THE EFFECT OF ACUTE AND CHRONIC EXERCISE ON MITOCHONDRIAL RESPIRATORY SENSITIVITY TO ADP IN HUMAN SKELETAL MUSCLE

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Abstract

ADP/ATP is believed to either diffuse slowly between mitochondria and the cytosol or be substituted with fast diffusing creatine (Cr) and phosphocreatine (PCr) 'phosphate shuttling' catalyzed by mitochondrial creatine kinase. Previous studies assessing Cr-dependent and independent ADP-stimulated respiratory kinetics showed acute exercise had no effect or impairment on ADP/ATP diffusion but promoted phosphate shuttling. Conversely, chronic exercise impaired both models of energy exchange. In an attempt to reconcile these seemingly diverse findings, we employed a longitudinal study design to assess the acute and chronic effects of exercise on energy exchange in human skeletal muscle when modeling *in vivo* PCr/Cr conditions *in vitro*. Our findings demonstrate an impairment in energy exchange when modeling *in vivo* concentrations of PCr/Cr found during high intensity exercise, despite an increase in mitochondrial content. These results contradict classical models of endurance exercise adaptations which hypothesize that greater oxidative capacity coincides with improved energy exchange.

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List of Abbreviations

$[ADP]_f$	Free adenosine diphosphate
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ANT	Adenine nucleotide translocator
ATP	Adenosine triphosphate
BLEB	Blebistatin
BMI	Body mass index
cCK	Cytosolic creatine kinase
$cCK^{-/-}$	Cytosolic creatine kinase knockout
CK	Creatine Kinase
$CK^{--/--}$	Double creatine kinase knockout
Cr	Creatine
Δp	Proton motive force
ETC	Electron transport chain
HIIE	High intensity interval exercise
IMM	Inner mitochondrial membrane
K_{mapp}	Apparent KM
LDH	Lactate dehydrogenase
mtCK	Mitochondrial creatine kinase
$mtCK^{-/-}$	Mitochondrial creatine kinase knockout
MyHC	Myosin heavy chain isoforms
NMR	Nuclear magnetic resonance
O_2	Oxygen
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
PCr	Phosphocreatine
Pi	Inorganic phosphate
PmFBs	Permeabilized muscle fibre bundles
ROS	Reactive oxygen species
SEM	Standard error of the mean
SNK	Student newman keuls
TBST	Tris buffered saline and Tween 20
VDAC	Voltage dependent anion carrier
V_{max}	Maximal rate of respiration
VO_2	Oxygen consumption
W	Watts
WT	Wildtype

Chapter 1: Introduction

The biochemical adaptations that occur in skeletal muscle as a result of endurance training have been studied extensively over the past 50 years with great emphasis being placed on the physiological consequences of these adaptations. While it was originally proposed that an increased capacity for endurance exercise at an exercise intensity below VO_2peak was a result of an improved ability to deliver oxygen (O_2) to the working muscles, other investigations deemed this unlikely given the finding that O_2 consumption (VO_2) at a given submaximal work rate is similar in both the trained and untrained state (23-25). This conclusion suggests that other factors may determine endurance capacity at submaximal exercise intensities and this led to a greater focus on the biochemical adaptations of skeletal muscle and furthermore, how these adaptations result in an increased endurance capacity.

It is well established that endurance exercise results in an increase in size and number of mitochondria, contributing to an overall rise in mitochondrial content (23, 26, 48). It has further been demonstrated that this increase in mitochondria is associated with a smaller disturbance in metabolic homeostasis within the muscle during exercise, specifically when looking at changes in adenosine triphosphate (ATP), adenosine diphosphate (ADP), creatine (Cr) and phosphocreatine (PCr). During exercise in trained skeletal muscle, ATP and PCr levels decrease less while ADP and Cr levels increase less compared to those in the untrained state (15, 19, 24, 25). The concentration of free ADP ($[ADP]_f$) has been established as the key regulator of oxidative phosphorylation (OXPHOS) in that as muscle contracts, ATP is hydrolyzed and ATP concentrations drop,

followed by a progressive rise in ADP and inorganic phosphate (Pi). This rise in ADP and Pi causes an increase in respiration in an attempt to produce more ATP (25). As previously mentioned, VO_2 for a given submaximal workload is the same in the trained and untrained state and additionally, the energy required to sustain this given workload is also the same across training states. With this in mind, it is thought that the increase in ADP required to drive OXPHOS in a trained individual with more mitochondria is lower than that of an untrained counterpart (23).

The degree to which mitochondria respond to a rise in ADP to produce ATP is known as mitochondrial respiratory sensitivity to ADP. Given the well-established finding that endurance training increases mitochondrial content, it has been hypothesized that this adaptation would coincide with a greater sensitivity to ADP. Interestingly, in recent years, studies investigating the effects of exercise on mitochondrial sensitivity to ADP in endurance-trained athletes found impairments in sensitivity following long-term exercise training (43, 80, 86). These somewhat perplexing findings can initially be rationalized when considering the fibre type composition of the athletes participating in these studies. For example, Kuznetsov et al., (1996) reported that type I fibres are less sensitive to ADP than type II fibres in rats (37). This is of relevance as the previously mentioned reports demonstrating impairments in sensitivity in endurance-trained athletes were associated with a significantly higher percentage of type I fibres compared to their sedentary counterparts (43, 80, 86).

Other investigations have attempted to characterize the effects of acute exercise on mitochondrial sensitivity to ADP following a variety of exercise protocols. The findings thus far have been inconsistent as certain exercise protocols and experimental

conditions showed improvements in sensitivity (69) while others showed no change or even impairments in sensitivity to ADP (53). To date, it remains difficult to reconcile the discrepancies seen within acute exercise and furthermore, the perplexing yet persistent findings indicating a lower sensitivity to ADP in trained individuals who have increased mitochondrial content and elevated oxidative capacity. Such inconsistent findings and surprising reductions in respiratory sensitivities in trained individuals further highlight the need for continued investigation into the effects of exercise on regulating mitochondrial respiratory sensitivity to ADP. With the use of a longitudinal study design we will assess the adaptations to energy exchange occurring as a result of exercise, with a specific focus on how human skeletal muscle respiratory sensitivity to ADP responds to 1) chronic exercise, 2) acute exercise in an un-trained state, and 3) acute exercise in a trained state. A short-term training protocol was utilized to examine adaptations independent of fibre type shifts.

Chapter 2: Literature Review

2.1 - ADP Sensitivity

2.1.1 - Oxidative Phosphorylation

Mitochondria are a double membrane structure, consisting of an ion permeant outer membrane, an intermembrane space rich with key physiological proteins and an inner mitochondrial membrane (IMM) that separates the mitochondrial matrix from the intermembrane space. Embedded within this inner membrane are proteins comprising the electron transport chain (ETC), a key component in mitochondrial energy transduction (Figure 1). The ETC is made up of five protein complexes (I-V) and two mobile electron carriers, which together catalyze the 'downhill' transfer of electrons through a series of redox reactions that produce free energy (50). The use of free energy in the production of ATP is based on a hypothesis proposed by eventual Nobel Prize winner Peter Mitchell in 1961 (45). Known as the chemiosmotic theory of oxidative phosphorylation, it is used to explain how the energy produced from redox reactions can be coupled to ATP synthesis through the use of proton pumps in an energy transducing membrane, such as the IMM. Within the mitochondria, this energy is used to generate an electrochemical gradient by pumping protons from the negative side (matrix) to the positive side (intermembrane space), producing Δp (proton motive force). This Δp is sufficient to force the secondary pump, known as the ATP synthase, to reverse and synthesize ATP from ADP and P_i, powered by the flow of protons against the chemical gradient back into the matrix through complex V (49). The coupling of the energy produced through oxidation and phosphorylation of ADP is known as oxidative phosphorylation (OXPHOS) and contributes 95% of the ATP produced within the cell (50).

OXPHOS is stimulated when there is a rise in $[ADP]_f$ in an attempt to maintain a steady state balance between the amount of ATP being hydrolyzed and the amount of ATP being produced. As $[ADP]_f$ progressively increases, so does the rate of mitochondrial respiration (71). The greater the sensitivity of mitochondria to ADP, the more efficiently they can respond to this rise in $[ADP]_f$ to produce ATP. There are several key modulators of ADP sensitivity within the mitochondria that each play a role in the transfer of cytosolic ADP to the mitochondrial matrix, including adenine nucleotide translocator (ANT), voltage dependent anion carrier (VDAC) and mitochondrial creatine kinase (mtCK).

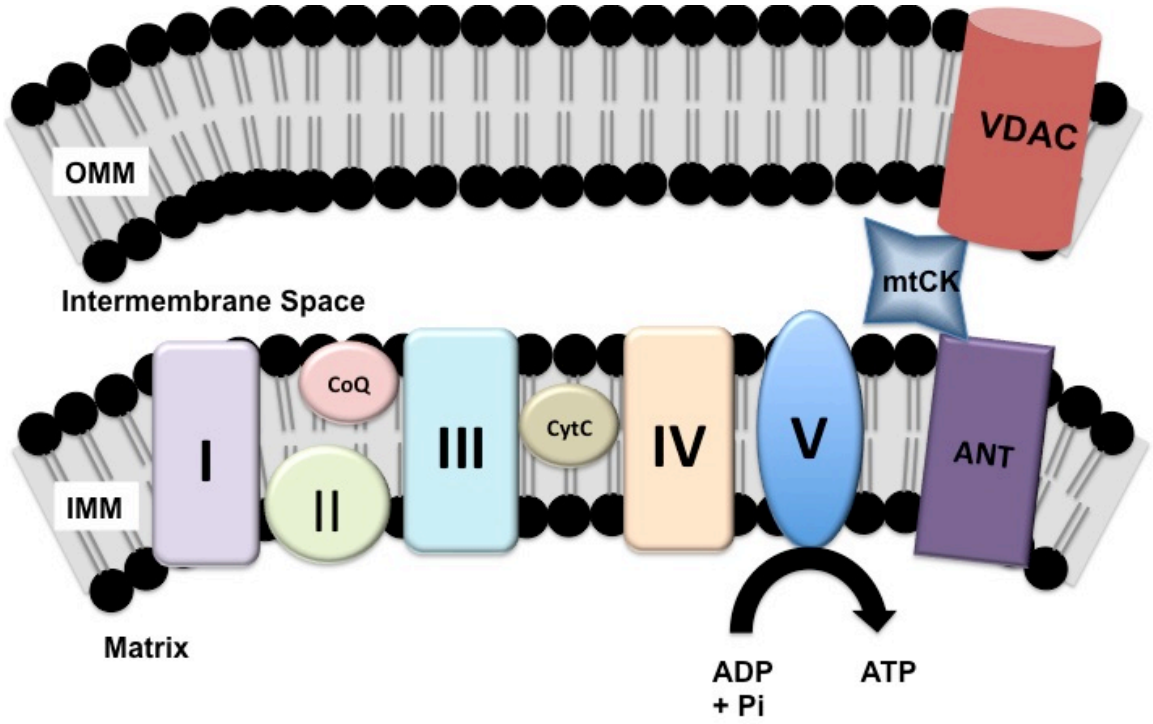

Figure 1. Schematic of Key Components of ETC. Embedded within the IMM, the ETC consists of five protein complexes (I-V) and two mobile electron carriers which catalyze the transfer of electrons downhill through redox reactions that release free energy. This free energy is used to pump protons from the mitochondrial matrix to intermembrane space, establishing an electrochemical gradient that can be used to power the phosphorylation of ADP to produce ATP at complex V.

2.1.2 – Adenine Nucleotide Translocator

ANT, the most abundant protein in the mitochondria (7, 13, 42), is embedded within the IMM and is responsible for the exchange of ATP and ADP between the intermembrane space and the mitochondrial matrix (Figure 1). Existing in four isoforms in human, ANT 1 is expressed predominately in heart and skeletal muscle and contributes to approximately 10% of total mitochondrial protein content while ANT 2 is found predominately in lymphoid cells and liver (8, 42). ANT 3 is expressed in very low levels across all tissues and ANT 4 has only been found in human brain (8). ANT is highly selective for adenine nucleotides and plays a key role in regulating the ATP/ADP ratio within the mitochondrial matrix (13). Through the use of the previously mentioned electrochemical gradient that exists across the IMM, ANT can drive the transfer of ATP produced through OXPHOS into the cytosol while also bringing ADP back into the matrix to be phosphorylated (42). Not surprisingly, in mice with an ANT 1 deficiency, ADP stimulated respiration (state III) was significantly lower compared to wild-type (WT) controls which is consistent with an inability to exchange ADP and ATP (21).

ANT plays a key role in the regulation of ADP sensitivity, specifically in regards to its functional coupling to mtCK which will be described in greater detail in section 2.1.4. Briefly, it has been demonstrated that ANT's active center is sterically close to the active centre of mtCK and can facilitate the guiding of ATP to mtCK for transphosphorylation into PCr (47, 62). This functional coupling is essential for efficient energy exchange within the mitochondria and ANT dictates the direction of this reaction by thermodynamically raising the chemical potential for ATP and lowering the chemical potential for ADP within the vicinity of mtCK to ensure the reaction is driven towards the

production of PCr. As will be described in detail in section 2.2 – Leading Models of Energy Exchange, PCr is a much more efficient transporter of high energy phosphates and can overcome diffusion limitations experienced by ATP, better meeting the demands of the working muscle. Through the transport of ATP out of the matrix and ADP into the matrix ANT maintains a level of homeostasis that maximizes efficient energy production within the mitochondria and therefore, plays a regulatory role in ADP sensitivity.

2.1.3 – Voltage Dependent Anion Carrier

VDAC, known as the mitochondrial gatekeeper, is expressed in three isoforms in human and is found on the outer mitochondrial membrane (OMM) (Figure 1) while VDAC 1 is also found on the sarcolemma membrane. VDACs form pores spanning the outer mitochondrial membrane creating a channel for metabolites to move between the mitochondria and the cytosol. These pores can exist in a high conducting state, in which the 3nm pore is completely open and the movement of metabolites, with a preference for anions, can occur across the membrane (34, 57, 58). It is in this open state that VDAC is responsible for the transport of ATP out of the mitochondria and ADP into the mitochondria to facilitate the process of maintaining energy homeostasis (14, 57). However, when membrane potential is altered, the pore will close and become preferentially selective towards smaller cations while the previously free moving anions see a great reduction in flux. In fact, the closed state of VDAC is essentially impermeable to ATP demonstrating the key role VDAC plays in the regulation of mitochondrial function (14, 34, 41, 58).

To further elucidate the ability of VDAC to control the fate of energy production within the mitochondria a VDAC 1 deficient mouse was generated. By measuring the

mitochondrial sensitivity to ADP in permeabilized muscle fibres (see 2.3.3 – Permeabilized Muscle Fibre Bundles) the importance of VDAC in the transfer of ADP and ATP was further demonstrated. In both gastrocnemius and ventricle muscle, the apparent K_M (K_{mapp}) for ADP was increased by 30% in the VDAC 1 deficient mouse compared to WT control indicating a lower sensitivity to ADP, likely due to the increased barrier for ADP to diffuse into the mitochondria (3). Interestingly, soleus of the same VDAC 1 deficient mice actually became more sensitive to ADP but this improvement was accompanied by an impairment in maximal rate of respiration (V_{max}) indicating that differences in the role of VDAC exists between muscle types (3). In addition, a VDAC 3 knockout was generated and in this model, heart tissue was less sensitive to ADP but neither oxidative or glycolytic skeletal muscle was affected (4). To date, VDAC 2 deficient mice have not been created because VDAC 2 deficient embryos are not viable (12). These fibre type differences that appear when examining the role of VDAC in the facilitation of energy exchange coincide with tissue specific roles of creatine kinase (CK), a system of enzymes that facilitate the transfer of high energy phopshates.

2.1.4 – Creatine Kinase

Creatine kinase (CK), an important enzyme in cellular energetics, is comprised of a system of isoenzymes strategically located at sites of energy production (mitochondria, mtCK) and utilization (cytosolic, cCK) within the cell (1, 79). CK catalyzes the reversible transfer of the phosphoryl group between PCr and ATP through the reaction: $MgADP^- + PCr^{2-} + H^+ \leftrightarrow MgATP^{2-} + Cr$. The most common and well accepted function of the CK system is to act as a "temporal energy buffer" maintaining high intracellular ATP/ADP ratios at the expense of PCr (78). cCK has been found to be bound to myofibrils and

functionally coupled to myosin ATPase as well as Sarcoendoplasmic Ca^{2+} ATPase and Na,K-ATPase where it's main role is to maintain a high ATP/ADP ratio during periods of elevated activity (76). More recently, the importance of mtCK in the movement of ADP and ATP between the cytosol and matrix has been established (1, 22, 63). mtCK has a strong affinity for acidic phospholipids, much like those comprising the IMM and OMM. In fact, mtCK directly binds to VDAC on the OMM and to cardiolipin of the IMM. Given the high abundance of ANT on the IMM, this affinity for cardiolipin results in contact sites between mtCK and ANT and a direct binding to VDAC (65-67). These three proteins create a functional substrate channel within the mitochondria that allows for the efficient transport of energy into and out of the mitochondria (Figure 2).

Interestingly, the relative activities of cCK and mtCK vary between fibre types within the skeletal muscle system. Gastrocnemius and psoas muscle, both of which are predominately fast twitch glycolytic muscles, have high levels of PCr and 98% of CK activity is cCK based. On the other hand, soleus and diaphragm, slow twitch, oxidative muscles, show less total CK activity overall but 25% of activity is mtCK based (17). The relative importance of both forms of CK were studied through the use of creatine kinase deficient mice lacking cCK ($cCK^{-/-}$), mtCK ($mtCK^{-/-}$) and a model deficient in both forms of CK ($CK^{--/--}$). Given the necessity of ATP for muscle contraction, force production and fatigue kinetics were examined at length in these CK deficient models. In the $mtCK^{-/-}$ mice, no impairment in twitch force, tetanic force or time to peak force in the hind limb muscle compared to WT mice was observed (68). Similarly, in the $cCK^{-/-}$ model there was no impairment in maximal twitch force (72) however, the drop in force was much

more rapid compared to WT controls and this was accompanied by a lower tetanic force and a longer half relaxation time (68). The $CK^{--/--}$ model showed impaired voluntary running capacity, through both decreased running speed and decreased distance covered compared to WT controls (46). This finding is not surprising, given the hind limb showed impaired force production after just 60ms compared to 150ms in WT as well as lower tetanic and twitch force production, a longer half relaxation time and lower tetanic force per cross sectional area (16, 68). Interestingly, when measuring fatigue resistance in the $CK^{--/--}$ mice, fibre type differences became evident. While the soleus was very quick to fatigue relative to controls, the EDL was actually more resistant to fatigue indicating a potential adaptation within this glycolic muscle (16). Overall, the strength differences between the 3 models indicate that cCK is the dominant regulator of acute force production and burst activity within the muscle. Furthermore, a deficiency in both forms of CK impairs both contractile strength and total body movement capabilities to a much greater degree than either single isoform deficiency, demonstrating the separate roles that the specific CK isoforms play as well as the combined effect of a functional CK system.

A second important set of measurements within the three CK deficient models were the changes in mitochondrial protein content and activity of mitochondrial enzymes. In $mtCK^{-/-}$ soleus muscle, where mtCK activity would normally dominate, there was a significant increase in total lactate dehydrogenase (LDH) activity as well as a shift toward the more glycolytic isoforms of LDH. This was accompanied by an increase in the relative percentage of type IIb fibres in mutant soleus as well as increased cCK activity (6). On the contrary, in $cCK^{-/-}$ gastrocnemius muscle, where cCK activity would be highest, there was an increase in the activity of electron transport chain enzymes

cytochrome c oxidase (COX), citrate synthase (CS) and succinate dehydrogenase (SDH) by 50-80% relative to WT controls (68, 72) as well as a shift toward more oxidative isoforms of LDH (17, 31). These increases in $cCK^{-/-}$ gastrocnemius are likely explained by the appearance of more intermyofibrillar mitochondria that are larger in size and form densely packed rows within the cell (31, 72). It is evident from these adaptations within the specific muscles of mutant mice that oxidative fibres from $mtCK^{-/-}$ mice adapt by taking on a more glycolytic role. Conversely, glycolytic fibres from $cCK^{-/-}$ mutants do exactly the opposite by becoming more oxidative as well as shorten the distance between the mitochondria and cytosolic ATPases to allow for better control of the sites of ATP consumption (31).

The impairments caused by a lack of mtCK, cCK or both are most evident when measuring mitochondrial function, and specifically mitochondrial sensitivity to ADP directly. In oxidative muscle, $mtCK^{-/-}$ mutants showed no difference in V_{max} or sensitivity to ADP in the absence of Cr, however, when Cr was added to the system to maximally activate mtCK, sensitivity was improved but less so compared to the improvement seen in WT (6). Other investigations used ATP to stimulate respiration by driving normal metabolic processes within the muscle. PmFBs have a powerful ADP regenerating system given that bound cCK and ATPases are still present following permeabilization. As a result, the addition of ATP can stimulate the regeneration of ADP and consequently, drive OXPHOS (33). Using this method, ATP stimulated respiration in the presence of Cr was significantly lower in the mutant mice accompanied by a significantly lower $[ADP]_f$ further demonstrating the impaired transport of PCr/Cr within the mitochondria (33). While it is surprising that Cr was capable of increasing sensitivity to any extent in

oxidative muscle of mtCK$^{-/-}$, the lowered effectiveness is to be expected given the heavy reliance on mtCK within oxidative tissue. In glycolytic muscle of mtCK$^{-/-}$ mutants there is little effect on mitochondrial function or sensitivity to ADP. ATP stimulated respiration in the absence of Cr was the same when compared to WT and the addition of Cr increased respiration rates to the same extent in both mutants and WT (33). The null effect of a lack of mtCK within glycolytic tissue highlights the low percentage of mtCK activity within normal tissue and demonstrates the high reliance on cCK of glycolytic muscle for the regulation of energy exchange.

This high reliance on cCK in glycolytic tissue is most evident in the cCK$^{-/-}$ mice. ADP sensitivity is impaired in gastrocnemius muscle in both the presence and absence of Cr despite an increased V_{max} (74). The previously described shift towards a more oxidative form does not result in an increase in mtCK activity within glycolytic tissue, which likely explains the inability of mtCK to rescue Cr-dependent ADP sensitivity within this muscle (74). The importance of a functional CK system comprised of both cCK and mtCK is highlighted when examining the effects of cCK deficiency in oxidative tissue. Despite functional mtCK, Cr has less of an effect on submaximal respiration stimulated by both ADP or ATP in both soleus and ventricle muscle of cCK$^{-/-}$ mutants compared to WT controls (33, 74). However, in the absence of Cr, ADP sensitivity was significantly higher compared to WT indicating a potential improvement in ATP/ADP diffusion as a compensation (74). While it is difficult to reconcile why cCK would affect respiration with intact mtCK, when considering the CK system as a whole, the function of mtCK is likely impaired when cCK is missing. In essence, one end of the PCr/Cr shuttle is eliminated causing an impairment in the ability of PCr to transfer high energy

phosphates and as a result, the mitochondria must rely more heavily on ATP/ADP diffusion. This is a feasible option for the mitochondria given the improved sensitivity to ADP seen in the absence of Cr in oxidative tissue (74). The summation of findings in muscle of $cCK^{-/-}$ and $mtCK^{-/-}$ mutants demonstrates the necessity of a functional CK system within this tissue in order for phosphate shuttling to occur properly. While it is evident some compensation is occurring, specifically in efficiency of ATP/ADP diffusion, neither model can overcome the disruption on the phosphate shuttling system of energy exchange highlighting the importance of both CK isoforms in the regulation of ADP sensitivity.

It is within the $CK^{--/--}$ mice that the greatest impairments in respiratory sensitivity are seen and occurring in all muscle types. In oxidative tissue, ATP stimulated respiration is significantly lower in the presence of Cr as Cr had no effect on respiration unlike the $mtCK^{-/-}$ mutants. Furthermore, $[ADP]_f$ in the presence of Cr was significantly lower in the mutant mice compared to WT (33). This further highlights the importance of a functional CK system in order for phosphate shuttling to occur properly. In glycolytic fibres, there was a 60% increase in basal respiration and 90% increase in V_{max} compared to WT, likely due to the dramatic increase in mitochondrial content and enzyme activity (31). However, similar to the glycolytic fibres of $cCK^{-/-}$ mice this did not manifest into improvements in ADP sensitivity as submaximal respiration rates stimulated by ATP were significantly lower in both the presence and absence of Cr and this was accompanied by significantly lower $[ADP]_f$ compared to WT (33). The findings from the three models of CK deficiency make it clear that while a lack of mtCK is detrimental to ADP sensitivity in oxidative fibres and a lack of cCK is detrimental to both force production and

mitochondrial function in glycolytic fibres, these two models find ways to adapt and rely more heavily on the remaining form of CK in an effort to preserve overall function. However, when neither form of CK is present in the tissue, global problems in force production, fatigue and energy production are evident. This brings to light the key role the CK system plays in energy transfer demonstrating the importance in the regulation of ADP sensitivity within the mitochondria.

2.2 – Leading Models of Energy Exchange

As previously mentioned, mtCK forms an energy exchange channel through contact sites with VDAC on the OMM and ANT on the IMM. This channel is the key component in what is known as the Cr-dependent model of energy exchange as depicted in Figure 3. Specifically, as ATP is produced through OXPHOS, ANT shuttles this ATP into the intermembrane space. mtCK can trans-phosphorylate the phosphate group on ATP to Cr, producing ADP and PCr. PCr can then be shuttled out of the intermembrane space through VDAC until it comes in contact with cCK. cCK phosphorylates cytosolic ADP to produce ATP for the working muscle. Additionally, the resulting ADP from the phosphorylation of Cr in the intermembrane space can be shuttled back into the matrix through ANT to be used for the production of ATP through OXPHOS (66, 78, 83). The high reliance on CK to facilitate the transfer of these high energy phosphates makes this model Cr-dependent and it is estimated that 80% of energy transfer occurs through this model of phosphate shuttling in cardiac muscle (1, 22). While this is the main pathway for energy exchange, it is important to note, ADP and ATP can also freely diffuse through ANT and VDAC, a model of energy exchange, which is Cr-independent in nature as mtCK is not involved in this transfer of energy.

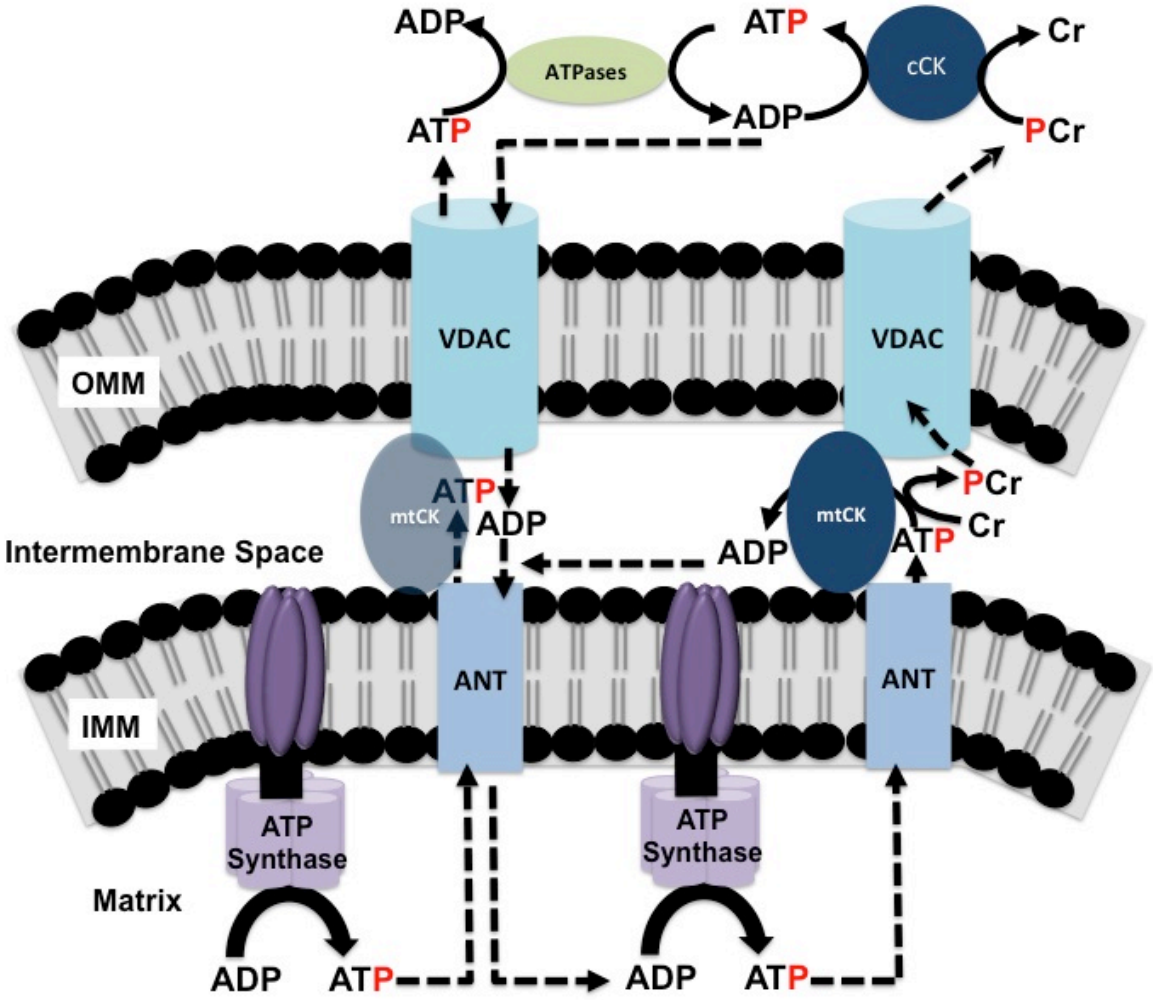

Figure 2. Schematic representation of leading models of energy exchange. The left hand side depicts the Cr-independent model of energy exchange in which miCK is inactivated and ADP/ATP rely solely on diffusion to transfer from the mitochondrial matrix to the cytosol. The other pathway, believed to transport about 80% of energy (1) is depicted on the right hand side and is Cr-dependent in nature as miCK is activated and ATP produced through OXPHOS in the mitochondrial matrix diffuses out through ANT into the intermembrane space where it meets miCK. miCK then facilitates the transfer of the high energy phosphate from ATP to PCr which can easily diffuse out of the mitochondria through VDAC to be used by cytosolic CK and ATPases at sites of high energy demand.

Using spermatozoa, a species with a vast distance between the site of energy production and site of energy utilization, it was established that PCr/Cr can overcome diffusion limitations experienced by ATP/ADP, better meeting the demands of the working muscle (77, 78). Specifically, PCr can diffuse at a rate seven times faster than

ATP and Cr can diffuse at a rate 2000 times faster than ADP, further demonstrating the improved capability of energy transfer when mtCK plays an active role (78). Given the previously described fibre type differences in reliance on mtCK, different techniques have been employed to directly assess the kinetics of energy exchange in various tissues in both rodents and humans following contraction or exercise.

2.2.1 – ^{31}P-NMR

Nuclear magnetic resonance (NMR) magnetization transfer is a commonly used technique for measurements of enzyme-catalyzed fluxes *in vitro.* In human skeletal muscle, during exercise there is a drop in PCr levels that is matched equally by a rise in Pi levels. There is minimal change in [ATP] but the calculated $[ADP]_f$ show a significant increase from rest and overall the sum of phosphorylated compounds does not change during exercise, indicating no loss of phosphate at submaximal exercise intensities (20). When viewing the NMR spectra, there is a clear transfer of the phosphate group from PCr to ATP. This has also been shown in rodent studies of hind limb muscle subject to isometric contraction (68, 72) and at length in perfused rat cardiac muscle (18, 29, 30, 36). The attribution of this phosphate shuttling between PCr and ATP to CK was confirmed through the previously mentioned models of CK deficiency. Indeed, $CK^{--/--}$ mutants demonstrate no change in peak areas on the NMR spectra indicating the inability for PCr to be hydrolyzed and the lack of phosphate transfer to ATP (68, 72). While this is a powerful, noninvasive *in vivo* method to determine concentrations of metabolically relevant phosphate compounds, the corresponding link to mitochondrial function is more difficult to infer and therefore, is a useful tool to accompany direct measurements of mitochondrial bioenergetics *in vitro* (70).

2.2.2 – Isolated Mitochondria

Chance and Williams (1955) and Lardy and Wellman (1952) were the first to determine that mitochondrial respiratory kinetics were altered in the presence and absence of phosphate acceptors and demonstrated the now classical finding that respiration rates are [ADP] dependent producing a curve that fits the now identified Michaelis-Menten model of enzyme kinetics (11, 39). By measuring oxygen consumption in isolated mitochondria, the K_{mapp} for ADP can be determined through the Michaelis–Menten enzyme kinetics – fitting model ($Y=V\max \times X/(K\mathrm{m}+X)$), where X is $[ADP]_f$ and Y is J_{O2} (rate of oxygen consumption) at $[ADP]_f$ and demonstrates the concentration of ADP that stimulates half maximal respiration. Isolated cardiac and liver mitochondria in the absence of Cr demonstrate a K_{mapp} for ADP of 17-18μM and that this value drops slightly when Cr is added to the medium to maximally activate miCK (11, 39, 47, 61, 64). However, estimations of $[ADP]_f$ within the cardiac cell are in the range of 30-50μM (73). This would suggest resting muscle operates near maximal respiratory capacity but it is well established that respiratory rates in resting muscle *in vivo* are a small fraction of capacity (64). Given the degree of manipulation that occurs during the isolation procedure, it was hypothesized that a component of functional importance was being lost and therefore, isolated mitochondria were not depicting the nature of ADP sensitivity that occurs *in vivo, in vitro.*

2.2.3 – Permeabilized Muscle Fibre Bundles

Isolated mitochondria have historically provided certain limitations when measuring the functional parameters of mitochondria given the high quantity of muscle required (>150mg) for the isolation process and the manipulation of the mitochondria that

occurs throughout the process (38, 54, 71). In 1986, Veskler et al., established a new method for assessing mitochondrial function in intact fibres through the use of saponin, a detergent that attacks areas that are cholesterol dense, including the cell membrane, while leaving areas low in cholesterol, such as the mitochondrial membrane, intact (75). By permeabilizing the cell membrane, access to the mitochondria is gained while leaving the morphological structure intact as well as maintaining organelle interaction within the cell. Additionally, this method requires a significantly lower amount of tissue (~2-3mg) making it a more attainable method for many researchers (75). Interestingly, when this method was employed to measure mitochondrial sensitivity to ADP, drastic differences between isolated mitochondria and permeabilized muscle fibre bundles (PmFBs) were seen.

In rat cardiac PmFBs, the K_{mapp} for ADP in the absence of Cr has been found to be 297μM, indicating much lower sensitivity relative to isolated mitochondria counterparts. Additionally, a much greater affect of Cr is evident, lowering the *K*m to 85μM (60, 61, 64). Similarly, in rat soleus, the K_{mapp} for ADP with no treatment has been found to be in the 350μM range while the addition of creatine lowered this to 105μM (Table 1) (37). Evidently, there are striking differences between mitochondrial respiratory kinetics in isolated mitochondria and PmFBs. As previously mentioned in section 2.1.3 – Voltage Dependent Anion Carrier, this is potentially due to the influence of cytoskeletal proteins on the permeability of the OMM, an interaction that is lost during the mitochondrial isolation process (22, 64). Importantly, in oxidative muscle, PmFBs are capable of capturing the effect of activated mtCK, as demonstrated through the considerably lower K_{mapp} in fibres treated with Cr. Moreover, in rat white gastrocnemius

muscle, the K_{mapp} for ADP with no treatment is 14μM, similar to that of isolated mitochondria and the addition of Cr lowers it to just 13μM once again indicating the little influence mtCK has on glycolytic fibres (Table 1) (37). While it is difficult to investigate fibre type differences in human skeletal muscle, the differences between Cr-independent and Cr-dependent respiratory kinetics are still evident in vastus lateralis muscle. The addition of 20mM Cr, known to elicit maximal effects, increased ADP sensitivity by 83% from the Cr-independent sensitivity to ADP. 20mM PCr was found to have exactly the opposite effect, decreasing sensitivity to ADP by 84%. Neither Cr nor PCr had any effect on $V_{max,}$ however, the concentration of PCr (1mM) required to reach half maximal effect was lower than the concentration of Cr (5mM) indicating that PCr is potentially more inhibitory to ADP sensitivity than Cr is enhancing (Table 1) (81).

Species - Muscle	**K_{mapp} – No Cr**	**K_{mapp} – 20 – 25mM Cr (μM)**	**K_{mapp} – PCr/Cr (μM)**
Rat - Cardiac	297 ±35 μM[1,2]	85 ±5 μM[1]	
Rat - Soleus	354 ±346 μM[1]	105 ±15 μM[1]	
Mouse – Soleus	222 ±80 μM[2]	90 ±0 μM[2]	
Rat – Gastrocnemius	14.4 ±2.6 μM[1]	13 ±10 μM[1]	
Human – Vastus Lateralis	140 μM[3]	63 ±7 μM[4]	300 μM[3]

Table 1. Apparent K_m for ADP in PmFBs from different muscles. Values are expressed as Mean ± SEM (when given). [1] (37), [2] (64), [3] (81), [4] (69).

It is evident from the combined findings of both rodent and human reports that the activation of mtCK is imperative to improved mitochondrial sensitivity to ADP. These findings coincide with the previously mentioned studies in spermatozoa indicating the heightened efficiency of the Cr-dependent model of energy exchange compared to the Cr-independent. While it is possible these two models of energy exchange work in conjunction with one another given the sustained ability of mitochondria to produce ATP

in the absence of Cr, it would seem intuitive that in times of high energy demand the more efficient Cr-dependent system would take over. Not surprisingly, given the lack of data to support this theory, exercise physiologists took an interest in exploring the effects of exercise, both acute and chronic, on the regulation of energy exchange in both rodent and human models.

2.3 – Exercise and ADP Sensitivity

2.3.1 – The effects of acute exercise on ADP sensitivity

In an attempt to better understand the relationship between exercise and mitochondrial sensitivity to ADP different investigations into the effects of a single bout of exercise on ADP sensitivity were performed. Tonkonogi and colleagues (1998) had participants complete a workout at 75% of VO_{2peak} until exhaustion and measured changes in sensitivity in the presence and absence of Cr as a tool to target the 2 models of energy exchange. In the absence of Cr, where ADP sensitivity is solely reliant on ATP/ADP diffusion, a single bout of exercise did not change K_{mapp} to ADP. However, with the addition of 20mM Cr, known to be maximally activating of mtCK, a single bout of exercise improved ADP sensitivity (69). Perry et al., who had participants perform exercise at 60% of VO_2peak for 2 hours, followed up this work in 2012. In this case, Cr-independent ADP sensitivity was impaired following exercise and Cr-dependent ADP sensitivity was unchanged (53). Additionally, Perry et al., measured ADP sensitivity in the presence of 12mM PCr and 24mM Cr to mimic the concentrations of the metabolites typically found in resting muscle. Surprisingly, this submaximal activation of mtCK resulted in an impairment in ADP sensitivity following a single bout of exercise (53). To date, the discrepancies within the acute exercise literature have not been resolved but it is

possible that setting in vitro concentrations of Cr:PCr to match in vivo conditions may be a critical experimental parameter to control.

2.3.2 – The effects of chronic exercise on ADP sensitivity

The first study to examine the effects of long term exercise on ADP sensitivity was performed by Walsh et al., in 2001. Participants completed 6 weeks of endurance training with 4, 1 hour sessions per week where the first 30 minutes was steady at 30% of VO_{2mac} and the second 30 minutes involved 2 minute high intensity intervals at 100% VO_2peak. Following 6 weeks of endurance training K_{mapp} for ADP was significantly increased in the trained individuals relative to sedentary in both the Cr-independent and Cr-dependent conditions, indicating an impairment in both models of energy exchange following long term training (80). Two additional studies compared ADP sensitivity in life-long trained athletes versus sedentary individuals and both found that the endurance trained athletes had a significantly higher Cr-independent K_{mapp} for ADP (43, 86). However in the Cr-dependent condition, one study found K_{mapp} for ADP was also significantly higher than sedentary counterparts (43) while the other found that Cr fixed the impairment in K_{mapp} such that there were no differences in sensitivity to ADP between active and sedentary groups (86). These somewhat perplexing findings can likely be attributed to an increase in the relative percentage of type I fibres versus type II fibres in the endurance trained athletes given the well documented and previously mentioned findings that type I fibres are less sensitive to ADP than type II fibres (37, 74).

Further work in rodents characterized changes in K_{mapp} in specific fibre types following training. Burelle and Hochachka (2002), measured changes in ADP sensitivity in both soleus and red gastrocnemius from rats that underwent 4 weeks of endurance

training with 5 sessions per week following a progressive training program. While the V_{max} in trained soleus muscle significantly increased, there were no differences in K_{mapp} to ADP in both the Cr-independent and Cr-dependent condition. Yet, in the red gastrocnemius muscle, while there was no change in V_{max} following training, K_{mapp} for ADP decreased in the Cr-independent condition while there was no change in the Cr-dependent condition following exercise (9). This suggests there is no effect of exercise on ADP sensitivity in oxidative fibres but an improved capacity for ATP/ADP diffusion in glycolytic fibres following endurance training.

Zoll et al., (2002), used the superficial and deep portions of rat plantaris muscle after rats underwent 8 weeks of voluntary running exercise. The superficial portion of the plantaris is glycolytic in nature while the deep portion is oxidative. In the absence of Cr, endurance training increased the K_{mapp} to ADP in both the deep and superficial portions of the plantaris while in the presence of Cr, the deep portion showed no difference in *K*m to ADP compared to sedentary controls while the superficial portion once again had a significantly higher K_{mapp} to ADP relative to control (85). It is of interest to note that in the sedentary group, the deep plantaris had a significantly higher K_{mapp} to ADP compared to the superficial portion in both the presence and absence of Cr. This result is to be expected given previous comparisons of oxidative versus glycolytic fibres. It was surprising to find in the trained rats, there was no difference in K_{mapp} to ADP when comparing the deep and superficial portions regardless of mtCK activation. This was accompanied by increased citrate synthase activity in the pooled plantaris and a slight elevation in type IIa fibres relative to type IIb and IIx. These findings indicate training shifts the superficial portion of the plantaris to a more oxidative role explaining the

impaired K_{mapp} to ADP and resemblance to deep plantaris sensitivity to ADP (85). While both studies confirm that endurance training has no effect on Cr-dependent sensitivity to ADP in oxidative fibres, there is conflicting data when considering Cr-independent sensitivity across all muscle types as well as Cr-dependent sensitivity in glycolytic tissue, drawing to light the extent of the work still needed to be done to understand this complex system of energy exchange.

Chapter 3: Rationale and Hypotheses

3.1 – Rationale

The effects of endurance exercise on mitochondrial content and metabolite flux have been characterized in depth over the years dating back to 1967 when John Holloszy reported that chronic exercise increased mitochondrial content in association with higher ATP/ADP ratio during exercise. This relationship has been repeated in numerous investigations, including humans, and supports the Holloszy proposal that muscle with greater mitochondrial content requires less of a rise in $[ADP]_f$ to stimulate a given rate of ATP synthesis, thereby minimizing the rise in ADP during exercise as seen in trained muscle (15, 23-25, 52). However, these investigations did not directly determine if mitochondrial respiration in response to a given [ADP] is altered post-training. In recent years, the determination of ADP sensitivity through the measurement of mitochondrial respiratory kinetics has led to a second and opposing theory, which suggests that endurance training decreases mitochondrial sensitivity to ADP despite an increase in mitochondrial content in order to elicit a tighter control of energy homeostasis by preventing erratic fluxes in ATP production in response to a rise in ADP during contraction (43, 85, 86). This model, while supported by direct measurements of ADP-stimulated respiration, is difficult to reconcile with the known improvements in ATP/ADP and mitochondrial content in trained muscle during exercise that support the Holloszy model. However, the studies measuring mitochondrial respiration post-training compared 20mM Cr to the absence of Cr conditions but have never controlled in vivo phosphate shuttling conditions. There is never a time when PCr or Cr are completely absent *in vivo,* the total Cr content remains constant and the relative concentrations of the

2 metabolites fluctuate based on the energy state of the tissue. Consequently, by maximally saturating mtCK with Cr, or completely removing it from the system, key components involved in the regulation of mtCK *in vivo* are missing and this may alter the detection of true changes in the regulation of respiratory control by ADP post-exercise. Therefore, in order to gain a better understanding of how acute and chronic exercise regulate both energy exchange systems we employed a longitudinal study design to assess the effect of both acute and chronic exercise on mitochondrial respiratory sensitivity to ADP when modeling *in vivo* concentrations of PCr and Cr. The short-term nature of this longitudinal study is designed to permit detection of respiratory adaptations independent of fibre type shifts in human muscle with training.

3.2 – Specific Objectives

This thesis compared Cr-independent (ADP/ATP diffusion model) to Cr-dependent conditions that maximally activate phosphate shuttling (20mM Cr) or reflecting *in vivo* exercising conditions (20mM Cr, 2.4mM PCr). In this context, the objectives of this thesis were four-fold:

1. To determine the effects of a single bout of high intensity interval exercise on mitochondrial respiratory sensitivity to ADP
2. To determine how basal mitochondrial respiratory sensitivity is effected by short-term chronic high intensity interval exercise (9 sessions over 3 weeks)
3. To determine how training alters the acute effects of exercise on respiratory sensitivity to ADP to exercise

4. To determine whether changes in respiratory sensitivity to ADP with training are related to altered mitochondrial oxidative capacity (electron transport chain) or contents of proteins regulating energy exchange (ANT, VDAC, mtCK)

3.3 – Hypotheses

We hypothesize that *in vivo* concentrations of PCr and Cr will reveal that acute exercise improves mitochondrial sensitivity to ADP measured post-exercise in PmFBs, and furthermore, that training will improve 'pre-exercise' respiratory sensitivity to ADP in conjunction with greater mitochondrial content, fitting with the classical findings of Holloszy and others. Additionally, given the short-term endurance training protocol, we hypothesize that improvements in resting muscle ADP sensitivity with 9 training sessions will occur independent of fibre type changes and will be associated with greater content of ANT, VDAC and/or mtCK as well as mitochondrial content. Finally, we hypothesize that training will reduce the acute effects of exercise on ADP sensitivity owing to an already improved sensitivity in 'pre-exercise' resting muscle.

Chapter 4: Materials and Methods

All experimental procedures with human participants were approved by the Research Ethics Board at York University and conformed to the Declaration of Helsinki.

Human Participants, exercise testing and muscle biopsies

Eleven healthy, recreationally active men were recruited to participate in this investigation. Their mean ± standard error of the mean (SEM) age, height, weight, Body mass index (BMI) and VO_2peak were 24.8 ± 1.0 years, 180.4 ± 1.8cm, 75.5 ± 3.4kg, 23.2 ± 0.8kg m^{-2} and 51.9 ± 1.9 ml kg^{-1} min^{-1} respectively (Table 1). All participants were non-smokers, free of disease and not taking prescription medications or supplements. Participants were given both oral and written information about experimental procedures before giving their informed consent. All experimental procedures with human participants were approved by the Research Ethics Board at York University and conformed to the Declaration of Helsinki.

Participants initially completed a standardized graded VO_2peak test on a cycle ergometer. After at least 72 h, participants returned to the laboratory and completed a practice high intensity interval exercise (HIIE) session. The session involved 10 x 4 minute intervals at 91% maximum heart rate (~83% VO_2peak) with 2 minutes of rest in between (Figure 3). After 1-2 weeks, participants reported to the clinical laboratory for the first experiment (Figure 3). With the subject lying supine on a bed, a single skeletal muscle sample was obtained from the lateral aspect of the vastus lateralis by percutaneous needle biopsy technique using a spring-loaded 14 gauge Medax Biofeather disposable needle (San Possidonio, MO, Italy) under local subcutaneous anaesthesia (~2ml of 2% xylocaine without norepinephrine). A 12 gauge cannula was used to

puncture the skin at ~45° to a depth of 2 cm and guide the needle to an additional depth of 2 cm approximately parallel to longitudinal direction of muscle fibres. Four to five cuts (10-20mg each) were sampled with the needle rotating ~30° between cuts over a period of ~1 min. Each cut was removed from the needle with sterile forceps or surgical blades before the subsequent cut was made. The first two samples were used for preparation of fibre bundles, and the remaining used for western blotting (described below).

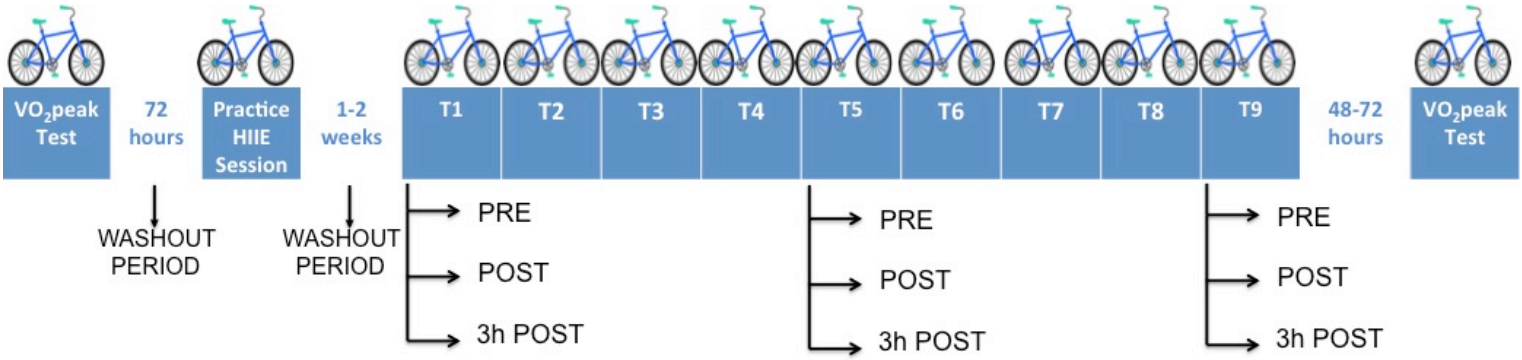

Figure 3. Longitudinal study design. Subjects first performed a baseline VO_2peak test followed by at least 72 hours as a washout period. Subjects were then brought back to complete a familiarization session and completed their first HIIE training session at 83% of their previously determined VO_2peak. After 1-2 weeks to ensure there were no residual effects of the familiarization session, subjects then began the training protocol completing 9 sessions of HIIE over the course of 3 weeks. Biopsies were taking prior to exercise, immediately following exercise and 3 hours post exercise.

After applying a band-aid to the ~2mm diameter biopsy site, participants were moved to the cycle ergometer and performed the exercise session at the pre-determined wattage to maintain 91% maximum heart rate. Immediately following this exercise session (T1), the subject moved back to the bed and a second biopsy was taken in the opposite leg as the pre-exercise biopsy. Participants remained at rest in the sitting position for 3 h after which a third and final biopsy was taken on the initial leg sampled with participants lying supine on the bed. Participants completed 8 additional exercise sessions over 3 weeks (alternating Mondays, Wednesdays, Fridays) with the same biopsy

procedure completed again in exercise sessions 5 (T5) and 9 (T9) (Figure 3). Throughout the 9 exercise sessions, cycling workload (Watts) was increased when required to maintain targeted heart rate as fitness improved. VO_2peak was re-assessed 48-72 hr after the 9^{th} session.

Preparation of permeabilized muscle fibres

This technique is partially adapted from previous methods (37, 69) and has previously been described (2, 53, 55). Briefly, small portions (~25 mg) of muscle were dissected from each biopsy and placed in ice-cold BIOPS, containing (in mM): 50 MES, 7.23 K_2EGTA, 2.77 CaK_2EGTA, 20 imidazole, 0.5 dithiothreitol (DTT), 20 taurine, 5.77 ATP, 15 PCr, and 6.56 $MgCl_2 \cdot 6\ H_2O$ (pH 7.1). The muscle was trimmed of connective tissue and fat and divided into several small muscle bundles (~2–7 mm, 1.0–2.5 mg wet weight). Each bundle was gently separated along the longitudinal axis with a pair of anti-magnetic needle-tipped forceps under magnification (Zeiss, Germany, 2000). Bundles were then treated with 30 μg/ml saponin in BIOPS and incubated on a rotor for 30 min at 4° C. Saponin at 30 μg/ml has previously been shown to optimize respiration in human skeletal muscle (32). Saponin is a mild, cholesterol-specific detergent that selectively permeabilizes the sarcolemmal membranes while keeping mitochondrial membranes, which contain little cholesterol, intact (38, 75). Following permeabilization, the PmFBs were placed in MiR05 containing (in mM): 0.5 EGTA, 10 KH_2PO_4, 3 $MgCl_2$.6 H_2O, 60 K-lactobionate, 20 Hepes, 110 sucrose and 1 mg/ml fatty acid free BSA (pH 7.1). PmFBs were washed at 4° C (<30 min) in MiR05 until the respiratory measurements were initiated.

Mitochondrial respiration in permeabilized fibres

High-resolution O_2 consumption measurements were conducted in 2 ml of respiration medium (MiR05) using the Oroboros Oxygraph-2k (Oroboros Instruments, Corp., Innsbruck, Austria) with stirring at 750 rpm. Respiration medium contained one of three combinations of Cr and PCr which modulates the mtCK-dependent phosphate shuttling system (5, 63, 64, 81): no creatine to model ADP/ATP diffusion, 20mM Cr to saturate miCK and phosphate shuttling, or 2.4 mM PCr and 20mM Cr to mimic in vivo phosphate shuttling conditions. The latter condition is concentrations reported in un-trained human muscle homogenate prepared from biopsies 5 min into exercise at 95% maximum heart rate which is similar to the present investigation (52). For ADP-stimulated respiratory kinetics, 5 mM glutamate and 5 mM malate were added as complex I substrates (via generation of NADH to saturate electron entry into complex I) followed by ADP titrations in step-wise increments. All experiments were completed before the oxygraph chamber [O_2] reached 150 μM. PmFBs spontaneously contract in assay medium, a phenomenon which can be prevented by the myosin II-specific inhibitor (BLEB) (55). BLEB at 25 μm, dissolved in DMSO (5 mM stock), was used to prevent spontaneous contraction in PmFB experiments. Polarographic oxygen measurements were acquired in 2 s intervals, with the rate of respiration derived from 40 data points, and expressed as pmol/s. Cytochrome *c* was added to test for mitochondrial membrane integrity, with all experiments demonstrating <10% increase in respiration.

The apparent K_{mapp} for ADP was determined through the Michaelis–Menten enzyme kinetics – fitting model ($Y=V\text{max}\times X/(K\text{m}+X)$), where X is [free ADP] (ADPf) and Y is J_{O_2} at [ADPf] – using Prism (GraphPad Software, Inc., La Jolla, CA, USA), as

published previously (55).

Western blot analyses

An aliquot of frozen muscle (10-30 mg) from each pre-training biopsy was homogenized in a plastic microcentrifuge tube with a tapered teflon pestle in ice cold buffer containing (in mM) 40 HEPES, 120 NaCl, 1 EDTA, 10 $NaHP_2O_7 \bullet 10H_2O$ pyrophosphate, 10 β-glycerophosphate, 10 NaF and 0.3% CHAPS (pH 7.1). Protein concentrations were determined using a BCA assay (Life Technologies, Carlsbad, CA, USA). Fifty micrograms of denatured protein was loaded for Western blotting and resolved by SDS-PAGE on 6–12% polyacrylamide gels, depending on the molecular weight of the protein, and transferred to a Low Fluorescence PVDF membrane. Membranes were blocked with Licor Odyssey Blocking Buffer (Licor, Lincoln Nebraska, USA) and immunoblotted overnight (4° C) with antibodies specific for each protein. A commercially available monoclonal antibody was used to detect electron transport chain proteins (human OXPHOS Cocktail, Abcam, Toronto, ON, Canada) at a concentration of 1:208. Commercially available polyclonal antibodies were used to detect VDAC isoforms 1 (1:500), 2 (1:500) and 3 (1:1000) (Sigma-Aldrich, St Louis, MO, USA), ANT isoforms 1 (1:40) and 2 (1:1000) (Abcam, Cambridge, UKa) and miCK (generous gift from Dr. Uwe Schlattner, Grenoble, France). An average of β-tubulin (Sigma Aldrich) and α–Actinin (Abcam) were used as a loading control given each individual protein demonstrated variability across all time points (Appendix A, Table 1). The cause for this variability is unclear but may be due to training responses or the percutaneous muscle biopsy procedure itself given a previous report demonstrated up to 25% variability in content for a variety of proteins across repeated biopsies within a subject (10). We do not

attribute this variability to methodological error in western blotting given the coefficient of variation of 6.4% for loading multiple lanes of a single homogenate. Furthermore, the western blots were repeated on newly prepared homogenate preparations but similar results were obtained.

Following overnight incubation in primary antibodies, membranes were washed 3x5 min in TBST and incubated for 1 h at room temperature with the corresponding infrared fluorescent secondary antibody (Licor, Lincoln, Nebraska, USA). Immunoreactive proteins were detected by infrared imaging (Licor CLx, Licor, Lincoln, Nebraska, USA) and quantified by densitometry (Image J).

Fibre Type: Myosin Heavy Chain Isoforms (MyHC)

MyHC isoforms were resolved using polyacrylamide gel electrophoresis (PAGE) adapted from methods previously described (35, 51). Briefly, 10 μg of vastus lateralis from the 3 h post exercise biopsies at T1, T5 and T9 proteins were resolved using a 0.75mm SDS/PAGE gel consisting of a stacking gel with 4% bis-acrylamide and a separating gel with 10% bis-acrylamide (final concentration separating: 30% glycerol, 0.2 M Tris-HCl pH 8.8, 0.1 M glycine, 0.4% SDS, 0.1% ammonium persulfate, 0.05% TEMED; stacking: 0.125 M Tris-HCl pH 6.8, 0.1% SDS, 0.1% ammonium persulfate, 0.05% TEMED). The electrophoresis buffer consisted of 100 mM Tris, 150 mM glycine, 0.1% SDS and 0.12% 2- mercaptoethanol (35). The gels were run on a Minigel electrophoresis system (Biorad Laboratories, Rockford, IL) at 4 °C at constant current of 20 mA until proteins entered the stacking gel and then at 140mV for a further 20 h to separate type I bands from type II bands. Different isoforms of type II fibres (IIa and IIx) were not resolved as this was not necessary for our determination of fibre type shifts between type I and type II fibres.

Following electrophoresis, gels were silver stained using Silver Stain for Mass Spectrometry kit following manufacturers' recommendations (Pierce, Rockford, IL). Bands were identified as described (35).

Statistics

Results are expressed as mean ± SEM. The level of significance was established at $P < 0.05$ for all statistics. A one-way ANOVA with repeated measures was used to test for differences in 1) K_{mapp} between each Pre across the training period (T1 Pre vs. T5 Pre vs. T9 Pre) 2) K_{mapp} within a training session (pre, post, 3h post) 3) the change in K_{mapp} from Pre to Post ('delta K_{mapp}' within each session) across T1, T5 and T9, and 4) densitometry values between the pre-exercise biopsies across T1, T5 and T9. When a significant *F*-ratio was obtained, *post hoc* analyses were completed using a LSD *post hoc* analysis. Missing data at two T5 Pre samples due to insufficient tissue availability were replaced with a regression imputation analyses in order to facilitate complete comparisons within all participants by 1 way ANOVA with repeated measures.

Chapter 5: Results

The mean power output during the training sessions increased by 9% between T1 and T9 (240 ± 13 to 260 ± 4 W, $p<0.05$ data not shown). Average exercise heart rate throughout 10 intervals did not change with training and averaged 91% across training sessions. $\dot{V}O_{2\,max}$ increased by 8.7% following training (51.9 ± 1.9 to 56.4 ± 2.1 $mL \cdot kg^{-1} \cdot min^{-1}$; $p < 0.05$; Table 1).

Effects of acute and chronic exercise on mtCK-independent ADP sensitivity

We first determined the effect of an acute bout of HIIE on mitochondrial respiratory sensitivity to ADP in PmFBs. When relying solely on ADP/ATP diffusion (Cr-independent), exercise increased respiratory sensitivity to ADP (decreased K_{mapp}) in both post-exercise biopsies (Pre: 735 ± 122μM, Post: 512 ± 103μM, 3h post: 469 ± 94μM, $p<0.05$ vs Pre; Fig. 4). This acute effect of exercise was not observed in T5 (Pre: 678 ± 124μM, Post: 603 ± 92μM, 3h post: 664 ± 83μM; Fig. 3) or T9 (Pre: 422 ± 77μM, Post: 330 ± 71μM, 3h post: 404 ± 93μM; Fig. 4). However, chronic exercise increased respiratory sensitivity to ADP in Pre muscle 40% by T9 (T9 Pre: 422 ± 77μM vs T1 Pre: 735 ± 122μM, $p<0.05$, Fig. 4).

Effects of acute and chronic exercise on Cr-dependent ADP sensitivity

In contrast to the Cr-independent system, there was no effect of a single bout of exercise in the presence of 20mM Cr (Cr-dependent) (Pre: 89 ± 30μM, Post: 64 ± 13μM, 3h post: 92 ± 31μM; Fig. 5). However, following T5, the Cr-dependent condition showed a significant decrease in sensitivity 3h post exercise relative to T5 Pre (Pre: 88 ± 24μM, Post: 118 ± 34μM, 3h post: 185 ± 50μM, $p<0.05$; Fig. 5). This impairment in sensitivity was abolished by T9, as T9 Pre K_{mapp} was significantly lower relative to T1 Pre and T5

Pre, indicating an improvement in sensitivity to ADP (T9 Pre: 63 ± 27μM, $p<0.05$, Fig. 5). No further improvements in sensitivity were seen following the ninth training session (T9 Post: 69 ± 16μM, T9 3h Post: 83 ± 24μM; Fig. 5).

Effects of acute and chronic exercise on PCr/Cr-dependent ADP sensitivity

Similar to the Cr-dependent condition, in the presence of typical muscle concentrations of Cr and PCr found during this specific high intensity exercise, there was no effect of a single bout of exercise on ADP sensitivity (Pre: 879 ± 201μM, Post: 962 ± 151μM, 3h post: 973 ± 212μM; Fig. 6). The PCr/Cr-dependent condition also showed a significant decrease in sensitivity (higher $K_{mapp)}$ following exercise at T5 (T5 Pre: 1071 ± 144μM, T5 Post: 1356 ± 159μM, $p<0.05$, Fig. 6) however; this impairment in sensitivity was recovered 3h post exercise (T5 3h Post: 1214 ± 209μM; Fig. 6). Contrary to the Cr-independent and Cr-dependent conditions, by T9 sensitivity to ADP was significantly impaired relative to T1 Pre and T5 Pre (T9 Pre: 1305 ± 196μM, $p<0.05$, Fig. 6) and no further changes in sensitivity were seen following the T9 (T9 Post: 1276 ± 201μM, T9 3h Post: 1160 ± 216μM; Fig. 6).

Phosphate shuttling protein contents

The content of proteins involved in mitochondrial-cytosolic energy exchange were measured prior to exercise at all three time points. VDAC isoforms 1, 2 and 3 contents did not change with training (Fig. 7) nor did content of ANT 1 or ANT 2 (Fig. 8). mtCK protein content did not change as a result of 9 exercise sessions (Fig. 9). Finally, the contents of the electron transport chain proteins were measured using an OXPHOS cocktail. Complex I increased 56% by T5 ($p<0.05$) and 47% by T9 vs T1 ($p<0.05$) with no difference between T9 and T5. Complex II did not change with training.

Complex III at T9 increased 29% relative to T1 ($p<0.05$) with no difference between T5 and T1 or T5 and T9. Complex IV at T5 increased by 32% relative to T1 ($p<0.05$) and by T9 increased 36% relative to T1 ($p<0.05$) with no differences between T5 and T9. Complex V protein content was unchanged with exercise (Fig. 10). The average of all five complexes increased 24% from T5 to T1 ($p<0.05$) and 30% from T9 to T1 ($p<0.05$) with no change between T5 and T9 (Figure 10).

Myosin Heavy Chain Isoforms (MyHC)

Changes in percentage of type I fibres relative to type II fibres were measured 3h post exercise at T1, T5 and T9 (Fig. 11). Relative proportions were unchanged over the 9 exercise sessions (T1: 41% Type I, 59% Type II; T5: 34% Type I, 66% Type II; T9: 44% Type I, 56% Type II).

Chapter 6: Discussion

In this study, we questioned whether modeling *in vivo* exercising concentrations of Cr and PCr *in vitro* reveals increases in respiratory sensitivity to ADP following training in keeping with the model proposed by Holloszy et al (19, 23-25) that predicts increased mitochondrial contents are associated with greater ADP sensitivity. Previous investigations compared +/- Cr *in vitro* to determine the effect of acute and chronic exercise on ADP/ATP diffusion vs phosphate shuttling models of energy exchange. Surprising impairments in ADP sensitivity +/- Cr following training directly challenge the Holloszy model of mitochondrial content being a critical determinant of respiratory sensitivity to ADP. By controlling 'exercising' [Cr:PCr]-*in vitro* to capture critical regulatory parameters of mtCK, we expected to detect improved ADP sensitivities post-training which would highlight the importance of controlling *in vivo* conditions during *in vitro* assessments. Instead, our findings demonstrate controlling exercising [Cr:PCr] *in vitro* revealed lower ADP sensitivity following training despite increases in sensitivity +/- Cr. These observations challenge our understanding of the relationship between mitochondrial content and ADP sensitivity as well as the role of mtCK and energy exchange pathways in mediating improved mitochondrial respiratory control by ADP. These findings also highlight the importance of modeling in vivo exercising Cr:PCr conditions during *in vitro* assessments of respiratory kinetics.

6.1 - Acute exercise: no change in mtCK-dependent respiration despite evidence for improved Cr-independent adenine nucleotide diffusion

Our findings suggest acute exercise does not alter the regulation of energy exchange in human skeletal muscle. This observation was made when modeling *in vivo*

Cr:PCr concentrations during the assessments of respiratory sensitivity to ADP *in vitro* using permeabilized muscle fibres. Of note, acute exercise improved Cr-independent sensitivity, suggesting improved ADP/ATP diffusion. However, this improvement was not seen in conditions where mtCK was maximally activated with 20mM Cr or when modeling *in vivo* exercise concentrations of PCr/Cr, making the importance of this response difficult to interpret as Cr is never absent in the muscle during exercise.

The lack of change in both Cr-dependent and Cr:PCr-dependent respiration suggests acute exercise does not alter mtCK-mediated phosphate shuttling, in contrast to previous findings (53, 69). Specifically, following an acute 2h bout of exercise at 60% VO_2peak, Cr-independent and Cr:PCr-dependent sensitivity to ADP was impaired while Cr-dependent was unchanged (53). On the contrary, following an acute bout of exercise at 75% VO_2peak until fatigue, there was no change in Cr-independent sensitivity to ADP and an improvement in Cr-dependent sensitivity (69). While it is difficult to determine the reason for the discrepancies in findings following acute exercise, it is possible that the varied responses are related to the exercise intensities adopted (Fig. 12). The contractile state of the muscle *in vitro* should also be considered when comparing findings to previous literature. As will be discussed in greater detail in section 6.4 – Importance of experimental paramaters, in the present investigation, PmFBs were in the relaxed state through the prevention of ADP-induced contraction using blebbistatin (BLEB), a myosin ATPase inhibitor. This is an important experimental parameter to consider as it was recently established that fibre contraction increases sensitivity to ADP (55). Prior to this finding, conclusions regarding sensitivity to ADP were made based on measurements in contracted muscle while the more recent findings from Perry et al (2012) and the present

investigation reflect measurements in relaxed muscle. This further indicates the importance of considering the experimental parameters when investigating this model of energy exchange.

6.2 - Chronic exercise: impaired sensitivity to ADP when modeling Cr:PCr concentrations present during exercise *in vivo* despite improvements +/- Cr

Following chronic exercise, sensitivity to ADP appears impaired when modeling concentrations of Cr:PCr found during exercise *in vivo*. This impairment was despite improvements in sensitivity in both the Cr-independent and Cr-dependent conditions. Previous investigations measuring changes in sensitivity to ADP following chronic exercise found a decrease in sensitivity in Cr-independent conditions (43, 80, 86) and either no change (86) or impaired sensitivity in Cr-dependent conditions (43, 80). These previous perplexing impairments in sensitivity were in the Cr-dependent and Cr-independent conditions are likely attributed to an increased proportion of type I relative to type II fibres in trained individuals as it has previously been shown that type I fibres have a lower sensitivity to ADP relative to type II (37). In the present investigation, 9 exercise sessions did not elicit changes in the relative proportion of type I to type II fibres. Therefore, this is the first report to demonstrate changes in sensitivity post-training occurring in the absence of fibre type shifts. This important finding reveals that adaptations occur within one or both of the fibre types whereas previously it has been suggested that muscle respiratory sensitivity is dependent solely on fibre type (9, 37, 85).

Furthermore, the altered sensitivities in all Cr/PCr conditions occurred without overall changes in the proteins involved in energy exchange (ANT, VDAC, mtCK). This suggests post-translational regulation of one or more proteins involved in both ADP/ATP

diffusion and phosphate shuttling of energy exchange resulting in an enhancement in the overall sensitivity of this energy exchange pathway as a result of exercise training.

6.3 - *In vivo* implications of exercise training on energy homeostasis

It is well established that exercise training increases oxidative capacity as a result of increased mitochondrial content and furthermore, it has previously been proposed that mitochondrial sensitivity increases as mitochondrial content increases (19, 23, 24). While this conclusion is most intuitive in nature, it does not help to explain the more recent findings demonstrating impaired sensitivity to ADP following training under various experimental conditions (43, 86). A second theory has been developed in an attempt to reconcile this decreased sensitivity despite increased mitochondrial content after training. It has been proposed that trained individuals have a tighter coupling between energy production and energy utilization due to an increased sensitivity to mtCK, indicating that in trained individuals, it is perhaps the PCr/Cr ratio as opposed to the ATP/ADP ratio that drives oxidative phosphorylation (80, 85, 86). Through this tighter control of energy production, a higher K_{mapp} contributes to the efficient production and utilization of energy and is activated through an unknown signaling pathway (86).

Contrary to our original hypothesis, our data supports this second theory given the impairment in sensitivity observed following 9 training sessions when modeling *in vivo* concentrations of PCr and Cr. Admittedly, this decreased ADP sensitivity is difficult to reconcile given the well-known finding that ATP/ADP_f ratio decreases less during exercise in a trained individual (19, 52, 56), which has classically been interpreted as reflecting an increase in sensitivity in individuals with greater mitochondrial content. Translating this impaired sensitivity with [Cr:PCr] is further complicated by the fact the

improved ADP sensitivity in the presence or absence of Cr supports the Holloszy model of mitochondrial content being a critical factor regulating respiratory control by ADP (19, 23-25). These improved sensitivities also completely contradict previous reports of impaired sensitivity but suggests the increased proportion of type I fibres following training (9, 43, 86) leading to overall decreased ADP sensitivity in mixed muscle PmFBs may have masked improvements within individual fibres. Nevertheless, despite being a novel observation that is consistent with improved ATP/ADP_f during exercise (19, 23-25), the physiological relevance of the improvements in ADP sensitivity +/- Cr in the present study are difficult to reconcile with the lower sensitivity in [Cr:PCr] conditions.

While the reason for these divergent responses across conditions is unclear, one possible interpretation is that mtCK sensitivity to both Cr and PCr regulation is increased following training. Hence, Cr-activation of mtCK appears improved as does PCr-inhibition of its activity. Indeed, the only difference between +Cr and the Cr:PCr condition is the addition of 2.4 mM PCr as 20mM Cr was used in both conditions. The physiological implications are counter-intuitive if mtCK sensitivity to PCr inhibition increased in addition to sensitivity to Cr activation, as the [Cr:PCr] used *in vitro* reflect *in vivo* exercising conditions. Ideally, additional comparisons to 'resting' muscle [Cr:PCr] and 'exercising' [Cr:PCr] in T1 vs T5 vs T9 would be performed to more accurately model the phosphate shuttling conditions that change with training. However, this would not alter the observation that ADP sensitivity still worsened at a fixed [Cr:PCr] assessed at each time point. To translate these findings to *in vivo* conditions is difficult as the implication would be that training results in impaired ADP sensitivity *in vivo*, which as noted above is inconsistent with improved ATP/ADP*f* post-training (19, 23-25). Thus,

when modelling *in vivo* conditions of [Cr:PCr], despite increased mitochondrial content and the absence of fibre type shifts, our findings do not support the known improvements in metabolic control (ATP/ADP_f) during exercise nor the model that increased mitochondrial content is associated with increased respiratory sensitivity to ADP. Future work addressing the mechanisms by which these changes in sensitivity occur following exercise would provide greater insight into the regulation of energy exchange *in vivo* and may help further explain these perplexing findings regarding regulation of ADP sensitivity following endurance training.

6.4 - The importance of additional experimental parameters

As previously mentioned, this investigation was performed using PmFBs in the relaxed state *in vitro*. BLEB has been used to prevent ADP-induced contraction that occurs during respiratory assessments in PmFBs (40, 53, 55, 82). The importance of modulating contractile state post-exercise on phosphate shuttling assessments was achieved by comparing BLEB vs. no BLEB in acute exercise but only with Cr-dependent conditions (53). We attempted to repeat this comparison across varying concentrations of Cr and PCr. However, we observed complete disintegration of PmFB in the absence of BLEB using small micro-punch biopsies (2.1mm length fibres) which has recently been noted by others (28) that was not evident previously with larger bergstrom biopsies (<5 mm length fibres). Consequently, we obtained very low respiratory rates in the absence of BLEB whereas BLEB preserved fibre integrity, which permitted accurate respiratory assessments compared to K_{mapp} of ADP of previous reports in humans (53, 55). This is of significant relevance as previous findings demonstrating an improvement in Cr-dependent sensitivity following acute exercise were measured in contracted fibres (No

BLEB) *in vitro* (53, 69) while the findings demonstrating no change in sensitivity were measured in relaxed fibres (with BLEB) *in vitro* (53). To date, no study has investigated the importance of +/- BLEB in detecting changes following training. Indeed, this study was designed to compare contractile states *in vitro* but this was not possible given the disintegration of PmFBs and minimal respiratory kinetics in the absence of BLEB. As such, the present findings must be interpreted in the context of the acute and chronic effects of exercise *in vivo* on muscle mitochondrial respiratory kinetics assess in relaxed muscle *in vitro*.

With this in mind, the advantages of PmFB relaxation are to optimize diffusion of ADP, substrates and oxygen into the core of each fibre and prevent fibre disintegration by the magnetic stirring during respiratory assessments, both of which prevent loss of respiratory kinetics as noted above. The specific disadvantage is we cannot rule out possible side effects of BLEB that may mask the effects of exercise. However, we have previously shown BLEB increases maximal respiration likely through improving diffusion gradients by exposing as many relaxed fibres as possible to the assay media. We therefore expect BLEB facilitated accurate detection of exercise effects on K_{mapp} to ADP rather than interfere with the assessments.

Chapter 7: Conclusions and Future Directions

7.1 – Conclusions

In the current study determining K_{mapp} for ADP while modeling concentrations of PCr/Cr found *in vivo* provides a new perspective on the effects of both acute and chronic exercise on mitochondrial sensitivity to ADP. Based on the findings from the present investigation, and contrary to our hypothesis, we found that acute exercise has no effect on phosphate shuttling-dependent respiratory kinetics while chronic exercise impairs kinetics when measured with *in vivo* [Cr:PCr] conditions. These chronic adaptations occurred in the absence of changes in content of proteins involved in the energy exchange pathway (ANT, VDAC, mtCK) and furthermore, in the absence of fibre type changes, indicating that another mechanism of regulation is at play. Once again, we are left with the perplexing finding that endurance exercise impairs mitochondrial sensitivity to ADP despite the increased mitochondrial content and oxidative capacity that accompanies long-term training. Given the improvements in sensitivity seen in the Cr-independent and Cr-dependent conditions, this study highlights the necessity of controlling experimental parameters to mimic conditions found *in vivo* in order to gain better insight into the regulation of energy exchange in skeletal muscle.

7.2 – Future Directions

Given that total content of the proteins involved in the energy exchange pathway did not change with endurance training, other potential sites of regulation should be explored. Specifically, when considering post-translational modifications, ANT, VDAC and mtCK are all potential sites. Proteomics techniques have determined that many mitochondrial proteins are acetylated and in fact, ANT is abundantly acetylated,

specifically at lysines 10, 23 and 92 (44). Interestingly, the affinity of ANT of ATP/ADP is much lower upon acetylation and furthermore, exercise decreases acetylation at lysine 23 indicating a potential regulatory mechanism for energy exchange within ANT (44). VDAC on the other hand, though it can also be acetylated, is predominately phosphorylated. While the phosphorylation sites of the three VDAC isoforms are well established, less is known about the physiological consequences of this modification (34). A study by Yoo et al (2001), reported phosphorylation of VDAC 1 in post-mortem brains of Alzheimer's and Down Syndrome patients but the specific sites of phosphorylation were unknown (84). Other work has attempted to correlate apoptosis and VDAC phosphorylation but very contradictory findings have been reported (34). While no work to date has looked directly at the effect of phosphorylation of VDAC on energy exchange within the mitochondria, given the substantial evidence for the potential detrimental effects of this post-translational modification, future work should attempt to characterize the effects of phosphorylation on mitochondrial function.

Other areas of research have focused investigations into the cellular compounds that may regulate the permeability of VDAC. In recent years, dimeric tubulin, a cytoskeletal protein in large abundance was found to reversibly block VDAC, resulting in impaired mitochondrial sensitivity due to the inability of ADP to reach the mitochondrial matrix accompanied by a build up of ATP within the mitochondria (22, 58). Specifically, experiments in brain and heart isolated mitochondria demonstrated 1-10μM tubulin resulted in 10x lower mitochondrial sensitivity to ADP due to blockage of VDAC (59). This is consistent with previous findings that mitochondrial sensitivity to ADP is much higher when measured in isolated mitochondria due to damage of the OMM and removal

of key cytoskeletal components during the isolation process (64). When compared to PmFBs, where structural and morphologic interactions remain more intact vs isolated mitochondria, sensitivity to ADP is significantly decreased, indicating a functioning OMM where VDAC controls the movement of nucleotides in and out of the mitochondria. It is clear that VDAC is of functional relevance when discussing the regulation of mitochondrial sensitivity to ADP and it's role within the mitochondria, and future work should be directed towards examining the potential sites of regulation of VDAC. Until then, understanding the role of each isoform in mediating exercise adaptations in respiratory sensitivity will be limited.

An interesting characteristic of mtCK is its ability to exist in two different oligomeric forms: dimeric and octameric. While mtCK is mostly found in it's octameric form, there can be a dynamic shift between the two depending on cellular pH, temperature and concentration. Importantly, mtCK must be in the octameric form in order for phosphate shuttling to occur properly and yet, the ratio of octameric/dimeric mtCK in humans is still uncharacterized (67). Future work investigating the effect of exercise on the ratio of octameric/dimeric mtCK could provide greater insight into the regulation of energy exchange in the untrained vs trained state. Additionally, it has previously been determined that mtCK is redox sensitive however, the effect of reactive oxygen species (ROS) generated by the mitochondria on mtCK enzyme activity has yet to be established (27). As a result, the role of ROS in exercise and the subsequent regulation of mtCK activity is of great importance.

Aside from the mechanisms by which exercise elicits changes in sensitivity to ADP, there are still many remaining questions to address when considering the ability of

exercise to regulate K_{mapp} for ADP. Burelle and Hochachaka (2002) (9) and Zoll et al., (2002) (85) investigated the effects of endurance exercise on sensitivity to ADP in both oxidative and glycolytic fibre types. While these investigations had somewhat conflicting data, both showed minimal adaptations occurring in the oxidative tissue and the majority of changes occurring in glycolytic tissue. This is somewhat surprising given the previously mentioned fact that glycolytic tissue relies very minimally on mtCK for energy exchange. However, neither study mimicked physiological concentrations of PCr/Cr and when considering the polar opposite conclusions that can be drawn from the present investigation when considering Cr-dependent and Cr-independent vs PCr/Cr-dependent, an important next step will be to determine the effects of chronic exercise on specific fibre types when modeling *in vivo* concentrations of PCr and Cr. Not only will this provide the most physiologically relevant conditions to draw conclusions from, this combined with previous work will allow for a greater understanding of the fibre type specific adaptations that occur as a result of endurance training to help further understand the adaptations occurring in human skeletal muscle.

Chapter 8: Tables and Figures

Age (years)	**24.8 ± 1.0**
Weight (kg)	75.5 ± 3.4
Height (cm)	180.4 ± 1.8
BMI (kg/m^2)	23.2 ± 0.8
Heart Rate (% of max)	91.4% ± 0.5
Pre VO_2peak ($ml\ kg^{-1}\ min^{-1}$)	51.9 ± 1.9
Post VO_2peak ($ml\ kg^{-1}\ min^{-1}$)	56.4 ± 2.1
Power Output (W)	239.8 ± 12.9
Power Output Increase (W)	20.5 ± 4.2

Table 2. Group Characteristics. All values are represented as mean ± SEM.

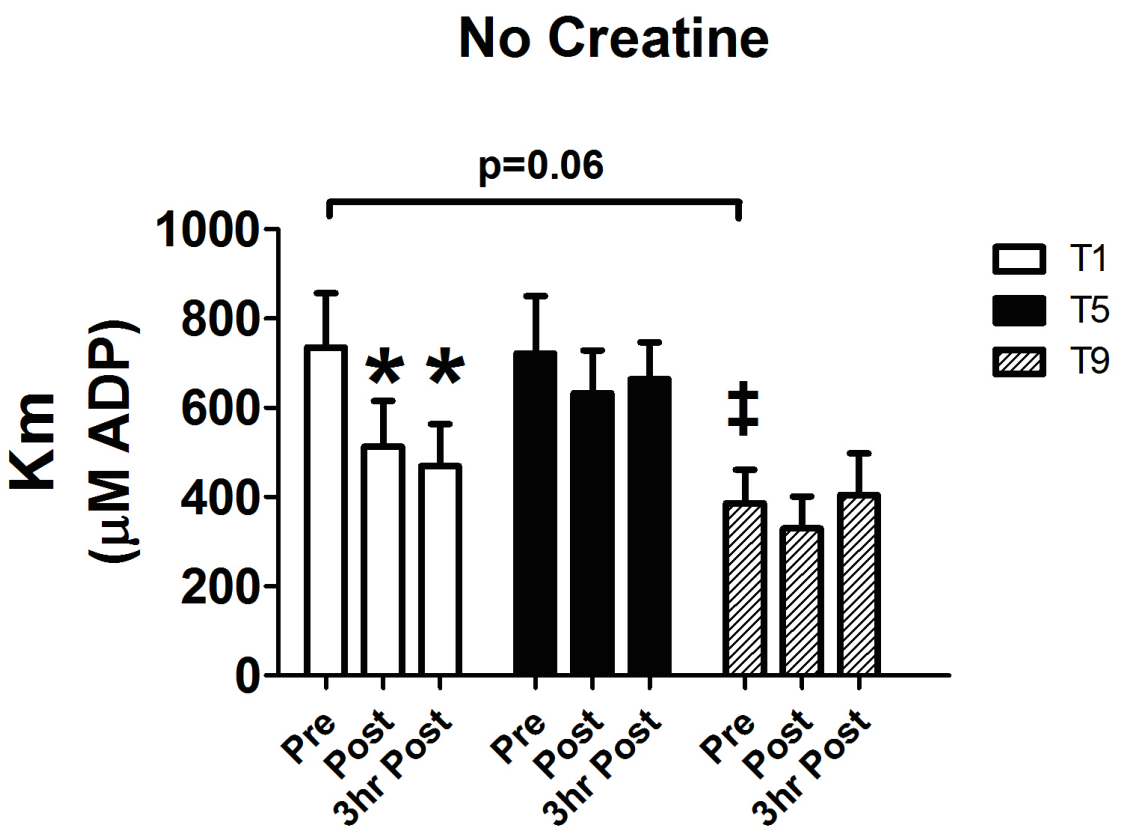

Figure 4. The effect of exercise on creatine-independent sensitivity to ADP. The K_{mapp} to ADP was determined in the absence of creatine. Results represent mean ± SEM for 11 participants. *, < T1 Pre, $p<0.05$. ‡, T9 Pre <T5 Pre, $p<0.05$.

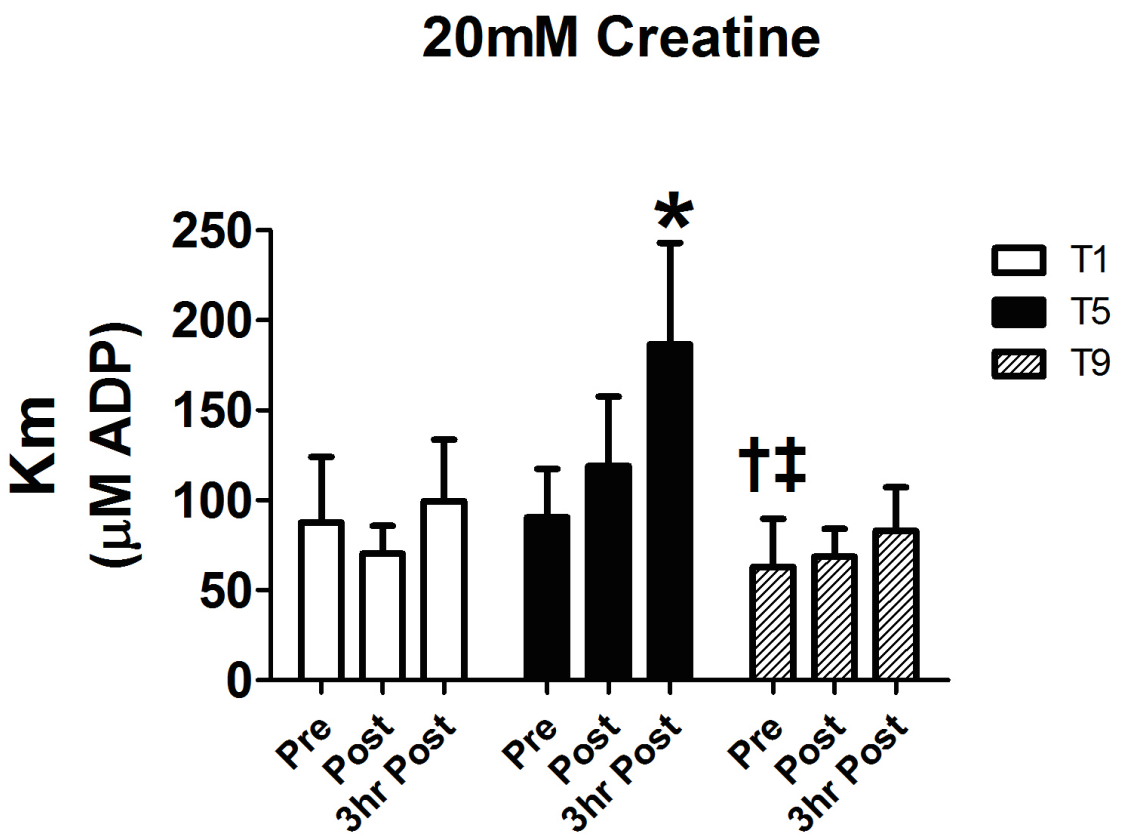

Figure 5. The effect of exercise on creatine-dependent sensitivity to ADP. The K_{mapp} to ADP was determined in the presence of 20 mM creatine. Results represent mean ± SEM for 11 participants. *, 3h Post> Pre, $p<0.05$. ‡, T9 pre < T5 pre, $p<0.05$. †, T9 pre < T1 pre, $p<0.05$.

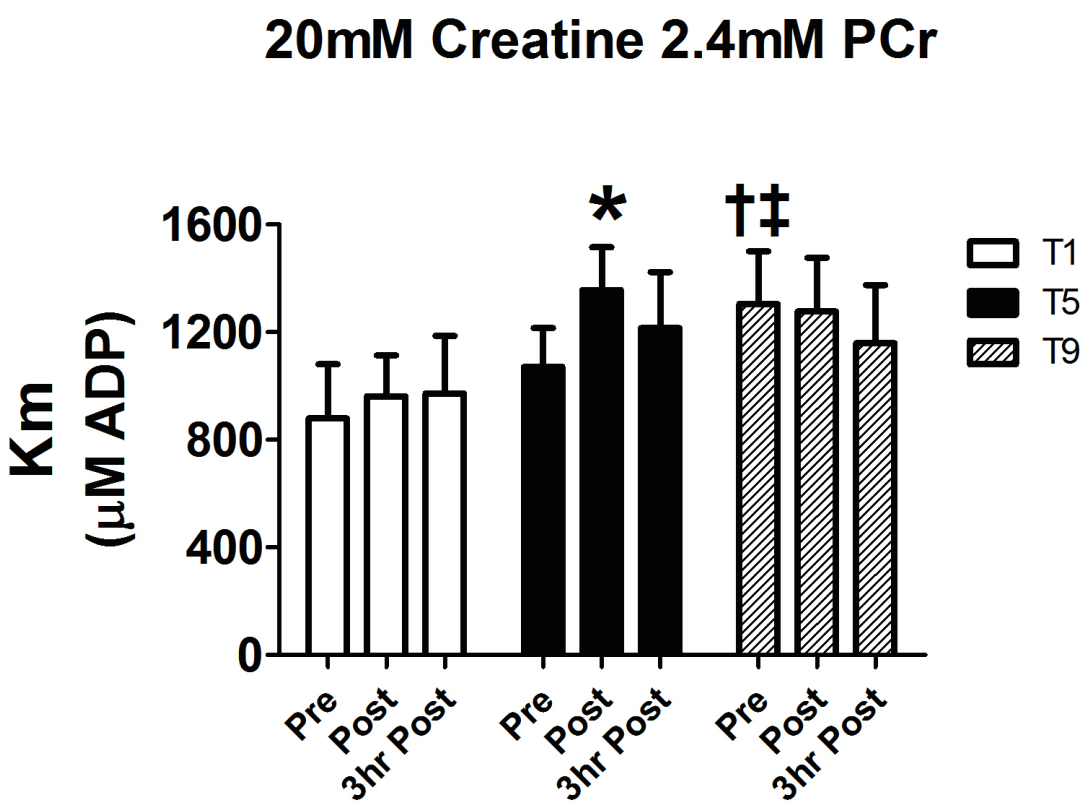

Figure 6. The effect of exercise on PCr/Cr dependent sensitivity to ADP. The K_{mapp} to ADP was determined in the presence of 2.4 mM PCr and 20 mM Cr, concentrations present in muscle during high intensity exercise. Results represent mean ± SEM for 11 participants. *, Post> Pre, $p<0.05$. ‡, T9 pre > T5 pre, $p<0.05$. †, T9 pre > T1 pre, $p<0.05$

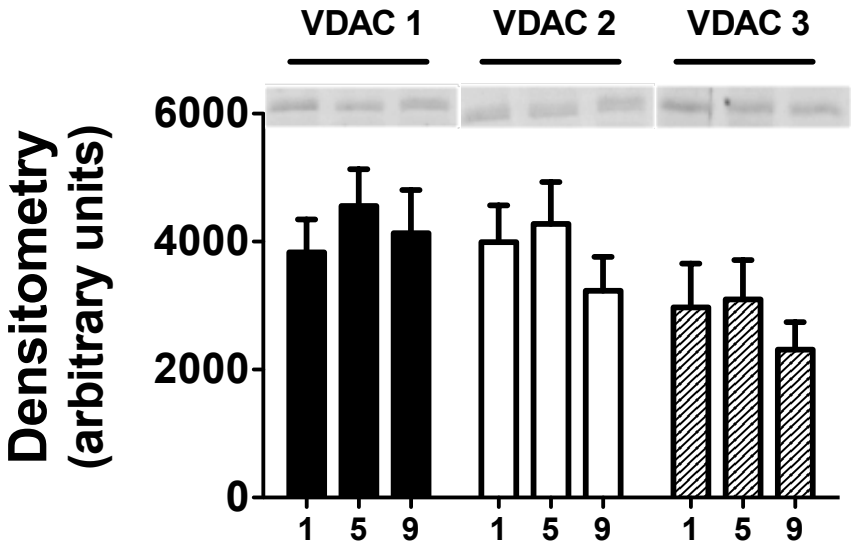

Figure 7. Effects of chronic exercise on VDAC protein content. Total content of VDAC isoforms 1, 2 and 3 were measured from the muscle biopsies taken prior to exercise sessions 1, 5 and 9. Results represent mean ± SEM for 10 participants.

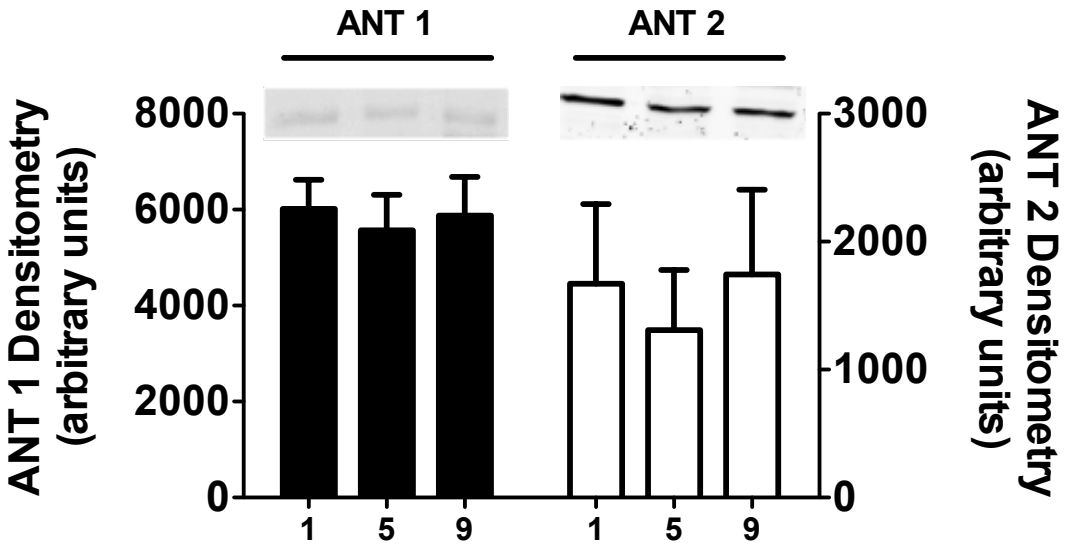

Figure 8. Effects of chronic exercise on ANT protein content. Total content of ANT isoforms 1 and 2 were measured from the muscle biopsies taken prior to exercise sessions 1, 5 and 9. Results represent mean ± SEM for 10 participants.

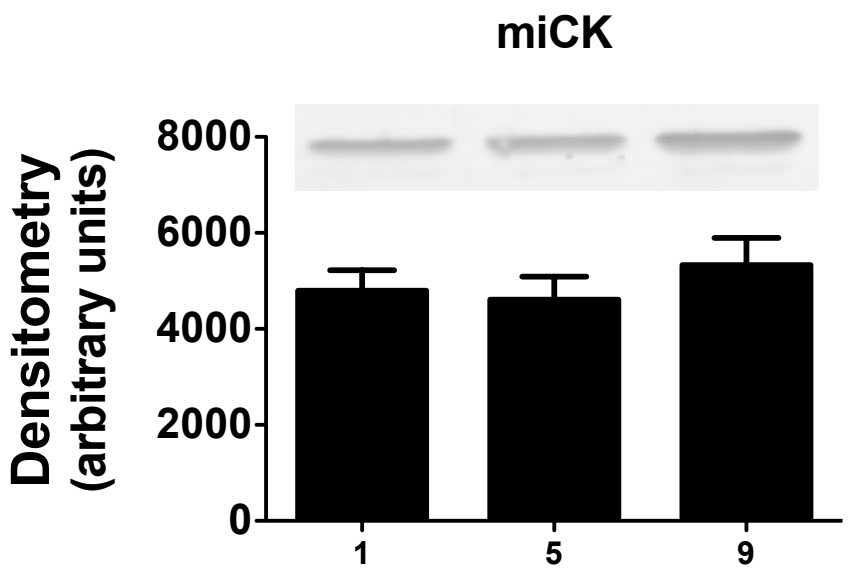

Figure 9. Effects of chronic exercise on mtCK protein content. Total content of mtCK was measured from the muscle biopsies taken prior to exercise sessions 1, 5 and 9. Results represent mean ± SEM for 10 participants.

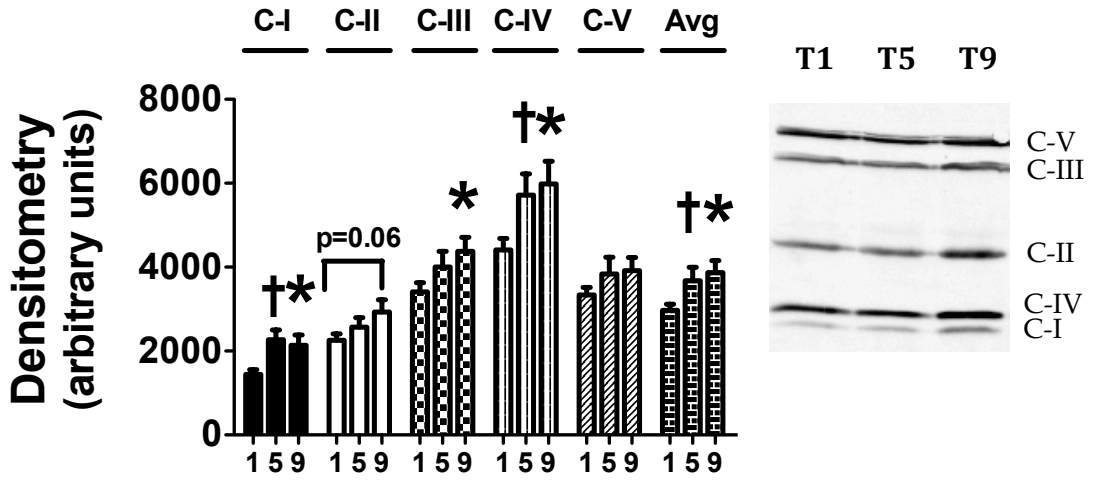

Figure 10. Effects of chronic exercise on total protein content of complexes I – V of the electron transport chain. Total content of electron transport chain proteins was measured from the muscle biopsies taken prior to exercise sessions 1, 5 and 9. Results represent mean ± SEM for 10 participants. *, T9 > T1, $p<0.05$. †, T5 > T1, $p<0.05$.

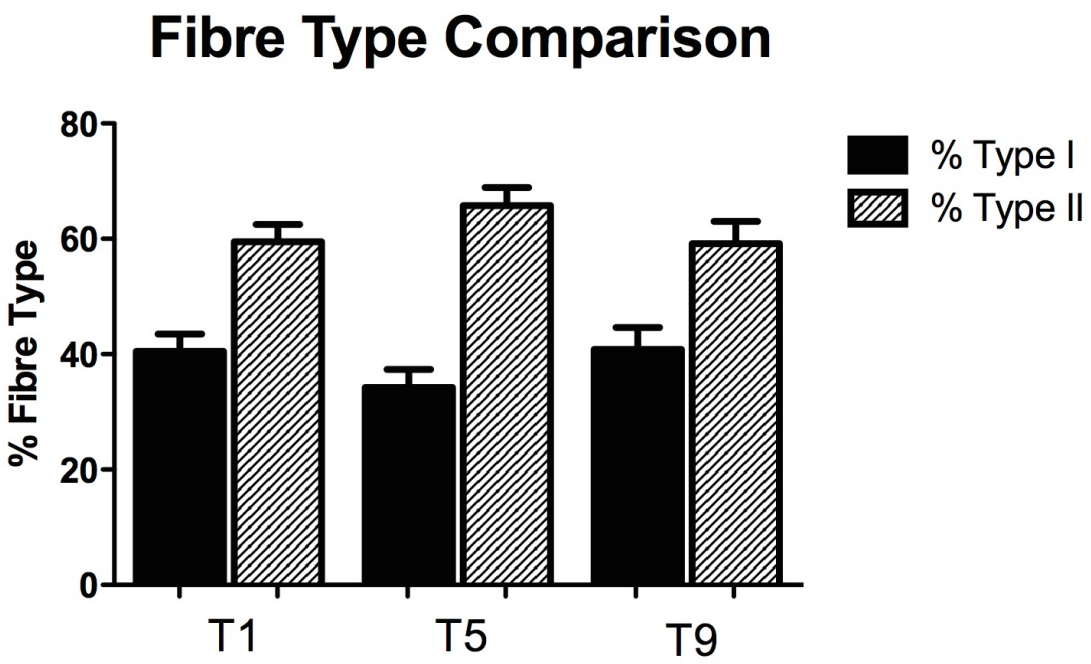

Figure 11. Percentage of Myosin Heavy Chain Type I vs Type II fibres. Changes in percentage of type I fibres relative to type II fibres were measured 3h post exercise at T1, T5 and T9. Results represent mean ± SEM for 10 participants.

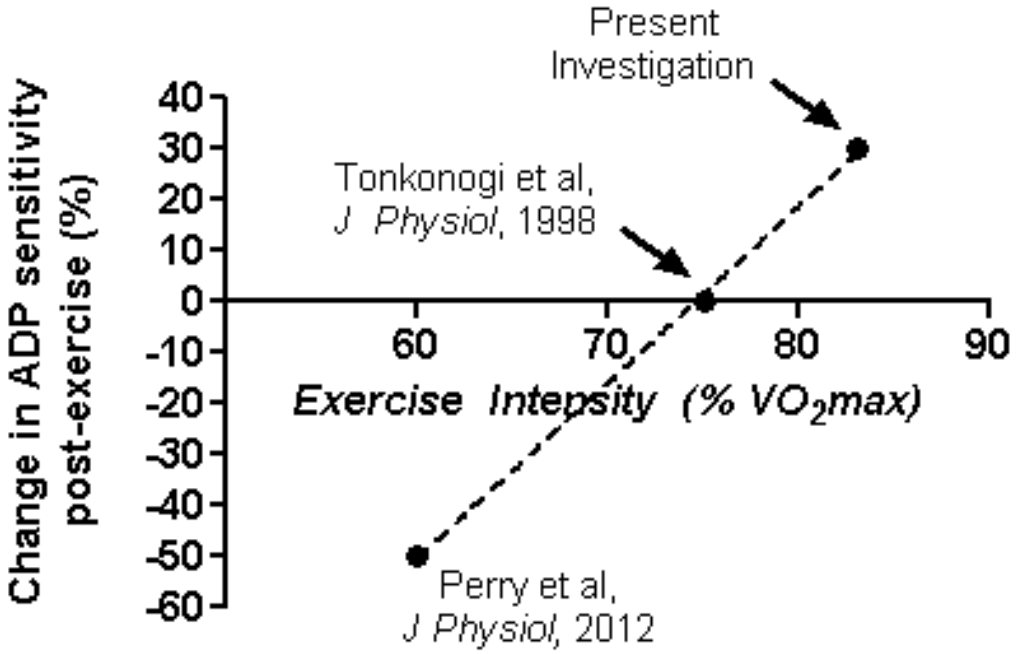

Figure 12. Correlation between exercise intensity and change in sensitivity. As exercise intensity increases, the positive change in sensitivity to ADP following exercise also increases indicating a potential correlation that should be examined further.

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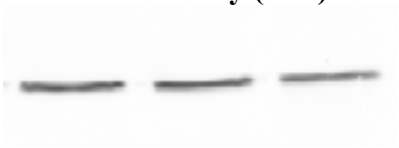
Appendix A

Western Blot Normalization Procedure

Table 1. ß-Tubulin raw densitometry (A.U) with representative blot



Subject	T0	T5	T9
1	2924	2744	3851
2	2179	3315	3501
3	2732	1559	2032
4	2618	2040	2952
5	2252	1434	2739
6	2645	1687	2431
7	2409	2813	1524
8	4136	2828	4196
9	3364	4221	3372
10	3233	4226	3591
11	3594	3235	2725
MEAN	2917	2737	2992
SEM	607	984	804
		p=0.26	p=0.37

Table 2. α -actinin raw densitometry (A.U) with representative blot

Subject	T0	T5	T9
1	2163	2149	243
2	497	837	1970
3	1419	1409	2595
4	3002	2553	1743
5	1353	1153	529
6	1055	1219	1502
7	2248	2442	1008
8	2579	768	1478
9	Image not useable	Image not useable	Image not useable
10	2822	2380	1563
11	719	2825	743
MEAN	1786	1773	1337
SEM	894	854	711
		p=0.98	p=0.13

Table 3. ß-Tubulin normalized relative to T0			
Subject	**T0**	**T5/T0**	**T9/T0**
1	1.00	0.94	1.32
2	1.00	1.52	1.61
3	1.00	0.57	0.74
4	1.00	0.78	1.13
5	1.00	0.64	1.22
6	1.00	0.64	0.92
7	1.00	1.17	0.63
8	1.00	0.68	1.01
9	1.00	1.25	1.00
10	1.00	1.31	1.11
11	1.00	0.90	0.76
MEAN	1.00	0.95	1.04
SEM	0.00	0.34	0.28
		p=0.29	p=0.32

Table 4. α -actinin normalized relative to T0			
Subject	**T0**	**T5/T0**	**T9/T0**
1	1.00	0.99	0.11
2	1.00	1.69	3.97
3	1.00	0.99	1.83
4	1.00	0.85	0.58
5	1.00	0.85	0.39
6	1.00	1.15	1.42
7	1.00	1.09	0.45
8	1.00	0.30	0.57
9			
10	1.00	0.84	0.55
11	1.00	3.93	1.03
MEAN	1.00	1.30	1.09
SEM	0.00	1.05	1.14
		p=0.21	p=0.40

Table 5. Correction Factor Numbers - Average of α -actinin and ß-Tubulin normalizations relative to T0

Subject	T0	T5/T0	T9/T0
1	1.00	0.97	0.71
2	1.00	1.60	2.79
3	1.00	0.78	1.29
4	1.00	0.81	0.85
5	1.00	0.74	0.80
6	1.00	0.90	1.17
7	1.00	1.13	0.54
8	1.00	0.49	0.79
9	1.00	1.25	1.00
10	1.00	1.08	0.83
11	1.00	2.42	0.90
MEAN	1.00	1.12	1.06
SEM	0.00	0.55	0.61
		p=0.26	p=0.37

Appendix B

Mitochondrial Function in Permeabilized Fibres

BIOPS Buffer

Chemical	Stock Solution	Molecular Weight	Final Concentration	Addition to *2 Litre* final volume
CaK_2EGTA*	100mM		2.77mM	55.4mL
K_2EGTA*	100mM		7.23mM	144.6mL
Na_2ATP		555.1	5.77mM	6.28g
$MgCl_2 \bullet 6H_2O$		203.3	6.56mM	2.67g
Taurine		125.1	20mM	5.02g
Na_2Phosphocreatine		327.14	15mM	9.81g
Imidazole		68.1	20mM	2.72g
Dithiothreitol (DTT)		154.2	0.5mM	0.154g
MES Hydrate		195.2	50mM	19.52g

** prepare stock solutions (see instructions below)*

BIOPS contains the following ion concentrations:	
Ca^{2+} Free	0.1 uM
Mg^{2+} Free	1mM
MgATP	5mM
Ionic Strength	160mM

CaK_2EGTA: Dissolve 2.002g $CaCO_3$ in 100mM hot (80°C) solution of EGTA (7.608g of EGTA in 200mL ddH_2O). Add 2.3g of KOH and adjust pH to 7.0 using KOH. Freeze unused portions

K_2EGTA: dissolve 7.608g EGTA and 2.3g KOH into 200mL ddH_2O. Adjust pH to 7.0 using KOH. Freeze unused portions

To make BIOPS:

1. Add approximately 1500mL of ddH_2O to 2000mL beaker
2. While constantly stirring add stock solutions of CaK_2EGTA and K_2EGTA
3. Weigh and add all powder chemicals
4. Adjust pH to 7.1 using KOH pellets
5. Using graduated cylinder, bring total volume to 2000mL
6. Filter and then aliquot into 50mL falcon tubes
7. Freeze falcon tubes

MiRO Buffer

Chemical	Stock Solution	Molecular Weight	Final Concentration	Addition to *2 Litre* final volume
EGTA		380.4	0.5mM	0.38g
$MgCl_2 \bullet 6H_2O$		203.3	3mM	1.22g
K-Lactobionate*	0.5M	358.3 free acid	60mM	240mL
Taurine		125.1	20mM	5.02g
KH_2PO_4		136.1	10mM	2.72g
HEPES		238.3	20mM	9.54g
Sucrose		342.3	110mM	75.3g
BSA		154.2	1g/L	2g

** prepare stock solution (see instructions below)*

MiRO contains the following ion concentrations:	
Ca^{2+} Free	0.0 uM
Mg^{2+} Free	2.1mM
K^+	90mM
Na^+	0
EGTA free	0.46mM
Osmolarity	330 mOsm
Ionic Strength	95mM

K-Lactobionate: Dissolve 35.83g lactobionic acid in 100mL ddH_2O, adjust pH to 7.0, adjust final volume to 200mL with ddH_2O

To make MiRO:

1. Add approximately 1500mL of ddH_2O to 2000mL beaker
2. While constantly stirring add stock solution of K-Lactobionate
3. Weigh and add all powder chemicals
4. Adjust pH to 7.1 using KOH pellets
5. Using graduated cylinder, bring total volume to 2000mL
6. Filter and then aliquot into 50mL falcon tubes
7. Freeze falcon tubes

Protocol 1 - ADP Titration FOR HUMAN – YORK TIMECOURSE				
Substrate	**Event Code**	**STOCK**	**Titration Volume (μl)**	**Final Concentration in chamber**
Miro5 Lights out BLEB Fibre	 F10 BLEB Fibre	No Cr 10 mM BLEB	1	5 μM
Stop stir bar, turn on lights and verify fibre is in chamber/not stuck on side wall Hit F10 (Lights out)				
100% O_2	O		Injection	250-275 μM
Glutamate Malate	G M	**1M** **1M**	**10** **1**	5 mM 0.5 mM
ADP	25 μM D	**50 mM**	**1**	25 μM D
ADP	50 μM D	**50 mM**	**1**	50 μM D
ADP	125 μM D	**50 mM**	**3**	125 μM D
ADP	200 μM D	**50 mM**	**3**	200 μM D
ADP	275 μM D	**50 mM**	**3**	275 μM D
ADP	500 μM D	**500 mM**	**1**	500 μM D
ADP	1 mM D	**500 mM**	**2**	1 mM D
ADP	2 mM D	**500 mM**	**4**	2 mM D
ADP	4 mM D	**500 mM**	**8**	4 mM D
ADP	8 mM D	**500 mM**	**16**	8 mM D
ADP	16 mM D	**500 mM**	**32**	16 mM D
Cyto c	cyto c	**4 mM**	**5**	10 μM
Succinate	20mM S	**2 M**	**20**	20mM S

*** Protocol 2 = MiRO + 20mM Cr * Protocol 3 = MiRO + 20mM Cr/2.4mM PCr**

Appendix C

Western Blotting

1. Homogenization and Protein Assay

CHAPS Homogenization Buffer

For 200mL solution at pH 7.5

Amount	Reagent	Concentration	Molecular Weight
1.906g	HEPES pH 7.5	40mM	238.3
1.403g	NaCl	120mM	58.44
74.4mg	EDTA Na2	1mM	372.2
892mg	NaHP2O7.10H2O pyrophosphate	10mM	446.1
432mg	B-glycerophosphate	10mM	216.04
420mg	NaF	50mM	41.99
0.6g	CHAPS	0.30%	

Muscle Homogenization

1. Place muscle in metal dish filled with liquid nitrogen
2. Using pre chilled spatula chip piece of muscle of desired size
3. Using pre chillsed forceps quickly place muscle on scale to get rough weight (do not wait for steady weight). While weighing, place forceps back in liquid nitrogen to remain cool
4. After getting weight, place muscle back in liquid nitrogen storage dewer until all samples have been weighed
5. Using the following dilution protocol place muscle in desired volume of homogenization buffer that contains:
    a. 1:100 Phosphatase Inhibitors 2 and 3
    b. 1:200 Protease Inhibitors
6. Dilutions: 400uL CHAPS buffer/15-20mg of muscle
    - 10-15 mg = 350uL
    - 5-10 mg = 250uL
7. Fill 50mL beaker with ice and place eppendorf with muscle securely in ice
8. Homogenize using tapered pestle for 3x10 seconds
9. Centrifuge at 800g for 10 minutes at 4°C
10. Save supernatant at -80°C

Protein Assay – BCA

1. Create standards by diluting BSA in homogenization buffer
    Standards (BSA):
    0 mg/mL (buffer)
    0.0625 mg/mL
    0.125 mg/mL

0.25 mg/mL
0.50 mg/mL
0.75mg/mL
1.00 mg/mL

2. Dilute 5 µL of each sample in 45 µL of homogenization buffer
3. Using a 96 well plate load in triplicates each standard and each sample
4. Add 190uL of mixed solution (50:1 Solution A:SolutionB) into each well of sample
   * Amount needed: (# of standards + # of unknowns) x (3 replicates) x (200uL)
5. Heat in over for 30 minutes at 37°C

2. Polyacrylamide Gel Electrophoresis (SDS PAGE)

10 X Running Buffer:

	500 ml	**1 L**	**2 L**
Tris	15 g	30 g	60 g
Glycine	72 g	144 g	288 g
SDS	5 g	10 g	20 g

1 X Running Buffer: 10 X Running buffer 100 ml
dH_2O 900 ml

---

10 X TBS (Tris Buffered Saline):

	500 ml	**1 L**
Tris	12.1 g	24.2 g
NaCl	40 g	80 g
Adjust to pH 7.6 with HCl		

1 X TBST:

	1 L	**2 L**
10 X TBS	100 ml	200 ml
dH_2O	900 ml	1800 ml
Tween 20	1ml	2 ml

Note: Ensure solution is stirring when adding Tween

Gel Preparation

	Stacking	Running				
		5%	6%	8%	10%	12%
dH_2O	6.8 ml	11.4	10.6 ml	9.4 ml	8 ml	6.7 ml
1.5M Tris-Base, pH 8.8	***	5 ml	5 ml	5 ml	5 ml	5 ml
1M Tris-HCl, pH 6.8	1.25 ml	***	***	***	***	***
30 % Acrylamide	1.70 ml	3.4 ml	4 ml	5.3 ml	6.7 ml	8 ml
10 % SDS	100 µl	200 µl	200 µl	200 µl	200 µl	200 µl
10 % APS	100 µl	200 µl	200 µl	200 µl	200 µl	200 µl
Temed	**20 µl**	**20 µl**	**20 µl**	**20 µl**	**20 µl**	**20 µl**

1. In 50mL falcon tubes make running gel and stacking gel (1 column makes 2 gels) ***DO NOT ADD TEMED UNTIL READY TO LOAD***
2. Take 1 short plate and one spacer plate and clean both sides of glass with methanol and kimwipe
3. Place clean short plate on top of spacer place and place both in mounting apparatus, ensuring the edge of the plates are lined up against the bench top
4. Place plates in apparatus on foam piece in gel stand
5. Place 3 transfer pipettes and falcon tube with methanol close to gel stand
6. Add TEMED to running gel and invert falcon tube 1-2 times
7. Using transfer pipette, fill space between glass plates with running gel until solution reaches the top of the green doors
1. Using new transfer pipette add methanol to top of plate to create an even line along the top of the gel removing any bubbles
2. Let sit until remaining running gel has hardened in the falcon tube
3. Once gel has set, invert gel to remove any excess methanol
4. Add TEMED to stacking gel and invert 1-2 times
5. Using transfer pipette, add stacking gel to top of glass plates, use methanol transfer pipette to remove any bubbles
6. Add comb to top of gel and allow to set

7. Prepare diluted samples by combining sample, water and Laemmeli's buffer + 2-mercaptoehtanol (100 µl 2-mer: 900 µl Lam)
    a. **Example of dilution to load 50µg protein at a concentration of 1µg/ µl**

Sample	Corr Prot (ug/ul)	Aliquot (ug)	Sample (ul)	H20	4X Laem (1:9 2-Mercap)	Vol for 1 Well
C7.5-A	4.599	50	10.9	26.6	12.5	50

8. Boil samples at 95 °C for 5 minutes
9. Spin down samples for 5 seconds
10. Make 1X running buffer in 1000mL graduated cylinder

11. Remove combs from gels and place gels in gasket
12. Place gasket with gels in tank and add 1X running buffer until it fills the top of gasket and tank
13. Load wells with desired concentration of samples
14. Run for desired time (40-75 minutes) at 160mV (or desired voltage)

15. Fill 2 plastic "biorad" dishes, one with methanol and one with transfer buffer
16. Place 2 transfer packs in dish of transfer buffer and place membrane in dish of methanol
17. After 2-3 minutes place first transfer pack on bottom of transfer case
18. Place membrane in transfer buffer for 10-15 seconds
19. Place membrane on top of transfer pack ensuring no air bubbles
20. Remove short plate from gel and place gel face down on membrane
21. Place second transfer pack on top of membrane
22. Using roller, roll from one side of transfer pack to the other to remove air bubbles
23. Hold firmly down in the middle of the transfer pack and remove excess liquid
24. Put lid on transfer case and run transfer (standard setting is 1.5mm gel for 10 minutes)

25. Following transfer remove membrane and place in dish with blocking buffer (50% Odyssey Blocking Buffer, 50% TBS) for 1 hour, rocking at room temperature
26. After 1 hour pour blocking buffer back into falcon tube (it can be resused) and add primary antibody
27. Place membrane in primary in fridge overnight, rocking
28. In the morning remove primary and pour back into falcon tube (can be resued)
29. Add TBST to dish and complete 3x5 minute TBST washes rocking at room temperature.
30. Add secondary antibody and leave rocking at room temperature for 1 hour
31. Remove secondary and place back in falcon tube (can be resused)
32. Complete 3 more 3x5 minute TBST washes
33. Detect using LiCOR Infrared Imager

Protein	Concentration	% Gel	Molecular Weight (KDa)	Important Notes
ANT 1	1:40	12%	32	
ANT 2	1:1000	12%	32	
VDAC 1	1:500	10%	33	
VDAC 2	1:500	10%	33	
VDAC 3	1:1000	10%	33	
mtCK	1:1000	12%	42	
OXPHOS	1:208	12%	50 and below	Do not boil samples
β - Tubulin	1:1000		52	
α -actinin	3.5 µg/mL		103	

Appendix D

Myosin Heavy Chain Quantification – Silver Stain

Sample Preparation

1. Homogenize tissue in CHAPS buffer following "Homogenization Protocol"
2. Prepare sample with standard H_2O:Sample:Sample Buffer ratio as you would for a regular western
    a. Will need to load 10ug protein
3. **2 MINUTE BOIL AT 95 DEGREEES CELCIUS**

Gel Preparation

10% Acrylamide Gel using **0.75mm Plates**

- 99:1 Acrylamide:Bis Ratio
    - to prepare: dissolve30g acrylamide with 0.3g BIS in 75mL ddH_2O
    - using graduated cylinder top up volume to 100mL with ddH_2O
    - decant into glass bottle, cover with foil (light sensitive) and store in fridge
        - SOLUTION IS GOOD FOR UP TO 1 MONTH

Running Gel Recipe:

Stock Concentration	Amount to Add	Final Concentration
100% Glycerol	3mL	30%
30% Acrylamide (99:1)	3.37mL	10%
ddH_2O	0.795mL	
AFTER ADDING GLYCEROL, ACRYLAMIDE AND WATER VORTEX TO MIX GLYCEROL TO AVOID IT SETTLING ON BOTTOM OF TUBE		
1.5M Tris (pH 8.8)	1.33mL	0.2M
1M Glycine	1.00mL	0.1M
10% SDS	0.4mL	0.4%
10% APS	0.1mL	0.1%
TEMED	10uL	0.1%

***Makes enough for 2 0.75mm gels**

Stacking Gel Recipe:

Stock Concentration	Amount to Add	Final Concentration
30% Acrylamide (99:1)	1.7mL	4%
ddH_2O	6.8mL	
1.0M Tris (p.H 6.8)	1.25mL	0.125M

10% SDS	0.1mL	0.1%
10% APS	0.1mL	0.1%
TEMED	20uL	0.1%

***makes enough for 2 0.75mm gels**

Running Buffer Recipe (10x)

Chemical	**Amount to add for 1L**	**Final Concentration**
1M Tris	**121.1g**	**100mM**
1.5M Glycine	**112.6g**	**150mM**
1% SDS	**10g**	**0.1%**
0.12% B-ME	****add fresh to 1X before running**	**0.12%**

1. Dilute 10x to 1x with 900mL H_2O and 100mL 10x Running Buffer. Add 120uL B-ME to 1X solution

Electrophoresis

1. Load samples on most inner part of gel as possible…try to avoid first and last well as the gel is very flimsy and the edges tend to stretch
2. Place in fridge on stir plate with magnetic stir bar in running tank
    a. Turn stirring on low
3. Run at 20mA (constant amperage) for 40 minutes (until samples are out of stacking)
4. Run at 140V (constant voltage) for 20 hours
    a. Make sure to check on running buffer levels in the gasket as they tend to leak over time
5. THE GEL WILL SLIP OUT THE BOTTOM OF THE PLATES A LITTLE BIT, THIS IS OKAY
    a. Make sure to not let the 150KDa marker run too low as the bottom of the gel gets damaged from the slipping
6. After approximately 20 hours when 150KDa marker is about 2cm from bottom of gel stop and proceed with silver stain procedure

From this point on everything you do needs to be with clean gloves*

Obtain fresh ddH_2O (ultrapure) in a sterile bottle…do not use ddH_2O from the big bench top container

1. Wash out square petri dish and larger biorad plastic dish with soap, ethanol and 2 rinses of ddH_2O
2. Cover bottom of each with fresh ddH_2O
3. Take short plate off front of gel and flip spacer plate face down so gel is resting in water in biorad plastic dish…with a bit of assistance gel should just fall off the spacer plate into the water
4. Carefully transfer gel to square petri dish
    a. Cut top corner above standard so ensure you know orientation of gel as it tends to move when being rocked

Silver Stain Protocol

Complete all steps on a rocker rocking very gently

1. wash gel in ultrapure water for 5 minutes. Replace the water and wash for another 5 minutes
2. Decant water and add Fixing Solution to the gel. Incubate for 15 minutes. Replace solution and fix for another 15 minutes. Gel can remain in fixing solution overnight without affecting stain performance.
3. Wash gel with Ethanol Wash for 5 minutes. Replace solution and wash for another 5 minutes.
4. Wash gel in ultrapure water for 5 minutes. Replace water and wash for another 5 minutes
5. Just before use, prepare sensitizer working solution by mixing 1 part Silver Stain Sensitizer with 500 parts ultrapure water (50uL Sensitizer with 25mL Water)
6. Incubate gel in sensitizer working solution for exactly 1 minute, then wash with two changes of ultrapure water for 1 minute each
7. Mix 1 part Silver Stain Enhancer with 100 parts Silver Stain (0.25mL enhancer with 25mL stain) and immediately add it to gel. Incubate gel for 5 minutes
8. Prepare developer working solution by mixing 1 part Silver Stain Enhancer with 100 parts Silver Stain Developer (0.25mL enhancer with 25mL Developer)
9. After 5 minute incubation remove petri dish from rocker and place on bench with white paper underneath
10. Quickly wash gel with two changes of ultrapure water for 20 seconds each
11. Immediately add developer working solution and incubate until protein bands appear
12. Once bands appear replace with water
    a. Bands will continue to intensify when developer is off the gel so replace with water before desired band intensity is reached
13. Do two 2 minute washes with water and replace with stop solution for 10 minutes
14. Replace stop solution with fresh stop solution and image gel.