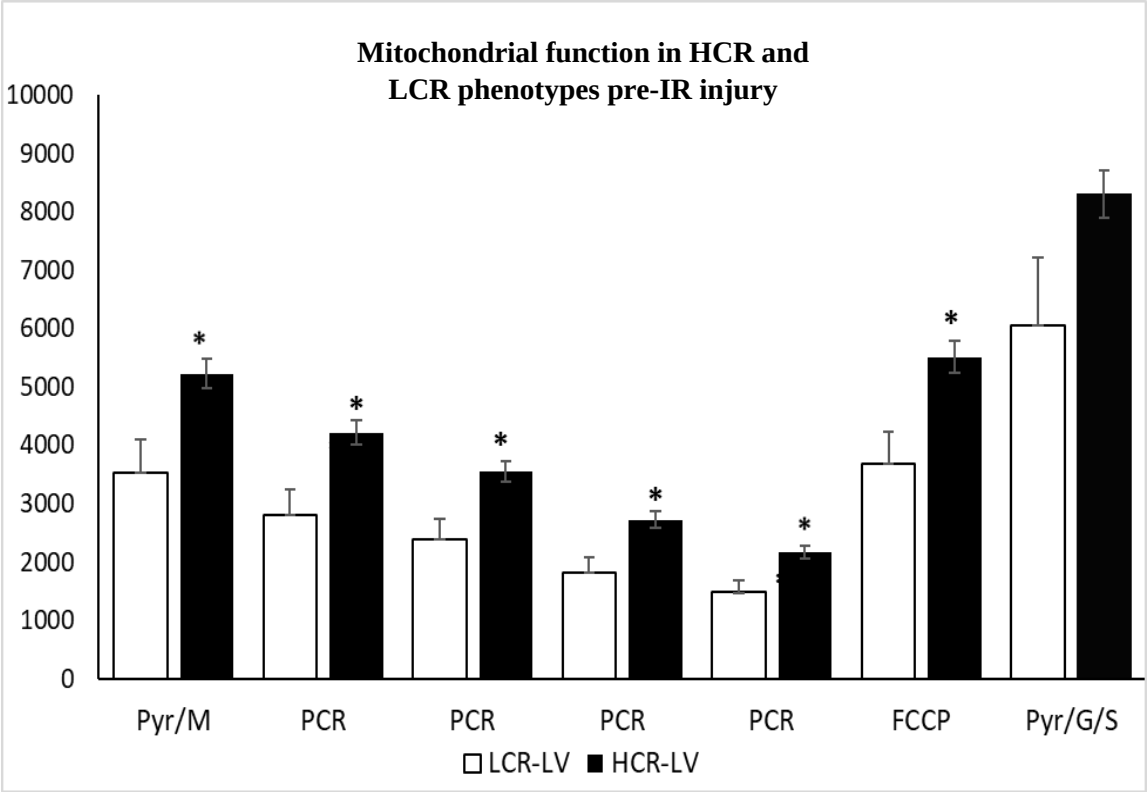


Figure 5.5. Comparison of LV mitochondria respiratory rate between HCR and LCR phenotypes pre-IR injury. Upper image represents the curves shape in Oroboros Instruments. Lower image shows the differences of LV mitochondria respiratory rate under different substrates between HCR and LCR before IR injury. Pyr/M (Pyruvate/Malate), PCR (Phosphocreatine), FCCP (Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone), Pyr/G/S (Pyruvate/Glutamate/Succinate). Paired t-test was used to analyze the data, * indicates $p < 0.05$. The error bars represent standard error. HCR (n=8), LCR (n=8).

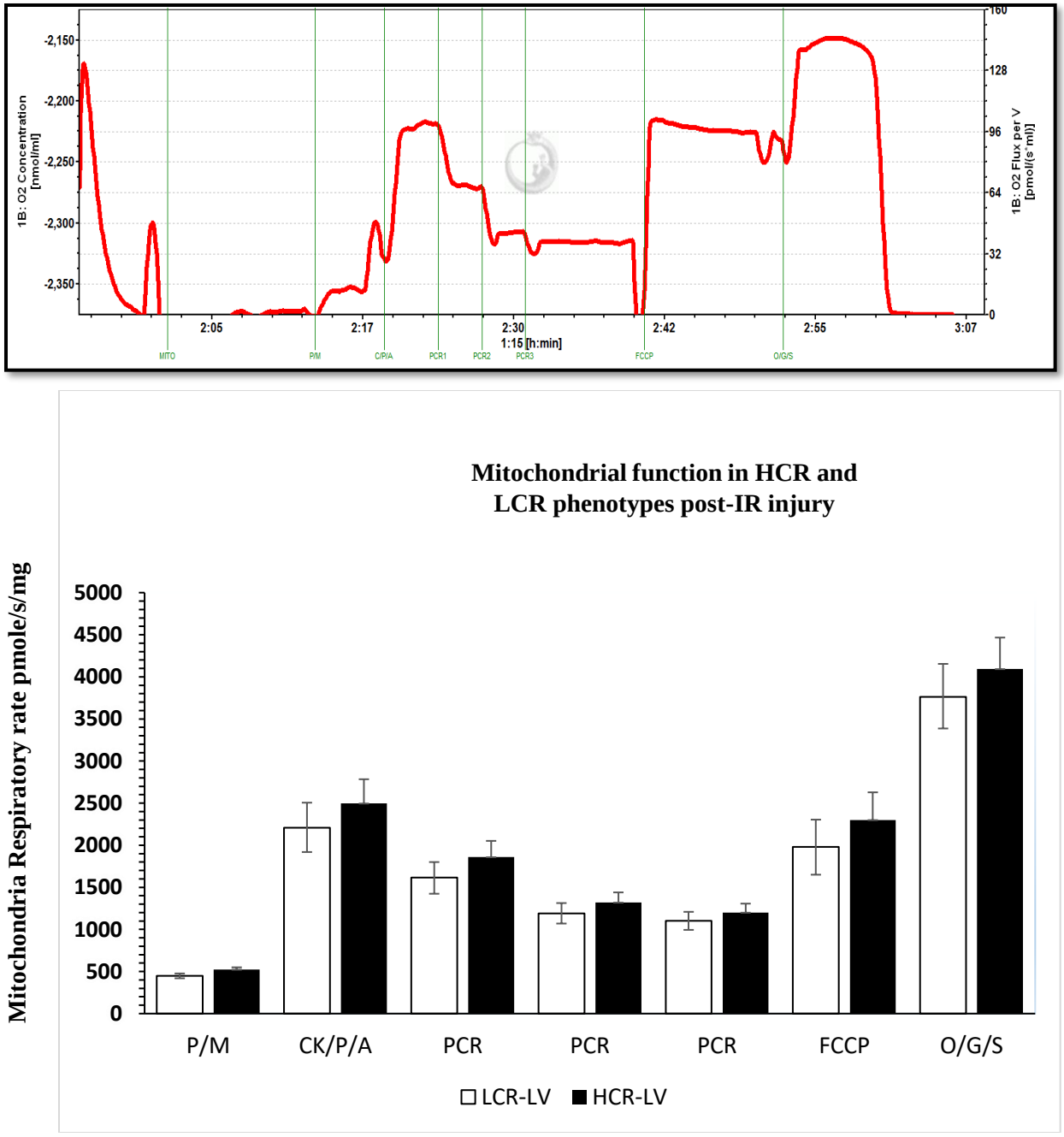


Figure 5.6. Comparison of LV mitochondria respiratory rate between HCR and LCR phenotypes post-IR injury. Upper image represents the curves shape in Oroboros Instruments. Lower image shows the differences of LV mitochondria respiratory rate under different substrates between HCR and LCR after IR injury. Pyr/M (Pyruvate/Malate), CK/P/A (Creatine kinase/ Phosphocreatine/ATP, PCR (Phosphocreatine), FCCP (Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone), O/G/S (Oxalate/Glutamate/Succinate). Paired t-test was used to analyze the data, * indicates $p < 0.05$. The error bars represent standard error. HCR (n=9), LCR (n=9).

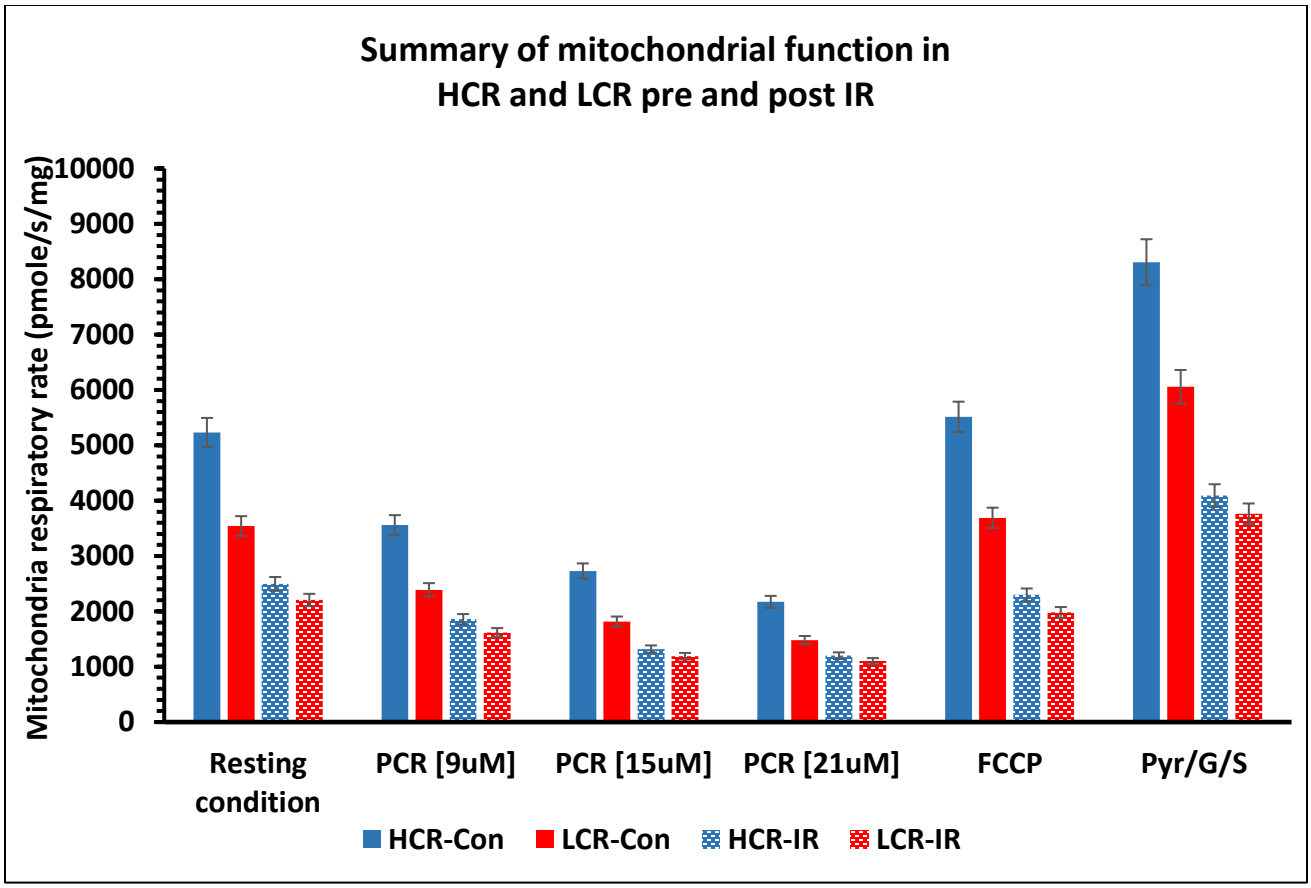
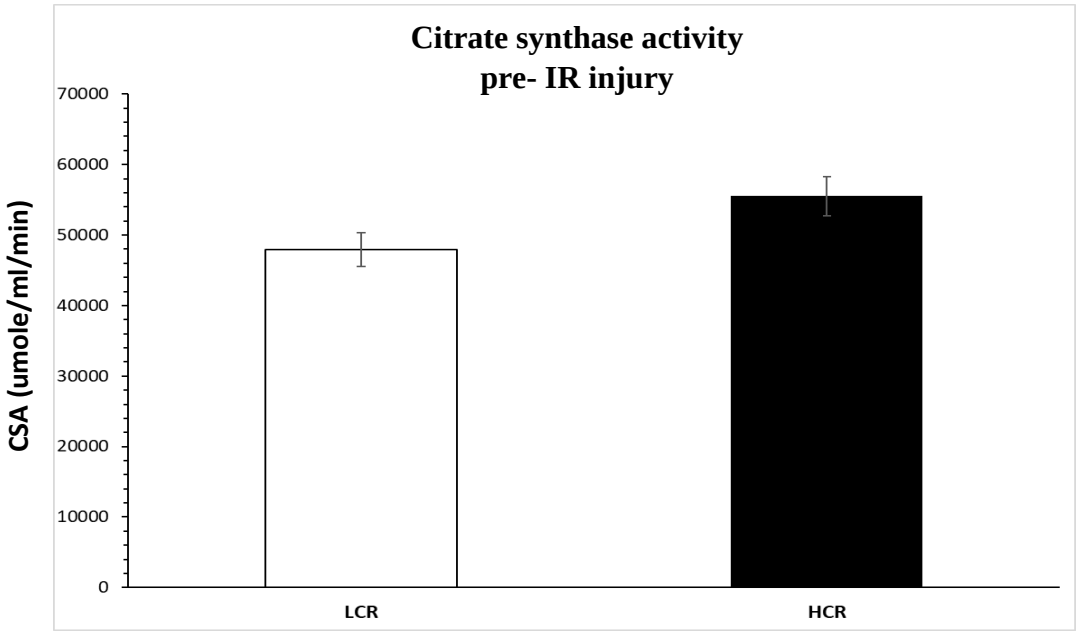


Figure 5.7. Summary comparison of LV mitochondria respiratory rate between HCR and LCR phenotypes, Control and IR injury. Paired t-test was used to analyze the data, * indicates $p < 0.05$. The error bars represent standard error.

A



B

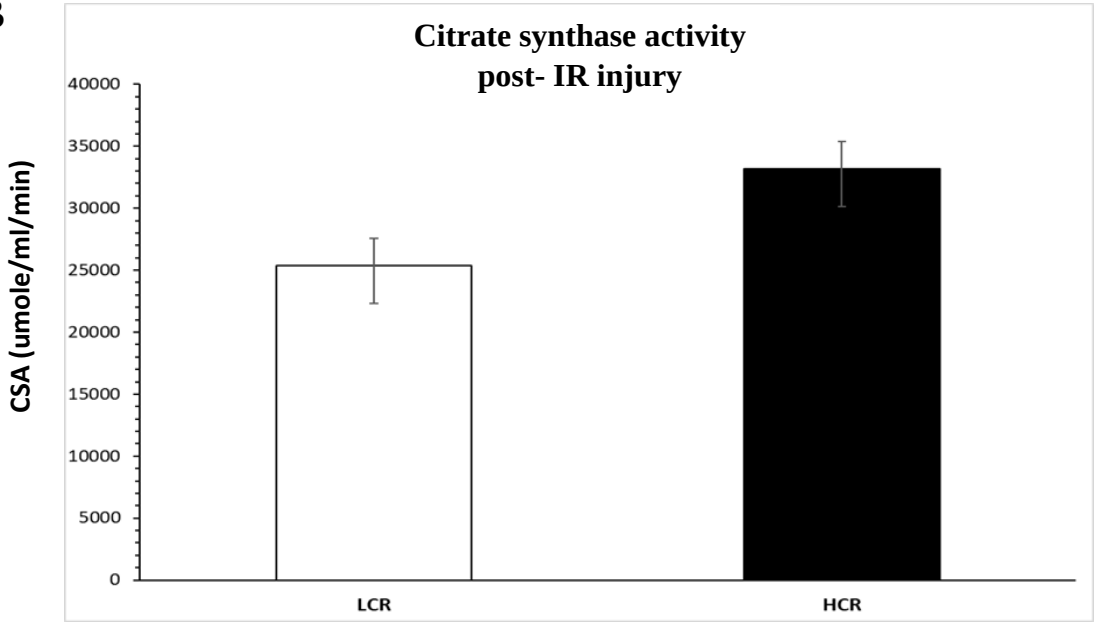


Figure 5.8. Comparison of citrate synthase activity between HCR and LCR phenotypes. A, shows the differences of citrate synthase activity in mitochondria from HCR and LCR rats before IR injury. B, shows the differences of citrate synthase activity in mitochondria from HCR and LCR rats after IR injury. Paired t-test was used to analyze the data, * indicates $p < 0.05$. The error bars represent standard error. HCR pre-IR (n=8), LCR pre-IR (n=8), HCR post IR (n=9), LCR post-IR (n=9).

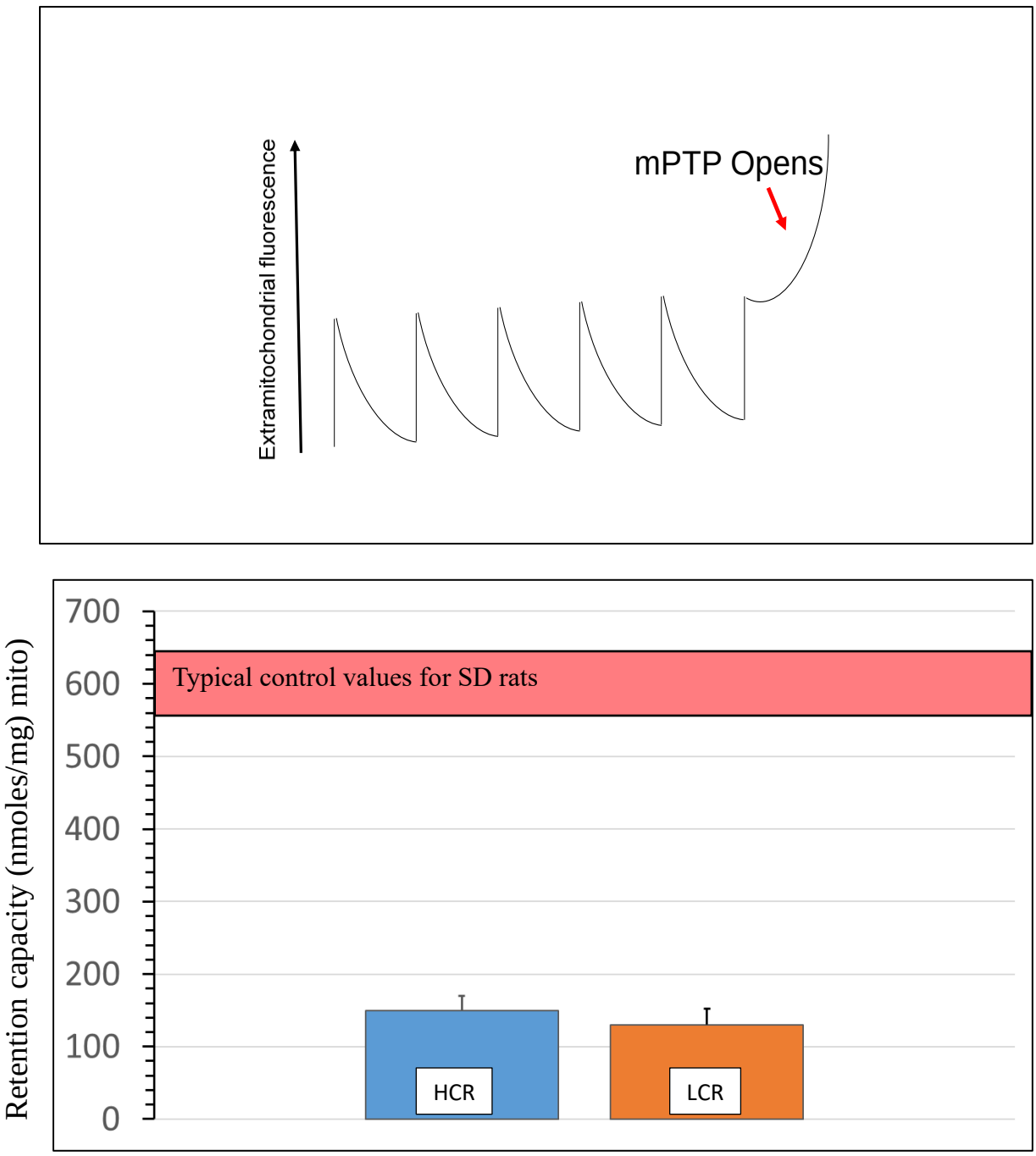


Figure 5.9. Comparison of calcium retention capacity after IR injury between HCR (blue) and LCR (orange) phenotypes. Upper image represents the concept of how Mitochondrial PTP opened by subjecting mitochondria to sequential 50 nmol CaCl2 pulses every 3 minutes, which causes repeated decreases in the fluorescent signal as Ca2+ is taken up by the mitochondria. Lower image shows the differences of calcium retention capacity in mitochondria from HCR and LCR rats after IR injury. Paired t-test was used to analyze the data, * indicates $p < 0.05$. The error bars represent standard error. HCR post IR (n=9), LCR post-IR (n=9).

CHAPTER 6: RELATIVE PROFIBROTIC GENE EXPRESSION IN CARDIAC FIBROBLASTS FROM LOW AEROBIC CAPACITY "DISEASE PRONE" RATS FOLLOWING ISCHEMIC REPRFUSION

ABSTRACT

Background: Ischemic heart disease is a major cause of morbidity. Regular active exercise is therapeutic after myocardial infarction, but up to 70% of individual exercise capacity is due to an intrinsic genetic component. Intrinsic capacity can be studied using high (HCR) and low (LCR) aerobic running capacity rat strains. The phenotypes differ by more than 5-fold in sedentary average running distance and time. The HCR rats have been characterized as "disease resistant", while the LCRs are characterized as "disease prone". In the heart, cardiac fibroblasts play a critical role in healing post myocardial infarction, but little is known about the effect of intrinsic aerobic capacity on early fibroblast gene expression following ischemia reperfusion.

Methods: On each study day, one HCR/LCR pair (n = 8 each) was anesthetized and hearts were rapidly excised. The hearts were immediately flushed with iced hyperkalemic, hyperosmotic, cardioplegia solution, and subjected to cold global ischemic arrest (80 min). Following arrest, the hearts underwent warm reperfusion (120 min) using a Langendorff style perfusion system. Following reperfusion, the heart was weighed, and the LV was isolated. A mid ventricular ring was obtained to estimate infarction size (TTC), and part of the remaining tissue (~150 mg) was transferred to 1xHBSS buffer on ice for subsequent isolation of fibroblasts. Cardiac fibroblasts from the LV of HCR and LCR rats were isolated, cultured and characterized. Immunohistochemistry were done to verify fibroblasts/myofibroblasts markers and the presence of BMPR2 in fibroblasts using cells cultured in 24 well cultured plates. Cells were also grown in series of 6-well plates and confluent were treated with BMP7, Angiotensin II and combination of

both, with and without receptor antagonists (BMP2R, noggin; Agtr1a, losartan). RNA was isolated from the respective six-well plates of cultured fibroblasts from various treated and controls groups. RNA (0.5µg) was used for cDNA synthesis followed by qPCR to determine baseline and inducible gene expression for Collagen I and III, MMP2, TIMP1, BMPR2, ATR1, SMAD5, and Bag3 in each phenotype.

Results: Immunocytochemistry successfully confirmed the presence of both fibroblasts and myofibroblasts markers. In these post IR fibroblast/myofibroblasts, LCRs showed significantly higher baseline type I collagen as well as the ratio of collagen I/collagen III ratio but the baseline expression of extracellular matrix regulatory genes such as MMP2, TIMP1, and SMAD5 were lower compared to HCRs counterparts. Both BMPR2 and Agtr1a (AT1R) receptor expressions were decreased in LCR, and the expression of anti-apoptotic gene, Bag3, was significantly higher in LCRs. Treated with BMP7, Angiotensin II or combination of both, with and without receptor antagonists (BMP2R, noggin; Agtr1a, losartan) confirmed the phenotypic selectivity of the inducible collagen I pathways. LCRs-CFBs antifibrotic gene expression were enhanced once treated with BMP7 however HCRs-CFBs Anti-fibrotic gene expression did not change once treated with either AngII or BMP. BAG3 gene expression was decreased in HCRs-CFBs once treated with AngII but not BMP7.

Conclusion: These results indicate that the LCR phenotype is predisposed to expressing a more fibrotic character in fibroblasts harvested immediately following IR and may indicate poorer long-term outcome/earlier onset heart failure following acute ischemic injury to the heart. The findings also clearly suggest that HCRs phenotype response through apoptosis and LCRs response through fibrosis post IR injury. These results suggest that Angiotensin II receptor (AT1R) pathway is responsive only in post-ischemic LCR cardiac fibroblasts, and the BMP receptor (BMPR2)

pathway is responsive only in post-ischemic HCR cardiac fibroblasts which could shed new light on individuals who develop post-infraction fibrosis despite therapeutic dosing with AT1R blockers (Angiotensin II type 1 pathway antagonists).

INTRODUCTION

Cardiovascular disease (CVD), including ischemia reperfusion (IR) injury, is the main cause of morbidity and mortality in developed countries. Ischemic heart disease is the most common type of CVD and the prevalent counting more than one million deaths in the United States alone every year (228). Moreover, CVD is the most widespread and costly health problems in the world with a price tag of $555 billion U.S. dollars in 2016 (229). Ischemic heart disease results as consequence of cut off heart muscle from oxygen-rich blood due to narrowed or blocked coronary arteries. Ischemic heart disease may lead to critical cardiac complications such as ventricular arrhythmias and congestive heart failure. Over recent decades, scientists and researchers show that ischemic pathology consist of injurious events experienced during ischemia and reperfusion which together termed as ischemia-reperfusion (IR) injury (230,231).

Myocardial ischemia reperfusion injury results in detrimental cardiovascular outcomes after myocardial ischemia, cardiac surgery or circulatory arrest. Mostly, depriving the heart from blood flow due to coronary occlusion causes an imbalance between oxygen demand and supply, named *ischemia*, which in turn leads to cardiac tissue damage or dysfunction (232). Intuitively, early and fast restoration of blood flow has been considered the treatment of preference to protect against more tissue injury. Unfortunately, restoring blood flow to the ischemic myocardium, named reperfusion, while necessary, can also aggravate or even induce an irreversible injury and be more deleterious than proceeding ischemia. This phenomenon was therefore termed *myocardial ischemia reperfusion injury* (232).

The major risk factors for IR that cannot be prevented including aging, male gender, and genetic factors. However, other significant risk factors can be adjusted or controlled, including smoking, hyperlipidemia, hypertension, physical inactivity, obesity, metabolic syndrome, and diabetes mellitus (290).

Ongoing research is aimed to explore therapeutic interventions against IR injury. Although numerous pharmacological and preconditioning approaches to cardioprotection have been explored, regular exercise participation is recognized as an important, cost effective, convenient, non-invasive and safer lifestyle intervention in the prevention and treatment of IR injury (1). Redundant protective effects are evident in the exercised heart including increased levels of heat shock proteins, altered nitric oxide (NO) signaling, enhanced Ca2+ handling proteins, improved ATP-sensitive potassium channels, and enhanced endogenous antioxidant (233). Moreover, exercise preconditioning has been reported to have anti-fibrotic and anti-apoptotic effects on the ischemia-reperfusion myocardium cells, which could lead to the protection of cardiac cells(291).

Myocardial IR injury is a complex event with multiple connected processes involving oxidative stress, cardiac metabolism, inflammation, hypertrophy, cardiomyocyte apoptosis, fibrosis, intracellular Ca+ overload and subsequent progression into irreversible cell death by apoptosis and necrosis (292–294). Each of these is changed in a time-dependent manner following to ischemia reperfusion injury (IR). A better understanding of the contribution of these processes to IR injury is important to open the way for new therapeutic strategies that can help decrease the myocardial infarct size and cardiac remodeling which are critical elements of IR prognosis (295).

In response to cardiac stresses, such as IR, the left ventricle (LV) undergoes adverse structural and functional remodeling, with cardiomyocyte hypertrophy and excessive production of the extracellular matrix (ECM) as typical features of IR insults. Although featured remodeling

events may be favorable in the beginning of the insult as they are started to compensate for failing cardiac function, this remodeling will eventually cause transition to heart failure over time (295). Myocardial fibrosis is one of the major IR injury hallmarks and remains an important target of therapy for patient with myocardial infarction, however, signaling pathways that regulate cardiac fibroblast remain a major knowledge gap of our understanding of cardiac remodeling. Therefore, researchers seek to uncover the molecular mechanism underlie cardiac fibrosis and all other contributed factors (295).

Cardiac fibroblasts (CFBs) are the largest population, in terms of interstitial cell numbers, in the heart (296). They are responsible for producing and maintaining the extracellular matrix surrounding myocytes (297). Although not contractile, fibroblasts are directly connected to myocytes via cell junctions and bridging the spaces between myocardial tissue layers (297). Cardiac fibroblasts are predominantly resistant to hypoxia and frequently involved in the cascade events after ischemic injury (298). After ischemic injury, cardiac fibroblasts can differentiate into myofibroblasts marked by increased amounts of α-smooth muscle actin (α-SMA) and characterized to be more mobile and contractile with a stronger ability produce ECM proteins. Myofibroblasts play a key role in cardiac remodeling by forming a collagen-rich scar that allows the infarcted area to preserve structural integrity after cardiomyocyte death (299). Transformation of cardiac fibroblasts to myofibroblasts shifts the balance in ECM turnover, promoting secretion and accumulation of fibrotic depositions such as I and III collagen (COL I and III) which in turn replace the myocytes or disturb the myocyte–myocyte connections in the myocardium leading eventually to general impairment of cardiac function (299).

Abnormal ECM turnover results in activation of cellular and molecular signaling cascades which can cause structural instability. ECM turnover and remodeling is mainly regulated via the

function of ECM-degrading enzymes, matrix metalloproteinases (MMPs), and their physiological inhibitors, tissue inhibitor of metalloproteinases (TIMPs) (300,301). CFBs can produce a number of MMPs and TIMPs whereby they can impact different aspects of ECM homeostasis and remodeling(302),(303).

Angiotensin II (AngII) is important for cardiovascular homeostasis (304). AngII works either through Ang II type-1 receptor (AT1R) or Ang II type-2 receptor (AT2R) and plays an important role in cardiac remodeling (305). This peptide involved in cardiac myocyte hypertrophy as well as myocardial fibroblast interstitial fibrotic changes associated with left ventricular hypertrophy, post myocardial infarction and congestive heart failure (304,306). AngII plays an important role in cardiac fibrosis as stimulation it induced the differentiation of cardiac fibroblasts into myofibroblasts, as indicated by increased expression of α-smooth muscle actin (α-SMA) and collagen types I and III (307). Losartan is an AT1R blocker and is widely used treatment for hypertension and has been confirmed to prevent IR-induced cardiac injury via inhibiting reactive oxygen species (ROS)-induced apoptosis (308,309).

One of the most powerful pro-fibrogenic systems involved in cardiac fibrosis is the transforming growth factor beta (TGFβ) superfamily, which includes TGF-β1 and bone morphogenetic proteins (BMPs) (310,311). BMPs binds directly to their receptors on the surface of target cells to form a heterotetramic complex containing of two dimers of type I (e.g. BMPR 1) and type II (e.g. BMPR2) receptors. This heteromeric complex formation allows the active type II receptor to transphosphorylate and activate the type I receptor, which in turn, causes conformational changes that lead to phosphorylation of downstream proteins known as R-Smads (Smad1, 5, and 8) (312). Activated, phosphorylated R-Smads in the cytoplasm then form complexes with co-Smad (Smad4), and together, they translocate into the nucleus to regulate gene

expression and begin cellular activity such as differentiation, apoptosis, angiogenesis, and extracellular matrix synthesis (313). Because of the close relationship between inflammation and fibrosis, BMP signaling may initially play an indirect positive role through promoting the recruitment of infiltrating leukocytes by endothelial cells into the tissue and consequently enhancing the degree of the profibrotic signal produced by these cells (314). BMPs through activation of SMAD-1/5/8, are able to inhibit TGF-β mediated fibrotic gene expression (315). Thus, hyper-activation of activin/TGF-β-mediated SMAD-2/3 signaling promotes fibrosis, whereas increased activity of BMP/SMAD-1/5/8 signaling is likely to facilitate anti-fibrotic. Both pathways compete for use of the same SMAD4, and so reduced use of SMAD4 by BMP may result in increased availability for TGF-β signal. It has been shown that BMPR2 mutations result in a disturbed BMP/TGF-β balance leads to an overactivated TGF-β signaling, which stimulates the fibrotic processes (316).

Bcl-2–associated athanogene 3 (BAG3) is a conserved protein expressed at high levels in the heart. It is a member of BAG family and the only member of the family to be induced by stressful stimuli (317). BAG3 is an anti-apoptotic protein that plays a critical role in several widespread diseases including cardiovascular diseases (318). Altered BAG3 expression has been associated with cardiac dysfunction, and overexpression of BAG3 has been reported to reduce infarct size and improve LV function as well as markers of autophagy and apoptosis in mice after IR injury (319).

Although regular exercise training is recognized as an important lifestyle intervention in the prevention and treatment of CVD and IR injury, not all individuals experience the same benefits from participating in exercise. It has been estimated that up to 60-70% of the variation in exercise capacity is due to the intrinsic genetic component (89). Some people have many risk

factors, don't exercise, and don't get CVD, while some people have no risk factors, exercise regularly, and still experience diseases. Thus, studying the differential impacts of intrinsic aerobic exercise capacity after cardiac ischemic reperfusion injury using intrinsic aerobic phenotype rats bred for low and high aerobic running capacity would provide a better platform for understanding the influences of intrinsic aerobic capacity on cardiac remodeling and fibroblast adaptive response, which represent long term injury, after ischemic reperfusion injury in these phenotypes.

Using a rat model to determine how intrinsic aerobic exercise capacity responds and adapts to several diseases has been developed by Koch and Britton (66). This novel rodent model created by selective breeding of rats that showed either high or low treadmill endurance running capacity. These strains are referred to as high-capacity runners (HCR) or low-capacity runners (LCR). Both strains were developed using rats that were untrained (caged activity-only condition) to exclude possible confounding influences of daily exercise training. By generation six, there was a 171% divergence in running capacity, with most of the change in running capacity relative to the founder population occurring in the HCR group (13% in LCR and 136% in HCR) (66,320). The high capacity running (HCR) animals are characterized broadly as "disease resistant", while the low capacity running (LCR) animals are characterized as disease prone. The low aerobic capacity rats are a genetic model for metabolic syndrome that contribute to an increased risk of diabetes and cardiovascular disease. A consistent characteristic of the LCR exhibit metabolic and cardiovascular phenotypes (i.e., overweight, insulin resistance, elevated blood pressure, microvascular endothelial dysfunction, and dyslipidemia) (321). Furthermore, LCR have a 45% shorter median lifespan than the high capacity running (HCR) animals (157). In the heart, cardiac fibroblasts play a critical role in healing post myocardial infarction, but little is known about the

effect of intrinsic aerobic capacity on early fibroblast gene expression following ischemia reperfusion.

In this study, we set out to uncover the influence of intrinsic (untrained) aerobic exercise capacity on stress induced cardiac fibrosis (fibrosis-related gene expression) post-acute myocardial ischemic reperfusion injury between HCR and LCR. Specifically, we tested the hypothesis that inherited low-aerobic capacity in LCR rats is predisposed to expressing a more fibrotic character in fibroblasts harvested immediately following IR. Our model utilizes cold cardioplegia as global ischemic arrest (80 min) and warm reperfusion (120 min) using a Langendorff style perfusion system.

MATERIALS AND METHODS

Animal Strains. 16 HCR and LCR, female rats, 40 weeks, generation 32, housed under sedentary conditions, and fed normal chow. LCR and HCR animals were randomly assigned to either HCR or LCR ischemia reperfusion (n = 8 each). HCR and LCR rats were obtained from the parent colony at the University of Toledo. This novel rodent model has been developed by Drs. Lauren Koch and Steven Britton at the University of Michigan. They have previously described the artificial selection used for the generation of the HCR and LCR strains (92). Briefly, each sex was selected from the founder population (N: NIH stock). Two-way artificial selective breeding was used to create low capacity runner (LCR) and high capacity runner (HCR) strains that were divergent for treadmill running capacity (run time until exhaustion on a graded treadmill exercise test). The 13 lowest and 13 highest running capacity rats of each sex were randomly paired for mating. This pattern was repeated over subsequent generations to produce the divergent strains using a rotational breeding scheme. In the present study animals from generation 32 were used. All the animals were exposed to treadmill exercise for the 5 days at 11 weeks of age when the

animals were phenotyped for treadmill running capacity. Upon verifying the phenotypes of the animals, animals were prepared for shipping at 14 weeks of age, or soon after as weather conditions (airport tarmac temperatures < 85°F) permitted. Once received by the Department of Comparative Medicine at ECU, the animals were maintained under mandatory quarantine for 10 weeks before they were released for study. Rats were provided standard rat chow and water ad libitum and were kept on a 12 h light/ 12 h dark time schedule until sacrifice. Animal procedures were conducted in accordance with American Physiological Society guidelines for the humane and safe use of animals, and all protocols involving animals used for these experiments were approved by the East Carolina University Animal Care and Use Committee.

Cardiac ischemic-reperfusion injury. Rats were anesthetized with an intraperitoneal injection of ketamine (80mg/kg; Cenvet Australia, Kings Park, NSW, Australia) and xylazine (10mg/kg; Provet, Eastern Creek NSW, Australia).The heart was harvested after being arrested with 1,000 mL of iced St. Thomas' cardioplegic solution (NaCl 110.0 mM, NaHCO3 10.0 mM, KCl 16.0 mM, MgCl2 16.0 mM, CaCl2 1.2 mM, pH 7.8) at 4°C, infused at a pressure of 60 mm Hg to 65 mm Hg into the aortic root, and the hearts were then stored in this solution at the same temperature for 80 min. After 80 min of cold global ischemic arrest, the heart was cannulated and immediately perfused retrogradely on a Langendorff perfusion apparatus with Krebs-Henseleit buffer (KHB) for 120 mins at 37°C (composition (mM): NaCl 118; KCl 4.7; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25; $CaCl_2$ 1.4; glucose 11; pH 7.3–7.4) and the a hydrostatic pressure maintained at 90 mm Hg (range, 80 to 95 mm Hg) by adjustment of the pump speed. Coronary flow (CF) was measured manually at the baseline and every 30 min of reperfusion.

Tissue isolation and infarct size quantification. Following reperfusion, the heart was taken off the cannula and weighed and trimmed at the AV groove and LV was then isolated. A mid

ventricular ring was obtained for infarct size quantification and the rest of LV was sliced into 3 approximately equal sections. One of the LV three slices was used for fibroblast experiments. For infarct size quantification, a mid-ventricular ring was then placed in a 0.1 % triphenyltetrazolium chloride (TTC) solution and incubated at 37 °C for 10 minutes in a shaking water bath. Following incubation both sides the slice were photographed with a digital camera attached to a dissecting microscope. Images were quantified using Image J software where total area (TA), lumen area and infarcted area (IA) were measured. The area at risk (AAR) was considered to be the entire left ventricle and was calculated by taking the TA and subtracting the lumen area. The infarcted area was determined by measuring the white appearing tissue in the AAR. Areas were converted to weights by multiplying the mean of the AAR and IA of both sides of the slice by the weight of each slice. The total weight of the IA from all sections was then divided by the total weight of the AAR from all sections to obtain a percentage of the heart that was infarcted.

Fibroblasts isolation and culture. Cardiac fibroblasts (CFs) were isolated as described previously (322). Briefly, left ventricles (LVs) were washed with 1X PBS to remove any remaining blood. LVs were then minced and digested with collagenase II containing DNase at 37°C. Digested tissues underwent two rounds of centrifugation (300x*g* for 10 minutes) with removal of supernatant. The pellet is then re-suspended in 5 mL of 10% FBS media to remove any remaining collagenase and debris, followed by incubation at 37°C + 5% CO2 in 1X Dulbecco's modified Eagle's medium DMEM/F-12 (1:1) media supplemented with 20% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 mg/mL). Isolated cells allowed to be cultured in a T_{25} flask containing a medium of DMEM+10% fetal calf serum (FCS) and maintained in a humidified 5% CO_2/95% air atmosphere at 37°C. The cells were allowed to attach the bottom of the flask and observed under the microscope. The medium was replaced every 2–3 days and cells

were washed with DPBS when necessary. Cells were grown to confluency and routinely split at a 1:3 ratio.

Immunocytochemistry staining in cardiac fibroblasts. Immunocytochemistry staining were done to detect alpha smooth muscle actin, alpha SMA marker for myofibroblast, vimentin and DDR2 markers for fibroblasts, and BMPR2 receptor of BMPs as previously described with slight modification. It is also done to quantify the expression of alpha SMA and BMPR2 by measuring the intensity using ImageJ software. Briefly, square cover slips (18x18-1, ThermoFisher) were placed in each well of a 24-well plate and kept in the cell culture hood under ultraviolet rays for one hour before plating cells. Cells were seeded in a density of 4000 cells per well and sat undisturbed overnight to allow for cell attachment to the bottom of the wells. Culture media were then removed from each well and cells were then rinsed three times with PBS and fixed with ice-cold 100% ethanol and incubated for 15 min. The cells cultured were then permeabilized with 0.5% Triton X-100 in PBS. Cells were blocked with blocking buffer (5% BSA in PBS) and kept at room temperature for 1 hour. Blocking buffer was then removed and cells cultured were then incubated with primary antibody against alpha smooth muscle actin (Goat) (1:250, Novus Biologicals, NB300-978), Vimentin (chicken) (1:250, Novus Biologicals, NB300-223), DDR2 (Rabbit) (1:500, Novus Biologicals, NBP2-14927, or BMPR2 (Mouse) (1:200, Abcam, ab130206) overnight at 4°C. Cells were washed with PBS and then incubated with secondary IgG antibodies conjugated to Alexa 555, or 488 (5 ug/mL, Invitrogen, Carlsbad, CA, USA) in dark room for 1 hour at room temperature. The nuclei were counterstained with DAPI. Excess antibody was removed by three washes and cells were imaged with confocal microscopy using relative reading of mean intensity per field area, keeping parameters constant throughout the acquisition process (Figure 6.2).

Cell culture treatments. Cells from passage 1 were allowed to be grown in T_{75} flasks with normal culture media until reached 80% or 90% confluency. Once reached confluence, cells were trypsinized with 0.5% Trypsin-EDTA phenol red-free solution (~4ml/each T75 flasks) and incubated for 3-4 minutes at 37 °C (time of incubation is based on the cells dispersion monitored under microscope). Cells were then neutralized using similar volume of Trypsin Neutralizer Solution and mixed gently. For cells counting, an equal volume of the cell suspension and Trypan blue dye was mixed in microtube (20 µl:20 µl) and 10 µl of dye/cells mixture was used to count cells using Automated Cell Counters. After counting cells, Cell suspension was then centrifuged (300Xg for 8-10 min using 8K Marathon) and Cell pellets were then re-suspended in 5-10 ml media (serum-free DMEM/F12 + 10% FBS and 1X antibiotic + antimycotic). Cell number was counted again using the previous mentioned method. Cells were then plated at a density of about 50,000 cells per well via 300µl aliquots into 6-well plate in duplicate. Cells were then incubated in cell culture incubator at 37 °C with 5% CO2. For each animal (3 experiment X 6 wells plate), Cells were treated with drug as following: for first 6 well plate: first duplicate were control (cells without any treatment), second duplicate were cells treated with AngII, (10^{-7} M), the third duplicate were cells treated with AngII + Losartan (10^{-5} M). For second 6 well plate: first duplicate was control (cells without any treatment), second duplicate were cells treated with BMP7, (50 ng/ml), the third duplicate were cells treated with BMP7+ Noggin (500 ng/ml). For third 6 well plate: first duplicate control (cells without any treatment), second duplicate were cells treated with AngII + BMP7. (Figures 6.8, 6.9, and 6.11)

RNA isolation and Real time-PCR. Total RNA was isolated from cultured fibroblasts using Trizol (Invitrogen, Carlsbad, CA) and Direct-zol RNA miniprep kit (Zymo Research, Irvine, CA) following manufacturer's instruction. cDNA was synthesized using the High-Capacity cDNA

Reverse Transcription kit from Applied Biosystems (Thermofisher) following the manufacturer's protocol. qReal-Time PCR was performed using Tackman 5̀ fluorescent labeled primers for Collagen I, Collagen II, MMP2, TIMP1, BMPRII, Ang1, Smad5, and Bag3 utilizing TaqMan Fast Advanced Master Mix (Life Technology) following the manufacturer's protocol. Beta-Actin was used as the reference gene since CT values for this gene did not change with treatment. The qReal-Time PCR detection system used was the ABI Quant Studio 6 Flex q-PCR (Applied Biosystems, Foster City, CA). Expression levels were reported as $^{2-\Delta CT}$.

Statistics. Statistical analysis and graphs were performed using data analysis software in Microsoft Excel and graph pad prism worksheet (Windows 10). Data is expressed as means ± SEM, and a p value <0.05 was considered statistically significant. Statistical analysis was performed Single factor ANOVA tests were used to compare gene expression between HCR and LCR to determine statistical significance among the groups (two groups at a time) comparing two different ways: control (baseline) data of HCR-Fbs with the counterpart of LCR-CFs group, and treated (inducible) HCR-CFBs with its counterpart of LCR-CBFs Differences in infarct size and coronary flow between HCR and LCR were analyzed using paired t-test.

RESULTS

Myocardial infarct size

After 80 min of cold global ischemic arrest and 120 mins of warm reperfusion, myocardial infarct size was not significantly different between HCRs and LCRs. Although HCR and LCR animals showed different pattern of infarct area, both have relatively similar size of myocardial infarction. There was high variability in infarct size between the animals in both phenotypes (Figure 5.3; P > 0.05).

Coronary Artery Flow

Coronary artery flow has been taken manually at the beginning, after 30 min, and after 60 minutes of reperfusion. Values from each measurement were averaged to obtain a value for each animal. Although the HCRs trend to have higher coronary artery flow than LCRs, the differences were not significantly different in all time points between HCRs and LCRs following IR injury. (Figure 5.4; $P > 0.05$).

Immunocytochemistry

Immunocytochemistry successfully confirmed the presence of both fibroblasts and myofibroblasts markers (Figure 6.3). It also confirmed the expression of BMPR2 (type II receptor of BMPs) in the cardiac fibroblasts (Figure 6.4). The expression of alpha smooth muscle actin was significantly higher (38 %) in LCRs comparing to HCRs (Figure 6.5; $P > 0.05$). The Immunocytochemistry data also revealed that HCRs' cardiac fibroblast expressing higher level of BMPR2 protein (34%) comparing to LCRs' cardiac fibroblast (Figure 6.6; $P > 0.05$). Cells counting revealed that LCR rats had higher number of fibroblasts/myofibroblasts (45%) comparing to HCR counterparts post-acute ischemic reperfusion (Figure 6.7; $P > 0.05$).

Baseline (non-treated) fibroblast gene expression after ischemic reperfusion injury

In these post IR fibroblast/myofibroblasts, LCRs showed significantly higher gene expression (54%) in the collagen I/collagen III ratio but lower expression of extracellular matrix regulatory genes including MMP2 (33%), TIMP1 (16%), and SMAD5 (20%) compared to HCRs counterparts. BMPR2 (40%), Agtr1a (28%), the main investigated receptors for BMPs and AngII respectively, also were both significantly decreased, and the anti-apoptotic gene, Bag3, was significantly (38%) higher in LCRs. Expression levels were reported as $^{2\text{-}\Delta\ CT}$ (Figure 6.10; $P > 0.05$), (Table 6.1).

Inducible (treated) fibroblasts gene expression after ischemic reperfusion injury

ECM gene expression. Regarding ECM gene expression**,** Collagen I gene expression was significantly higher in LCRs more than HCRs, but the activating pathways were phenotype dependent. Treating with Ang II induced gene expression for collagen I in LCR fibroblasts but not HCR and was completely blocked with losartan. By Contrast, treating with BMP7 induced gene expression of collagen I in HCRs fibroblasts but not LCRs, and was completely blocked by noggin. Treating the fibroblasts with a combination of receptor agonists confirmed the phenotypic selectivity of the inducible pathways. (Figure 6.10; $P > 0.05$).

Collagen III gene expression was significantly higher in HCRs more than LCRs. Treating with AngII did not change collagen III expression in both strains. However, adding losartan increased Col III expression in LCRs but not HCR fibroblasts (possible media expression of AngII, losartan effect). Treating with BMP7 induced Col III gene expression in LCRs and was completely blocked with noggin. However, in HCRs, BMP7 attenuated Col III expression and even more decreased once treated with noggin (possible tissue expression of BMP7, noggin effect), BMP7 inhibitor. Treating the fibroblasts with a combination of receptor agonists showed no differences in LCR fibroblasts but increased Col III gene expression in HCRs (suggesting negative effect of AngII on BMP7). (Figure 6.11; $P > 0.05$).

Like Col III, MMP2 gene expression was significantly higher in HCRs comparing to LCRs. Treating with AngII did not change MMP2 expression in both strains. However, adding losartan increased MMP2 expression in HCRs but not LCR fibroblasts (possible tissue expression of AngII, losartan effect). Treating with BMP7 induced MMP2 gene expression in LCRs and was completely blocked with noggin. However, in HCRs, BMP7 did not change MMP2 gene expression. Treating the fibroblasts with a combination of receptor agonists showed no differences in LCR fibroblasts

but increased MMP2 gene expression in HCRs (suggesting negative effect of AngII on BMP7). (Figure 6.12; $P > 0.05$).

TIMP1 gene expression was significantly higher in HCRs comparing to LCRs. Treating with BMP7, AngII or both, with and without receptor antagonists (BMP2R, noggin; Agtr1a, Losartan) showed no effect in TIMP1 gene expression in both phenotypes. (Figure 6.13; $P > 0.05$).

Although not significantly different, Smad5 gene expression was relatively higher in HCRs more than LCRs fibroblasts. Treating with either AngII or its antagonist, losartan, had no significant effect in Smad5 gene expression in both strains. However, Treating with BMP7 promoted Smad5 gene expression and even more enhanced once treated with noggin (possible tissue expression of BMP7, noggin effect), BMP7 inhibitor in LCRs. In HCRs, neither BMP7 nor its antagonist, noggin, showed any significant effect of Smad5 gene expression. Treating the fibroblasts with a combination of receptor agonists showed no differences in HCRs but increased Smad5 gene expression in HCRs (may be due to BMP7 effect). (Figure 6.14; $P > 0.05$).

Receptors gene expression. Regarding the gene expression of the main investigated receptors, BMPR2, BMPs receptor, gene expression was significantly higher in HCRs comparing to LCRs counterparts. In LCRs fibroblasts, treating with AngII induced BMPR2 and even more pronounced once treated with its antagonist, losartan, (possible tissue expression of AngII, losartan effect). However, treating with BMP7 also increased the gene expression of BMPR2 and was decreased with noggin, the BMPs blocker. Moreover, treating with combination of AngII and BMP7 brought the gene expression of BMPR2 to about the baseline level suggesting the antagonist effect of BMP7 on AngII and vice versa (reciprocal effect between BMP7 and AngII). In HCRs fibroblasts, treating with BMP7, AngII or both, with and without receptor antagonists (BMP2R, noggin; Agtr1a, losartan) showed no influence in BMPR2 gene expression. (Figure 6.15; $P > 0.05$).

Agtr1a, AngII type-I receptor, gene expression was significantly higher in HCRs comparing to LCRs counterparts. In LCRs, fibroblasts Agtr1a gene expression was reduced once treated with AngII and increased once treated with losartan. Moreover, treating with combination of AngII and BMP7 brought the gene expression of Agtr1a to about the baseline level suggesting the antagonist effect of BMP7 on AngII. However, treating with BMP7 induced the gene expression of Agtr1a, and was decreased with noggin, the BMPs blocker. Moreover, treating with combination of AngII and BMP7 brought the gene expression of Agtr1a, to about the baseline level suggesting that BMP7 antagonized the effect of AngII on Agtr1a gene expression and vice versa (reciprocal effect between BMP7 and AngII). In HCRs fibroblasts, treating with BMP7, AngII or both, with and without receptor antagonists (BMP2R, noggin; Agtr1a, Losartan) showed no influence in BMPR2 gene expression. (Figure 6.16; $P > 0.05$).

Anti-apoptosis gene expression. BAG3 gene expression was significantly higher in LCRs comparing to HCRs counterparts. In LCRs fibroblasts, treating with BMP7, AngII or both, with and without receptor antagonists (BMP2R, noggin; Agtr1a, Losartan) showed no impact in BAG3 gene expression. However, in HCR fibroblasts, treating with AngII decreased the gene expression of BAG3 and adding losartan increased its expression. Treating with either BMP7 or its antagonist, noggin, had no effect on BAG3 expression in HCRs fibroblasts. Treating the fibroblasts with a combination of receptor agonists, BMP7 and AngII exhibited increasing of BAG3 expression (may be due to BMP7 effect) (Figure 6.17; $P > 0.05$).

DISCUSSION

Myocardial IR injury is a complex event with multiple connected processes involving oxidative stress, cardiac metabolism, inflammation, hypertrophy, cardiomyocyte apoptosis, fibrosis, intracellular Ca^{++} overload and subsequent progression into irreversible cell death by

apoptosis and necrosis (292–294). In response to cardiac stresses, such as IR, the left ventricle (LV) undergoes adverse structural and functional remodeling, with cardiomyocyte hypertrophy and excessive production of the extracellular matrix (ECM) such as collagen I and collagen III typical features of IR insults. Although featured remodeling events may be favorable in the beginning of the insult as they are started to compensate for failing cardiac function, this remodeling will eventually cause transition to heart failure over time (295). Preventing adverse LV remodeling after acute IR injury by a simple, safe, cheap, and effective modality such as exercise will prolong a good quality of life by promoting systemic and local myocardial health. It is well established that increased levels of physical activity, exercise, and fitness decrease cardiovascular mortality by modifying the expression of genes that are linked to left ventricle (LV) health, function, and structure (289,323). Exercise has been shown to reduce arrhythmia, decrease myocardial stunning, and improve vascular reactivity in hearts exposed to ischemia reperfusion (267,268). Moreover, exercise training after MI improves the balance between MMPs and TIMPs and significantly declined collagen deposition which ultimately decrease cardiac fibrosis and preserve post-MI cardiac function (323).

It is well know that aerobic exercise capacity has two main components, genetic and environmental components (2,166). The genetic component defines the intrinsic endurance exercise capacity and appears to have two parts, the genes that regulate adaptive responses to exercise training and the genes that determine intrinsic exercise capacity (166).

Although aerobic exercise training has beneficial effects on several of cardiovascular diseases, the big variation in the physiological response to exercise training suggests potential impact of the genetic composition. It has been estimated that up to 60%-70% of the variation in exercise capacity is due to the genetic component (2). It is not clear if the genetic component for

enhanced exercise capacity alone can result in protection from cardiovascular diseases or whether the training stimulus is necessary to produce the positive results, or a combination of both.

Numerus evidence suggesting that low intrinsic aerobic capacity is associated with increased risk factors for cardiovascular disease has existed from a novel rat model of inherited LCR and impaired metabolic health. Using this model would help to understand the impact of these phenotypes on LV remodeling following acute cardiac stress such as myocardial ischemic reperfusion injury. Therefore, the goal of this study was to determine the effect of intrinsic aerobic capacity on early fibroblast gene expression and on the inducible, receptor-mediated fibroblast gene expression pathways following acute ischemia reperfusion.

The results of this study showed that the left ventricle of rats selectively bred for high endurance running capacity (HCR) and low endurance running capacity (LCR) were comparable in myocardial infarct size after 80 min of cold global ischemic arrest and 120 mins of warm reperfusion. Moreover, the results showed that coronary artery flow was not significantly different between HCR and LCR animals at all selected time points of reperfusion. These results were somewhat unexpected as active exercise training successfully preserve coronary artery flow and reduces infarct size after ischemia-reperfusion in rat heart and HCR rats have at least the intrinsic potential for increased physical activity. One previous study evaluated the impacts of swim training on myocardial infarct size after irreversible coronary artery ligation in the rat (32). Their results showed a reduction in infarct size after training and suggested that the cardioprotective effect of exercise was mediated by increased myocardial vascularity which might act to minimize the area of myocardium at risk during coronary artery obstruction (32). Another study confirmed that a 5-day program of exercise training regimen was adequate to confer infarct sparing in hearts subjected to a transient regional ischemia and followed by reperfusion

(269). They hypothesized that the improved tolerance to ischemia-reperfusion insult was caused by exercise-induced increases in myocardial antioxidant defenses (269). In 2003, David A. Brown and his colleagues have demonstrated that prolonged endurance training confers a cardioprotective effect against infarction in myocardium subjected to severe ischemia and subsequent reperfusion (270). In addition, they observed that during severe ischemia, coronary flow to regions of the myocardium outside the ischemic area at risk was better maintained in hearts isolated from endurance-trained rats. Furthermore, on reperfusion of the area at risk, the increase in flow to the previously ischemic region of the heart was markedly higher in hearts isolated from trained rats (270). Still, it also has been demonstrated that the genetic predisposition for exercise capacity is a different profile than the genetic predisposition to respond to active exercise, and our own earlier results suggest that vascular responses differences between phenotypes was less pronounced within the females (Chapter 2). Together, all these results suggest the importance of exercise to maintain a good coronary artery flow in order to reduce the infarct size after IR insult. Our results showed that all coronary flow has impaired at the same level in both HCR and LCR animals.

One explanation is that injury was too massive to maintain the cardioprotective impact of HCR phenotype. This concept is supported by the results in acute IR (Chapter 4) demonstrating phenotypic differences in myocardial infarction size with short time (15 min) but not with longer time (30 min) ligation, suggesting that the increased aerobic capacity phenotype may not be able to resist the severity of oxidative stress and reduction of endogenous antioxidant capacity and the impairment of protective signaling pathway related to the activation of STAT3 and Akt, the major mechanisms for the increased myocardial injury after prolonged ischemic insult.

Myocardial extracellular matrix (ECM) plays a critical role in cardiac development, homeostasis, and remodeling (324,325). The major structural components of the ECM are

collagens, predominantly collagen type I and collagen type III, which are produced mainly by fibroblasts(326), and its synthesis and degradation is vital for normal cardiac structure and function (297). It is worth to mention that collagen I/III ratio plays an essential role in the functional integrity of various tissues. Increasing I/III ratio has been linked to increased cardiac stiffness & rigidity which lead to Compromised physiological heart function (e.g. impaired cardiac filing decreased CO and ultimately heart failure). On the other hand, decreasing I/III ratio can lead to increased tissue cardiac elasticity, flexibility and ultimately cardiomyopathy (e.g impaired cardiac emptying) (327,328). During pathological conditions, cardiac fibrosis is a critical aspect of cardiac remodeling following myocardial infarction, hypertension, and other cardiovascular diseases (297,299,329). Differentiation of cardiac fibroblasts into myofibroblasts, characterized by expression of α-smooth muscle actin (α-SMA) and production of extracellular matrix (ECM) components such as collagen types I and III, is a critical event in cardiac fibrosis (330). During ECM remodeling, the balance between matrix metalloproteinases (MMPs) and their inhibitor, the tissue inhibitors of metalloproteinases (TIMPs), is as very important as the proper ECM production (331). TIMPs and MMPs are strongly regulated in normal wound healing, and their imbalance has been related to pathologic fibrosing diseases.

Our current results clearly showed the LCR-CFBs after IR insult have significantly higher baseline gene expression (54%) in the collagen I/collagen III ratio than HCR-CFBs. Moreover, LCR-CFBs showed lower expression of the main extracellular matrix regulatory genes including MMP2 (33%) and TIMP1 (16%) compared to HCR-CFBs. The results also indicated that MMP2/TIMP1 ratio was smaller in LCRs than HCRs. This finding provide evidence that lower intrinsic exercise capacity is accompanied by early LV remodeling, excessive cardiac collagen deposition, as well as imbalance between MMP2 and TIMP1 ratio, a key factor for the progression

of cardiac dysfunction. Smad5, is also one of the ECM regulatory proteins involved in the transforming growth factor beta signaling pathway that cause inhibition of the proliferation of hematopoietic progenitor cells (332). It is one of the BMPs downstream proteins known as R-Smads that able to inhibit TGF-β mediated fibrotic gene expression (333). Although not significant, our data showed that Smad5 gene expression were relatively lower in LCR-CFBs imposing more profibrotic feature to this phenotype after IR injury.

Regarding the receptors, BMPR2 is one of the main BMPs receptors that involved in many physiological and pathological conditions. It has been shown that BMPR2 mutations result in a disturbed BMP/TGF-β balance leads to an overactivated TGF-β signaling, which stimulates the fibrotic processes (316). Furthermore, BMPR2 expression has been reported to be reduced in the bleomycin model of lung fibrosis and pulmonary hypertension, compared with control animals (334). On another hand, Agtr1a, is one of the major two receptors of Ang II, the main powerful fibrotic factors which plays a key role in cardiac inflammation and fibrosis in chronic heart diseases (335). Consistent of what we have seen in ECM gene expression, our results revealed that baseline BMPR2 gene expression was lower in LCR-CFBs further suggesting a profibrotic phenotype. Moreover, lower BMPR2 mRNA expression in LCR-CFBs was consistent with the BMPR2 protein level as determined by immunocytochemistry stain. A similar phenotypic difference in BMPR2 expression also was seen in a previous work in our lab using a hind limb peripheral ischemia model. Pulmonary fibroblasts are known to express BMP2, BMP4, and BMP7. Further, it has been demonstrated that treatment of fetal lung fibroblasts with BMP4 could activates canonical and noncanonical BMPR2 pathways, which in turn, prevents proliferation, and increases expression of t alpha SMA (336). To our knowledge, we are the first in isolation, culturing and characterization of fibroblasts from IR hearts and showing the

expression of BMPR2. Although previous studies have revealed that BMP-7 can antagonize fibrosis in different tissues and organs, it is unidentified whether BMP-7 has similar impacts on myocardial fibrosis. However, one previous study has investigated the expression of BMP-7 in atrial fibrillation and the role of BMP-7 and TGF-β/Smads signaling in myocardial fibrosis. Their results indicated that BMP-7 can control TGF-β1/Smad3 by targeting Smad1/5 to antagonize fibrosis in cardiac fibroblasts as a result of atrial fibrillation (337).

Ang II-dependent cardiac fibrosis promotes collagen production, accumulation, and matrix remodeling through the up-regulation of Col I1 expression in the cardiac tissue (327,338). Inconsistent of results of gene expression that are consistent with each other to stigmatize LCRs as a profibrotic phenotype after IR injury, Agtr1a gene expression was higher in HCRs than LCRs. It is well known that expression level of the Agtr1a defines the biological efficacy of Ang II; hence, overexpression of the Agtr1a is one possible mechanism by which Ang II can contribute to cardiovascular disease (339). Therefore, the possible explanation is that detected gene expression of Agtr1a in our study was extremely low in both strains, LCR & HCR, and not enough to pick up the actual differences between the two investigated phenotypes. Another explanation is that Agtr1a might be expressed to play another role than to be profibrotic as the outcome of our results showed that HCRs seems to be antifibrotic phenotype. Supporting to this view, Ang II was recently found to activate the nuclear factor erythroid-2–related factor 2 in cardiac myocytes, a protein that enhances expression of several antioxidant genes (340).

Regarding the anti-apoptosis gene, BAG3, our results showed that LCR-CFBs have significantly higher mRNA expression of BAG3 than HCR-CFBs after IR injury. This result is consistent with a remodeling advantage for HCRs as myofibroblast apoptosis is essential for normal resolution of wound repair, including cardiac infarction repair. Moreover, impaired cardiac

myofibroblast apoptosis is associated with excessive extracellular matrix (ECM) deposition, which could be responsible for pathological cardiac fibrosis (341). Expressing higher level of BAG3 in LCRs will allow myofibroblast to live longer which ultimately elongate the fibrosis (adverse remodeling) and promote early heart failure. This view is even clearly supported from cell number data which revealed that LCRs have higher number of cells compared to HCRs counterparts post IR. Another evident is that alpha-SMA, the biomarker of myofibroblasts, was higher expressed in LCRs more than HCRs.

Taken collectively, our results strongly suggested that LCR exhibit a predisposition for LV fibrosis after acute IR which is in line with our overall hypothesis and the findings of previous proteomic and array studies using the same rodent model (342,343) and suggested that LCR may show a predisposition for LV remodeling in old animals, another type of stress. Another work has suggested that more mature LCR rats (~30 weeks of age) exhibit upregulated cardiac collagen deposition and increased myocardial fibrosis and diastolic dysfunction. Overweight female rats selectively breed for low aerobic capacity exhibit increased myocardial fibrosis and diastolic dysfunction (321). Following enzymatic isolation, cardiomyocytes from older LCR also tend to be shorter and wider than those from their HCR counterparts (343,344). Rebecca et al, demonstrated that the onset of cardiac fibrosis in the LCR phenotype occurs considerably earlier and is evident already by 12 weeks (345). Given that LCR rats are generally heavier in nature may explain also why they are more prone to cardiac fibrosis as clinical findings constantly demonstrated that cardiac fibrosis is strongly associated with obesity and contributes to cardiac dysfunction in obese women. Kosmala et al. demonstrated that the left ventricular dysfunction are caused by the changes in the MMP/TIMP system that might promote the attenuation of ECM degradation, mainly due to the downregulation of MMP-2 in obese women (346).

These results were not surprising to us as the dynamic (inducible) aerobic exercise has been proved to improve cardiac fibrosis and HCR rats have at least the intrinsic potential for increased physical activity. Kwak et al. investigated the alterations of collagen profile in response to exercise training and proved that physical training protected against age-related downregulation of active MMPs (347). Renáta et al demonstrated that a 12-week voluntary exercise significantly increased the MMP-2 activity, indicating the protective effects of exercise against collagen accumulation and fibrosis (324). MMP-2 degrades the ECM proteins which are responsible for cardiac remodeling. In addition, they also found that exercise training caused a reduction in collagen type I content and improved the MMP/TIMP profile resulting in protective effects against cardiac injuries (324). Experimental protocol with exercise training in both sham-operated and ovariectomized rats resulted in a significant increase in the MMP-2 activity and reduced the collagen deposition in the heart, suggesting an important protective approach to treat CVD (324). The mechanism associated with induced collagen turnover and cardioprotection is due to the MMP/TIMP profile and the result of the activation pathways of MMP.

Fibrosis is multifactorial, and the molecular mechanisms related to the regulation of ECM metabolism involve multiple signaling pathways. Interestingly, our results revealed that activating pathways for collagen I gene expression was distinct and seems to be phenotype dependent. Treating with Ang II induced gene expression for collagen I in LCR CFBs but not in HCR CFBs and was completely blocked with losartan. By Contrast, treating with BMP7 induced gene expression of collagen I in HCR fibroblasts but not LCRs, and was completely blocked by noggin. Treating the fibroblasts with a combination of receptor agonists confirmed the phenotypic selectivity of the inducible pathways. LCRs seem to follow the widely accepted pathway for collagen I expression, AngII. However, HCRs seems to have a unique pathway for collagen I

expression through BMPs. Our results suggest that HCRs prefer to use BMPs pathway and LCRs select to use AngII pathway which could answer the question of why HCRs are more antifibrotic and LCRs are more pro-fibrotic phenotype. There are increased evidences about antifibrotic impact of BMP7 in several tissue such as liver, lung, and kidney (348–350). Also, it is well known that AngII is strong mediator for myocardial fibrosis after cardiac injury (335).

Regarding Col III, LCRs-CFBs increased Col III expression when treated with BMP7 and was completely blocked with noggin. However, treating with combination of agonists, AngII and BMP7 reduced Col III mRNA expression suggesting negative effect of AngII on BMP7. Consistent with the literature, HCRs-CFBs improve Col III expression when treated with BMP7. Our results showed that both strains did not change gene expression of TIMP1 once treated with BMP7, AngII or both, with and without receptor antagonists which suggest that TIMP1 expression may use different way to be activated other than AngII or BMP7. TGF-b1 has been suggested to be an alternative pathway for TIMP1 gene expression (351).

Regarding the antifibrotic gene expression, our results indicated that MMP2, Smad5 gene expression were enhanced in LCRs-CFBs but not in HCRs-CFBs once treated with BMP7 but not AngII. Regarding receptor expression, our results revealed that BMPR 2 expression was induced in LCRs-CFBs but not in HCRs-CFBs once treated with either AngII or BMP7 and that expression was antagonized once treated by combination of the agonists suggesting reciprocal effects between BMP7 and AngII in LCR-CFBs. On other hand, Agtr1a (AT1R) expression was reduced in LCRs-CFBs but not HCRs-CFBs once treated with AngII and significantly increased once treated with BMP7.

These results together suggest that LCR-CFBs were trying to resist the imposed fibrosis by the injury via a reciprocal pathway through BMPR2 pathway in order to enhance anti-fibrotic

effect. As a baseline BMPR2 protein and gene expression was already high, HCRs appeared to have been physiologically primed and therefore did not need an adaptive compensatory response. Regarding the anti-apoptosis gene expression, BAG3 gene expression was decreased in HCRs-CFBs but not LCR-CFBs once treated with AngII but not BMP7 suggesting that AngII may play a key role in HCR-CFBs apoptosis.

CONCLUSION

It is widely accepted that inactivity or sedentary lifestyle can increase morbidity and mortality of CVD. Physical exercise has become a nonpharmacological therapeutic option in the prevention and treatment of CVD in both genders. However, not all individuals experience the same benefits from participating in exercise. It has been estimated that up to 70% of the variation in exercise capacity is due to the intrinsic genetic component. In this study, we investigated the impact of intrinsic (genetic) aerobic capacity (as represented by genetic models of intrinsic (i.e., untrained) low-capacity runners (LCR) and high-capacity runners (HCR)) on the early LV remodeling (as represented by fibroblasts response) after subjected to acute IR injury. Our finding indicates that the LCR phenotype is predisposed to expressing a more fibrotic character in fibroblasts harvested immediately following IR and may indicate poorer long-term outcome/earlier onset heart failure following acute ischemic injury to the heart. Moreover, the Angiotensin II receptor pathway is responsive only in post-ischemic LCR cardiac fibroblasts, and the BMP receptor pathway is responsive only in post-ischemic HCR cardiac fibroblasts which could shed new light on individuals who develop post-infraction fibrosis despite therapeutic dosing with Angiotensin pathway antagonists. HCRs appeared to have been physiologically primed and therefore did not need an adaptive compensatory response. Thus, our data clearly support a

cardioprotective effect of higher inborn aerobic capacity and that lower inborn aerobic capacity rats are more prone for cardiac fibrosis after ischemic reperfusion injury.

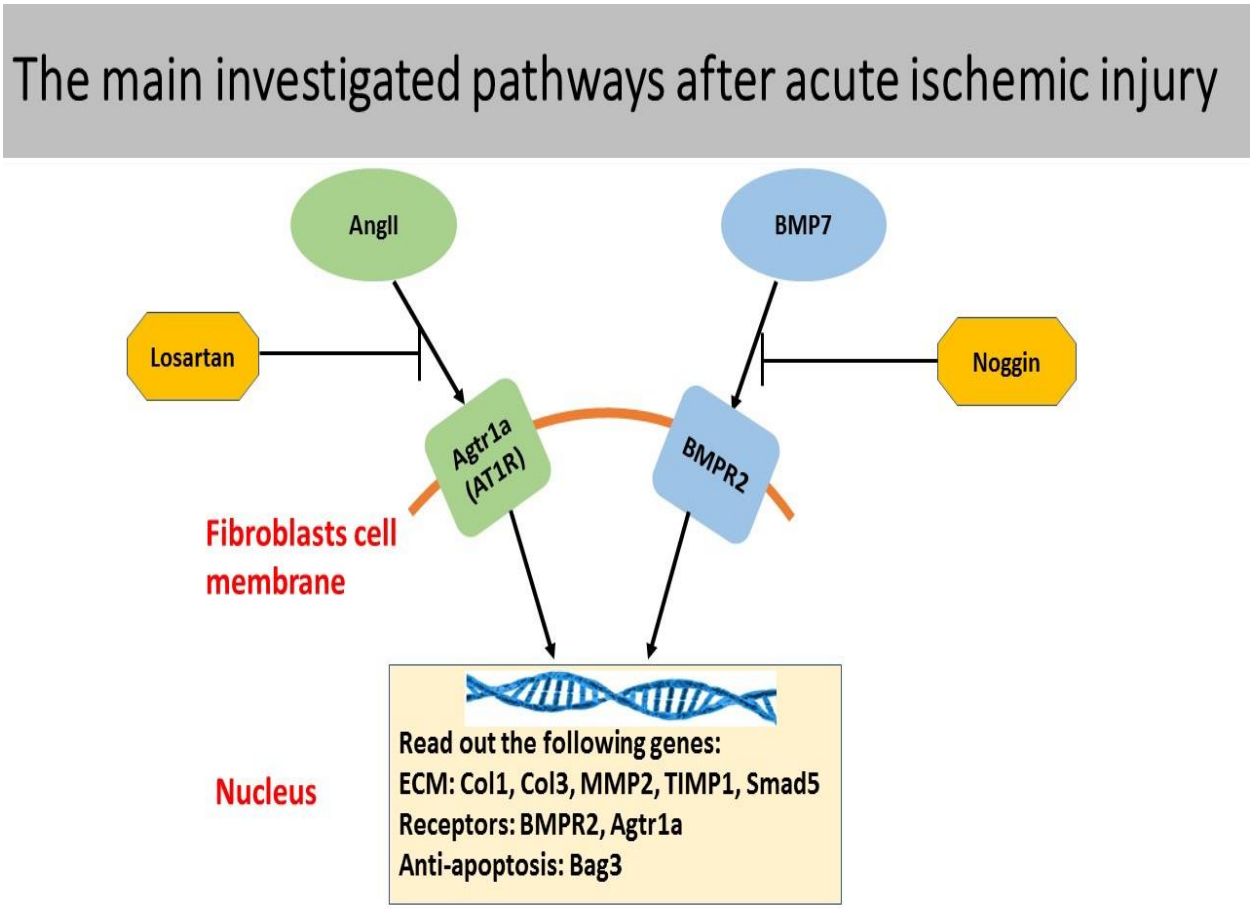


Figure 6.1 Schematic of the main investigated pathways after acute ischemic injury.
After acute IR injury, cardiac fibroblasts from HCR and LCR animal were treated with BMP7, Angiotensin or both, with and without receptor antagonists (BMP2R, noggin; Agtr1a, Losartan).

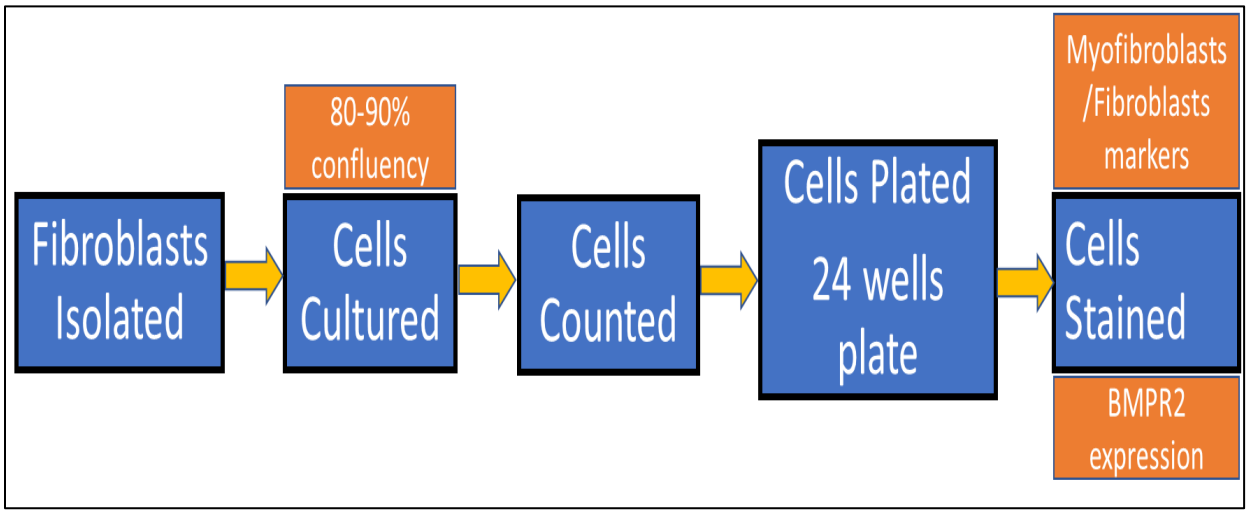


Figure 6.2. Cardiac fibroblast culture protocol for morphology purpose.

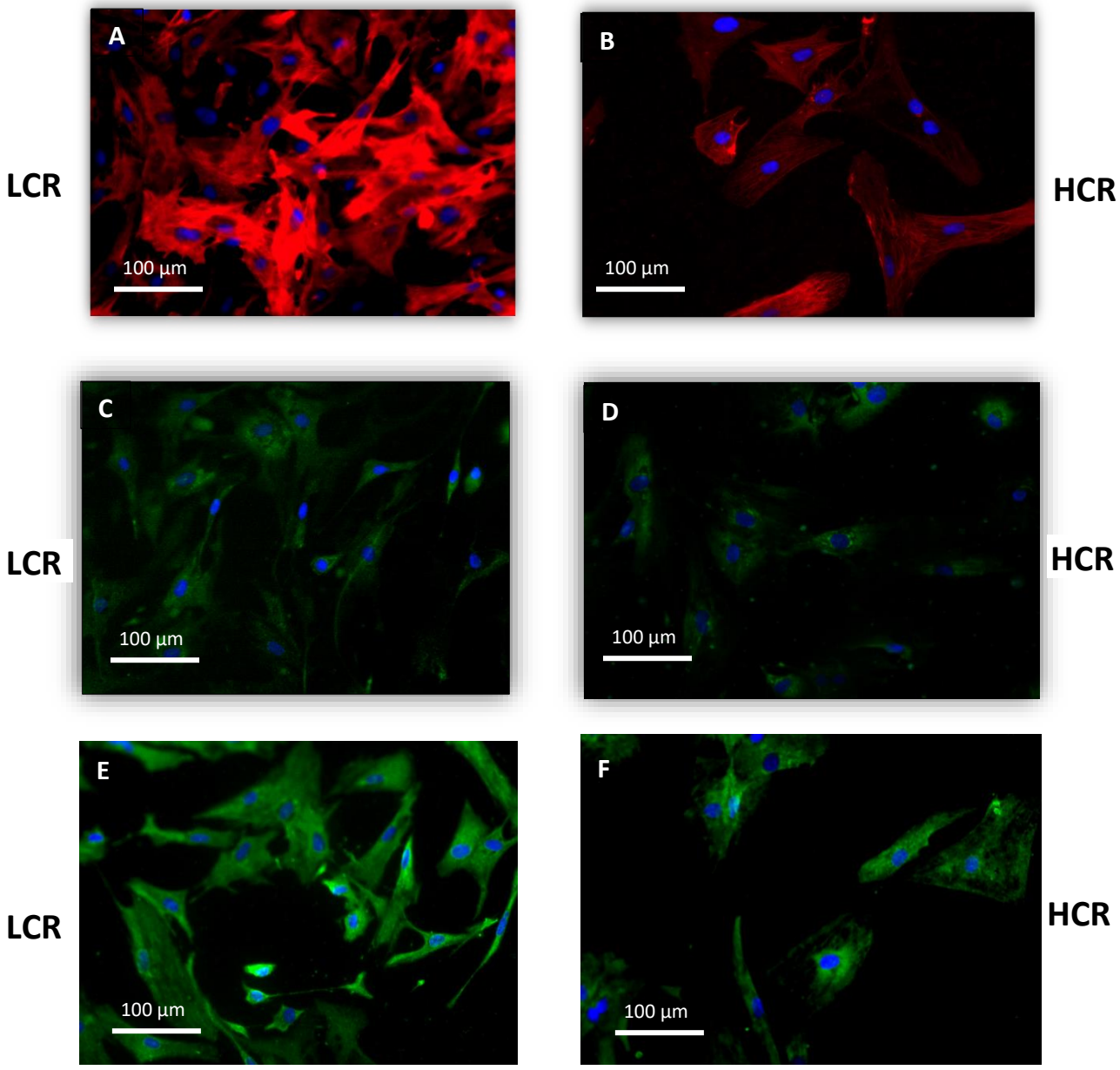


Figure 6.3. Immunocytoemistry stain for cardiac fibroblasts and myofibroblasts markers after acute IR injury. A, alpha SMA in LCR. B, alpha SMA in HCR. C, vimentin in LCR. D, Vimentin in HCR. E, DDR2 in LCR. F, DDR2 in LCR. HCR (n=8), LCR (n=8).

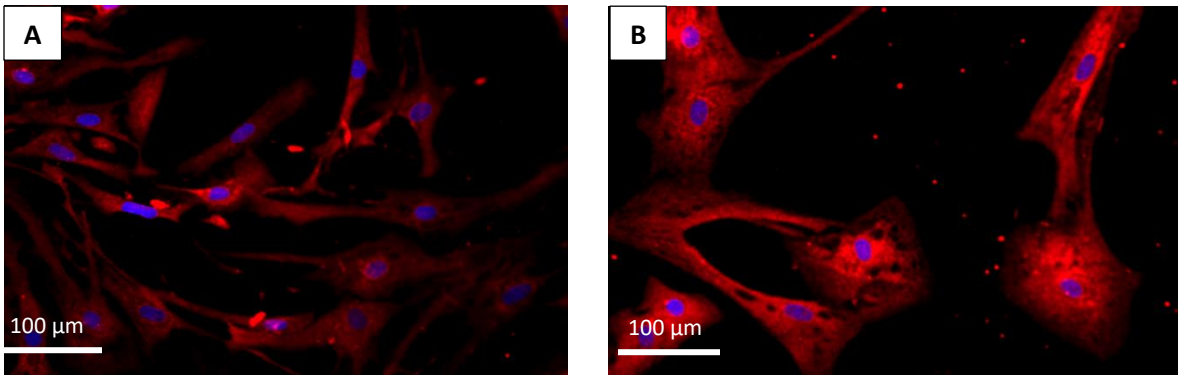


Figure 6.4. Immunocytochemistry stain for the expression of BMPR2 in the cardiac fibroblasts after acute IR injury. A, BMPR2 expression in LCRs. B, BMPR2 expression in HCRs. HCR (n=8), LCR (n=8).

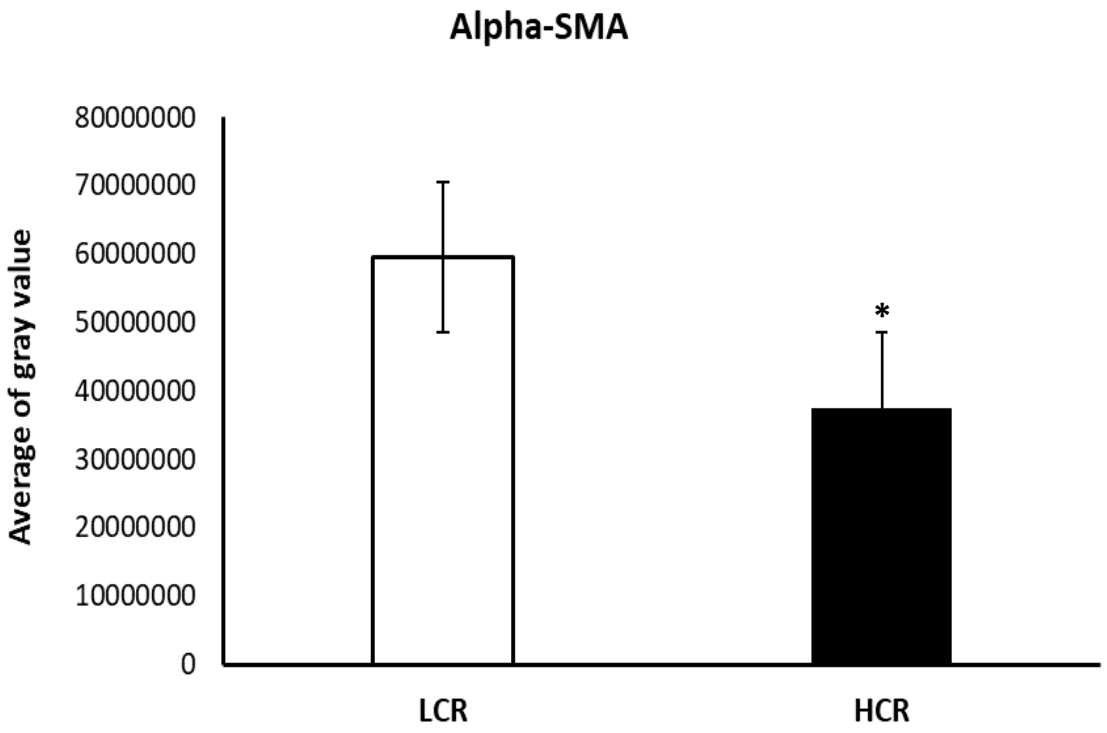


Figure 6.5. Comparison of alpha smooth muscle actin (αSMA) expression between HCR and LCR phenotypes after acute IR injury. α-SMA average area and intensity were measured using NIH imageJ software calibrated to transform the number of pixels (viewed on a computer monitor) into micrometers from an image of the immunocytochemistry stain. Single Paired t-test was used to analyze the data, * indicates $p < 0.05$. The error bars represent standard error. HCR (n=8), LCR (n=8).

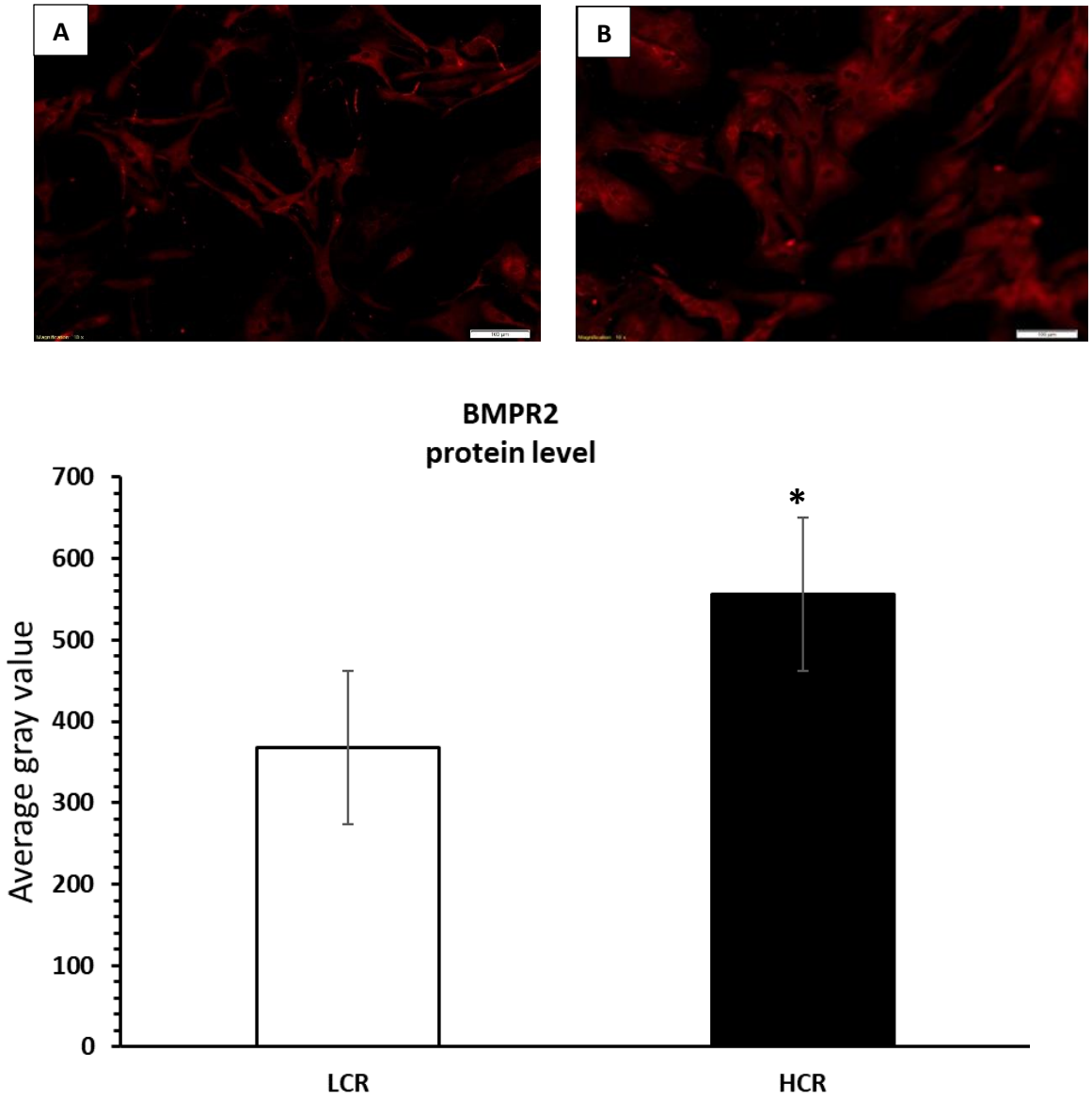


Figure 6.6. Comparison of BMPR2 protein expression in fibroblast between HCR and LCR phenotypes after acute IR injury. The upper images represent the immunocytochemistry images for BMPR2 expression. A, BMPR2 expression in LCR fibroblasts. B, BMPR2 expression in LCR fibroblasts. Lower graph represents the differences of BMPR2 expression between the two phenotypes. BMPR2 protein average area and intensity were measured using NIH imageJ software calibrated to transform the number of pixels (viewed on a computer monitor) into micrometers from an image of the immunocytochemistry stain. Single Paired t-test was used to analyze the data, * indicates $p < 0.05$. The error bars represent standard error. HCR (n=8), LCR (n=8).

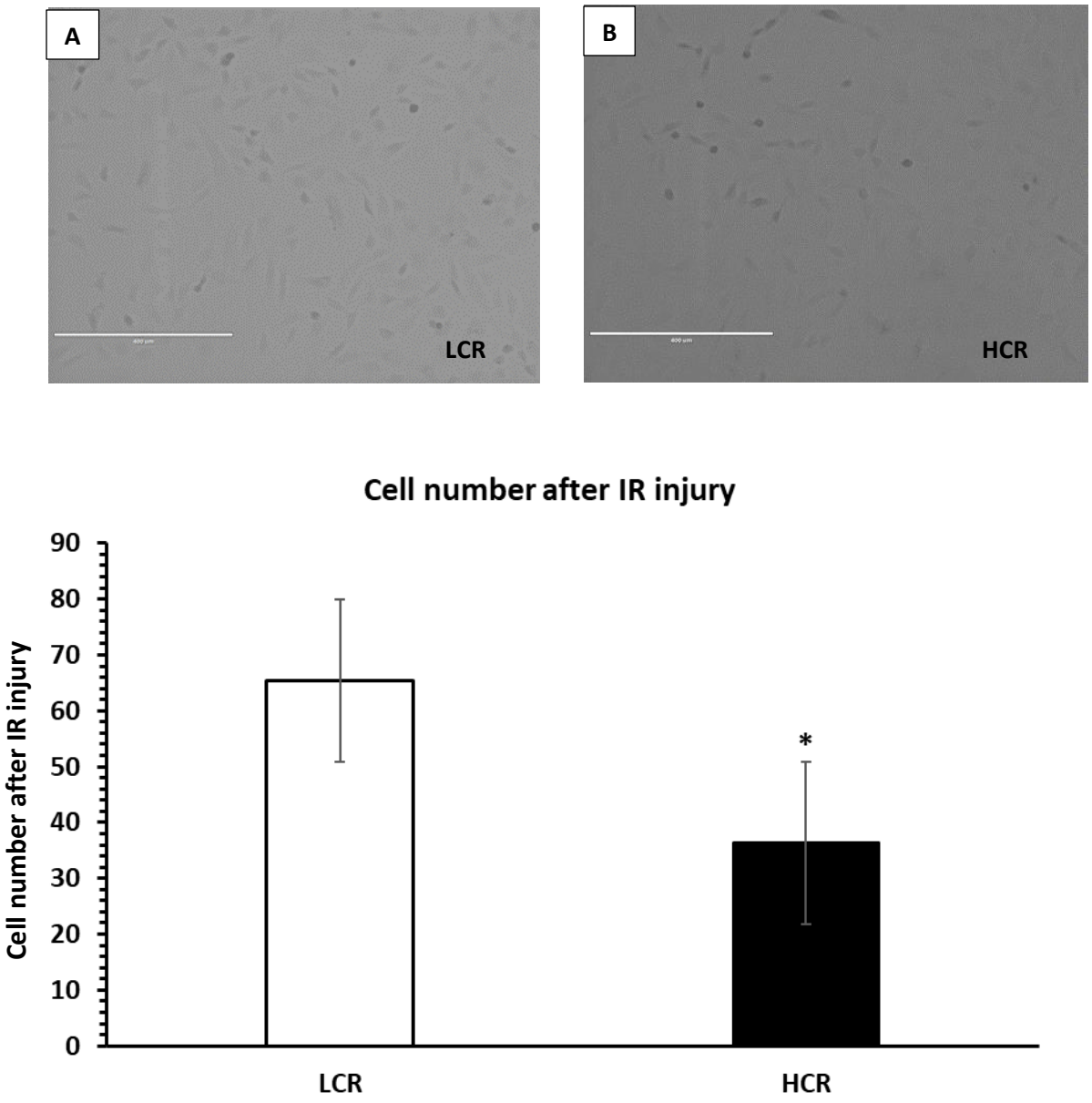


Figure 6.7. Comparison of cell number between HCR and LCR phenotypes after acute IR injury. The upper images represent the images of cell culture post 14 days of IR injury. A, cell culture from LCRs. B, cell culture from HCRs. Lower graph represents the differences of cell number between the two strains after acute IR injury. Cell number were counted using NIH imageJ software from an image of the immunocytochemistry stain in the same settings. Single Paired t-test was used to analyze the data, * indicates $p < 0.05$. The error bars represent standard error. HCR (n=8), LCR (n=8).

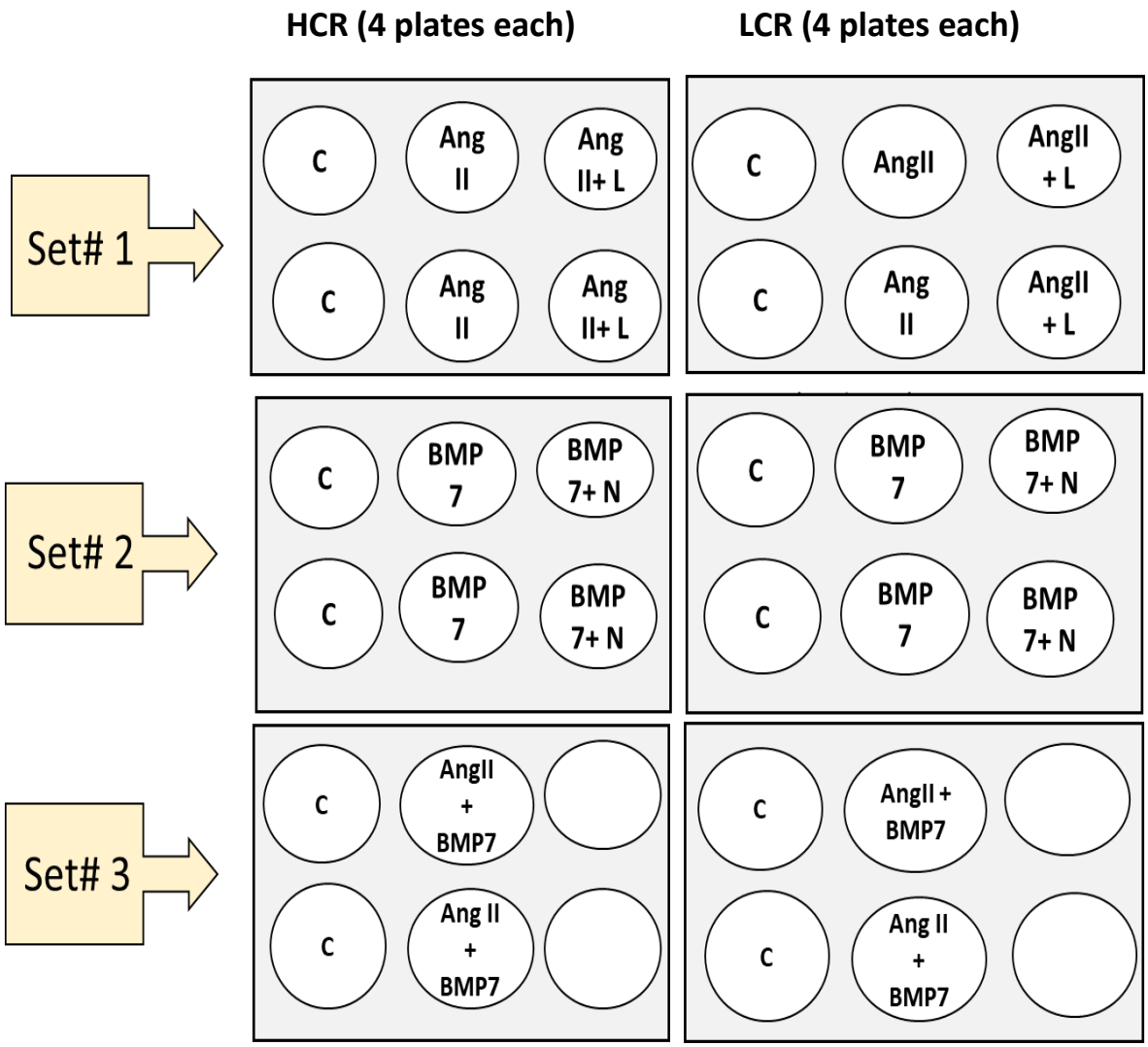


Figure 6.8. General plates set up for cardiac fibroblasts treatment. Set #1, Control, AngII, AngII + Losartan. Set#2, Control. BMP7, BMP7+Noggin. Set#3, Control, AngII+BMP7.

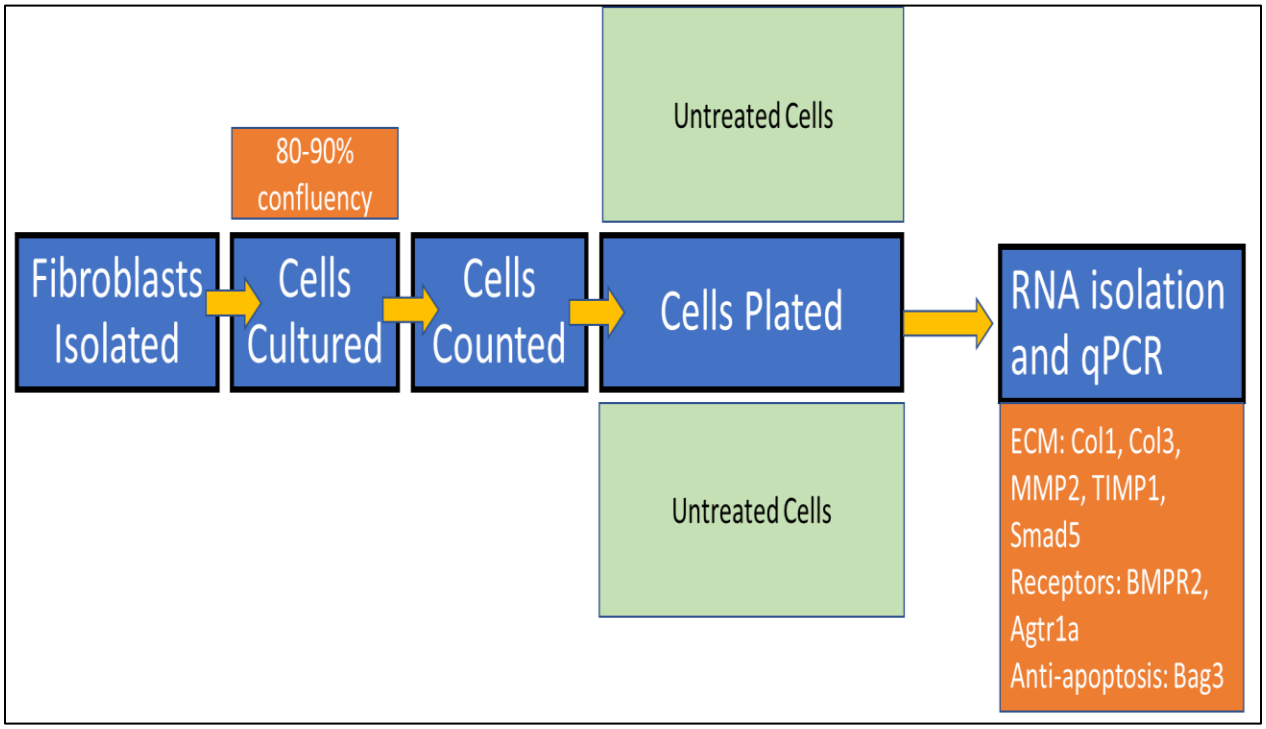


Figure 6.9. Cardiac fibroblast culture protocol for baseline gene expression purpose.

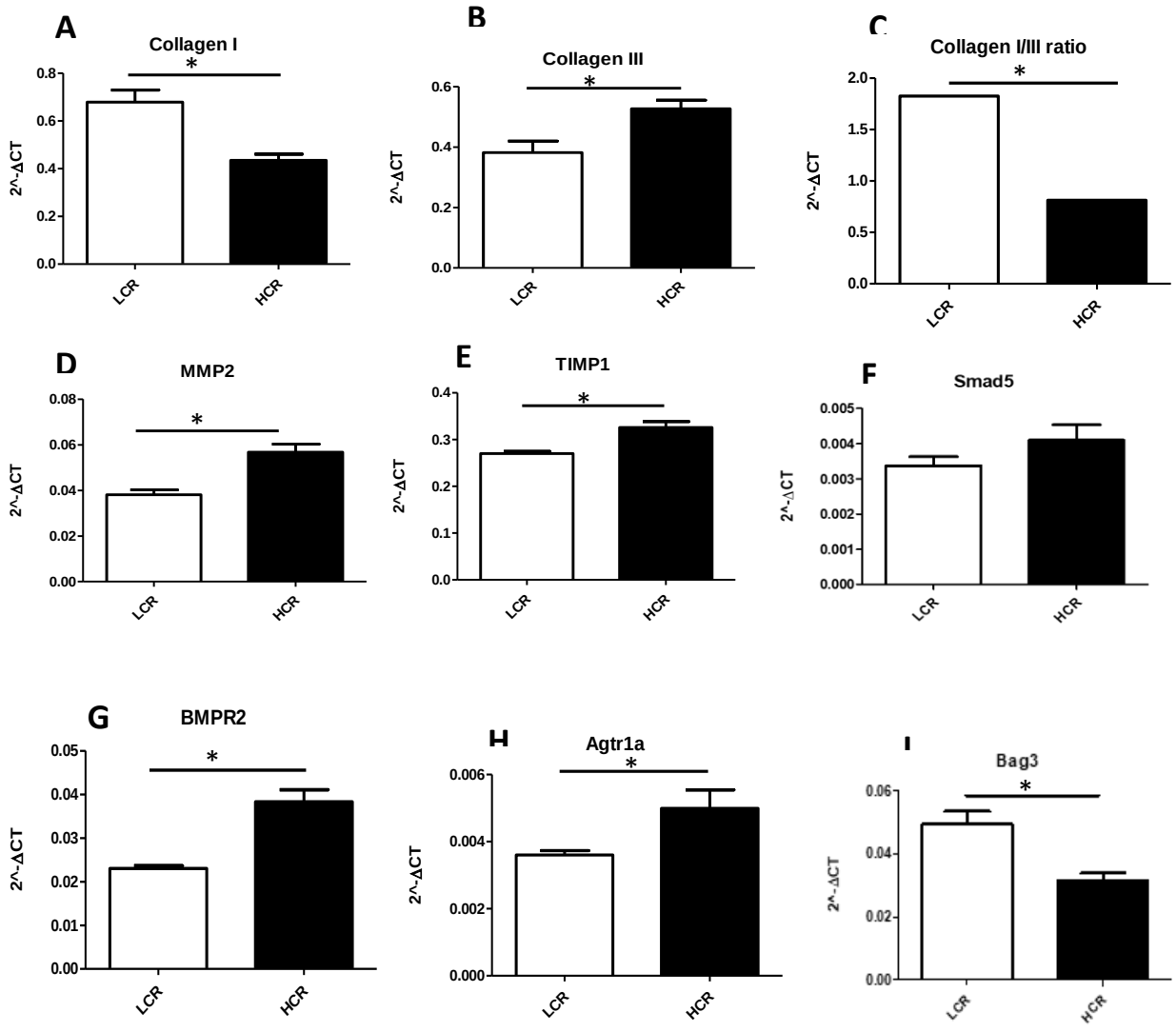


Figure 6.10. Comparison of the baseline gene expression between HCR and LCR phenotypes after acute IR injury. A, B, C, D, E, F represent ECM genes expression. G and H represent the gene expression of the receptors. I, represent anti-apoptosis gene expression. Single factor ANOVA test was used to analyze the data, * indicates $p < 0.05$. The error bars represent +/- SEM. HCR (n=8), LCR (n=8).

Table 6.1. Summary of basal gene expression in both HCR and LCR cardiac fibroblasts after IR.

Extracellular Matrix Regularity Genes	**Phenotypic differences**
Collagen I	LCR > HCR
Collagen III	HCR > LCR
Collagen I/III Ratio	LCR > HCR
MMP2	HCR > LCR
TIMP1	HCR > LCR
SMAD5	HCR = LCR (HCR relatively higher but not significant)
ECM Receptors	
BMPR2	HCR > LCR
Agtr1a (AT1R)	HCR > LCR (too small amount)
Anti-apoptosis Gene	
BAG3	LCR > HCR
Summary	After I/R injury, baseline gene expression in LCR fibroblasts shows more pro-fibrotic characteristics; while HCRs show more pro-apoptotic expression

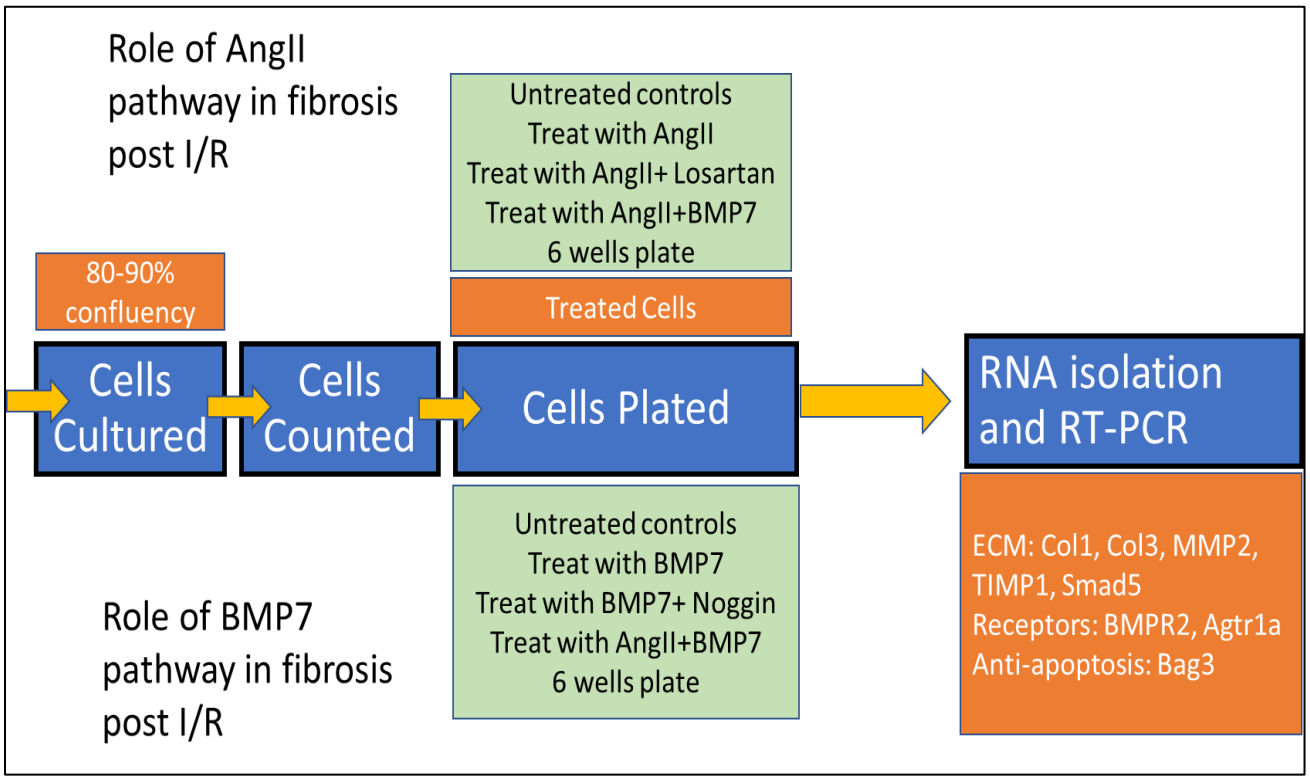


Figure 6.11. Cardiac fibroblast culture protocol for stimulated gene expression purpose.

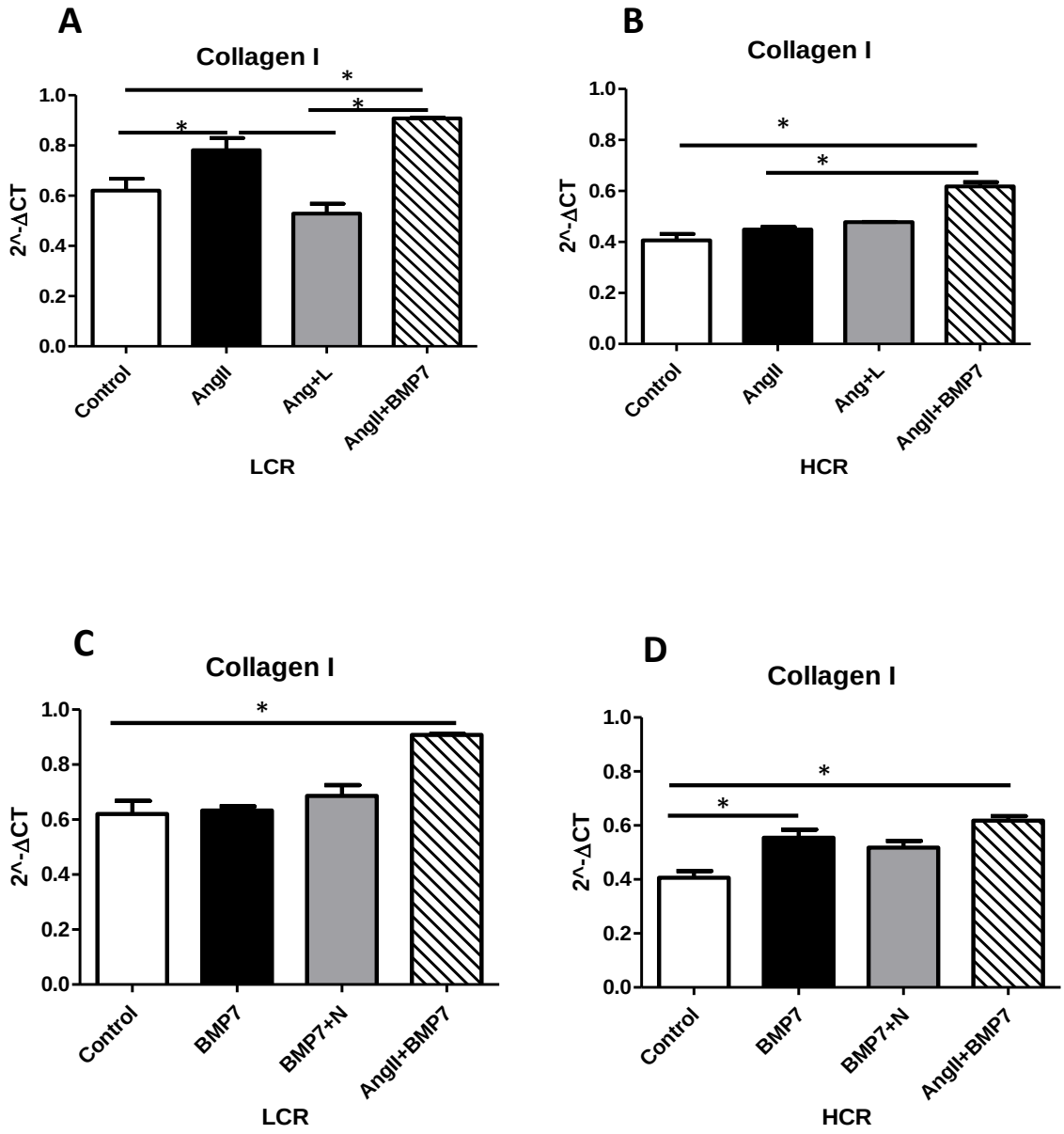


Figure 6.12. Comparison of inducible collagen I gene expression between HCR and LCR phenotypes after acute IR injury. A, represents gene expression in LCR-CFBs post-IR injury treated with AngII, AngII+L, or AngII+BMP7. B, A, represent gene expression in HCR-CFBs post-IR injury treated with AngII, AngII+Losartan, or AngII+BMP7. C, represents gene expression in LCR-CFBs post-IR injury treated with BMP7, BMP7+noggin, or AngII+BMP7. D, represents gene expression in HCR-CFBs post-IR injury treated with BMP7, BMP7+noggin, or AngII+BMP7. Single factor ANOVA test was used to analyze the data, * indicates $p < 0.05$. The error bars represent $+/-$ SEM. HCR (n=8), LCR (n=8).

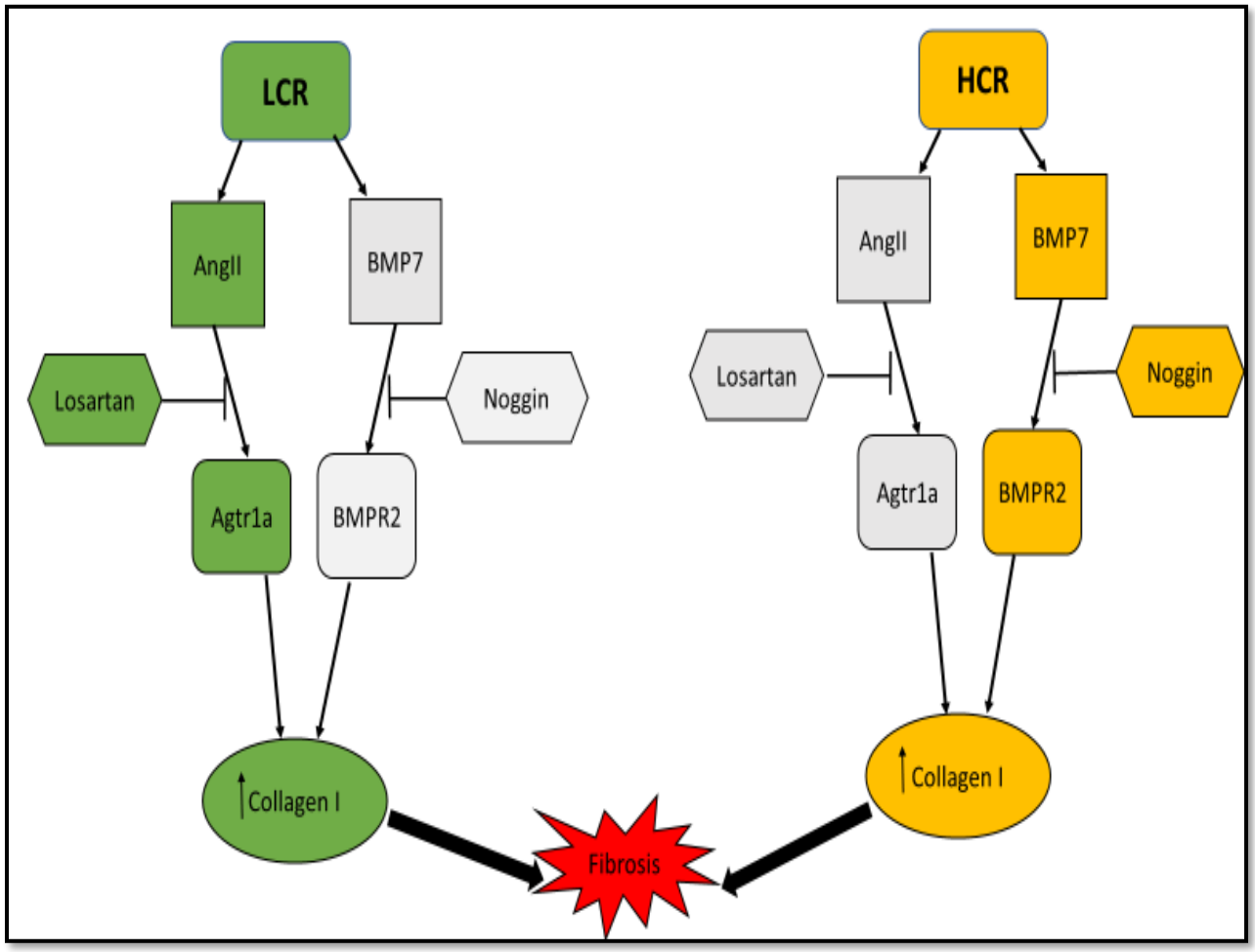


Figure 6.13. Distinct activating pathways for collagen I gene expression in LCR and HCR phenotypes in cardiac fibroblasts after IR injury.

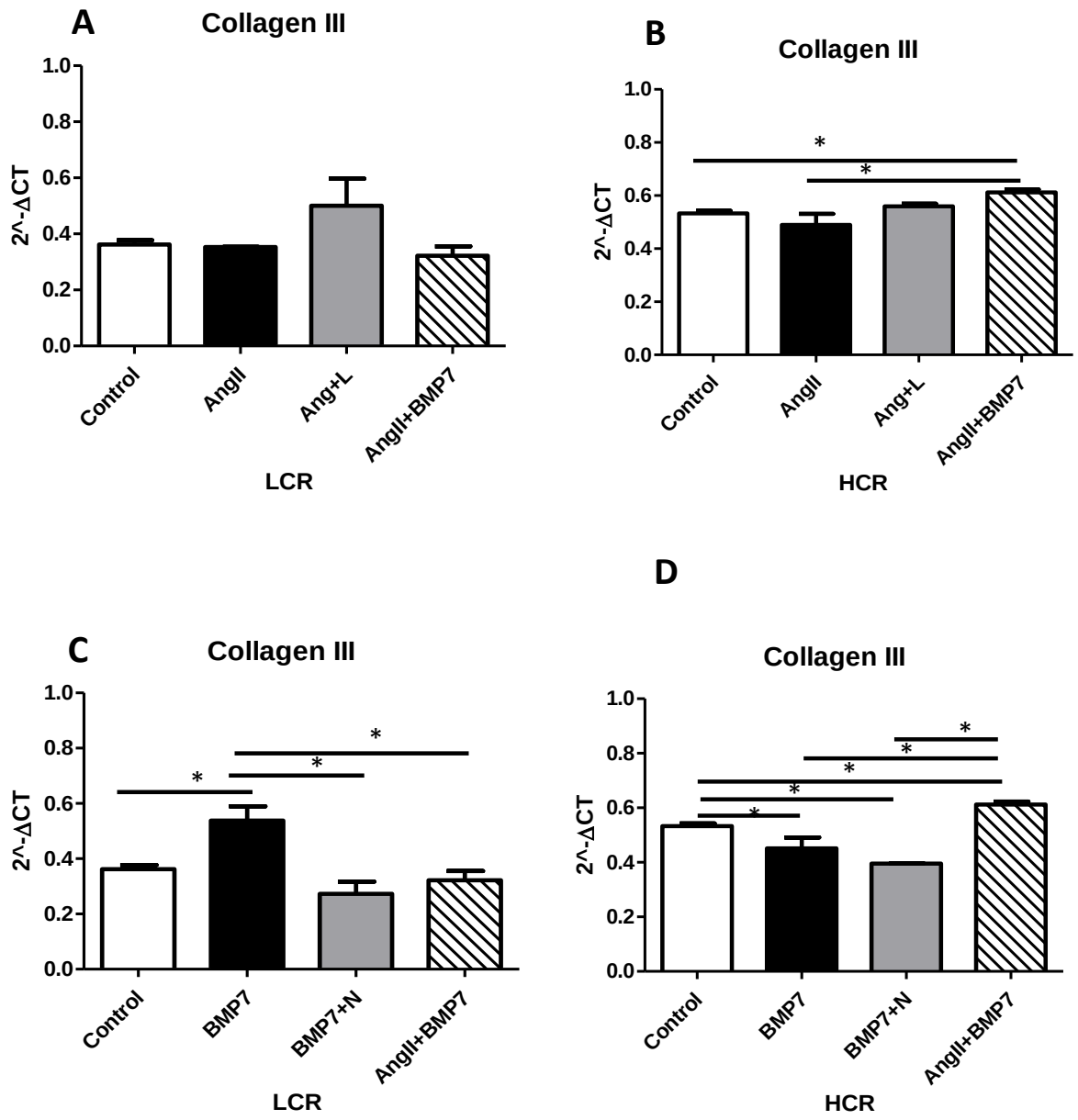


Figure 6.14. Comparison of inducible collagen III gene expression between HCR and LCR phenotypes after acute IR injury. A, represents gene expression in LCR-CFBs post-IR injury treated with AngII, AngII+L, or AngII+BMP7. B, A, represent gene expression in HCR-CFBs post-IR injury treated with AngII, AngII+Losartan, or AngII+BMP7. C, represents gene expression in LCR-CFBs post-IR injury treated with BMP7, BMP7+noggin, or AngII+BMP7. D, represents gene expression in HCR-CFBs post-IR injury treated with BMP7, BMP7+noggin, or AngII+BMP7. Single factor ANOVA test was used to analyze the data, * indicates $p < 0.05$. The error bars represent $+/-$ SEM. HCR (n=8), LCR (n=8).

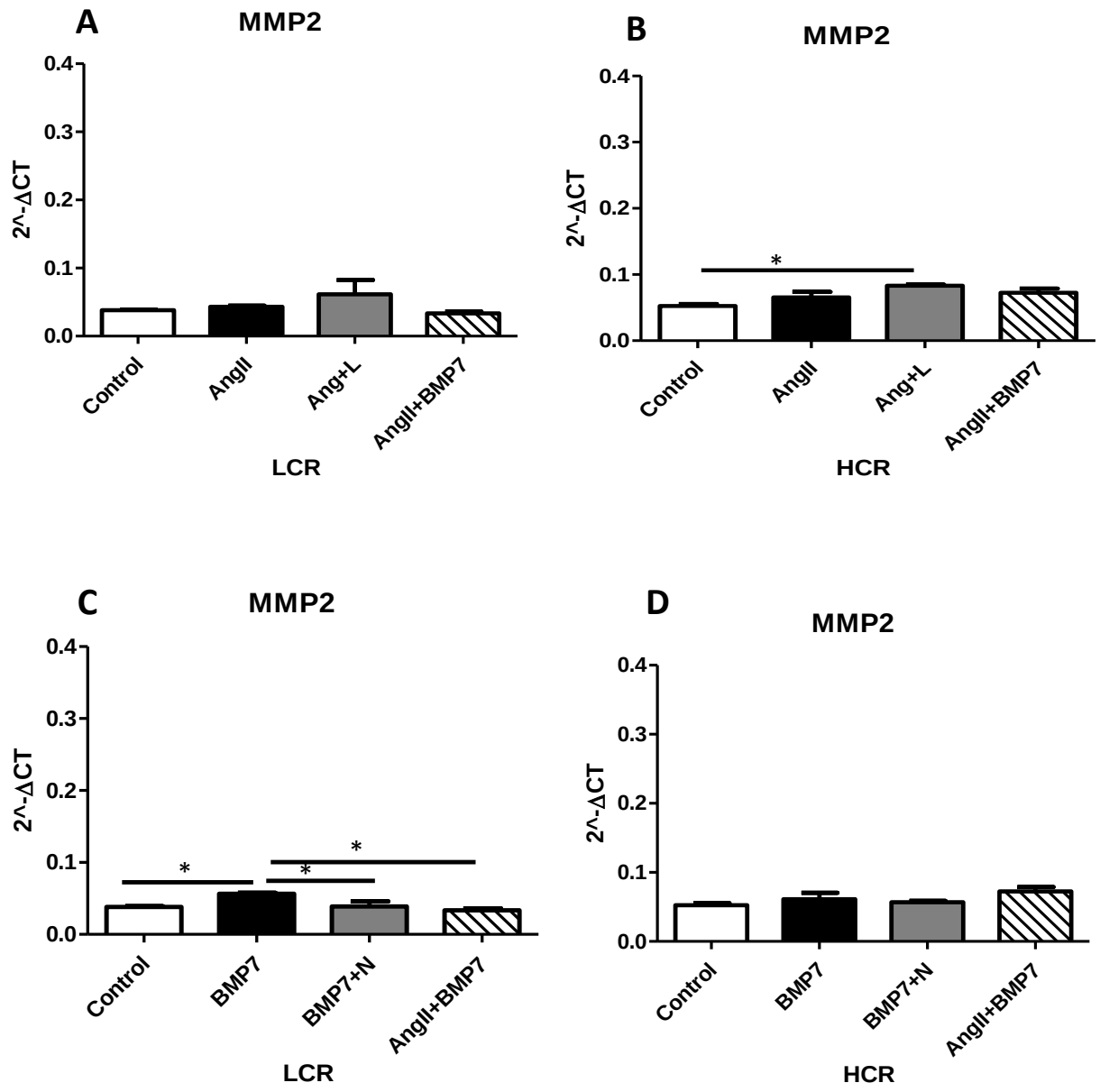


Figure 6.15. Comparison of inducible MMP2 gene expression between HCR and LCR phenotypes after acute IR injury. A, represents gene expression in LCR-CFBs post-IR injury treated with AngII, AngII+L, or AngII+BMP7. B, A, represent gene expression in HCR-CFBs post-IR injury treated with AngII, AngII+Losartan, or AngII+BMP7. C, represents gene expression in LCR-CFBs post-IR injury treated with BMP7, BMP7+noggin, or AngII+BMP7. D, represents gene expression in HCR-CFBs post-IR injury treated with BMP7, BMP7+noggin, or AngII+BMP7. Single factor ANOVA test was used to analyze the data, * indicates $p < 0.05$. The error bars represent $+/-$ SEM. HCR (n=8), LCR (n=8).

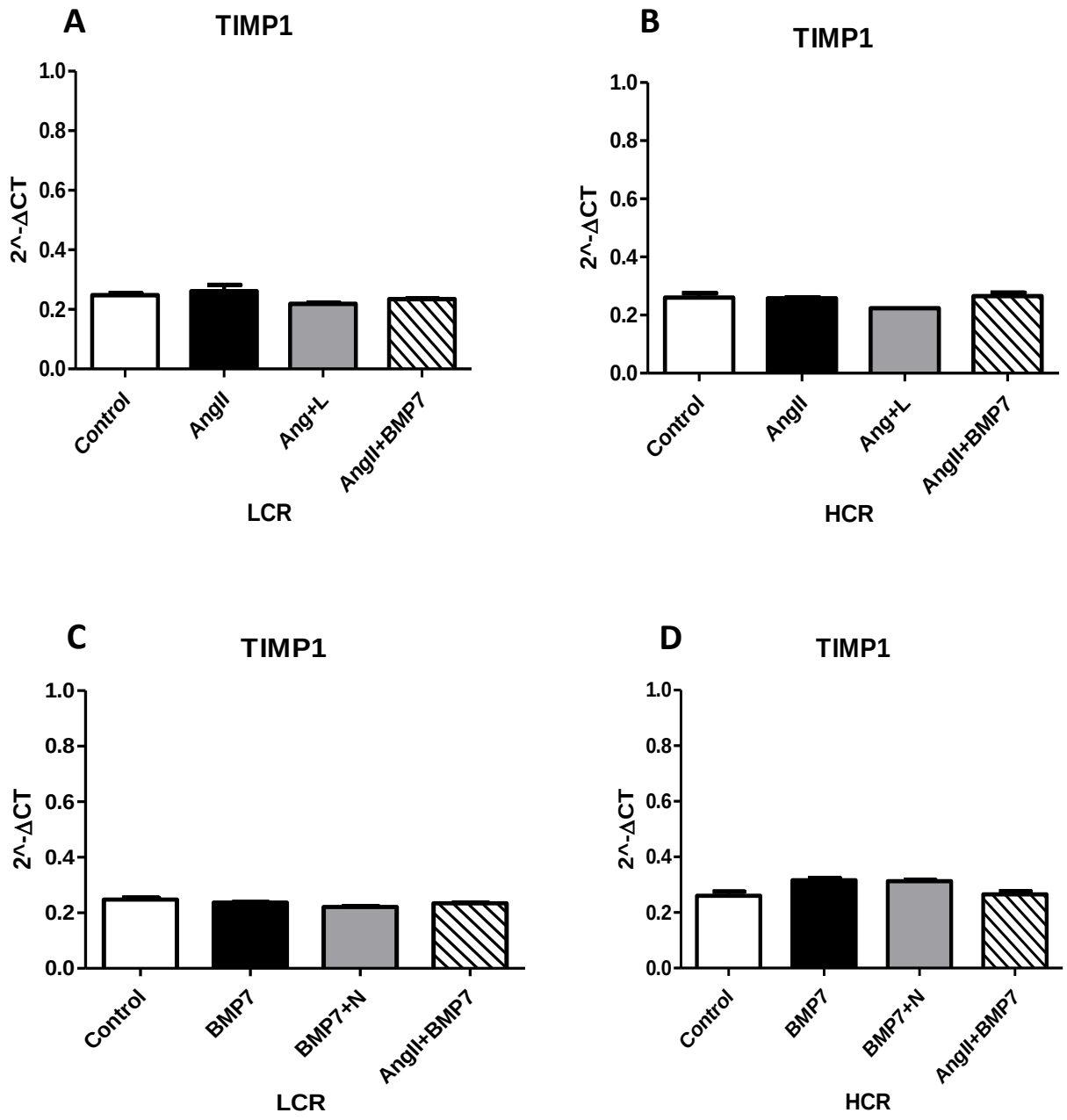


Figure 6.16. Comparison of inducible TIMP1 gene expression between HCR and LCR phenotypes after acute IR injury. A, represents gene expression in LCR-CFBs post-IR injury treated with AngII, AngII+L, or AngII+BMP7. B, A, represent gene expression in HCR-CFBs post-IR injury treated with AngII, AngII+Losartan, or AngII+BMP7. C, represents gene expression in LCR-CFBs post-IR injury treated with BMP7, BMP7+noggin, or AngII+BMP7. D, represents gene expression in HCR-CFBs post-IR injury treated with BMP7, BMP7+noggin, or AngII+BMP7. Single factor ANOVA test was used to analyze the data, * indicates $p < 0.05$. The error bars represent $+/-$ SEM. HCR (n=8), LCR (n=8).

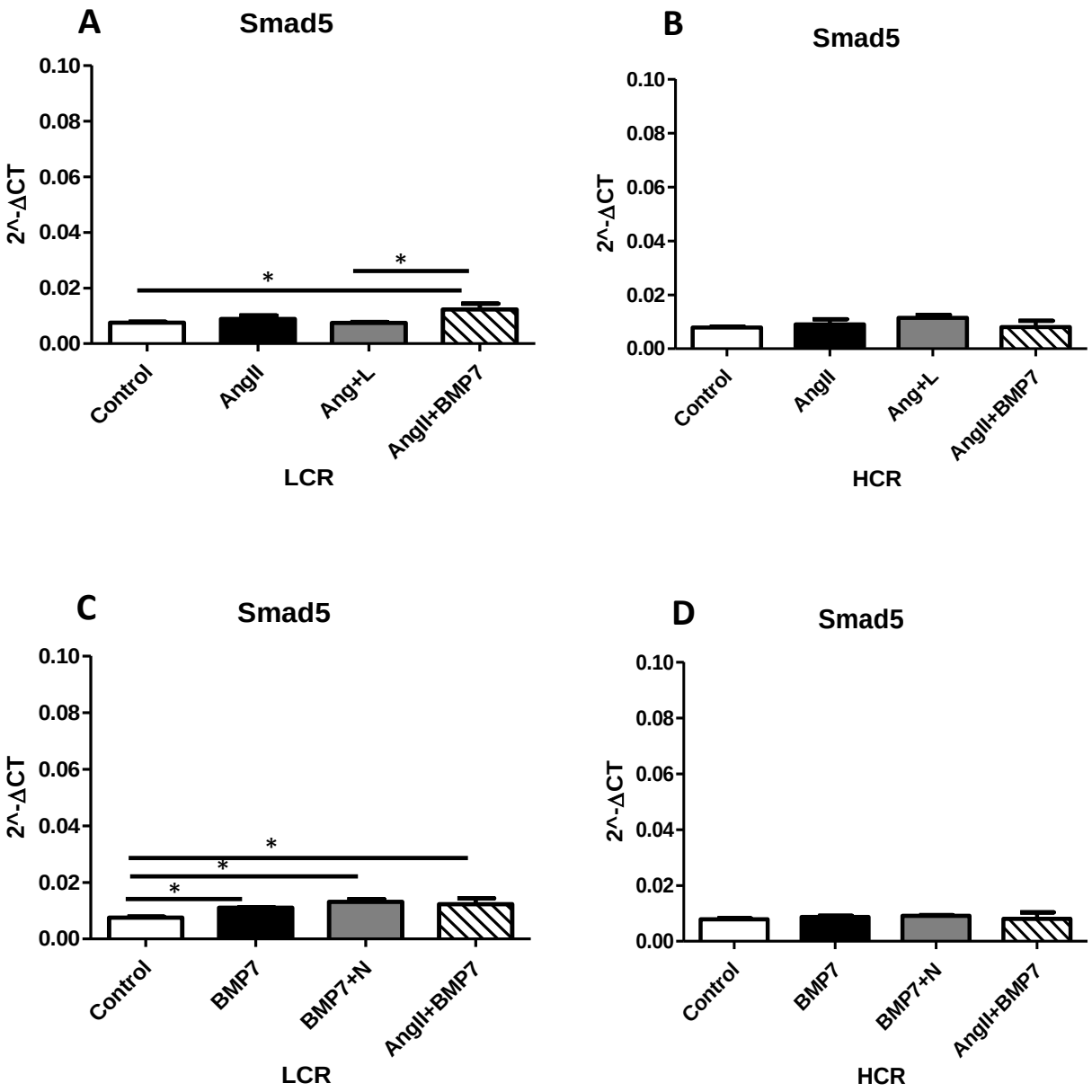


Figure 6.17. Comparison of inducible Smad5 gene expression between HCR and LCR phenotypes after acute IR injury. A, represents gene expression in LCR-CFBs post-IR injury treated with AngII, AngII+L, or AngII+BMP7. B, A, represent gene expression in HCR-CFBs post-IR injury treated with AngII, AngII+Losartan, or AngII+BMP7. C, represents gene expression in LCR-CFBs post-IR injury treated with BMP7, BMP7+noggin, or AngII+BMP7. D, represents gene expression in HCR-CFBs post-IR injury treated with BMP7, BMP7+noggin, or AngII+BMP7. Single factor ANOVA test was used to analyze the data, * indicates $p < 0.05$. The error bars represent $_{+/-}$ SEM. HCR (n=8), LCR (n=8).

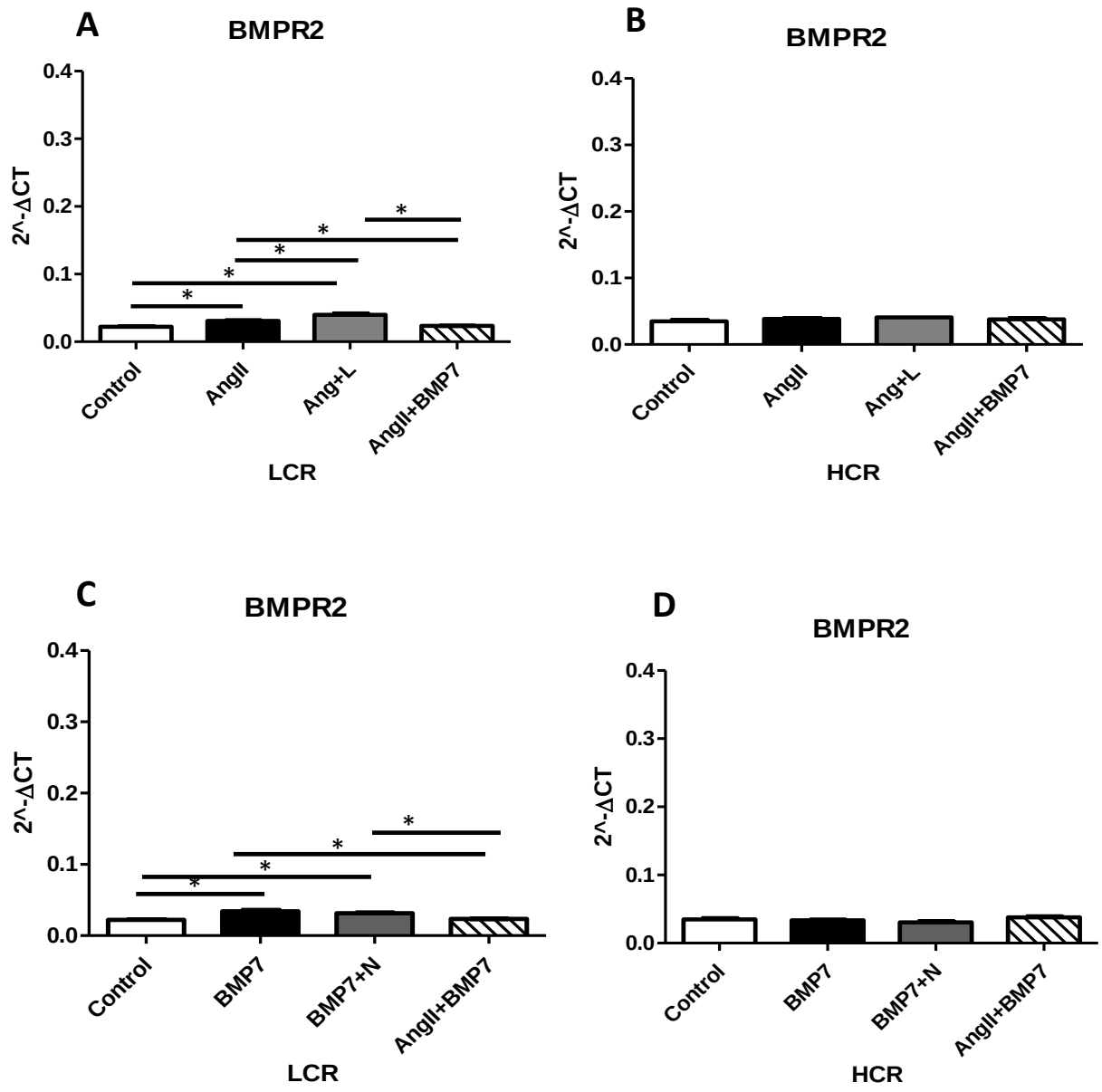


Figure 6.18. Comparison of inducible BMPR2 gene expression between HCR and LCR phenotypes after acute IR injury. A, represents gene expression in LCR-CFBs post-IR injury treated with AngII, AngII+L, or AngII+BMP7. B, A, represent gene expression in HCR-CFBs post-IR injury treated with AngII, AngII+Losartan, or AngII+BMP7. C, represents gene expression in LCR-CFBs post-IR injury treated with BMP7, BMP7+noggin, or AngII+BMP7. D, represents gene expression in HCR-CFBs post-IR injury treated with BMP7, BMP7+noggin, or AngII+BMP7. Single factor ANOVA test was used to analyze the data, * indicates $p < 0.05$. The error bars represent $+/-$ SEM. HCR (n=8), LCR (n=8).

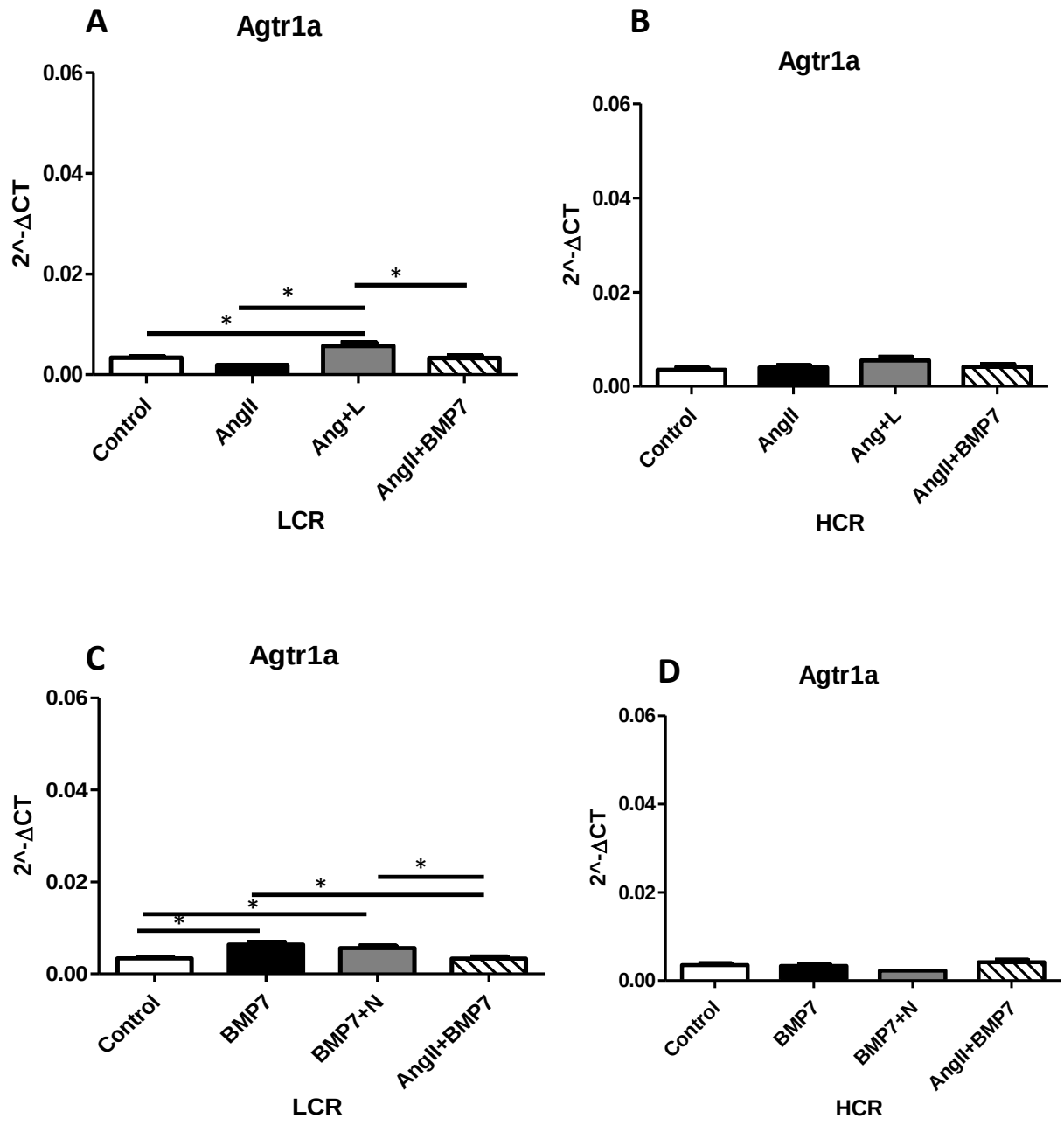


Figure 6.19. Comparison of inducible Agtr1a gene expression between HCR and LCR phenotypes after acute IR injury. A, represents gene expression in LCR-CFBs post-IR injury treated with AngII, AngII+L, or AngII+BMP7. B, A, represent gene expression in HCR-CFBs post-IR injury treated with AngII, AngII+Losartan, or AngII+BMP7. C, represents gene expression in LCR-CFBs post-IR injury treated with BMP7, BMP7+noggin, or AngII+BMP7. D, represents gene expression in HCR-CFBs post-IR injury treated with BMP7, BMP7+noggin, or AngII+BMP7. Single factor ANOVA test was used to analyze the data, * indicates $p < 0.05$. The error bars represent $+/-$ SEM. HCR (n=8), LCR (n=8).

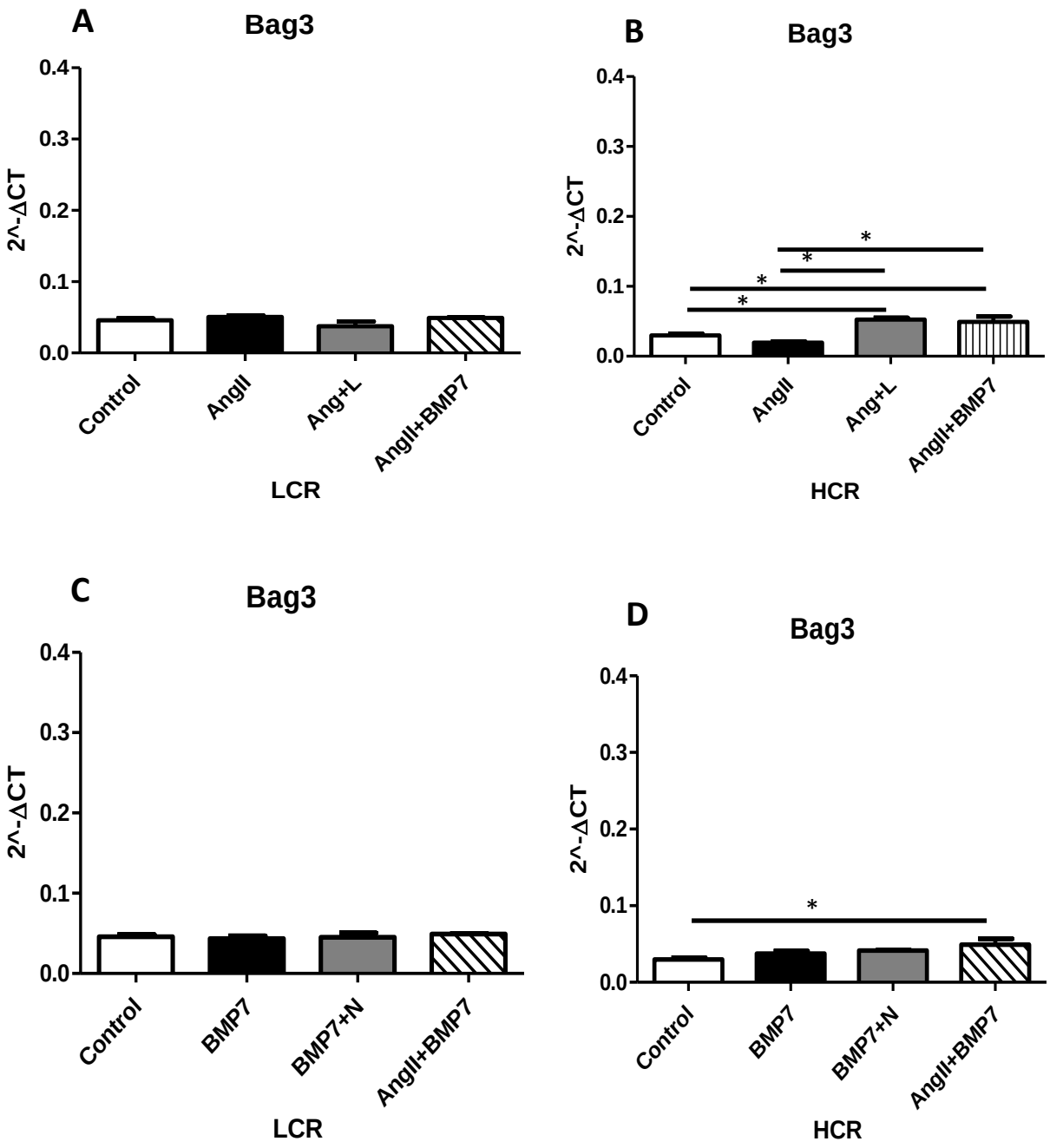


Figure 6.20. Comparison of inducible Bag3 gene expression between HCR and LCR phenotypes after acute IR injury. A, represents gene expression in LCR-CFBs post-IR injury treated with AngII, AngII+L, or AngII+BMP7. B, A, represent gene expression in HCR-CFBs post-IR injury treated with AngII, AngII+Losartan, or AngII+BMP7. C, represents gene expression in LCR-CFBs post-IR injury treated with BMP7, BMP7+noggin, or AngII+BMP7. D, represents gene expression in HCR-CFBs post-IR injury treated with BMP7, BMP7+noggin, or AngII+BMP7. Single factor ANOVA test was used to analyze the data, * indicates $p < 0.05$. The error bars represent $+/-$ SEM. HCR (n=8), LCR (n=8).

Table 6.2. Summary of stimulated gene expression in both HCR and LCR cardiac fibroblasts after IR.

Gene	LCR	HCR
Col I	↑ by Ang II	↑ By BMP7
Col III	↑ by BMP7	↓ By BMP7
MMP2	↑ By BMP7	Not changed
TIMP1	Not changed	Not changed
SMAD5	↑ By BMP7	Not changed
BMPR2	↑ by both angII and BMP7	Not changed
Agtr1a (AT1R)	↓ by AngII and ↑ by BMP7	Not changed
Bag3	Not change	↓ by angII
Summary	LCR fibroblasts have generally more responsiveness to stimulation. LCR fibroblasts are induced via the Ang II system in Col 1 gene expression	HCR cardiac fibroblasts are not stimulated via the Ang receptor, but Col1 is inducible via the BMP pathway HCR appears innately less prone to fibrotic stimulation

CHAPTER 7: DISCUSSION OF IMPACT OF INTRINCIC AEROBIC EXERCISE CAPACITY ON CARDIOVASCULAR OUTCOMES FOLLOWING VARIOUS FORM OF CARDIOVASCULAR STRSS

Cardiovascular disease (CVD) continues to be a leading cause of morbidity and mortality worldwide (268). In the united states, nearly half of all adults have at least one key risk factors for development of CVD (i.e., high blood pressure, high cholesterol, or smoking) (352). There are numerous risk factors causing the development and progression of CVD, but one of the most common is a sedentary lifestyle and physical inactivity (12). Indeed, exercise is recognized as one of the most important therapeutic treatment for patients who have cardiovascular diseases. However, some people respond well to exercise, while others respond poorly or have no response at all to exercise, even when controlling other factors such as age, sex, and ethnic origin. Exercise response in humans is determined mainly by intrinsic aerobic capacity (70%) and secondly by active exercise (10-40%) (56). This suggests the potential contribution of genetic predisposition or intrinsic aerobic in cardiovascular injury pathogenesis and remodeling. Therefore, studying the influence of intrinsic (inherent) aerobic exercise on cardiovascular responses following acute or chronic cardiovascular injuries is crucial for two reasons: first it helps in optimizing exercise to receive beneficial outcomes and therefore better structuring of therapeutic exercise programs to achieve intended outcomes in limiting consequences associated with cardiovascular disease risk factors.; and second, it may provide insight into of novel mechanisms in the development of some critical cardiovascular diseases in intrinsic aerobic phenotypes that can contribute to the pathophysiology of the disease process.

This thesis evaluated the potential impact of intrinsic aerobic exercise capacity on response to various form of cardiovascular stress including vascular reactivity to age and gender, cardiac

function and mitochondrial respiration to cardiotoxicity agent (Doxorubicin) and age, as well as cardiac myocyte, metabolic and fibroblast responses following different forms of acute ischemic-reperfusion injury. The data presented in this thesis is built on a strong body of knowledge that high intrinsic aerobic capacity running phenotype is a disease resistant while low intrinsic capacity running phenotype is a disease prone to several models and risk factors of metabolic and cardiovascular diseases. This includes factors such overweight, insulin resistance, high blood pressure (68), dyslipidemia (353–355), and previous unpublished work on peripheral occlusive disease. We hypothesized that the low aerobic endurance running capacity (LCR) phenotype will show more deterioration in vascular, metabolic, and cardiac adaptive responses following various forms of cardiovascular stress than the high aerobic endurance running capacity (HCR) phenotype. Herein we discovered that rats selectively bred for high endurance running capacity (HCR) and low endurance running capacity (LCR) have different responses to some cardiovascular stress. Most of current results also provide distinct elements supporting notion that the low endurance running capacity phenotype has a higher risk for developing cardiovascular diseases due to insufficient priming in the anatomical structure for adapting to any of tested cardiovascular insults. However, with some results, the story did not entirely straight forward “Disease Prone” LCR and “Disease Resistant” HCR characterization didn’t always hold up.

Given that age and sex are one of the most cardiovascular health modulators (356), and knowing that compared to the male HCRs, the male LCR rats have a 13% higher blood pressure, a significantly lower endothelial-dependent vascular relaxation as induced by acetylcholine, and an impaired regulation of oxidative pathways in mitochondria (68). Therefore, we started our thesis project by testing whether there are differences in vascular reactivity using peripheral thoracic aorta rings between the two phenotypes, HCR and LCR that are either age or sex dependent. For

this study, we obtained male and female HCR and LCR rats, 12 month vs 30 month, and by using organ bath chambers of myograph, the cumulative relaxation and contraction concentration-response curves were generated from thoracic aortic rings to the dilators; sodium nitroprusside (SNP), forskolin (FSK), HA1077 and acetylcholine (ACH), or constrictors; phenylephrine (PE), (5-HT), endothelin-1 (ET-1), norepinephrine (NE) and K^+.

Aged HCR and LCR rats showed significant gender differences for both the vasodilatation and vasoconstriction responses in the LCRs whereas phenotype difference predominantly present in males. The gender difference in vascular reactivity was maintained with the breading selection for running capacity. However, the differences in the aortic responses between male and female was more pronounced in LCRs than in the HCRs. The LCR males had the poorest aortic responses, while the females, in both strains, retained the best overall dynamic responses for vasoconstriction (such as ET-1) and vasodilation (such as ACH). This finding suggests that selection for higher aerobic capacity may provide protection within the vasculature with aging. Thus, these findings are strongly contributing to the characterization of the LCRs as "disease prone" and support the finding that state "HCRs generally show greater longevity in spontaneous aging comparing to the LCRs". These results, in turn, aroused our curiosity to test wither the innate HCR phenotypic "protection" against acute stress would be preserved with age or not.

In probing the effects of intrinsic aerobic capacity on cardiac protection against myocardial stress in aged rats, we obtained old female HCRs and LCRs, with age of 32 months, and treated them with Doxorubicin. Thus, we hypothesized that aged low aerobic endurance running capacity (LCR) rats will show more deterioration in metabolic and cardiac function after doxorubicin treatment when compared to the aged high aerobic endurance running capacity counterparts. We used Doxorubicin to induce acute cardiotoxicity in these animals for several reasons. It is one of

the most widely prescribed anticancer agents (136) and age is the greatest risk factor for developing cancer. Moreover, Dox-induced cardiotoxicity has been well recognized and vary from vasodilation, and hypotension to left ventricular dysfunction and congestive heart failure (137). Furthermore, patients with cardiomyopathy due to Dox-induced toxicity have an especially poor prognosis, as their survival chances are worse than patients with ischemic cardiomyopathy (138). We were interesting to investigate the phenotypic differences in cardiac function and mitochondrial respiration because the drug seems to be retained in cardiomyocytes more than in other cell types (140) and that the chemical structure of doxorubicin is prone to the generation of free radicals, leading to an increase in toxic reactive oxygen species (ROS) produced by the mitochondria, which trigger DNA damage and induces intrinsic mitochondria-dependent apoptotic pathways in cardiomyocytes (151).

The control animals that were not treated with doxorubicin did not perform significantly different in either test which was a somewhat surprising to us. Increased cardiac performance has been observed in young adults, suggesting perhaps that some of the cardiac associated aspects of intrinsic aerobic capacity are not durable throughout age. A possible explanation to the demise of this benefit is that oxidative damage to the mitochondrial DNA and electron transport chain accumulates with age (173,174). HCRs decreased EF (lost contractility) and increased HR (Reflex tachycardia) 10 days after Dox. In this study, LCRs treated with doxorubicin showed higher mitochondrial respiration, especially in the glutamate-based protocol. Also, the LCR animals responded by increasing their cardiac volumes but failed in increasing their cardiac output, while the HCRs increased their heart rate and cardiac output in the absent of exercise training. When compared to the control responses, doxorubicin increased mitochondrial respiratory capacity 2-5-fold in LCRs in the glutamate protocol, however, HCR isolates did not increase respiratory

capacity in response doxorubicin treatment in any substrate protocol. The explanation of these results is more likely because of their genetic endowment, the HCR rats have less need to adapt to the doxorubicin injury than the LCR rats. The HCRs may have been intrinsically prepared to handle the doxorubicin insult. Thus, HCRs treated with Dox did not show any increasing in the mitochondrial respiration because the insult was not as impactful for this phenotype in comparison to the LCRs. So, the increasing in LCRs mitochondrial respiration in LCRs after Dox treatment seems to be not efficient but choose this adaptive response in trying to compensate the reducing in mitochondrial content. Our results indicate that animals with low inherent aerobic capacity were susceptible to doxorubicin insult as evidenced by an adaptive mitochondrial response, while the high aerobic capacity animals appeared to have been physiologically primed and therefore did not exhibit an adaptive compensatory response. It also consistent with previous finding which suggested that the LCR phenotype scored high on cardiovascular risk factors and the HCR score high for health factors. Moreover, it provides evidence that the inherent HCR phenotypic "protection" against acute stress are preserved with age. The studies in chapter 2 and 3 has differentiated the baseline vascular reactivity (male vs. female) and cardiac and mitochondrial functions (female only) following Dox-induced cardiotoxity in aged HCR and LCR phenotype. So, we use aging as a risk factor for cardiovascular function decline.

As almost half of American adults have at least one key risk factor for development of CVD (352) and given that coronary artery disease is currently the leading cause of mortality and morbidity in the western world, we sensed the importance of studying the effect of inherent aerobic exercise capacity on cardiac response to different forms of myocardial ischemic reperfusion.

As this model has demonstrated consistently difference in adaptive responses to acute peripheral artery ligation, little is known about whether the same differences will be still exists

with the same model in cardiac tissue. For that reason, we have become curious to investigate the influences of intrinsic aerobic exercise on the myocardial response after acute ischemic reperfusion injury (AIRI) and weather the gender differences will be preserved in the two phenotypes. To test this hypothesis, female and male HCR and LCR rats, 16-18 months of age, from generation 17 were used and myocardial AIRI was induced by either 30 or 15-min ligation of the left anterior descending coronary artery, followed by 2 hr of reperfusion.

Our results show that with 30 min ligation, Myocardial infarct was not altered by phenotype in this model of endurance exercise capacity, but female rats in both the HCR and LCR groups exhibited smaller infarct size compared to their male counterparts. Moreover, the protein expression of the reperfusion injury salvage kinase (RISK) did not significantly different between the two phenotypes. Actually, all phenotypes demonstrated the ability to increases total AKT, PKCE and Xanthine Oxidase (RISK pathway enzymes) levels and the increase in these RISK pathway enzymes did not differ between the phenotypes. Consistent with these results, histology results indicated that myocardial neutrophil infiltrate following AIRI increased in all groups and there was no differ with infiltrate according to phenotype or gender. These results together indicate that selection for higher insurance capacity does not confer cardiac protection against this situation of injury and, therefore, don't support the central hypothesis of this project. However, take in consideration that 30 min of ligation is appropriate time to test the therapeutic effect of developed drugs for this condition but not ideal to mimic the real case of ischemic reperfusion injury, we used a shorter time of ligation (15 min). with 15 min ligation, LCRs heart are more vulnerable to AIRI as indicated by the larger infarct size compared to HCRs. Collecting together, it become clear to us that the protection of high inherent aerobic capacity against AIRI seems to be time-dependent protection and the severity of the injury determines the limit of protection.

Gender has been recognized as an important factor in determining the risk for cardiovascular diseases (73) and cardioprotective effects of sex hormones have been reported in both experimental and clinical studies (111). Sex based differences are present which indicates phenotypic selection based on aerobic capacity did not alter sex-based patterns of response to AIRI. This result is also in consistent with our results in chapter 2 as sex differences were maintained in both phenotypes. The LCR males had the poorest aortic responses, while the females, in both strains, retained the best overall dynamic responses. Therefore, these differences seem to be genetic differences and not sex-dependent differences.

Some of the most interesting findings in this overall project resulted from our study of the impact of intrinsic aerobic capacity on the mitochondrial (discussed in chapter 5) and fibroblasts (discussed in chapter 6) adaptive responses after acute ischemic reperfusion (IR) injury. A consistent characteristic of the LCR is reduced metabolic capacity in several tissues (68,271,272), but metabolic capacity in cardiac tissue is not well studied in these phenotypes, particularly following the metabolic stress of ischemia and reperfusion. Hence, we were interested in understanding the differential changes in mitochondrial adaptive response pre and post cardiac ischemic reperfusion injury between HCRs and LCRs. Our hypothesis is that LCR rat's mitochondrial adaptive response will be more deteriorated by IR injury than HCRs. To test this hypothesis, we obtained 30 HCR and LCR, female rats, 40 weeks, generation 32. Our model utilizes cold cardioplegia as global ischemic arrest (80 min) and warm reperfusion (120 min) using a Langendorff style perfusion system. We thoroughly investigated mitochondria because mitochondrial dysfunction is considered to be an early event in the cardiovascular injury (357).

The baseline assessment of cardiac mitochondria respiratory rates indicates that HCR mitochondria show respiratory rates 32% higher at rest and more than 40% higher under

maximally stimulated conditions, compared to LCR mitochondria. Comparing these results with the results obtained from chapter 3 which show that the basal and maximal oxygen consumption rates were not different between aged LCR and HCR phenotypes supporting our view that some of the cardiac associated aspects of intrinsic aerobic capacity are not durable throughout age. Although HCR may provide better baseline respiratory rate than LCR phenotype, the phenotype advantage was lost after this IR insult. Furthermore, we observed no phenotypic differences in overall infarct size, calcium retention capacity, or citrate synthase activity after IR injury. One possibility is that the stress was greater than any protection the phenotype might have afforded. Once a certain threshold is passed, the HCRs actually show proportionally greater loss of respiratory capacity compared to LCRs suggesting that HCRs are more vulnerable to the acute IR insult, in the absent of oxygen. Together, these data could suggest limits in the extent to which the HCR phenotype might be "protective" against acute tissue stressors. Our results from chapter 4 may support this view as shorter time of coronary artery ligation showed phenotypic differences in infarct size while with longer time ligation, the differences have disappeared. Thus, our data do not clearly support a cardioprotective effect of higher inborn aerobic capacity. If there is an effect, it can be overwhelmed.

Desiring for further investigation about the impact of inherent aerobic capacity on the cardiac remodeling after acute I/R injury (or at least the prediction about long-term outcome post-IR injury), 16 HCR and LCR, female rats, 40 weeks, generation 32, were obtained and acute IR were done utilizing the same model we mentioned in chapter 5. Then, cardiac fibroblasts from the LV of HCR and LCR rats were isolated, cultured and characterized (chapter 6). RNA was isolated from the cultured fibroblasts, and RT-PCR was used to determine baseline and inducible gene expression for Collagen I and III, MMP2, TIMP1, BMPR2, ATR1, SMAD5, and Bag3 in each

phenotype. We were interested to study fibroblasts response because cardiac fibroblasts play a critical role in healing post myocardial infarction, as they are differentiate into myofibroblasts which, in turn, play a key role in cardiac remodeling by forming a collagen-rich scar that allows the infarcted area to preserve structural integrity after cardiomyocyte death (299).

In vitro study showed that intrinsic aerobic capacity is an important factor in regulating fibroblasts response after IR injury. In these post IR fibroblast/myofibroblasts, LCRs showed significantly higher gene expression in the collagen I/collagen III ratio. Thus, LCRs could be more vulnerable to the cardiac rigidity and stuffiness which lead to impaired cardiac filing and accelerate heart failure (327). Moreover, LCRs had lower extracellular matrix regulatory genes including MMP2, TIMP1, and SMAD5 compared to HCRs counterparts. BMPR2, Agtr1a, also were both decreased, and the anti-apoptotic gene, Bag3, was significantly higher in LCRs. These results indicate that the LCR phenotype is predisposed to expressing a more fibrotic character in fibroblasts harvested immediately following IR and may indicate poorer long-term outcome/earlier onset heart failure following acute ischemic injury to the heart. These results are in consistent with the results from previous studies using the same animal model (HCR& LCR) and showing that LCR rats are more prone to cardiac fibrosis against different kind of stress such as obesity and aging (321,345). Furthermore, our results suggest that HCR fibroblasts are likely to response to this injury by promoting apoptosis signaling pathway, while LCR fibroblasts prefer to select fibrosis pathway in response to this kind of insult. This result is supported by our immunocytochemistry results which show that cell culture and alpha smooth muscle actin are much higher in LCRs comparing to HCRs phenotype.

Interestingly and one of the novel findings of this project, our results show that activating pathways for collagen I gene expression is distinct and seems to be phenotype dependent. Treating

with Ang II induced gene expression for collagen I in LCR-CFBS but not HCR and was completely blocked with losartan. By contrast, treating with BMP7 induced gene expression of collagen I in HCR fibroblasts but not LCRs, and was completely blocked by noggin. Treating the fibroblasts with a combination of receptor agonists confirmed the phenotypic selectivity of the inducible pathways. How the intrinsic aerobic phenotype governs these pathways is unknown but could shed new light on individuals who develop post-infarction fibrosis despite therapeutic dosing with Angiotensin pathway antagonists. Moreover, treating with BMP7, Angiotensin or both, with and without receptor antagonists (BMP2R, noggin; Agtr1a, Losartan) show that LCRs anti-fibrotic gene expression were enhanced once treated with BMP7 however HCRs anti-fibrotic gene expression did not change once treated with either AngII or BMP7. HCRs seems to be antifibrotic phenotype as they have higher BMPR2 gene expression which was also matching the protein level as confirmed by immunocytochemistry results. These results suggest that LCR cardiac fibroblasts were trying to resist the imposed fibrosis by the injury via a reciprocal pathway through BMPR2 pathway in order to enhance anti-fibrotic effect.

Taken collectively, our results strongly suggest that LCRs exhibit a predisposition for LV fibrosis after acute IR and HCRs appeared to have been physiologically primed and therefore did not need an adaptive compensatory response which is together in a line with our central hypothesis for overall project.

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APPENDIX: ANIMAL CARE AND USE COMMITTEE APPROVAL LETTER

East Carolina University
Tomorrow starts here.

Animal Care and
Use Committee
212 Ed Warren Life
Sciences Building
East Carolina University
Greenville, NC 27834-4354

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

May 1, 2018

Robert Lust, Ph.D.
Department of Physiology
Brody 6N-98
East Carolina University

Dear Dr. Lust:

Your Animal Use Protocol entitled, "Cardiovascular Consequences of Artificial Selection Based on Aerobic Running Capacity" (AUP #Q330a) was reviewed by this institution's Animal Care and Use Committee on May 1, 2018. The following action was taken by the Committee:

"Approved as submitted"

Please contact Aaron Hinkle at 744-2997 prior to hazard use

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

Sincerely yours,

SBMcRae

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

SM/jd

Enclosure