THE EFFECTS OF EXERCISE TRAINING ON MYOCARDIAL RESISTANCE TO ISCHEMIA-REPERFUSION INJURY IN THE RAT HEART

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ABSTRACT

Coronary artery disease (CAD) remains a leading cause of death worldwide. Myocardial ischemia-reperfusion (IR) injury is a major pathological event leading to morbidity and mortality in those with CAD. It is well established that repeated bouts of exercise improve myocardial tolerance to IR, termed exercise-induced cardioprotection. However, gaps remain in our understanding of this cardioprotection, including: sex differences, dose-response, and the cellular mechanisms involved. Therefore, we examined the cardioprotective effects of short- and long-term exercise against myocardial ischemia-reperfusion injury in male and female rats, while investigating unexplored cellular mechanisms.

Male and female rats were subjected to 0 days (Sed), 5 days (EX5d), or 8 weeks (EX8w) of treadmill running. Maximal capacity exercise tests were performed before and after the training protocols. EX5d animals did not improve exercise capacity, while EX8w animals did, with no difference between the sexes in % improvement. Following exercise training, rats underwent regional myocardial ischemia-reperfusion (45min/24hr), and infarct size was measured for each heart. Sed females had smaller

infarcts than Sed males. Short-term running reduced infarct size in males and females compared to Sed. Long-term running did not reduce infarct size in males compared to EX5d, but EX8w females exhibited smaller infarcts compared to Sed and EX5d females. Myocardial calpain and matrix metalloproteinase-2 (MMP-2) protein levels were not different between experimental groups, but calpastatin and Tissue inhibitor of metalloproteinase-2 (TIMP-2) levels were increased in EX groups compared to Sed, with no difference between sexes. Females had higher protein levels of both subunits of the K_{ATP} channel (K_{ir}6.2, SUR2A) compared to males across all experimental groups. We also identified expression level changes of several microRNAs in response to IR and modifications to these changes associated with exercise. The major findings of these studies are: (1) changes in exercise capacity are not required for exercise-induced cardioprotection against IR injury; (2) long-term exercise augmented cardioprotection against IR injury compared to short-term exercise, but only in females; (3) exercise increased levels of TIMP-2; (4) exercise altered expression levels of microRNAs involved with IR injury; and (5) expression of MMP-2 was upregulated in the ischemic region compared to the unaffected area.

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These past six years of graduate school have been some of the most challenging, yet fulfilling, times in my life. I've been pushed and challenged in ways I didn't know were possible, yet I always received support from those around me. My doctoral training has left me with the skillset and confidence required to achieve my career goals in academia, and for that I am deeply indebted to several outstanding individuals. The saying "no man is an island" has become abundantly clear to me in the pursuit of my doctoral degree.

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CHAPTER I

Review of the literature

Specific Aims:

Myocardial ischemia-reperfusion (IR) injury remains a major cause of morbidity and mortality worldwide. Outcome after IR injury varies, but it is highly dependent on the extent of myocardial damage. Therefore, protecting the heart against IR injury by limiting infarct (cell death) size is vital to improving the quality of life and longevity of millions around the world. To date, the only pragmatic method of conferring cardioprotection against IR-induced myocardial injury is endurance exercise training; a phenomenon known as exercise-induced cardioprotection. Repeated bouts, as few as three consecutive days, of endurance exercise have been shown to confer a cardiac phenotype that resists injury during an IR insult. This reduces cardiac remodeling as well as improves contractile function following the insult. Unfortunately, the precise cellular adaptations responsible for exercise-induced cardioprotection are unclear and remain a topic of debate.

One reason the cellular adaptations leading to exercise-induced cardioprotection have been difficult to elucidate is because of the different durations of exercise training utilized in previous studies. Evidence from the literature indicates that as few as three consecutive days of treadmill running results in improved resistance to IR injury, and other studies have shown that long-term exercise training (weeks) also provides a cardioprotective effect. However, there has been no study to date that has definitively examined the effect of short-term versus long-term exercise in the ability to protect the myocardium from IR injury. There is a need to understand the dose-response of cardioprotection provided by exercise in order to help elucidate the mechanisms

responsible for cardioprotection. In this project, we accomplished this by exposing rats to one of two different exercise training protocols: short-term training (5 days) or long-term training (8 weeks).

Also, despite being well-established that exercise provides protection against IR injury, it is unclear if this cardioprotection is sex-dependent. No previous work has directly compared the effects of long-term exercise training on the ability to confer protection against myocardial IR between male and females.

Another complicating factor to our understanding of exercise-induced cardioprotection is a lack of regional resolution for molecular analysis of ischemic myocardial tissue versus unaffected tissue. Clinically, regional IR is much more common than global (whole-heart) IR, yet many studies use a global IR model to study molecular changes. Of the possible molecular mechanisms involved with exercise-induced cardioprotection, the roles of microRNAs and proteases remain largely unexplored. microRNAs (miRNAs) may regulate a myriad of cellular processes, including cell death during ischemia-reperfusion injury. Additionally, increases in myocardial cytosolic Ca^{2+} levels have been observed during both myocardial ischemia and reperfusion, and growing evidence suggests that cytosolic free Ca^{2+} overload is a major contributor to IR-induced injury. This damage appears to be largely due to the activation of the protease calpain. However, most studies of IR looking at miRNAs and Ca^{2+} overload have used global ischemia of the whole heart rather than the more relevant regional IR. Additionally, there is emerging evidence for the role of another protease, MMP-2, that contributes to myocardial dysfunction and myocyte death following IR. Furthermore,

little is known about the effects of prolonged endurance exercise on either cardiac levels of miRNAs or myocardial protein levels of enzymes involved in proteolysis, and if these are related to exercise-induced cardioprotection.

To address these issues, we had the following specific aims for this study:

1. To determine the cardioprotective effects of short-term versus long-term endurance exercise training against ischemia-reperfusion injury in rats.

Following the completion of an exercise training protocol, determine whether there is a difference in myocardial infarct size in rats subjected to short-term (5 days) and long-term (8 weeks) of exercise training.

We hypothesize that rats subjected to long-term (8 weeks) exercise training will exhibit smaller myocardial infarcts compared to the hearts from rats subjected to short-term (5 days) exercise training. Both short-term and long-term groups will exhibit smaller infarct sizes compared to sedentary counterparts. We hypothesize that there will be no difference in cardioprotection between male and female rats.

2. Identify differences in the expression levels of miRNAs, previously shown to be affected by IR, between the unaffected area and the zone at risk of hearts following ischemia-reperfusion injury.

Assess the regional differences in miRNA levels between the zone at risk and the unaffected area following IR injury. Relate these differences with infarct size.

We hypothesize that the expression levels of miRNAs related to IR injury will differ in the unaffected area and the zone at risk. Exercise training will mitigate these changes in exercised rats but not sedentary rats.

3. To determine whether endurance exercise training provides cardioprotection through changes in protease (calpain and MMP-2) expression.

We hypothesize that exercise training will result in an increased calpastatin to calpain ratio in the heart. This ratio will be further increased in rats exercised for 8 weeks compared to rats exercised for 5 days. Both exercise groups will have increased ratios compared to sedentary rats.

We hypothesize that exercise training will result in a decrease in the protein levels of MMP-2 in the heart. MMP-2 protein levels will be lower in rats exercised for 8 weeks compared to rats exercised for 5 days. Both exercise groups will have decreased MMP-2 protein levels compared to sedentary rats.

RESEARCH STRATEGY

A. SIGNIFICANCE

Coronary artery disease (CAD) remains a leading cause of morbidity and mortality worldwide. Over 16 million Americans currently have CAD, and economic costs attributed to CAD are estimated to be $200 billion annually in the US alone (72). Myocardial infarction (MI), which is the result of ischemia-reperfusion (IR) injury, is the major pathology associated with CAD and a leading cause of death. Approximately every 40 seconds, an American will have an MI (72). Since survival after an MI is

inversely related to the size of the infarction, interventions aimed at reducing infarct size are of significant clinical relevance (29). In this regard, it is well established that regular endurance exercise training provides robust cardioprotection against IR injury (11, 17, 27, 46, 55).

Exercise is arguably the most clinically relevant preconditioning (cardioprotective) stimulus that has been studied to date given its availability (economically affordable), sustainable duration of protection (11, 16, 42), and strong evidence that it is protective of human hearts as well as other mammalian hearts (as opposed to some classical models of preconditioning) (19, 49). Also, numerous animal studies provide direct evidence that endurance exercise protects the heart from IR injury (8, 32, 37, 51). Although much has been learned about exercise-induced cardioprotection through these studies, the precise cellular adaptations responsible for the resistance to IR injury remain a topic to be understood. In addition, it is unclear if there is a sex difference in the ability for exercise to protect the heart from IR injury (12, 50), or if different mechanisms exist between the sexes that contribute to cardioprotection.

As mentioned previously, the mechanisms responsible for exercise-induced myocardial protection against IR injury remain a topic of debate, and numerous possible mediators have been proposed. A few mechanisms that have been explored are: reactive oxygen species (ROS) (51), heat shock proteins (27, 28, 53), and cellular energy status (10, 54). However, there are a variety of possible mechanisms that contribute to exercise-induced cardioprotection against IR injury that have not been explored.

Recent research suggests that mircroRNAs (miRNAs) may play an important role in many cellular processes, including apoptosis and cardiac remodeling, through gene regulation by repressing target mRNAs (65). Interestingly, emerging evidence now strongly supports that miRNAs have a role in regulating cell death during ischemia-reperfusion injury (2). However, most studies of IR on miRNAs have used global ischemia of the whole heart rather than the more relevant regional IR. Interpreting such results is complicated, as it is difficult to determine if particular miRNAs are protective of myocytes, or have a deleterious effect. Furthermore, very little is known how the expression levels of miRNAs associated with IR change in response to endurance exercise training. There has been no definitive study that has looked at the effects of endurance exercise on miRNAs and cardioprotection.

There is evidence that proteolysis plays an important role in myocardial injury during IR (52). Increases in myocardial cytosolic Ca^{2+} levels have been observed during both myocardial ischemia and reperfusion (38, 39, 43), and growing evidence suggests that cytosolic free Ca^{2+} overload is a major contributor to IR-induced injury by activating a proteolytic pathway (48). The Ca^{2+}-activated protease calpain has been shown to play a deleterious role in the heart during the progression of IR injury, which ultimately impacts cardiac function and can lead to cell and organismal death. However, surprisingly little is known about the effects of prolonged endurance exercise on cardiac levels of calpain, or its endogenous inhibitor calpastatin. Recently, a protease has been identified for its potential contribution to intracellular IR injury: the matrix metalloproteinase-2 (MMP-2) (35). There is emerging evidence that MMPs contribute

to cardiac myocyte death and dysfunction during and following IR (58). A comprehensive assessment of the changes in the myocardial levels of these proteins in response to exercise and IR is still lacking, and that assessment will aid in our understanding of cardioprotection.

We hypothesized that endurance exercise training provides cardioprotection against IR injury by reducing the protein levels of calpain and MMP-2, and also mitigates miRNA changes in the heart. Further, we hypothesized that additional endurance exercise (5 days vs 8 weeks) will provide additional resistance to IR injury. Herein we utilized two different exercise training protocols (5 days vs. 8 weeks) to assess the adaptations to endurance exercise responsible for cardioprotection against IR injury in male and female rats. We linked this change in cardioprotection to differences in protease and miRNA levels. The success of this research promises to provide new insight into the mechanisms underlying exercise-induced cardioprotection and lead to new clinical targets for better resistance to IR injury.

B. ADDRESSING GAPS IN OUR UNDERSTANDING OF CARDIOPROTECTION

There are three main factors that have contributed to our lack of understanding of the mechanisms behind exercise-induced cardioprotection: (1) there is a gap in the literature in regards to a “dosing” effect of exercise and its ability to confer a cardioprotective phenotype; (2) lack of assessment of regional changes in hearts exposed to IR (versus whole heart IR); and (3) a lack of direct comparison of sex differences in cardioprotection against IR injury. By using regional IR techniques, two exercise protocols of differing durations (5 days vs. 8 weeks), and directly comparing

levels of myocardial injury following IR in male and female rats, we will be able to determine more precisely the mechanisms behind exercise-induced cardioprotection.

We therefore designed an innovative study in order to address these gaps in our understanding of cardioprotection. We employed two different exercise protocols (short-term and long-term) to study exercise-induced cardioprotection against IR injury, while utilizing an innovative technique allowing for better spatial resolution of the injured myocardium, explored novel mechanisms that contribute to cardioprotection, and conducted these experiments in both male and female rats. Below are the major innovative aspects.

B1. An innovative approach to measure regional changes in the myocardium in response to IR injury:

In humans, myocardial ischemia is most commonly regional in nature, where only part of the ventricle experiences a blockage in blood flow. Interestingly, much of our understanding of exercise-induced cardioprotection comes from studies that have looked at the response of the heart to global IR, where the entire heart undergoes an ischemic insult (36, 42, 55). This approach has its benefits, but a major limitation is that extrapolation of the results to regional ischemia should be used with extreme care. For example, global ischemia eliminates the interaction between cells of different regions of the heart, and must be conducted outside of the body (69). To address this issue, we will employ a regional IR approach, unique to the study of exercise-induced cardioprotection against IR. Furthermore, spatially resolved molecular analysis of infarcted myocardial tissue has not been properly addressed in previous exercise

studies. Routinely, following IR, the entire infarcted heart is homogenized for molecular analysis, preventing a regional assessment of the molecular changes that occur in response to IR (55). Interpreting results from IR studies using homogenized tissue is complicated, as it is difficult to determine if changes in various molecular markers are protective, deleterious, or coincidental. One example is Heat Shock Protein (HSP) 72, which was speculated to be an important mediator of exercise-induced cardioprotection, but has recently been repudiated following improved methodology (53). To address this important limitation in the regional analysis of infarcted tissue, we developed a unique method for accurate dissection of the zone at risk from the unaffected area by cryosectioning the infarcted heart. The unique advantages of this technique over the traditional methodologies include: (1) better spatial resolution for the measurement of the size of the infarct as a percentage of the area at risk; (2) effective separation of the area at risk from the unaffected area; (3) identification of molecular changes in response to IR unique to the area at risk; and (4) allowing for a comparison of molecular changes in the area at risk to the unaffected area.

B2. Varying the amount of endurance exercise:

Interestingly, some previous studies indicate that as few as 3-5 consecutive days of endurance exercise are required to achieve a significant level of cardioprotection against IR-induced myocardial infarction (27). Hamilton et al. demonstrated that exercising rats for 5 consecutive days, 60 min per day, resulted in a significant reduction in infarct size compared to sedentary rats. A separate study concluded that 20 weeks of treadmill running also reduced infarct size in rats exposed to IR compared to sedentary

counterparts (11). This is a significant finding, as it identifies exercise as an optimal cardioprotective strategy due to its sustainable effects, unlike other cardioprotective interventions. For example, cardioprotection resulting from ischemic preconditioning loses its effectiveness with multiple applications (16). However, complete details about the dose-response impact of endurance exercise on cardioprotection remain unknown. It is unclear if there is a maximal amount of cardioprotection that can be achieved through endurance exercise. Importantly, no previous studies have compared the degree of cardioprotection afforded by short-term versus long-term exercise training in the same study. By utilizing exercise protocols of different durations in this project, we were be able to determine if the exercise-induced cardioprotection against IR injury can be increased with additional exercise training (short-term exercise training versus long-term exercise training). It is well established that a multitude of adaptations occur with additional exercise training compared to acute exercise training. Hence, it seemed likely that additional exercise training would result in an increased level of cardioprotection against IR injury. By creating two separate exercise training protocols (5 days of treadmill running versus 8 weeks of treadmill running), we utilized a novel training approach in order to determine if additional exercise training yields an increase in cardioprotection.

B3. Sex differences in cardioprotection:

Exercise-induced cardioprotection has been observed in both male (11) and female (27) animals. However, it is unclear if, or to what extent, exercise alters myocardial susceptibility to IR injury in a sex-dependent manner. Some previous studies

in rats have shown that female hearts are more resistant to IR injury following exercise training than male hearts (8, 11, 32), while others have not observed this same effect (43, 53). The reason for these discrepancies is unclear, but warrants further investigation. One reason for this lack of clarity regarding the role of sex differences is that previous studies have generally not directly compared the exercise cardioprotection response in males compared to females in the same study. Therefore, another primary objective of this study was to directly determine if the ability for endurance exercise to alter the susceptibility of rat hearts to IR injury is sex-dependent.

B4. miRNA and exercise-induced cardioprotection:

miRNAs, small noncoding RNA molecules, are emerging as important regulators of myocardial injury during IR through their repression of target mRNAs (18, 56). Because of the recent discovery of their importance, much is left to be elucidated on the role of miRNAs in IR injury. One miRNA that appears to play a critical role in IR injury is miRNA-214. miRNA-214 plays a protective role against IR injury by attenuating Ca^{2+} overload, and genetically modified mice lacking miRNA-214 have been shown to exhibit significantly larger infarcts in response to IR as compared to wild-type littermates (2). Transfection of select miRNA inhibitors and mimics, thought to be implicated in IR injury, into cardiac myocytes subjected to IR showed a significant protective effect, further establishing that miRNAs play a role in cardioprotection and thus may have promise as a future pharmacological intervention (68). miRNA-125b has been shown to confer cardioprotection by attenuating myocyte apoptosis during IR injury, which has been a proposed mechanism for exercise-induced cardioprotection (71). Surprisingly, to

our knowledge no previous studies have been conducted looking at the potential role of miRNAs in exercise-induced cardioprotection. Thus, we reason that determining the effects of endurance exercise on the levels of miRNAs implicated in IR injury is an important step forward for the field. Additionally, our regional IR model will provide better spatial resolution on the protective or deleterious roles that miRNAs play during IR compared to information from whole-heart ischemia studies.

B5. Protease expression contributing to IR injury:

It is well established that intracellular Ca^{2+} overload plays a critical role in the development and progression of cellular injury and death during IR (48). Increases in myocardial cytosolic Ca^{2+} have been observed during both ischemia and reperfusion, and evidence strongly suggests that this is a major contributor to IR injury (61). Altered Ca^{2+} homeostasis may trigger processes that directly cause cell death such as hypercontracture, mitochondrial damage, and proteolysis (25). Surprisingly, very few studies have been conducted looking at the effects of endurance exercise on myocardial calcium handling during IR. An intriguing target is the role that proteolysis plays in IR-induced injury. Damage to the myocyte during IR is inflicted, in part, through activation of the Ca^{2+}-dependent protease calpain. Calpain is activated by prolonged exposure to elevated cytosolic Ca^{2+} levels (26), and activation during IR plays a significant role in myocyte cell death. This became evident after studies showed strong evidence indicating that pharmacological inhibition of calpain significantly attenuates myocardial infarction (31, 67, 73). Calpastatin is an endogenous inhibitor of calpain, and the ratio of calpastatin to calpain in cardiac myocytes is physiologically important because this ratio

greatly impacts the ability of Ca^{2+} to activate calpain (26). That is, an increased calpastatin to calpain ratio would favor calpain inhibition. A possible mechanism of exercise-induced cardioprotection is that exercise training could increase the cardiac levels of calpastatin, or decrease the cardiac levels of calpain. In turn, this would increase the calpastatin to calpain ratio, which would provide cardioprotection by inhibiting calpain activation. However, there is sparse evidence on the effects of endurance exercise on the levels of calpain and calpastatin in the heart, with one study finding a decrease in calpain activity in response to IR in hearts from rat hearts that underwent exercise training compared to sedentary rats (23). Therefore, we measured the changes in myocardial calpain and calpastatin in sedentary rats and rats subjected to two different amounts of endurance exercise (5 days vs 8 weeks).

In addition to calpain/calpastatin, recent work has demonstrated that another protease, MMP-2, may play an important role in myocardial IR injury (35). MMP-2, a zinc-dependent endopeptidase, has been shown to be activated in response to oxidative stress, such as that experienced by cardiac myocytes during IR (35). To date, there has been little attention paid to inhibition of MMP-2 activity following IR injury as a possible mechanism of exercise-induced cardioprotection.

Matrix metalloproteinases (MMPs) are known to be involved in the degradation of the extracellular matrix in both physiological and pathological conditions, and play a key role in scar formation following myocardial infarction (58). While this extracellular role of MMP-2 in myocardial infarction has been well established, recent studies have suggested an additional, intracellular, role of MMP-2 activation in IR injury (15, 35).

MMP-2 has been shown to have a number of intracellular proteolytic targets in cardiac myocytes, among them cardiac troponin I (TnI) (5, 70), myosin light chain-1 (57), α-actinin (62), and titin (1). Degradation of these crucial myofilament proteins by MMP-2 would potentially account for the myocardial contractile dysfunction observed following IR injury (24).

Tissue inhibitor of metalloproteinases (TIMPs) are major cellular inhibitors of the MMPs, and TIMPs have been found in cardiomyocytes (3). It is thus possible that exercise provides cardioprotection against IR injury through 1) decreasing MMP-2 protein levels, or 2) inhibiting MMP-activation by increasing the protein levels of the TIMPs. Therefore, we measured the changes in myocardial MMP-2 and TIMP-2 protein content in sedentary rats and rats subjected to two different amounts of endurance exercise (5 days vs 8 weeks).

C. THE APPROACH

The primary objectives of this project were: (i) to determine the adaptations to long-term endurance exercise that contribute to exercise-induced cardioprotection against IR injury, (ii) to elucidate the roles that miRNAs and proteases play in exercise-induced cardioprotection, (iii) to study the regional changes in these markers following IR, and (iv) investigate sex differences in cardioprotection against IR injury.

C1. Preliminary Data: We previously acquired preliminary data that demonstrated the feasibility of this project.

C1.1. Improved spatial resolution for the measurement of infarct area and the area at risk:

We have successfully developed a method for improving the efficacy of quantifying the total area, infarct area, and the zone at risk of heart slices following IR injury (Figure 1.1). Determination of the degree of cardioprotection relies heavily on accurate quantification of the infarct size, zone at risk, and unaffected area. Existing techniques have a high degree of variability in these measurements. We have developed a technique involving freezing of the sample prior to imaging the infarct area. Using this method, our lab has successfully shown the ability for 3 separate observers to report statistically similar measurements of infarct size, total area, and zone at risk (ZAR).

C1.2. Regional analysis of molecular changes: Previous studies of exercise-induced cardioprotection against IR have induced global ischemia on the heart. Furthermore, the myocardium is commonly homogenized for molecular analysis, potentially obstructing a clear view of the changes that occurred in areas of the heart exposed to IR (the ZAR), and the unaffected area. Using the unique approach described in B1.1, we have effectively separated the ZAR from the unaffected area and measured statistically significant changes in miRNAs associated with IR injury (miRNAs 92a and 484; p-value <0.05 with fold change $>\pm 2.0$)(Table 1.1).

C1.3. Analysis of changes in proteins involved with proteolysis:

We have successfully shown that endurance exercise training in non-stressed hearts is associated with increased calpastatin to calpain ratios compared to sedentary

counterparts ($p < 0.05$) (Figure 1.2). The improved calpastatin/calpain ratio exhibited by exercised animals appears to be due to a significant decrease in calpain levels compared to sedentary animals ($p < 0.05$). Our results show that endurance exercise is associated with an increase in the ratio of calpastatin to calpain, which is physiologically important. In Aim 3 of our study we set out to determine if this improved ratio is maintained in response to IR, if it is augmented with additional exercise training (8 weeks compared to 5 days), and if that ratio is correlated with improved cardioprotection. Additionally, we set out to determine the effects of endurance exercise on the myocardial protein levels of MMP-2 and TIMP-2, a separate proteolytic pathway than calpain/calpastatin.

D. Conclusion

In summary, this is the first study to directly compare the effects of short-term and long-term exercise in the ability to provide cardioprotection against myocardial ischemia-reperfusion injury. We have identified novel mechanisms that contribute to improved myocardial tolerance against IR injury that are a result of exercise. Additionally, this is one of the first studies to directly compare cardioprotection against IR injury between male and female rats.

Each chapter herein will contain a detailed introduction to the topic of the chapter, an in-depth methods section for the new methods used in that chapter, as well as a brief re-iteration of methods used in previous chapters, a results section with will include new results for that chapter in addition to a brief reference to results from previous chapters that are pertinent to the understanding of the new data, followed by a detailed discussion section. The hope is that the findings of this dissertation will

contribute to a better understanding of the cellular mechanisms that protect the myocardium from ischemia-reperfusion injury, with the ultimate goal of developing new therapeutic interventions to help reduce the staggering impact of cardiovascular disease in our society.

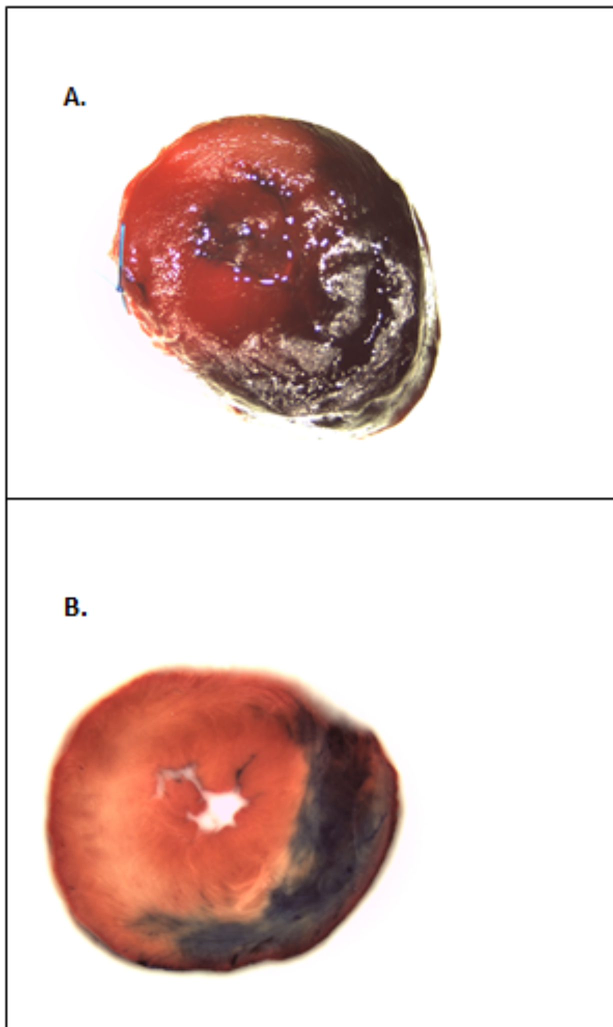

Figure 1.1: Quantification of infarct size. (A&B) Representative myocardial tissue samples following IR, staining, and slicing. The blue area (Evans blue dye) represents the unaffected area, the red area represents the area at risk, and the white/pale red represents the infarction. Image of a wet sample (A) and frozen sample (B) to display the improved visual resolution achieved from flash-freezing the samples. This will allow for effective regional assessment of changes in the myocardium in response to IR.

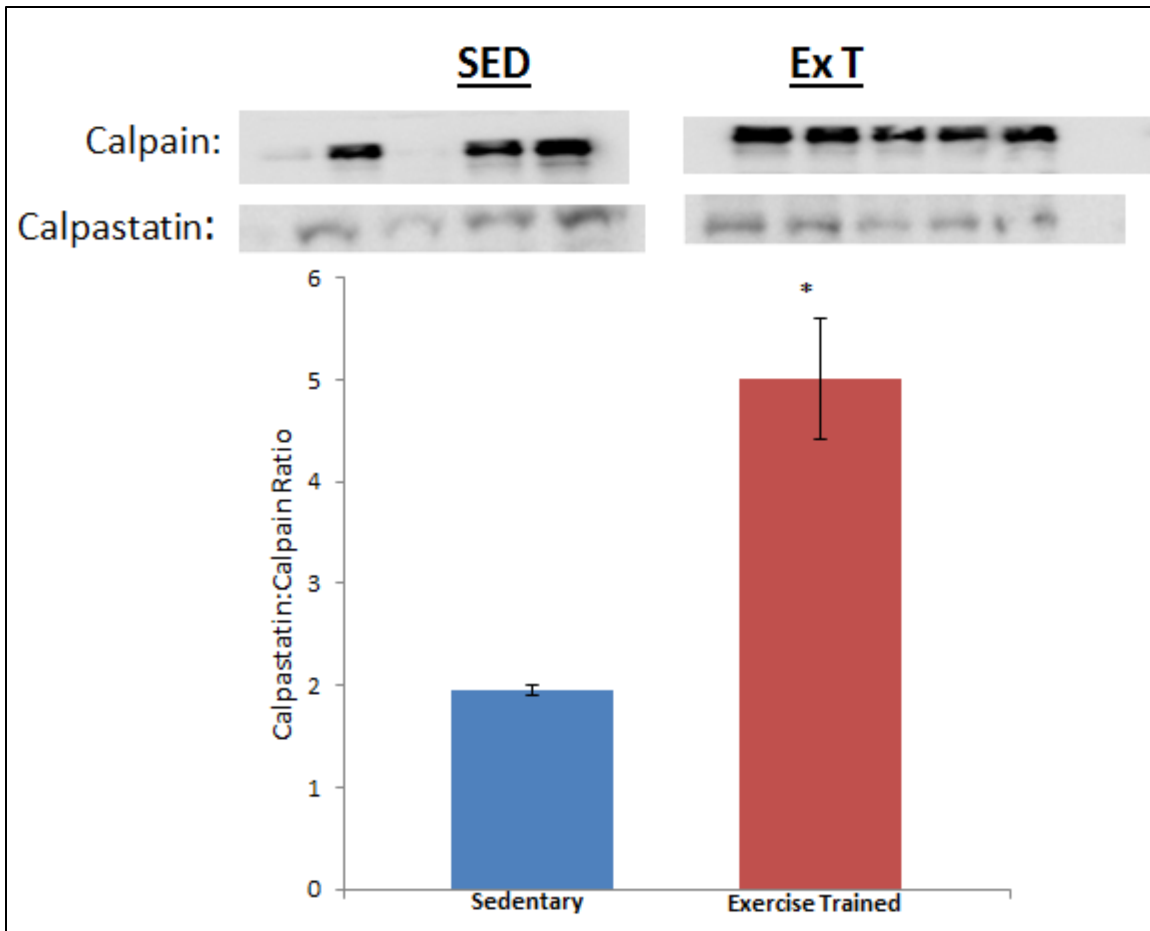

Figure 1.2. Calpastatin:Calpain protein ratio. Following12 weeks of exercise training, hearts from exercise-trained rats exhibited significantly higher calpastatin:calpain ratios compared to sedentary counterparts (p= 0.0083). Data are integrated from multiple Western blots.

Table 1.1: Expression level changes in response to IR for select miRNAs previously implicated in IR injury. Fold regulation changes are comparing the ZAR to the UA.

microRNA	**p-value**	**Fold Regulation** (in zone at risk vs. unaffected)
347	0.096619	-4.72
484	0.048772	-2.81
92a	0.041943	-11.34

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CHAPTER II

Exercise-induced cardioprotection against myocardial infarction in the rat heart is sex-dependent

Introduction

Coronary artery disease (CAD) remains the leading cause of morbidity and mortality of both men and women worldwide. Over 16 million Americans currently have CAD, and economic costs attributed to CAD are estimated to be $200 billion annually in the US alone (2). The most common manifestation of CAD is myocardial infarction (MI), a result of ischemia and/or reperfusion (IR) injury of the myocardial tissue. Numerous interrelated events occur during IR that, if severe and prolonged, result in cardiac myocyte damage and death (infarct). These include: decreased cellular [ATP], increases in reactive oxygen species (ROS), increases in cytosolic [Ca^{2+}], and activation of calcium-activated proteases (19, 31).

Each year over 1.2 million Americans suffer an MI, which damages the myocardium and leaves the individual susceptible to additional cardiac events. Since infarct size is inversely related to survival following MI, strategies to reduce infarct size are of enormous clinical value (18). Interestingly, sex appears to influence both the occurrence of MI, with the incidence being much lower in premenopausal women than age-matched men (23), as well as the severity of the MI in response to the same degree of IR, with hearts from females having smaller infarct sizes following ischemia compared to males (12, 25, 28). However, the mechanisms for these sex differences in IR injury are not well understood.

It is well documented that exercise training improves myocardial tolerance to IR, a phenomenon referred to as exercise-induced cardioprotection (reviewed in 31).

Exercise confers resistance against several different forms of IR injury, including myocardial stunning (3, 27, 37), arrhythmia (1, 10, 34), and infarction (17, 26). Evidence from the literature demonstrates that both chronic (weeks)(5) and acute (as few as 1-3 consecutive days)(8, 16) endurance exercise yields resistance to IR injury. While both short-term and long-term exercise training have been shown to confer cardioprotection against IR injury, there have been no direct comparisons of the magnitude of exercise protection with different durations of exercise training. Therefore, a primary objective of the present study was to test the hypothesis that long-term (8 week) exercise training results in a greater degree of cardioprotection against IR injury compared to short-term exercise.

Exercise-induced cardioprotection has been observed in both male (7) and female (17) animals. However, it is unclear if, or to what extent, exercise alters myocardial susceptibility to IR injury in a sex-dependent manner. Previous studies in rats have shown that female hearts are more resistant to IR injury following exercise training than male hearts (4, 6, 21), while others have not observed this same effect (29, 38). Therefore, another primary objective of this study was to determine if the ability for endurance exercise to alter the susceptibility of rat hearts to IR injury is sex dependent.

While the exact mechanism(s) responsible for exercise-induced cardioprotection have not been fully elucidated, it is possible that cardioprotection is achieved by attenuating the intracellular damaging events that occur during IR (31). For example,

there is strong evidence suggesting a role in cardioprotection for two main classes of proteins proposed to be important mediators of IR injury: 1) Ca2+-activated proteases and their inhibitors, and 2) proteins that aid in sensing cellular energetic stress. Therefore, we examined the myocardial protein content of the Ca^{2+}-activated protease, Calpain, and its endogenous inhibitor, Calpastatin, as well as the sarcolemmal ATP-sensitive potassium (K_{ATP}) channel. Using a rat model of IR injury and MI, we examined the effect of short- versus long-term exercise training on the expression of these proteins. In addition, we examined sex differences in this exercise effect.

Methods

Animals. Three-month-old male and female Sprague-Dawley rats were obtained from Envigo (locally maintained colony in Madison, WI, USA). We selected Sprague-Dawley rats for this experiment because it is a well-accepted model for studying exercise-induced myocardial adaptations and myocardial responses to IR. Upon arrival, rats were randomly assigned to one of three groups: a five day exercise training group (EX5d; n=20), an eight week exercise training group (EX8w; n=20), or a sedentary control group (Sed; n=20). Each group contained equal numbers of males and females. All animals were housed in pairs in clear plastic cages in the same temperature controlled facility with a 12:12-h light/dark reverse cycle under the care of a full-time veterinarian. The rats were provided standard rat chow and water *ad libitum*. All animals were killed at least 24 h after the last training (or handling control) session. The experimental protocol and euthanasia were approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee (IACUC) and followed the guidelines established by the American Physiological Society for the use of animals in research.

Treadmill exercise training. Training was started one week after the rats' arrival to reduce the stress associated with shipping and acclimation to a new location. Animals assigned to the exercise groups were habituated to treadmill exercise on a daily basis for 5 consecutive days. This habituation period involved a gradual increase in running time beginning with 5 min/day (first day) and ending with 25 min/day (5th day)

at 12 m/min at 0% grade, an amount below that which is likely to produce a significant change in aerobic capacity.

Following the habituation period, animals were given 2 days of rest and then underwent a speed-ramped test to assess maximal running capacity. This protocol was previously developed to pattern clinical stress tests as a standard screen for exercise capacity (22). Briefly, rats exercised on a motorized treadmill set at a constant grade of 15% and an initial speed of 18 m/min. The speed was progressively ramped 1 m/min every 2 min until exhaustion occurred. Exhaustion was defined as the third time the rat remained in contact with the shock grid for 2 s. Total distance run was calculated from belt speed (m/min) and duration (min) of the run.

Following the initial max running test, animals in the EX8w group were run on a treadmill for 60 min/day, 5 days/week, for 8 weeks. The treadmill speed was gradually increased every few weeks, with females running at the following maximal intensities: weeks 1-2: 19 m/min, weeks 3-4: 21 m/min, weeks 4-8: 23 m/min. Male rats ran at the following maximal intensities: weeks 1-2: 15 m/min, weeks 3-4: 18 m/min, weeks 4-8: 21 m/min. This training protocol was developed to maximize compliance and reduce animal stress based off of previous exercise studies in our laboratory, and to account for the innate differences in exercise capacity between male and female rats. At the conclusion of the 8-week exercise protocol rats underwent a second maximal exercise capacity test. Sed rats were placed on the treadmill for 5 min/day twice per week in order to control for the effects of handling.

After the initial max running test, animals in the EX5d group were trained for 3 days for the same duration and speed as the EX8w rats, immediately followed by a second max running test, amounting to 5 consecutive days of exercise training.

Ischemia-reperfusion protocol. Animals were deeply anesthetized using isoflurane gas. After left thoracotomy, a suture was threaded through the tissue surrounding the left anterior descending coronary artery close to its origin (3-5 mm distal to the aorta). Transient regional ischemia was induced by tightening the suture to occlude the artery with the use of a removable snare. Electrical activity of the heart (continuous ECG monitoring) and visualization of cyanotic heart tissue were used to confirm the presence of ischemia. Ischemia lasted for 45 min. At the end of ischemia, the snare was loosened and reperfusion ensued for 24 hr while animals recovered in their cage. The durations of ischemia and reperfusion were chosen to ensure myocardial infarction, and the protocol was adapted from previous work conducted at the Cardiovascular Research Center at UW-Madison (24). For sham surgery, left thoracotomy was performed but the coronary artery was not occluded. Five animals developed severe fibrillation or did not survive the IR surgery (four Sed males, one EX8w female), and their tissue was discarded from the study. Following euthanasia, the plantaris muscle was removed and flash frozen using liquid nitrogen for subsequent citrate synthase assay.

Measurement of infarct size. At the end of reperfusion (24 hr), the snare was retightened around the coronary artery, and 100 µl of 0.5% Evans blue dye solution was

injected into the aorta and perfused through the heart. Hearts were then quickly excised, rinsed in cold saline, and sliced transversely from base to apex into slices of equal width. Each of the slices was then immersed in 100 mM phosphate-buffered saline with 0.1% triphenyltetrazolium chloride (TTC) and incubated for 10 min at 37°C. After incubation, each slice was placed in Optimal Cutting Temperature (OCT) compound, flash frozen using liquid nitrogen, and immediately stored at -80°C. Using a cryomicrotome, frozen slices were carefully sectioned until the OCT was trimmed away, exposing the embedded heart slice. Each slice was then imaged using a digital microscope camera. The microscope lighting and magnification were kept constant throughout the procedure for experimental consistency.

Images of the slices were quantified using ImageJ imaging software, and two separate individuals performed analyses in a single-blind manner to avoid experimenter bias. The zone at risk (ZAR) was identified as the area of each slice that did not turn blue (i.e., not perfused with dye) after perfusion with the solution containing Evans blue dye. The portion of the ZAR that did not turn red in response to TTC incubation and remained white was classified as the infarct area. Any tissue that was stained blue was defined as the unaffected area (UA). ZAR and infarct size were obtained from each slice, and slices from the same heart were added together. The size of the infarct area was expressed as a fraction of the ZAR by taking the sum of the infarct area and dividing by the sum of the ZAR. Thus, all fractional infarct size values are reported as a percentage of the ZAR.

Western blots. The LV free wall was dissected from the frozen heart sections, and the ZAR and UA were carefully separated. ZAR samples were then homogenized in QIAzol Lysis Reagent (Qiagen USA, Germantown, MD, USA) using a motorized homogenizer (PowerGen 700; Thermo Fisher Scientific, Waltham, MA, USA). The homogenates were immediately frozen at -80°C and used for subsequent Western blot assay. Western blots were performed using SDS-PAGE in a 4-15% polyacrylamide gel with 20 µg of homogenate per lane. Only homogenate from the ZAR was used for the Western blots. Blots were first probed using a monoclonal antibody against Vinculin (Cell Signaling Technology, Danvers, MA, USA) at a dilution of 1:1,000. After a secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (Cell Signaling Technologies) and then a chemiluminescent substrate (Bio-Rad Laboratories, Hercules, CA, USA) were applied, blots were imaged using ImageQuant LAS 4000 (GE Healthcare, Boston, MA, USA). Blots were then stripped using standard methods and reprobed for K_{ir}6.2 (Cell Signaling Technologies, 1:1,000), Calpain I (Cell Signaling Technologies, 1:1,000), Calpastatin, (Cell Signaling Technologies, 1:1000), SUR2A (Sigma-Aldrich USA, St. Louis, MO, USA, 1:1,000), or Dystrophin (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:1,000). All secondary antibody used was horseradish peroxidase-conjugated goat anti-rabbit, with the exception of the secondary antibody used against SUR2A, which was anti-mouse. Band density was analyzed using Image Studio Lite. Western blot band densities are reported as normalized to Vinculin.

Citrate synthase assay. Samples (65-80 mg) were cut from the frozen plantaris muscles and homogenized. Total muscle protein was determined by Bradford protein

assay, and the protein concentration of all samples was equalized. Citrate synthase activity was then determined using an assay kit (Sigma-Aldrich, USA) and spectrophotometer.

Data analysis. All statistical comparisons were made using GraphPad Prism (version 7). Data were analyzed using a 2 (sex) x 3 (training group) ANOVA. When appropriate, effects of training (within sex) were evaluated with a one-way ANOVA. Values are means ± SE, and significance is reported at $p < 0.05$.

Results

Maximal exercise capacity. Changes in maximal exercise capacity, comparing pre-exercise to post-exercise max running tests, are reported as absolute change (m) and relative change (%), and are presented in Figure 2.1. Female and Male EX8w animals had significantly higher exercise capacities compared to EX5d counterparts. Female EX8w rats had significantly higher pre and post-exercise capacities in absolute terms compared to male EX8w rats (post-training: 668.9 ± 87.0 m vs. 310.4 ± 46.5 m, respectively, $P < 0.05$). However, the relative (% change) improvement from baseline measurements (pre-exercise training) was not different between EX8w females and males (235.4 ± 21.3% vs. 201.6 ± 22.5%), suggesting an equally effective exercise training protocol between males and females.

Citrate synthase activity. Citrate synthase activity in the plantaris (a mixed muscle) was determined as a measure of endurance exercise training effect and results are shown in Figure 2.1. Citrate synthase activity was significantly increased following 8 weeks of training in both male and female rats compared to sedentary animals (EX8w males: 24.01 ± 0.50 vs. Sedentary males: 15.33 ± 0.4 μmol·g wet wt^{-1}·min^{-1}; EX8w females: 25.18 ± 0.67 vs. Sedentary females: 15.71 ± 0.34 μmol·g wet wt^{-1}·min^{-1}, $P < 0.05$). There was no difference in citrate synthase activity between EX8w males and EX8w females, corroborating our maximal exercise capacity test results. Additionally, there was no difference in citrate synthase activity in male or female EX5d rats (15.09 ± 0.41 and 15.25 ± 0.39 μmol·g wet wt^{-1}·min^{-1}, respectively) compared to sedentary.

Increased citrate synthase activity indicates that the animals were endurance trained as a result of the 8 week exercise protocol.

Infarct size. Data on infarct size, normalized for ZAR, are summarized in Figure 2.2. The infarct size was significantly smaller in hearts from EX5d animals compared to Sed animals (27.5 ± 1.0% vs. 37.2 ± 2.3%, $P < 0.05$; Fig. 2.2.A), and infarct size was significantly smaller in hearts from EX8w animals compared to hearts from EX5d animals (21.4 ± 1.0% vs. 27.5 ± 1.0%, $P < 0.05$; Fig. 2.2.A). The increased cardioprotection in response to additional exercise training only existed in female rats. Infarct size was significantly smaller in hearts from EX8w females compared to EX5d females (19.4 ± 1.1% vs. 25.1 ± 0.9%, $P < 0.05$; Fig. 2.2.B), but infarct size was not significantly different between hearts of EX8w males and EX5d males (23.5 ± 1.1% vs. 29.9 ± 1.1%; Fig. 2.2.B). Hearts from female rats exhibited significantly smaller infarcts compared to hearts from male rats across every experimental group (Female: Sed 32.6 ± 1.2%, EX5d 25.1 ± 0.9%, EX8w 19.4 ± 1.1%; Male: Sed 41.8 ± 3.3%, EX5d 29.9 ± 1.1%, EX8w 23.5 ± 1.1%, $P < 0.05$; Fig. 2.2). These data indicate that female rats are intrinsically more protected from IR injury than male rats, and that female rats can strengthen their level of cardioprotection against IR injury with long-term exercise training compared to that afforded by short-term exercise training.

Calpain and Calpastatin protein levels. Intact calpain I, calpastatin, and dystrophin protein levels were measured in the myocardium from the ZAR using Western blot analysis. The results, displayed in Fig. 2.3, reveal that calpain protein

content was not altered with exercise training in hearts from female or male rats (Fig. 2.3.A). Myocardial calpastatin protein levels were significantly increased in both EX5d and EX8w groups compared to Sed animals ($P < 0.05$; Fig. 2.3.B), while there were no difference in calpastatin protein levels between EX5d and EX8w animals. There was no significant different in myocardial calpastatin levels between female and male rats in any of the experimental groups (Fig. 2.3.B). There were no differences in dystrophin protein levels between any of the groups (data not shown). All protein levels were normalized to vinculin.

K_{ATP} channel expression. Protein expression of K_{ATP} channel subunits is presented in Fig. 2.4. Protein levels of the pore-forming subunit $K_{ir}6.2$ increased in response to exercise training in both female and male hearts compared to Sed counterparts ($P < 0.05$, Fig. 2.4.A). There was no significant difference in $K_{ir}6.2$ protein levels between EX5d animals and EX8w animals in either sex, but there was a trend towards higher protein levels in EX8w females compared to EX5d females ($P = 0.0725$). $K_{ir}6.2$ protein levels from female hearts were significantly higher compared to males between all experimental groups ($P < 0.05$). Protein levels of the sulphonylurea receptor subunit (SUR2A) significantly increased in response to exercise training in both female and male rat hearts compared to sedentary counterparts ($P < 0.05$, Fig. 2.4.B). There was no significant difference in SUR2A protein levels between EX5d males and EX8w males. Hearts from EX8w females had significantly higher protein levels of SUR2A compared to EX5d females ($P < 0.05$, Fig. 2.4.B), and SUR2A protein levels were from female hearts

were significantly higher compared to males between all experiments groups ($P < 0.05$).

All protein levels were normalized to vinculin.

Discussion

Sex-dependent cardioprotection against myocardial infarction. A significant finding of our research was that myocardial infarcts produced by ischemia-reperfusion (IR) injury were smaller in hearts from rats that underwent long-term (8 weeks) endurance exercise training compared to rats that underwent short-term (5 days) endurance exercise training. This effect of exercise duration was sex-dependent, with only hearts from female rats displaying improved tolerance to IR injury following 8 weeks of exercise training compared to 5 days. Male rats did exhibit cardioprotection against IR injury following long-term exercise, but this degree of cardioprotection was not significantly different than the protection conferred by short-term exercise. While previous studies have shown that both short-term (17) and long-term (5) endurance exercise training results in resistance to IR injury, to our knowledge, this is the first study to directly determine that the exercise-induced cardioprotection afforded by short-term exercise can be increased with additional exercise training, and show that this effect is sex-dependent.

Another finding of the current study was that the myocardium from female rats was more resistant to IR damage compared to their male counterparts across all experimental groups (Sed F vs. Sed M, EX5d F vs. EX5d M, and EX8w F vs. EX8w M). The majority of IR studies performed provide strong evidence that hearts from females, relative to males, are intrinsically more protected from IR injury (6, 25, 28, 38) although this effect is not universally seen (33). The data from this study (Fig. 2.2) is in agreement

with these previous findings, supporting the notion that females are inherently more protected than males. However, there is considerable debate as to whether female hearts are more resistance to IR damage following exercise training. Some previous studies have shown that hearts from female rats are more resistant to IR injury following exercise training than male rats (4, 6, 21), while others have not observed this same effect, with hearts from female rats exhibiting more damage than males following an ischemic episode (29, 38). It is unclear what is responsible for this discrepancy in findings. One possible explanation is the primary measure of myocardial damage used in the study. In our current research, we used infarct size as the primary marker of damage, as infarct size is highly predictive of future mortality and morbidity following MI (18). This is a common marker of myocardial damage, and is in agreement with previous studies that have shown females are more tolerant to IR following exercise (6). However, Thorp and colleagues (38) used mechanical work as their primary measure of myocardial damage following ischemia, and hearts from exercised females had decreased left ventricular mechanical performance following ischemia than hearts from male rats. This finding is not necessarily in disagreement with our current findings. It is possible that hearts from exercised females are more susceptible to myocardial stunning following an ischemic episode, but are still more resistant to infarction.

It is possible that our observation of the sex-dependence of the ability of 8 weeks of exercise to confer additional protection against MI compared to 5 days was due to the greater exercise capacity of females compared to males. Indeed, on average, females ran a greater distance during both pre-training and post-training maximal

exercise tests, as well as completing a greater overall training volume compared to male rats. However, there was no significant difference between males and females in either the percent improvement in maximal running distance after 8 weeks of training, or in skeletal muscle citrate synthase activity, both common markers of standard endurance training adaptations (9, 22). Thus, there was no evidence of greater adaptation to the 8 weeks of training in females compared to males, despite the improved cardioprotection in females. In fact, we noted a disconnection between exercise-induced cardioprotection and standard endurance adaptations. The 5-day exercise training conferred some cardioprotection in both males and females despite the lack of improvement in maximal distance run or in skeletal muscle citrate synthase activity. Also, in males, 8 weeks of exercise training resulted in improvements in these markers of endurance training but did not results in significant further cardioprotection compared to 5 days. These results suggest that the sex-difference in improved cardioprotection with 8 weeks of training was not directly related to improved exercise capacity.

Mechanisms of exercise-induced cardioprotection. The exact mechanism(s) responsible for exercise-induced cardioprotection are unclear and remain a topic of debate (31). There is strong evidence that the protein expression of the sarcolemmal ATP-sensitive potassium (K_{ATP}) channel is higher in hearts from female rats than males (6, 36), and previous studies have demonstrated the importance of the sarcolemmal K_{ATP} channel in cardioprotection (4, 14, 15, 30). Although the precise role of this ion channel in cardioprotection is unknown, it has been suggested that it may act as a

cellular energy sensor that is involved in sustaining cellular ATP levels during metabolic stress. Furthermore, pharmacological blockade of these channels negates the cardioprotective benefits of exercise (4). Our findings, that protein levels of both subunits of the K_{ATP} channel ($K_{ir}6.2$ and SUR2A) are increased in response to exercise training (Fig. 2.4), support previous results (6). Importantly, we found that protein levels of these subunits appear to plateau following short-term exercise in male hearts, but continue to increase with long-term exercise in female hearts. This increase is associated with increased resistance to IR injury with long-term exercise training in hearts from female rats, but not males. Prior research has shown that increased expression of SUR alone is sufficient to increase the assembly of functional sarcolemmal K_{ATP} channels in cardiac myocytes (36), and hearts from EX8w females had significantly higher levels of the SUR2A protein than any other experimental group. These results provide strong evidence of the role of the K_{ATP} channel and the SUR2A subunit in exercise-induced cardioprotection and suggests that the improved cardioprotection resulting from 8 weeks compared to 5 days of exercise training in female rats is partially due to the increased myocardial expression of the K_{ATP} channel resulting from long-term compared to short-term exercise.

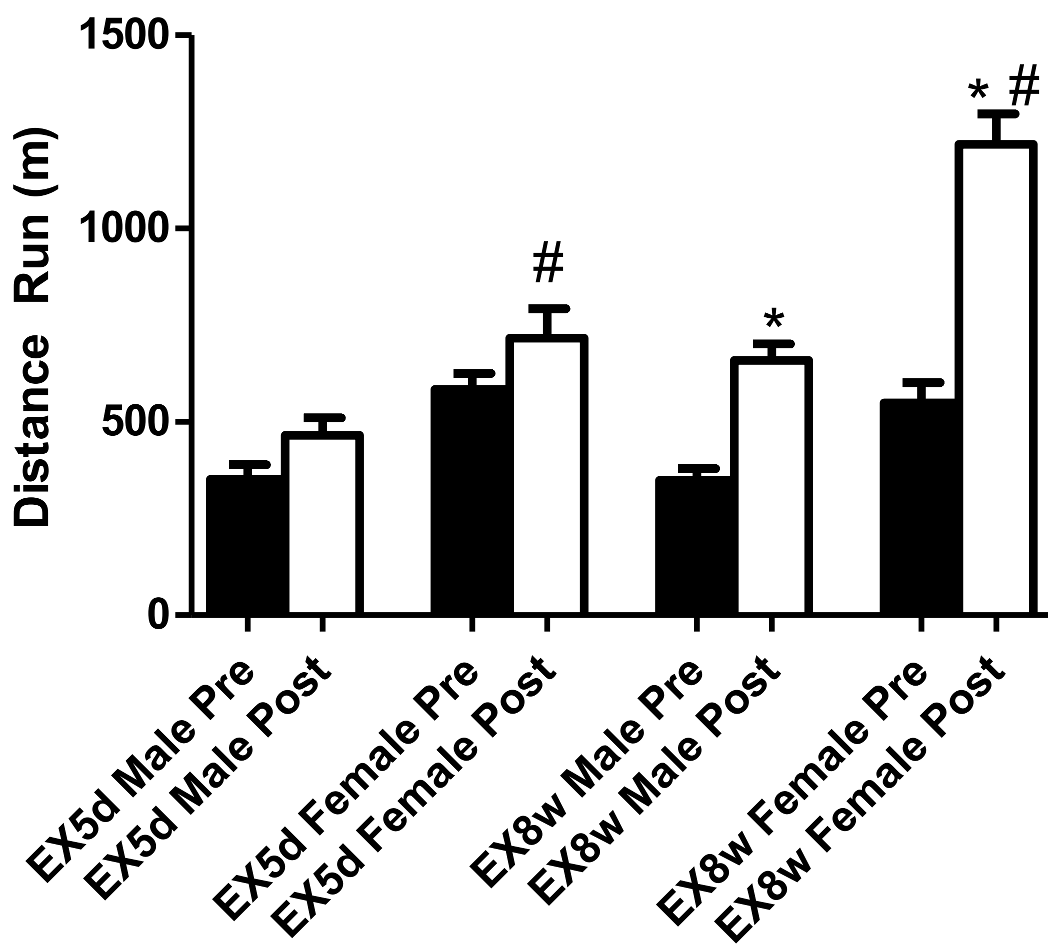
It has been suggested that activation of calpain, a Ca^{2+}-activated protease, can contribute to myocardial injury during IR (13), and previous research indicates that inhibition of calpain can attenuate tissue damage (20, 39, 40). Calpastatin is an endogenous inhibitor of calpain, and the ratio of calpain to calpastatin in cells affects the ability of Ca^{2+} to activate calpain. It is possible that exercise provides

cardioprotection through decreasing levels of calpain protein. However, our current data do not support this hypothesis, as there was no difference in calpain levels between any of our experimental groups (Fig. 2.3), which has also been seen previously (11). However, our results show that protein levels of calpastatin were increased in the hearts of EX5d and EX8w animals compared to Sed rats. This is again in agreement with previous research regarding the effects of short-term exercise (11), but is a novel finding for long-term exercise training. Interestingly, there was no difference in the ratio of calpain to calpastatin between male and female rats, despite differences in infarct size. These results likely indicate that this proteolytic system plays an important role in IR injury, and in exercise-induced cardioprotection, but does not seem to be involved in the sex differences observed in these effects. Additionally, the protein levels of dystrophin, a potential target for degradation by calpain which can lead to cardiac myocyte membrane instability and cell death (32), was not significantly different between any of the experimental groups.

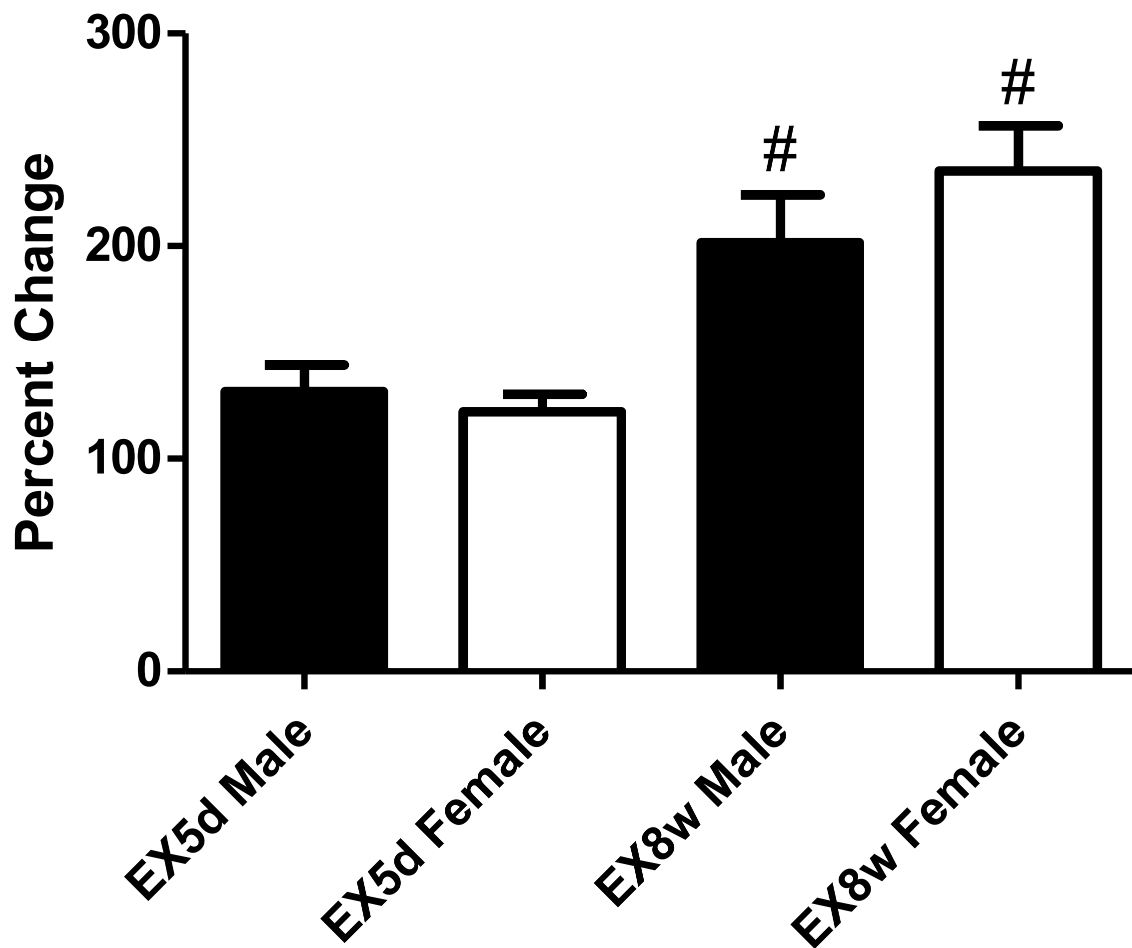
Conclusion. In summary, we have demonstrated that both short-term and long-term endurance exercise training significantly reduced infarct size in both sexes, but protection against infarction was augmented with additional exercise training (long-term) in female rats but not male rats. These data indicate that exercise-induced reductions in infarct size are sex-dependent, with females being more protected against IR injury by exercise. Sex difference in cardioprotection conferred by endurance exercise may be related to increased expression of the sarcolemmal K_{ATP} subunits, $K_{ir}6.2$ and SUR2A.

Figure 2.1. Effects of exercise training.

A: Distance run (meters) for maximal exercise test. *Pre*= Pre-training max test. *Post* = Post-training test. Data are means ± SEM for n= 10 animals per group. Group abbreviations are as described in METHODS. *= Significant difference (P < 0.05) in post-training value compared to Pre-training value for that sex and exercise group. # = Significant difference (P <0.05) in female compared to male for that same condition.



B: Change in maximal running capacity (% change in distance in post-training test vs. pre-training test). Data are means ± SEM for n= 10 animals per group. # = Significant difference (P <0.05) in female compared to male.



C: Plantaris muscle Citrate synthase activity. Individual data points for each animal are shown along with mean and SEM for n= 10 animals per group.

* Significant difference (P < 0.05) compared to SED.

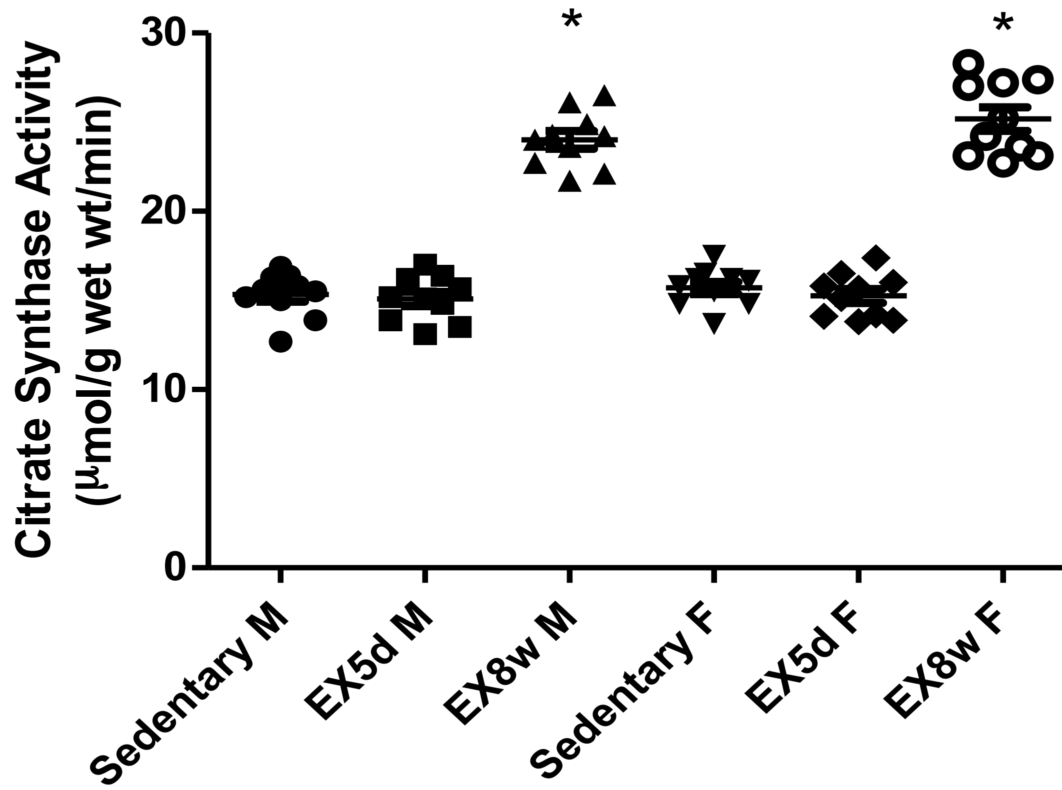
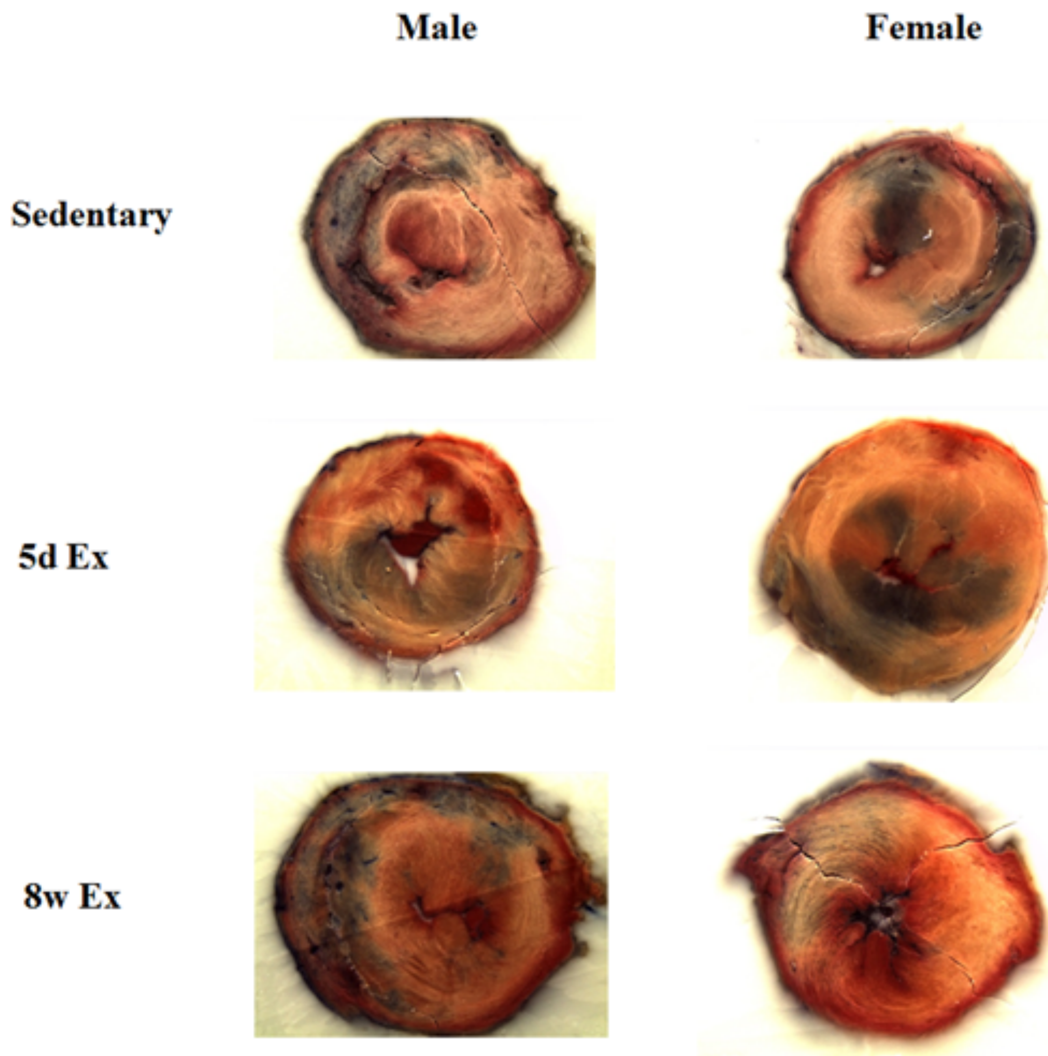
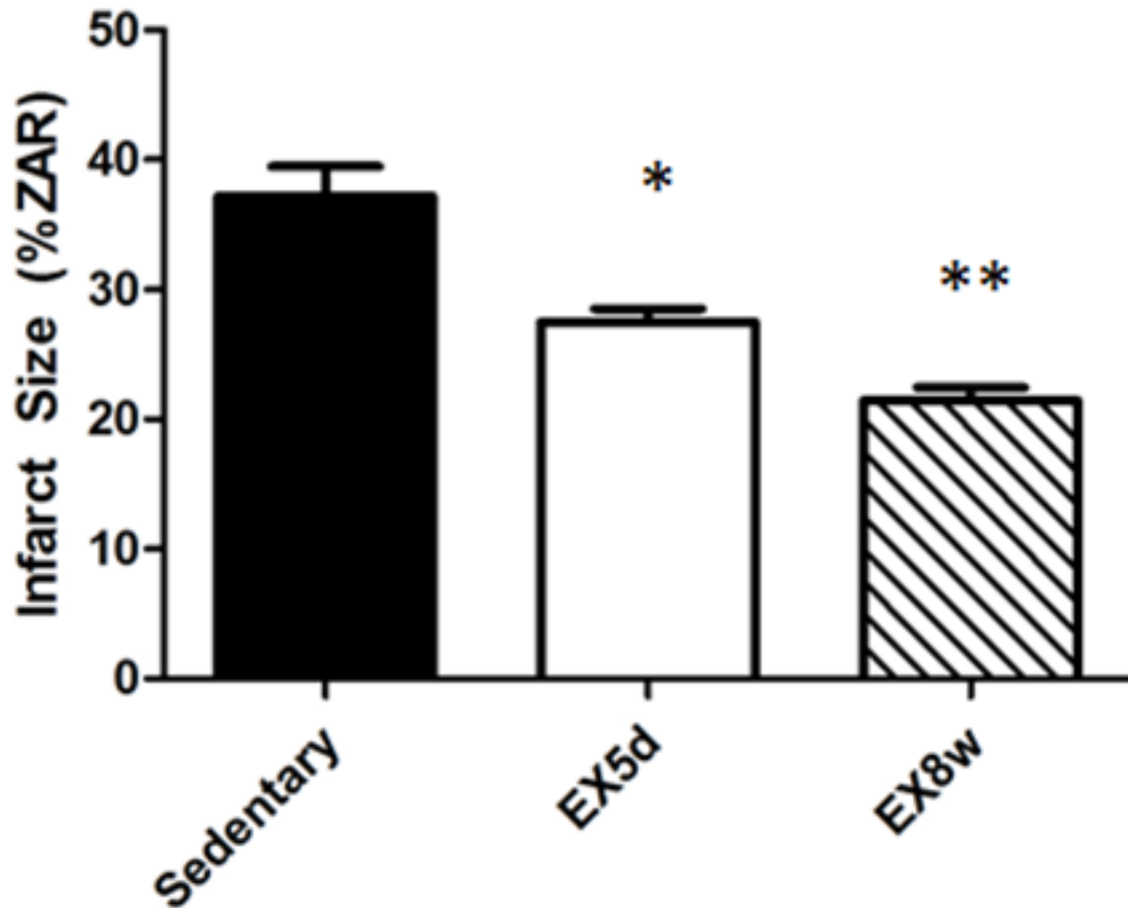


Figure 2.2. Myocardial infarct size.

A: Representative digital images of stained heart slices used to quantify infarct size as a percentage of the ischemic zone at risk (ZAR). Regions of the slice stained blue were defined as the unaffected area; pink-red tissue represents area not receiving flow (i.e. ZAR); white tissue represents infarct.



B: Quantification of average infarct size expressed as a percentage of ischemic zone at risk (ZAR) for sedentary (Sed) and Exercise Trained (EX5d and EX8w) groups (n=5/group). Infarcts were significantly smaller in both Ex groups than in Sed hearts (* P < 0.05). Infarcts were significantly smaller in Ex8w than in Ex5d hearts (** P < 0.05).



C: Infarct size by sex. Hearts from female rats had smaller infarcts compared to male hearts in every experimental group (# P < 0.05). Hearts from EX5d female rats had significantly smaller infarcts than Sed female rats (* P < 0.05), and EX8w females had significantly smaller infarcts than EX5d females (** P < 0.05). Hearts from EX5d male rats had significantly smaller infarcts than Sed male rats (* P < 0.05). Infarct size in EX8w males was not significantly different than EX5d males.

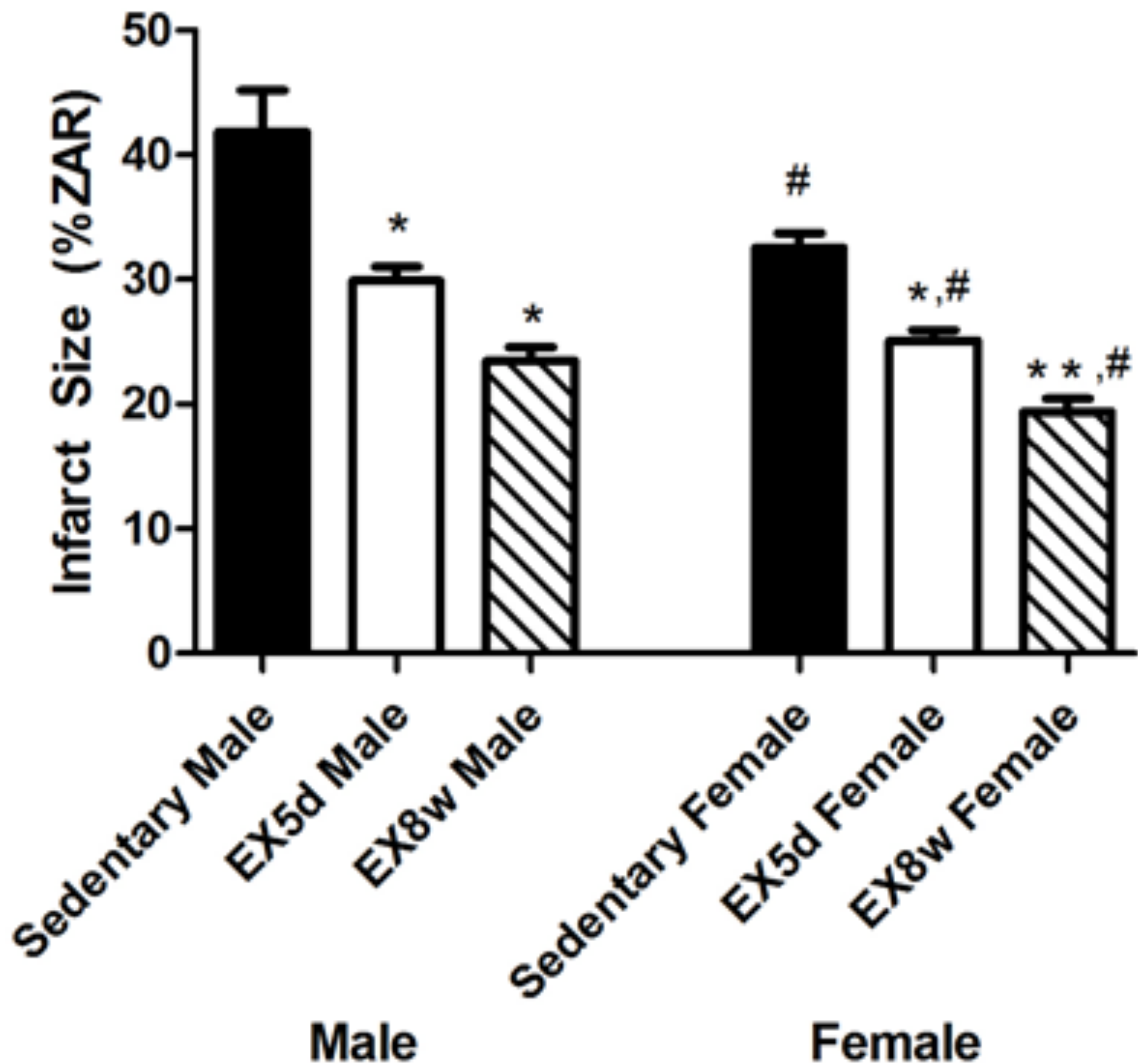
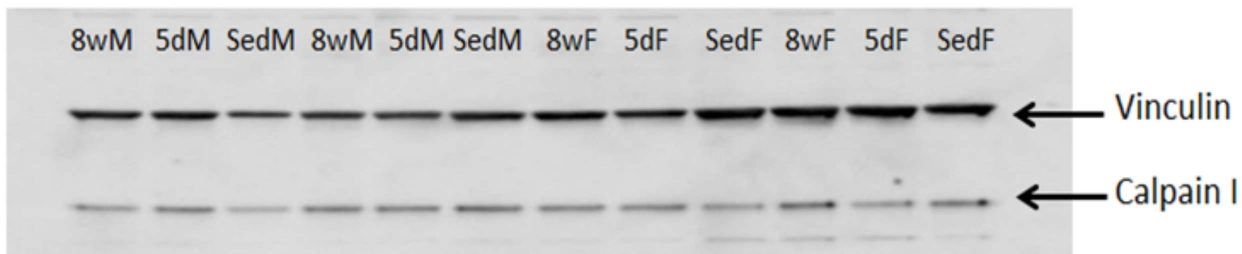
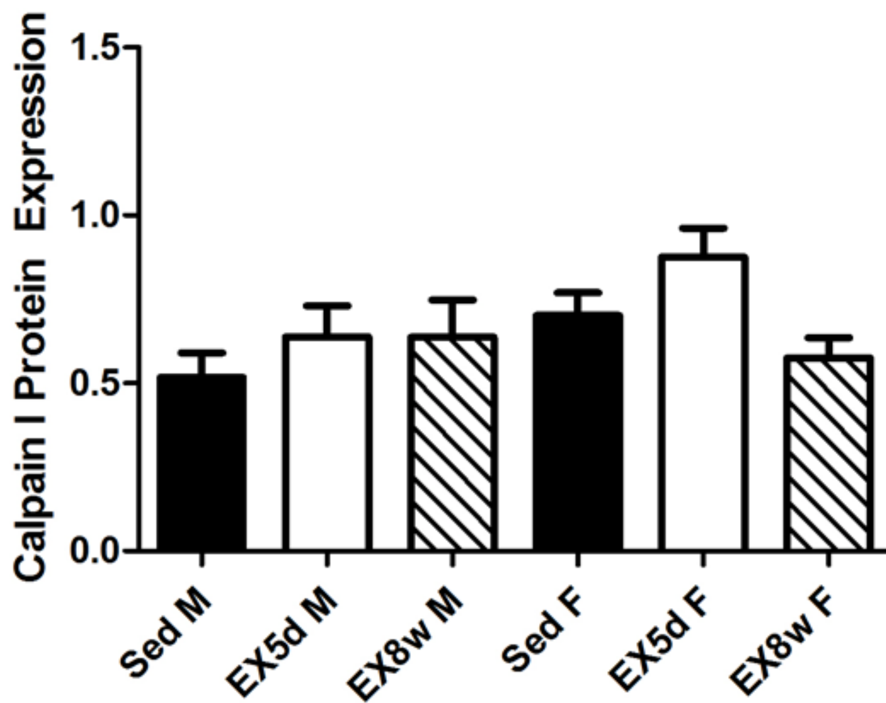


Figure 2.3. Analysis of calpain I protein and calpastatin protein.

A: Representative Western blot bands and quantification of calpain I protein from all experimental groups, normalized to vinculin. Note that calpain I protein was not altered in any of the experimental groups. Data are integrated from multiple Western blots.

A





B: Representative Western blot bands and quantification of calpastatin protein, an endogenous inhibitor of calpain, from all experimental groups (normalized to vinculin). * = significantly different from sedentary ($P < 0.05$). Calpastatin levels did not differ between exercised hearts from male or female rats. Data are integrated from multiple Western blots.

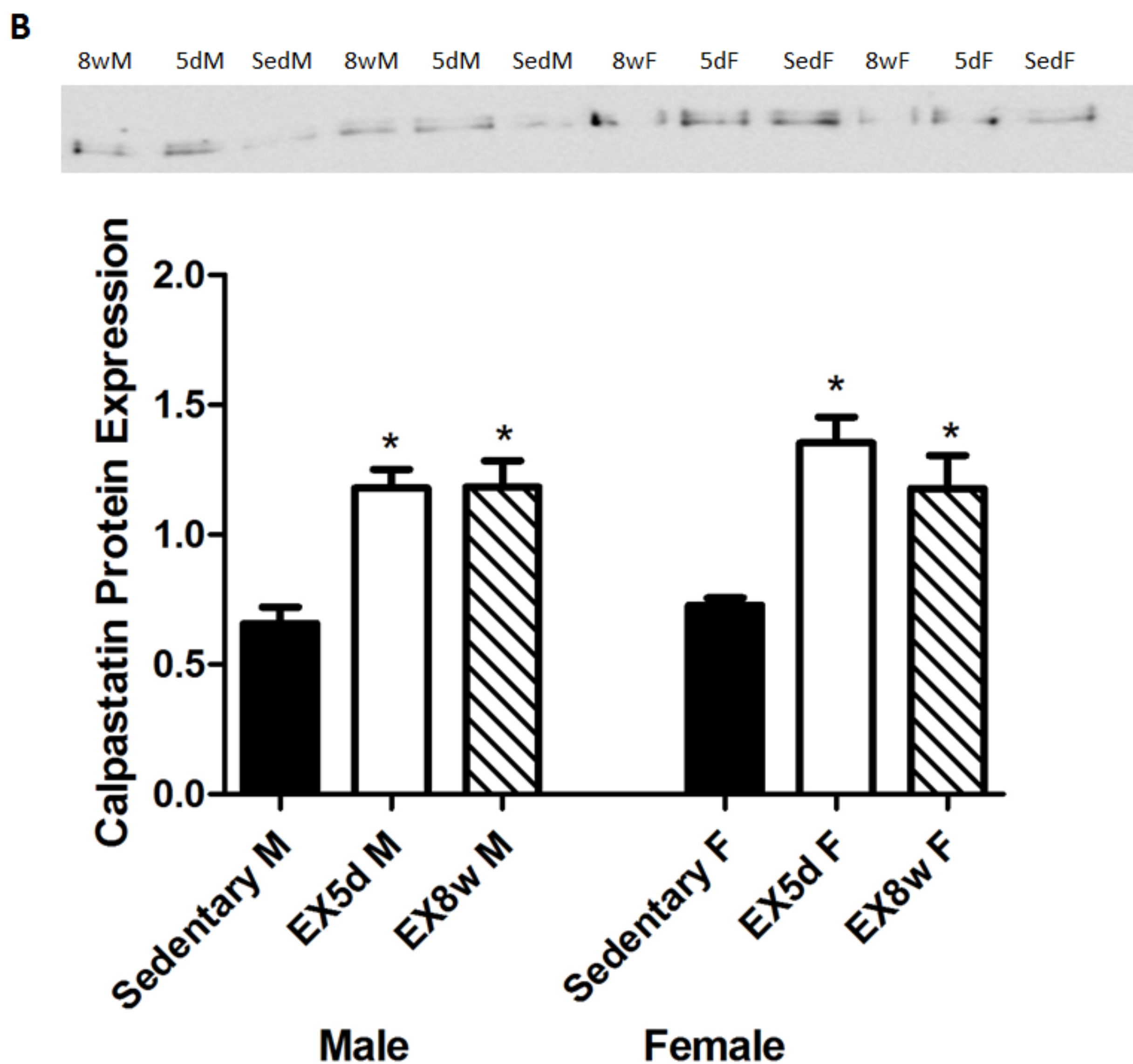
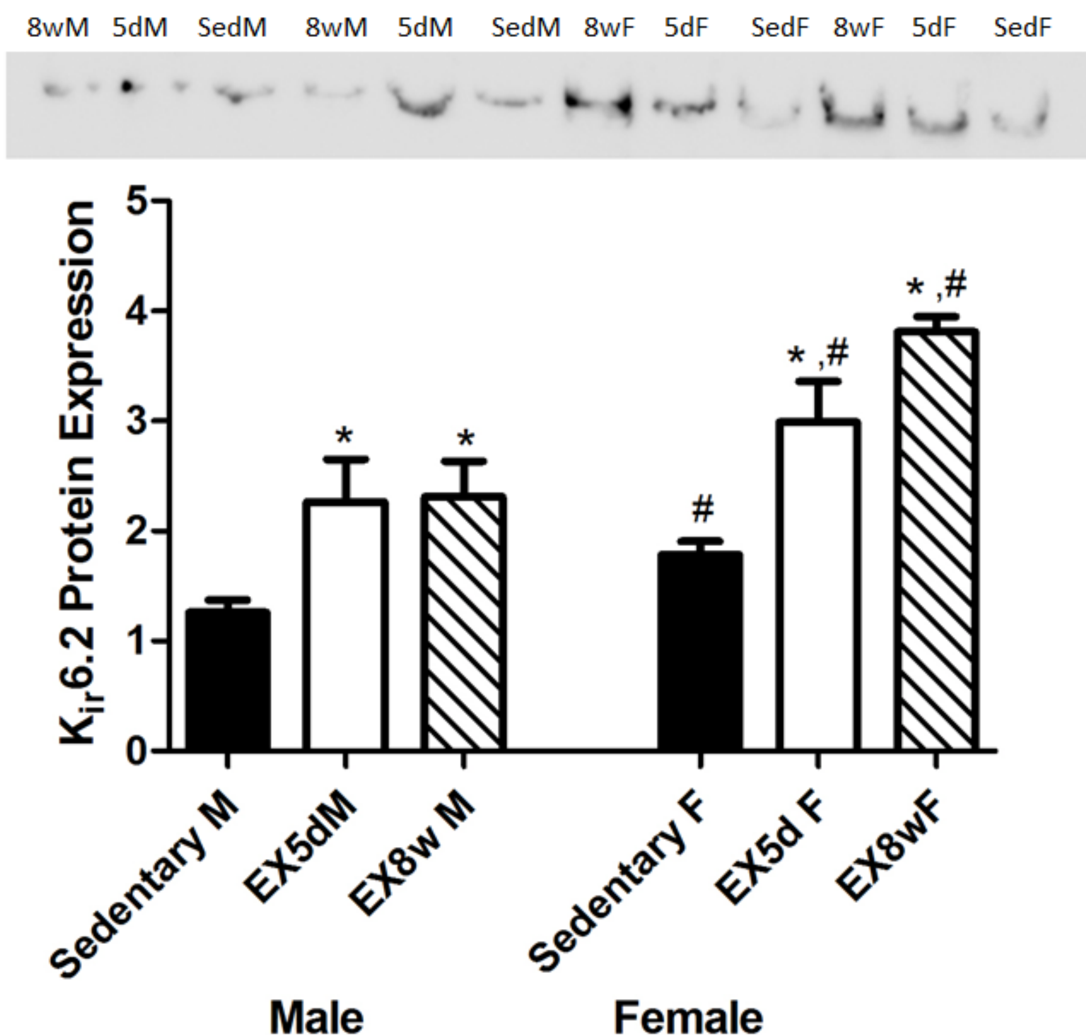
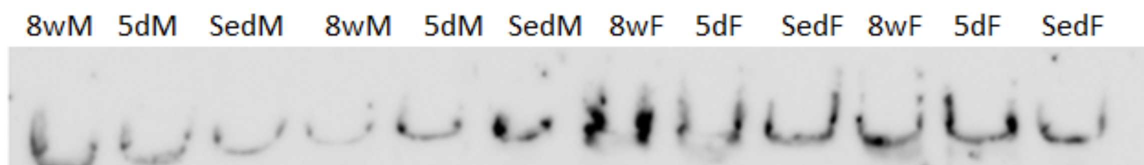


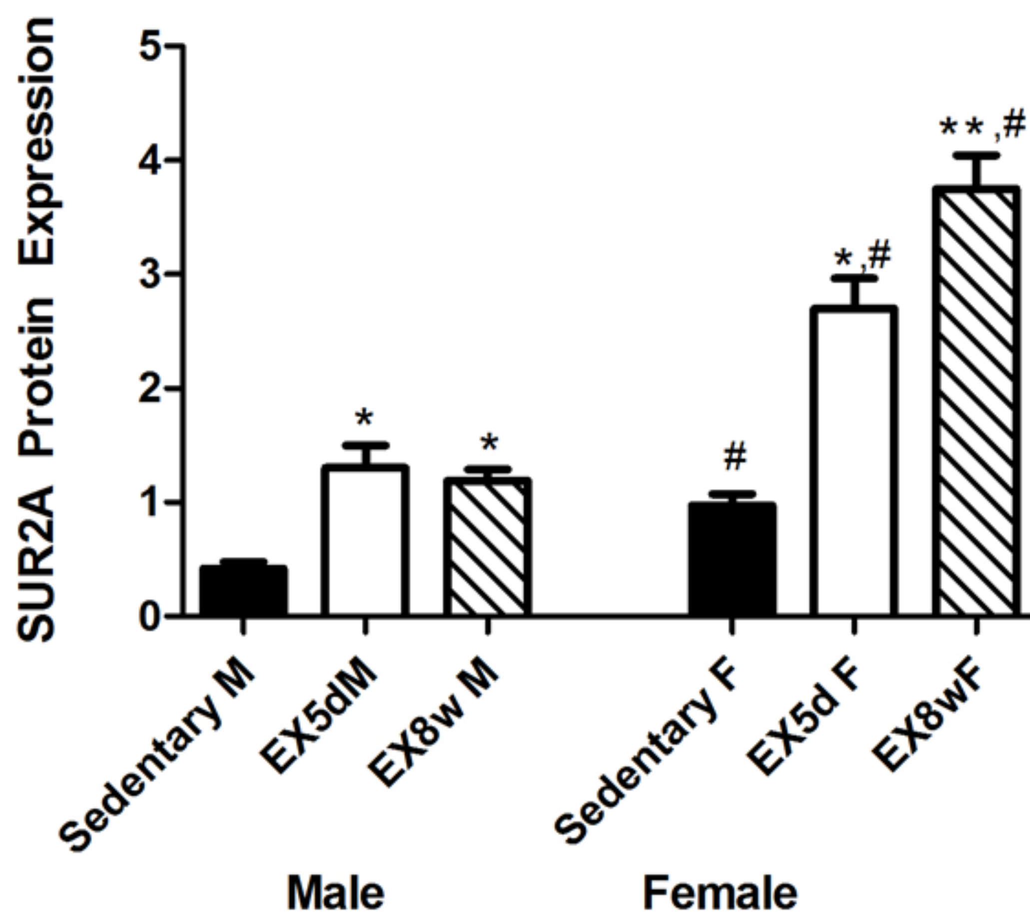
Figure 2.4. Myocardial ATP-sensitive K^+ (K_{ATP}) channel subunit expression for all experimental groups.

A: Representative bands and quantification for the pore-forming subunit of the K_{ATP} channel, $K_{ir}6.2$ (normalized to vinculin). Female $K_{ir}6.2$ protein levels were significantly higher than male protein levels at every time point (# $P < 0.05$). Myocardial $K_{ir}6.2$ protein levels from EX5d and EX8w animals were higher than Sed hearts, regardless of sex (* $P < 0.05$). Myocardial $K_{ir}6.2$ protein levels from EX8w females trended towards significant difference compared to EX5d females ($P = 0.0725$), while EX8w male protein levels were identical to EX5d males. Data are integrated from multiple Western blots.



B: Representative bands and quantification for the sulphonylurea receptor (SUR2A), the regulatory subunit of the K_{ATP} channel (normalized to vinculin). Female SUR2A protein levels were significantly higher than male protein levels at every time point (# $P < 0.05$). Myocardial SUR2A protein levels from EX5d and EX8w animals were higher than Sed hearts, regardless of sex (* $P < 0.05$). Myocardial SUR2A protein levels from EX8w females were significantly higher compared to EX5d females (**$P < 0.05$), while EX8w male protein levels were similar to EX5d males. Data are integrated from multiple Western blots.





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CHAPTER III

Role of inhibition of matrix metalloproteinase-2 in exercise-induced cardioprotection against ischemia-reperfusion injury

Introduction

Coronary artery disease remains a major cause of death worldwide (3, 36). Myocardial infarction (MI), the major pathology associated with coronary artery disease, is commonly the result of an ischemia-reperfusion (IR) insult to the myocardium. Although many factors may contribute to cellular injury during IR, growing evidence suggests that reactive oxygen species (ROS) production and cytosolic free Ca^{2+} overload are major contributors (6, 17). Given the high rate of coronary artery disease and the associated cardiac injury, developing measures to protect the myocardium against IR injury is of extreme importance. To that end, it is well established that repeated bouts of endurance exercise training provides robust cardioprotection against IR injury (21). As few as 3-5 days consecutive exercise training significantly improves myocardial tolerance to IR injury, a process which is commonly referred to as exercise-induced cardioprotection (12).

Endurance exercise training promotes numerous cellular adaptations in cardiac myocytes (13). However, the precise cellular adaptations that result in cardioprotection against IR injury remain a topic of debate, and numerous putative mediators have been proposed (reviewed in 22). Of these proposed mechanisms, considerable attention has been given to the role of the proteolytic system (9, 10, 23). Surprisingly, despite evidence for a clear role of the proteolytic systems in IR injury, the effects of exercise on this system have not been fully investigated. For example, recent work has linked IR injury to the activation of the matrix metalloproteinase-2 (MMP-2), a zinc-dependent endopeptidase that is activated in response to oxidative stress (reviewed in 14). To date, there has been little attention paid to inhibition of

MMP-2 activity following IR injury as a possible mechanism of exercise-induced cardioprotection.

Matrix metalloproteinases (MMPs) are known to be involved in the degradation of the extracellular matrix in both physiological and pathological conditions, and play a key role in scar formation following myocardial infarction (29). While this extracellular role of MMP-2 in myocardial infarction has been well established, recent studies have suggested an additional, intracellular, role of MMP-2 activation in IR injury (Fig. 3.1)(7, 14). MMP-2, thought to be activated by oxidative stress during IR (34), has been shown to have a number of intracellular proteolytic targets in cardiac myocytes, among them cardiac troponin I (TnI) (4, 35), myosin light chain-1 (26), α-actinin (30), and titin (1). Degradation of these crucial myofilament proteins by MMP-2 would potentially account for the myocardial contractile dysfunction observed following IR injury (11).

MMP-2 activity is regulated by many factors, including intracellular oxidative stress (34) and phosphorylation (24, 25). Tissue inhibitor of metalloproteinases (TIMPs) are major cellular inhibitors of the MMPs, and TIMPs have been found in cardiomyocytes (2). It is thus possible that exercise provides cardioprotection against IR injury through 1) decreasing MMP-2 protein levels, or 2) inhibiting MMP-activation by increasing the protein levels of the TIMPs. However, no study to date has specifically investigated the effect of endurance exercise training on the protein levels of MMP-2 and TIMP-2 following IR injury. Therefore, the specific aim of this study was to determine the effects of exercise training on MMP-2 and TIMP-2 protein

expression. We hypothesized that hearts from exercised rats would have decreased levels of MMP-2 and increased levels of TIMP-2 compared to sedentary rats.

Methods

Animals. Three-month-old male and female Sprague-Dawley rats were obtained from Envigo (locally maintained colony in Madison, WI, USA). We selected Sprague-Dawley rats for this experiment because it is a well-accepted model for studying exercise-induced myocardial adaptations and myocardial responses to IR. Upon arrival, rats were randomly assigned to one of three groups: a five day exercise training group (EX5d; n=20), an eight week exercise training group (EX8w; n=20), or a sedentary control group (Sed; n=20). Each group contained equal numbers of males and females. All animals were housed in pairs in clear plastic cages in the same temperature controlled facility with a 12:12-h light/dark reverse cycle under the care of a full-time veterinarian. The rats were provided standard rat chow and water *ad libitum*. All animals were killed at least 24 h after the last training (or handling control) session. The experimental protocol and euthanasia were approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee (IACUC) and followed the guidelines established by the American Physiological Society for the use of animals in research.

Treadmill exercise training. Training was started one week after the rats' arrival to reduce the stress associated with shipping and acclimation to a new location. Animals assigned to the exercise groups were habituated to treadmill exercise on a daily basis for 5 consecutive days. This habituation period involved a gradual increase in running time beginning with 5 min/day (first day) and ending with 25 min/day (5th day) at 12 m/min at 0% grade, an amount below that which is likely to produce a significant change in aerobic capacity.

Following the habituation period, animals were given 2 days of rest and then underwent a speed-ramped test to assess maximal running capacity. This protocol was previously developed to pattern clinical stress tests as a standard screen for exercise capacity (15). Briefly, rats exercised on a motorized treadmill set at a constant grade of 15% and an initial speed of 18 m/min. The speed was progressively ramped 1 m/min every 2 min until exhaustion occurred. Exhaustion was defined as the third time the rat remained in contact with the shock grid for 2 s. Total distance run was calculated from belt speed (m/min) and duration (min) of the run.

Following the initial max running test, animals in the EX8w group were run on a treadmill for 60 min/day, 5 days/week, for 8 weeks. The treadmill speed was gradually increased every few weeks, with females running at the following maximal intensities: weeks 1-2: 19 m/min, weeks 3-4: 21 m/min, weeks 4-8: 23 m/min. Male rats ran at the following maximal intensities: weeks 1-2: 15 m/min, weeks 3-4: 18 m/min, weeks 4-8: 21 m/min. This training protocol was developed to maximize compliance and reduce animal stress based off of previous exercise studies in our laboratory, and to account for the innate differences in exercise capacity between male and female rats. At the conclusion of the 8-week exercise protocol rats underwent a second maximal exercise capacity test. Sed rats were placed on the treadmill for 5 min/day twice per week in order to control for the effects of handling.

After the initial max running test, animals in the EX5d group were trained for 3 days for the same duration and speed as the EX8w rats, immediately followed by a second max running test, amounting to 5 consecutive days of exercise training. See Figure 3.2 for a timeline of the exercise training protocols and IR procedure.

Ischemia-reperfusion protocol. Animals were deeply anesthetized using isoflurane gas. After left thoracotomy, a suture was threaded through the tissue surrounding the left anterior descending coronary artery close to its origin (3-5 mm distal to the aorta). Transient regional ischemia was induced by tightening the suture to occlude the artery with the use of a removable snare. Electrical activity of the heart (continuous ECG monitoring) and visualization of cyanotic heart tissue were used to confirm the presence of ischemia. Ischemia lasted for 45 min. At the end of ischemia, the snare was loosened and reperfusion ensued for 24 hr while animals recovered in their cage. The durations of ischemia and reperfusion were chosen to ensure myocardial infarction, and the protocol was adapted from previous work conducted at the Cardiovascular Research Center at UW-Madison (16). For sham surgery, left thoracotomy was performed but the coronary artery was not occluded. Five animals developed severe fibrillation or did not survive the IR surgery (four Sed males, one EX8w female), and their tissue was discarded from the study. Following euthanasia, the plantaris muscle was removed and flash frozen using liquid nitrogen for subsequent citrate synthase assay.

Measurement of infarct size. At the end of reperfusion (24 hr), the snare was retightened around the coronary artery, and 100 µl of 0.5% Evans blue dye solution was injected into the aorta and perfused through the heart. Hearts were then quickly excised, rinsed in cold saline, and sliced transversely from base to apex into slices of equal width. Each of the slices was then immersed in 100 mM phosphate-buffered saline with 0.1% triphenyltetrazolium chloride (TTC) and incubated for 10 min at 37°C. After incubation, each slice was placed in Optimal Cutting Temperature (OCT) compound, flash frozen using liquid nitrogen, and immediately stored at -80°C. Using a cryomicrotome, frozen slices were carefully

sectioned until the OCT was trimmed away, exposing the embedded heart slice (Figure 3.3). Each slice was then imaged using a digital microscope camera. The microscope lighting and magnification were kept constant throughout the procedure for experimental consistency.

Images of the slices were quantified using ImageJ imaging software, and two separate individuals performed analyses in a single-blind manner to avoid experimenter bias. The zone at risk (ZAR) was identified as the area of each slice that did not turn blue (i.e., not perfused with dye) after perfusion with the solution containing Evans blue dye. The portion of the ZAR that did not turn red in response to TTC incubation and remained white was classified as the infarct area. Any tissue that was stained blue was defined as the unaffected area (UA). ZAR and infarct size were obtained from each slice, and slices from the same heart were added together. The size of the infarct area was expressed as a fraction of the ZAR by taking the sum of the infarct area and dividing by the sum of the ZAR. Thus, all fractional infarct size values are reported as a percentage of the ZAR.

Western blots. The LV free wall was dissected from the frozen heart sections, and the ZAR and UA were carefully separated from one another (Figure 3.3). ZAR and UA samples were then homogenized separately in QIAzol Lysis Reagent (Qiagen USA, Germantown, MD, USA) using a motorized homogenizer (PowerGen 700; Thermo Fisher Scientific, Waltham, MA, USA). The homogenates were immediately frozen at -80°C and used for subsequent Western blot assay. Western blots were performed using SDS-PAGE in a 4-15% polyacrylamide gel with 20 µg of homogenate per lane. Blots were first probed using a monoclonal antibody against Vinculin (Cell Signaling Technology, Danvers, MA, USA) at a dilution of 1:1,000. After a secondary

horseradish peroxidase-conjugated goat anti-rabbit antibody (Cell Signaling Technologies) and then a chemiluminescent substrate (Bio-Rad Laboratories, Hercules, CA, USA) were applied, blots were imaged using ImageQuant LAS 4000 (GE Healthcare, Boston, MA, USA). Blots were then stripped using standard methods and re-probed for MMP-2 (Cell Signaling Technologies, catalog number 13132S, 1:1,000) and TIMP-2 (Cell Signaling Technologies, catalog number 5738S, 1:1,000). All secondary antibody used was horseradish peroxidase-conjugated goat anti-rabbit. Band density was analyzed using Image Studio Lite. Western blot band densities are reported as normalized to Vinculin.

Citrate synthase assay. Samples (65-80 mg) were cut from the frozen plantaris muscles and homogenized. Total muscle protein was determined by Bradford protein assay, and the protein concentration of all samples was equalized. Citrate synthase activity was then determined using an assay kit (Sigma-Aldrich, USA) and spectrophotometer.

Data analysis. All statistical comparisons were made using GraphPad Prism (version 7). Data were analyzed using a 2 (sex) x 3 (training group) ANOVA. When appropriate, effects of training (within sex) were evaluated with a one-way ANOVA. Values are means ± SE, and significance is reported at $p < 0.05$.

Results

Maximal exercise capacity. Changes in maximal exercise capacity, comparing pre-exercise to post-exercise max running tests, are reported as absolute change (m) and relative change (%), and are presented in Table 1. Female and Male EX8w animals had significantly higher exercise capacities compared to EX5d counterparts. Female EX8w rats had significantly higher pre and post-exercise capacities in absolute terms compared to male EX8w rats (post-training: 668.9 ± 87.0 m vs. 310.4 ± 46.5 m, respectively, $P < 0.05$). However, the relative (% change) improvement from baseline measurements (pre-exercise training) was not different between EX8w females and males (235.4 ± 21.3% vs. 201.6 ± 22.5%), suggesting an equally effective exercise training protocol between males and females.

Citrate synthase activity. Citrate synthase activity in the plantaris (a mixed muscle) was determined as a measure of endurance exercise training effect and results are shown in Table 1. Citrate synthase activity was significantly increased following 8 weeks of training in both male and female rats compared to sedentary animals (EX8w males: 24.01 ± 0.50 vs. Sedentary males: 15.33 ± 0.4 µmol·g wet wt^{-1}·min^{-1}; EX8w females: 25.18 ± 0.67 vs. Sedentary females: 15.71 ± 0.34 µmol·g wet wt^{-1}·min^{-1}, $P < 0.05$). There was no difference in citrate synthase activity between EX8w males and EX8w females, corroborating our maximal exercise capacity test results. Additionally, there was no difference in citrate synthase activity in male or female EX5d rats (15.09 ± 0.41 and 15.25 ± 0.39 µmol·g wet wt^{-1}·min^{-1}, respectively) compared to sedentary. Increased citrate synthase activity indicates that the animals were endurance trained as a result of the 8 week exercise protocol.

Infarct size. Data on infarct size, normalized for ZAR, is summarized in Figure 2. The infarct size was significantly smaller in hearts from EX5d animals compared to Sed animals (27.5 ± 1.0% vs. 37.2 ± 2.3%, $P < 0.05$; Fig. 3.4), and infarct size was significantly smaller in hearts from EX8w animals compared to hearts from EX5d animals (21.4 ± 1.0% vs. 27.5 ± 1.0%, $P < 0.05$; Fig. 3.4). The increased cardioprotection in response to additional exercise training only existed in female rats. Infarct size was significantly smaller in hearts from EX8w females compared to EX5d females (19.4 ± 1.1% vs. 25.1 ± 0.9%, $P < 0.05$; Fig. 3.4), but infarct size was not significantly different between hearts of EX8w males and EX5d males (23.5 ± 1.1% vs. 29.9 ± 1.1%; Fig. 3.4). Hearts from female rats exhibited significantly smaller infarcts compared to hearts from male rats across every experimental group (Female: Sed 32.6 ± 1.2%, EX5d 25.1 ± 0.9%, EX8w 19.4 ± 1.1%; Male: Sed 41.8 ± 3.3%, EX5d 29.9 ± 1.1%, EX8w 23.5 ± 1.1%, $P < 0.05$; Fig. 3.4). These data indicate that female rats are intrinsically more protected from IR injury than male rats, and that female rats can strengthen their level of cardioprotection against IR injury with long-term exercise training compared to that afforded by short-term exercise training.

MMP-2 protein levels. Intact MMP-2 protein levels were measured in the myocardium from the ZAR and UA using Western blot analysis. The results, displayed in Figure 3.5, reveal that MMP-2 protein content was significantly increased in the ZAR compared to the UA across all experimental groups ($P < 0.05$). There was no difference in MMP-2 protein levels between the sexes, or between groups. All protein levels were normalized to vinculin.

TIMP-2 protein levels. Myocardial protein levels of TIMP-2 are presented in Figure 3.6. TIMP-2 protein levels were significantly increased in the hearts from EX5d and EX8w rats compared to Sed rats ($P < 0.05$). There was no difference in TIMP-2 levels between male and female rats. There was no difference in TIMP-2 levels between the ZAR and UA within any experimental group. All protein levels were normalized to vinculin.

Discussion

A novel method of exercise-induced cardioprotection against ischemia-reperfusion injury. A major finding of the present study is that endurance exercise training leads to an increase in myocardial TIMP-2 (Tissue inhibitor of metalloproteinases) protein levels. While several putative mediators of exercise-induced cardioprotection against IR injury have been proposed (22), the role of the matrix metalloproteinase proteolytic system has not been investigated. We (Chapter II) and others (9, 10) have previously observed that cardioprotection appears to involve alterations in the calpain to calpastatin ratio. However, to our knowledge, this is the first study to demonstrate that both short-term and long-term endurance exercise training increase TIMP-2 levels in the myocardium. Furthermore, increased myocardial TIMP-2 protein levels were associated with improved tolerance to IR injury.

It is well-established that matrix metalloproteinases (MMPs) are involved in the degradation of the extracellular matrix in both physiological and pathological conditions, and play a key role in scar formation following myocardial infarction (29). Interestingly, recent studies have suggested an additional, intracellular, role of MMP-2 activation in IR injury (7, 14). Previous work has demonstrated that MMP-2 has a number of intracellular proteolytic targets in cardiac myocytes, among them cardiac troponin I (TnI) (4, 35), myosin light chain-1 (26), α-actinin (30), and titin (1). Degradation of these crucial myofilament proteins by MMP-2 would potentially account for the myocardial stunning and cell death following IR injury (11). Therefore, since MMP-2 activation contributes to cardiac myocyte dysfunction and death, inhibition of MMP-2 would confer cardioprotection from IR.

Exercise increases TIMP expression. TIMPs are the major cellular inhibitors of MMPs (2), and TIMP-2 has been shown to have preferential binding for MMP-2 (32). Evidence suggests that an imbalance in the ratio between MMPs and TIMPs serves as a contributor to myocardial injury during and/or following IR (27, 28). During normal physiological conditions, the ratio between these proteins is tightly regulated in order to control myocardial proteolytic activity. However, under pathological conditions such as IR, this balance is thought to be perturbed and can lead to myocardial damage, such as decreased contractile function and infarction (14). Our current findings are among the first to demonstrate that endurance exercise training increases the myocardial protein levels of TIMP-2. Recently, Szabo et al. (31) reported that myocardial TIMP-2 levels were increased with wheel running in rats, but this study only utilized voluntary exercise and female rats. Our results strongly suggest exercise-induced increases in TIMP-2 expression may inhibit IR-induced MMP-2 activity and thus provide a potential mechanism that contributes to exercise-induced cardioprotection against IR injury.

Another potential mechanism through which endurance exercise training provides cardioprotection against IR injury could be by promoting a decrease in myocardial levels of MMP-2, as this would change the ratio of MMP-2:TIMP-2. Interestingly, our results indicate that, while IR itself increased MMP-2 levels in ischemic areas compared to non-ischemic areas, these levels were not different in exercise trained compared to sedentary hearts (Figure 3.5). Previous work by Posa et al. (20) found that exercise resulted in decreased levels of MMP-2 protein in the plasma following IR, but they did not assess levels in the myocardial tissue. It is well known that MMP-2 has extracellular functional activity, so MMP protein levels are often assayed in the extracellular fluid or in the plasma (29). However, the intracellular actions of

MMP-2 in IR injury do not require that MMP-2 be elevated in the extracellular fluid. Our results suggest that, if exercise training affects the role of MMP-2 in intracellular IR injury, it does not do so by changing MMP-2 protein expression.

MMP-2 is initially synthesized as an inactive, full-length 72-kD protein that must be activated by conformational changes. Therefore, it is likely that increased MMP-2 activity, not necessarily protein levels, contributes to myocardial damage (14). MMP-2 activity is regulated by many factors, including intracellular oxidative stress (34) and phosphorylation (24, 25). It is plausible that exercise provides cardioprotection through decreased MMP-2 activity and not by decreased protein levels, but MMP-2 activity was not directly tested in the current study. Furthermore, decreased MMP-2 activity would likely be the direct result of increased TIMP-2 levels, the protein's endogenous inhibitor, and not through a modification of the MMP-2 protein. This would corroborate our hypothesis that exercise confers cardioprotection through increasing TIMP-2 levels in the myocardium, but requires further investigation.

Additionally, it should be noted that although females exhibited smaller myocardial infarct size compared to male counterparts (Figure 3.4), we detected no difference between males and females in the effects of exercise on TIMP-2 levels. The role exercise has in providing improved tolerance against IR injury is likely multi-factorial, and additional, separate mechanisms may account for the sex differences observed in the study.

Ischemia-reperfusion increases MMP-2 levels in zone at risk. Another significant contribution of our current work is the discovery that protein levels of MMP-2 were higher in the ZAR compared to the UA in hearts subjected to IR injury, and this finding was true across all

experimental groups. The majority of previous research conducted on the proteolytic effects of MMPs has focused on the extracellular matrix proteins (29). Interestingly, there is emerging evidence that suggests that the myocardial injury associated with IR caused by MMP-2 activation may actually occur primarily within the myocyte (8, 34, 35). Various studies have provided compelling evidence for the localization of MMP-2 to the sarcomere of cardiac myocytes (28, 35) and other subcellular compartments, including the mitochondria (18, 33, 35), which strongly indicates the important role of the MMPs in myocardial stunning and infarction following IR. However, since most previous studies of MMP involvement in IR injury have been conducted using the global ischemia model, there has not been a definitive demonstration that MMP-2 is upregulated in the ischemic area compared to the non-ischemic area of the same heart.

Our findings, that MMP-2 protein levels were increased in the ZAR compared to the UA, supports the notion that MMP-2 has intracellular roles following IR, and is not simply localized to the extracellular matrix. Although it is probable that MMP-2 levels were increased in the extracellular fluid surrounding the ZAR compared to the UA following IR, our handling of the samples (washing in phosphate buffer) would likely minimize these differences. Intracellular levels of MMP-2, however, would not be affected by our handling of the tissue. Therefore, we can safely conclude that our data suggest that intracellular levels of MMP-2 are increased following IR, which may contribute to myocardial injury.

Summary and conclusion. This is one of the first studies to show that exercise training leads to increased myocardial TIMP-2 levels. Furthermore, to our knowledge, this is the first

study to show that TIMP-2 levels are increased in response to both short and long-term exercise training. It has been well-established that short-term exercise training, in addition to long-term training, improves myocardial tolerance to IR. Our data suggest a novel mechanism, increased levels of TIMP-2, which contributes to this cardioprotection.

Another major finding of the current study was the localization of increased levels of MMP-2 protein to the ZAR. This finding corroborates other work suggesting the important intracellular roles of MMP-2 in IR injury. Although its role in myocardial IR injury is becoming clearer, MMP-2's importance in cellular function, in both health and disease, requires further work.

Figure 3.1. Role of MMP-2 in ischemia-reperfusion injury in cardiac myocytes.

Generation of reactive oxygen species during ischemia-reperfusion activates MMP-2 within cardiac myocytes. The activated MMP-2 degrades intracellular proteins including TnI, leading to contractile dysfunction. MMP-2 activation can also lead to apoptotic and/or necrotic cell death. The degree of intracellular damage is related to the duration of ischemia. Adapted from Kandasamy et al., 2010 (14).

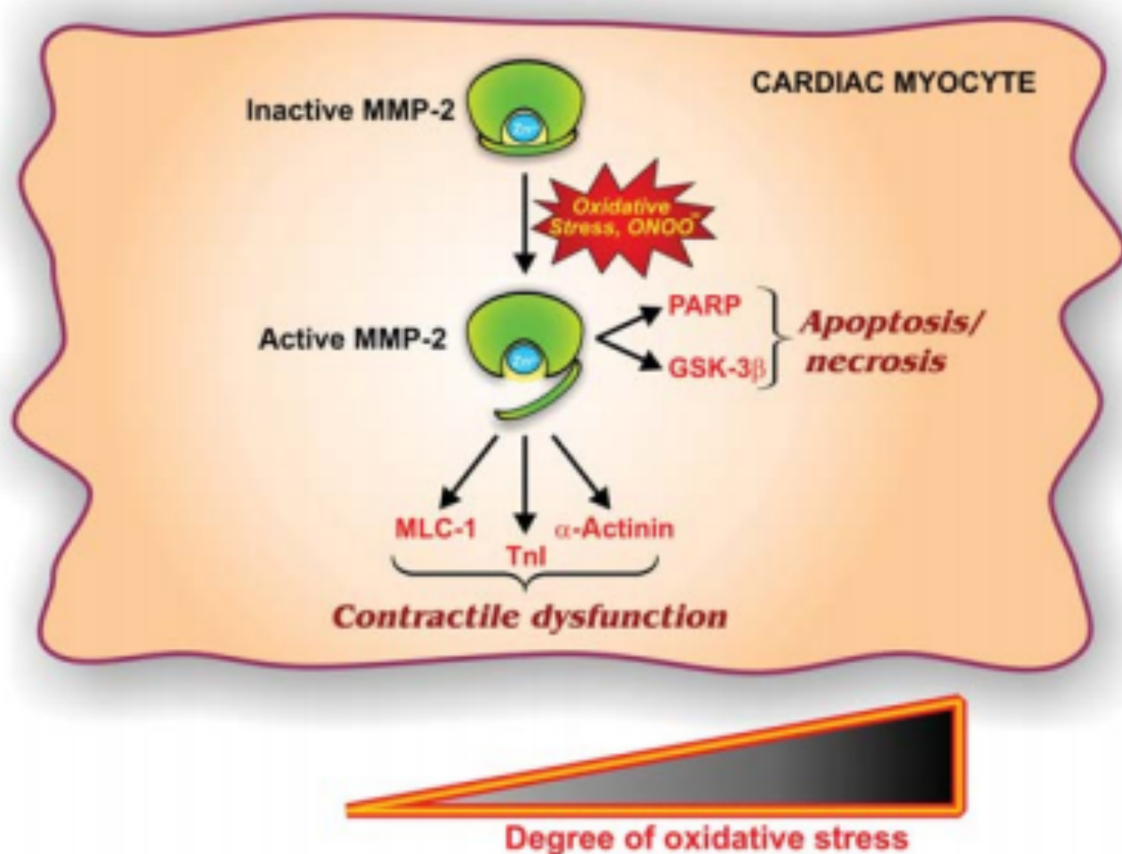


Figure 3.2. Timeline of Exercise Training

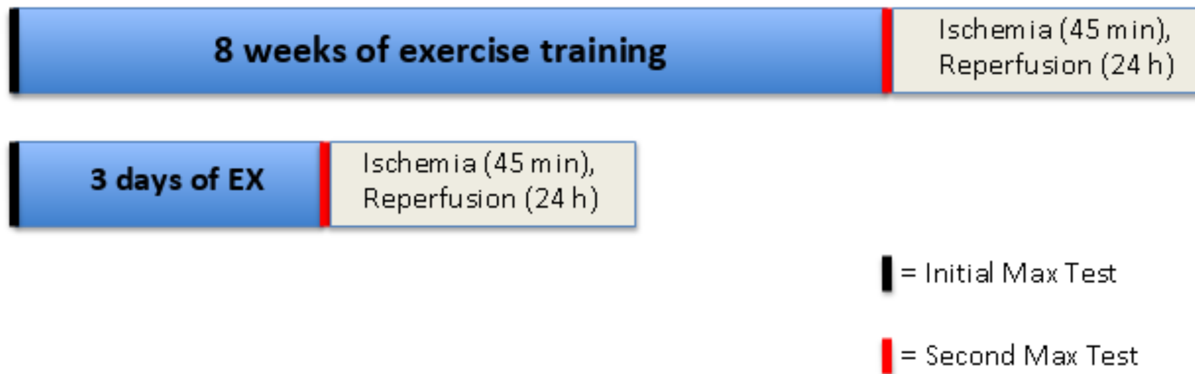
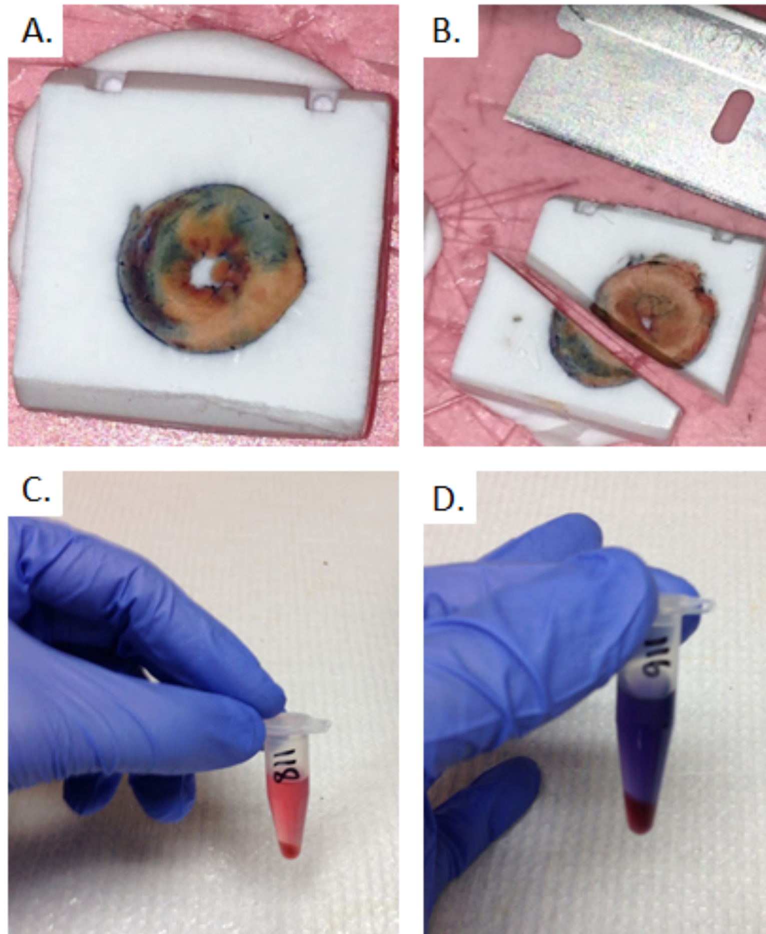


Figure 3.3. Preparation of Frozen Heart Slices



A: Heart slice embedded in OCT, exposed after trimming away OCT on surface with cryomicrotome.

B: Embedded heart slices were dissected in the cryomicrotome to ensure samples were kept cold the entire time. Samples were separated into the ZAR and the UA.

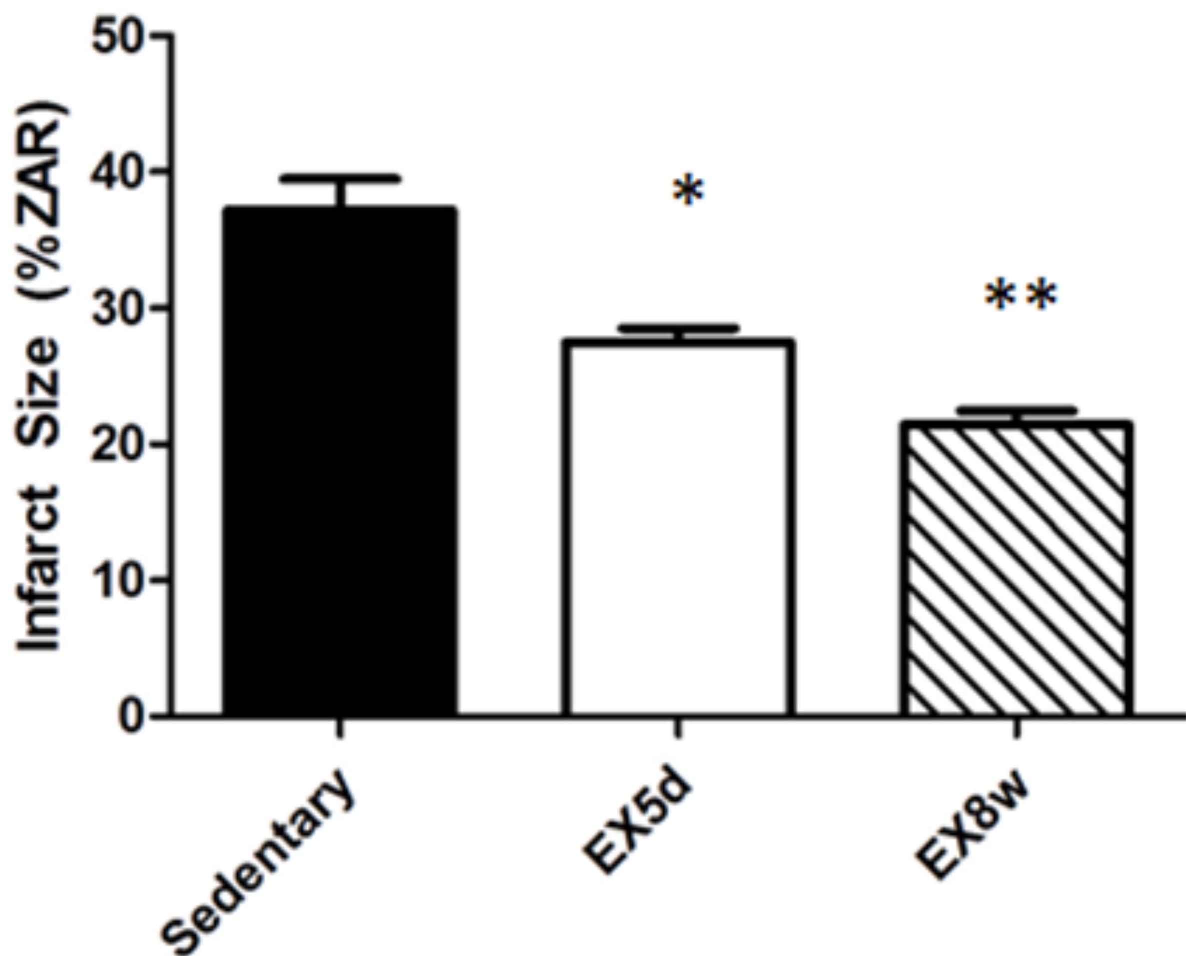
C: Homogenized heart sample from UA. Notice the lack of Evans blue dye in the homogenate, indicating accurate dissection.

D: Homogenized heart sample from ZAR. Notice dark blue coloring, indicating the presence of Evans blue dye and an accurate dissection.

Figure 3.4. Myocardial infarct size.

A: Quantification of average infarct size expressed as a percentage of ischemic zone at risk (ZAR) for sedentary (Sed) and Exercise Trained (EX5d and EX8w) groups (n=5/group). Infarcts were significantly smaller in both Ex groups than in Sed hearts (* P < 0.05). Infarcts were significantly smaller in Ex8w than in Ex5d hearts (** P < 0.05). Referenced from Chapter II.

A.



B: Infarct size by sex. Hearts from female rats had smaller infarcts compared to male hearts in every experimental group (# P < 0.05). Hearts from EX5d female rats had significantly smaller infarcts than Sed female rats (* P < 0.05), and EX8w females had significantly smaller infarcts than EX5d females (** P < 0.05). Hearts from EX5d male rats had significantly smaller infarcts than Sed male rats (* P < 0.05). Infarct size in EX8w males was not significantly different than EX5d males. Referenced from Chapter II.

B.

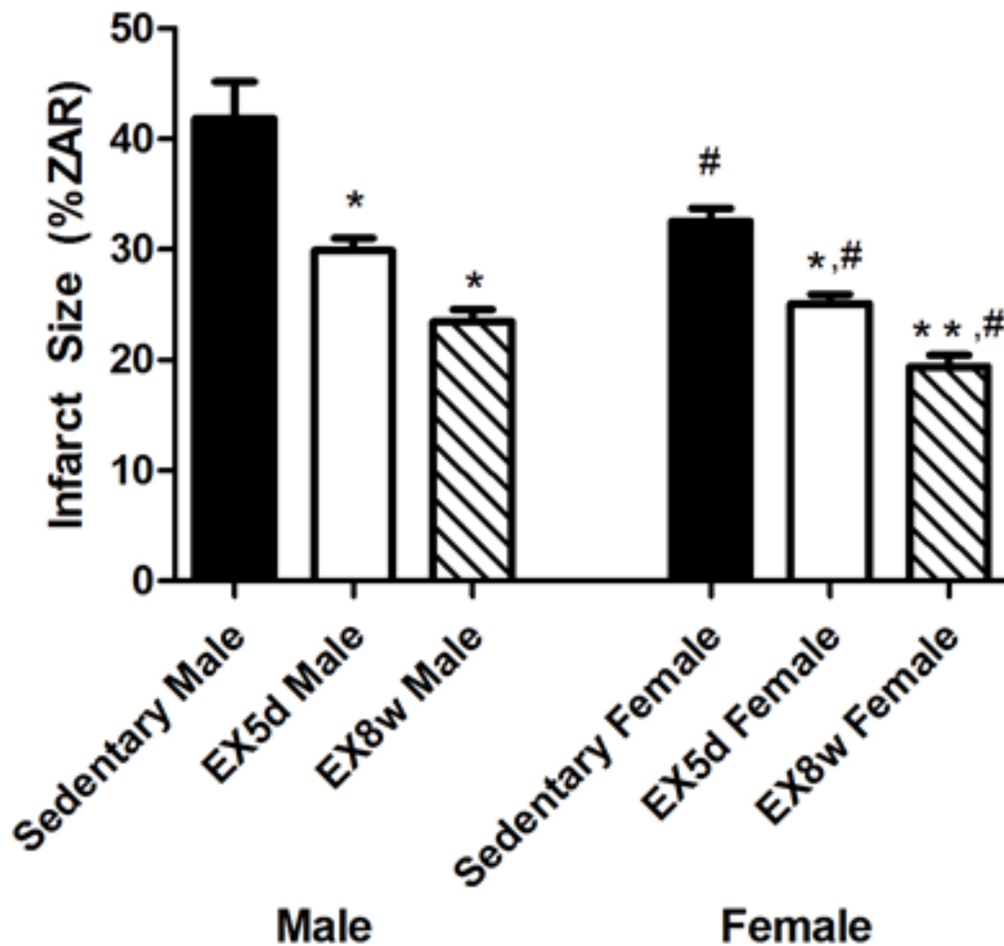


Table 3.1. ***Effects of exercise training***

Group	Δ DIST (m)	Δ DIST (%)	Citrate synthase activity (µmol • g wet wt^{-1} • min^{-1})
Sedentary			
Male	-	-	15.33 ± 0.40
Female	-	-	15.71 ± 0.34
EX5d			
Male	103.7 ± 36.2	131.7 ± 12.4	15.09 ± 0.41
Female	132.5 ± 52.2	122.2 ± 8.2	15.25 ± 0.39
EX8w			
Male	310.4 ± 46.5*	201.6 ± 22.5*	24.01 ± 0.50*
Female	668.9 ± 87.0*,#	235.4 ± 21.3*	25.18 ± 0.67*

Values are means ± SEM. Response to 5 days (EX5d) or 8 weeks (EX8w) of exercise training (n=10/group/sex). Change in maximal running capacity (Δ DIST) is a pre to post training comparison, and is expressed as either absolute change (m) or percent change (%). Maximal running capacity was significantly improved from 5d to 8w in both males and females (*P < 0.05). EX8w Females exhibited a greater absolute (m) change in maximal running capacity compared to EX8w males (# P < 0.05), but there was no difference in relative (%) change. Plantaris muscle Citrate synthase activity was significantly increased (P < 0.05) in EX8w animals compared to EX5d and Sed animals.

Figure 3.5. Myocardial MMP-2 protein levels in the ZAR and UA for all experimental groups.
A: Representative Western blot bands and quantification of MMP-2 protein from all experimental groups, separated into the ZAR and UA, and normalized to vinculin. Note that MMP-2 protein levels were significantly higher in the ZAR compared to the UA across all experimental groups (P < 0.05). Data are integrated from multiple Western blots.

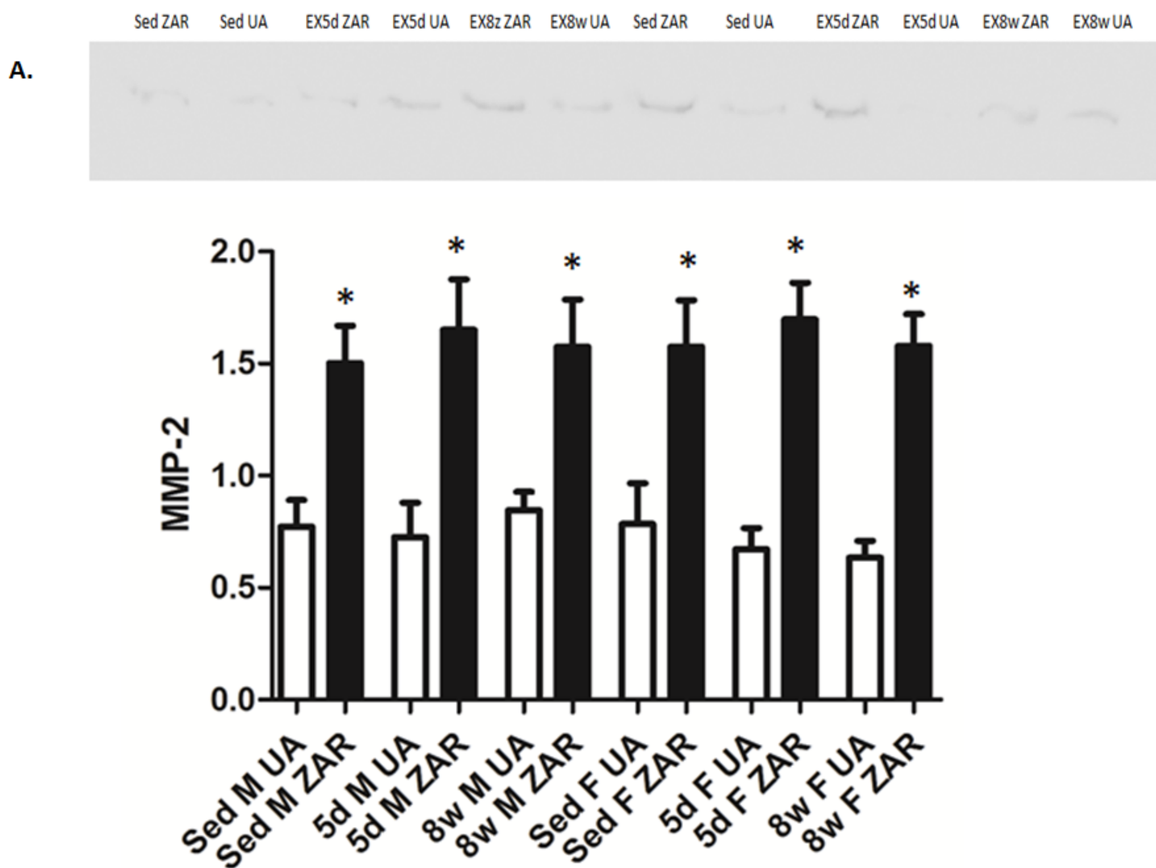
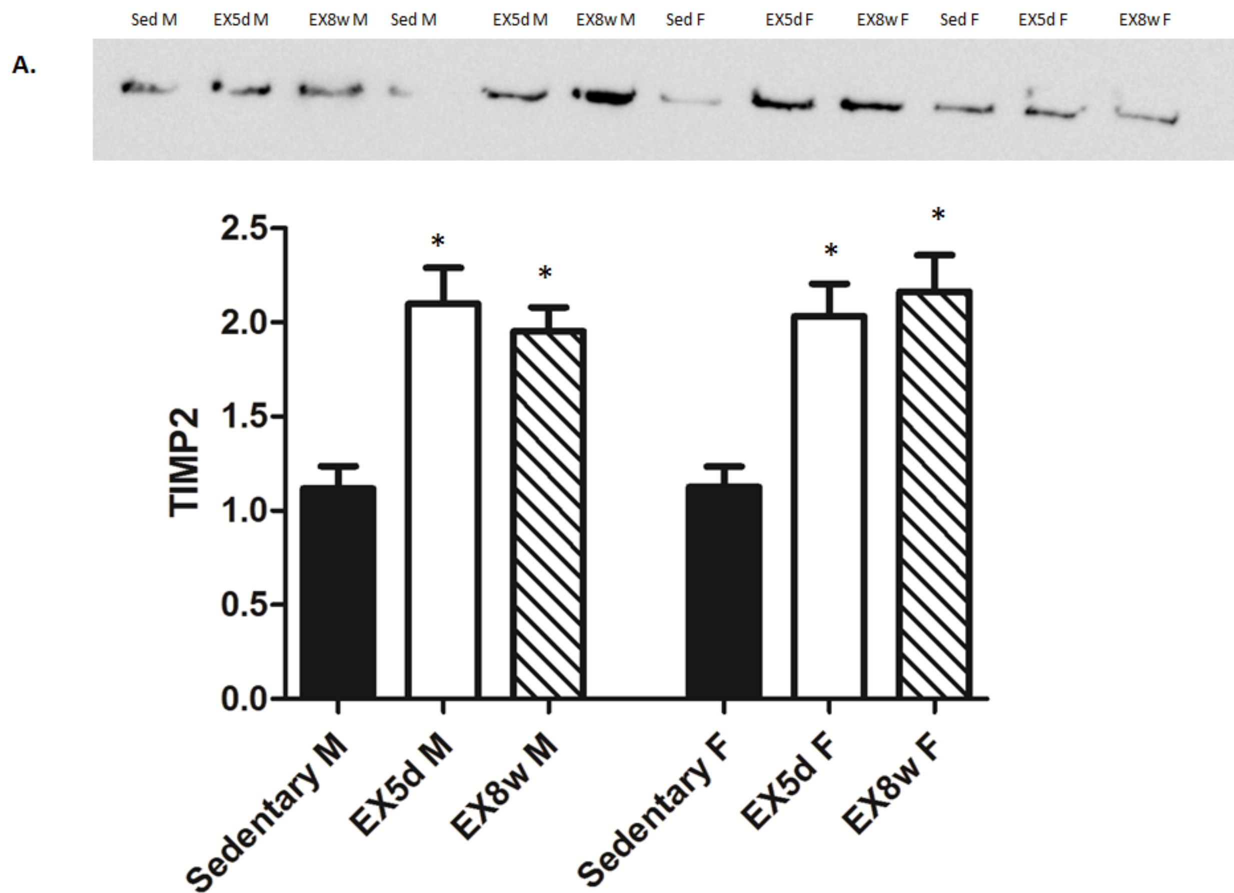


Figure 3.6. Myocardial TIMP-2 protein levels for all experimental groups.

A: Representative Western blot bands and quantification of TIMP-2 protein from all experimental groups. Note that TIMP-2 protein levels were significantly increased in all EX groups compared to Sed (P < 0.05). There was no difference in TIMP-2 protein levels between the ZAR and UA. Data are integrated from multiple Western blots.



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CHAPTER IV

MicroRNAs: a novel mechanism of exercise-induced cardioprotection against ischemia-reperfusion injury in the rat heart

Introduction

MicroRNAs (miRNAs) are small (19-23 nucleotides) noncoding RNA molecules that have recently been recognized as endogenous regulators of gene expression. miRNAs participate in post-transcriptional regulation of mRNA expression by repressing mRNA translation, or by promoting mRNA degradation, and are now known to be essential in numerous molecular regulatory pathways (Figure 4.1)(35). miRNAs are known to play important roles in many physiological and pathological processes, and several cellular and molecular pathways are affected by the regulatory function of miRNAs, including aging, cancer, and cardiovascular disease (3, 12, 22, 29).

However, little is known on the impact of miRNAs in myocardial ischemia-reperfusion (IR) injury. There is emerging evidence that a number of miRNAs have a role in modulating the cell death process during IR (7, 25, 36), but previous results have not been clear as to if these miRNAs promote or inhibit cell death. Furthermore, modulation of miRNA expression in vivo with pharmaceutical interventions appears to be a feasible therapeutic approach to a number of pathologies (27), thus application of both miRNA inhibitors and miRNA mimics (promoters) offer a potential therapeutic option for cardioprotection (15, 28). For these reasons, a better understanding of the roles of miRNAs during IR injury is clearly necessary.

Very little is known about the effects of exercise on the expression levels of miRNAs in the myocardium. It is well established that exercise confers a protective effect against myocardial IR injury (24). However, despite intensive research in the last decades, the exact physiological mechanisms underlying the cardioprotective effect remain largely unclear. The

potential role of miRNAs in exercise-induced cardioprotection has been largely unexplored. Given the emerging role of miRNAs in IR injury, it is possible that exercise exerts, at least in part, its cardioprotective effects through changes in the expression levels of miRNAs. Therefore, in the present study, we aimed to identify miRNAs involved with exercise-induced cardioprotection against IR injury.

Methods

Animals. Three-month-old male and female Sprague-Dawley rats were obtained from Envigo (locally maintained colony in Madison, WI, USA). We selected Sprague-Dawley rats for this experiment because it is a well-accepted model for studying exercise-induced myocardial adaptations and myocardial responses to IR. Upon arrival, rats were randomly assigned to one of three groups: a five day exercise training group (EX5d; n=20), an eight week exercise training group (EX8w; n=20), or a sedentary control group (Sed; n=20). Each group contained equal numbers of males and females. All animals were housed in pairs in clear plastic cages in the same temperature controlled facility with a 12:12-h light/dark reverse cycle under the care of a full-time veterinarian. The rats were provided standard rat chow and water *ad libitum*. All animals were killed at least 24 h after the last training (or handling control) session. The experimental protocol and euthanasia were approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee (IACUC) and followed the guidelines established by the American Physiological Society for the use of animals in research.

Treadmill exercise training. Training was started one week after the rats' arrival to reduce the stress associated with shipping and acclimation to a new location. Animals assigned to the exercise groups were habituated to treadmill exercise on a daily basis for 5 consecutive days. This habituation period involved a gradual increase in running time beginning with 5 min/day (first day) and ending with 25 min/day (5th day) at 12 m/min at 0% grade, an amount below that which is likely to produce a significant change in aerobic capacity.

Following the habituation period, animals were given 2 days of rest and then underwent a speed-ramped test to assess maximal running capacity. This protocol was previously

developed to pattern clinical stress tests as a standard screen for exercise capacity (16). Briefly, rats exercised on a motorized treadmill set at a constant grade of 15% and an initial speed of 18 m/min. The speed was progressively ramped 1 m/min every 2 min until exhaustion occurred. Exhaustion was defined as the third time the rat remained in contact with the shock grid for 2 s. Total distance run was calculated from belt speed (m/min) and duration (min) of the run.

Following the initial max running test, animals in the EX8w group were run on a treadmill for 60 min/day, 5 days/week, for 8 weeks. The treadmill speed was gradually increased every few weeks, with females running at the following maximal intensities: weeks 1-2: 19 m/min, weeks 3-4: 21 m/min, weeks 4-8: 23 m/min. Male rats ran at the following maximal intensities: weeks 1-2: 15 m/min, weeks 3-4: 18 m/min, weeks 4-8: 21 m/min. This training protocol was developed to maximize compliance and reduce animal stress based off of previous exercise studies in our laboratory, and to account for the innate differences in exercise capacity between male and female rats. At the conclusion of the 8-week exercise protocol rats underwent a second maximal exercise capacity test. Sed rats were placed on the treadmill for 5 min/day twice per week in order to control for the effects of handling.

After the initial max running test, animals in the EX5d group were trained for 3 days for the same duration and speed as the EX8w rats, immediately followed by a second max running test, amounting to 5 consecutive days of exercise training.

Ischemia-reperfusion protocol. Animals were deeply anesthetized using isoflurane gas. After left thoracotomy, a suture was threaded through the tissue surrounding the left anterior descending coronary artery close to its origin (3-5 mm distal to the aorta). Transient regional

ischemia was induced by tightening the suture to occlude the artery with the use of a removable snare. Electrical activity of the heart (continuous ECG monitoring) and visualization of cyanotic heart tissue were used to confirm the presence of ischemia. Ischemia lasted for 45 min. At the end of ischemia, the snare was loosened and reperfusion ensued for 24 hr while animals recovered in their cage. The durations of ischemia and reperfusion were chosen to ensure myocardial infarction, and the protocol was adapted from previous work conducted at the Cardiovascular Research Center at UW-Madison (17). For sham surgery, left thoracotomy was performed but the coronary artery was not occluded. Five animals developed severe fibrillation or did not survive the IR surgery (four Sed males, one EX8w female), and their tissue was discarded from the study. Following euthanasia, the plantaris muscle was removed and flash frozen using liquid nitrogen for subsequent citrate synthase assay.

Myocardial dissection. At the end of reperfusion (24 hr), the snare was retightened around the coronary artery, and 100 µl of 0.5% Evans blue dye solution was injected into the aorta and perfused through the heart. Hearts were then quickly excised, rinsed in cold saline, and sliced transversely from base to apex into slices of equal width. Each of the slices was then immersed in 100 mM phosphate-buffered saline with 0.1% triphenyltetrazolium chloride (TTC) and incubated for 10 min at 37°C. After incubation, each slice was placed in Optimal Cutting Temperature (OCT) compound, flash frozen using liquid nitrogen, and immediately stored at -80°C. The zone at risk (ZAR) was identified as the area of each slice that did not turn blue (i.e., not perfused with dye) after perfusion with the solution containing Evans blue dye. The portion of the ZAR that did not turn red in response to TTC incubation and remained white was

classified as the infarct area. Any tissue that was stained blue was defined as the unaffected area (UA). ZAR and UA sections were dissected apart and stored for later miRNA isolation.

MicroRNA Arrays. RNA was isolated from ZAR and UA segments of the left ventricular free wall. MicroRNA isolation was performed according to the protocol as supplied in the manufacturer's kit (miRNeasy Isolation Kit, Qiagen). Briefly, RNA quality and quantity were assessed using a spectrophotometer (DU 800, Beckman Coulter). Following RNA isolation and quantification, RNA samples from the same sex and experimental groups were pooled into one sample per group, and cDNA was prepared following the manufacturer's protocol (miScript II RT Kit, Qiagen). Template cDNA was then fluorescently labeled and prepared for PCR (SYBR Green PCR Master Mix, Qiagen). miRNA PCR array plates specific for cardiovascular disease were used to perform PCR (miScript miRNA PCR Array Rat Cardiovascular Disease, Qiagen). PCR was performed according to the manufacturer's protocol (Qiagen) using a thermocycler (Step One Plus, Applied Biosystems). Table 4.2 provides a list of all of the miRNAs present on this PCR array.

Threshold values were set using a logarithmic amplification plot. Threshold values were chosen within the lower half of the log-linear range of the amplification plot. Changes in gene expression were determined by the number of cycles required for the signal intensity of a given PCR product to reach threshold (C_T). C_T values, normalized to housekeeping genes provided on the miRNA array, were used to calculate fold change values, and these values were used to make relative comparisons on the expression levels of miRNAs between samples.

Citrate synthase assay. Samples (65-80 mg) were cut from the frozen plantaris muscles and homogenized. Total muscle protein was determined by Bradford protein assay, and the protein concentration of all samples was equalized. Citrate synthase activity was then determined using an assay kit (Sigma-Aldrich, USA) and spectrophotometer.

Identification of miRNAs. A fold change of at least ± 2.0 was required for a change in expression to be considered significant, per the manufacturer's protocol (Qiagen). We first selected miRNAs that were significantly affected by IR by comparing miRNA expression in IR tissue versus that from sham operation tissue, and dividing these miRNAs into three groups according to the effect of IR compared to sham: down-regulated by IR, up-regulated by IR, not affected by IR. These categories were then further subdivided into two additional categories according to the direction of miRNA changes in response to exercise: inhibited by exercise (either 5 days or 8 weeks), or enhanced by exercise (either 5 days or 8 weeks). This analysis resulted in the following categories of miRNA expression:

- *Down-regulated by IR*: Sed ZAR vs. Sed Sham
- *Up-regulated by IR*: Sed ZAR vs. Sed Sham
- *Not affected by IR*: Sed ZAR vs. Sed Sham
- *Inhibited by EX (either 5d or 8w)*: EX ZAR vs. EX Sham, then compare to those up-regulated in Sed ZAR
- *Enhanced by EX*: EX ZAR vs. Sed Sham, and also had to be up-regulated in Sed ZAR

Data analysis. Statistical comparisons were made using GraphPad Prism (version 7). Data were analyzed using a 2 (sex) x 3 (training group) ANOVA. When appropriate, effects of training (within sex) were evaluated with a one-way ANOVA. Values are means ± SE, and significance is reported at $p < 0.05$.

miRNA PCR array analyses were performed using the GeneGlobe Data Analysis Center software (Qiagen) by comparing fold change values. Expression levels were considered significantly different if they exhibited a fold change of at least ± 2.0. Positive values represent an up-regulation in expression; negative values represent a down-regulation in expression.

Results

Exercise training. Changes in maximal exercise capacity, comparing pre-exercise to post-exercise max running tests, are reported as absolute change (m) and relative change (%), and are presented in Table 4.1. Female and Male EX8w animals had significantly higher exercise capacities compared to EX5d counterparts. Female EX8w rats had significantly higher pre and post-exercise capacities in absolute terms compared to male EX8w rats (post-training: 668.9 ± 87.0 m vs. 310.4 ± 46.5 m, respectively, $P < 0.05$). However, the relative (% change) improvement from baseline measurements (pre-exercise training) was not different between EX8w females and males (235.4 ± 21.3% vs. 201.6 ± 22.5%).

Citrate synthase activity in the plantaris (a mixed muscle) was determined as a measure of endurance exercise training effect and results are shown in Table 4.1. Citrate synthase activity was significantly increased following 8 weeks of training in both male and female rats compared to sedentary animals (EX8w males: 24.01 ± 0.50 vs. Sedentary males: 15.33 ± 0.4 $\mu mol \cdot g\ wet\ wt^{-1} \cdot min^{-1}$; EX8w females: 25.18 ± 0.67 vs. Sedentary females: 15.71 ± 0.34 $\mu mol \cdot g\ wet\ wt^{-1} \cdot min^{-1}$, $P < 0.05$). There was no difference in citrate synthase activity between EX8w males and EX8w females, corroborating our maximal exercise capacity test results. Additionally, there was no difference in citrate synthase activity in male or female EX5d rats (15.09 ± 0.41 and 15.25 ± 0.39 $\mu mol \cdot g\ wet\ wt^{-1} \cdot min^{-1}$, respectively) compared to sedentary. Increased citrate synthase activity indicates that the animals were endurance trained as a result of the 8 week exercise protocol. These results are explained in further detail in Chapters II and III.

Identification of MicroRNAs associated with Ischemia-Reperfusion. In total, the expression levels of 31 microRNAs were altered as a result of IR. Of these 30 microRNAs, the expression levels of 30 were up-regulated compared to sham operated control, while the expression level of only one microRNA was down-regulated in response to IR. A comprehensive list of the microRNAs affected by IR can be found in Figure 4.2.

Identification of MicroRNAs associated with 5 days of exercise. In male rats, of the miRNAs that were up-regulated in response to IR, 9 were inhibited by 5 days of exercise (22, 122, 130a, 133a, 133b, 144, 208a, 29b, 451) and 1 was enhanced by 5 days of exercise (494) (Figure 4.3). Twelve miRNAs that were not affected by IR alone were down-regulated in the ZAR from hearts of male rats as a result of 5 days of exercise (100, 107, 199a, 23b, 26a, 30a, 30c, 30d, 30e, 497, 499, 99a), while 10 miRNAs that were not affected became up-regulated following 5 days of exercise (142, 182, 183, 18a, 21, 222, 223, 347, 406, 484) (Figure 4.3).

In female rats, of the miRNAs that were up-regulated in response to IR, 7 were inhibited by 5 days of exercise (122, 142, 18a, 24, 423, 466, 92a) and none were enhanced by 5 days of exercise (Figure 4.5). Two miRNAs that were not affected by IR alone were down-regulated in the ZAR from hearts of female rats as a result of 5 days of exercise (144, 451), while 19 miRNAs that were not affected became up-regulated following 5 days of exercise (7a, 126, 133a, 133b, 181a, 181b, 185, 206, 21, 26b, 27a, 27b, 29a, 29c, 30a, 322, 352, 378, 499) (Figure 4.5).

Identification of MicroRNAs associated with 8 weeks of exercise. In male rats, of the miRNAs that were up-regulated in response to IR, 8 were inhibited by 8 weeks of exercise (22, 122, 130a, 133a, 133b, 144, 208a, 29b, 451) while none were enhanced by 8 weeks of exercise

(Figure 4.4). Eighteen miRNAs that were not affected by IR alone were down-regulated in the ZAR from hearts of male rats as a result of 8 weeks of exercise (103, 107, 10b, 124, 125b, 142, 143, 145, 16, 181a, 199a, 19a, 27a, 30a, 30e, 497, 93, 99a), while 5 miRNAs that were not affected became up-regulated following 8 weeks of exercise (182, 183, 466, 484, 7a) (Figure 4.4).

In female rats, of the miRNAs that were up-regulated in response to IR, 3 were inhibited by 8 weeks of exercise (122, 142, 19a) and two were enhanced by 8 weeks of exercise (183, 494) (Figure 4.6). Five miRNAs that were not affected by IR alone were down-regulated in the ZAR from hearts of female rats as a result of 8 weeks of exercise (125, 133a, 199a, 22, 451), while zero miRNAs that were not affected became up-regulated following 5 days of exercise (Figure 4.6).

There were no miRNAs that were down-regulated by IR that were affected (either inhibited or enhanced) by 5 days or 8 weeks of exercise in either sex.

Discussion

In the present study, we have shown that the expression pattern of miRNAs in the myocardium is altered in response to ischemia-reperfusion compared to hearts from sham operated animals, and that this effect of IR injury is then modified by both short- and long-term exercise training. Both short-term and long-term exercise training resulted in improved cardioprotection against IR injury compared to sedentary rats, as measured by infarct size (Table 4.1). By conducting a systemic comparison of miRNA expression changes and the direction of the changes induced by IR, we have elucidated possible associations between miRNA changes and cardioprotection afforded by exercise.

MicroRNAs are known to be involved in multiple cellular pathways in the myocardium and in a number of cardiac pathologies (27, 28, 29) but, for the most part, the role of miRNAs in IR injury has not been fully explored (2, 9, 15, 25). Further, there have been very few studies on the role of miRNA's in exercise-induced cardioprotection against IR injury. We found a number of miRNAs whose expression was altered by IR as well as exercise training (Figure 4.3). The functional roles of these miRNAs have not been fully characterized, but there appear to be two main categories of cellular function that seem to be associated with the miRNAs that were identified in our study. These are discussed below.

miRNAs associated with apoptosis during IR. Some studies have previously reported IR injury-associated alterations in cardiac myocyte miRNA levels that regulate apoptosis (27), thereby showing a possible mechanism through which changes in miRNA levels can alter infarct size. Inhibition of miRNA-122 has been shown to reduce cell death during IR (20), and there is

compelling evidence that inhibition of miRNA-122 mitigates cell death by preventing the opening of the mitochondrial permeability transition pore (PTP), thus preventing apoptosis (18). In the current study, miRNA-122 expression was up-regulated following IR in both male and female sedentary rats, but 5 days and 8 weeks of exercise inhibited this up-regulation in both sexes. Thus, exercise-induced inhibition of miRNA-122 expression represents a potential mechanism for the cardioprotective effects of exercise during IR injury. Expression levels of several other miRNAs associated with apoptosis were down-regulated following exercise training as well. miRNA-130a represses the translation of Smad 4, which is a transcription factor that can exert anti-apoptotic effects in the cell (19). Inhibition of miRNA-130a has been shown to protect cardiac myocytes from IR-induced death (19). miRNA-130a expression was significantly decreased compared to the hearts from sedentary animals for both EX5d and EX8w males, which was associated with enhanced cardioprotection. This result was also true for miRNA-133a/b, which has also been linked to cellular death via apoptosis, with decreased expression leading to less cell death (6). Interestingly, miRNA-130a and miRNA-133a/b were not affected by exercise in female rats, indicating a sex-difference in miRNA-related cardioprotection.

miRNAs associated with autophagy, cell structure, and oxidative stress. Oxidative stress and its downstream consequences is commonly suggested as a mechanism for IR injury, and protection from oxidative stress presumably plays a role in exercise-induced cardioprotection (24). Several of the miRNAs identified in the present study are related to oxidative stress. For example, the exact role of miRNA-208a is yet to be fully elucidated, but previous work has shown its involvement in the heart in response to oxidative stress (5, 30). Interestingly, miRNA-

208a levels can be detected in the blood earlier than cTnT following acute MI, and this miRNA has been suggested as a possible diagnostic tool for the presence of infarcted tissue (10). Although its mechanism of action isn't fully understood, pharmacological inhibition of miRNA-208a resulted in a block of pathological cardiac remodeling and improved heart function following MI in rats (23). In our study, miRNA-208a expression levels were significantly decreased in both EX5d and EX8w males, but not in either female group. This again indicates a possible sex-difference in miRNA-related cardioprotection,

It is a somewhat surprising finding that expression changes in some miRNAs believed to confer cardioprotection from IR injury, such as miRNA-208a, were altered in the exercised hearts from male but not female rats. Although exercise training conferred cardioprotection against IR injury in both sexes in the current study, the hearts from female rats were better protected against IR injury than hearts from male rats. A likely explanation of this finding is that miRNAs are upstream of the eventual cellular effector, which in cardioprotection is often times a protein involved with apoptosis, oxidative/energetic stress, or autophagy. It is possible that these proteins are more robustly increased in female rats following exercise training than in male rats, and thus the stimulus for altering miRNA expression levels during IR in females may be reduced.

Other miRNAs that have previously been shown to be involved with oxidative stress and autophagy were impacted by exercise as well. miRNA-494, which protects against oxidative stress in the heart (31), was significantly increased in EX5d males and EX8w females. miRNA-22 inhibits autophagy, and expression of miRNA-22 has been shown to increase with age. This

results in decreased cardiac autophagy, and can lead to cardiac hypertrophy (11). Interestingly, inhibition of miRNA-22 in mice prevented post-infarction remodeling and improved cardiac function, as well as decreasing age-related cardiac hypertrophy compared to controls (11). In the present study, miRNA-22 levels were down-regulated following 8 weeks of exercise in male rats, but this trend did not hold true in female rats (no change). In addition, miRNA-16, miRNA-143, miRNA-145, and miRNA-484 have all been linked to cardiac autophagy and oxidative stress in response to IR (1, 13, 21, 32, 34, 39), and the expression levels of these miRNAs were all decreased by exercise training in the current study. Interestingly, the change in the levels of these miRNAs was predominantly in male rats, with few or no changes in the hearts from exercise females. This, in combination with the above data on sex-differences in myocardial miRNA levels following exercise, strongly indicates a sex-difference in the mechanisms through which exercise confers cardioprotection against IR injury.

Summary and conclusion. To our knowledge, this is the first study to investigate the effects of exercise training on miRNA expression during IR injury as a result of exercise training. Our results suggest that exercise training may confer cardioprotection, in part, through changes in expression of miRNAs known to be associated with apoptosis, autophagy, and oxidative stress. Furthermore, the effect of exercise on miRNA expression appears to be sex-dependent, with exercise affecting the expression of a greater number of miRNAs in male rats compared to that seen in females.

Figure 4.1. miRNA biogenesis and mechanisms of gene expression inhibition. Adapted from van Rooij, 2011 (26).

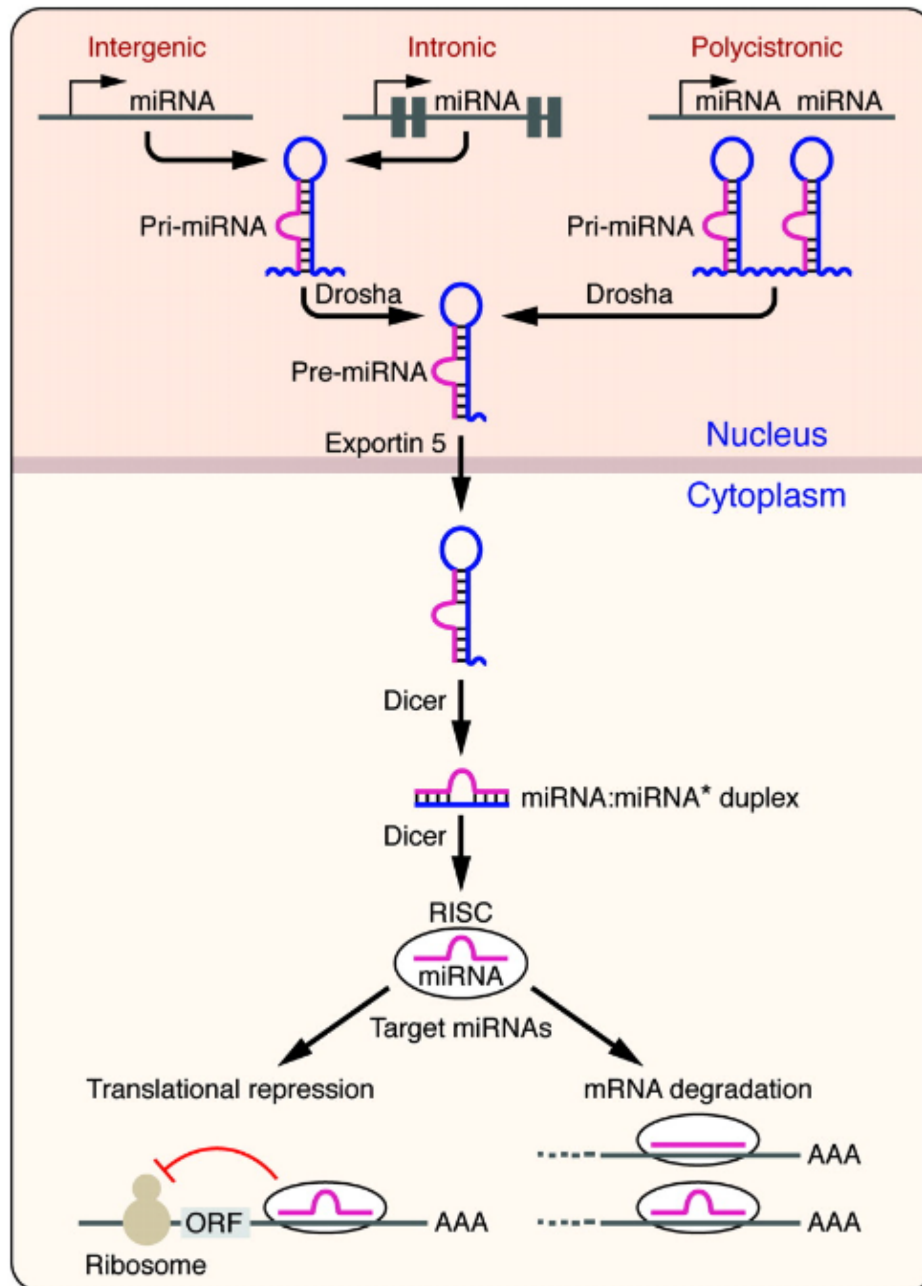


Table 4.1. ***Effects of exercise training***

Group	Δ DIST (m)	Δ DIST (%)	Citrate synthase activity (μmol • g wet wt^{-1} • min^{-1})
Sedentary			
Male	-	-	15.33 ± 0.40
Female	-	-	15.71 ± 0.34
EX5d			
Male	103.7 ± 36.2	131.7 ± 12.4	15.09 ± 0.41
Female	132.5 ± 52.2	122.2 ± 8.2	15.25 ± 0.39
EX8w			
Male	310.4 ± 46.5*	201.6 ± 22.5*	24.01 ± 0.50*
Female	668.9 ± 87.0*,#	235.4 ± 21.3*	25.18 ± 0.67*

Values are means ± SEM. Response to 5 days (EX5d) or 8 weeks (EX8w) of exercise training (n=10/group/sex). Change in maximal running capacity (Δ DIST) is a pre to post training comparison, and is expressed as either absolute change (m) or percent change (%). Maximal running capacity was significantly improved from 5d to 8w in both males and females (*P < 0.05). EX8w Females exhibited a greater absolute (m) change in maximal running capacity compared to EX8w males (# P < 0.05), but there was no difference in relative (%) change. Plantaris muscle Citrate synthase activity was significantly increased (P < 0.05) in EX8w animals compared to EX5d and Sed animals.

Table 4.2. List of all miRNAs present on the PCR array.

Rat Cardiovascular Disease miRNA PCR Array
miR-39-3p, let-7a-5p, let-7b-5p, let-7c-5p, let-7d-5p, let-7e-5p, let-7f-5p, miR-1-3p, miR-100-5p, miR-103-3p, miR-107-3p, miR-10b-5p, miR-122-5p, miR-124-3p, miR-125a-5p, miR-125b-5p, miR-126a-3p, miR-130a-3p, miR-133a-3p, miR-133b-3p, miR-140-5p, miR-142-3p, miR-143-3p, miR-144-3p, miR-145-5p, miR-146a-5p, miR-150-5p, miR-15b-5p, miR-16-5p, miR-17-5p, miR-181a-5p, miR-181b-5p, miR-182, miR-183-5p, miR-185-5p, miR-18a-5p, miR-195-5p, miR-199a-5p, miR-19a-3p, miR-206-3p, miR-208a-3p, miR-21-5p, miR-210-3p, miR-214-3p, miR-22-3p, miR-221-3p, miR-222-3p, miR-223-3p, miR-224-5p, miR-23a-3p, miR-23b-3p, miR-24-3p, miR-25-3p, miR-26a-5p, miR-26b-5p, miR-27a-3p, miR-27b-3p, miR-29a-3p, miR-29b-3p, miR-29c-3p, miR-30a-5p, miR-30c-5p, miR-30d-5p, miR-30e-5p, miR-31a-5p, miR-320-3p, miR-322-5p, miR-328a-3p, miR-342-3p, miR-347, miR-352, miR-365-3p, miR-378a-3p, miR-423-3p, miR-451-5p, miR-466d, miR-484, miR-494-3p, miR-497-5p, miR-499-5p, miR-7a-5p, miR-92a-3p, miR-93-5p, miR-98-5p, miR-99a-5p

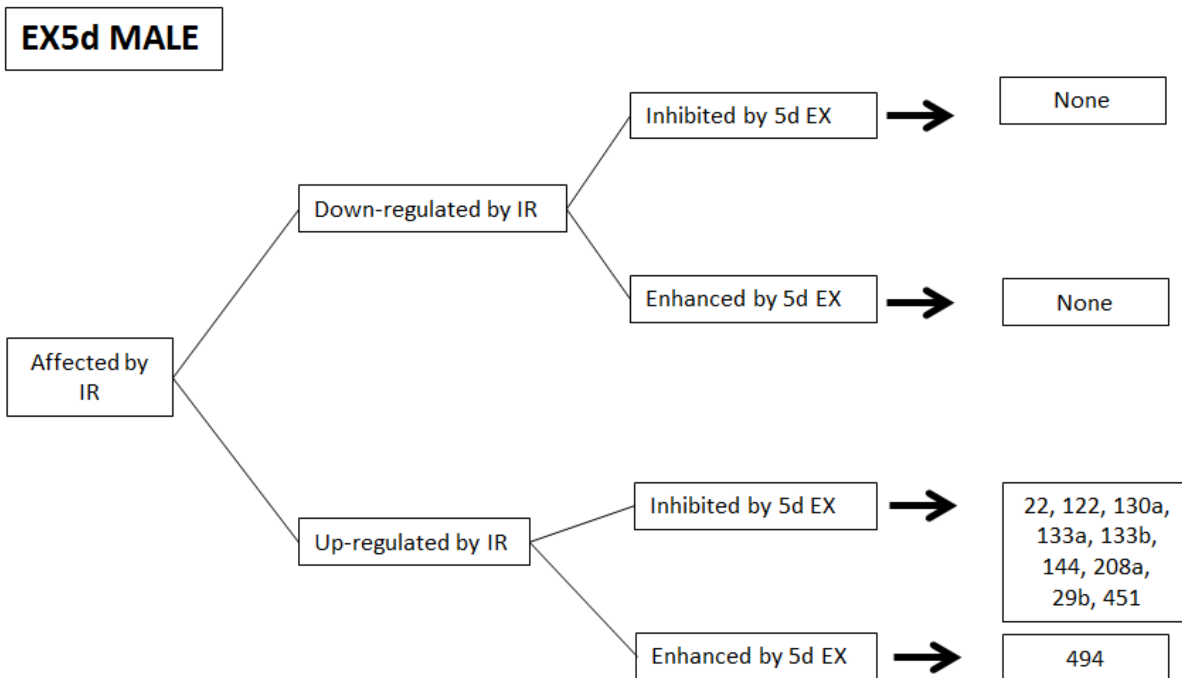
Figure 4.2. A. MicroRNAs that were significantly affected by IR. List determined by comparing miRNA expression levels in the ZAR compared to SHAM hearts across all experimental groups.

MicroRNAs Up-Regulated IR	MicroRNAs Down-Regulated by IR
17, 18, 19a, 22, 24, 29b, 122, 124, 130a, 133a, 133b, 142, 144, 146, 150, 182, 183, 208a, 210, 214, 221, 222, 223, 328, 347, 423, 451, 466, 484, 494	31

B. MicroRNAs that were not affected by IR, but were affected by exercise training (either 5d or 8w).

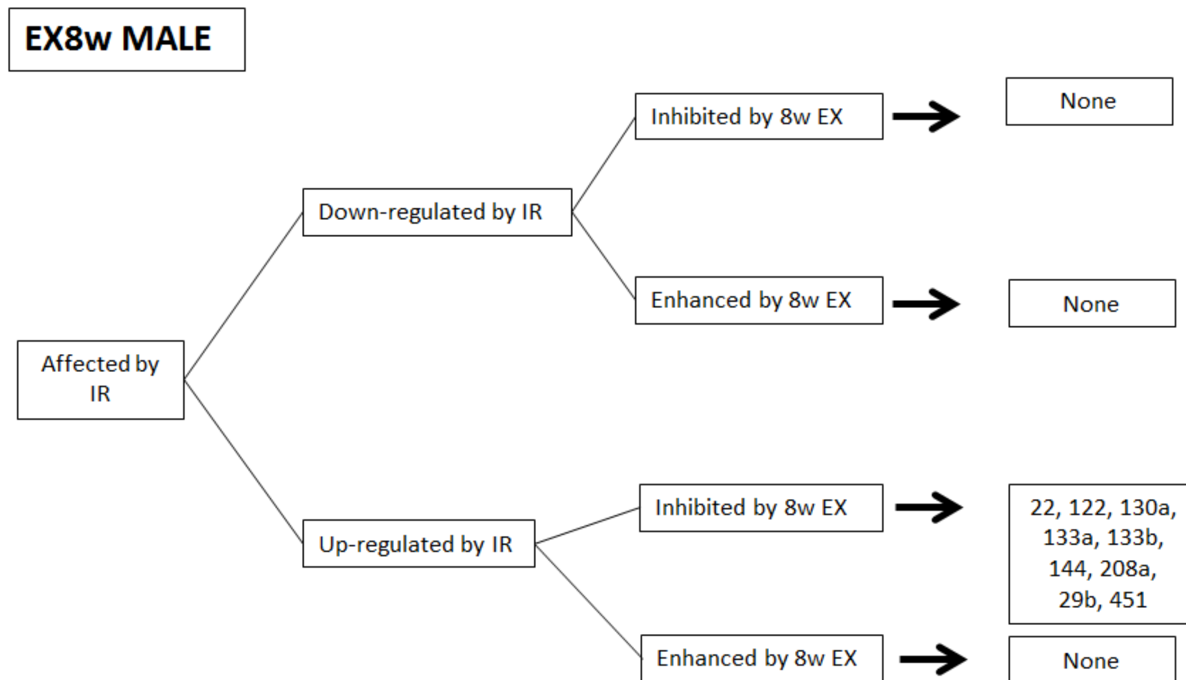
Not affected by IR, but affected by exercise
7a, 93, 99a 103, 107, 10b, 124, 125b, 126, 143, 145, 16, 181a, 181b, 185, 199a, 206, 222, 223, 19a, 21m 26b, 27a, 27b, 29c, 30a, 30e, 322, 347, 352, 378, 406, 497, 499

Figure 4.3. MicroRNAs that were significantly affected by IR and 5 days of exercise in male rats. MicroRNAs that were affected by IR and altered by exercise are categorized below.



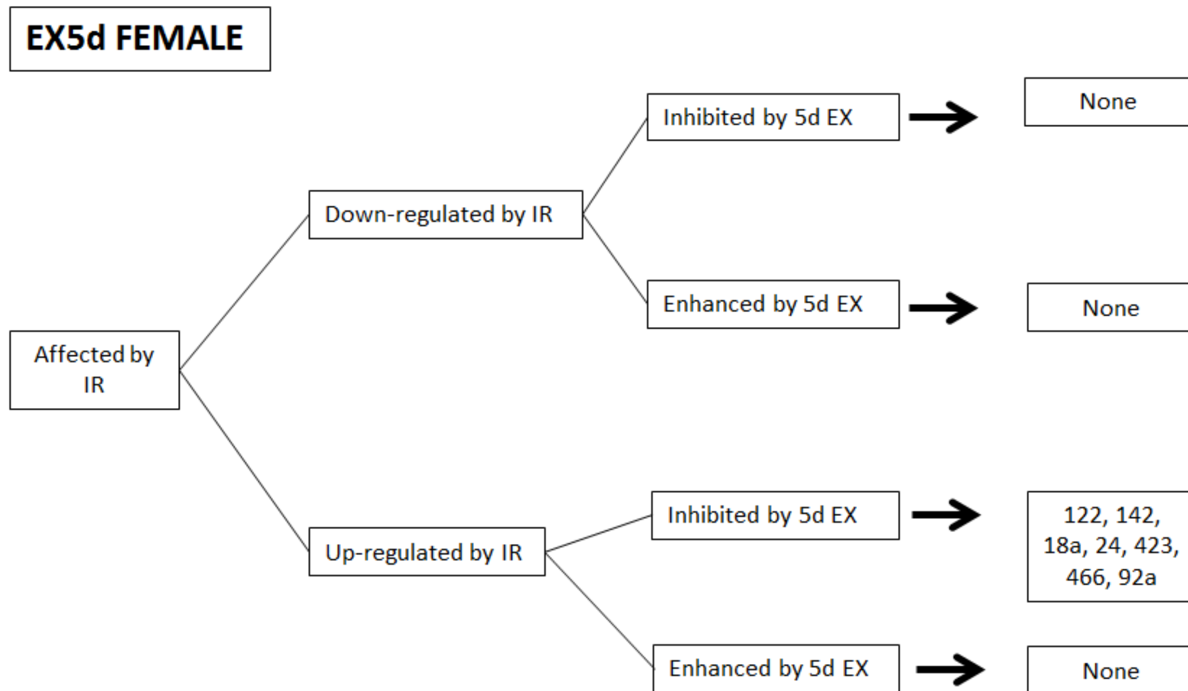
Apoptosis	Autophagy, Cell Structure, or Oxidative Stress	Unknown or Other
122, 130a, 133a, 133b	22, 208a, 494	29b, 144, 451

Figure 4.4. MicroRNAs that were significantly affected by IR and 8 weeks of exercise in male rats. MicroRNAs that were affected by IR and altered by exercise are categorized below.



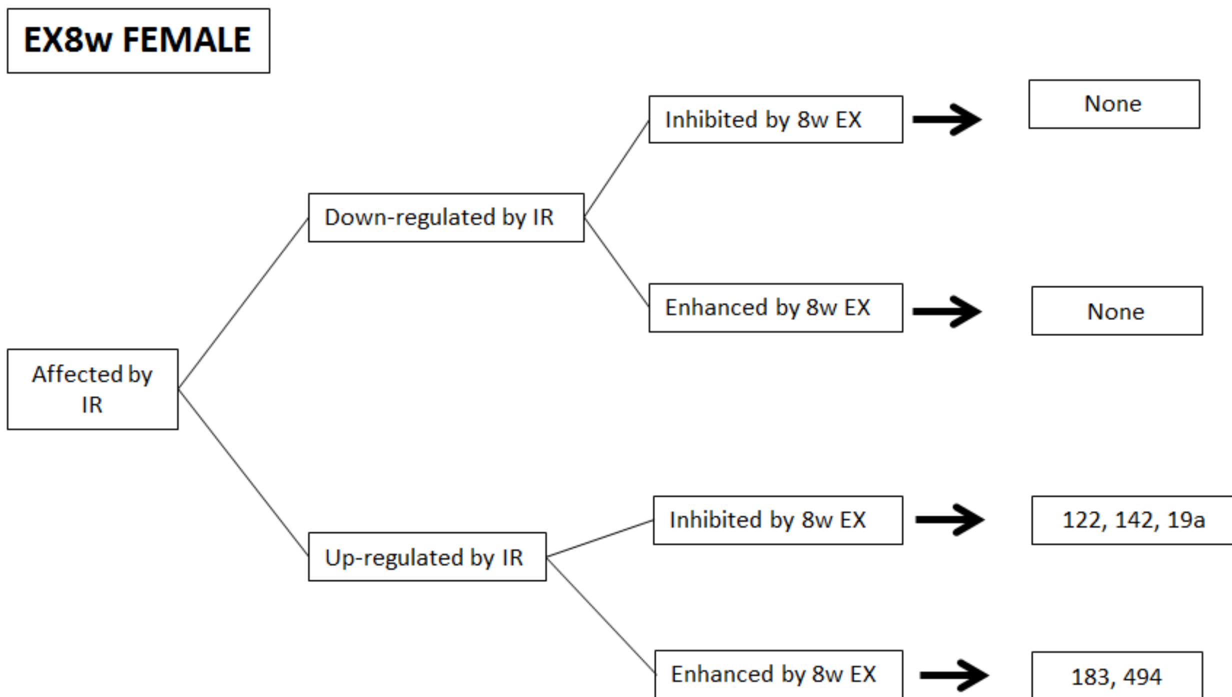
Apoptosis	Autophagy, Cell Structure, or Oxidative Stress	Unknown or Other
122, 130a, 133a, 133b	22, 208a	29b, 144, 451

Figure 4.5. MicroRNAs that were significantly affected by IR and 5 days of exercise in female rats. MicroRNAs that were affected by IR and altered by exercise are categorized below.



Apoptosis	Autophagy, Cell Structure, or Oxidative Stress	Unknown or Other
122		18a, 24, 92a, 142, 423, 466,

Figure 4.6. MicroRNAs that were significantly affected by IR and 8 weeks of exercise in female rats. MicroRNAs that were affected by IR and altered by exercise are categorized below.



Apoptosis	Autophagy, Cell Structure, or Oxidative Stress	Unknown or Other
122	494	19a, 142, 183

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CHAPTER V

Summary and Conclusions

Endurance exercise training is known to provide cardioprotection against myocardial ischemia-reperfusion injury, but the cellular mechanisms for this phenomenon are not completely understood (5). The ability for exercise to confer cardioprotection, commonly defined as a reduction in infarct size, has been shown following both short-term (7) and long-term exercise training (4). However, none of the previous studies on exercise-induced cardioprotection against IR injury have directly compared the effects of short-term versus long-term exercise training in the ability to reduce infarct size. Therapeutic tools to reduce infarct size are of extreme clinical value, as myocardial infarct size is inversely related to mortality following a myocardial infarction (9). Furthermore, it is unclear if the ability for exercise to confer cardioprotection against IR injury is sex-dependent. Thus, we designed and conducted the experiments described in Chapters I-IV in order to address these issues. In this project we have investigated the effects of short-term versus long-term exercise training on cardioprotection against IR-injury, examined the sex-dependency on the ability for exercise to increase myocardial tolerance to IR injury, and explored the role of novel mechanisms (MMP-2, TIMP-2, and micro-RNAs) involved in protecting the myocardium from IR-injury. The significant conclusions outlined in this dissertation are as follows:

1) In Chapter II, we describe the effects of short-term (5 days; EX5d) and long-term (8 weeks; EX8w) exercise training on conferring protection against myocardial ischemia-reperfusion injury in the hearts of male and female rats. While both exercise-training protocols yielded significant reductions in infarct size compared to sedentary animals, we found that 8 weeks of exercise training resulted in significantly smaller infarcts compared to rats exercised for just 5 days, but this effect was only true in the hearts of female rats. Hearts from exercised

male rats, while exhibiting significantly smaller infarcts compared to sedentary males, were no better protected from IR injury after 8 weeks of exercise training compared to 5 days of exercise training. These results suggest that long-term exercise training yields additional cardioprotection against IR-injury compared to the amount conferred following short-term exercise, but this effect is sex-dependent.

We then investigated potential mechanisms leading to the observed sex difference in cardioprotection. Hearts from female rats had significantly higher protein levels of both subunits of the myocardial K_{ATP} channel, $K_{ir}6.2$ and SUR2A, compared to hearts from male rats. Furthermore, protein levels of the SUR2A subunit, which is believed to be the rate-limiting subunit for proper K_{ATP} channel formation (16), was significantly increased in the hearts of EX8w females compared to EX5d females. There was no difference in myocardial SUR2A protein levels between EX8w and EX5d males, indicating that protein levels of this subunit plateaus after short-term exercise in male hearts. There was no difference in calpain levels between any of our experimental groups. However, calpastatin levels were significantly increased in the hearts from all exercise groups, with no difference between sexes. These data suggest that cellular energy sensors (K_{ATP} channel) and the proteolytic system (calpain/calpastatin) play an important role in IR injury, and in exercise-induced cardioprotection. However, it seems the proteolytic system is not involved in the sex differences observed in cardioprotection, while increased protein levels of the K_{ATP} channel was related to the increased cardioprotection observed in the hearts of female rats following exercise training.

2) In Chapter III, we describe a novel mechanism contributing to exercise-induced cardioprotection against IR injury. Matrix metalloproteinase-2 (MMP-2) is a proteolytic enzyme which is thought to degrade extracellular matrix proteins during cardiac stress. It has recently been proposed to have an intracellular role as well during IR (10), but levels of MMP-2 protein have not been directly compared between the zone at risk (ZAR) and unaffected myocardium during IR injury. We found significant elevation in the protein levels of MMP-2 between the ZAR and the unaffected area, which supports the idea that MMP-2 has an intracellular effect during IR. Further, we found that protein levels of Tissue inhibitor of metalloproteinases-2 (TIMP-2) were significantly increased following exercise training (both 5d and 8w) compared to levels seen in sedentary rats. Interestingly, there was no difference in TIMP-2 protein levels between male and female rats, or between EX5d and EX8w rats. These data suggest that increased levels of TIMP-2 play an important role in exercise-induced cardioprotection against IR injury, and provide further evidence of the important role of the proteolytic system in IR injury. However, this newly described mechanism does not appear to contribute to the sex-differences in cardioprotection described in Chapter II.

3) In Chapter IV, we describe, for the first time, significant changes in microRNA expression associated with exercise-induced cardioprotection against IR injury. We identified expression level changes of several microRNAs in response to IR as well as modifications to these changes associated with exercise training. Furthermore, we identified distinct categories of microRNAs related to exercise-induced cardioprotection against IR injury: 1) apoptosis, 2) autophagy, cell structure, or oxidative stress, and 3) other or unknown. Interestingly, we observed that in EX5d and EX8w males there were a larger number of expression level

alterations in microRNAs that have previously been shown to be protective against IR-injury compared to female rats. This is surprising, given that hearts from female rats exhibited smaller infarcts than male rats across all experimental groups. These data suggest a role of microRNAs in exercise-induced cardioprotection, and strongly indicates a sex-difference in the mechanisms through which exercise confers cardioprotection against IR injury.

Conflicting results

As noted above, we observed significant sex differences in the ability for exercise to confer improved resistance to IR injury, with hearts from females being more protected than males. We noted sex differences in expression of certain proteins associated with IR (subunits of the myocardial K_{ATP} channel) that appeared to support these proteins as possible mechanisms for these sex differences in exercise cardioprotection. However, it is worth noting that several of the molecular mechanisms we assessed yielded counter-intuitive results. For example, we observed an effect of exercise on the ratio of calpain to calpastatin expression but there was no sex difference in this exercise effect despite differences in infarct size between the sexes. That same trend held true for MMP-2 and TIMP-2 levels. A likely explanation is that exercise does confer cardioprotection against IR injury, in part, through alterations to the proteolytic systems. However, this mechanism of exercise-induced cardioprotection appears to be equally effective in both males and females. Additionally, expression levels of a number of microRNAs previously described to be beneficial in IR injury (17) were increased in the hearts of exercised males but not exercised females. This is a surprising finding due to the fact that males displayed larger myocardial infarct sizes compared to female rats. MicroRNAs, while

important regulators of gene expression, are upstream of the eventual effector proteins (8). Therefore, a possible explanation of this finding is that certain proteins involved in exercise-induced cardioprotection are more robustly altered in the hearts of female rats compared to males following exercise training. During IR, these proteins already exist in the hearts of female rats, and can provide immediate cardioprotection at the onset of ischemia. However, it is possible that these proteins are not altered to the same extent following exercise training in male rats, and at the onset of ischemia there is then a larger cellular drive to modify the expression levels of certain genes (through microRNA expression levels) to alter protein content within the cell.

Summary

This is the first study to directly determine that the exercise-induced cardioprotection afforded by short-term exercise can be increased with additional exercise training, and show that this effect is sex-dependent. Additionally, we identified novel mechanisms as potential mediators of exercise-induced cardioprotection. Exercise-induced increases in levels of TIMP-2 and alterations to the expression levels of microRNAs involved with IR injury are novel findings, and their effects on exercise-induced cardioprotection has not previously been explored. We also, for the first time, determined that expression of MMP-2 was upregulated during IR in the zone at risk compared to the unaffected area, providing further support to an intracellular role for this protease during IR.

Limitations

Exercise training. It is possible that our observation of the sex-dependence of the ability of 8 weeks of exercise to confer additional protection against MI compared to 5 days was due to the greater exercise capacity of females compared to males. Indeed, on average, females ran a greater distance during both pre-training and post-training maximal exercise tests, as well as completing a greater overall training volume compared to male rats. It could be argued that, ideally, male and female rats would have followed identical exercise training protocols. However, regardless of this possible limitation, there was no significant difference between males and females in either the percent improvement in maximal running distance after 8 weeks of training, or in skeletal muscle citrate synthase activity, both common markers of standard endurance training adaptations. Thus, there was no evidence of greater adaptation to the 8 weeks of training in females compared to males, despite the improved cardioprotection in females. Furthermore, the different exercise protocols used between male and female rats resulted in a much higher adherence and reduced stress levels to the exercise training, common problems of exercise studies (13).

Measurement of infarct size. We employed a unique method to quantify infarct size as a percentage of the zone at risk. Typically, following tissue staining of the infarcted heart with Evans blue dye and TTC, fresh hearts are sliced and then imaged (15). These wet tissue slices are difficult to image due to the glare caused by the moisture, which we believe contributes to a less accurate quantification of the infarct size. Therefore, we employed a new method whereby we followed the same staining protocols commonly used, but then after slicing the heart we froze the sections in OCT compound to reduce the glare. It is possible that freezing the sample in OCT altered the staining pattern and could have impacted our interpretation of

infarct size. However, preliminary work in our lab indicated that frozen samples were more accurately quantified, with significantly less variability between observers, than wet samples, and therefore do not believe this method impacted our analysis.

MicroRNA PCR Arrays. For each experimental group, we pooled together mRNA from the tissue of 5 animals to make cDNA for the microRNA PCR arrays. This practice is commonly done to help reduce the exorbitant price of the microRNA PCR array plates (12). However, pooling together the tissue samples reduced our statistical power. Also, it is possible that by pooling together our tissue samples we are artificially reducing the variability in the response to exercise training. However, our intention was to provide a profile of the alterations in microRNA expression levels in cardioprotected hearts, and pooling together our samples did not interfere with that goal.

Remaining Questions

Additional exercise training. While our citrate synthase assay and maximal exercise capacity tests clearly show an adaptation in both male and female rats to long-term (8 weeks) exercise training, it is unclear if cardioprotection plateaus after 8 weeks of exercise training. Therefore, it would be of great interest to examine whether additional exercise training, 12-20 weeks, would further augment cardioprotection against IR injury.

Mechanism(s) of sex differences. Our data show that exercise-induced cardioprotection against IR injury is sex-dependent, with the hearts of female rats being more protected than the hearts of male rats. We have proposed a few possible mediators of this sex-difference herein, but it is unclear what is ultimately responsible for sex differences in protein expression. This

work could thus be extended to study the effects of estrogen by including a group of ovariectomized females. It has been suggested that expression of the myocardial K_{ATP} channel is driven, in part, due to circulating estrogen (6). If estrogen is responsible for the increased levels of the myocardial K_{ATP} channel observed in this study, and if this channel contributes to the observed sex differences in cardioprotection, then ovariectomized females should have similar levels of cardioprotection as those seen in males.

Activity of MMP-2. Our results suggest that, if exercise training affects the role of MMP-2 in intracellular IR injury, it does not do so by changing MMP-2 protein expression. MMP-2 is initially synthesized as an inactive, full-length 72-kD protein that must be activated by conformational changes. Therefore, it is likely that increased MMP-2 activity, not necessarily protein levels, contributes to myocardial damage (10). MMP-2 activity is regulated by many factors, including intracellular oxidative stress (20) and phosphorylation (18, 19). It is plausible that exercise provides cardioprotection through decreased MMP-2 activity and not by decreased protein levels, but MMP-2 activity was not directly tested in the current study. Furthermore, decreased MMP-2 activity would likely be the direct result of increased TIMP-2 levels, the protein's endogenous inhibitor, and not through a modification of the MMP-2 protein. This would corroborate our hypothesis that exercise confers cardioprotection through increasing TIMP-2 levels in the myocardium, but requires further investigation.

Single-cell contractile measurements. Myocardial stunning is a common consequence of IR injury (3), and exercise has been shown to reduce myocardial stunning following IR (11). However, it is not known if there is a difference in the ability of short-term versus long-term

endurance exercise training to reduce myocardial stunning following IR. Furthermore, it is unclear if there is a sex difference in the ability of exercise to reduce myocardial stunning following IR (14). Previous work has suggested that, during IR, MMP-2 specifically degrades contractile proteins such as TnI and titin (1, 2). Our observation of increased MMP-2 expression with IR, as well as exercise-induced increases in the expression of the MMP-2 inhibitor TIMP-2, suggests that cardiac myocyte contractile function would be diminished as a result of IR, but exercise training should minimize the decrease in contractile function.

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