Impact of exercise training on stress signaling pathway and purine metabolism in healthy and disease models and the influence of ribose supplementation

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Abstract

Regular exercise improves physical fitness and general health via significant muscle molecular adaptations. ATP (energy) and its regulation is critical to metabolic homeostasis. ATP hydrolysis and re-synthesis are typically balanced, disrupted when the rate of ATP re-phosphorylation can no longer support extreme exercise workload, such as high-intensity training (HIT), or production is compromised (metabolic disease). Consequently, purine degradation to terminal purine metabolites (hypoxanthine and xanthine) occurs, which diffuse across the sarcolemmal membrane into the blood converted to uric acid and eliminated by the kidneys. This process generates reactive oxygen species (ROS) that can damage skeletal muscle fibres and the local capillary network.

Extreme metabolic insult triggers three possible responses: (1) oxidative stress; (2) endoplasmic reticulum (ER) instability; and (3) hypoxic stress. Cellular oxygen consumption and subsequent ROS production are implicit in the activation of these signaling pathways. ROS stimulates beneficial muscle adaptations associated with regular exercise as per the concepts of hormesis. However, overexposure (acute or chronic) can drive muscle dysfunction and pathology. Elucidation of the signaling pathways altered during intense metabolic stress, e.g. HIT or exercise in metabolically compromised muscle, is needed. Furthermore, supporting cellular metabolism under intense pressure could prevent excessive stress signaling and these negative consequences. Ribose supports purine recovery and biosynthesis, and supplementation may protect metabolically challenged muscles, or prevent formation of the critical signals required to induce beneficial adaptations within skeletal muscle, e.g., ROS.

Study 1 examined the molecular stress signaling response to HIT in mice. Following HIT, the levels of Keap1, SOD, and PGC-1α were elevated in the skeletal muscles of female mice, yet the expression of HO-1, Sirt1 and PGC-1α, which can be induced by hypoxic stress protein, HIF-1, was upregulated in male skeletal muscle. This indicates female skeletal muscle may be more responsive to oxidative stress, while male muscle may be more responsive to hypoxic stress.

Study 2 examined (1) oxidative, metabolic and hypoxic stress signaling in metabolically challenged dystrophic (*mdx*) mouse model exposed to regular normo-tensive exercise; and (2) whether ribose supplementation could protect muscles from exacerbated myopathy. Ribose treatment enhanced resistance to fatigue and forelimb muscle strength in *mdx* mice by inducing hypoxia stress signaling. Additionally, we observed that ribose supplementation reduced purine degradation, leading to potential adaptations in ER stress signaling pathways by promoting fat metabolism. Therefore, ribose could be a therapeutic adjunct to treat muscle fatigue in DMD patients and female carriers of the dystrophin gene mutation.

Study 3 focussed on exercise performance and stress signaling adaptations with HIT in humans. Impacted heavily by COVID-19, this small pilot study indicated that HIT training induces the Nrf2-mediated antioxidant program and Sirt1which may safeguard muscle cells against oxidative damage caused by exercise.

This thesis examined models of stress in healthy and disease states to better metabolic signaling dynamic and influence of ribose supplementation on the adaptation pathways.

Declaration of Authenticity

I, BO QI, declare that the Ph. D thesis entitled “Impact of exercise training on skeletal muscle stress signaling pathway and purine metabolism in healthy and disease models and the influence of ribose supplementation” is no more than 80,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously in whole or in part, for the award of any other academic degree or diploma. Except where otherwise, this thesis is my own work.

I have conducted my research in alignment with the Australian Code for the Responsible Conduct of Research and Victoria University’s Higher Degree by Research Policy and Procedures.

Signatur  Date

Ethics Declaration

“All research procedures reported in the thesis were approved by the Animal Ethics Committee and 15-007 and 20-006 and Human Research Ethics Committee, 17-003.

Signat  Date

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List of Abbreviations

ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
AMPK	AMP-activated Proteins Kinase
APS	Ammonium Persulphate
ATP	Adenosine Triphosphate
ATF4	Activating Transcription Factor 4
ATF6	Activating Transcription Factor 6
BMI	Body Mass Index
CaMMK2	Calcium Calmodulin Kinase Kinase-2
Ca^{2+}	Calcium
CAT	Catalase
CHOP	CCAAT-Enhancer-Binding Protein Homologous Protein
Ck	Creatine Kinase
CP	Creatine Phosphate
CREB	cAMP Response Element-Binding
Cr	Creatine
CS	Citrate Synthase
DMD	Duchenne Muscular Dystrophy
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic Acid
EGTA	Etheylen Glycol-bis (β-aminoethyl ether)-N, N, N', N'-Tetraacetic Acid
eIF2α	Eukaryotic initiation factor 2α
ER	Endoplasmic Reticulum
ERO1	Endoplasmic Reticulum Oxidoreductin 1
ETC	Electron Transport Chain
FAD	Flavin Adenine Dinucleotide
GSH	Glutathione

GSSG	Glutathione Oxidized
HDAC3	Histone Deacetylase 3
HIF1α	Hypoxia-inducible Factor 1-alpha
HIF1β	Hypoxia-inducible Factor 1-beta
HIT	High Intensity Training
HO1	Heme Oxygenase 1
HOCL	Hypochlorous Acid
Hx	Hypoxanthine
IDH	Isocitrate Dehydrogenase
IMP	Inosine Monophosphate
Keap1	Kelch-like ECH-associated Protein 1
LKB1	Liver Kinase B1
LDH	Lactate Dehydrogenase
$\Delta\Psi m$	Mitochondrial Membrane Potential
mdx	Muscular dystrophy x-linked on c57BL/10 background (mouse)
MPTP	Mitochondrial Permeability Transition Pore
MQ	Milli Q Ultrapure Water
NAD	Nicotinamide Adenine Dinucleotide
NAD^+	Nicotinamide Adenine Dinucleotide: Oxidized Form
NADH	Nicotinamide Adenine Dinucleotide: Reduced Form
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NOX(s)	NADPH Oxidase(s)
NQO1	NAD(P)H Dehydrogenase Quinone 1
Nrf2	Nuclear Factor Erythroid 2–related Factor 2
O_2^-	Superoxide
OGDH	Oxoglutarate Dehydrogenase
OXPHOS	Oxidative Phosphorylation
PMSF	Phenylmethylsulfonyl Fluoride
Phosph-p62	Phosphorylated p62

PDH	Pyruvate Dehydrogenase
PDI	Protein Disulfide Isomerase
PGC-1α	Peroxisome proliferator-activated receptor-gamma coactivator-1alpha
PK	Pyruvate Kinase
PNC	Purine Nucleotide Cycle
PPAR	Peroxisome proliferator-activated receptor
PRPP	5-phoshphoribosyl-1-pyrophosphate
ROS	Reactive Oxygen Species
PrN	Purine Nucleotide Pool
PVDF	Polyvinylidene Difluoride
SDH	Succinate Dehydrogenase
SDS	Sodium Dodecyl Sulfate
SIT	Sprint Interval Training
SOL	Soleus
SR	Sarcoplasmic Reticulum
SSE	Steady State Exercise
TA	Tibialis Anterior
TCA	Tricarboxylic Acid
TCr	Total Intramuscular Creatine
TEMED	Thermo Scientific Pierce Tetramethylethylenediamine
TP62	Total Protein p62
WIK	Western Immunoprecipitation Kinase
WT	Wild-Type
XO	Xanthine Oxidase

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Chapter 1
Review of Literature

1. Introduction

Skeletal muscle is a highly dynamic and energetic tissue primarily adapted to provide locomotion. Its cellular and molecular mechanisms have evolved to cater for extremes of physical work ranging from short and powerful rates of contractile work to long sustaining endurance activity. The plasticity of skeletal muscle is dynamic and remodelling is relatively rapid from a cellular perspective (Mukund and Subramaniam, 2020).

Regular exercise improves muscle function and physical fitness, which coincides with an increase in general health and well-being and significant molecular adaptations at the muscle level (Lambert, 2016, Rivera-Brown and Frontera, 2012). Training adaptions occur as a consequence of molecular remodeling within the skeletal muscle and are relative to the exercise performed and the intracellular stress incurred. These adaptations include, for example, angiogenesis (Kwak et al., 2018) and mitogenesis (Yoboue and Devin, 2012), which improve oxygen and nutrient delivery to the muscles and escalate energy production capacity to improve endurance or facilitate muscle hypertrophy for the muscle force development (Olfert et al., 2016). Physiologically, stress signaling ultimately results in the necessary adaptations to increase the endurance and/or strength potential of the muscle such that the tissue (and organism) can survive under pressure. A lack of exercise or a pathologically compromised cellular function can result in disuse, or diseased-related atrophy/wasting, respectively. This is highlighted by the muscle mass reductions observed: 1) in the gravity-free environment of space where there is a reduced force requirement (Raj et al., 2010); 2) de-training, where there is the partial or complete loss of training adaptions (Tokmakidis et al., 2009); and 3) injury immobilisation, resulting in a decrease in muscle strength and function (Wall et al., 2015). These circumstances reduce the molecular stimuli to sustain muscle mass. Pathological circumstances such as muscular dystrophy, cancer and metabolic diseases also result in reduced muscle mass and function but appear driven more by chronic activation of stress signaling pathways (Appell, 1990, Martin and Freyssenet, 2021, Moon et al., 2015). Thus, optimal muscle mass and function is

dependent upon a critical level of activity-based stress signaling where too much or too little has detrimental consequences to the organism.

The effect of exercise intensity and frequency on skeletal muscle plasticity can be largely contextualized within the concept of hormesis. Hormesis is a biological principle whereby repetitive low dose exposure to an otherwise toxic insult induces adaptations that are generally favourable to an organism's survival (Kourakis et al., 2021). Outside of the boundaries of hormesis, acute high dose, or long-term moderate dose exposure to a toxic insult can incur damage to the organism (Gordon, 2010, Petejova et al., 2019). For example, frequent exercise within physiological boundaries as administered during training induces repetitive stress signals such as reactive oxygen species (ROS) (He et al., 2016), which stimulate protective molecular responses that are beneficial to resist other toxic insults. Conversely, acute exercise outside of physiological durability or intensity e.g, running a marathon, may result in sufficient ROS to cause oxidative damage, which could be toxic and/or fatal (Belhadj Slimen et al., 2014). In this way, regular normo-intensive exercise acts as a medicine by inducing adaptive cellular responses that prevent chronic disease (Radak et al., 2005).

The post-exercise molecular remodelling within skeletal muscle is mediated by a complex interplay between a myriad of signaling pathways coupled to downstream regulators of transcription and translation (Egan and Zierath, 2013, Mesquita et al., 2021). The hormetic response is driven by the extent and type of exercise stress: the frequency, cadence and intensity of the stress repetition are all important factors (Lucas et al., 2015, Slaght et al., 2017). Aerobic, low intensity endurance or high-intensity, explosive exercise are the extremes of the spectrum and play a role in determining the remodelling outcomes within the muscle tissue (Egan and Zierath, 2013). To this effect, this review will explore the molecular signaling pathways involved in interpreting and integrating stress signals caused by exercise to effect adaptations, with a focus on the role of purine metabolism in initiating the signals.

1.1. Metabolism/ Metabolic Stress

Metabolic stress is the physiological process that occurs during the stressful conditions (such as exercise or disease) in response to low energy (mainly ATP) levels, which is insufficient for long-term cell survival in normal condition (Johnson et al., 2019). ATP production is quite important during metabolic stress. The mitochondria, an organelle founded in the cell of most eukaryotes, plays a crucial role in metabolism. Mitochondria are the primary source of ATP production through the complex interplay of metabolic pathways (Spinelli and Haigis, 2018, Nolfi-Donegan et al., 2020), which exerts in tandem to convert the glucose, fatty acids and other energy sources into ATP.

1.1.1. Cellular energy balance is maintained by integrated metabolic systems in skeletal muscle

The cellular energy balance is maintained through the complex metabolic pathways that are present within these cellular organelles (Aon and Cortassa, 2015, Martínez-Reyes et al., 2016, Ahmad et al., 2018). Creatine phosphate (CP) is a mediate energy source that buffers acute ATP demand (Guimarães-Ferreira, 2014). Glycolysis occurs in the cytoplasm and is the first step in the metabolic pathway that converts glucose into ATP (Melkonian and Schury, 2019, Naifeh et al., 2021). This process results in the conversion of one molecule of glucose into two molecules of pyruvate. The pyruvate produced in glycolysis is then transported into the mitochondria, where it is further metabolized through the tricarboxylic acid (TCA) cycle and the electron transport chain (Anraku, 1988, Ahmad et al., 2018).

Mitochondrial metabolism also involves the TCA cycle, which is a closed loop of reaction that generate energy in the form of reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$), which are then used in oxidative phosphorylation to produce ATP (Martínez-Reyes et al., 2016, Martínez-Reyes and Chandel, 2020). The cycle starts with the condensation of acetyl-CoA with oxaloacetate to form citrate, and then proceeds through various reactions that involve

the oxidation of intermediate compounds and the release of carbon dioxide (Martínez-Reyes et al., 2016). The energy generated from the TCA cycle is used to power cellular processes such as growth, reproduction, and response to stimuli. The TCA cycle involves a series of enzyme-catalysed reactions, each of which is specific to a particular intermediate compound. Some of the key enzymes involved in the TCA cycle. These enzymes are all located in the mitochondria and are essential for the proper functioning of the TCA cycle. For instance, one of the key enzymes of the TCA cycle, IDH, increases its activity in response to exercise, which enhances the rate of the TCA cycle and the production of ATP (Zhang et al., 2011). The TCA cycle also generates intermediates that are used to produce additional ATP through anaerobic processes, such as glycolysis. Collectively, those processes contributes to generate energy for the cell and produce essential metabolic intermediates (Fernie et al., 2004, Williams and O'Neill, 2018).

Oxidative phosphorylation is the major source of ATP in most cells, and it is a crucial component of cellular respiration (Slater, 1977, Kadenbach et al., 2010). It allows cells to generate large amounts of ATP from the energy stored in fuels, such as glucose and fatty acids, and helps to maintain cellular energy levels. Electrons are transferred from energy-rich molecules, such as NADH and $FADH_2$, to electron acceptors (such as O_2) through a series of protein complexes in the inner mitochondrial membrane (Schulz et al., 2016). The transfer of electrons generates a proton gradient across the inner mitochondrial membrane, which is used to produce ATP through ATP synthase. The ATP synthase enzyme uses the energy stored in the proton gradient to add a phosphate group to ADP, converting it into ATP.

Overall, the maintenance of cellular energy balance in mitochondria is crucial for the normal functioning of cells and the organism as a whole. Disruptions in this balance can lead to a variety of metabolic disorders and diseases.

1.1.2. Cytosolic energy systems buffer metabolic stress

1.1.2.1. Purine Metabolism and biosynthesis

The central role of purine nucleotide metabolism is to support of energy supply for muscle contraction. This is well regulated by the purine nucleotide pool (PNP) at rest and periods of nonstrenuous activity (in healthy individuals) (Hellsten et al., 2004). The PNP is made up of ATP, ADP, adenosine-5-monophosphate (AMP), inosine-5-monophosphate (IMP) and inosine. The PNP at rest consists of predominantly (>90%) ATP (Miller et al., 2019), ensuring an immediate and effective energy production for cellular function. However during periods of stress an elevated ATP hydrolysis and imbalance of ATP turnover results in significant and rapid muscular ATP reductions, a single 30 second sprint bout results in a 30~40% decrease in muscle ATP levels (Stathis et al., 1994, Hellsten et al., 2004), especially in type II fibres (Karatzaferi et al., 2001b). As ATP is dephosphorylated to ADP, when muscle PCr levels are sufficient ATP can be restored through the addition of an inorganic phosphate (from PCr) to ADP. However, after a short while (sec), PCr is rapidly depleted at maximal ATP turnover (Mendez-Villanueva et al., 2012) and subsequent glycolysis and OXPHOS cannot resynthesize ADP as effectively and accumulated ADP is dephosphorylated to AMP, before deamination to IMP (Sahlin, 1986, Jacobs et al., 1982, Fischer et al., 2007). The majority of IMP is then re-aminated to ATP within the muscle via the purine nucleotide cycle (PNC) during recovery (Holeček, 2022), however a portion is further de-phosphorylated to inosine or hypoxanthine (Hx), traversing the sarcolemma and enabling entry into the bloodstream (Hellsten-Westing et al., 1993, Hellsten et al., 1998, Hellsten et al., 1999). Purines nucleotides that we lost to blood can no longer be salvaged and are excreted within the urine (Ardawi and Newsholme, 1983, Gerber et al., 2014, Stathis, 2006) thus resulting in a loss of cellular energy requiring energy intensive recovery process (Hellsten et al., 1998, Hellsten et al., 1999). Bangsbo and colleagues showed purine efflux is responsible for a 5% loss of the muscle adenine nucleotide pool within 10 min following a 30 s “all out” intense exercise (Bangsbo et al., 1992). More recently, purine efflux was shown to account for a 9% adenine nucleotide

loss for a single exercise protocol (Hellsten et al., 1999). The metabolic cost of the complete reconstitution of an adenine nucleotide molecule is far greater than intramuscular salvage from Hx (Zieliński and Kusy, 2012, Gerber et al., 2014).

Uric acid is the end-product of purine metabolism and xanthine oxidase (XO) is the rate-limiting enzyme of uric production form Hx and xanthine during purine metabolism (Doehner and Landmesser, 2011). In this process, XO produces ROS by generating hydrogen peroxide and superoxide (Tabet and Touyz, 2007, Furuhashi, 2020). Various publications have demonstrated that allopurinol treatment, an XO inhibitor, reduces plasma uric acid concentrations (Stathis et al., 2005, Negi et al., 2020), regulated the ROS production (Engberding et al., 2004, Kang et al., 2006), and promotes ATP resynthesis through the purine salvage pathway (Peglow et al., 2011). Therefore, inhibition of XO had a potential beneficial influence on balance purine metabolism and suppress the ROS production.

Purine nucleotide recovery occurs in two distinct stages, salvage and resynthesis. The purine nucleotide salvage pathway is the first opportunity for adenine nucleotide recovery, acting within the muscle to resynthesize IMP (which can be re-aminated to ATP) by combining the purine base Hx, with 5-phoshphoribosyl-1-pyrophosphate (PRPP). IMP formation via purine salvage however appears to be rate limited by the bioavailability of PRPP (Harmsen et al., 1984). This process acts within the muscle, preventing purine escape, because once the purine nucleotides exit the muscle they can no longer be re-salvaged and are excreted in the urine (Stathis et al., 1994, Stathis et al., 2005). Increasing the availability of PRPP, however enhances IMP reformation and limits purine nucleotide loss, enhanced by the provision of ribose, subsequently demonstrating enhanced purine and muscular ATP recovery (Harmsen et al., 1984, Brault and Terjung, 2001, Hellsten et al., 2004). The purine *(de-novo)* bio-synthesis pathway on the other hand is a recovery pathway that replenishes the ATP pool from simple molecule sugars and amino acids (Ardawi and Newsholme, 1983). ATP *(de-novo)* synthesis in humans is slow and limited by PRPP availability. Rat studies demonstrating a synthesis rate of ~35 $\mu mol.kg^{-1}$ dry $wt^{-1}.hr^{-1}$ (Tullson et al., 1988), providing a rationale for lower ATP concentrations following periods of intense exercise training in humans (Hellsten-Westing et al., 1993, Stathis et al., 1994). It has been postulated that the purine *(de-novo)* synthesis pathway can be

enhanced in recovery subsequently increasing intramuscular ATP content, achieved through oral ribose supplementation (Tullson and Terjung, 1991, Hellsten-Westing et al., 1993). A higher level or more rapid restoration of intramuscular ATP may be effective in allowing an enhanced exercise recovery rate between training sessions, resulting in an improved or better sustained performance with repeated sprint interval training (SIT) sessions over time, and consequently, enhanced physiological adaptations. Furthermore, it has been shown that SIT training better preserves muscular ATP during exercise, has a lower production of muscular IMP and Hx during exercise and recovery, and a significantly higher level of PCr during recovery (Stathis, 2006).

1.1.2.2. Creatine Phosphagen System

Creatine phosphate (PCr) is a high energy phosphate stored in skeletal muscle that is hydrolysed to generate ATP using ADP as a substrate catalysed by creatine kinase in cytosol and mitochondria (Figure 1.1). The creatine phosphagen system acts as an energy buffer and synthesises a pool of ATP. During short-term intense activities. (e.g. high intensity interval training (HIT) and SIT), the phosphagen system is the quickest way to produce a large amount of energy and maintain muscle contractions (Wells et al., 2009, Baker et al., 2010). Once the storage of the phosphagen system are used up, the glycolytic system becomes the primary source of energy, because glycolysis rate is lower than phosphagen system during the short time intensity exercise (Baker et al., 2010). It is reported that increasing the capacity of PCr system in training (Jones et al., 2007, Forbes et al., 2008) and supplements (Francaux et al., 2000, Zarzeczny et al., 2001, Forbes et al., 2022) contributed to replenish the ATP levels and prevent ADP loss (Forbes et al., 2008, Forbes et al., 2022), so PCr had potential to inhibit the production of IMP, subsequently leading to less production of Hx, xanthine and uric acid, which indirectly reduced the ROS production during purine metabolism (Holloway et al., 2018).

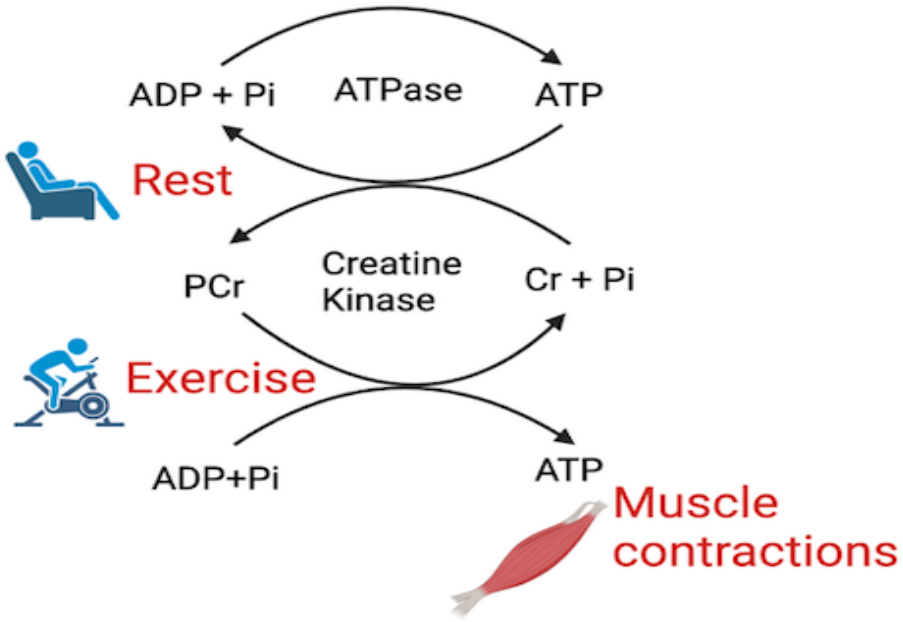


Figure 1-1 Creatine phosphagen system. ATP/CP cycle is important to maintain ATP concentration. During the rest, ATP is degraded to ADP and phosphate is transferred to creatine to generate phosphocreatine in mitochondria. During metabolic stress (e.g. exercise), stored phosphocreatine in muscle will reattach the phosphate group, the production of this reaction is creatine and ATP, which is the energy source for muscle contraction.

1.1.3. Signaling pathways in response to degrading ATP levels

Metabolic equilibrium is controlled predominantly by transcriptional mechanisms that induce changes to gene expression and transcriptional co-regulators. Dysfunction or lack of action by these transcriptional coregulators is thought to contribute to the pathogenesis of common metabolic disorders (Spiegelman and Heinrich, 2004). During supramaximal exercise ATP turnover exceeds ATP resynthesis, in an attempt to maintain homeostasis during exercise challenge the AMPK complex senses energy level changes (increased AMP and ADP) and initiates signaling processes that regulate both short- and long-term adaptations (Hardie, 2015). Following exercise, AMPK senses the low energy status and indirectly increases NAD+ via enhanced fat oxidation (Cantó et al., 2009).

Furthermore, AMPK induces mitochondrial biogenesis not only by enhancing PGC-1α activity but also inducing transcription and co-transcription. Mitochondrial biogenesis occurs in response to increased ATP demands (Hees and Harbauer, 2022). PGC-1α is enriched in skeletal muscle. In most tissues, PGC-1α regulate mitochondria biogenesis and functions and mediate oxidative phosphorylation in muscle cells (Chan and Arany, 2014). Low energy levels can stimulate and increase the expression of PGC-1α (Fernandez-Marcos and Auwerx, 2011), which promotes the mitochondrial capacity.

1.1.3.1. AMPK signaling pathway

AMP-activated protein kinase (AMPK) is one of the key modulators' of oxidative metabolism in response to metabolic stress (Moldogazieva et al., 2020). A decreased oxygen level leads to hypoxic condition during the exercise training, hypoxia could activate AMPK in brain (Rousset et al., 2015, Dengler, 2020) and skeletal muscle (Kjøbsted et al., 2018, Siques et al., 2018). Furthermore, imposing additional tissue hypoxia with exercise, even very light day-to-day activities could exacerbate ATP depletion in diseased states, which can activate AMPK signaling pathway. AMPK is highly conserved sensor of the intracellular ATP levels and plays a role in muscle tissue adaption (Herzig and Shaw, 2018). Once activated, AMPK stimulates catabolic pathways to produce ATP, while turning off anabolic

pathways that consume ATP, to maintain cellular energy stores (Carling, 2004). Besides, AMPK phosphorylates several downstream substrates and subsequently regulates the ATP levels. it contributes to switch on ATP-generating pathways through fatty acid oxidation by phosphorylation of the acetyl-CoA carboxylase1 (ACC-1) (Dzamko et al., 2008) and glycolysis (Kishton et al., 2016) and switch off ATP-consuming pathways through fatty acid synthesis by phosphorylation of the acetyl-CoA carboxylase2 (ACC-2) (Hardie and Pan, 2002).

There exists other proteins and factors involves in AMPK signaling pathway. The upstream kinases calcium calmodulin kinase kinase-2 (CaMMK2) and liver kinase B1 (LKB1) play a key role in phosphorylating and then activating AMPK (Fogarty et al., 2016, Jeon, 2016). The O_2 deprivation could damage the oxidative phosphorylation in mitochondrial and then leads to elevate the ratio of ADP/ATP, which can activate the canonical pathway of AMPK activation by LKB1, AMPK could also be phosphorylated by CaMMK2 subsequently to increase of calcium under hypoxia stress. Impaired oxidative phosphorylation in mitochondrial cause the production of ROS, which might not only induce these pathways, but also activate the AMPK directly (Figure 1.2).

Interestingly, activation of AMPK may have a beneficial role in dystrophic muscle. In the dystrophin deficient muscle, there exist lots of cellular defects, such as increased oxidative stress, abnormal fragility of sarcolemma, and enhanced calcium concentration, also exhibit mitochondrial dysfunction and diminished expression of exergy-producing metabolic genes (Gao and McNally, 2015). Therapeutic activation of AMPK might stimulate autophagic removal of defective mitochondrial in mdx mice, therefore leading to beneficial effects on overall muscular dystrophy phenotype (Kuznetsov et al., 1998, Chen et al., 2000) because autophagy has been demonstrated to be crucial in clearing dysfunction organelles and in preventing tissue damage, so it might be a target for new therapeutic intervention in dystrophic muscle.

Therefore, activation of AMPK in the cells could be a potential target for therapeutic strategies for the treatment of metabolic disease.

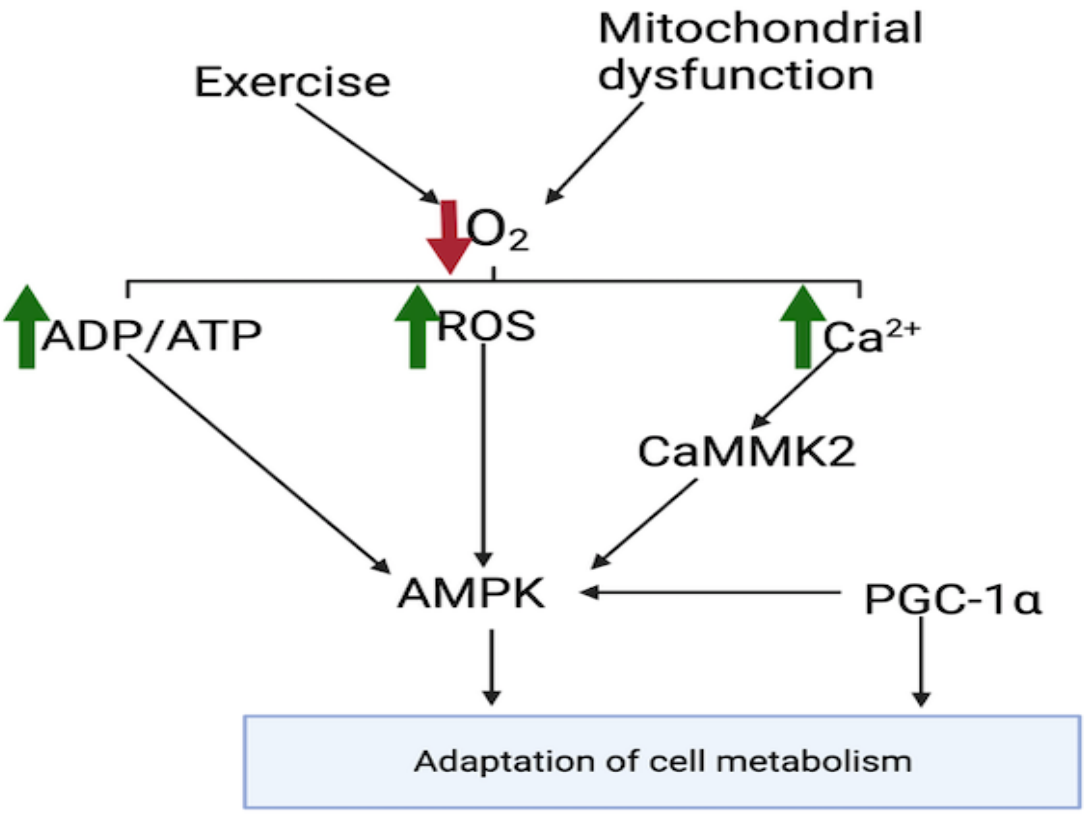


Figure 1-2 Adaptive response of activation AMPK to exercise-induced stress. Exercise and mitochondrial dysfunction, a feature of Duchenne Muscular Dystrophy (DMD), impact the energy metabolism and leads to decreased oxygen levels in cells. Increased ratio of ADP to ATP, production of ROS and unbalanced Ca^{2+} induced the AMPK and drive the response.

1.1.3.2. PGC-1α

Regular exercise could modulate adaption by enhancing the antioxidants capacity in active muscle, HIT or extreme exercise, however, lead to the increased ROS levels (mostly, H_2O_2), which trigger the PGC-1α expression. PGC-1α is the key molecule that both improve mitochondrial biogenesis and antioxidant defence capacity in response to increased ROS levels induced by exercise or damaged muscle. It is reported that PGC-1α could control SOD through Nrf2 activation (Lu et al., 2010, Rius-Pérez et al., 2020) and strongly induce the mitochondrial biogenesis by coactivation of Nrf2 and other cofactors (like Nrf1).

Handschin demonstrated that expression of PGC-1α could alleviate DMD progression through improving the parameter characteristics of DMD, such as muscle damage and exercise performance (Handschin et al., 2007). In addition, PGC-1α has a strong correlation with utrophin in muscle cells, which is *an* autosomal *an*d functional paralogue of dystrophin (bLjubicic and Jasmin, 2015, Suntar et al., 2020) and protects against dystrophy independently of utrophin. The induction of utrophin by PGC-1α is vital to alleviate muscle damage mdx mice (Chan and Arany, 2014).

It is reported that PGC-1α could trigger the AMPK signaling pathway and drive the response and maintain the cell metabolism. It is not surprising that PGC-1α be involved in the cellular response to hypoxic state (Shoag and Arany, 2010). In addition, overexpression of PGC-1α in mice have been investigated to have an increased life span and to be protected from sarcopenia. Therefore, activation of AMPK-related genes with exercise may have beneficial effects in muscle wasting disease by attenuating the severity of the disease progression.

1.2. Stressing the system

1.2.1. Exercise

Exercise increases the demand of ATP to fuel actin-myosin ATPase and maintain sarcoplasmic reticulum (SR) calcium uptake during cross-bridge cycling. The greater the exercise intensity, the higher the ATP turnover rate and subsequent demand on mitochondria to drive ADP re-phosphorylation to sustain the workload. This leads to an increased consumption of oxygen. A point of maximal oxygen uptake of the active muscle will arise as the exercise intensity increases (maximal oxygen consumption) which reflects physiologically: 1) the limitation of the capacity for delivering oxygen to the active muscle or 2) the metabolic capacity of the mitochondria to utilise the oxygen and produce energy at the required rate. Under reduced oxygen availability, there is a restricted capacity for ADP phosphorylation and electron flow in the ETC becomes imbalanced, resulting in the formation of ROS (Selivanov et al., 2011).

Exercise adaptations for improved health and performance appear to respond better to intensity of exercise (on time comparison) compared with volume of work performed. Therefore, a time effective exercise protocol such as HIT (high-intensity training) or SIT (sprint interval training) may result in significant enhancements with reduced work output and time commitment and greater compliance rates (Santos et al., 2020). HIT could provide performance benefits for athletes involved in intermittent activity and improve the health of recreational exercisers. It may also be a suitable alternative to endurance training, continuous aerobic exercise, and improve compliance of inactive individuals initiating exercise training for better health. HIT programs have demonstrated potent and effective metabolic adaptations to enhance health and physical fitness (Burgomaster et al., 2005) with mitochondria appearing to be the key determining factor within skeletal muscle (Wang et al., 2010, Larsen et al., 2015). Whilst the exercise intensity of effort in the HIT exercise protocols may not directly impacted by the mitochondria, the major energy producer at rest and during recovery between exercise bouts will be influenced by the mitochondrial function. Understanding its regulatory

mechanisms and capacity may enable the enhancement of energy efficiency. Many mitochondrial adaptations appear to be related to intensity of exercise performed, including mitochondrial function (Lundby and Jacobs, 2016, Hood et al., 2019) and mitochondrial biogenesis, reflected by changes in peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) (mRNA and protein) expression (Egan et al., 2010). It has been reported that mitochondrial content (balance between mitochondrial degradation and biogenesis) is elevated following SIT and may be influenced by mitochondrial ETC efficiency (Gillen et al., 2016). HIT and steady state exercise (SSE) however appear to enhance its expression, promote greater fat utilisation and enhance aerobic capacity (Bassett and Howley, 2000, Stisen et al., 2006). Granata and colleagues found that 4 weeks of SIT elicited greater and more rapid mitochondrial adaptations (PGC-1α, protein content, and mitochondrial respiration) than HIT and SSE (Granata et al., 2016, Granata et al., 2021). The upregulation of mitochondrial adaptations could enhance the supply and utilisation of ATP during SIT, thus having a positive effect on exercise performance and energy efficiency (Hoppeler et al., 1985, Drake et al., 2016).

Furthermore, adaptations to improve mitochondrial function and enhance ATP re-phosphorylation potential may greatly impact health and disease, with metabolic disorders demonstrating low levels of mitochondrial function and content (Kelley et al., 2002, Mogensen et al., 2007).

1.2.2. Disease: Duchenne muscular dystrophy

DMD is a severe X-linked recessive disorder with an incidence of 1:5,000 (Emery et al., 2015), making it one of the most common recessive disorders in the human population. It is a chronic muscle-wasting disorder which leads to progressive muscle weakness and atrophy, caused by mutations within the dystrophin genes. DMD is currently without an effective therapy. The *mdx* mouse, which lacks functional dystrophin expression due to a point mutation in dystrophin gene (Rodrigues et al., 2016, Yucel et al., 2018), has been widely studied as a model for DMD research. The absence of dystrophin in muscle cells cause numerous pathology issues, such as impaired energy homeostasis,

mitochondrial dysfunction and increased calcium influx, leading to the muscle damage, worsening the muscle pathological (Figure 1.3).

Impaired energy systems have identified as a key factor in the degeneration of dystrophin-deficient muscle (Timpani et al., 2015). Insufficient metabolic energy had been documented both in DMD patients (Willi et al., 2022) and *mdx* mice (Rybalka et al., 2014). PCr, contains high-energy phosphate to produce the ATP, is significantly decreased in skeletal muscle both in DMD female and male adults (Willi et al., 2022), which is also supported by Pulido study, which found that PCr contents was decreased about 50% in *mdx* myotubes of those in control mice (Pulido et al., 1998). Additionally, various publications have demonstrated that deficiencies of TCA enzymes and metabolites (Dulloo et al., 2004, Martínez-Reyes and Chandel, 2020), glycolysis (Chi et al., 1987, Pant et al., 2015) and purine metabolism (Camina et al., 1995) in DMD muscle. TCA cycle related enzymes are essential for maintain the normal cell functions, whereas in dystrophin-deficient muscle, IDH, succinate dehydrogenase (SDH) and aconitase in TCA cycle are dysfunctional (Chen et al., 2000, Martínez-Reyes and Chandel, 2020). Pant demonstrated that the activities of glycolytic enzymes, pyruvate kinase and hexokinase, in *mdx* EDL muscle was higher than WT mice (Pant et al., 2015). Purine metabolism (also impacts ATP production). It is reported that DMD involves alterations leading to blockage of IMP formation (Sahlin et al., 1990), which is associated with high ATP turnover and increased ADP and AMP in the muscle (detailed described in the Section 1.5.6). Therefore, muscle dystrophin leads to severe perturbation in myocellular energy homeostasis with resting ATP levels, consistently demonstrated as 50% below that of healthy skeletal muscle at rest (Tamari et al., 1982, Shuttlewood and Griffiths, 1982, Vignos Jr, 1983, Cole et al., 2002). This infers that dystrophin-deficient muscle consistently maintains sub-threshold ATP levels to sustain daily function which are likely incompatible with long-term cell survival.

Mitochondria dysfunction is a known pathological feature of DMD and impairs processes involved in Ca^{2+} homeostasis. Rybalka et al. demonstrated that DMD pathology is worsened by a Complex I deficiency, which partly contributes to reduce in ATP production (Rybalka et al., 2014). Additionally, In DMD patients and mdx mice studies, the absence of dystrophin causes disruption of

the dystrophin-glycoprotein complex (DGC), which is critical for maintaining sarcolemma integrity and the activity of signaling complexes and ion channels. DGC disruption induces direct calcium influx and impairs abnormal cytosolic calcium homeostasis, causing membrane leakage and increased vulnerability of myofibers to necrosis. The high levels of intracellular calcium are directly related to the increase of oxidative stress and exacerbated inflammation (Whitehead et al., 2008, Shin et al., 2013). Moreover, calcium is also involved in the regulation of ATP production indirectly (Kuo and Ehrlich, 2015). Calcium exerts control on all the mitochondrial dehydrogenases, which regulates the ATP production. Calcium can activate the creatine phosphate kinase (Morris and Cole, 1979), which has been shown to catalyze the process of PCr breakdown, then promoting ATP production. Calcium uptake contributes to aerobic metabolism by activating all the TCA cycle dehydrogenase. i.e. IDH, an important TCA enzymes, converts isocitrate to a-ketoglutarate, this TCA enzyme is suppressed when the ATP/ADP and $NADH/NAD^+$ ratio is increased. The other two calcium dependent TCA dehydrogenase, pyruvate and the a-ketoglutarate dehydrogenases, also shared IDH in the TCA cycle. It is reported that ATP/ADP ratio determined how sensitively Ca^{2+} binds to IDH, a reduction in the ratio of ATP to ADP increases Ca^{2+} binding to IDH, which in turn cause the decrease of the Km for isocitrate (Rutter and Denton, 1988). Another TCA enzyme oxoglutarate dehydrogenase (OGDH), which catalases the a-ketoglutarate to succinyl-CoA, the Ca^{2+} binding to OGDH can cause a drop in the Km for a-ketoglutarate (Denton, 2009). In addition, increased Ca^{2+} is a biomarker of metabolic stress, mitochondrial calcium may promote ROS production by activating mitochondria relevant enzymes, like activating nitric oxide synthase (NOS) and nitric oxide (NO) production, leading to excessive mitochondrial ROS formation (Gupta Kapuganti et al., 2010), which is detrimental and can result in muscle dysfunction and pathology.

In animal models of DMD, reduced endothelial and neuronal NOS (eNOS and nNOS) expression has been reported (Loufrani et al., 2004, Dabiré et al., 2012). Reduction in NOS activities may contribute to pathology in muscular dystrophin, because NOS is the only compound of dystrophin complex that is selectively abundant in fast-twitch muscle cells, which preferentially degradation in DMD (Webster et al., 1988). It is reported that reduced NO signaling also contributes to contraction-

induced injury of dystrophin-deficient muscle. *Mdx* muscle exhibits progressive damage post-contraction that can be alleviated by treatment with a NO donor (Asai et al., 2007). In addition, NOS plays an important role in the regulation of blow flow, which decreased in skeletal muscle in DMD patient (Emery and Schelling, 1965, Dietz et al., 2020). Taken together, modulation of NOS activities could be considered a potential new approach to slow disease progression in DMD patients.

The ER is composed of an extended network of tubules. This membrane-bound organelle is responsible for the regulated release of Ca^{2+} into the cytoplasm to trigger muscle contraction (Bohnert et al., 2018). In addition to its vital role in calcium homeostasis, this dynamic structure is also responsible for the proper folding and packaging of proteins. This dysfunction causes an accumulation of unfolded and misfolded proteins in the ER lumen, which may affect cellular function and create a toxic environment in the cell, leading to its death. In a bid to cope with ER stress, eukaryotic cells elicit a conserved adaptive mechanism, the unfolded protein response (UPR), aiming to increase production of ER chaperones, to clear damaged proteins, and to re-establish ER homeostasis (Zhang and Kaufman, 2006).

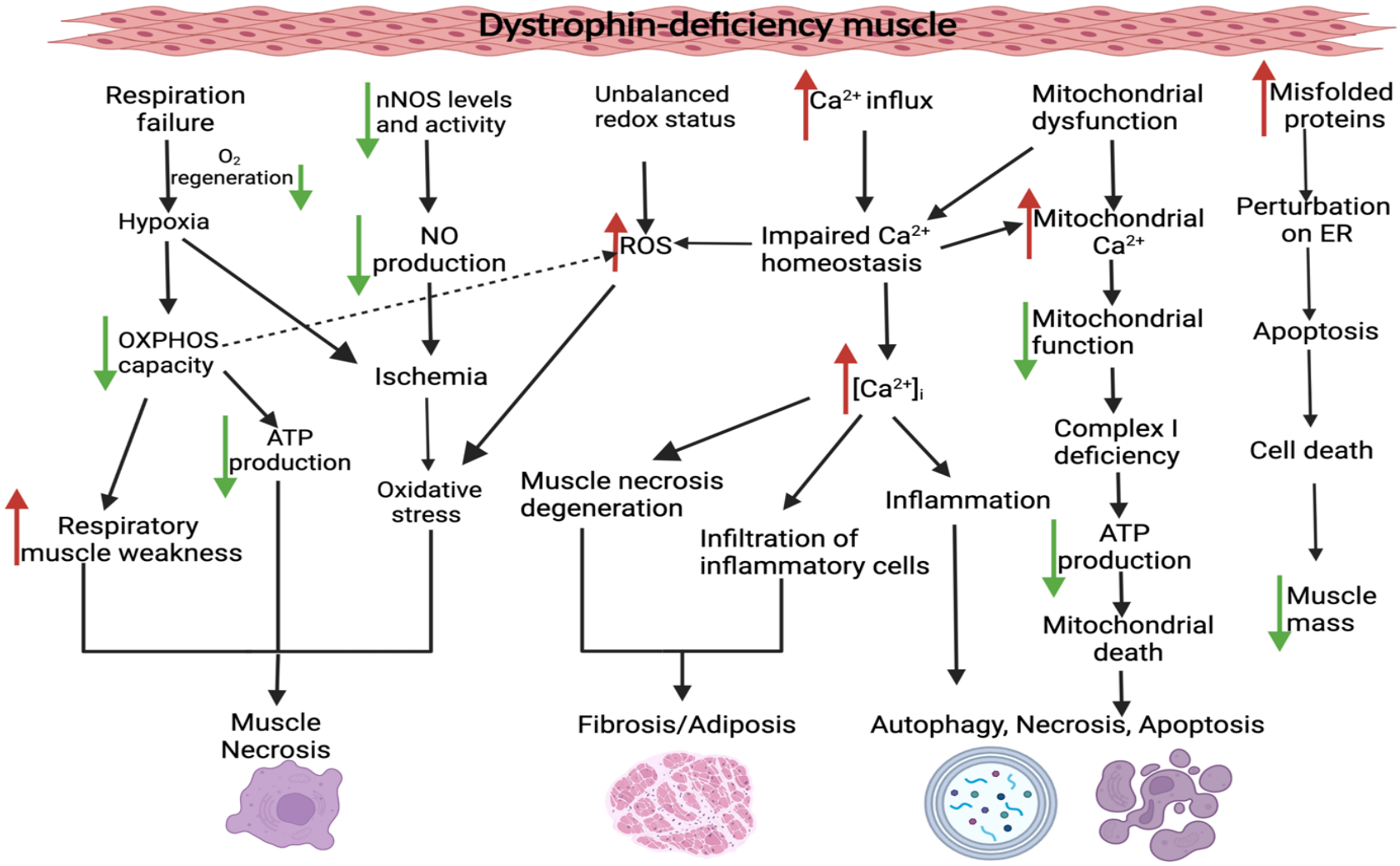


Figure 1-3 Pathophysiology of dystrophin-deficiency muscle

1.3. Molecular signature of metabolic stress

1.3.1. Metabolites as signaling molecules

Purines compounds, perform a wide range of crucial intracellular and external biological functions (Huang et al., 2021, Díaz-Muñoz et al., 2022). ATP and adenosine (in particular) control a wide range of cellular and systems-level physiological processes. Starting with ATP, which serves as the cellular energy currency, to adenosine, which is produced when cellular ATP is depleted and reacts to lower energy demand and thereby preserve ATP during times of metabolic stress. Therefore, response of purine metabolites (such as IMP, Hx, xanthine and uric acid) may act as indicators of metabolic stress in skeletal muscle.

IMP is the first product of purine metabolism (Brooke et al., 1986). Studies have demonstrated that IMP production during ischemia contraction was frequently accompanied by high levels of muscle lactate, the depletion of PCr, and elevated levels of ADP and AMP (Sahlin et al., 1990, Tullson et al., 1996). For instance, during long-term (2-4 h) anoxia, the rate of IMP formation was ~4% of that during contraction, despite similar changes in PCr, lactate, ADP, and AMP (Sahlin et al., 1990). Bonagurio have identified that dietary IMP increased the capacity of oxidative phosphorylation via enhancing the mitochondrial respiration rate to support energy requirements in response to low-energy levels in pig (Bonagurio et al., 2022).

Hx is an intermediate metabolites of purine metabolism and is increased in the blood and skeletal muscle during the metabolic stress of high-intensity exercise (Domaszewska et al., 2020, Yin et al., 2021). Various studies on energy metabolism have demonstrated that an increase of Hx level that can range from 2-10 times higher than the resting levels in response to different exercise intensity (Zieliński et al., 2013, Zielinski and Kusy, 2015). Hx is also the main purine metabolites involves in the purine salvage pathway, which recycled the PRPP and stored Hx to the formation of IMP via hypoxanthine-guanine phosphoribosyltransferase (HGPRT). During high-intensity exercise, there is an increase in ATP consumption, which is accompanied by the production of Hx by AMP and IMP

(Gerber et al., 2014, Zieliński et al., 2019). The muscle releases the stored Hx into the circulation, which is then carried to other tissues. Besides, Hx plays an important role in the skeletal muscle metabolism. Hx induced the increased uncoupling proteins UCP2, which degraded more glycogen into lactate in the via elevated capacity of glycolysis (Brand et al., 2010, Vozza et al., 2014). Hx is degraded to xanthine via xanthine oxidase (XO) and guanine deaminase converts guanine to xanthine, which is oxidized by XO to generate the end product of purine metabolism uric acid (Maiuolo et al., 2016). Several studies have demonstrated that high levels of uric acid and its association with metabolic stress, such as high intensity exercise and disease models (Green and Fraser, 1988, Soltani et al., 2013, Gicchino et al., 2023).

The intramuscular accumulation of lactic acid has long been considered as another indicator during the metabolic stress of high-intensity exercise (Sahlin, 1986, Fitts, 1994, Green, 1997). During low-intensity exercise or at rest, the body creates additional proteins that help absorb and convert lactic acid to energy, which means there is an even rate of lactic acid production and blood lactate removal (Nalbandian and Takeda, 2016). As intensity of exercise increases, more ATP degraded to AMP, a greater glycogen breakdown to lactic acid and a decline of muscle force or power output occurs leading to impairment of whole-body exercise performance because of the development of fatigue (Cairns, 2006).

1.3.2. Oxidative stress & Inflammation

Exercise is increasingly present in the lives of humans and becoming the primary approach for maintaining health, longevity and reducing the risk of some chronic disease such as diabetes, cardiovascular diseases and muscular disorders (Yavari et al., 2015). Intense exercise might exceed the maximum antioxidant capacity in the body, resulting in the production of free radicals. Free radicals derived from oxygen are called ROS. Oxidative processes are ever present as O_2 is crucial to life and plays a large role in the production of ROS and exercise elevates ROS production and challenges the antioxidant capacity in muscle cells. Oxidative stress occurs when ROS is excessively produced and antioxidant systems are unable to balance the response, then leading to oxidative stress (Ďuračková,

2010). Oxidative stress is an impaired balance between the production of free radicals and antioxidant defence systems in the body (Debevec et al., 2017). The imbalance between the production of ROS and the removal of these compounds by the antioxidant defence system causes oxidative stress (Figure 1.4). Early studies focused on the side effects of ROS production in the muscle, such as muscle damage and dysfunction as oxidative stress can damage cells and proteins and is also linked to some chronic diseases, such as cancers (Lu et al., 2007, Liou and Storz, 2010), and metabolic diseases (Giacco and Brownlee, 2010, Akhigbe and Ajayi, 2021). In contrast, more recent studies have focussed on the adaptive potential of the oxidative process and indicate that this acute transient production of ROS with exercise plays a crucial role in hormesis and is required process for maintaining healthy muscles. This highlights that ROS generation during the exercise is programmed and necessary for major signaling pathways, which may contribute the muscle adaption (Powers et al., 2010, Powers et al., 2020, Wang et al., 2021).

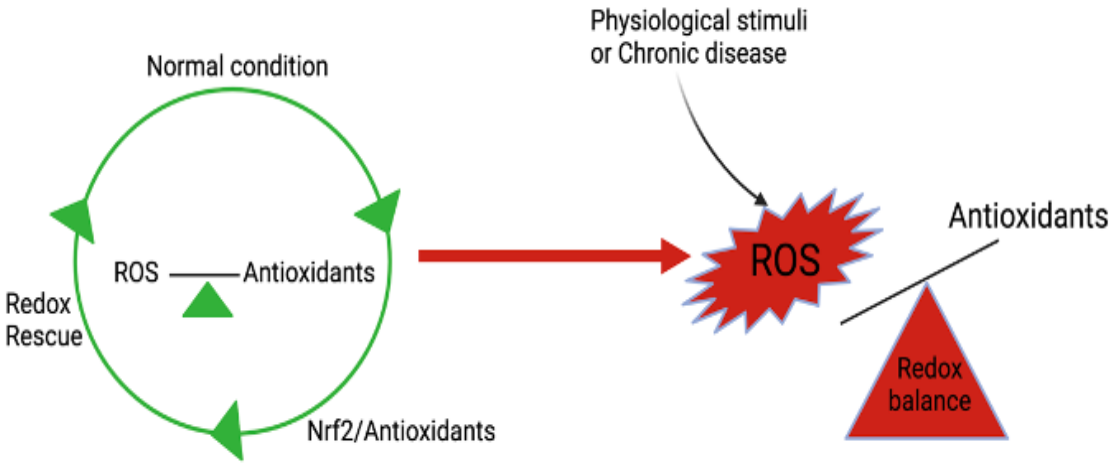


Figure 1-4 Redox balance in normal condition and oxidative stress conditions in living organism

1.3.2.1. ROS production

ROS are highly unstable molecules that have important cell signaling functions but also the propensity to cause damage when generated in high enough concentrations (Schieber and Chandel, 2014). Molecular oxygen (O_2) is the final acceptor of electrons in cellular metabolism and mitochondrial bioenergetics. A consequence of elevated mitochondrial O_2 consumption, and an increased flux through sequential redox reactions in the inner mitochondrial membrane is the increased production of ROS. Complementary antioxidative enzyme systems have evolved to buffer these ROS and protect the mitochondria against ROS accumulation and oxidative toxicity. Along with mitochondria, numerous other organelles contribute to ROS production, including the endoplasmic reticulum (ER), lysosomes, and peroxisomes (Figure 1.5) (Zeeshan et al., 2016, Di Meo et al., 2016, Le Moal et al., 2017). For example, inside the ER, ROS are produced from catalytic processes by ER oxidoreductin 1 (ERO 1) and from delivering electrons to O^2 by NADH-cytochrome P450 reductase to form O^{2-}, with electrons delivered to O^2 by the electron transport chain on the nuclear membrane, assisted by NADH (Zeeshan et al., 2016). Peroxisomes are other major sites of ROS, which are mainly for the production of superoxide and hydrogen peroxide through the catalyse of XO, it also involves in different metabolic pathways including fatty acid b-oxidation, nucleic acid and polyamine catabolism, etc (Sandalio et al., 2013). In addition, there are key enzymes within the cytosol, the sarcolemma and the extracellular muscle environment that generate ROS. Beside the respiration chain, NADPH oxidases (NOXs) are the predominant sources of ROS in cells and regulate numerous redox-dependent signaling pathway. XO and nitric oxide synthase (NOS) also contribute to the redox potential (Roy et al., 2015). XO is involved in last two reaction of purine degradation pathway, converting hypoxanthine (Hx) to Xanthine and then to uric acid (Hille and Nishino, 1995, Christen et al., 2001), XO uses oxygen as an electron acceptor and produce superoxide. NOS has been reported to catalyse superoxide anion production depend on substrate (L-arginine and molecular oxygen) (Crane et al., 1998, Alderton et al., 2001) and cofactor (flavin adenine dinucleotide, FAD) (Andrew and Mayer, 1999, Förstermann and Sessa, 2012).

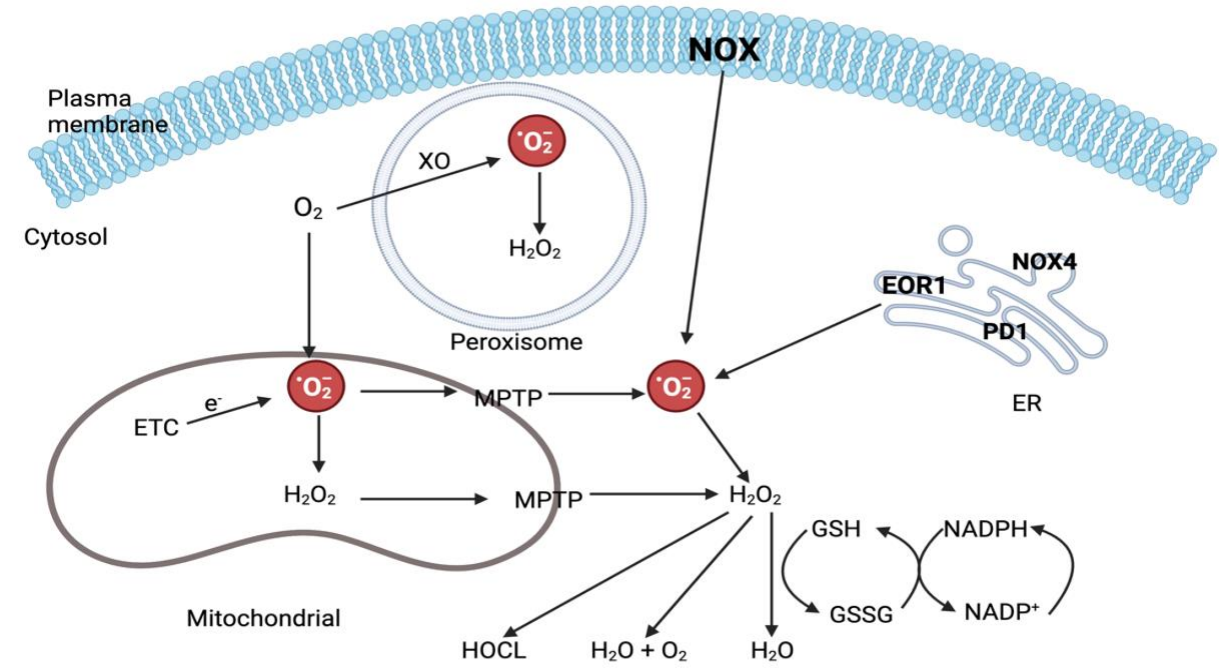
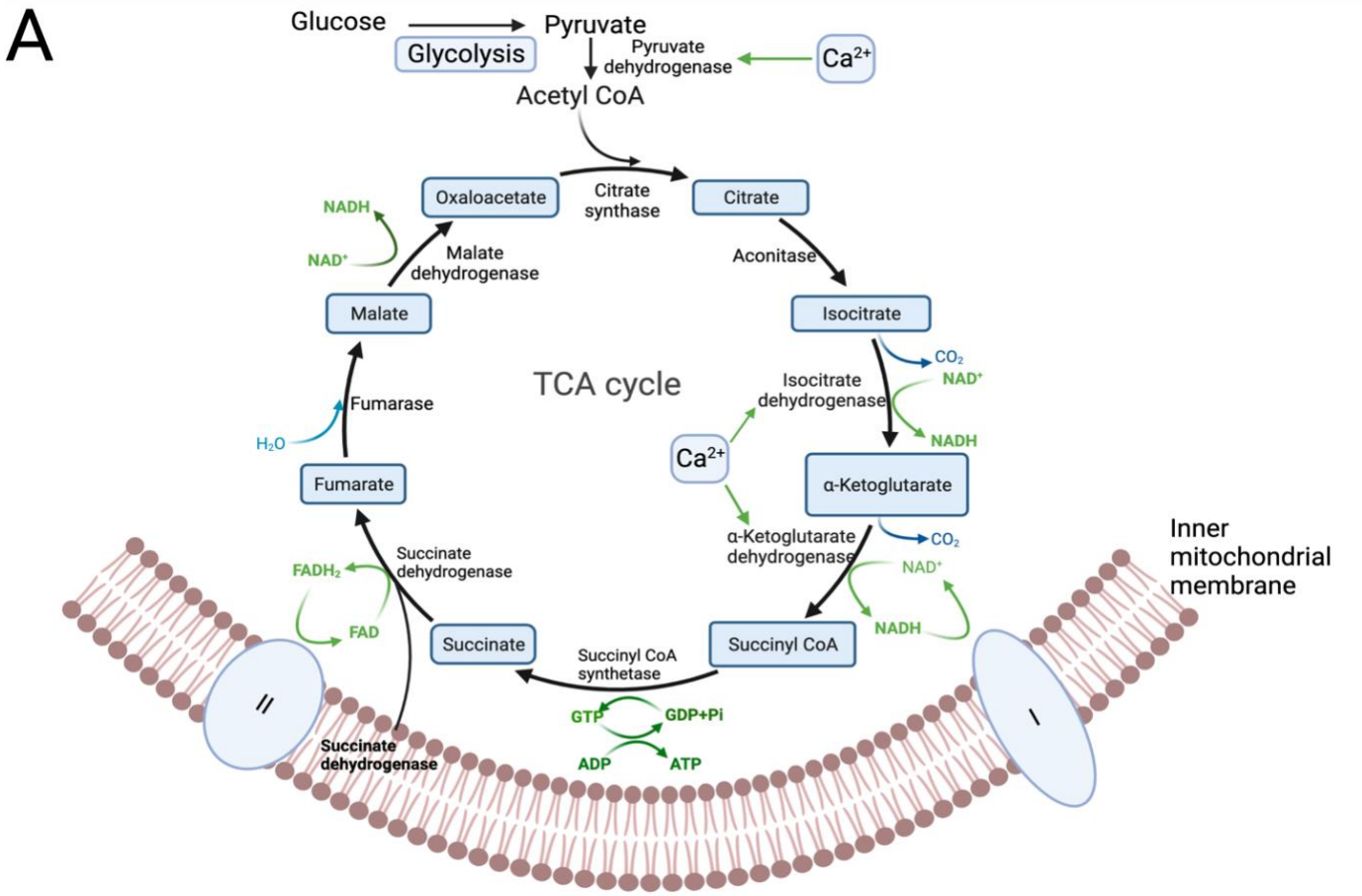


Figure 1-5 Major source of ROS inside of muscle cell. The main source of ROS are mitochondria, ER, peroxisome and the NOX complex in the cell membrane. In normal condition, overproduction of ROS is scavenged by the antioxidants defence system in the muscle cell. ERO1: endoplasmic reticulum oxidoreductin 1; ETC: electron transport chain; GSH: glutathione; GSSG: glutathione oxidized; H_2O_2: hydrogen peroxide; HOCL: hypochlorous acid; MPTP: mitochondrial permeability transition pore; NADPH: nicotinamide adenine dinucleotide phosphate reduced; $NADP^+$: nicotinamide adenine dinucleotide phosphate oxide; NO: nitric oxide; NOX: NADPH oxidase; O^{2-}: superoxide radical; PDI: protein disulfide isomerase; SOD: superoxide dismutase; XO: xanthine oxidase.

Mitochondrial complexes play a vital role in the production of ATP, which is necessary for cell survival and is also an important source of ROS (Tirichen et al., 2021). ROS production of mitochondria is controlled by various factors (such as TCA substrates, calcium and oxygen availability). Studies have documented that mitochondrial respiration can be supported either by substrates linked to NAD^+ (such as glutamate, pyruvate, or malate) (Stein and Imai, 2012) or substrates linked to FAD (such as succinate, α-glycerophosphate) (Heikal, 2010), which influence the CoQ (an intermediate during the ROS production) in the respiration chain (Alcázar-Fabra et al., 2016).

The mitochondria electron transport chain (ETC), which is composed of four transmembrane protein complexes (I to IV) with a multitude of redox reactions (Figure 1.6B). Complex I is the first complex in the ETC and is responsible for transferring electrons from NADH to CoQ (ubiquinone). Complex I produces ROS when electrons circulate in the forward direction and when complex I linked substrates are used to feed the TCA (Figure 1.6A), where electrons circulate through complex I in the ETC. During this process, electrons mainly leak to produce superoxide from the I_F site of complex I during the oxidation of NADH to NAD^+. Besides, ROS also produced in complex III and complex IV, electrons in these complexes can escape and react with O_2, leading to the formation of ROS, such as superoxide. In normal conditions, these ROS are efficiently neutralized by the antioxidant defences within the mitochondria, such as SOD and Catalase. However, when the levels of ROS production become excessive, or the antioxidant defences become overwhelmed, an imbalance occurs, which can lead to oxidative stress, and cellular damage cellular, including DNA, proteins, and lipids. Over time, this oxidative stress can contribute to the development of a variety of diseases, including neurodegenerative disorders, cardiovascular disease, and cancer.


A
Glucose
Glycolysis
Pyruvate
Pyruvate dehydrogenase
Ca2+
Acetyl CoA
Oxaloacetate
Citrate synthase
Citrate
NADH
NAD+
Malate dehydrogenase
Aconitase
Malate
Isocitrate
TCA cycle
CO2
NAD+
Isocitrate dehydrogenase
NADH
H2O
Fumarase
Ca2+
α-Ketoglutarate
Fumarate
α-Ketoglutarate dehydrogenase
CO2
NAD+
FADH2
Succinate dehydrogenase
FAD
NADH
Succinate
Succinyl CoA synthetase
Succinyl CoA
GTP
GDP+Pi
ADP
ATP
Succinate dehydrogenase
Inner mitochondrial membrane

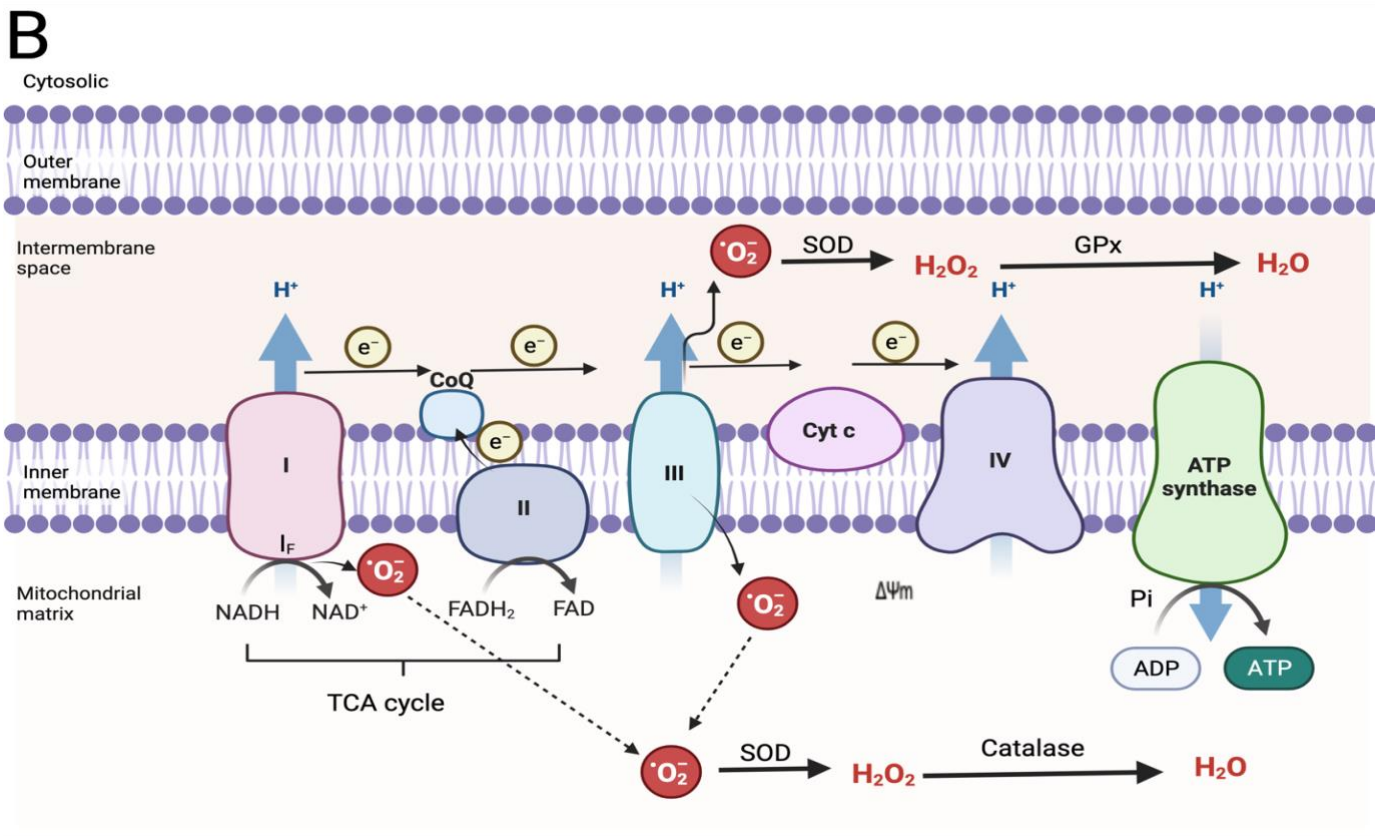


Figure 1-6 TCA cycle metabolites (A), generation and elimination of ROS in ETC (B). Glucose enters the cells through glycolysis and finally break down to pyruvate in the cytoplasm. Pyruvate is transported into mitochondrial matrix, where it can be oxidized into acetyl-CoA by pyruvate dehydrogenase. In these enzymatic reactions of the TCA cycle, $FADH_2$ and NADH are generated and then transfer electrons into the mitochondrial transport chain (also known as ETC). As the electrons are funneled through the complex I, III and IV in the inner mitochondrial membrane, a functional ETC form a mitochondrial membrane potential (ΔΨm), which generates transfer membrane potential of the hydrogen ion that is utilized to make ATP. As we can see from diagram B, ROS is the product of ETC, mitochondria produce ROS mainly O_2^-. When O_2^- leaks, it moves into inner membrane space, they may escape to cytosolic, where they can convert into H_2O_2 by SOD. H_2O_2 is further break down into H_2O by catalase and GPx. (cited from (Zhao et al., 2019)).

Oxidative stress generated during the elevated metabolic conditions (like exercise, hypoxia and chronic diseases), may lead to damage DNA and proteins as a consequence of disrupted cellular processes. Once antioxidant defences are overwhelmed, the increased ROS levels stimulate a signal cascade to initiate an adaptive response and develop an enhanced cellular function to protect the muscle cell from harmful oxidative damage. A key stress activated factor that is key to the antioxidant defense system is Nrf2, Nrf2 is the transcription factor responsible for the modulation of the cellular redox balance and antioxidant system which activates a multitude of signal responses with exercise-induced oxidative stress in the skeletal muscle. Downstream genes of Nrf2 signaling pathway could contribute to increase antioxidants levels and muscle adaptation in response to oxidative stress. Nrf2 target genes and their functions are presented in Table 1.1.

1.3.2.2. The Nrf2

Nrf2 is a key oxidative stress signaling molecule with multiple protein response outcomes. Physical activities induce the production of ROS in skeletal muscles due to the increase in oxygen consumption and energy demand (Powers et al., 2020, Wang et al., 2021). Nrf2 acts as a crucial transcription factor, in response to oxidative stress, that can modulate the expression of phase II enzyme genes expression by binding with the antioxidant response elements (AREs) sequence in the nucleus (Itoh et al., 1997, Motohashi and Yamamoto, 2004, Tebay et al., 2015). The Nrf2/AREs binding also can activate the ROS defence and supress the ROS production.

Under normal condition, nuclear levels of Nrf2 are low, Nrf-2 is sequestered in cytoplasm and its activity is principally regulated by Keap1 through the complex Keap1-Nrf2, which promotes the ubiquitination and degradation of Nrf2. Upon exposure to several stressors (like high-intensity exercise, drugs and specific disorders), nuclear accumulation of Nrf2 increases, because Nrf2 is released and directly translocates to the nucleus where its antioxidant functions occur (Figure 1.7) (Kobayashi et al., 2004).

Physical exercise induces autophagy, autophagy deficiency leads to the accumulation of p62, a multifunctional cargo receptor that can sequester Keap1 and stabilize Nrf2, resulting in Nrf2

activation (Komatsu et al., 2010). p62, a protein encoded by the sequestosome 1 gene (SQSTM1), is a multifunctional protein induced by oxidative stress and is involved in the activation of the Nrf2 signaling pathway (Jiang et al., 2015). Under oxidative stress, an autophagic pathway was found to be maintained by a Keap1–Nrf2 feedback loop through p62. In response to oxidative stress, Nrf2 is disassociated from Nrf2-Keap1 compounds, through p62 binding to Keap1 competitively, and then translocated to nuclear, where it can activate the downstream target genes (Taguchi et al., 2011). This promotes autophagy in a positive feedback loop. Autophagy is a controlled process shown to be a crucial role in clearing dysfunctional organelles and preventing tissue damage. Additionally, p62 senses the saturation of ROS buffering systems, which leads to the redox unbalance, redox sensitivity is important to increase autophagy and the survival of cells under oxidative stress conditions. Thus, p62 and Nrf2 create a positive feedback loop to regulate a plethora of cellular functions (Taniguchi et al., 2016, Jain et al., 2010).

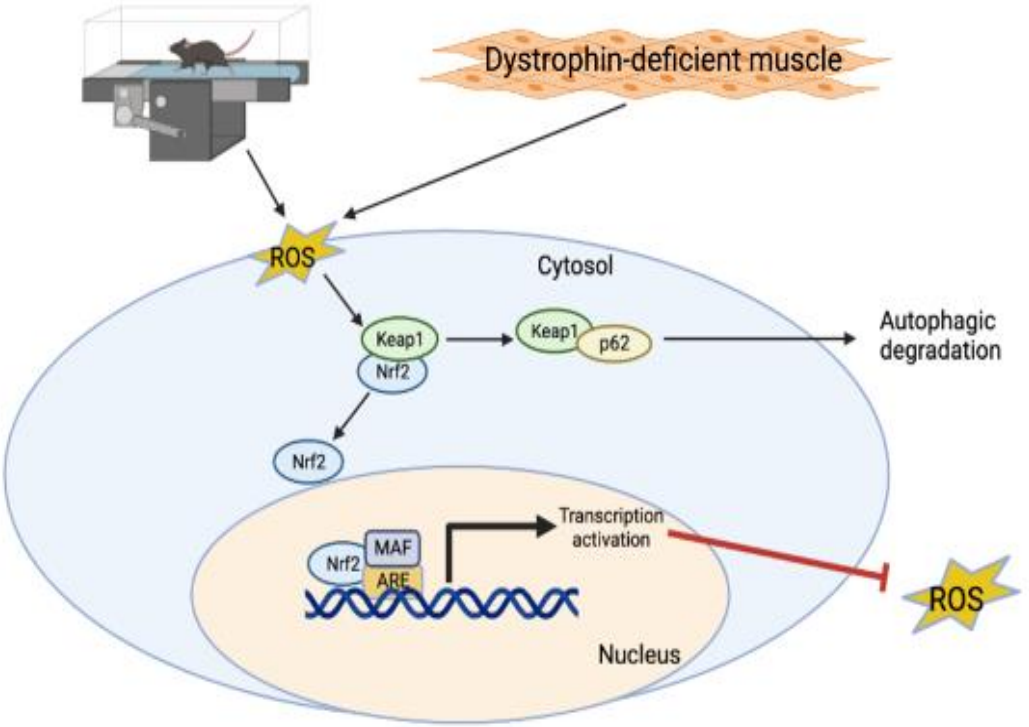


Figure 1-7 Nrf2 signaling pathway in response to increased ROS levels. HIT could activate the Nrf2 pathway, Nrf2 disassociated from Nrf2/keap1 bind protein, and translocated to nucleus, where it makes antioxidant response to HIT.

Table 1-1 Few Nrf2 target genes and their functions

Target genes	Functions	References
NQO1	FAD-binding protein reduce quinones to hydroquinone	(Mondal et al., 2018)
SOD	Catalyse the dismutation of the superoxide radical (O_2^-) into molecular oxygen or hydrogen peroxide.	(Younus, 2018, Wang et al., 2018)
Catalase	Destroying cellular hydrogen peroxide to form water and oxygen	(Nandi et al., 2019)
HO1	Catalase the degradation of heme and generates the antioxidant molecules, Really important for preventing disease caused by oxidative stress	(Loboda et al., 2016, Fomusi Ndisang, 2017)
p62	Result in hyperactivation of Nrf2 and delineates unexpected roles of autophagy in controlling the transcription of cellular defense enzyme genes	(Komatsu et al., 2010)
NF-kB	Nrf2 pathway inhibits the activation of NF-κB pathway by increasing HO-1 expression and preventing the degradation of IκB-α	(Bellezza et al., 2012) (Liu et al., 2017)

To protect the biological system from ROS toxicity, several antioxidant defence enzymes including NAD(P)H quinone dehydrogenase 1, superoxide dismutase (SOD), catalase, HO1, glutathione peroxidase (GPx), exist to buffer the production of ROS. For example, SOD is a class of enzymes that catalyses the removal of superoxide by dismutation into oxygen and hydrogen peroxide. HO1 could catalyse the degradation of heme and generates the antioxidant molecules (Ryter et al., 2006, Loboda et al., 2016), it is also really important for preventing disease caused by oxidative stress in skeletal muscle (Deshane et al., 2005, Fomusi Ndisang, 2017). Therefore, the balance between ROS production and antioxidant response is important for redox homeostasis and has potential implications for the long-term cellular function and health of the individual.

1.3.2.3. NAD(P)H quinone dehydrogenase 1

NAD(P)H quinone dehydrogenase 1 (NQO1) is a member of the NAD(P)H dehydrogenase (quinone) family and encodes a cytoplasmic 2-electron reductase. It is reported that NQO1 could regulate the production of ROS and is able to alleviate oxidative stress (Mondal et al., 2018) induced by many factors (including exercise or metabolic disease). Induction of NQO1 is developed as a component of the Nrf2-induced adaptive response to oxidative stress and is activated as part of the aromatic hydrocarbon receptor induced response (Dinkova-Kostova and Talalay, 2010). It is reported that NQO1 is a conserved target gene of Nrf2 and can serve to monitor the activity of the Nrf2 pathway (Mutter et al., 2015). The activation of Nrf2/AREs results in the activation of ROS defence system and supress the ROS production. Furthermore, NQO1 can catalyze the two-electron reduction of quinones, it escapes the one-electron reduction and avoids subsequent redox cycling of the quinone derivatives and the formation of ROS (Lee et al., 2021b).

NQO1 has been reported to potential benefit in the modulation of DMD patient. NQO1, in particular, appears to slow down its antioxidant buffering function in older DMD patients despite being strongly expressed in very early-stage patients. NQO1 can catalyse the reduction of quinone to hydroquinone, then preventing the toxic accumulation of quinone by-products that associated with

cellular process (Cavelier and Amzel, 2001, Asanuma et al., 2003). Therefore, upregulation of NQO1 may be a potential therapeutic strategy for protection from oxidative stress induced by exercise or metabolic disease via activation of Nrf2 activation and inhibition of ROS production.

1.3.2.4. Superoxide dismutase

Another downstream enzyme of Nrf2 signaling pathway is SOD. SOD has three different isoforms include Cu/Zn-SOD (SOD1) (Dell'Orco et al., 2016), Mn-SOD (SOD2) (Sun et al., 2015), and extracellular SOD (SOD3) (Wang et al., 2018), which are important phase II enzymes and functions as a natural defensive mechanism for cells (Zhang et al., 2013, Dell'Orco et al., 2016). Activation of Nrf2 signaling pathway leads to the overexpression of SOD1 (Dell'Orco et al., 2016, Robertson et al., 2020). Stimulation of SOD2 have been reported as the mechanism that Nrf2/ARE (antioxidant response elements) bonds protect mitochondria that are exposed in oxidative stress (Sun et al., 2015) because it can break down the superoxide ion into oxygen and hydrogen peroxide, then decreasing the ROS production (Younus, 2018). SOD2 has Studies have also shown that SOD involved in the regulation of several disease states. Overexpression of SOD protects against pro-oxidant insults brought on by inflammatory cytokines (Chen et al., 2017), hyperoxic damage (Case et al., 2017), cancer (Robbins and Zhao, 2014) and ischaemia/reperfusion (Joseph et al., 2008). For instance, SOD has benefits in reducing the lung inflammation by inhibiting the activation of proinflammatory cytokines (IL-1, and IL-6) and enhancing the anti-inflammatory cytokine (IL-10) action (Koo et al., 2005). SOD3 is primarily found in extracellular spaces and is particularly important for protecting the extracellular matrix and preventing damage to tissues (Wang et al., 2018).

1.3.2.5. Heme oxygenase 1

One of the Nrf2 regulated antioxidant genes is HO1, which catalyses the degradation of heme and generates the antioxidant molecules and is important for protecting against oxidative stress induced by exercise or disease (Smith et al., 1994, Kurucz et al., 2018). It is reported that deficiency

of HO1 causes atrophy, mitochondrial dysfunction and inability to exercise adaption in skeletal muscle (de Souza et al., 2021).

The chronic inflammatory response further exacerbates the dystrophic phenotype impinging the antioxidant defence. The existence of a molecular borderline between the 'presymptomatic' phase of the disease and the later stage of DMD suggests that a pharmacological approach, specifically acting in the presymptomatic phase of the pathology, should ameliorate the efficacy of later therapeutic interventions on DMD patients. A combined impairment of HO1 in younger DMD children may contribute to the early onset of inflammation and to the progressive increases of ROS production (Petrillo et al., 2017). The temporal progression of muscle oxidation has been reported in dystrophic-deficient muscle and in aged *mdx* mice (Prosser et al., 2013), where an amplified ROS signaling was also found (Terrill et al., 2013). The elevation of oxidative stress during the period of physical exercise could trigger the activation of Nrf2 to induce increased the level of antioxidant protein HO1 and thus produced the anti-inflammatory and antioxidant effects. HO1 plays pivotal role in regulating the immune response at early stage, Kapturczak demonstrated observed the high-level of pro-inflammatory cytokines (interleukin1: IL 1 and interleukin 6: IL 6) in HO knock out mice (Kapturczak et al., 2004). A decrease of HO1 level were found in young DMD patient compared to control group, which indicates the enzyme's positive anti-inflammatory action does not entirely manifest itself in the younger children. In young DMD patient (2- to 9-year-old), this HO1 partial deficit is linked to a upregulation of IL6 (Petrillo et al., 2017). Therefore, an efficient active antioxidant system is critical to guarantee a prompt and stable response against the elevated ROS production in DMD.

1.3.2.6. NF-κB

It is assumed that Nrf2 and NF-κB signaling pathways cooperate to maintain the physiological homeostasis of cellular redox status and to regulate the cellular response to oxidative stress. However, the molecular mechanisms underlying this functional interaction appear to be tissue specific are still being elucidated.

ROS play a major role in regulating cellular defence mechanisms and physiological functions. During the period of HIE, ROS concentration increases in skeletal muscle, excessive ROS production will cause the imbalance of redox status leading to the cell and tissue damage (Ma and He, 2012). NF-κB is a redox sensitive transcription factor, which may have a protection role under oxidative stress by inhibiting the ROS accumulation (Lingappan, 2018).

Nrf2 pathway inhibits the activation of NF-κB pathway by increasing antioxidant defences and HO-1 expression, which efficiently neutralizes ROS to detoxify toxic chemicals and hence, reduces ROS mediated NF-κB activation (Soares et al., 2004). Nrf2 pathway also inhibits NF-κB mediated transcription by preventing the degradation of IκB-α (Yerra et al., 2013). Similarly, NF-κB mediated transcription reduces the Nrf2 activation by reducing the ARE gene transcription. This decreases free CREB binding protein (CBP) by competing with Nrf2 for cysteine/histidine 1-kinase inducible domain of CBP (Liu et al., 2008). NF-κB also enhances the recruitment of histone deacetylase3 (HDAC3) to the ARE region by binding to Mafk and hence interferes with the transcriptional facilitation of Nrf2 (Wakabayashi et al., 2010). NF-κB also plays a vital role in muscle wasting disorder. Muscle degradation in DMD is exacerbated by increased oxidative stress (Lawler, 2011). Kumar and colleagues report that a skeletal muscle-specific activation of NF-κB have been demonstrated even before the onset of dystrophic damage (Kumar and Boriek, 2003). Increased oxidative stress and NF-κB activation occur in *mdx* mice and that their inhibition significantly ameliorates functional, morphological, and biochemical parameters (Messina et al., 2006, Messina et al., 2009). Activation of Nrf2 has a favourable role in ameliorating inflammation of *mdx* mice via inhibition of the NF-κB signaling pathway (Gillard et al., 2015).

To summarize, Nrf2 and NF-κB individually influence many signaling cascade to maintain redox homeostasis. However, their interaction could further influence key redox modulation in health and disease.

1.3.3. Endoplasmic reticular (ER) stress

The ER is an organelle responsible for the folding, modification and synthesis of secretory and membrane bound proteins and calcium homeostasis (Hong et al., 2017). Protein synthesis and protein folding is highly controlled and is influenced by alterations in ER homeostasis (Cao and Kaufman, 2014). Changes in ER homeostasis can be triggered by factors such as Ca^{2+} depletion (Mekahli et al., 2011, Seo et al., 2020), hypoxia (Díaz-Bulnes et al., 2020, Akman et al., 2021), altered glycosylation (Shu et al., 2019, Kerselidou et al., 2021) or viral infection (Li et al., 2015, Hassan et al., 2012). As a consequence of the accumulation of unfolded proteins during ER stress, a cellular response mechanism exists to limit and prevent further and more damaging accumulation of unfolded proteins.

UPR is a protective cellular mechanism that limits the accumulation of unfolded proteins within the endothelium by increasing the folding ability of the ER or removing some of the unfolded proteins, thus alleviating ER stress (Hetz, 2012). The number of folded proteins is reduced and the balance of protein folding is restored if the UPR is successful. Alternatively, unfolded proteins accumulate and consequently result in programmed cell death. Increased ROS and oxidative stress has also been linked to UPR signaling pathway and ER stress (Figure 1.8) (Ozgur et al., 2014). During ER stress, accumulation of misfolded proteins in ER lumen could drive the UPR, leading to adaption or apoptosis (Malhotra and Kaufman, 2007). UPR is activated by three signaling pathways, IRE1, PERK and ATF6, which all play a role in re-establishing basal levels of unfolded proteins following ER stress (details below). Another mechanism that contributes to alleviate the ER stress is the formation of disulfide bonds. Disulfide bond formation has been demonstrated to couple protein folding and stability (Feige and Hendershot, 2011, Qin et al., 2015), then decreasing the number or structure of folding protein or misfold proteins. Protein disulfide isomerase (PDI) plays a vital role in the formation of disulfide bonds in the ER, ER oxidoreductin 1 (ERO1) could transfer electron from PDI to molecular oxygen through the process FAD reaction, which cause the overloaded of folding system and generation of hydrogen peroxide (Moilanen and Ruddock, 2020, Shergalis et al., 2020). Protein overload and ROS production cause the ER stress, that might trigger cell death (Redza-Dutordoir and Averill-Bates, 2016). In

response to increased ROS levels in ER lumen, UPR signaling pathway and antioxidant enzymes (such as GSH/GSSG system) could be activated and drive adaptive changes that converts hydrogen peroxide into water and oxygen.

The occurrence of ER stress in the sarcomere can be induced by increased energy expenditure i.e. exercise: The positive effect of exercise on ER stress depends on the intensity and duration of exercise, which has been shown to mitigate the relevant ER stress proteins. Scientific studies show that HIT or extreme exercise can induce ER stress and activate the UPR pathways (Hart et al., 2019). On the other hand, regular moderate-intensity exercise can attenuate the responses of genes and proteins related to ER stress. ER stress can also occur with reduced energy generation capability i.e. disease muscular dystrophy of which the downstream consequences of dystrophin deficiency could trigger of ER stress (Bohnert et al., 2018, Shin et al., 2013).

We mentioned that ER plays an essential role in calcium homeostasis, disturbance in calcium concentration decreases the protein folding capacity, causing accumulated unfolded proteins resulting in ER stress. Disruption of Ca^{2+} homeostasis is an indicator of DMD pathogenesis. In dystrophin-deficient muscles, muscle contraction is impaired with an alter Ca^{2+} handling. Potential mechanisms of this contribution could be a result of the sustained increase in cytosolic Ca^{2+} in dystrophic muscle exerting a Ca^{2+} overload on the mitochondrial transition pore, which eventuates in apoptosis (Crompton, 1999). Therefore, modulation of calcium concentration might reduce the muscle damage caused in dystrophin-deficient muscle. Exercise training has the potential to be beneficial to endothelial dysfunction and maintain cardiovascular homeostasis via increased in antioxidative response and a reduction of inflammatory cytokines expression (Xia et al., 2017, Davies, 2018).

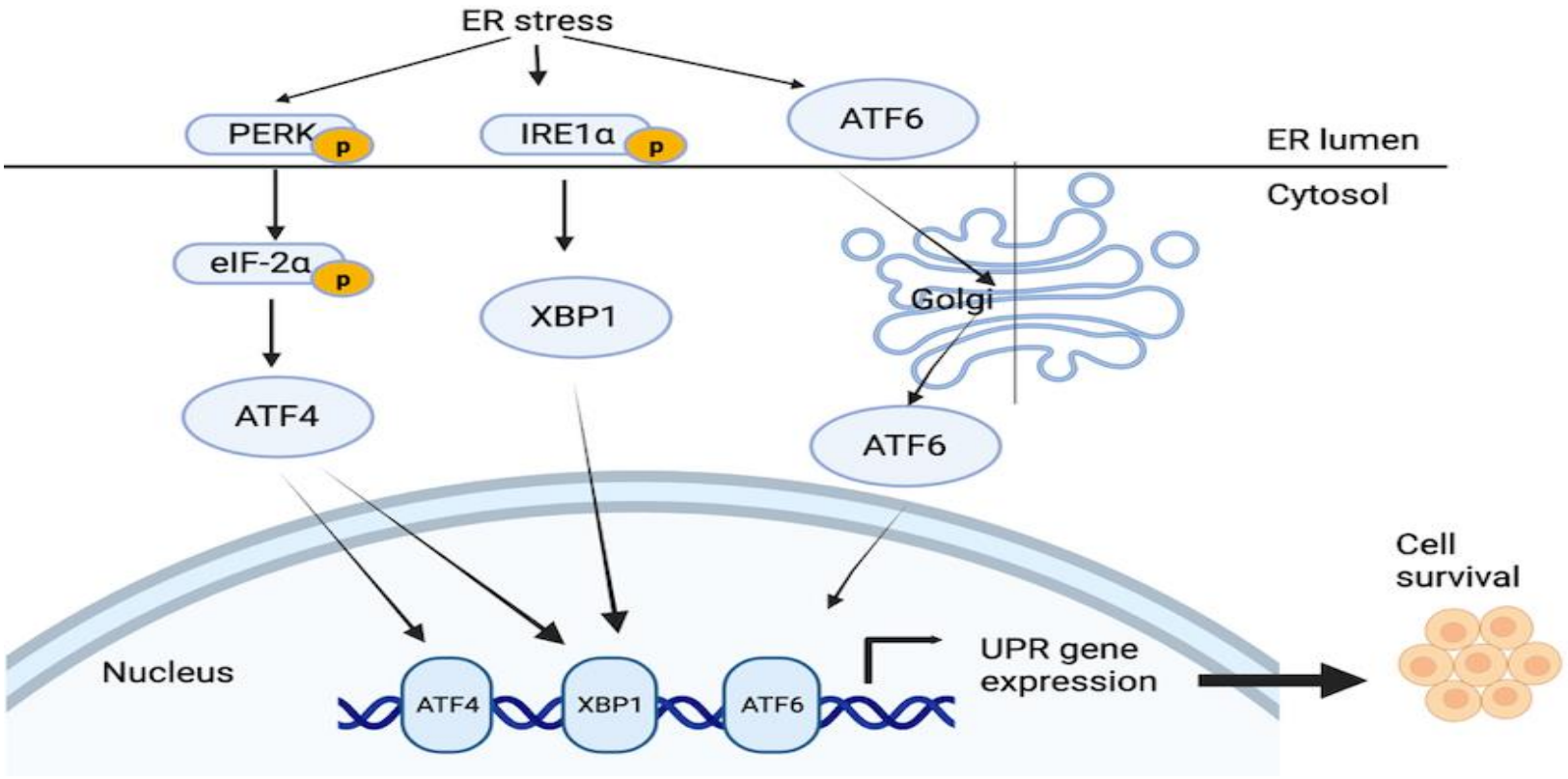


Figure 1-8 Activation of UPR activators proteins in response to ER stress. There exists three UPR activators: PERK, IRE1α and ATF6. These three ER sensors could contribute to improve the folding capacity of ER and alleviate the misfolded or unfolded proteins, which is important to maintain the ER protein homeostasis. ER: endoplasmic reticulum. ATF4: activating transcription factor 4, ATF6: activating transcription factor 6, CHOP: C/EBP homologous protein, eIF-2α: eukaryotic translation initiation factor 2α, IRE1α: inositol requiring enzyme 1α, UPR: unfolded protein response, XBP1: X-box binding protein 1.

1.3.3.1. ATF6 *and* ATF4

ATF6 and ATF4 are master regulators of the cellular stress response that promotes adaptation in cells. ATF6 is an UPR sensor, which is functioned to regulate the transcription expression and emerging as a major regulator of organogenesis and tissue homeostasis (Hillary and FitzGerald, 2018). ATF4 is frequently upregulated in muscle cells and controls the expression of a wide range of adaptive genes that help muscle cells get through periods of stress.

Exercise reduces phosphorylation of the ER stress sensors and inhibits or decreases ER stress-associated apoptosis (Kim et al., 2014). Protein Kinase RNA-like ER Kinase (PERK, a UPR sensory protein) can be activated by disruptions in protein folding. The ensuing PERK phosphorylation of the α subunit of eIF2 enhances cell survival and represses global protein synthesis, thereby reducing influx of nascent polypeptides into the overloaded ER (Dey et al., 2010). Eukaryotic initiation factor 2α (eIF2α) induces ATF4 translational expression in response to a range of environmental and physiological stresses including those afflicting the ER. The ATF4-directed regulatory scheme has been referred to as the integrated stress response (Figure 1.7) (Dey et al., 2010).

Unlike the PERK/eIF-2α/ATF4 pathway, ATF6 is proteolytically cleaved in golgi under ER stress, which allows the N-terminal cytosolic parts to be transported into the nucleus and act as a transcription factor to upregulation of target genes, such as CCAAT-enhancer-binding protein homologous protein (CHOP) (Ye et al., 2000).

ATF4 serve to enhance transcriptional expression of genes involved in amino acid metabolism and resistance to oxidative stress (Roybal et al., 2005) including transcription of transcription factor-CHOP, ATF4 and CHOP independently or in combination are believed to coordinate key facets of the UPR transcriptional and translational control directed by PERK. ATF4 and CHOP also function in a feedforward loop to induce expression of a related transcription factor, which is a central network for determining cell fate in response to ER stress. ATF6 has been demonstrated to upregulate catalase gene expression and reduce ROS concentration to protect cardiac myocyte against oxidative stress (Jin et al., 2017).

ATF4 has been proposed as a potential mediator of muscle weakness and atrophy, PERK and eIF2α are increased in dystrophic muscle of *mdx* mice (Moorwood and Barton, 2014, Hulmi et al., 2016). They report that ATF4 has a strongly positive correlations with PERK and eIF2α levels, and that ATF4 could be a positive regulator in *mdx* mice. Another study investigated that ER stress inhibitors restore ER-mitochondria links, mitochondrial calcium uptake, and improve contractility of the diaphragm in mdx mice (Pauly et al., 2017), further implying that heightened ER stress and UPR pathways contribute to the dystrophic phenotype.

1.3.3.2. Sestrins

Sestrins are transcriptionally induced by increased ROS during HIT, and its up-regulation is vital in attenuating muscle damage and maintaining cellular viability and cellular functions (Ro et al., 2016). Shin and colleagues indicated that Nrf2 activators specifically upregulate the expression of Sestrins in a time- and dose- dependent manner and that Nrf2–ARE pathway activation seemed to be essential for Sestrins induction (Shin et al., 2012). In turn, Sestrins might act as a positive modulator of Nrf2 signaling, which shows a beneficial effect in exercise induced stress. Interestingly, it has been reported that Sestrin2 is closely related to both Keap1 and the autophagy adapter p62/SQSTM1 (Bae et al., 2013). They also revealed that Sestrin2 protects against oxidative damage by enhancing Keap1 degradation mechanistically via p62-mediated autophagic degradation and consequent Nrf2 activation. As mentioned above, the dystrophin-deficient muscle results in ER stress, ER stress can induce Sestrins as a protective mechanism (Ding et al., 2016). It is reported that Sestrins are central nutrient status sensors and could alleviate ER stress by inhibiting mammalian target of rapamycin (mTORC) through AMPK activation (Budanov and Karin, 2008). Sestrins can eliminate ROS by inducing antioxidant enzymes and activating Nrf2 through p62-dependent degradation of Keap1 pathways (Bae et al., 2013).

Muscle dystrophy results in muscle atrophy, which involves the loss of muscle mass strength and function (Schiaffino and Mammucari, 2011, Egerman and Glass, 2014). Overexpression of sestrin1 can maintain the muscle mass and strength in the atrophic condition (Segalés et al., 2020).

In summary, ER stress induced by exercise or disease can induce Sestrin expression which potentially act as a defence regulator against stress to protect cells through complex signaling pathway and its antioxidant functions.

1.3.4. Hypoxic stress

Hypoxia is a condition where the body is starved of sufficient O_2 supply that ultimately impact at the tissue level. O_2 is an essential substrate in cellular metabolism and bioenergetics required for energy production in cells (Nakazawa et al., 2016). However, tissues confront periods of insufficient O_2 availability during specific condition such as exercise or mitochondrial disease. This can occur in situations of extreme and rapid metabolic demand and blood flow limitations such as intense exercise or limitations to O_2 supply or metabolic capacity for energy production. O_2 deprivation is also paradoxically linked to the improper accumulation of free radicals, which cause additional stress on relevant proteins in the cells (Blokhina et al., 2003). For example, H_2O_2 is produced as a consequence of the disruption of the mitochondrial electron transport chain, caused by low oxygen levels (Lee et al., 2011). In hypoxia, complex I (the first enzyme complex of the respiratory chain) deactivate, which induces the superoxide burst in the cell (Saldana-Caboverde et al., 2020). The production of superoxide and H_2O_2 influence the biological processes of cells and tissues. During hypoxic conditions, cells activate adaptive responses to stimulate changes of cellular capacity for improvements of matching O_2 supply with metabolic and redox demands (Majmundar et al., 2010). These metabolic parameters could activate the related hypoxia-induced signaling pathway, therefore contributing to alleviate the hypoxia stress via the regulation of the levels of proteins involved in these pathways.

Increasing O_2 demand can lead to more severe localised muscle tissue hypoxia, which results in impaired muscle function (Dipla et al., 2017), mitochondrial function (Kueh et al., 2013, Mori et al., 2021), molecular alteration (Byrne et al., 2020, Serano et al., 2022), and apoptosis (Brunelle and Chandel, 2002). Under normal condition, sufficient O_2 is delivered into muscle cell, which contributes to energy generation and maintaining muscle functions (Pittman, 2000). Under hypoxic condition, less oxygen is available to supply the increased oxygen demands of muscles and maintaining muscle

functions. Furthermore, oxygen is not just for cell survival, but also is important for redox-mediated adaptation. i.e. NADH/NAD+ ratio plays an important to participant in the process of glycolytic and oxidative phosphorylation (Yang and Sauve, 2016, Xie et al., 2020, Stein and Imai, 2012). During the initial phase of hypoxia, NAD pool decreases in muscle cells, a direct consequence of this process is the accumulation of NADH because of oxygen limitation. Once ratio of NADH of NAD+ is decreased, it causes the inhibition of enzymes (such as pyruvate dehydrogenase), this process inhibits the acetyl-CoA entering the TCA cycle and pyruvate oxidation in the cytosol (Fisher-Wellman et al., 2015, Klimova et al., 2019), which might trigger molecular signaling pathway and drive the adaptive response to regulate the NADH/ NAD^+ ratio. Accumulated NADH levels lead to the increase of electron leakage and ROS production (Xiao et al., 2018, Korge et al., 2016), which can activate the Nrf2 signaling pathway and antioxidants defence system to response. It is reported that impaired mitochondrial oxidative phosphorylation is a characteristic feature of dystrophin-deficient muscle (Percival et al., 2013). Oxidative phosphorylation is important for generating energy through the formation of ATP. Molecular oxygen acts as the final electron receptor during the process of oxidative phosphorylation (Slater, 1977). It is reported that Hypoxia-mediated signaling pathway could be activated to alleviate the stress in the dystrophin-deficient muscle (Nguyen et al., 2021).

1.3.4.1. HIF1

Under normoxic conditions, HIF1α is constantly expressed but quickly degraded by a complicated process. Initially, hydroxylated by the specific enzyme call Prolyl Hydeoxylase Domain protein (PHD), then hydroxylated HIF1α could be recognised by the Von Hippel-Lindau (VHL), which can activate the HIF1α ubiquitination leading to the HIF1α degradation by the proteasome (Strowitzki et al., 2019). This process is oxygen-dependent involves three co-factors (Kuiper and Vissers, 2014), because the hydroxyl cluster is derived from the oxygen molecule (Figure 1.8).

Reduced oxygen levels in skeletal muscle during exercise are a consequence of increased oxygen consumption in the face of limited supply. HIF1α is the key regulator in the metabolic adaption

to exercise. HIF1α protein level is increased in skeletal muscle after exposure to low oxygen for one hour and bout of acute exercise can activate HIF1α in skeletal muscle (Stroka et al., 2001). HIF1α is the major transcription factor regulating the expression of genes related to anaerobic metabolism in the adaption to HIT (Evans et al., 2010, Lee et al., 2021a). HIF1α was elevated 3 hr after an acute HIT bout in gastrocnemius muscle. They also found that long-term HIT increased the basal levels of HIF1α as well as the glycolytic capacity in gastrocnemius muscle. In healthy muscle, hypoxia condition and HIF1 activation are known to inhibit oxygen consumption (Lindholm and Rundqvist, 2016), and influence muscle metabolism (Nguyen et al., 2021). HIF1α is also a regulator of myogenesis, including mitogenesis and angiogenesis (Egan and Zierath, 2013, Elashry et al., 2022).

The impact of HIF1α pathway in dystrophin-deficient muscle remains to be demonstrated. HIF1α activation might be influenced by multifactorial pathological mechanism in skeletal muscle in dystrophin-deficient skeletal muscle. Hypoxic stress in tissues is characteristic of many pathological settings, and the HIF1α is a key factor in hypoxia and directs critical adaptations to enable cells, tissues, and organisms to survive and thrive in these conditions (Mazumdar et al., 2010). Several studies revealed that HIF1α could inhibit the metabolic diseases.

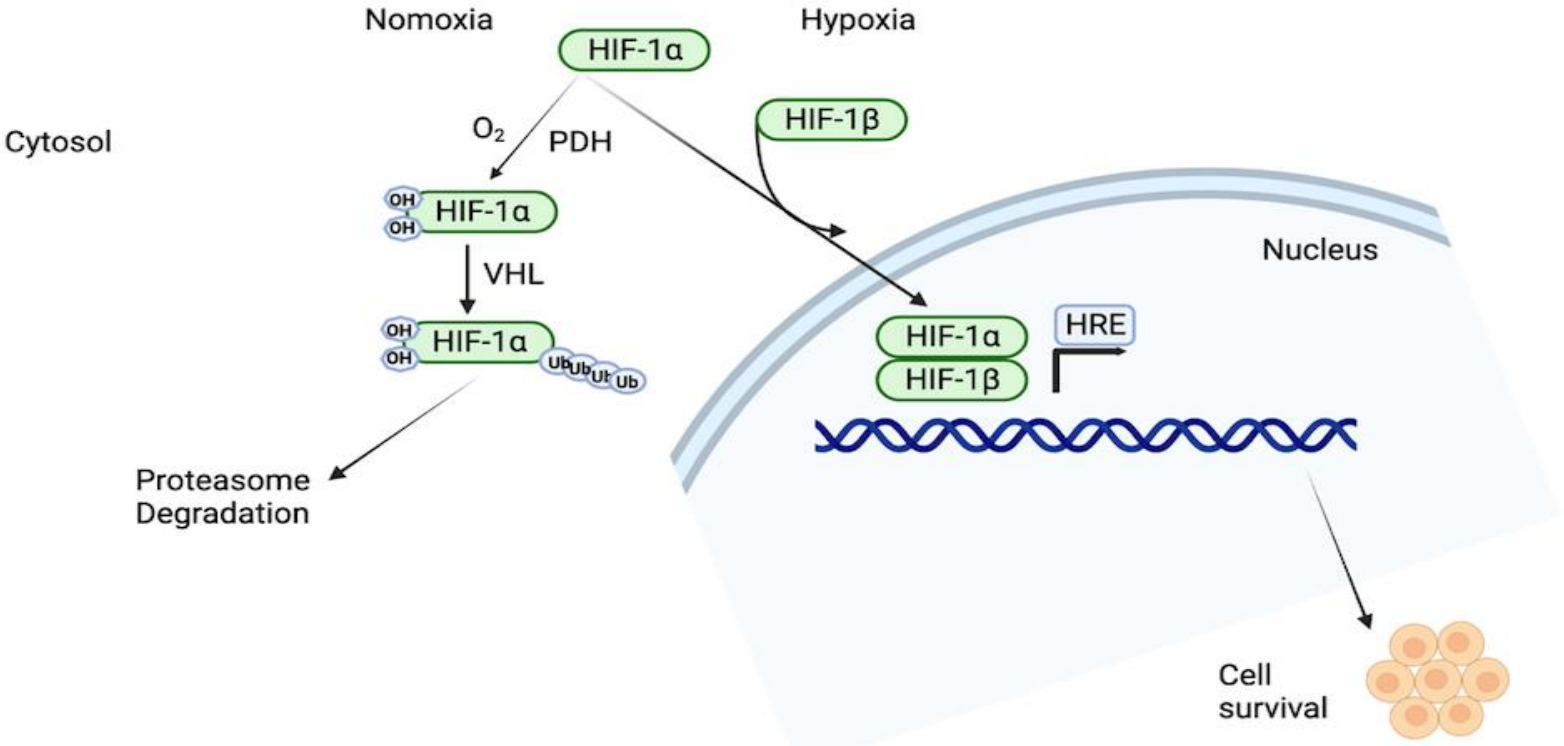


Figure 1-1 Representation of HIF1 signaling pathway in normoxia and hypoxia conditions. In normal conditions, HIF1α is hydroxylated by PHD, resulting in the binding with VHL. This process leads to the polyubiquitination and proteasomal degradation of HIF1α. In hypoxia condition, oxygen deprivation and increasing ROS could inhibit PHD activity, then preventing HIF1α degradation, accumulation of HIF1α translocated to nucleus, where it binds HIF1β and hypoxia response elements (HRE), then promoting the transcription of HIF1target to protect cell (cited from (Manuelli et al., 2021)).

1.3.4.2. Sirt1

Sirt1 is highly expressed in many mammalian tissues including skeletal muscle. It is a sensor and regulator for the maintenance of energy and redox balance. During HIT, sufficient production of ATP is a big metabolic challenge for skeletal muscle. HIT leads to the reduction of ATP levels, which induce increased levels of NAD^+ from NADH and ratio of NAD^+/NADH, which further induces Sirt1 activity (Elashry et al., 2022). Sirt1 is not only vital to maintain the metabolism of healthy muscle (Laemmle et al., 2012), but also positively resolved oxidative stress. Houtkooper demonstrated that Sirt1 could modulate the proteins functions by NAD+-dependent post-translational modification (Houtkooper et al., 2012), thereby representing a metabolic sensor and activation of Sirt1 could be used to treated specific mitochondrial disease.

In addition, Sirt1 activity is also involved in the treatment of dystrophin-deficient muscle (Kuno and Horio, 2016). Chalkiadaki and colleagues demonstrated that overexpression of Sirt1 in dystrophic muscle, caused by vigorous regeneration following muscle damage and necrosis, reverses the phenotype of *mdx* mice, as determined by histology, creatine kinase release into the blood, and endurance in treadmill exercise (Chalkiadaki et al., 2014). In addition, Sirt1 overexpression also caused elevated contents of utrophin, a functional analogue of dystrophin. Taken together, Sirt1 activation might be the new approach for targeting some specific muscle diseases.

1.5. Targeting purine homeostasis to overt metabolic stress signaling

1.5.1. Background of Ribose

D-Ribose, a pentose monosaccharide used by all the cells of the body, is an essential compound in energy metabolism and provides the structural backbone of genetic material, DNA and RNA, certain vitamins and other important cellular compounds. Ribose can be synthesised from intermediates of the glycolytic pathway via the pentose phosphate pathway, but the process is slow and different organs produce different amounts. Ribose can be sourced naturally from foods (plants and

animals), like mushroom, beef and poultry, milk and eggs. People take extra ribose for several reasons, including improving cardiac myopathies and physical performance in diseased and healthy states, respectively. Ribose supplementation could not only increase energy recovery during the exercise training sessions (Kreider et al., 2003), but improve recovery of muscle damage induced by exercised and/or disease models (Peveler et al., 2006, Cao et al., 2020).

1.5.2. Recovery of energy store

Following exercise, rodents recover adenine nucleotides in approximately half a day, believed to be due to increased PRPP availability (Tullson and Terjung, 1991). The role of PRPP is proposed to be of critical importance to the *(de-novo)* synthesis process (Zoref-Shani et al., 1982, Tullson and Terjung, 1991, Brosh et al., 1976, Becker et al., 1986). PRPP is a product of ribose, and increased availability enhances nucleotide resynthesis (Gross et al., 1983, Hellsten et al., 2004), increasing intramuscular ATP stores potentially improves subsequent exercise performance and/or recovery (Boobis et al., 1983, Jones et al., 1985, Wilson et al., 2013). Although the mechanisms underlying fatigue are still widely debated, ATP availability is purported as a potential causal effect (Hellsten et al., 2004, Wilson et al., 2013). A number of animal studies have investigated the effect of ribose perfusion in skeletal and cardiac muscle (Tullson and Terjung, 1991, Van Gammeren et al., 2002, Seifert et al., 2017, Cao et al., 2020). One study found that hindquarter perfusion of 5 mM ribose in rats enhanced PRPP and subsequent ATP synthesis (Tullson and Terjung, 1991) with a 3-4 fold rise in the *de-novo* synthesis rates. However, it should be noted that although effective in ATP recovery, ribose concentrations were supraphysiological. Oral supplementation has produced mixed effects (Op't Eijnde et al., 2001, Berardi and Ziegenfuss, 2003, Hellsten et al., 2004). Op't Eijnde examined the effects of oral ribose supplementation in repeated maximal exercise in 20 healthy male participants. Ribose or placebo was supplemented for 4 days prior, during and for 4 hrs following intermittent knee extension. Muscular ATP was significantly depleted in both groups immediately following exercise, and no significant difference in ATP repletion or performance benefit was noted in or between groups at 24 hrs post exercise (Op't Eijnde et al., 2001). The significant depletion of muscular ATP and total

adenine nucleotide pool (TAN) is well represented in repeated high intensity exercise training (Stathis et al., 1994, Hellsten et al., 2004), with ATP and TAN concentrations still below baseline levels for several days post training (Stathis, 2006, Hellsten et al., 2004). Furthermore, studies that have previously examined the effects of ribose supplementation on adenine nucleotide resynthesis did not accurately account for the slow rate of purine synthesis (72 hrs) (Hellsten et al., 2004, Tullson et al., 1988) and as such saw little to no significant effect on exercise performance or ATP replenishment (Berardi and Ziegenfuss, 2003, Kreider et al., 2003, Kerksick et al., 2005, Wagner et al., 1991). Currently, a misconception exists between the acute ATP hydrolysis and salvage vs the chronic ATP degradation and *de-novo* synthesis, which ribose supplementation impacts (Op't Eijnde et al., 2001, Berardi and Ziegenfuss, 2003). Furthermore, an acute exercise challenge without prior training could further limit the effects of ribose in ATP recovery, as the resynthesis of IMP from Hx is limited by the activity of HGPRT. HGPRT is an enzyme that catalyses the reaction between Hx and PRPP to form IMP, and at rest is estimated to be responsible for 75% recovery of intramuscular Hx production (Edwards et al., 1979), thus reducing irreversible efflux into the bloodstream and subsequent impact of the slower and more costly purine biosynthesis pathway (Edwards et al., 1979). Sprint training has been shown to elevate HGPRT activity (Hellsten-Westing et al., 1993, Ziegenfuss et al., 2002, Stathis, 2006) and subsequently reduce purine efflux from the muscle, therefore if you can increase not only the availability of PRPP but the catalyst responsible for greater flux (HGPRT), significant ATP repletion that improves recovery between training sessions and subsequent exercise performance, was not accounted for in a number of studies (Berardi and Ziegenfuss, 2003, Kerksick et al., 2005).

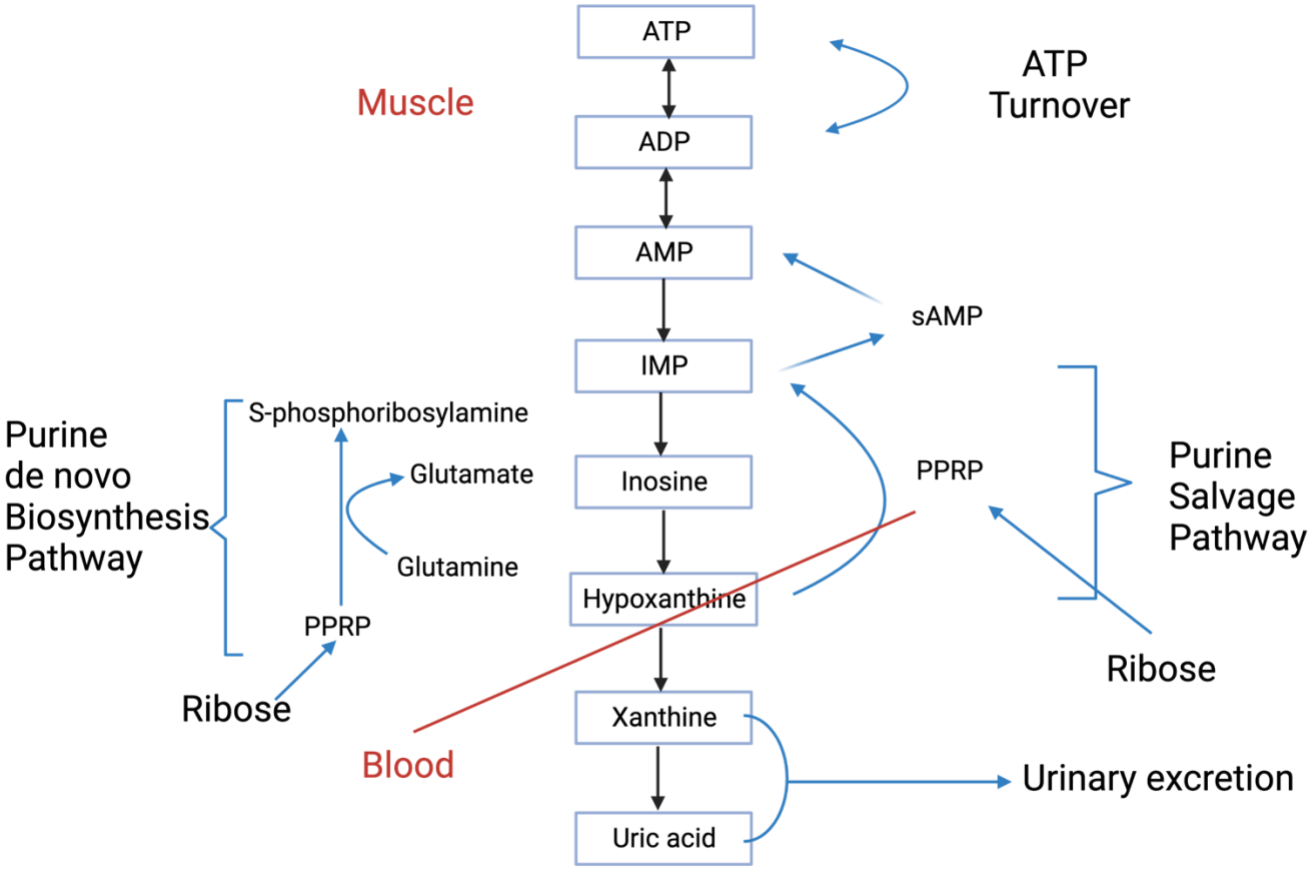


Figure 1-9 Purine metabolism pathways. It displays the degradation, recycling and urinary excretion of purines, when still in the muscle purines can be salvaged by the purine salvage pathway. However, once they enter the blood stream they can no longer be recycled and are thus excreted within the urine. The loss of metabolites induces a metabolic cost and replacement of lost purines is done via the energy intensive, de-novo synthesis pathway, which can be replenished through ribose supplementation via PRPP.

The potential “energy boosting” role of ribose has been tested for the improvement of some diseases, such as coronary heart disease (CHD), heart failure, chronic fatigue syndrome and fibromyalgia (Perlmutter et al., 1991, Teitelbaum et al., 2006). Teitelbaum have reported that approximately 66% of patients (with chronic fatigue syndrome or flbromyalgia) experienced significant improvement while on ribose, with an average increase in energy perceived on the visual analog scale of 45% and an average improvement in overall well-being of 30%. Moreover, d-ribose also reduces clinical symptoms in patients suffering from fibromyalgia and chronic fatigue syndrome (Teitelbaum et al., 2006). Furthermore, ribose supplementation produced an increased rate of adenine nucleotide synthesis following ischemia (Zimmer and Gerlach, 1978). Another study from Zimmer revealed that infusion of supplemental ribose during reperfusion restored ATP pools to normal within 12 hrs, whereas 72 hrs were required without d-ribose supplementation (Zimmer and Ibel, 1984). Shecterle and his colleague found that d-ribose supplementation was effective in regenerating depressed ATP levels and improved cardiac function following myocardial ischemia (Shecterle and St Cyr, 2012). D-ribose perfused working rat hearts subjected to 15 min of ischemia demonstrated improved recovery of myocardial ATP levels and in functional capacity during recovery following ischemia (Pasque et al., 1982). This indicates the potential for improved myocardial energy levels and function in the area of ischemic cardiovascular diseases.

There is limited research on the effect of ribose supplementation on recovery of energy metabolism and muscle function in myopathies both in humans and animal models. Coley have treated normal and myositis mice with 4 mg/kg ribose daily and found treatment with ribose had no significantly effects on muscle function in mice (Coley et al., 2013). However, ribose supplementation could reduce muscle soreness, which is a symptom of exercise induced muscle damage, improve recovery time of muscle damage and inhibit lipid oxidation (Cao et al., 2020). The activity of these pathways (*de novo* purine synthesis and salvage pathway) is limited by the availability of ribose and it has been demonstrated that ribose supplementation can improve the resynthesis of ATP in skeletal muscle in

rat hindlimb perfusion (Tullson and Terjung, 1991) and in the recovering muscle of sprint trained humans (Hellsten et al., 2004).

ATP depletion is a hallmark response of metabolic remodeling in dystrophic- deficiency muscle (Percival et al., 2013) and may be a clue to potential adaptations that may occur in more acute and intermittent energy-consuming environments in skeletal muscle. During intense exercise, ATP hydrolysis is greater and exceeds the re-synthesis rate and consequently reduces the adenine nucleotide pool, which potentially limits energy availability for subsequent muscle contraction and exercise performance. Remodeling in dystrophic-deficiency muscle and may be a clue to potential adaptations that may occur in more acute and intermittent energy-consuming environment in skeletal muscle. Imbalance between ATP turnover both occur in cardiac model and high-intensity exercise condition with skeletal modes for different reasons with the same net consequence. Therefore, investigating different models of chronic metabolic stress and the potential of concurrent supplementation of ribose may produce accelerated recovery of ATP, improve metabolic and molecular adaptations and stimulate better performance outcomes in health and disease.

1.6. Summary

Adaptations to stress conditions induced by exercise or metabolic disease are modulated by complex signaling pathways. Relevant markers involved in these signaling pathways could identify processes responding to the stress and protect the muscle cell. Superimposing a challenge of exercise and metabolic disease to investigate the subsequent response to metabolic stress on these signaling pathways has not been reported. This thesis investigates the stress associated with chronic aerobic exercise training on skeletal muscle stress signaling in healthy and metabolically-stressed (*mdx*) mice and test whether ribose supplementation can alleviate stress and protect against metabolic stress signaling.

Chapter 2
Aims and hypotheses

The principal aim of this thesis is to investigate the molecular stress signaling pathways that are active in skeletal muscle during metabolic challenge associated with exercise and disease. We aimed to: (1) compare exercise training stress alone (in mice and humans) and superimposed with a muscle disease state (metabolically-stressed *mdx* mice); and (2) investigate whether nutritional supplementation of ribose, a sugar moiety fundamental to the *de novo* synthesis and recycling/salvage of purines (e.g., ATP) can protect against exercise-induces stress in skeletal muscle. Exercise training could lead to the purine loss from skeletal muscle alongside with ROS production, subsequently stimulating the signaling pathway that might limit the purine degradation. For example, transient 30-40% reduction in the skeletal muscle ATP level after 30 s sprint exercise has been demonstrated from previous study (Stathis et al., 1994, Hellsten et al., 1998). Numerous studies have indicated that ribose could improve the process of ATP production via stimulating *de novo* synthesis or salvage pathway (Tullson and Terjung, 1991, Salerno et al., 1999, Hellsten et al., 2004). The replenished ATP in the body might contribute to protecting against exercised-induce stress. Therefore, the overarching hypothesis for the thesis is that exercise training stress and ribose supplementation will provoke better purine (ATP) recovery via enhancing the capacity of *de novo* synthesis or salvage signaling pathway, improve exercise performance/ muscle function in mice via activation of stress signaling pathways in mice and humans.

The specific aims of the thesis are:

Study 1: The aim of study 1 (Chapter 4) was to determine the molecular stress signaling that occurs in response to HIT in mice (tissue samples collected from a previously published study; Wilson et al., 2018). We hypothesised that HIT would alter the expression of Nrf2 mediated cytoprotective enzymes, upregulate of ATF4 and Sestrins of ER stress markers, and activate the HIF-1 mediated hypoxia stress signaling in female and male mice, these adaptations might protect the body against the stress induced by HIT.

Study 2: The primary aim of study 2 (Chapter 5) was to determine the involvement of metabolic stress-induced signaling on skeletal muscle adaptations to moderate intensity exercise training, and the ability of ribose supplementation to circumvent this stress signaling (oxidative stress markers: Nrf2, Keap1, phosphorylated p62, total p62, HO1, NQO1, catalase, ER instability markers : ATF6, ATF4, Sestrin1 and Sestrin2 and hypoxia stress markers HIF1α, HIF-1β, PGC-1α and Sirt1) in healthy wild-type (WT) and metabolically-stressed mdx mice. It is reported that moderate intensity exercise training (12m/min for 30 min, twice a week) could worsen exercise performance/muscle function in metabolically-stressed mdx mice (De Luca et al., 2003). Ribose, a simple sugar, has been reported to elevate the rate of ATP resynthesis via improving the capacity of de novo synthesis and purine salvage pathway, which is important for optimising exercise training. We expected that the ribose supplementation could improve the exercise performance and muscle function in healthy mice, the predominant and hypothesised combination of ribose supplementation and exercise training stress could increase the capacity of purine salvage and the rate of ATP synthesis, which would protect against exercise-induced stress in skeletal muscle resulting in improved exercise performance/muscle function in metabolically-stress *mdx* mice.

Study 3: The aim of study 3 (Chapter 6) was to determine the role of stress signaling in the adaptations induced by SIT training on exercise performance in humans. We hypothesised interaction of exercise training and ribose supplementation could enhance the exercise training performance, increase the rate of ATP synthesis, drive adaptation and protect against the exercise induce stress through alteration the expression of Nrf2 mediated cytoprotective enzymes, upregulation of ATF4 and Sestrins of ER stress markers, and activation the HIF-1 mediated hypoxia stress signaling in humans compared to untrained people.

Chapter 3
Methods

This chapter describes the general methods used in studies in the thesis. Western blot method were shared in all three studies (Chapter 4, 5 and 6). Methods of muscle metabolites HPLC were used in both Chapter 5 and Chapter 6.

3.1. Western blot

To assess the biomarkers of different signaling pathways (oxidative stress, ER stress and hypoxia stress) we were exploring, proteins (Table 3.1) involved in these signaling pathways were analysed through semi-quantitative western blot analysis. Gastrocnemius muscles were utilised for this analysis.

Table 3-1 List of antibodies for western blot analyses

Antibody name	Molecular weight (KDa, Observed)	Host	Dilution for western blot	Blot	Supplier	Catalogue no.
Nrf2	97-100	Rabbit	1:1000	PVDF	Cell signaling	12721S
Keap1	60-64	Rabbit	1:1000	PVDF	Cell signaling	8047S
Phosphorylated p62	62	Rabbit	1:1000	PVDF	Cell signaling	16177S
p62	62	Rabbit	1:1000	PVDF	Cell signaling	5114S
HO1	28	Rabbit	1:1000	PVDF	Cell signaling	26416S
NQO1	29	Rabbit	1:1000	PVDF	Cell signaling	62262S
SOD1	20	Rabbit	1:1000	PVDF	Cell signaling	2770S
Catalase	60	Rabbit	1:1000	PVDF	Cell signaling	12980S
ATF6	90-100	Rabbit	1:1000	PVDF	Cell signaling	65880T
ATF4	45-50	Rabbit	1:1000	PVDF	Protein-Tech	1835-1-AP
Sestrin1	66-68	Rabbit	1:1000	PVDF	Protein-Tech	21668-1-AP
Sestrin2	54-60	Rabbit	1:1000	PVDF	Protein-Tech	10795-1-AP
HIF-1α	120	Rabbit	1:1000	PVDF	Cell signaling	14179S
HIF-1β	87	Rabbit	1:1000	PVDF	Cell signaling	5537S

Sirt1	120	Rabbit	1:500	PVDF	Cell signaling	9475S
PGC1-α	120-130	Rabbit	1:1000	PVDF	Cell signaling	2178S
AMPKα	62	Rabbit	1:1000	PVDF	Cell signaling	2532S
Phosphorylated AMPK	62	Rabbit	1:1000	PVDF	Cell signaling	2535S

Frozen muscles were homogenized with an OMNI tissue Homogenizer (OMNI International, Kennesaw, Georgia, USA) for 13 seconds in ice-cold western blot buffer, which contains 600mM b-Glycerophosphate, 40mM Tris, pH 7.5, 1mM ethylenediaminetetraacetic acid (EDTA), 5mM etheylen glycol-bis (β-aminoethyl ether)-N, N, N', N'-tetraacetic acid (EGTA), 0.5% Triton X-100, 1M NaF, 1M Na_3VO_4 10 mg/ml leupeptin, and 0.2 M phenylmethylsulfonyl fluoride (PMSF). Crude homogenates were used for further analysis.

Next, total protein concentration were determined in crude homogenates using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equivalent amounts of protein (30µg) from each homogenate were dissolved in the Laemmli buffer which includes western immunoprecipitation kinase (WIK) buffer and 2x sodium dodecyl sulfate (SDS) buffer and 50 mM dithiothreitol (DTT), heated for 5 min at 95°C and loaded to electrophoretic separation on SDS-acrylamide gels containing 1.5mm resolving gels (30% Acrylamide; 4x Tris/SDS at pH 8.8; Milli-Q water; fresh 10% Ammonium persulphate (APS) and Thermo Scientific Pierce Tetramethylethylenediamine (TEMED)) and1.5mm stacking gels (30% Acrylamide; 4x Tris/SDS at pH 6.8; Milli-Q water; fresh 10% APS and TEMED) filled with running buffer containing Tris-base, Glycine, SDS and Milli-Q water. Thereafter, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane placed into transferring buffer solutions containing Tris-base, Glycine, Methanol and Milli-Q water, then blocked with 5% or 3% powdered milk in TBST, which contains 3M NaCl, 1M Tris at pH 7.5. Tween 20 and Milli-Q water) for 1 hr and allowed to incubate overnight at 4°C with primary antibody (as per Table 2.1 above) dissolved into 1% bovine serum albumin (BSA)/TBST. After overnight incubation, the membranes were washed for 30 min in TBST and then probed with the appropriate peroxidase-conjugated secondary antibody for 1 hr at room temperature. Following 30 min of washing in TBST, the blots were developed with a DARQ CCD camera mounted to a Fusion FX imaging system (Vilber Lourmat, Germany) using ECL Prime reagent containing 50/50 (vol/vol) two different Substrates: Luminol/enhancer solution and Peroxide solution (Amersham, Piscataway, NJ, USA). Once the image was captured, the membranes were stained with Coomassie Blue containing Methanol, Acetic Acid, Brilliant Blue and Milli-Q water to verify equal loading amount of total protein in all lanes. Densitometric measurements were carried

out using Fusion CAPT Advance software (Vilber Lourmat, Germany). For all data, the protein of interest was normalised to the signal intensity of Coomassie Blue.

3.2. Muscle metabolites

ATP, PCr, Cr and lactate levels were quantitated using a method adapted from the Lowry's Laboratory (Lowry, 2012) For all metabolites, a NADH standard curve (concentration at 50, 100, 200 and 400 mM) was established by using a UV-visible spectrophotometer at 340 nm to compare the change in fluorescence of the internal standards (metabolites standard) against the standard curve. Analyses were performed on a 96 well plates and measured using a XMrak spectrophotometer (Serial number: 10048, Bio-Rad Laboratories, Hercules, CA, USA).

3.2.1. Freeze drying

The muscle which is to be analysed is stored in liquid nitrogen in labelled cryules with punctured lids. Samples were freeze dried under vacuum (Edwards Modulyo, Edwards High Vacuum, Britain, England) for approximately 48 hrs before rapidly transfer the dried muscle samples into a desiccator at room temperature.

3.2.2. Weighing freezed dried muscle

Dried samples were weighed to determine the water content and then physically crushed ready for extraction and weighed into aliquots.

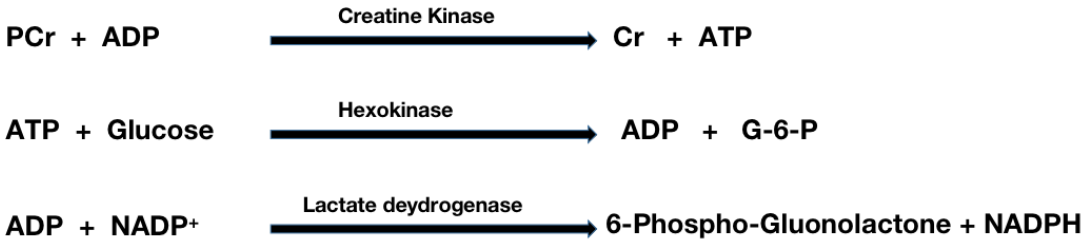
3.2.3. Metabolite extraction

250 µL of 0.5 M perchloric acid (PCA) and 1 mM EDTA were then added after 2 mg (± 0.01 mg) of the powdered sample was weighed into an eppendorf tube. Prior to being spun for 2 min at 28,000 rpm at 0°C in a centrifuge, tubes were vortexed and allowed to sit on ice for 10 min. A new eppendorf tube was filled with 200 µl of supernatant and 50 µL of 2.1 M $KHCO_3$, and the tube was

centrifuged again at the same settings after 5 min on ice. Supernatant was gathered and kept in cryule tubes at -80°C until needed.

3.2.4. ATP-PCr

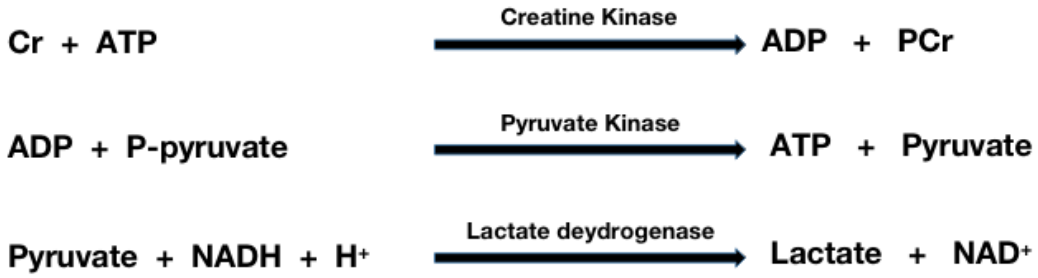
ATP and PCr content was determined in samples using a three-step enzymatic process (diagram cited from Cara Timpani (Timpani, 2017)):



After the standard curve was created, 10 µL of the reagent cocktail (50 mM Tris Buffer at pH 8.1; 1 mM $MgCl_2$; 0,5 mM DTT; 100 mM glucose; 50 mM NADP), ATP, PCr, and NADH standards, as well as the samples, were pipetted into 96 well plates. The first reading reflected the NADPH content by the presence of G-6-P dehydrogenase in the reagent mixture. Following the addition of diluted hexokinase, which yields G-6-P, the substrate for the third reaction, and a 30 min incubation at room temperature, the second reading was taken. Readings one and two were subtracted to reveal the variation in NADPH concentration, which coincides with ATP concentration. The third reading was taken after adding creatine kinase (CK) and ADP to the well and giving it a 60 min incubation at room temperature. This reaction generates NADPH, which is a reflection of the PCr content in the sample.

3.2.5. Cr

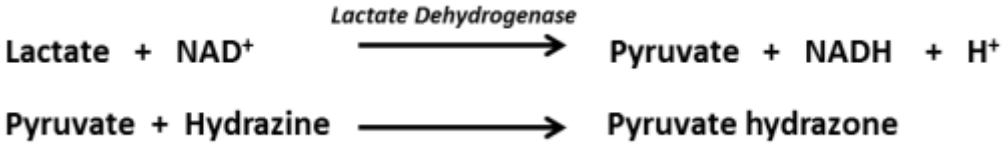
Cr content was determined in samples using a three-step enzymatic process (diagram cited from Cara Timpani (Timpani, 2017)):



Following the creation of the standard curve, 30 µL of the reagent cocktail (50 mM Imidizole at pH 7.4; 5 mM $MgCl_2$; 30 mM KCl; 0.1 mM phosphoenolpyruvic acid (PEP); 0.2 mM ATP; 1 µg/ml lactate dehydrogenase (LDH); 5 µg/ml pyruvate kinase (PK)) and the PCr standard or sample were pipetted into 96 well plates. After 15 min of incubation at room temperature, the first reading shows residual NADH concentration. After adding CK (in 0.05% BSA) and allowing the mixture to sit at room temperature for 60 min, the second measurement was taken. Pyruvate, the substrate for the third reaction, was produced and measured 15 min after the second reading.

3.2.6. Muscle lactate

A two-step enzymatic procedure was used to determine the lactate content of the samples (diagram cited from Cara Timpani (Timpani, 2017)):



Before the first reading without any samples, the plates were incubated for 30 min using just the reagent cocktail (1 M hydrazine; 1 M glycine; 0,1 M NAD; 25 mg/ml LDH) that had been pipetted onto 96-well plates. After that, a reagent blank, lactate standard, or a sample volume of 5 µL was pipetted into the plate, and it was allowed to sit at room temperature for 60 min before a second reading was taken.

3.3. High performance liquid chromatography (HPLC)

Whole blood samples were collected into lithium heparin tubes and centrifuged at 3,500 rpm for 10 min at 4°C to separate the plasma and haematocrit portions. Subsequently, 100 µl of plasma was added to 200 µl of ice-cold 3 M PCA, centrifuged at 3,500 rpm for 4 min at 4°C and the supernatant was stored at -80°C before analysis for lactate. The remaining plasma was stored in liquid nitrogen for later analysis of inosine, hypoxanthine (Hx), xanthine and uric acid. Prior to analysis 100 µl of plasma was thawed and deproteinised 50 µl of 1.5 M PCA and subsequently neutralised with 37 µl of 2.1 M $KHCO_3$ immediately prior to analysis.

Internal standard calibration curve was established measure from HPLC software, (Composition of internal calibration curve: Standard 1: 150 uM ATP and 8 uM of other stock solutions; Standard 2: 180 uM ATP and 5 uM of other stock solutions; Standard 3: 200 uM ATP and 2.5 uM of other stock solutions; Standard 4: 250 uM ATP and 1.0 uM of other stock solutions; Table 3.2). Before the internal standard calibration curve was made, each subject (ATP, ADP, AMP, IMP, Inosine, Hx, xanthine) of Internal standard requires to be injected into column and read the retention time.

Plasma Hx, xanthine, inosine and uric acid were determined on neutralised PCA extracts, using a modification of the reverse phase HPLC technique described by Wynants and Van Belle using a Shimazu chromatography system (model: LC-2030, Shimadzu Corporation, Kyoto, Japan). A Phenomenex Luna 5 µm C18/100A (250 x 4.6 mm) analytical column (Phenomenex, Torrance, CA, USA) was used to perform the analysis. The mobile phases used for separation consisted of 0.15 M ammonium dihydrogen phosphate ($NH_4H_2PO_4$), pH 6.00 and a mixture of acetonitrile and methanol (50/50, v/v). The eluent was monitored at 254 nm. The retention time of Hx, xanthine, inosine and uric acid standard were showed in Figure 3.1.

Table 3-2 Molecular weight and concentration of different stock solution

Subject	Molecular weight (g/mol)	Concentration	Supplier	Observed retention time (min)
ATP	605.2	10mM	Sigma-Aldrich	6.099
ADP	471.2	1mM	Sigma-Aldrich	6.662
AMP	347.2	1mM	Sigma-Aldrich	9.426
IMP	500.2	1mM	Sigma-Aldrich	4.939
Inosine	268.2	1mM	Sigma-Aldrich	14.586
Hx	136.1	1mM	Sigma-Aldrich	8.025
Xanthine	152.1	1mM	Sigma-Aldrich	8.880
Uric acid	168.1	1mM	Sigma-Aldrich	4.379

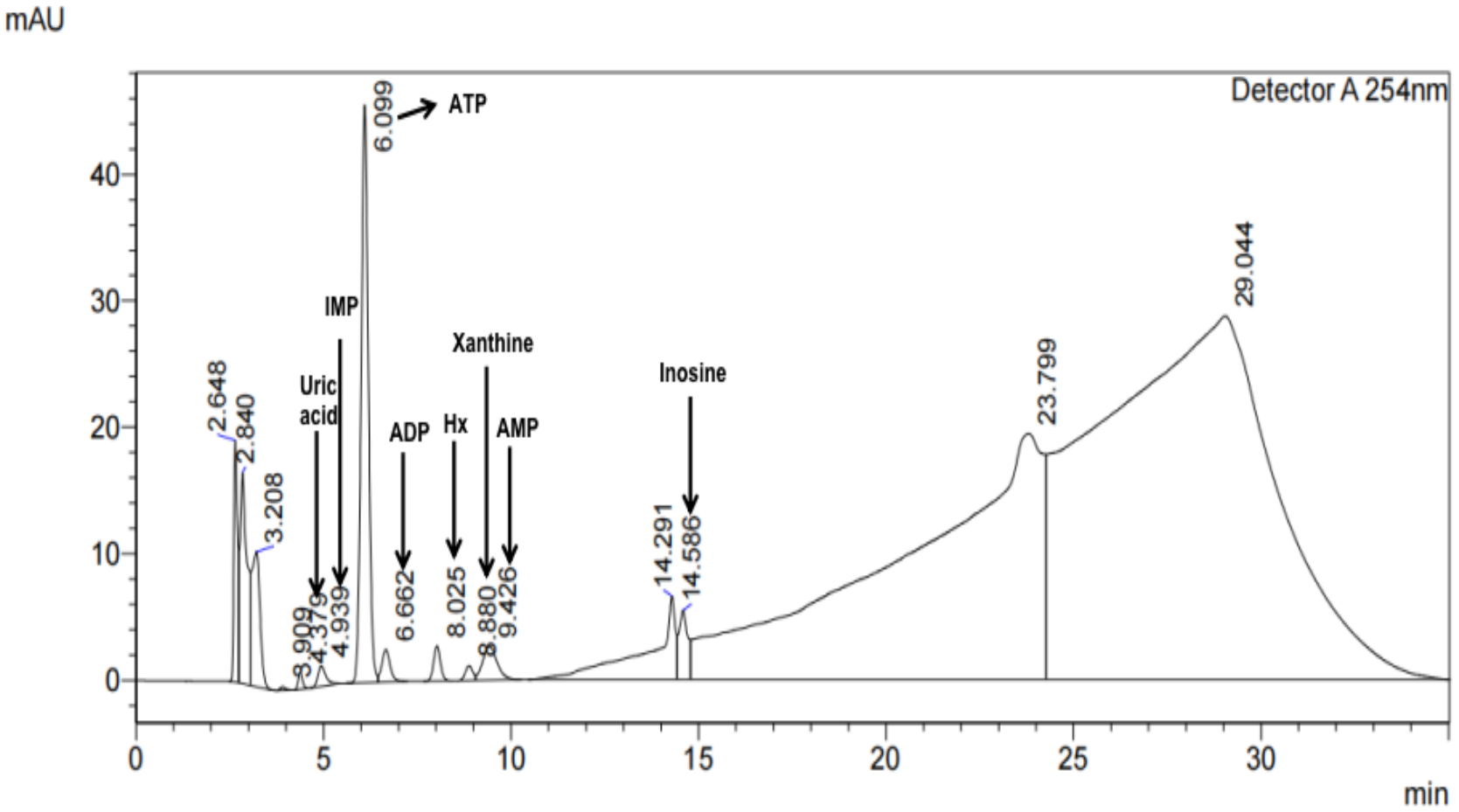


Figure 3-1 Retention time of ATP, ADP, AMP, Inosine, Hx, Xanthine and Uric acid in plasma. Separation was achieved on a 100CH-18 column using the methanol as the only organic modifier. Column flow rate: 1 ml/min.

Chapter 4

Effect of HIT training on skeletal muscle stress signaling in female and male mice

4.1. Introduction

In this chapter, we aimed to investigate whether oxidative, ER and/or hypoxic stress signaling pathways are induced in skeletal muscle by HIT training in healthy mice and whether sex differences exist in these responses. We leveraged skeletal muscle collected from a previously published study that subjected male and female mice to 6-8 repetitions of 20 s treadmill running bouts (at a previously determined top speed) interspersed by 40 s of active rest (at 5 cm/s; slope 0°) for three non-consecutive days per week over 12 weeks (Wilson et al., 2018). HIT is a cardiovascular exercise strategy that alternates short periods of very intense exercise with low-intensity or no intensity recovery periods (Biddle and Batterham, 2015). From a biochemical perspective, HIT should theoretically induce very rapid, intense bursts of metabolic stress resulting in the degradation of purines and their loss from skeletal muscle as end products, hypoxanthine and xanthine. As per our theoretical model (details in Section 1.1), the intensity of this metabolic stress may result in the activation of oxidative, ER and hypoxic stress signaling pathways geared toward driving the necessary adaptations to counter future challenges to homeostasis.

Compared to moderate and low intensity exercise, HIT has a greater potential to push the boundaries of hormesis and the magnitude of the adaptive response. The production of ROS has been proposed as an important mechanism in these adaptations. To this extent, antioxidant supplementation during exercise training has been shown to blunt training adaptations (Peternelj and Coombes, 2011, Merry and Ristow, 2016, Ferran et al., 2020, Mason et al., 2020). The biochemical pathways activated/amplified during metabolic stress are critical producers of these ROS (Schieber and Chandel, 2014, Zhao et al., 2017). Shifts in relative metabolite balances associated with metabolic stress also activate ER and hypoxia stress signaling pathways. For example: (1) HIT causes the rapid degradation of ATP to AMP, which via increased activity of AMP kinase (AMPK) (Chesser, 2007), may result in transient ER stress signaling to inhibit protein synthesis and conserve cellular energy (Marino et al., 2021); and (2) accumulation of TCA cycle intermediates such as fumarate, which is generated

cytosolically through intense PNC activity during metabolic stress, can independently activate HIF1α-mediated hypoxia signaling (Choudhry and Harris, 2018) and the Nrf2-mediated antioxidant response (Ashrafian et al., 2012). Collectively, but not exclusively, these stress responses promote the beneficial adaptations to skeletal muscle associated with exercise such as increased mitochondrial biogenesis and quality control to increase the overall energy producing capacity of the cell (Loy et al., 2013, Sorriento et al., 2021), increased capillarisation and blood delivery to the muscle to support these mitochondria (Hearon Jr and Dinenno, 2016, Liu et al., 2022), and a more capable antioxidant defence system to limit cellular oxidative damage associated with a higher metabolic rate (Morillas-Ruiz and Hernández-Sánchez, 2015).

HIT training has been reported to have sex diverse impacts (Zhang et al., 2022, Ramadan et al., 2022). HIT could enhance testosterone levels, which is a hormone documented to have greater protection on oxidative stress in males (Baumgartner et al., 1999, Zhang et al., 2022). One study found that high intensity exercise (20 m/min, 60 min, 5 day/weeks on a mice treadmill at 10° incline for 8 weeks from the age of week 7) increased serum testosterone levels in male mice (Zhang et al., 2022). In terms of females, HIT could increase estrogen levels (Hao et al., 2010, Ramadan et al., 2022), which might protect against oxidative stress via regulation of phosphorylated cAMP response element binding protein (Ni et al., 2015) . One study demonstrated that HIT elevated estrogen level by 143.16 % compared to controls in females (Ramadan et al., 2022). Moreover, different signaling pathway in response to exercise-induced stress might be involved in different sexes. Estrogen could supress oxidative stress (such as disrupted redox homeostasis) through inhibiting ROS production (Felty et al., 2005) and scavenging free radicals (Ruiz-Larrea et al., 2000). Whereas, testosterone induces the expression of unfolded protein response transcriptional factor CHOP (Azhary et al., 2019) and activates the HIF1α signaling pathway (Peng et al., 2019).

The purpose of this study was to evaluate classical biomarkers of oxidative, ER and hypoxic stress signaling in mouse skeletal muscle following HIT training. We hypothesised that HIT would upregulate the expression of Nrf2 mediated cytoprotective enzymes, stimulate of ER stress induced

UPR markers, and activate HIF-1 mediated hypoxia stress signaling in female and male mice. We also expected sex diverse stress signaling responses to HIT.

4.2. Materials and Methods

4.2.1. Animals

All experimental procedures were approved by the Victoria University Animal Ethics Committee (VUAEC-15/007) and conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Mice had *ad libitum* access to food (high fat diet) and water throughout the experiment.

4.2.2. HIT training

Eight week-old female (n=19) and male (n=15) C57BL/6 mice (Average weight: 17.6 ± 1.7g female, 22.2g ± 2.5 g males) were randomized to four different groups: female control, female HIT, male control and male HIT. Mice in the HIT groups commenced 3 days per week training of 6-8 repetitions of 20 s sprint at an individualised pre-determined maximum capacity, interspersed with 40 s walking at 8 cm/s on a rodent treadmill at 0° incline per 8-day cycle for 10 weeks. To determine maximum speed mice underwent: (1) an incremental warm-up procedure for 5 min until an average speed of 8 cm/s was reached; (2) an incremental speed increase from 8 to 35 cm/s, which was maintained for 20 s then slowly decreased back to 8cm/s for 40 s; (3) an incremental speed increase from 8 to 40 cm/s, which was maintained for 20 s and then repeated; (4) 20 s running and 40 s active walking cycles with incrementally increased running speeds (5 cm/s) until fatigue. The average maximum speed of all mice was 59 ± 2 cm/s.

4.2.3. Body Weight and Body Composition

Body weight and composition (fat mass and lean mass) was quantitated weekly using scales and EchoMRI (EchoMRI- body composition analyser, Houston, TX, USA), respectively, before and after exercise training. Percent change in response to 10 weeks exercise training in body mass was calculated as [(Before exercise mass – After exercise mass)/ Before exercise mass] x 100. Percent change in percent body fat/lean was calculated as [(Before exercise fat/lean mass- After exercise fat/lean mass/ Before exercise fat/lean mass)] x 100. These indices were used as crude indicators of training effect.

4.2.4. Muscle samples collection

At the experimental endpoint mice were anaesthetised with a 60 mg/kg intraperitoneal injection of pentobarbitone. Right gastrocnemius muscles were excised from mice and snap frozen in liquid nitrogen for further analysis (muscle samples were from previous PhD student in our lab).

4.2.5. Western Blot

Right gastrocnemius was used for determining the gene expression of metabolic stress markers. Oxidative stress markers (Nrf2, Keap1, total and phosphorylated p62, HO1, NQO1 and SOD), ER stress markers (ATF4, Sestrin1 and Sestrin2) and hypoxia stress markers (HIF1α, Sirt1 and PGC-1α) were measured as described in Section 3.1. in Method section). All protein expression data were normalized to female control group.

4.3. Statistical analysis

Data are presented as mean ± SEM. Two-way ANOVA (Prism 8) was utilised to detect sex (female and male) and exercise (control and HIT) differences. An α value of 0.05 was considered significant and an α value between 0.05 and 0.1 was considered as a statistical trend.

4.4. Results

4.4.1. Effect of HIT on body composition

As a crude indicator of HIT training adaptations, and especially, metabolic stress, we assessed HIT effects on body mass and composition (Figure 4.1). Following 10 weeks training, body mass (female:16.0%, $p<0.0001$ and male: 16.9%, $p<0.0001$, respectively.) and fat mass (female: 14.7%, $p<0.0001$ and male: 11.1%, $p<0.0001$, respectively) were significantly reduced by HIT. As expected, the body lean mass significantly increased (female: 9.6%, $p<0.0001$ and male: 8.9%, $p<0.0001$, respectively).

4.4.2. Effect of HIT on stress signaling

4.4.2.1. Oxidative stress markers

To investigate the effect of HIT on oxidative stress signaling in skeletal muscle we quantitated protein biomarkers of Nrf2 activation (Figure 4.2). While there was no effect of HIT training on Nrf2 protein expression per se ($p>0.1$, Figure 4.2A), Keap1 expression was increased ($p<0.001$, Figure 4.2B) and there was a strong trend for increased p62 phosphorylation ($p=0.059$, Figure 4.2D) in female skeletal muscle suggesting Nrf2 dissociation, Keap1 sequestering by p62 and activation of autophagic degradation. However, there was no statistically significant increase in protein expression of Phase II antioxidant enzymes that are normally induced by Nrf2 mediated ARE transcription (Figure 4.3) – albeit, there was a strong trend for increased SOD expression in female muscle ($p=0.055$; Figure 4.3C). There was no evidence of Nrf2 signaling or upregulation of Phase II antioxidant enzymes in male skeletal muscle, although the same upregulation of phosphorylated p62 (indicative of autophagy activation) was observed in male, as per female, skeletal muscle ($p<0.05$; Figure 4.2D). HO1 expression was increased in male skeletal muscle ($p<0.05$; Figure 4.3A), but apparently not through Nrf2 activation. Keap1 protein expression was positively correlated with maximum speed in HIT trained

female (p=0.048; Figure 4.2F) but not male mice (p>0.1; Figure 4.2G), although notably, both Keap1 expression and maximum running speed were lower in male compared to female mice (Figure 4.3F and G). There was no correlation between maximum running speed and protein expression of any other oxidative stress biomarkers (data not shown).

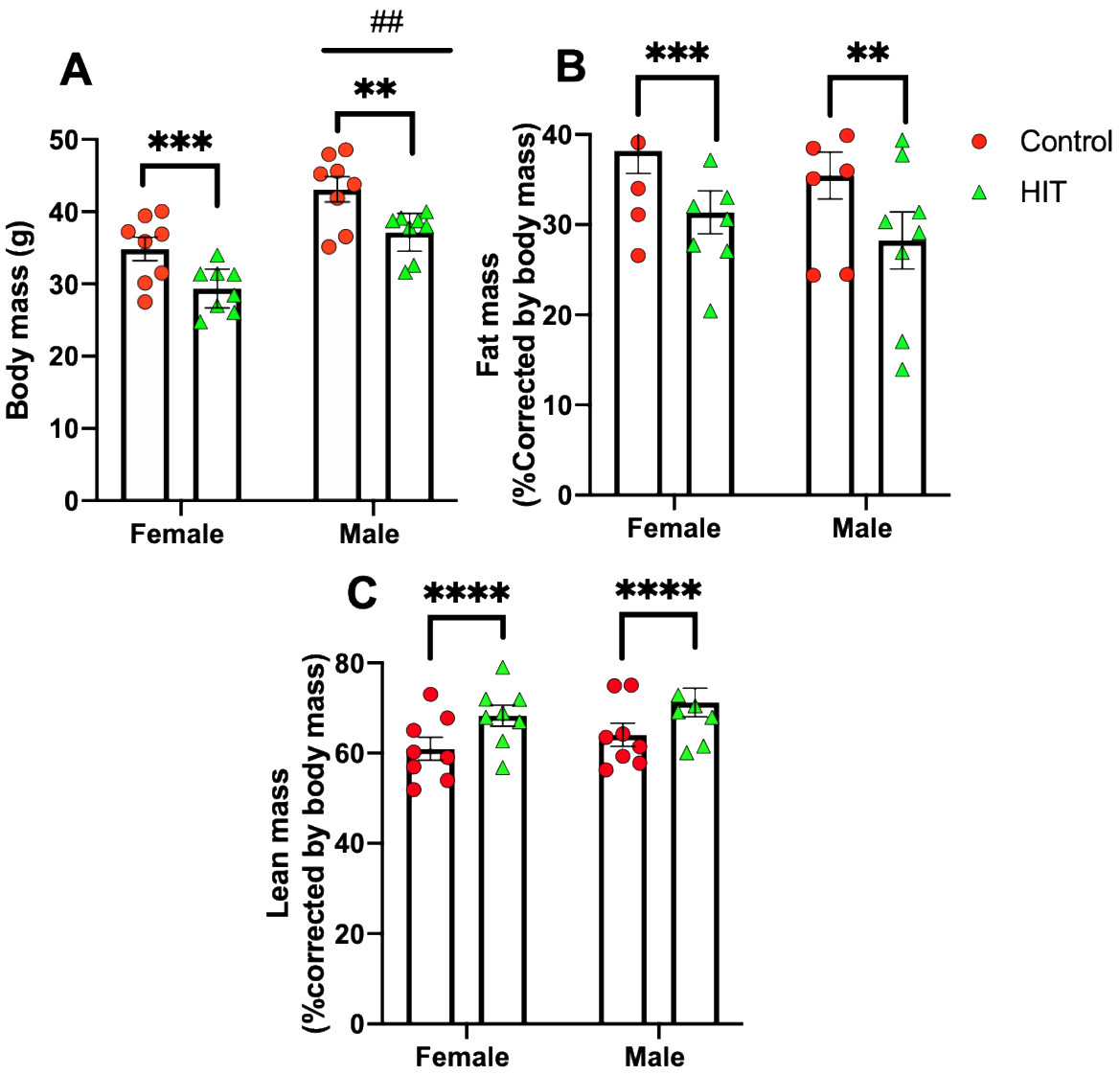


Figure 4-1 HIT training reduces body mass and changes body composition in mice. We compared the impact of HIT training between male and female mice on (A) body mass, (B) fat mass and (C) lean mass. Body mass and fat mass were decreased by HIT, indicating metabolic/caloric stress., whereas lean mass was increased. ***p<0.01, ***p<0.001 and ****p<0.0001 HIT effect compared to sedentary control group. $^{\#\#}p$<0.01 male significant difference from female group.

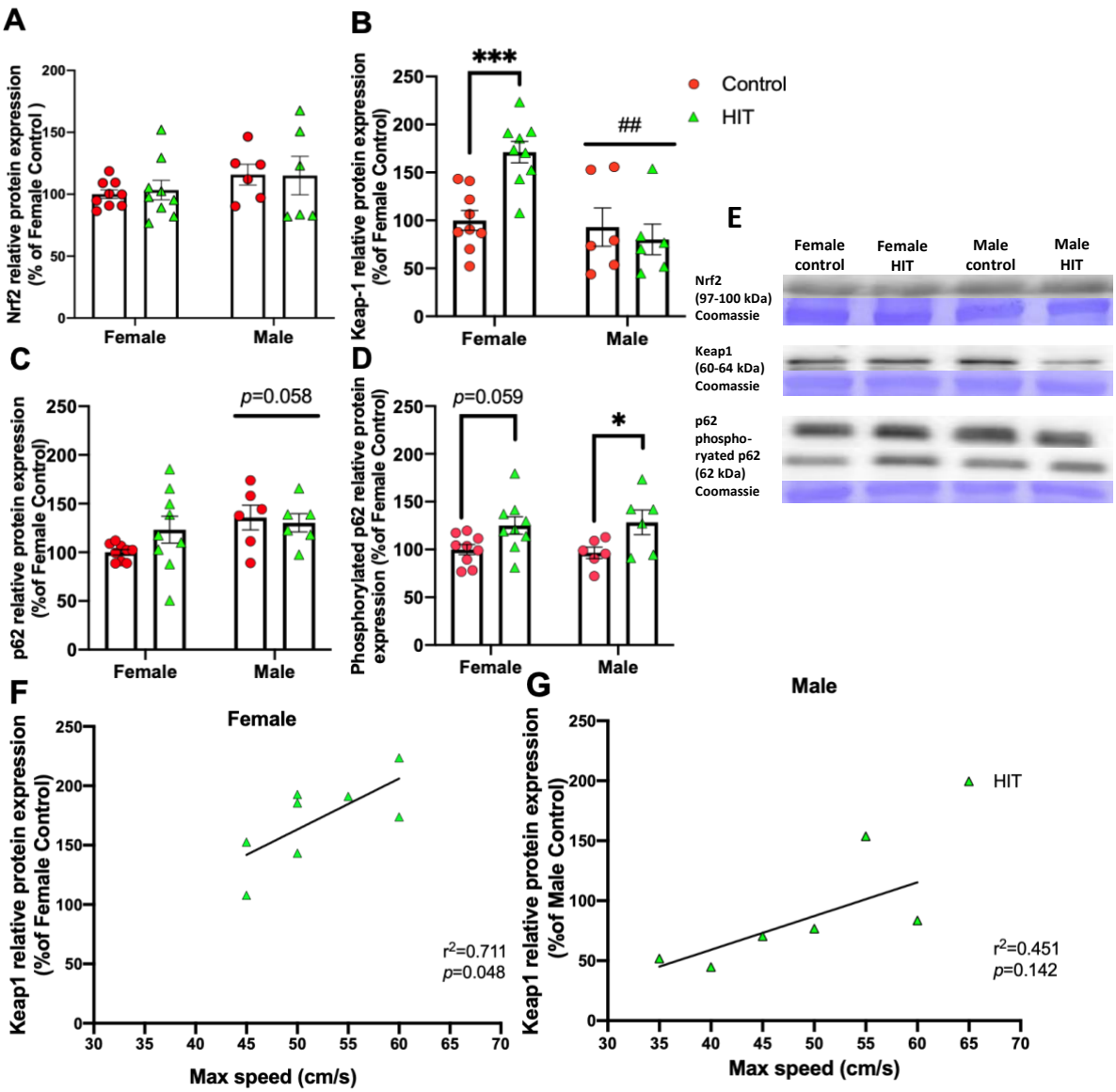


Figure 4-2 The effect of HIT training on the expression of Nrf2-mediated cytoprotective proteins. HIT increased (B) Keap1, the Nrf2 repressor protein and had a positive trend in (D) phosphorylated p62, an indicator of autophagic degradation of Keap1, in the female HIT group compared to the sedentary control group. In comparison male mice had lower keap1 levels compared to female mice and significantly increased phosphorylated p62 in response to HIT training. However, there were no distinct effect in the expression of (A) Nrf2 (as a major stress response transcriptional regulator for oxidative stress and (C) total p62, a sequester of Keap1 during Nrf2 activation in any group. Keap1 expression was positively correlated with maximum running speed in female (F) but not male (G) HIT mice. (E) Western blot representative images are displayed alongside with a Coomassie blue representative image, which was used as the protein loading control.*p<0.05, ***p<0.001 HIT effect compared to control group. ##p<0.01 male significant difference from female group. p values and correlation coefficients (r2) are shown for correlations.

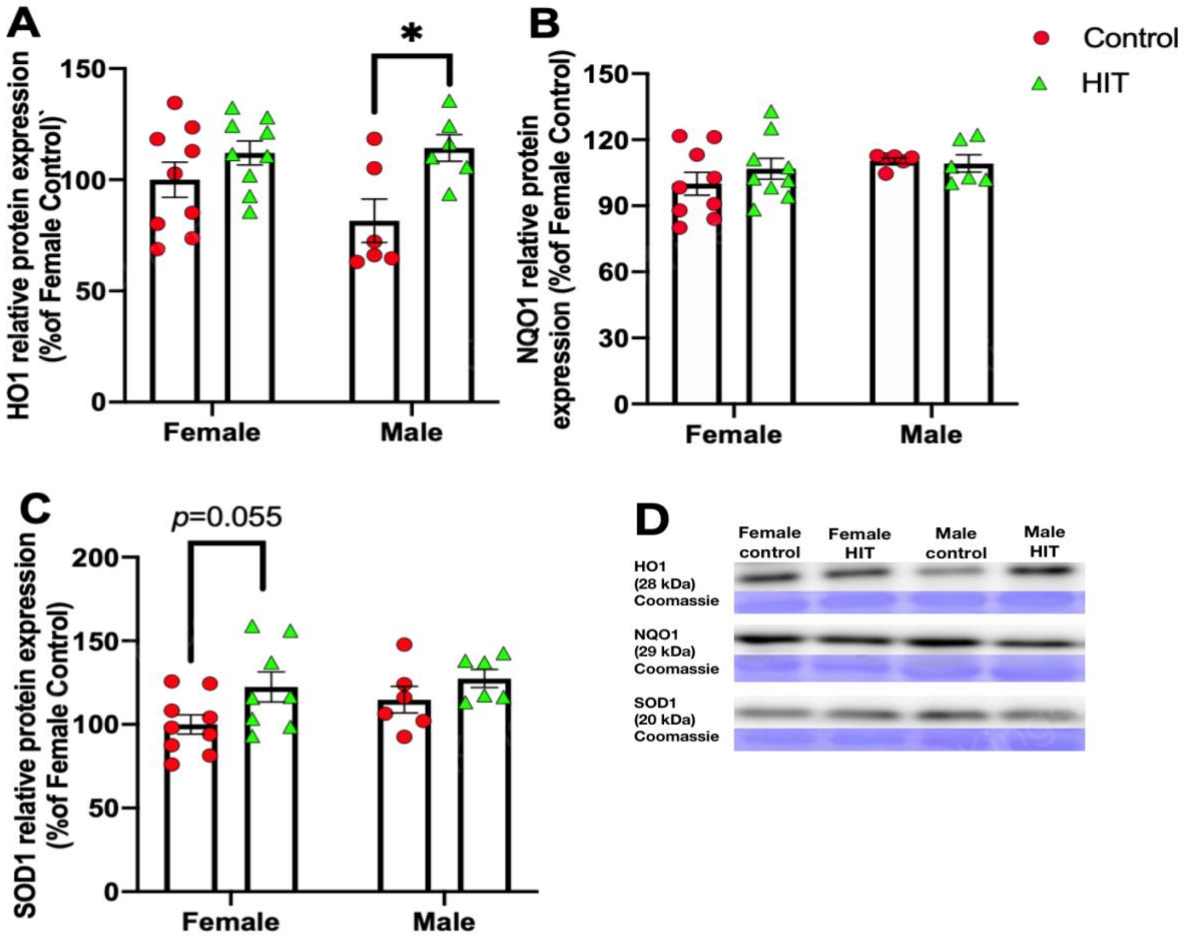


Figure 4-3 HIT training induces antioxidants response. HIT training increased (A) HO1 (Heme oxygenase 1), a cytoprotective enzyme in response to oxidative stress, in male groups and had a strong trend in the expression of (C) Superoxide dismutase 1 (SOD1), as a part of first defence line against oxidative damage, in female group compared to control group. No notable effect was observed in the expression of (B) NQO1 (NAD(P)H quinone oxidoreductase 1) as a readout parameter of Nrf2 activity. (D) Western blot representative images are displayed alongside with a Coomassie blue representative image, which was used as the protein loading control. *p<0.05 HIT effect compared to control group, trends of p<0.1 were reported.

4.4.2.2. ER stress markers

The response of protein markers of ER stress to HIT are presented in Figure 4.4. There was no effect of HIT on protein expression of ATF4 or Sestrin1 in either female or male skeletal muscle (p>0.1). In contrast, HIT trained skeletal muscle had significantly increased protein expression of Sestrin2 (p<0.05) in male skeletal muscle only.

4.4.2.3 Hypoxic stress markers

Alterations of hypoxic stress markers in response to HIT are presented in Figure 4.5. There was a strong trend for protein expression of Sirt1 and PGC-1α to be higher in male skeletal muscle (p=0.066, Figure 4.5B and p=0.08, Figure 4.5C, respectively) after HIT training. HIT significantly increased PGC-1α protein expression in female skeletal muscle compared to the control group (p<0.001, Figure 4.5C). PGC-1α expression was positively correlated with lean mass in female (p=0.034, Figure 4.5F) and male (p=0.049, Figure 4.5G) mice, whereas we didn't see any strong relationship between PGC-1α expression and max speed in female (p>0.1 Figure 4.5D) and male mice (p>0.1 Figure 4.5E. However, there was no distinct difference in the expression of HIF-1α in response to exercise training (p>0.1).

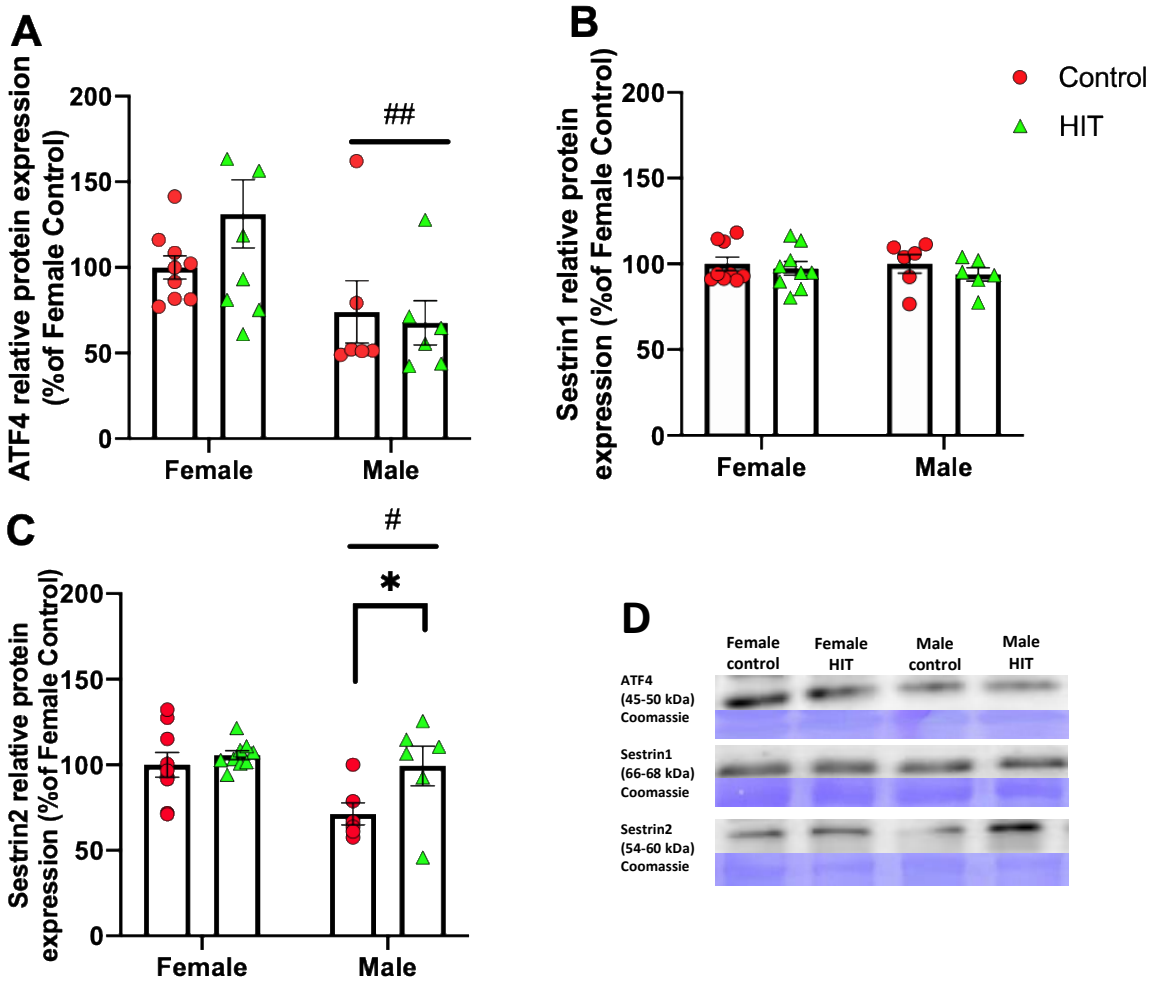


Figure 4-4 Effect of HIT training on ER stress markers. There was no difference in the expression of (A) ATF4, one of the regulators of ER stress, and (B) Sestrin1, regulation of the ER stress sensor IRE1 in response to HIT training. we found that HIT training increased the expression of (C) Sestrin2, a critical mediator of the unfolded protein response during ER stress. Male group had lower ATF4 and Sestrin2 content. (D) Western blot representative images are displayed alongside with a Coomassie blue representative image, which was used as the protein loading control. *p<0.05 HIT effect compared to control group. $^{\#}p$<0.05 and $^{\#\#}p$<0.01 male group significant difference from female groups.

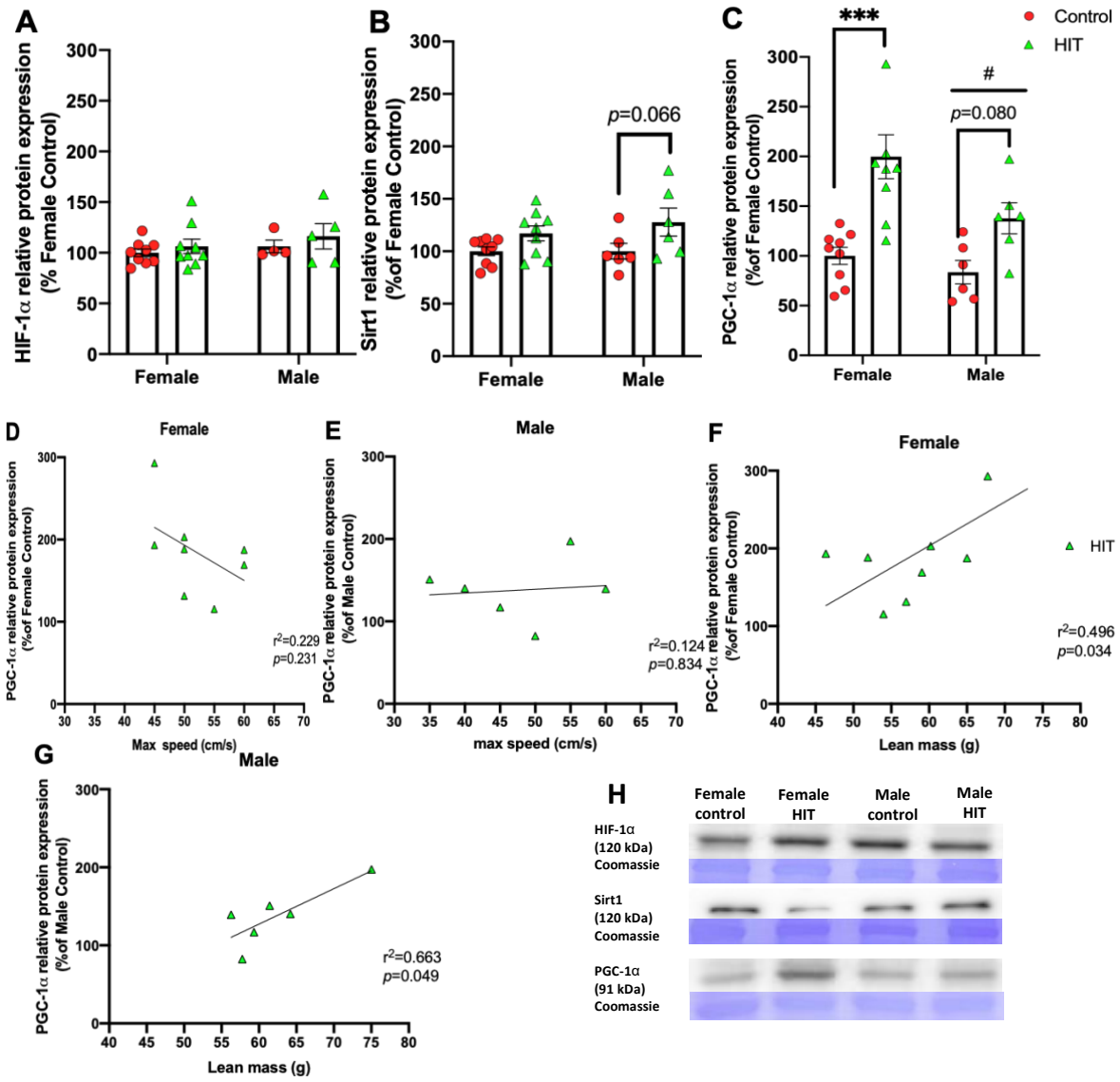


Figure 4-5 Effect of HIT on hypoxia stress markers in mouse gastrocnemius muscle. Western blots were probed for (A) HIF-1α, a key modulator of the transcriptional response to hypoxia; (B) Sirt1(sirtuins1) act as a sensor of HIF1 signaling; and (C) PGC-1α, a powerful transcription regulator of mitochondrial biogenesis and hypoxia-inducible genes. we didn't observe the relationship between max speed and PGC-1α in female (D) and male (E). PGC-1α expression was positively correlated with lean mass, an anthropometric training adaptation, in female (F) and males (G). (H) Western blot representative images are displayed alongside with a Coomassie blue representative image, which was used as the protein loading control.*p<0.05; trends of p<0.1 were reported. p value represents HIT training compared to control group in Figure 4.5D and E. r2 is represented for the correlation coefficients.

4.5. Discussion

Regular exercise training plays a major role in improving health and body composition (Chiu et al., 2017). HIT may provide additive benefits over steady-state exercise because it more rapidly challenges the metabolic systems, tissue oxygenation (increasing respiration) and blood flow, which supplies the muscle with fresh oxygen and blood (Ichinose et al., 2015, Willis et al., 2019). In the current study, 10 weeks of HIT was effective at reducing the body and fat mass of high fat-fed mice and at increasing the lean mass (Wilson et al., 2018). Previously, Wang et al. showed that 8 weeks of HIT, consisting of 10x4 min bouts at 85-90% VO_2 max on treadmill running with 2 min active recovery (slope 25º), reduced fat and body mass in males mice (Wang et al., 2017). Whereas Martinez-Huenchullan et al. demonstrated significantly reduced fat mass and increased lean mass but no change in body mass after 10 weeks of HIT (8x2.5 min bouts at 90% maximum running capacity) in male mice (Martinez-Huenchullan et al., 2019). Collectively, these studies suggest that fat mass is lost first due to intense stress on the anaerobic metabolic systems and higher demand for aerobic metabolism in the 24 hrs immediately after the HIT session to recover energy homeostasis (i.e., excess post-exercise oxygen consumption (EPOC)) (Balsom et al., 1993). Augmentation of aerobic capacity through mitochondrial biogenesis is necessary for subsequent accretion of lean (e.g., muscle) mass, an energy demanding process (Drigny et al., 2014). HIT involving 4 repetitions, 4 min 85-95% peak heart rate and 3 min active recovery was shown to increase muscle protein synthesis and satellite cell activation resulting in muscle mass accretion (Callahan et al., 2021).

Muscle protein synthesis is particularly sensitive to the mTOR-AMPK regulatory nexus where metabolic stress sufficient to induce AMPK phosphorylation results in the mTORC1-dependent inhibition of protein synthesis (Hong-Brown et al., 2010). Similarly, ER stress signaling can slow, or even stall, ribosomal protein synthesis. Thus, the induction of cellular stress defence mechanisms is crucial to accrete lean mass in response to HIT training – increased mitochondrial number and efficiency to protect against metabolic stress in the first instance, is a cornerstone of these adaptations. Of the three cell stress signaling pathways we probed, PGC-1α, a transcriptional regulator of the

mitochondrial biogenesis program induced by metabolic/hypoxic stress, and phosphorylated p62, an indicator of increased protein sequestration (e.g., Keap1) and autophagy induction, were the only proteins consistently upregulated by HIT training across male and female mice. Increased autophagy is a well-documented response to exercise training and is induced by metabolic and hypoxic stress. It functions to degrade and replenish, or recycle, damaged organelles, particularly mitochondria (He et al., 2012, Ju et al., 2016).

In our study, similar body composition adaptations were observed between female and male mice in response to HIT, even though male mice were significantly heavier than females. However, there were significant differences in stress signaling within female and male skeletal muscle, while we saw no evidence of active Nrf2 signaling in either sex, male skeletal muscle showed high HO-1 expression and female skeletal muscle trended higher SOD1 expression. Notably, SOD1 can be induced by hydrogen peroxide independently of Nrf2 (Dell'Orco et al., 2016) and while Nrf2 is the major transcriptional regulator of HO-1, several other redox sensitive regulators can independently control HO-1 transcription, including activator protein-1 (AP-1) (Harada et al., 2008), NF-κB (Li et al., 2020b), HIF-1 (Consoli et al., 2021) and prostaglandin metabolites (Gong et al., 2002). Thus, these proteins could be upregulated through an Nrf2-independent mechanism and in a sex specific manner. HIT female skeletal muscle expressed significantly more Keap1 than sedentary controls, yet this effect was not observed in male muscle. Our data suggest that if transient Nrf2 activity is involved in HO-1 transcription following HIT, male skeletal muscle may be more sensitive due to lower native repressor function. Whereas female skeletal muscle may be more efficient at exploiting Nrf2-independent antioxidation mechanisms due to a higher native Keap1 expression. Although Keap1 is a well-known repressor of Nrf2, recent evidence suggests it has many alternative functions and a broad cellular interactome – it is postulated that the balance of Nrf2-to-Keap1 is important and that Nrf2 is an important repressor of Keap1 hyperactivity (Robertson et al., 2020). It is interesting to speculate that the balance of Nrf2-to-Keap1 may be influenced by sex-related differences such as hormones. For example, HIT has been shown to increase testosterone levels in males (Baumgartner et al., 1999) but

balance estrogen levels in females (Van Pelt et al., 2015), which in addition to regulating body mass and composition, may be influential an Nrf2/Keap1 activity.

Another stress biomarker protein that was specifically upregulated in male but not female skeletal muscle in response to HIT in our study was Sestrin2. Upregulation of Sestrin2 has been reported in response to diverse cellular stresses including HIT (Delshad et al., 2021, Eslami et al., 2022). Delshad et al. reported that 8 weeks of HIT training (5-8 repetitions, 2 min at 80-100% HRmax with 2 min active recovery at 50% of HRmax) increased skeletal muscle Sestrin2 content in male mice compared to sedentary controls (Delshad et al., 2021). Previously, Sestrin2 upregulation coincided with activation of autophagy in the skeletal muscle of old mice (Lenhare et al., 2017). Our data show that Sestrin2 content was higher in female than male at rest, Sestrin2 indirectly controls mTORC1 activity as part of the unfolded protein response, preventing further exacerbation of ER stress (Jegal et al., 2017).

There was a strong trend for Sirt1 to be upregulated in male but not female mice also. 12 weeks HIT training, consisting of 13x4 min bouts at 85-90% VO_2 max on treadmill running with 2 min active recovery (slope: 20^{o}) elevated testosterone levels in male Wistar rats (Ambroży et al., 2021), which is the primary sex hormone in male and activates eNOS activity, and subsequently induce Sirt1 expression (Ota et al., 2012). Moreover, Sirt1 is an NAD+ dependent Class III histone deacetylase inhibitor which is involved in ROS production (Chen et al., 2016) and has been reported to inhibit the apoptosis and protect muscle cells from hypoxia stress (Luo et al., 2019). HIT training in female mice results in a more intense hypoxic response and quicker adaptations. Whereas in males, after 10 weeks of training, the adaptation still seems to be happening.

4.6. Conclusion

Current evidence indicates that HIT impacts the body composition and drives the molecular adaptations to exercise induced stress. To our knowledge, this is the first study to explore the relationship between protein biomarkers of different cellular stress responses in response to HIT and the impact of sex. We found the expression of Keap1, SOD and PGC1α were higher in female skeletal

muscle following HIT, yet the expression of HO-1, Sirt1 and PGC-1α, which can all be induced by hypoxic stress protein, HIF-1, were upregulated in male skeletal muscle. Our data suggest that female muscle may be more responsive to oxidative-type stress, while male muscle may be more responsive to hypoxia.

Chapter 5

Effects of moderate aerobic training and RIB supplementation on skeletal muscle stress signaling in healthy WT and *mdx* mice

5.1. Introduction

DMD is a fatal genetic disorder characterised by progressive muscle degradation and weakness (Goldstein and McNally, 2010, Yiu and Kornberg, 2015, Sinha et al., 2017). It is caused by loss of function of the cytoskeletal protein, dystrophin, which results in chronic muscle damage that eventually exceeds the capacity of muscles to repair (Yiu and Kornberg, 2015, Duan et al., 2021). ATP plays a vital role in resisting skeletal muscle degeneration (e.g., by buffering calcium influx and ROS production that stimulate degeneration of dystrophic muscles) and facilitating repair processes. Yet dystrophic muscles share many similarities with muscles from individuals afflicted by inborn errors of metabolism, in which the metabolic systems cannot keep up with energy demand. Human DMD patients and *mdx* mice, a commonly used animal model of DMD, have many metabolic disturbances (as reviewed by our group previously) (Timpani et al., 2015) including reduced ratio of phosphocreatine to phosphate (Zhang et al., 2008, Braun et al., 2001), 50% reduction in resting ATP concentration (Austin et al., 1992, Cole et al., 2002) and reduced mitochondrial ATP production capacity (Rybalka et al., 2014, Timpani et al., 2015). Mitochondria produce most of the cells ATP and mitochondrial dysfunction potentiates chronic metabolic stress in dystrophin-deficient muscles (Lindsay et al., 2021). This metabolic stress contributes significantly to the loss of muscle mass and function in DMD and for this reason, mitochondrial medicine development is an area of intense research. However, the precise mitochondrial defect and/or reason for dysfunction has not been definitely established making drug targeting difficult.

Replenishment of muscle ATP levels can be approached in other ways than through manipulating mitochondrial function. When ATP is degraded to ADP without the capacity for matched mitochondrial oxidative phosphorylation, ADP is progressively degraded to uric acid resulting in ROS formation and the net flux of purine bases from muscle (Stathis et al., 1994, Hellsten et al., 1999). Purines are essential to cell function and survival and when their depletion is threatened, *de novo* synthesis via the 5-phosphoribosyl-1-pyrophosphate pathway (PRPP), a slow and energetically

expensive process, predominates. Supporting purine salvage to prevent depletion and de novo synthesis to pre-empt depletion, could be a useful medicinal strategy to slow the progression of DMD. Ribose (RIB), a simple carbohydrate, plays a vital role in driving the synthesis of PRPP to maintain purine homeostasis (Cai et al., 2022), as well as supporting the production of energy co-factors (Mehta et al., 2013). Ribose supplementation has been reported to improve the resting ATP recovery following exercise- induced ATP deplete muscle (Hellsten et al., 2004). Hellsten demonstrated that muscle ATP concentration 72 hrs after a HIT exercise bout was higher with RIB supplementation compared to placebo. Few studies have explored the potential benefit of RIB supplementation on DMD patients. One clinical study involving 5 young DMD boys (aged 6.5 to 11 years) trialled 250 mg ribose twice daily for 12 weeks to support purine nucleotide synthesis and muscle energy levels, However, there were no effects on muscle ATP concentration nor improvement in muscle function (i.e. muscle strength) (Griffiths et al., 1985). Notably, disease course is already well established at this age and muscle pathology extensive. RIB's capacity to support muscle bioenergetics might be more effective if administered at diagnosis (generally 2-3 years of age), to protect against the deterioration of muscle mass and quality in the first instance. A 10-year clinical trial of the purine nucleotide cycle metabolite, adenylosuccinic acid (ASA), overarchingly demonstrated that disease could be slowed if administered early by protecting muscle from damage and wasting (Bonsett and Rudman, 1992). Whereas treatment of older children, especially those who had already lost the ability to ambulate, could not reverse the loss of muscle mass or overt pathology (Bonsett and Rudman, 1992). Thus, early intervention appears crucial for curbing disease progression using metabolism-targeted therapies.

The overarching aim of this study (chapter 5) was to investigate whether RIB supplementation could be useful to mitigate metabolic stress and disease severity in dystrophin deficient muscles when applied from infancy. Since the *mdx* phenotype is milder than in human DMD patients, we exercise trained *mdx* mice for 5 weeks to intensify metabolic stress and test RIB in a severe phenotype model. In particular, we were interested to examine (1) which stress signaling pathways were predominant in *mdx* muscles; and (2) whether ribose supplementation could circumvent stress in *mdx* mice. We

hypothesised that ribose supplementation would improve muscle function and fatigability in *mdx* mice secondary to reducing metabolic stress.

5.2. Methods

5.2.1. Ethics

All experimental procedures were approved by the Victoria University Animal Ethics Committee (AEETH 20/006) and conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Animals were derived from our inhouse breeding colonies (AEETH 20/005).

5.2.2. Animals and treatments

Wild type C57BL/10ScSn (WT; n=48) and *mdx* (C57Bl/10*;* n=48) male mice, were randomly assigned into 4 different groups: WT VEH (n=24), WT RIB (n=24), *mdx* VEH (n=24) and *mdx* RIB (n=24). Treatments commenced at 3 weeks of age (3 years human equivalence), just prior to a period of an intense growth-associated muscle damage phase in *mdx* mice and concluded at the experimental endpoint at 8 weeks of age. Mice were dosed daily based on body weight with either 0.5% methylcellulose or 1.6 g/kg D-ribose in VEH via oral gavage (Walker Scientific). A 22-gauge needle (25 mm) was used for small mice (body weight less than 15 g), whereas 25-gauge gavage needle (38 mm) was used for large mice (body weight more than 15 g). The ribose dosage was equivalent to that used previously in human studies with no adverse effect (Hellsten et al., 2004) corrected for mouse metabolism according to FDA guidelines (Volunteers, 2002). Each treatment group (n=24) was further separated into sedentary (SED; n=12) and exercised (EX; n=12) groups. Forced treadmill running (12 m/min for 30 min) was applied to a subset of all WT and *mdx* treatment groups from 4 weeks of age to prevent the spontaneous remission of muscle degeneration normally seen in *mdx* mice, and promote disease severity (De Luca et al., 2003), which concluded in the final (8^{th}) week. Food and water consumption were monitored throughout the entire experiment.

5.2.3. Moderate exercise training and exhaustion protocol test

Moderate exercise training commenced at 4 weeks age (WT and *mdx* EX mice only) using a rodent treadmill system (Panlab Harvard Apparatus, Barcelona, Spain). EX mice underwent forced treadmill running 2 x weekly with at least two days rest in between training sessions (total of 7 sessions). Mice were placed on the treadmill with no slope and acclimatised for 5-10 m at a slow pace, before ramping to 12 m/min for 30 min commenced. The final (8^{th}) session was replaced with an exhaustion/fatigue protocol test. Mice were acclimatised for 5 min, then the speed was slowly increased by 1 m/min until the mice were exhausted (refused to run despite gentle probing with an air jet). Stopping time was captured from the machine and recorded as the time to fatigue.

5.2.4. Forelimb grip strength and whole-body grip strength

Forelimb and whole-body grip strength were measured weekly from 3 weeks age until the experimental endpoint, as a functional marker of disease progression. Forelimb grip strength was assessed using a commercial rodent dynamometer (BioSeb, Vitrolle, France). Tests were performed in triplicate with 1 min rest between each test and data are expressed relative to body mass (g force. g^{-1} bw^{-1}). Whole body grip strength was assessed using a custom-built inverted mesh grid system. Mice were placed onto the mesh, inverted and the latency to fall was measured. This test was performed once, and data are expressed as the minimum holding impulse (latency. g^{-1} bw^{-1}).

5.2.3. Body weight and composition assessment

Body composition (fat and lean mass) was determined using echo MRI at the experimental endpoint (Echo Medical Systems, Houston, USA). Mice were placed into perspex tubes and the tubes inserted into the scanner for a non-invasive, silent scan of body composition. Data are expressed relative to body mass (g tissue. bw^{-1}).

5.2.4. Blood glucose and ketone concentrations

At the experimental endpoint, blood glucose and ketone levels were measured in the morning on blood droplets from the tail tip using the commercial handheld Rightest GM700SB glucometer (Bionime, Taiwan, China) and Lifesmart Twoplus blood ketone meter (Lifesmart Healthcare, Punjab, India).

5.2.5. Surgery

At the experimental endpoint at 8 weeks age, mice were deeply anesthetized via isoflurane inhalation (4% induction, 2% maintenance). Skeletal muscles of interest were removed in the following order: left and right flexor digitorum brevis (FDB) muscles for the assessment of mitochondrial function, left extensor digitorum longus (EDL) and soleus for the measurement of ex vivo contractile properties, left and right gastrocnemius (GAS) or western blot analyses, and quadriceps (QUADS) for quantitation of muscle metabolites. Other muscle and organs were excised in the following orders: tibialis anterior (TA), plantaris, diaphragm, heart, lungs, liver, spleen, duodenum colon, kidneys and brain, these muscle and organs immediately weighed and snap frozen in liquid nitrogen for further analysis.

5.2.6. Ex vivo contractile properties

Ex vivo assessment of skeletal muscle contractile function was performed as described by us previously (Timpani et al., 2020) using predominantly fast-twitch EDL and slow-twitch SOL muscles. Muscles were tied onto surgical silk loops (4-0), dissected from the hindlimb, then placed into baths within a DMT myography system (DMT, Denmark) containing Kreb's solution (NaCl 118 mM, $MgSO_4 \cdot 7H_2O$ 1 mM, KCl 4.75 mM, Na_2HPO 1 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 24 mM and glucose 11 mM; pH 7.4) bubbled with carbogen (5% CO_2 in O_2) at 30^{o}C. Once the optimal length for each muscle was determined, a force-frequency protocol was performed by stimulating muscles at increasing frequencies (i.e., 10, 20, 30, 40, 50, 60, 80, 100, 120, 150 and 180 Hz) with a 3 min rest period in between each stimulation. After force-frequency was determined, EDL and soleus was stimulated 3

times to analyse the basic contractile properties (I.e. peak twitch force (P_t), peak tetanic force (P_o), twitch to tetanic ratio (P_t/P_o), Time to peak (TTP) and half relaxation time (½ RT). The train duration of pulses was 350 ms and 500 ms for the EDL and SOL muscles, respectively.

5.2.7. Mitochondrial and anaerobic metabolism

Seahorse extracellular flux analysis (Agilent, Mulgrave, Australia) was used to quantitate mitochondrial and anaerobic metabolism as described by us previously (Timpani et al., 2020). FDB muscles were incubated in prewarmed dissociation media for 1hr and 45 min (37^{o}C, 5% CO_2), then FDB bundles were transferred into the incubated medium, triturated and plated onto Seahorse XF24 cell culture V7 microplates (Agilent, VIC, AUS). A Seahorse Bioscience XF24 Analyser were used to measure the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in response to inhibitor and uncoupler drugs. OCR was measured as an indicator of mitochondrial OXPHOS capacity, whereas ECAR was measured as an indicator of anaerobic glycolysis.

5.2.8. Western blotting

Western blot was performed to quantitate protein biomarkers of oxidative, ER and hypoxia stress signaling pathways in snap frozen GAS muscles as described in Section 3.1. All antibodies used are described in Table 3.1, Section 3.1.

5.2.9. Muscle and plasma metabolites

Following surgical excision of skeletal muscles, the remaining blood volume was taken via cardiac puncture and transferred to lithium heparin microtubes. Whole blood was transferred to eppendorfs and centrifuged for 5 min at 3,000g. Plasma was decanted and snap frozen. Plasma metabolites (inosine, Hx, xanthine and uric acid) were quantitated in thawed plasma via HPLC as described in Section 3.3. Snap-frozen QUADS were used to analyse muscle metabolites (creatine, PCr, TCr and ATP) using xMrak spectrophotometer as described in Section 3.3.

5.2.10. Statistics

All data are presented as mean ± standard error of the mean (SEM). Data were analysed using GraphPad Prism v8 (GraphPad Software, San Diego, CA, USA). A three-way ANOVA with Turkey's post-hoc test was utilised to detect main effects and interactions between strain (WT versus *mdx*), treatment (VEH versus ribose) and activity (SED versus EX). An α value of 0.05 was considered a significant difference and an α value between 0.05 and 0.1 was considered a statistical trend.

5.3 Results

5.3.1. Effect of EX training and RIB treatment on health indices

We assessed the effect of EX training and RIB treatment on body weight, body composition, food and water consumption in WT and *mdx* male mice (Figure 5.1 and Figure 5.2). Although EX was used in this study to promote metabolic stress and exacerbate muscle disease in *mdx* mice, it was pertinent to assess whether it also led to training adaptations in both WT and *mdx* mice. EX significantly reduced fat mass and trended to increase lean mass in both strains *(p*<0.01, Figure 5.1 C and *p*=0.073, Figure 5.1D, respectively) compared to SED groups. Compared to VEH groups, RIB treatment significantly reduced the fat mass of both WT and *mdx* mice (*p*<0.05, Figure 5.1C) but had no impact on lean tissue mass. *Mdx* mice had a lower proportional fat mass and higher lean mass than WT mice (*p*<0.0001). There were no significant differences in growth, e.g., body weight accumulation, between any groups during the experiment (*p*>0.1, Figure 5.1A and B) nor were there differences in food and water consumption (*p*>0.1, Figure 5.2 A, B, C, and D). These data demonstrate RIB treatment has no obvious adverse effects in mice over 5 weeks of oral treatment.

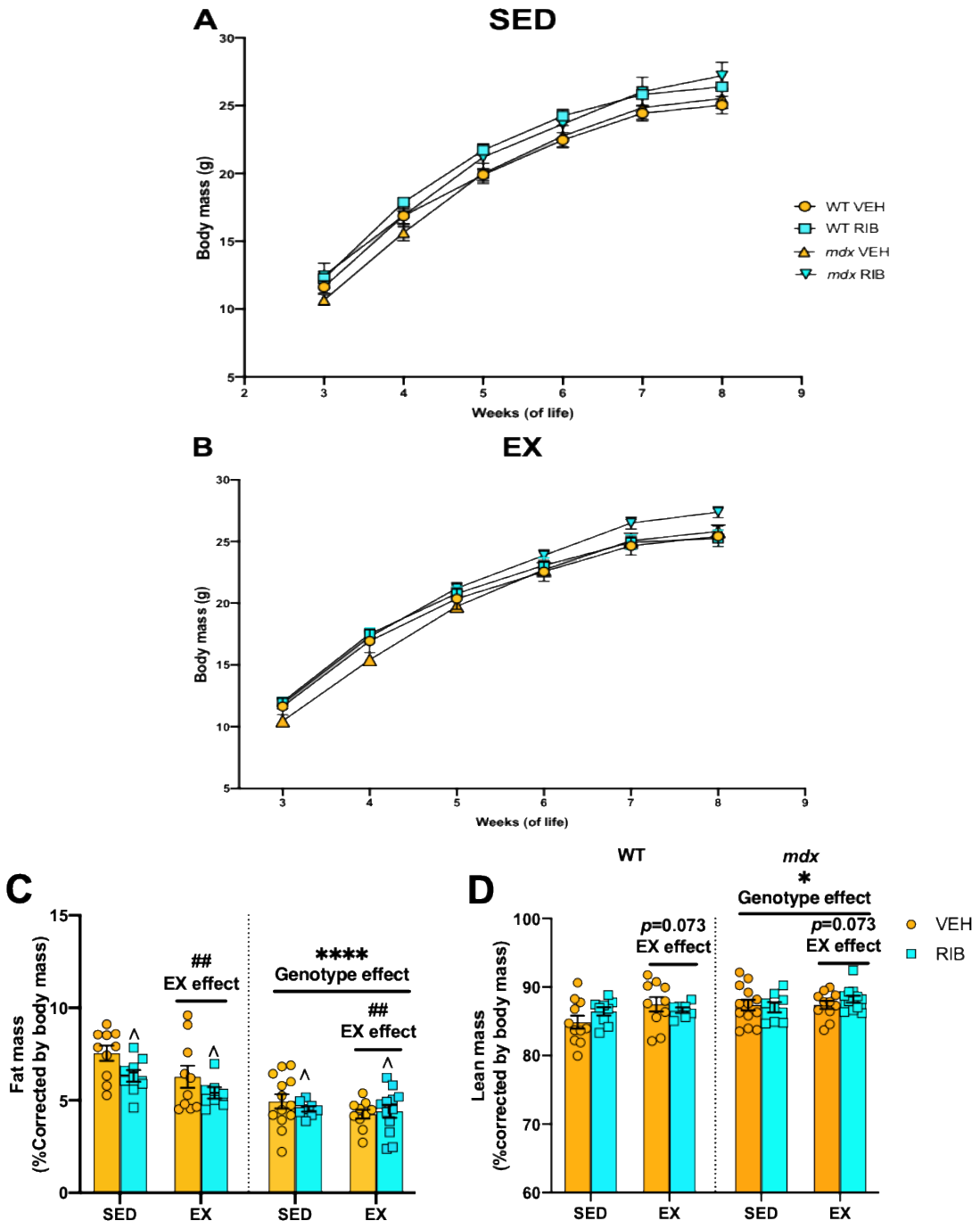


Figure 5-1 Body weight and body composition of RIB supplementation combined with moderate exercise training throughout the entire experiment period in WT and mdx male mice. There were no differences in body weight in any groups. Fat (C) and lean mass were (D) presented corrected for body mass. Data is presented as mean of each group and error bars represent standard error of the mean. *p<0.05, *****p<0.0001 WT versus mdx; $^{\#\#}p$<0.01 EX versus SED; ^p<0.05 RIB versus VEH; p<0.1 means there is a trend compared to SED mice.

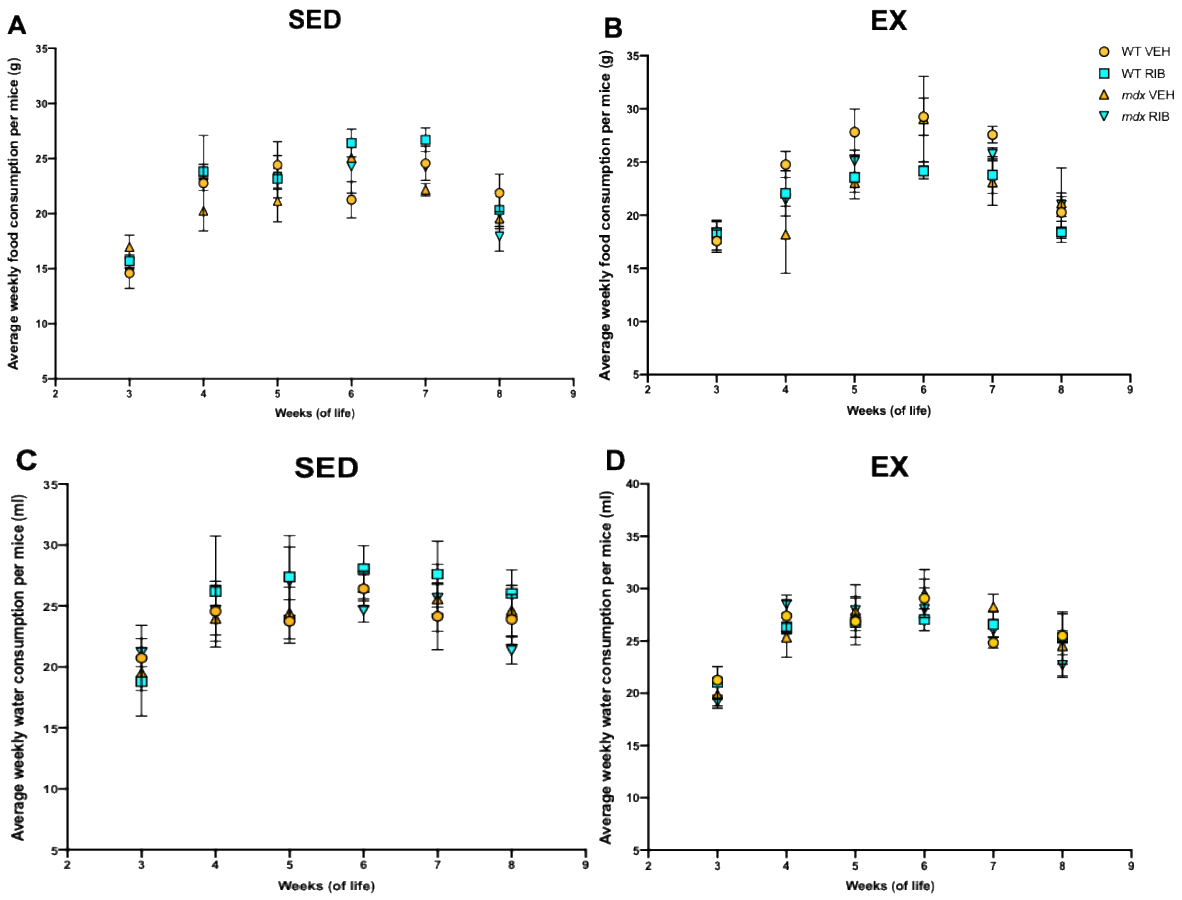


Figure 5-2 Average of food and water consumption of RIB supplementation combined with moderate exercise training throughout the entire experiment period in WT and *mdx* male mice. There are no differences in average food and water consumptions in any groups. n= 7-10 for RIB mice and n= 10-12 for VEH mice.

5.3.2. Effect of EX and RIB treatment on skeletal muscle and organ mass

At the experimental endpoint, muscle and organs mass were excised and weighed (Table 5.1). We found that RIB treatment induced higher EDL (~18.07% increase), soleus (~31.33% increase) and heart mass (~8.05% increased) compared to VEH groups (p<0.05). However, diaphragm mass was ~11.05% lower in RIB groups compared to VEH groups (p<0.05). Hindlimb muscle weights (GAS, TA and QUADS) and liver weights were significantly increased in *mdx* mice (p<0.05) compared to WT groups, whereas brain and lungs weights were significantly decreased in *mdx* mice (9.12% and 8.99%, respectively, p<0.05). There was no effect of RIB treatment on plantaris, colon, duodenum, kidney and spleen weights.t (p>0.1). Exercise training had no significant effect on the mass of any tissue (p>0.1) compared to SED group.

	WT				*mdx*				*p* value		
	VEH		RIB		VEH		RIB		Genotype	EX	Supplement
	SED	EX	SED	EX	SED	EX	SED	EX			
EDL	0.42± 0.03	0.36± 0.02	0.47± 0.04^^	0.48± 0.03^^	0.43± 0.03	0.45± 0.04	0.47± 0.04^^	0.54± 0.03^^	0.6246	0.1884	0.0037
Soleus	0.36± 0.06	0.37± 0.04	0.43± 0.03^^	0.45± 0.08^^	0.38± 0.03	0.39± 0.03	0.51± 0.06^^	0.58± 0.06^^	0.4252	0.1729	0.0012
GAS	5.63± 0.19	5.94± 0.15	5.51± 0.12	5.67± 0.13	6.76± 0.26****	6.62± 0.21****	6.18± 0.12****	6.20± 0.31****	<0.0001	0.7817	0.9400
TA	1.75± 0.10	1.78± 0.10	1.62± 0.12	1.72± 0.07	2.43± 0.11***	2.49± 0.06***	2.48± 0.08***	2.32± 0.06***	<0.0001	0.7684	0.3693
QUADS	6.12± 0.29	5.71± 0.24	6.03± 0.35	5.86± 0.25	8.33± 0.74***	7.86± 0.06***	6.11± 0.75***	7.25± 0.78***	<0.0001	0.5242	0.6106
Plantaris	0.83± 0.10	0.84± 0.05	0.84± 0.05	0.91± 0.06	0.93± 0.04*	0.92± 0.02*	0.93± 0.03*	0.94± 0.04*	0.0299	0.2232	0.2999
Heart	5.23± 0.12	5.26± 0.11	5.80± 0.17	5.66± 0.17	4.82± 0.15**	5.35± 0.11**	5.16± 0.12**	4.91± 0.06**	0.0056	0.2178	0.7002
Diaphragm	2.32± 0.34	2.13± 0.29	2.90± 0.21^^	3.09± 0.42^^	3.34± 0.24****	3.59± 0.24****	3.94± 0.21****^^	3.53± 0.35****^^	<0.0001	0.7625	0.0040
Liver	46.59± 1.73	48.24± 1.34	45.60± 2.17	46.27± 2.17	49.77± 2.07****	54.65± 1.22****	52.57± 1.42****	50.01± 3.38****	<0.0001	0.1634	0.6321
Spleen	3.34± 0.08	3.46± 0.06	3.29± 0.14	3.53± 0.23	3.30± 0.10	3.47± 0.07	3.61± 0.41	3.59± 0.26	0.6343	0.9428	0.1608
Duodenum	1.62± 0.25	1.65± 0.36	1.59± 0.26	1.49± 0.14	1.54± 0.20	1.17± 0.25	1.47± 0.37	1.28± 0.30	0.8590	0.2806	0.8785
Colon	1.15± 0.17	1.23± 0.20	0.94± 0.06	0.95± 0.05	1.21± 0.21	1.14± 0.17	1.06± 0.09	1.07± 0.22	0.3929	0.4252	0.3170
Brain	18.34± 0.40	18.82± 0.60	17.38± 1.09	17.63± 0.65	15.97± 0.48***	16.70± 0.64***	16.49± 0.28***	16.43± 0.24***	0.0004	0.8228	0.4280
Kidney	6.67± 0.23	6.94± 0.23	6.39± 0.39	6.99± 0.57	7.27± 0.14	7.12± 0.17	7.02± 0.13	6.81± 0.52	0.0572	0.4187	0.2168
Lungs	7.91± 0.64	8.00± 0.39	7.91± 0.63^^	8.55± 0.36^^	6.49± 0.33**	7.11± 0.39**	7.56± 0.31**^^	8.30± 0.42**^^	0.0034	0.4920	0.0046

Table 5-1 Weights of muscles and organs from VEH and RIB groups throughout the entire experiments. Endpoint muscle ad organs mass of WT and *mdx* mice treated with and without RIB and EX. All weights are expressed relative to body mass ratio (mg/g) and data are presented as mean ± SEM. EDL, Soleus, GAS: gastrocnemius, TA: tibialis anterior, QUADS: quadriceps, plantaris, kidney mass were presented as the means of left and right side. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, WT versus *mdx* mice, $^{\#\#}p$<0.01 EX versus SED mice, ^p<0.05, ^^p<0.01 RIB versus VEH mice, p<0.1 means there is a trend between individual groups. n= 7-10 for RIB mice and n= 10-12 for VEH mice.

5.3.3. Effect of EX and RIB treatment on muscle strength and fatigue threshold

DMD patients, and even maternal carriers of the dystrophin gene mutation that do not manifest progressive muscle wasting, are more susceptible to muscle weakness and fatigue during physical exertion (Duan et al., 2021). To assess the impact of metabolic stress on physical activity capacity, we subjected EX mice to a treadmill fatigue test to exhaustion in lieu of their final training run. *Mdx* mice were 1.5-times more fatigable than WT mice, consistent with reduced metabolic capacity ($p<0.001$, Figure 5.3C). RIB treatment significantly improved the time to exhaustion in *mdx* mice by 2-fold ($p<0.05$, Figure 5.3C), but had no effect in WT mice. We also found that RIB treatment significantly increased the forelimb grip strength of *mdx* mice ($p<0.01$, Figure 5.3A) and there was a positive trend for the same effect in WT mice ($p=0.073$, Figure 5.3A). However, there was no notable effect of RIB or genotype on the whole body holding impulse ($p>0.1$, Figure 5.3B).

5.3.4. Effect of EX and RIB treatment on contractile properties

To evaluate the muscle contractile function, excised EDL and soleus muscles were measured at the endpoint of the experiment. No significant differences were observed in any group in the force-frequency relationship of either EDL or soleus ($p>0.1$, Figure 5.4 A, B, E, F). Interestingly, there was a main effect of RIB treatment to increase the absolute force production of soleus muscle ($p<0.0001$, Figure 5.4 G) compared to VEH both in WT and *mdx* strains. There was no genotype or EX effect on muscle contractile functions though ($p>0.1$, Figure 5.4). As summarised in Table 5.2, RIB treatment significantly increased P_O both in WT and *mdx* mice compared to VEH groups ($p<0.001$). It was found that neither EX training nor RIB treatment impacted on L_O, P_t and ratio of P_t to P_O ($p>0.1$). As showed in Figure 5.4, fatigue resistance was not improved by RIB in the EDL ($p>0.1$, Figure 5.4I, G). However, in the soleus, fatigue resistance was improved during the later stage of fatigue run when RIB treatment and EX training were combined ($p<0.05$, Figure 5.4L).

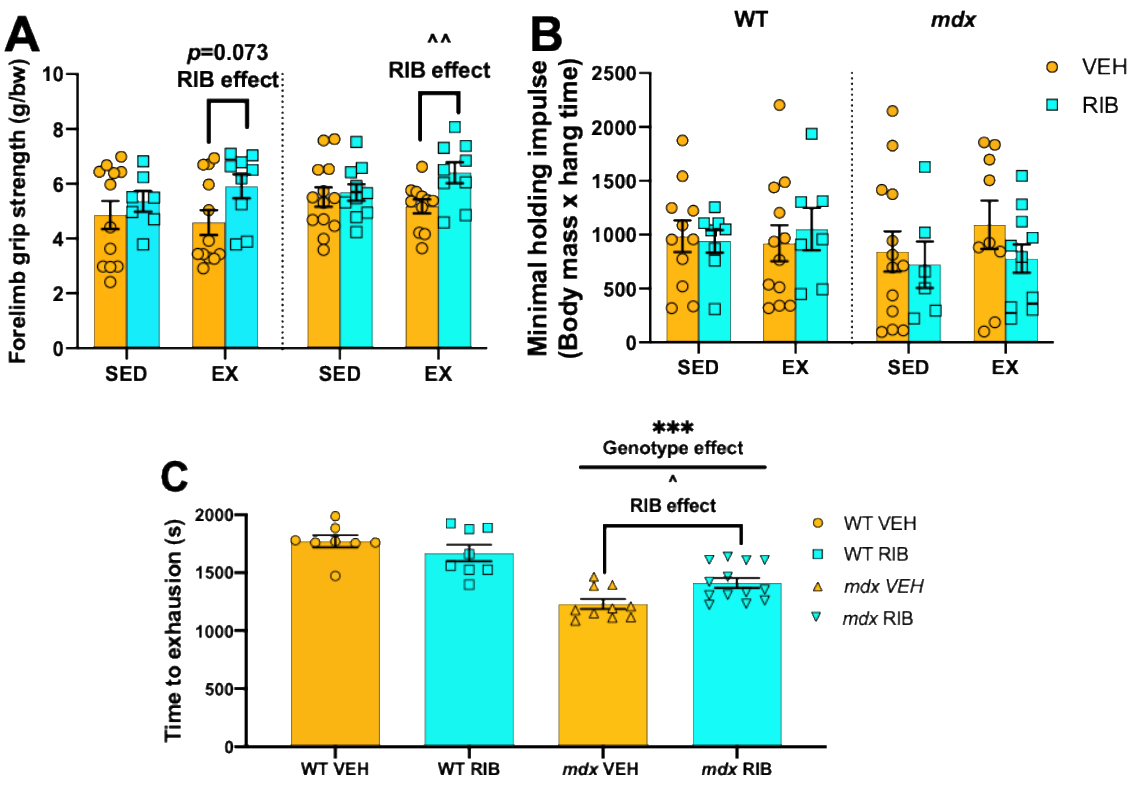


Figure 5-3 Effect of EX training and RIB treatment on clinical indices and fatigue run test. Forelimb (A) and Whole-body grip strength (B) was measured at the final (8th) week of entire experiment and all the mice from EX groups were forced to run on treadmill until exhaustion (C) at last training session. RIB significantly increased forelimb grip strength and fatigue resistance in *mdx* mice and trended to increase forelimb grip strength in WT EX group compared to VEH. WT mice run longer than *mdx* mice. Neither RIB nor EX had no effect on whole body holding impulse. ***p<0.001 WT versus mdx; ^p<0.05, ^^p<0.01 RIB versus VEH; p<0.1 means there is a trend between individual groups.

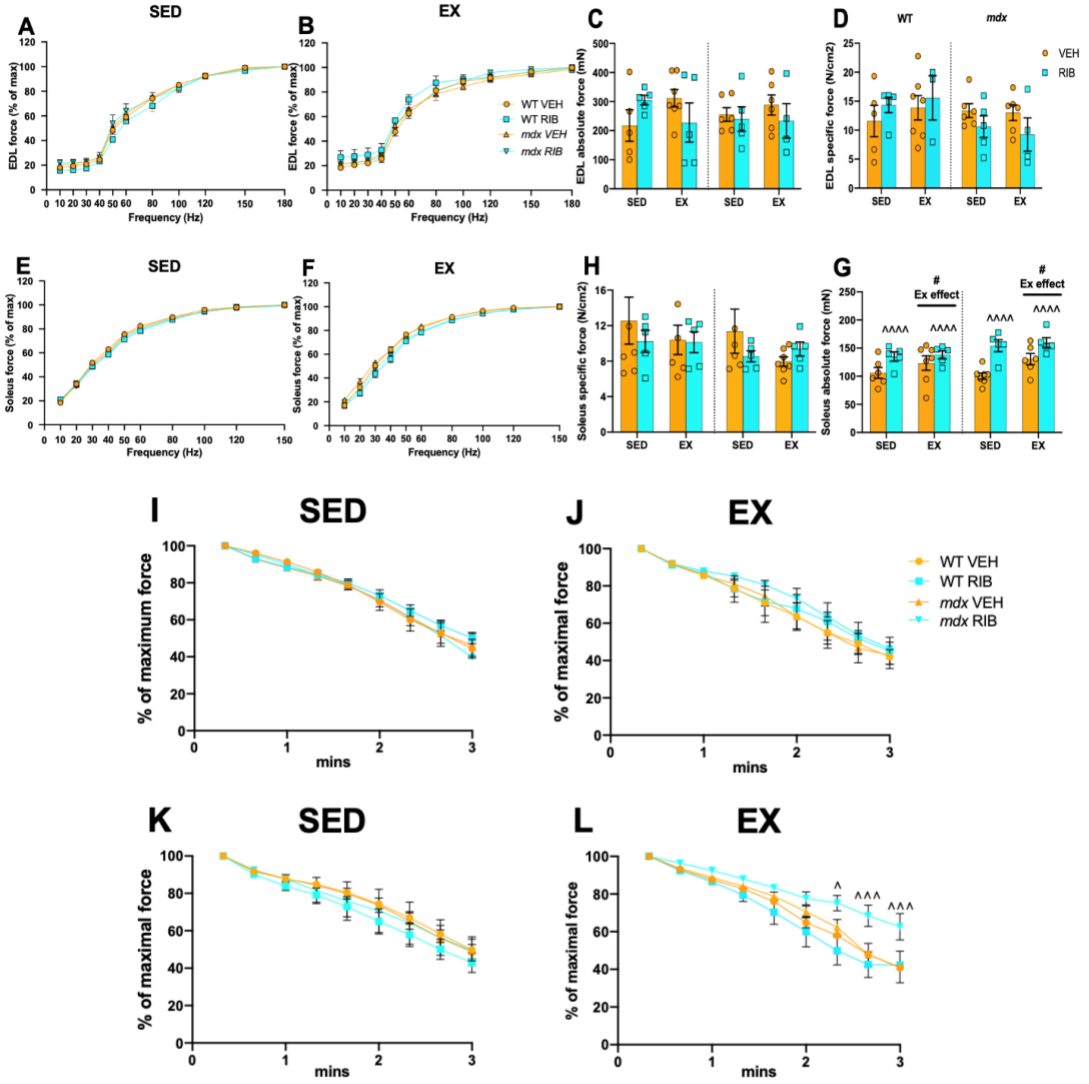


Figure 5-4 Effect of EX training and RIB treatment on skeletal muscle contractile function. EDL and soleus muscles underwent ex vivo assessment of contractile parameters, with force-frequency (A, B, E, F) and absolute (C, G) and specific force (D, H) were determined. Additionally, EDL (I, J) and soleus (K, L) muscle underwent fatigue protocol to evaluate the muscle fatigability. RIB treatment increased the absolute force and P_o in soleus and not EDL muscles, combination effect of EX training and RIB treatment improved force production in the later stage of fatigue run. All values are presented as mean ± SEM. ##p<0.01 EX versus SED; ^p<0.05, ^^^p<0.001 ^^^^p<0.0001 RIB versus VEH.

Parameters	WT				*mdx*				*p* value		
	VEH		RIB		VEH		RIB		Genotype	EX	Supplement
	SED	EX	SED	EX	SED	EX	SED	EX			
EDL											
L_o (mm)	11.3 ±0.03	11.6 ±0.03	11.5 ±0.03	11.4 ±0.05	11.4 ±0.04	11.6 ±0.02	11.3 ±0.01	11.7 ±0.03	0.9106	0.2735	0.1877
P_t (mN)	35.28 ±9.6	42.7 ±7.44	43.9 ±4.0	56.08 ±8.7	45.4 ±5.8	59.2 ±14.9	41.2 ±11.8	44.2 ±14.9	0.5331	0.2586	0.9687
P_o (mN)	278.5 ±44.3	278.2 ±34.2	285.0 ±16.6	246.7 ±48.1	254.6 ±23.7	238.3 ±42.2	240.0 ±42.4	233.12 ±59.7	0.4576	0.9255	0.7821
P_t/P_o	0.16 ±0.02	0.15 ±0.02	0.15 ±0.02	0.22 ±0.03	0.18 ±0.01	0.15 ±0.04	0.016 ±0.03	0.18 ±0.02	0.4569	0.1375	0.3475
TTP	22.22 ±3.78	23.55 ±3.28	21.98 ±4.40	25.59 ±3.90	22.24 ±3.85	21.40 ±3.61	19.85 ±2.23	25.35 ±4.45	0.1533	0.9182	0.3255
½ TP	13.84 ±0.65	13.86 ±0.58	13.24 ±1.03	12.01 ±0.70	13.63 ±0.62	14.63 ±1.10	14.23 ±2.03	14.60 ±1.07	0.4173	0.7990	0.3147
Soleus											
L_o (mm)	10.8 ±0.03	11.1 ±0.03	10.6 ±0.04	10.4 ±0.03	10.4 ±0.03	11.3 ±0.08	10.2 ±0.06	10.6 ±0.05	0.8472	0.2125	0.1670
P_t (mN)	19.9 ±3.3	17.1 ±1.9	17.1 ±2.9	18.4 ±1.5	16.1 ±2.4	17.0 ±1.7	18.5 ±2.7	22.6 ±2.7	0.6925	0.8356	0.9158
P_o (mN)	105.7 ±9.7	122.7 ±12.7	135.4 ±9.7^^^	137.9 ±7.2^^^	109.9 ±11.4	112.5 ±12.5	154.3 ±10.6^^^	159.8 ±8.7^^^	0.6286	0.6429	0.0005
P_t/P_o	0.16 ±0.01	0.13 ±0.02	0.13 ±0.02	0.14 ±0.01	0.16 ±0.03	0.12 ±0.03	0.12 ±0.02	0.14 ±0.02	0.8264	0.7546	0.2193
TTP	38.35 ±2.31	39.99 ±2.74	44.08 ±3.25	37.55 ±1.89	37.05 ±2.63	37.92 ±2.21	38.82 ±1.53	40.88 ±2.86	0.1884	0.7713	0.6550
½ TP	15.78 ±1.25	12.42 ±0.68	11.45 ±0.62	13.06 ±1.42	15.3 ±1.97	14.27 ±1.20	14.23 ±0.74	14.74 ±1.80	0.2027	0.3854	0.1653

Table 5-2 Comparison of contractile parameters between all groups. RIB treatment increased Po in soleus and not EDL muscles. Single twitch properties including EDL= extensor digitorum longus; Lo= optimal length; Pt= single twitch force; Po= absolute tetanic force production; Pt/Po= twitch to tetanus ratio were also evaluated for EDL and soleus muscles. ^^^ p<0.001 RIB versus VEH.

5.3.5. Effect of EX and RIB treatment on metabolic stress indices

5.3.5.1. Mitochondrial function

We examined the effect of EX and RIB treatment on parameters of mitochondrial functions in FDB fibres in WT and *mdx* mice (Figure 5.5). The oxidative metabolic potential (% of basal OCR, Figure 5.5A) and glycolytic metabolic potential (% of basal ECAR) were firstly determined. We found that RIB treatment trended to reduce the ECAR metabolic potential (% of basal ECAR, Figure 5.5B) in *mdx* mice compared to VEH in EX groups (p=0.074, Figure 5.5B), but neither EX nor RIB treatment had an effect on OCR (p>0.1, Figure 5.5A). Next, we generated metabolic phenograms depicting the response of metabolism to simulated metabolic stress (chemical mitochondrial uncoupling) (Figure 5.5E), where the run indicates bio-energetical capacity and the slope indicates whether oxidative of anerobic metabolism is more influential. *Mdx* FDB fibres were more energetic in the basal state than WT fibres and were more dependent on anaerobic glycolysis following EX, but overall, they were less responsive during simulated metabolic stress. RIB treatment increased the overall bio-energetical capacity of WT EX and SED groups but had no impact on either oxidative or anaerobic contribution. RIB treatment had no effect on metabolic capacity of *mdx* FDB fibres (Figure 5.5E). There were no differences in mitochondrial coupling efficiency (p>0.1, Figure 5.5C) or ATP production (p>0.1, Figure 5.5D) in FDB fibres in any groups.

5.3.5.2. Muscle metabolites

To investigate metabolic stress at the muscle level, we assessed ATP and metabolites of the creatine phosphagen system that recovers ADP to ATP in the cytosol and supports the exchange of ADP and ATP across the mitochondrial membranes in QUADS (Figure 5.6). ATP levels were significantly reduced in *mdx* QUADS (p<0.01, Figure 5.6A) consistent with metabolic stress but there was no effect of RIB on them. Total Cr (Cr + PCr) levels were normal in mdx mice and there was a tendency for RIB to increase them in the *mdx* EX group only (p=0.074, Figure 5.6B).

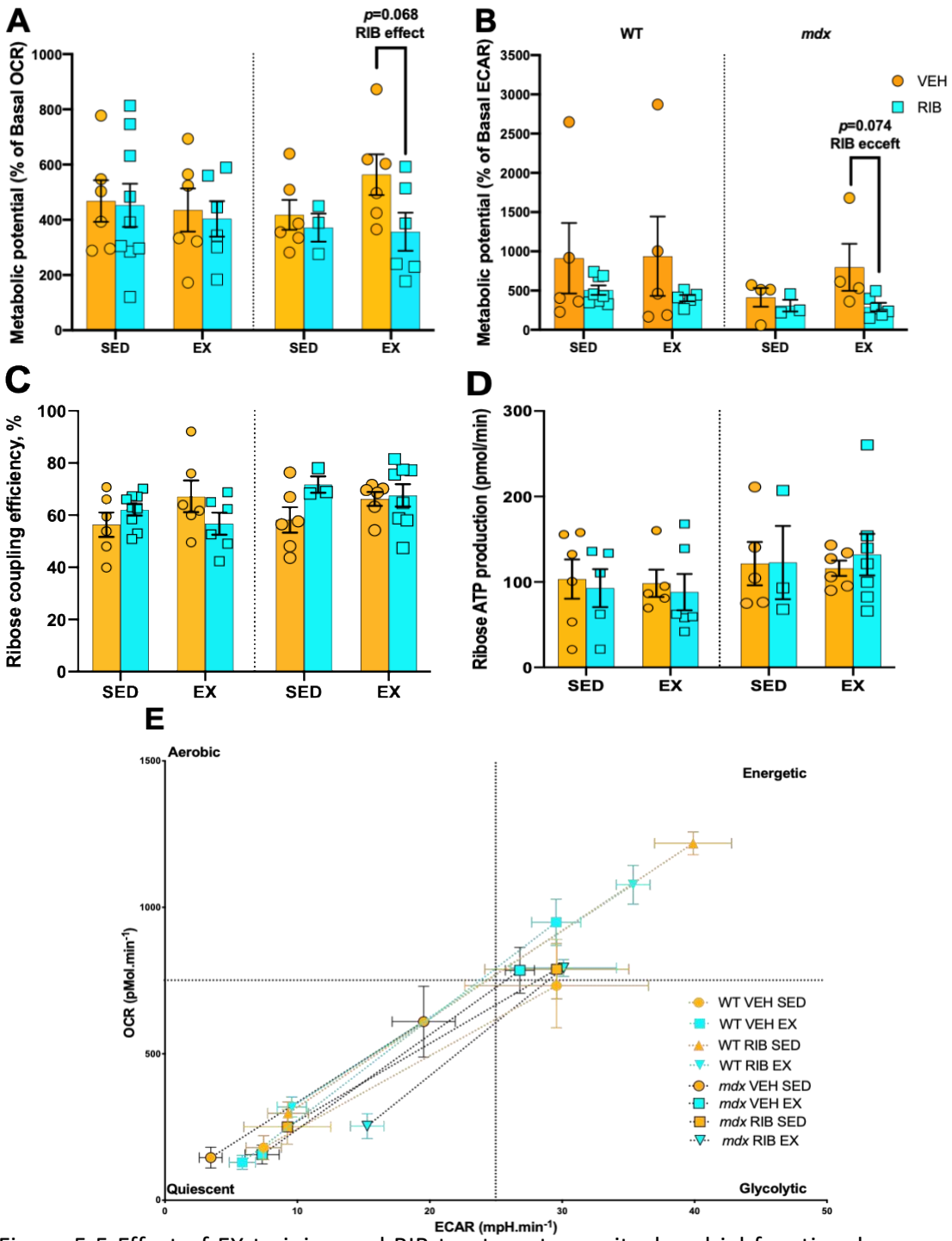


Figure 5-5 Effect of EX training and RIB treatment on mitochondrial functional parameters. No significant difference in oxidative (A), glycolytic (B) metabolic potential, coupling efficiency (C) and ATP production (D) were observed between EX training and RIB treatment mice, except RIB treatment decreased glycolytic metabolic potential (B) compared to VEH in *mdx* EX group. The overall gain in bio-energetical function induced by EX training or RIB treatment is observed in (E). $p<0.1$ there is a trend between individual groups.

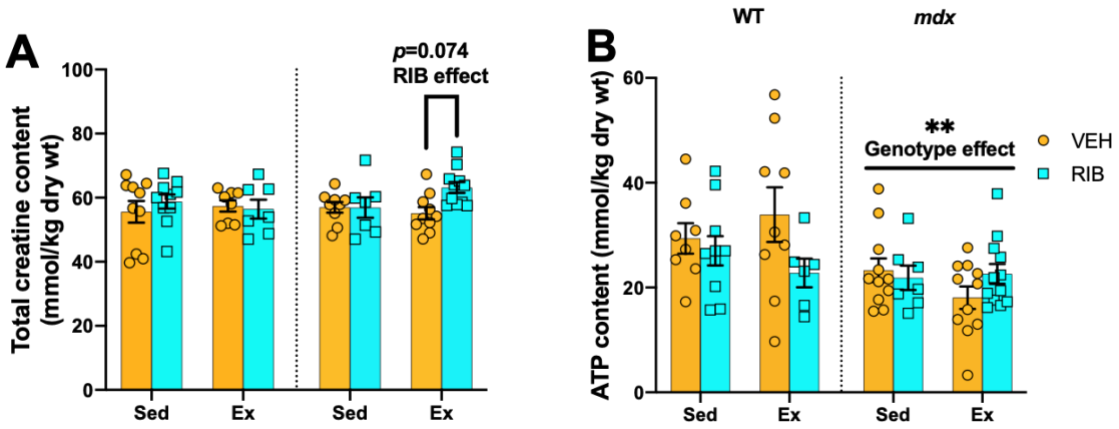


Figure 5-6 Effect of EX training and RIB treatment on muscle metabolites. Snap frozen QUADS muscle were used to determine TCr (Cr + PCr, A) and ATP levels (B). EX training had no effect in TCr and ATP contents. RIB had a positive trend in TCr level and no effect in ATP levels. We found that *mdx* mice had lower ATP levels compared to WT group. $^{**}p<0.01$ *mdx* versus WT. $p<0.1$ there is a trend between individual groups.

5.3.5.3. Blood glucose and ketones and plasma metabolites

To assess biofluid markers of systemic metabolic stress, we first assessed the effect of RIB and EX on unfasted a.m. blood glucose and ketone levels in WT and *mdx* mice (Figure 5.7). M*dx* mice had lower unfasted blood glucose and ketone levels than WT mice ($p<0.001$ main genotype effect, Figure 5.7A and $p<0.05$ main genotype effect, Figure 5.7B, respectively) indicative of a higher metabolic rate/energy expenditure in *mdx* mice following the nocturnal activity period. RIB significantly increased unfasted blood ketones levels in WT EX VEH mice ($p<0.001$, Figure 5.7B), but had no effect in the *mdx* groups, whereas EX significantly decreased unfasted blood ketones levels in WT groups compared to SED. There was no effect of EX training or RIB treatment on unfasted blood glucose levels in WT or *mdx* mice ($p>0.1$, Figure 5.7A and B).

The degradation of purines during metabolic stress results in the efflux of xanthine and hypoxanthine from muscle and accumulation of uric acid via xanthine oxidoreductase activity. Consistent with the lower ATP levels within – and increased purine degradation product flux out of – muscle, *mdx* mice trended to have higher plasma uric acid concentration compared to WT mice ($p=0.081$, Figure 5.7C). Although RIB had no effect on muscle ATP levels, it did significantly reduce plasma uric acid concentration in *mdx* mice ($p<0.05$, Figure 5.7C). There were no differences in xanthine or inosine concentration in *mdx* compared to WT mice ($p>0.1$, Figure 5.7D and E). Although we attempted, Hx could not be detected in plasma from any mice.

5.3.6. Effect of EX and RIB treatment on markers of stress cell signaling

5.3.6.1. Oxidative stress markers

Nrf2 can be activated by both metabolic and oxidative stressors (as well as others) and involves key events, including the sequestration of Keap1 by p62, and phosphorylation of p62 to stimulate autophagic degradation of the Keap1/p62 complex. This frees Nrf2 to enter the nucleus and transcribe the phase II antioxidant response, in which NQO1, catalase and HO1 are upregulated. We assessed the activation of the Nrf2 antioxidant response in GAS muscle via western blot (Figure 5.8).

*K*eap1 and p62 protein expression were increased in *mdx* compared to WT GAS ($p<0.0001$, Figure 5.8B and D) consistent with Nrf2 activation even though Nrf2 levels were unchanged per se ($p>0.1$, Figure 5.8A). This idea was supported by the higher Nrf2/keap1 and keap1/p62 ratios in *mdx* GAS ($p<0.0001$, Figure 5.8E and F). However, p62 phosphorylation did not synergistically increase in *mdx* compared to WT GAS ($p>0.1$, Figure 5.8C) suggesting Keap1 and p62 are complexing but autophagy is not being effectively activated to degrade the complexes. Keap1 and phosphorylated p62 expression were significantly increased in response to EX compared to SED GAS in WT or *mdx* mice ($p<0.01$, Figure 5.8B and C) suggesting, although not conclusively, effective induction of autophagy. However, p62 expression per se was not increased proportionately following EX ($p>0.1$, Figure 5.8D). As such, the Keap1 to p62 ratio was increased following EX ($p=0.098$, Figure 5.8F). Interestingly, RIB significantly increased the expression of Keap1 in *mdx* compared to VEH GAS ($p<0.001$, Figure 5.8B) although neither the Nrf2 to Keap1 nor the Keap1 to p62 ratios were changed ($p>0.1$, Figure 5.8E and F).

Importantly, none of the downstream phase II antioxidant enzymes that are induced by Nrf2 transcription of the ARE were upregulated in *mdx* GAS muscle ($p>0.1$; Figure 5.9). In fact, Catalase expression was downregulated in both SED and EX *mdx* GAS compared to WT control ($p<0.01$, Figure 5.9C)**.** In contrast, EX did induce upregulation of HO1 expression in WT and *mdx* GAS ($p<0.01$; Figure 5.9A), but not of NQO1 or Catalase ($p>0.1$, Figure 5.9B and C). RIB induced HO1 expression in *mdx* ($p<0.05$, Figure 9A) but not WT GAS ($p>0.1$; Figure 5.9) highlighting its capacity to manipulate the immunometabolic nexus.

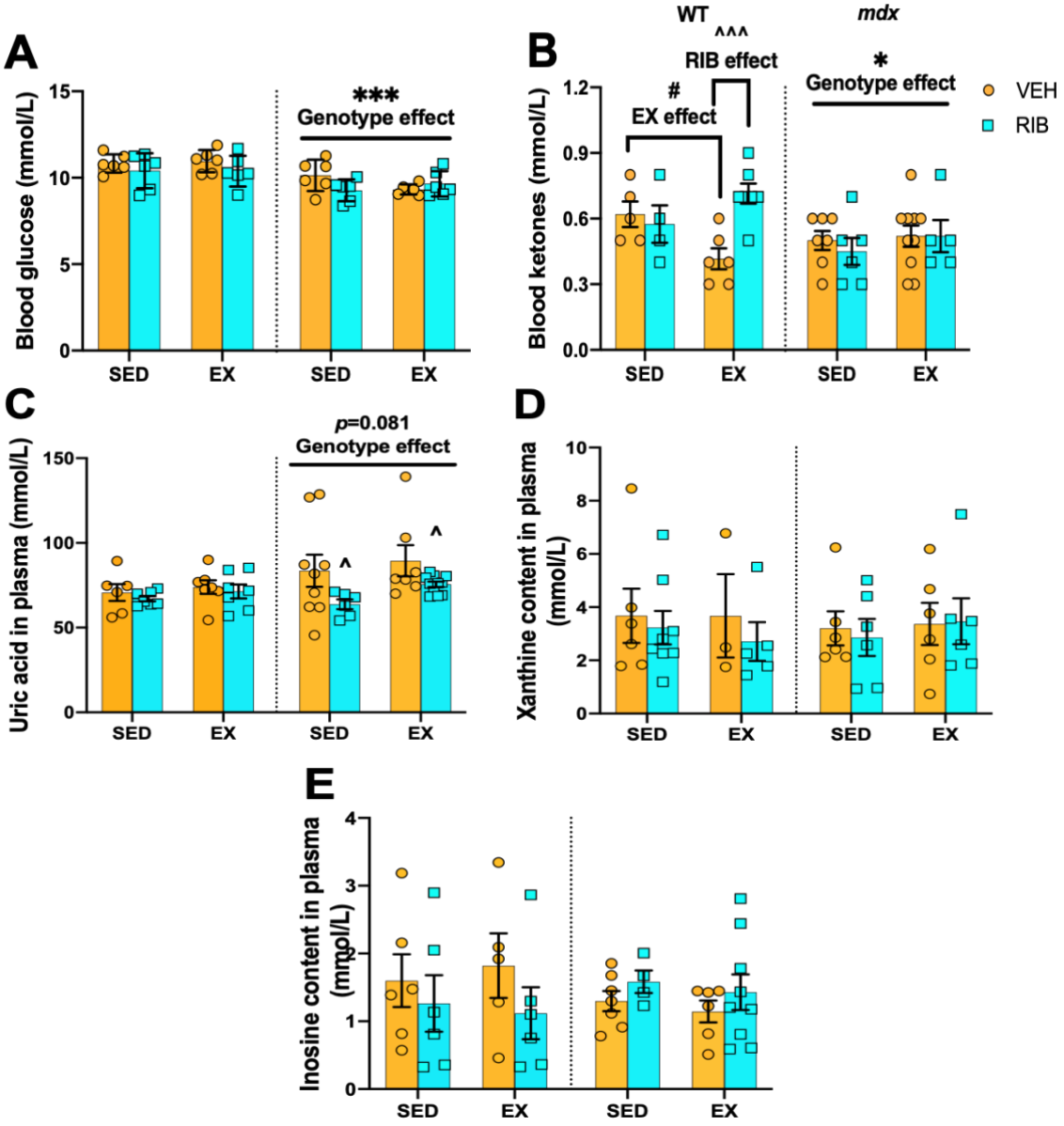


Figure 5-7 Effect of EX training and RIB treatment on unfasted blood glucose and ketone and purine metabolites. Blood glucose and ketones were measured in the morning on blood droplets from the tail tip using commercial handheld gluco-/keto-meters. Blood glucose (A) and ketone (B) levels were reduced in *mdx* mice. EX training decreased blood ketone level in WT group not in *mdx* mice. RIB treatment increased blood ketone levels in WT EX mice but not in any other group. Blood was collected by cardiac puncture for the measurement of plasma metabolites. Uric acid (C), xanthine (D) and inosine (E) were present. *mdx* mice trended to have higher uric acid and RIB treatment reduced plasm uric acid in *mdx* mice. Neither EX training nor RIB treatment impacted in plasma xanthine (D) and inosine levels (E). *p<0.05, ***p<0.001 *mdx* versus WT; $^{\#}p$<0.05 EX versus SED; ^p<0.05, ^^^p<0.001 RIB versus VEH.

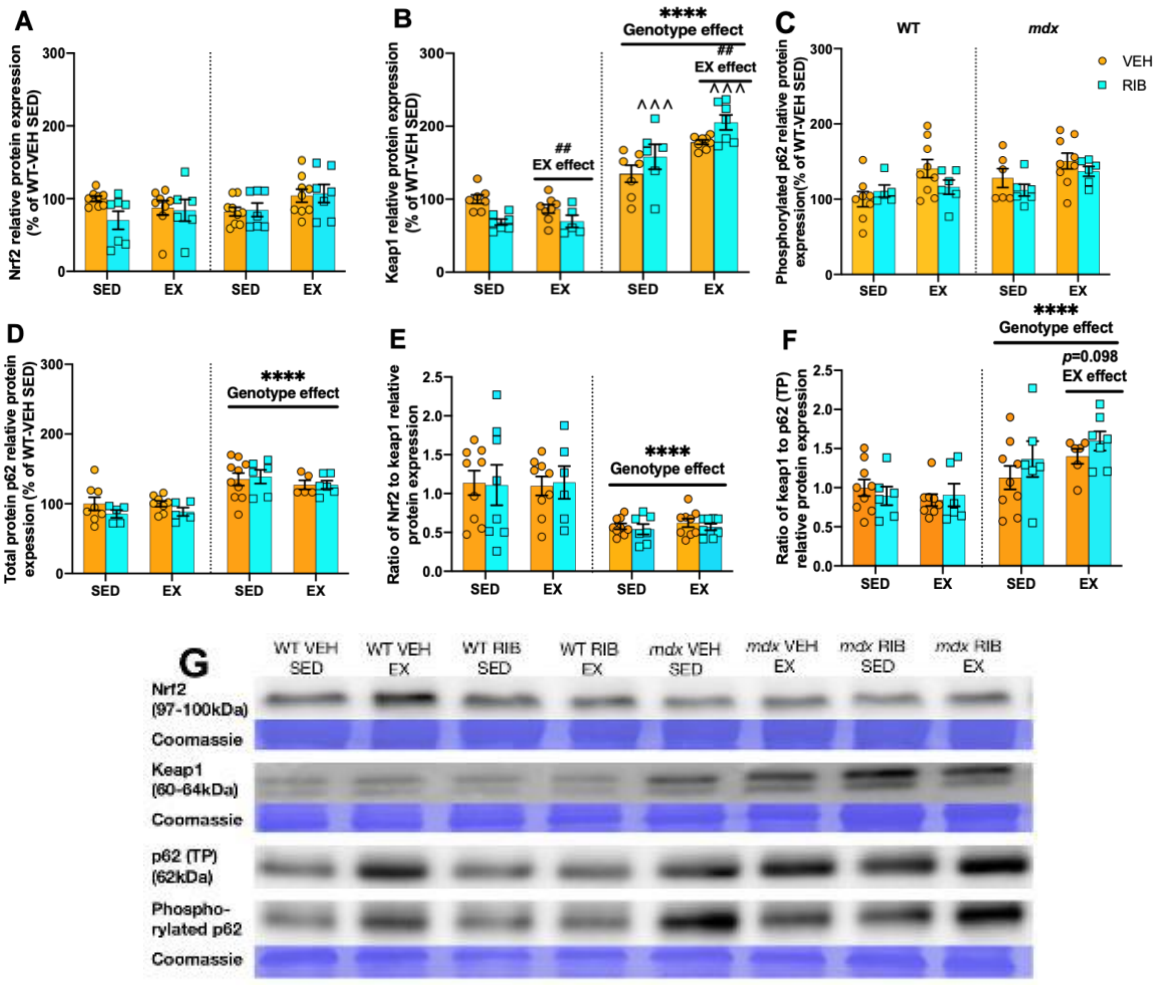


Figure 5-8 Effect of EX training and RIB treatment on the expression of Nrf2 mediated proteins in GAS muscle. Protein expression was normalised to WT VEH SED group. Neither EX training nor RIB treatment impacted in the expression of Nrf2 (A). *mdx* mice had higher Keap1 (B) and total protein p62 (D), EX training increased the expression of keap1 (B) in *mdx* mice and phosphorylated p62 (C) levels both in *mdx* and WT mice. EX training trended to increase the ratio of keap1 to p62 (F), not Nrf2 to keap1 ratio (E). Although RIB treatment increased keap1 levels (B) in *mdx* mice, had no impact on the ratio of Nrf2 to keap1 and keap1 to p62. (G) Western blot representative images are displayed alongside with a Coomassie blue representative image, which was used as the protein loading control. ****p<0.0001 mdx versus WT; $^{\#\#}p$<0.01 EX versus SED; ^^^p<0.001 RIB versus VEH; p<0.1 there is a trend between individual groups.

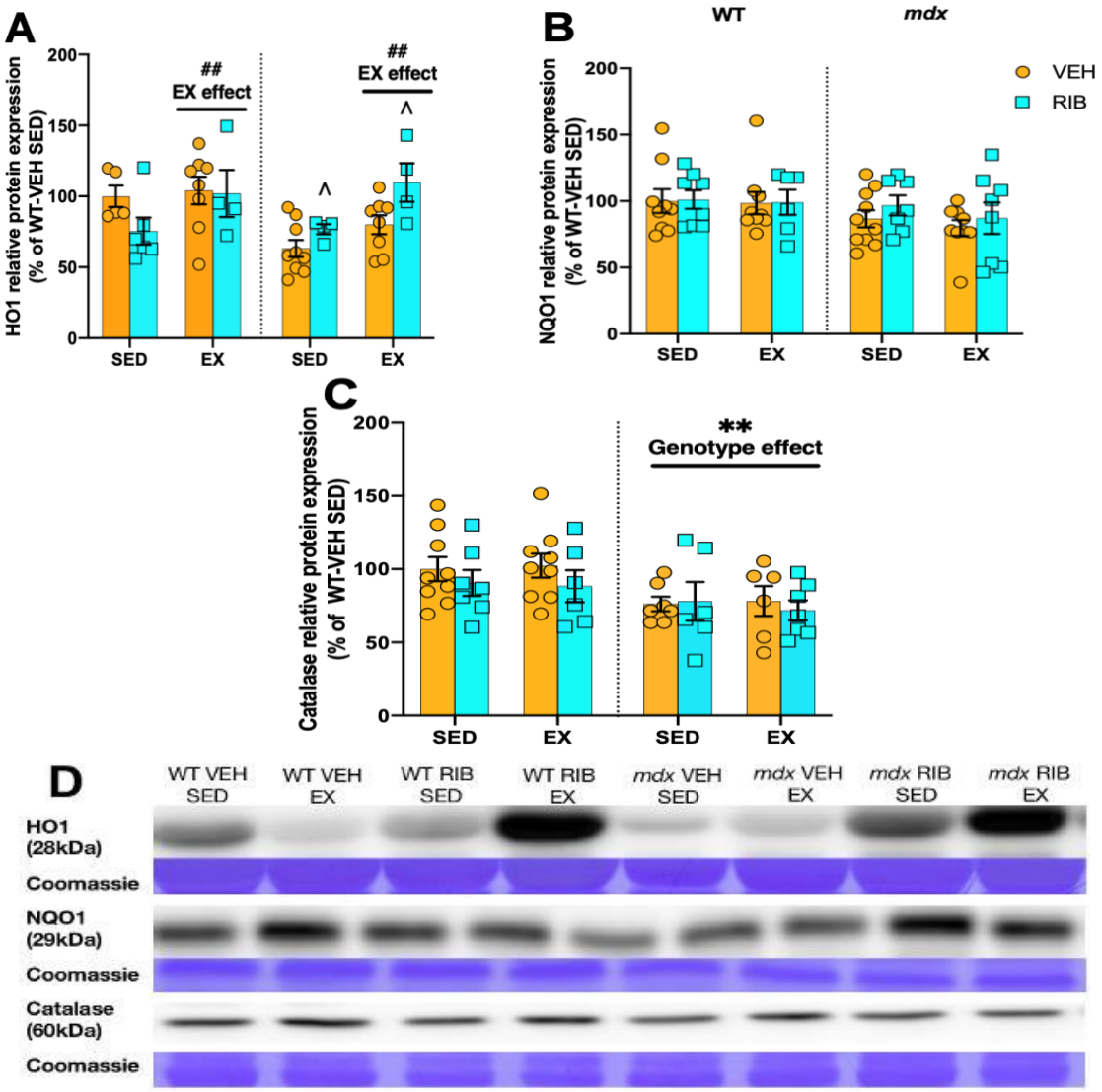


Figure 5-9 Effect of EX training and RIB treatment on Nrf2 downstream antioxidant enzymes. *mdx* mice had lower catalase (C) levels compared to WT mice. EX training increased the HO1 levels (A) both in WT and mdx GAS muscle. RIB treatment increased the expression of HO1 (A) in *mdx* mice, not in WT. Neither EX training nor RIB treatment impacted on phase II antioxidant enzymes NQO1 (B) and Catalase (C). (D) Western blot representative images are displayed alongside with a coomassie blue representative image, which was used as the protein loading control. $^{**}p<0.01$ mdx versus WT; $^{\#\#}p<0.01$ EX versus SED; $^{\wedge}p<0.05$ RIB versus VEH.

5.3.6.2. ER stress markers

ATF 4 and ATF6 are protein biomarkers of ER stress with roles in controlling the unfolded protein response and protein translation rate in response to cell stress, amongst others. Also involved in ER stress signaling, Sestrin1 and Sestrin2 facilitate cellular adaptations to exercise. These proteins were quantitated via western blot and are presented in Figure 5.10. ATF6 and Sestrin1 expression were upregulated in *mdx* GAS, however ATF and Sestrin2 expression were normal (p<0.0001, Figure 5.10A and p<0.05, Figure 5.10D, respectively). There was a strong trend for EX to induce ATF6 in WT and *mdx* GAS, however it did not induce any other ER stress protein (p=0.053, Figure 5.10A). There was a strong trend for RIB to induce ATF4 expression in *mdx* but not WT GAS (p=0.060, Figure 5.10B). Suprisingly, EX reduced Sestrin2 expression compared to the *mdx* SED VEH control (p<0.05 Figure 5.10C).

5.3.6.3. Hypoxia stress markers

Finally, we assessed protein biomarkers of hypoxia stress, including HIF-1α and-1β isoforms, Sirt1, PGC-1α, AMPK total and phosphorylated AMPK (Figure 5.11). HIF-1α (p<0.0001, Figure 5.11A), HIF-1β (p<0.001, Figure 5.11B) and Sirt1 (p<0.0001 Figure 5.11C) were all upregulated in *mdx* GAS. However, PGC-1α expression, which is typically induced in response to hypoxia and metabolic stress, was significantly reduced (p<0.05, Figure 5.11D)**.** EX trended to further induce Sirt1 (p=0.097, Figure 5.11C) in *mdx* GAS and significantly reduced total AMPK α expression both in WT and *mdx* GAS (p<0.5 Figure 5.11E). However, EX had no effect on any other protein or on WT GAS. RIB further induced HIF-1α (p<0.001, Figure 5.11B), Sirt1 (p<0.001 Figure 5.11C) and trended to increase phosphorylated AMPK expression (p<0.1, Figure 5.11F) in *mdx* but not WT GAS. Neither EX, nor RIB affected PGC-1α expression (p>0.1, Figure 5.11D).

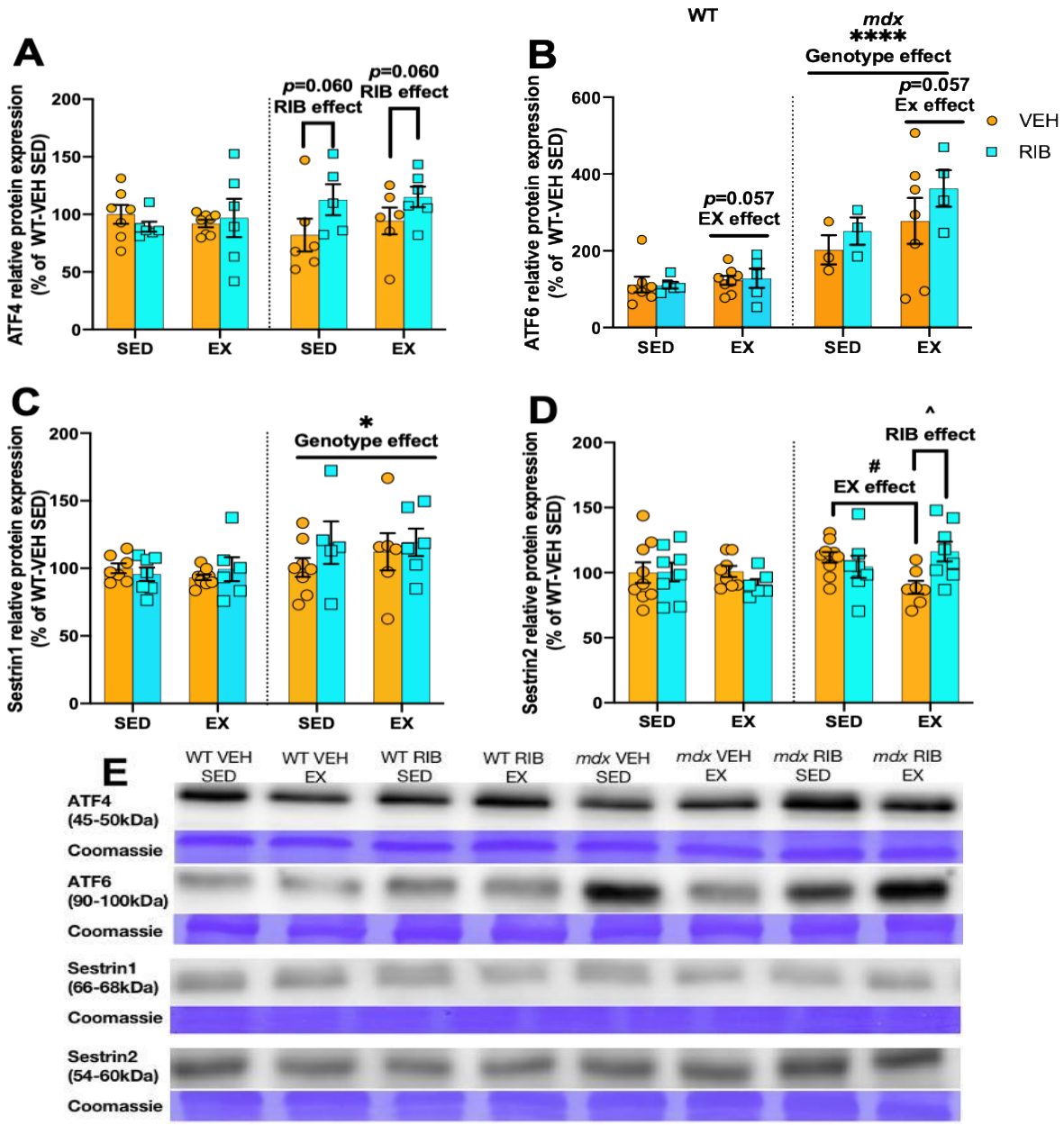


Figure 5-10 Effect of EX training and RIB treatment on ER related proteins. ATF4 (A), ATF6(B), Sestrin1(C) and Sestrin2 (D) were normalised to WT VEH SED group. *mdx* mice had lower catalase (C) levels compared to WT mice. *mdx* mice had higher ATF6 (B) and Sestrin1 (C) compared to WT mice. EX training trended to increase the ATF6 levels both in WT and *mdx* mice and significantly decreased Sestrin2 content (D) in *mdx* VEH EX. RIB treatment trended to increase the ATF4 levels in *mdx* mice both in SED and EX group and significant increased Sestrin2 levels in *mdx* EX group. Neither EX training nor RIB treatment impacted on the expression of Sestrin1 (C). (E) Western blot representative images are displayed alongside with a coomassie blue representative image, which was used as the protein loading control. *p<0.05, ****p<0.0001 mdx versus WT; $^{\#}p$<0.05 EX versus SED; ^p<0.05 RIB versus VEH. p<0.1 there is a trend between individual groups.

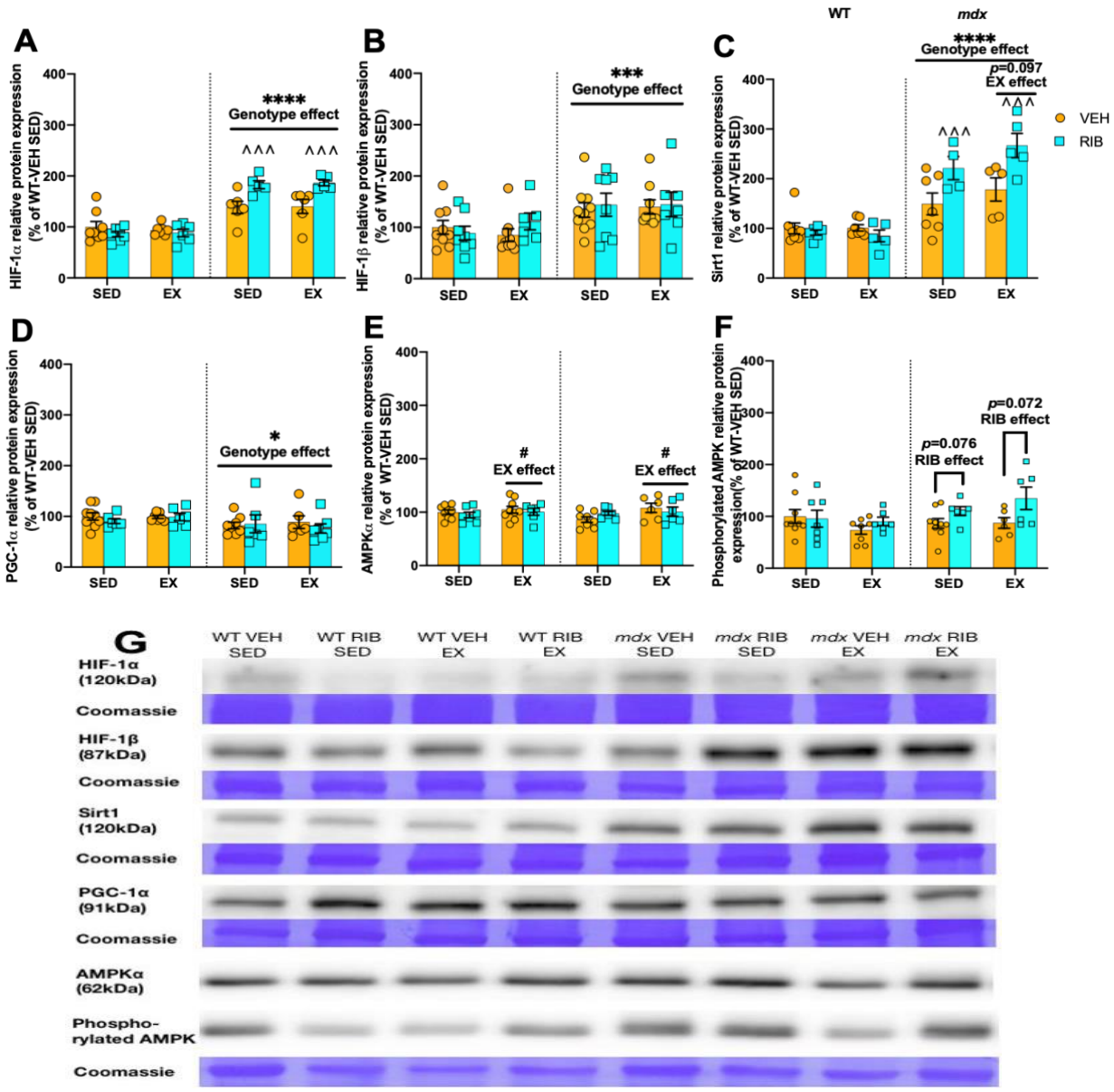


Figure 5-11 Effect of EX training and RIB treatment on metabolic related proteins. HIF-1α (A) and-1β (B) isoforms, Sirt1 (C), PGC-1α (D), AMPK total (E) and phosphorylated AMPK (F) were normalised to WT VEH SED group. *mdx* mice had higher levels of HIF-1α (A) and-1β (B) and Sirt1 (C) and lower levels of PGC-1α (D). EX training significantly increased the AMPKα (E) both in WT and *mdx* mice and trended to increase Sirt1 level in *mdx* mice. RIB treatment significantly elevated HIF-1α (A) and Sirt1 (C) in *mdx* mice and trended to increase phosphorylated AMPK in *mdx* mice. (G) Western blot representative images are displayed alongside with a Coomassie blue representative image, which was used as the protein loading control. *p<0.05, ***p<0.001, ****p<0.0001 *mdx* versus WT; $^{\#}p$<0.05 EX versus SED; ^^^p<0.001 RIB versus VEH. p<0.1 there is a trend between individual groups.

5.4. Discussion

This is the first study to explore the effect of RIB supplementation combined with moderate exercise training on muscle function and molecule signaling pathways in *mdx* mice. Our major findings in this chapter were that RIB could alleviate the moderate genotype-induced, exercise-aggravated-muscle wasting through improving muscle functions and improved the adaptation via activating the molecular signaling pathway.

In a bid to explore whether EX training and/or ribose had any potential benefit for muscle function, we assessed contractile characteristics of EDL, a predominantly fast-twitch muscle, and SOL, a predominantly slow-twitch muscle, from healthy WT and dystrophic *mdx* mice. The only effect that RIB had on contractile characteristics was that it increased the absolute force of the SOL muscle. However, it also increased the absolute and the ratio of SOL:body mass, highlighting that force improvements were due to more contractile elements. The mechanisms involved in this muscle/fibre-type specific effects are unclear. RIB flux into the muscle could result in hyperosmosis and muscle swelling, a known inducer of protein synthesis via mTOR (Marzuca-Nassr et al., 2019). The fact that this effect was only observed in SOL infers that slow-twitch muscle fibres may express more ribose transporters than fast-twitch fibres even though these fibres are physiologically more resistant to metabolic stress. Fibre type transitions from slow (smaller fibres) to fast (larger fibres) type appear not to be causal. While SOL muscles seem particularly sensitive to environmental cues that induce fibre-specific gene re-programming e.g., slow>fast transitions occur in response to microgravity (Shenkman, 2016), we saw no effect of RIB on specific force, which would be expected if slow>fast transitions were evident. Our in vivo measures showed RIB *mdx* mice were stronger through the forelimbs, yet there was no effect on the whole body holding impulse, further supporting the idea that specific muscles are affected more than others.

We were especially interested to determine the impact of RIB on the fatigability of the mice and their muscles. Muscle fatigue is a well characterised feature of DMD and is evident even in female carriers of the *Dmd* gene mutation who maintain dystrophin expression (albeit reduced) and do not

manifest the disease (reviewed in (Timpani et al., 2015)). Consistent with our hypothesis that RIB treatment could buffer metabolic stress by stimulating the PNC and increasing the overall muscle purine pool via *de novo* biosynthesis, we observed fatigue resistance of *mdx* SOL muscle during the later stages of the fatigue run, but only in EX mice. This corresponded with a 2-fold increase in the time to exhaustion in *mdx* EX mice during the treadmill fatigue test. Since we did not administer a fatigue test to SED mice (as we didn't want to perturb stress signaling pathways so close to tissue harvest), it is unclear whether the whole body effects of RIB are also EX-dependent. A purine homeostasis-supported mechanism of action is suggested by the fact that RIB increased TCr expansion, trended to reduced plasma uric acid levels, but did not improve mitochondrial OCR when metabolic stress was simulated through (chemical) uncoupling in *mdx* EX mice. These findings are consistent with our previous study investigating ASA treatment in *mdx* mice. ASA had no effect on mitochondrial function, yet significantly increased muscle TCr (particularly phosphorylated Cr (PCr) levels) (Timpani et al., 2020). The TCr pool expands muscle energy storage capacity for rapid re-phosphorylation of ADP, and its size directly corresponds with the muscle's ability to buffer dynamic fluctuations in ATP to protect against metabolic stress (Bonora et al., 2012). Although RIB did not increase ATP levels per se, the capacity for PCr-mediated ATP synthesis was impacted. Our data suggest that RIB is acting at the level of the PRPP/PNC in this instance to participate in the salvage and recycling of degrading purine nucleotides to expand the highest order of energy storage. Ribose has been shown to unlock the IMP pathway to replenish ATP via activation of PRPP in other disease states, e.g., chronic fatigue syndrome (Mahoney et al., 2018) also.

One of the consequences of metabolic stress-mediated purine degradation is the production of ROS by XO as hypoxanthine and xanthine are metabolised (Battelli et al., 2016, Lindsay et al., 2018). XO activity is increased in *mdx* muscles resulting in higher levels of circulating oxidative stress biomarker, orthotyrosine (Lindsay et al., 2018). The Nrf2/Keap1 signaling pathway is an essential regulator of the cytoprotective response to oxidative stress and its activation is intimately linked to purine degradation – first, by XO-produced ROS and second, by fumarate generated by adenylosuccinate lyase (ADSL), a multi-function enzyme controlling both *de novo* purine biosynthesis

and PNC function during metabolic stress. Nrf2 is activated through removal of repressor, Keap1, allowing it to translocate into the nucleus and transcribe the ARE. Keap1 is sequestered by p62 where it remains aggregated until autophagy mechanisms are enacted. To this effect, Nrf2 activation is not necessarily indicated by higher expression of Nrf2 protein, nor lower expression of Keap1, although protein levels of both might be modulated by repetitive hormetic stressors, such as that elicited by disease (DMD) or EX. We saw no evidence of Nrf2 activation (either protein levels, Nrf2:Keap1 ratio or expression of Nrf2 specific Phase II antioxidative enzymes) in *mdx* muscles though, which appeared to be linked to a dramatically higher Keap1 expression that was increased by EX and even more so by RIB treatment. Keap1 has important biological functions aside from Nrf2 repression, including the control of angiogenesis, an important adaptation to metabolic stress, exercise and remodelling in skeletal muscle. *Mdx* muscles have well demonstrated blood flow perturbations due to dysregulated nitric oxide (NO) signaling. Dystrophin anchors neuronal NO synthase (nNOS) beneath the sarcolemma to dynamically control blood flow proportionate to energy demand (Timpani et al., 2017a). In dystrophin-deficient muscles, unbound nNOS is targeted for degradation by calpains resulting in NO deficiency and loss of regulation between muscle fibres and their capillaries (Timpani et al., 2017a, Timpani et al., 2017b). Muscle fibres immediately proximal to capillaries are particularly prone to necrosis (Hernández et al., 2011) highlighting that nutrient and hypoxia stress are contributing factors to DMD pathology. We showed HIF1α/β and Sirt1 were upregulated in mdx muscles indicating hypoxia stress signaling was present in our mdx mice. Intriguingly, and consistent with our previous work documenting dysfunctional mitochondrial responses in the context of DMD (Timpani et al., 2015), PGC1α expression was reduced in *mdx* muscles, whereas the normal response to HIF1 induction (and AMPK phosphorylation) is PGC1α-orchestrated mitochondrial biogenesis (Shoag and Arany, 2010). In *mdx* muscles only, RIB enhanced HIF1α and Sirt1 expression (and trended to induced AMPK phosphorylation) but could not overturn the suppression of PGC1α protein levels. Metabolites are known to elicit important cell signaling functions (Baker and Rutter, 2023), and TCA cycle intermediates, malate and fumarate, specifically act as simulators of hypoxia stress by inducing

HIF1α/β (Martínez-Reyes and Chandel, 2020). RIB may directly interact with HIF or induce it secondary to stimulating de novo purine biosynthesis/PNC anaplerosis where ADSL generates more fumarate to induces HIF1 in a pseudo-hypoxia mechanism.

ER stress is well documented in DMD patients and *mdx* mice (Pauly et al., 2017, Gallot and Bohnert, 2021). ER stress can trigger the UPR to alleviate this stress using several different signaling pathways involving ATF's (Oslowski and Urano, 2011, Hetz, 2012). ATF6 is a master regulator of the UPR, although alternative inducers and regulators (such as Sestrin) exist to fine-tune the process. For example, Sestrin1 can be induced by nutrient depletion (amino acid, glucose) and oxidative stress amongst other stimuli, to participate in the UPR. While it is thought to respond to escalating ROS levels by inducing Nrf2, Sestrin 1 also possesses oxidoreductase activity by acting as a peroxide acceptor (Rai et al., 2017). ATF6 and Sestrin 1 expression were both elevated in *mdx* muscles and there was a strong trend for upregulation in EX muscles (WT and *mdx*; p=0.057) indicating that metabolic stress is involved in their induction and that *mdx* muscles are in a chronically stressed state. Since Catalase – a canonical antioxidant that dismutates hydrogen peroxide found in peroxisomes and mitochondria – expression was reduced and Nrf2 evidently repressed in *mdx* muscles, Sestrin 1 might upregulate to compensate for loss of these alternative antioxidant functions. There may be functional benefits of this specific antioxidant signature, for example, to facilitate muscle remodelling by muscle stem (satellite) cells.

Sestrins have been touted as "evolutionarily conserved mediators of exercise benefits" where their knockdown in various species results in failure to gain the beneficial adaptations of exercise (Kim et al., 2020). In our study, neither Sestrin 1 nor 2 expressions increased in EX muscles following twice-weekly moderate intensity treadmill running for 4 weeks (with the final session being a run-to-fatigue test), suggesting that the intensity and frequency of exercise activity is important for their induction. In fact, in *mdx* muscle, EX reduced the expression of Sestrin 2, which likely explains why PGC1α expression failed to increase in response to the activation of HIF-mediated hypoxia signaling. We maintained a relatively low level of EX "training" in our mice because *mdx* muscles are particularly sensitive to mechanical damage (De Luca et al., 2003), and this was perhaps too insufficient EX stimuli

to effect Sestrin expression in WT muscles. Importantly, our study was the first to investigate Sestrins in the context of DMD, and remarkably, when EX was applied to mdx mice, Sestrin 2 expression downregulated. There are few instances of pathology related Sestrin downregulation in the literature, but skeletal muscle wasting associated with disuse (immobilisation and denervation) (Segalés et al., 2020) and aging (i.e., sarcopenia) (Zeng et al., 2018) are two. While the mechanisms are currently unclear, a third pathological instance may shed light. Sestrins appear particularly sensitive to nutrients and are downregulated in the liver in response to high fat diet leaving the liver exposed to reduced ER stress responses, inflammation and fibrosis (Kim et al., 2021). It is well demonstrated that muscles from DMD patients and *mdx* mice have a higher lipid content than WT controls (as detected by Oil Red O staining (Timpani et al., 2015, Timpani et al., 2020), and quantitative MRI), which may result in downregulation of Sestrin 2. A higher muscle fat fraction is also characteristic of age-related sarcopenia, in which Sestrin 2 is downregulated (Zeng et al., 2018). However, it is unclear why this effect in mdx muscles is specifically related to the additional application of EX stress. Notably, RIB treatment normalised Sestrin 2 expression in *mdx* EX muscles and it is likely that this occurs via an ATF4-dependent mechanism since RIB trended to increased expression of ATF4 in *mdx* muscles. Our previous work demonstrated that treatment with PNC metabolite, ASA, resulted in a significant reduction of muscle neutral fat levels, suggesting that PNC products can reprogram metabolism in favour of fatty acid utilisation. PNC-generated fumarate seems a likely candidate since exogenous dimethyl fumarate treatment (in the context of multiple sclerosis) induces lipid metabolism (Bhargava et al., 2019).

Collectively, our data highlight perturbed stress responsivity and adaptations in *mdx* muscles, some of which could be normalised by modulation of purine metabolism via RIB. When EX was applied to already stressed *mdx* muscles, some of these perturbations were exacerbated but it is unclear whether this benefits or progresses the myopathy. With respect to the Nrf2ome, specifically the interaction between Nrf2 and Keap1, our data suggest that it is critical for cytoprotection against hypoxia and oxidative stress, but it cannot execute responses to both stress insults at the same time. In our study, hypoxia signaling was predominant in *mdx* muscles (and was exacerbated by EX and

RIB) and it is possible that this comes at the expense of oxidative stress responsivity. It is possible that orchestrated ER stress signaling compensates for Nrf2 repression when multiple stress insults are present. Our future work will more deeply probe this molecular crosstalk using a multi-omics approach and decipher whether particular signaling pathways can be manipulated for therapeutic value. In particular, we are keen to investigate the role of purines and their flux within this molecular conversation.

5.5. Conclusion

In summary, this is the first study to explore the potential of RIB supplementation to mitigate muscle dysfunction in dystrophic *mdx*, and particularly, metabolically stressed (via EX) mice. Our data show that RIB treatment improved fatigue resistance and fibre-type specific strength of *mdx* muscles via induction of the hypoxia response adaptations. Our data indicate that RIB attenuates purine degradation, which adapts ER stress signaling pathways potentially via inducing fat metabolism. RIB could be a therapeutic adjunct to effectively treat muscle fatigue in DMD patients and female carriers of the dystrophin gene mutation.

Chapter 6

Effects of combined HIT and RIB supplementation on skeletal muscle stress signaling and purine metabolism in humans: A pilot study

6.1. Introduction

High intensity intermittent exercise stimulates greater and broader adaptation in shorter time frames compared with low-moderate intensity protocols resulting in rapid improvements to health and performance (Egan and Zierath, 2013). SIT is the extreme intensity of HIT and the shorter and the more intense nature drives rapid perpetuations in metabolism to support high power outputs. This results in subsequent metabolic responses and consequential adaptations that may influence health and performance (Burgomaster et al., 2006, Burgomaster et al., 2008, Granata et al., 2016). Greater ATP turnover rates with SIT result in reductions in ATP. Impacted muscle ATP is degraded to purine bases and subsequently leeched from the muscle nucleotide pool following intense exercise, resulting in a slow and costly resynthesis process. The extent of the aggregated ATP loss with progressive sprint training combined with a slow rate of restoration may compromise the performance of subsequent sprint training sessions and recovery.

During intense exercise there is an increase in ATP turnover supplying contractile demand. Muscular ATP supply and utilisation is enhanced with SIT, primarily via adaptations to glycolytic capacity (Hargreaves and Spriet, 2020) and mitochondria functions (Oliveira and Hood, 2019), therefore maximising muscular ATP turnover capacity and supply may improve the ability to exercise and train at maximal capacities, thus enhancing adaptations to SIT. When the consumption of ATP exceeds resynthesis, a decline in ATP results due to unmatched turnover. This results in an elevation of ADP from basal levels (~10μM) to 100-300 μM, where it is readily hydrolysed to AMP and quickly deaminated to IMP and this can result in a decreased 30-40% ATP levels in mixed muscle fibres following short sprints up to 30 s (Cady et al., 1989, Stathis et al., 1994, Allen et al., 2008). Karatzaferi and her colleagues found that 25s of maximal exercise on a cycle ergometer resulted in an 80% reduction in ATP levels in type II fibres and no significant changed in type I fibres (Karatzaferi et al., 2001b). Power output was also found to be reduced by ~42.7% upon completion of the exercise bout. ATP recovered from the single bout to ~54% of its original content in 1.5min of recovery, however peak power output in the second bout (commencing after 1.5min passive recovery) was decreased by

~15.4% (Karatzaferi et al., 2001a). No studies have examined the influence of muscular ATP content on SIT or HIT performance, however the enhancement or maintenance of intramuscular ATP content or supply may enhance performance outcomes or fatigue resistance in intense exercise (Kichenin and Seman, 2000, Hellsten et al., 2004, Ferraresi et al., 2015). The supplementation of oral ATP has been previously been shown to have a low bioavailability, and has not promoted intramuscular ATP content and subsequent performance or support the metabolic processes in question (Jordan et al., 2004). However intramuscular ATP content can be increased through the support of purine salvage (synthesis) and precursor molecules of purine *de-novo* synthesis.

Ribose supplementation supports greater purine salvage and resynthesis, and enhanced ATP recovery from intense exercise following 7 days intense sprint training (Hellsten et al., 2004). By extension, a sustained supply of ribose during training potentially supports muscular bio-energetic recovery, maintains ATP levels and elevated ATP turnover during any subsequent exercise. An accumulated improvement in ATP recovery may consequently support muscle energetics and enhance progressive training performances and muscular adaptations to SIT in the longer term.

Both HIT and SIT can increase ROS production, and subsequently exaggerate the metabolic stress response. The high ATP turnover rates and ATP degradation results in elevated levels of xanthine oxidase, which catalase the Hx to xanthine and xanthine to uric acid along with ROS generation (McNally et al., 2003). Increased ROS have potential to damage the muscle (Di Meo et al., 2019). Ribose has been reported to improve the resting ATP recovery via activation of PRPP pathway (Mahoney et al., 2018), which might offer potential benefits by inhibiting the breakdown of adenine nucleotides and impacting the formation of xanthine and uric acid. Moreover, in response to exercise induced stress, multiple molecular signaling pathways are involved in the process of adaptation. Nrf2-regulated cytoprotective enzyme, UPR pathway and hypoxia induced stress pathway could be activated and contribute to mitigate the stress induced by exercise (Bogdanis et al., 2013, Kim et al., 2014, Li et al., 2020a). No study has explored the effect of ribose on these molecular signaling pathway during the period of exercise.

Therefore, we hypothesised that the interaction of exercise training and ribose supplementation could enhance the exercise training performance, increase the rate of ATP resynthesis, drive adaptation and protect against the exercise induce stress through altered expression of Nrf2 mediated cytoprotective enzymes, stimulation of ER stress induced UPR markers, and activation the HIF-1 mediated hypoxia stress signaling in humans compared to untrained people.

6.2. Method and analysis

6.2.1. General study overview

Five healthy volunteers (2 males and 3 females) with an average age 22.9 ± 1.4 years) with BMI of 25.6 ± 2.0 $kg.m^{-2}$ and active recreational fitness level (VO_2 peak, 36.4 ± 5.0 $ml.kg^{-1}.min^{-1}$), performed 8 weeks of sprint interval training (SIT). Prior to training participants performed a graded cycle exercise test (VO_2 max), AIS cycle power profile (Table 1) to determine their level of fitness and strength, respectively. They also assessed for body compositional analysis (fat mass, muscle mass, legs and arms mass) with using a dual-energy X-ray absorptiometry (DEXA, GE Medical System, Chicago, IIlinois, USA) scan and software Encore (Version 16), They also performed a pre-training sprint test protocol consisting of a 30 s “all out” cycle bout (Lode Excalibur, Groningen, Netherlands) and blood and muscle tissue samples were collected before and following exercise for analysis of metabolic stress. Upon completion of baseline testing participants were randomly (double blinded) separated into placebo (n=3) or ribose (n=2) supplementation groups and performed 8 weeks of SIT. Participants ingested the supplement 3 times daily during the 8 weeks period at breakfast, lunch and dinner. They completed 3 x SIT sessions per week progressively increasing the load over time (Table 6.2.2) under supervision at Victoria University. Upon completion of the 8 weeks training and supplementation period participants performed the same battery of tests as the baseline measures to determine differences in metabolic function and performance (VO_2 max, peak and mean power output) with training and supplementation of ribose.

Table 6-1 AIS power profile

Outline of power profile test protocol

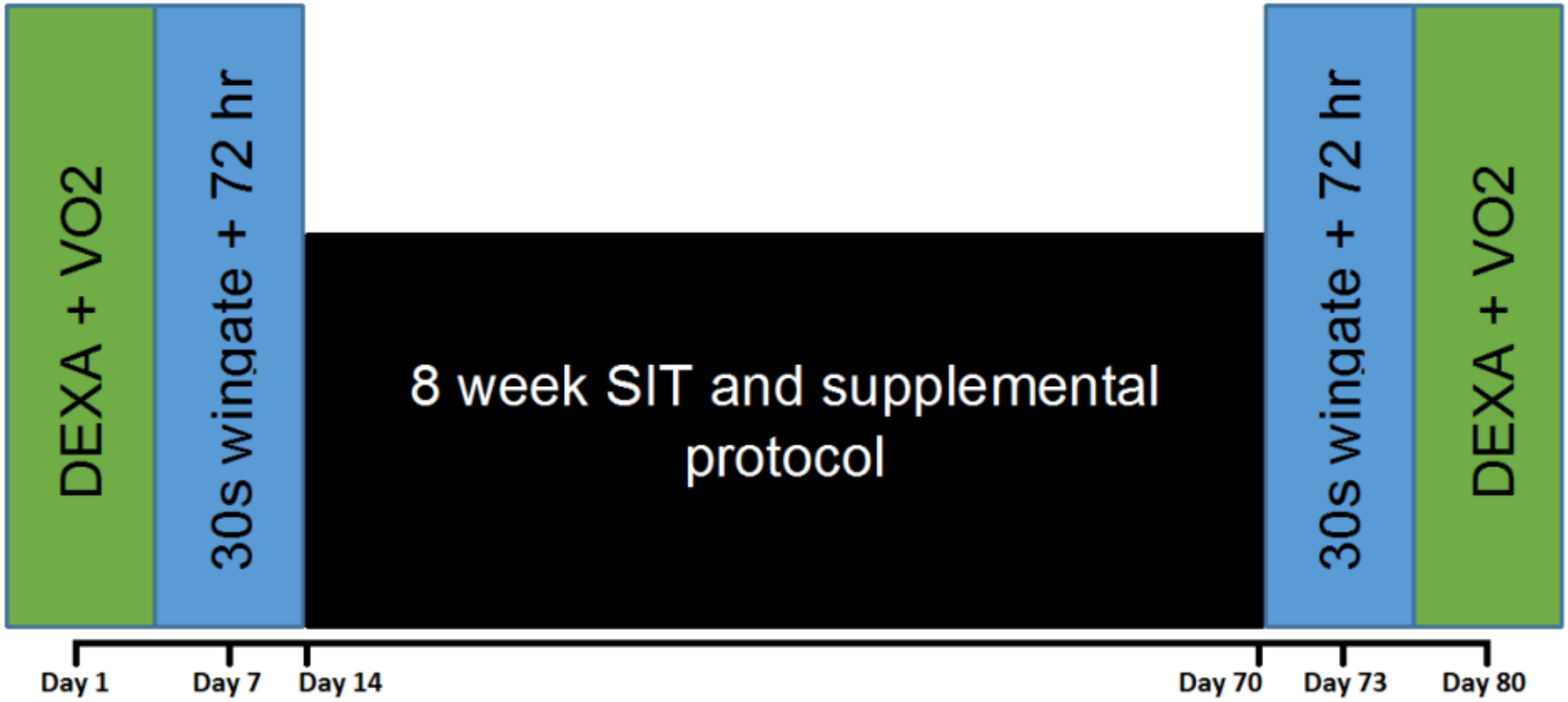
Time (min:sec)	Duration & gear selection	Power output	Heart rate	Notes
0:00-0:06	6s, small gear	*	*	Standing start
0:06-1:00	Active recovery (54s)	*	*	50-100 W
1:00-1:06	6s, big gear	*	*	Standing start
1:06-4:00	Active recovery (174s)	*	*	50-100 W
4:00-4:15	15s (self-selected)	*	*	Rolling start (70-80 rpm)
4:15-8:00	Active recovery (225s)	*	*	50-100 W
8:00-8:30	30s (self-selected)	*	*	Rolling start (70-80 rpm)
8:30-14:00	Active recovery (330s)	*	*	50-100 W
14:00-15:00	1 min (self-selected)	*	*	Rolling start (70-80 rpm)
15:00-23:00	Active recovery (480s)	*	*	50-100 W
23:00-27:00	4 min (self-selected)	*	*	Rolling start (70-80 rpm)

Table 6-2 SIT training timeline

Outlines the progression of the training design and where testing will occur

Week	Protocol	Training sessions per week	Power output	Heart rate	Blood sample	Biopsy
-1	1x30s	1 - Testing	*	*	0, Post, 30 min, 60 min, 24hrs and 72hrs	0, Post, 24hrs and 72 hrs
1	3x20s 120s recovery	2xSIT + 1x power profile	*	*		
2	4 x20s 120s recovery	3xSIT	*	*		
3	5 x20s 120s recovery	3xSIT	*	*		
4	6 x20s 120s recovery	2xSIT + 1x power profile	*	*		
5	8 x20s 120s recovery	3xSIT	*	*		
6	8 x20s 120s recovery	3xSIT	*	*		
7	8 x20s 120s recovery	3xSIT	*	*		
8	8 x20s 120s recovery	2xSIT + 1x power profile	*	*		
9	1x30s	1 - Testing	*	*	0, Post, 30 min, 60 min, 24hrs and 72hrs	0, Post, 24hrs and 72 hrs

Figure 6-1 Study design timeline



6.2.2. Participants

Five healthy active non-obese (BMI<30) volunteers (3 males and 2 females) between the ages of 18-35 years were sourced from the Victoria University staff and student population. Participants were free of cardiovascular, haematological and metabolic disease, and did not consume medications or supplements. Participants were fully informed of the nutritional supplement and experimental protocols, aims, risks and discomfort associated with the investigation, prior to providing written informed consent. The participant consumed either 200 $mg.kg^{-1}$ bw^{-1} ribose or 200 $mg.kg^{-1}$ bw^{-1} maltodextrin (control group) 3 times per day with food.

6.2.3. Graded exercise test protocol

A VO_2 max test of each subject was determined about one week prior to beginning the experimental trials. The exercise protocol involved riding on a cycle ergometer (Lode, Groningen Netherlands) for 3 min at three submaximal work rates, subsequently the work rate was increased every min thereafter until volitional exhaustion. Participants were encouraged to maintain a pedalling frequency of 80 revolutions per min. Expired air was measured for ventilation, oxygen and carbon dioxide content by gas analysers (Applied Electrochemistry S-3A O_2 and CD-3A CO_2). These analysers were calibrated before each test using commercially prepared gas mixtures. Oxygen consumption was calculated by a microprocessor using standard equations (Moxus, AEI technologies Texas. USA).

6.2.4. AIS profile

AIS profile following a standardised warm-up of 5 min self-paced, 5 min at 100 watts, participants underwent an AIS power profile (Table 6.2.1). This conducted in weeks 1, 4 and 8 to determine changes in sprint ability with training.

6.2.5. A single bout of 30 s “all out” protocol

Participants, following a standardised warm-up, complete a 1x 30 s maximal effort sprint bout on a Wattbike Pro (Wattbike, Nottingham, United Kingdom). Power output, speed, cadence, and peddling technique data had collected for the sprint bout.

6.2.6. Sprint interval training

The SIT bouts were performed on an electronically braked ergometer (Velotron, SRAM, Chicago Il. USA). Participants were instructed to remain seated and pedal as fast as possible for the duration of the test. In multiple sprint protocols, all recovery was passive rest. Subjects were familiarised with the respective sprint tasks for each study by performing the sprint bout (without sampling procedures) at least one week prior to the trials.

6.2.7. Blood & plasma samples and treatment

Blood was sampled from an antecubital vein, via an indwelling catheter, at rest, and during recovery following performance tests. For repeated sampling of blood, the catheter was kept patent by periodic flushing with small amounts of sterile isotonic saline. The blood was immediately placed into lithium heparin tubes and spun in a centrifuge and analysed for lactate using the YSI 2500 (John Morris group, Sydney, NSW, AU). The remaining plasma was stored at –80 ^{o}C for later analysis of inosine, hypoxanthine (Hx), xanthine and uric acid. Prior to analysis 100 µl of plasma was thawed and deproteinised with 50 µl of 1.5 M perchloric acid and subsequently neutralised with 37 µl of 2.1 M potassium hydrogen carbonate. The neutralised perchloric acid extracts, were measured by high performance liquid chromatography (HPLC) technique with a modified method (Wynants and Van Belle, 1985) using a Shimazu chromatography system (model: LC-2030, Shimadzu Corporation, Kyoto, Japan). A Phenomenex Luna 5 µm C18/100A (250 x 4.6 mm) analytical column (Phenomenex, Torrance, CA, USA) was used to perform the analysis. The mobile phases used for separation

consisted of 0.15 M ammonium dihydrogen phosphate ($NH_4H_2PO_4$), pH 6.00 and a mixture of acetonitrile and methanol (50/50, v/v). The eluent was monitored at 254 nm.

6.2.8. Muscle sample collection and storage

Muscle biopsies were obtained from the vastus lateralis under local anasthesia (1% xylocaine). An incision was made at the site in the skin (one incision for each sample per sprint test) and muscle samples were taken distal to proximal (3 cm apart) and the proximal incision was approximately 10 cm to the lateral epicondyle of the femur, using the percutaneous needle technique (Bergstrom, 1962) modified for suction (Evans et al., 1982). Leg selection was random and in the second trial the contralateral leg was biopsied. Muscle samples were frozen in liquid nitrogen within 5-10 s of obtaining biopsies. This small time delay in freezing would have minimal consequence on the metabolite levels of resting muscle samples. However, it may play a small role in the post exercised samples, in particular, the PCr and Cr levels where estimates of PCr resynthesised in a 5 s delay are significant (Sant'Ana Pereira et al., 1996). This is an unavoidable consequence of the methodology employed and is similar pre and post training for comparison.

Snap-frozen muscle biopsies were used to analyse the metabolites (ATP-PCr, creatine and lactate) analysis as described in “Section 3.2”.

6.2.9. Western blot

Western blotting was performed to quantitate protein markers of oxidative, ER and hypoxia stress signaling in snap-frozen muscle biopsies as described in section 3.1. All the antibodies used are listed in “Table 3.1, Section 3.1.”

6.2.10. Statistics

All the data presented as mean ± SEM. Data were analysed using the GraphPad Prism v8 (GraphPad software, San Diego, CA, USA). A two-way ANOVA with Turkey’s post hoc test was utilised

to detect training effect over time. An unpaired T-test was used to detect the effect between individual groups. An α value of 0.05 was considered significant and an α value between 0.05 and 0.1 was considered a statistical trend.

6.3. Results

6.3.1. Effect of SIT training and/or ribose on the alteration of body composition

DEXA machine was used to measure the body mass and body composition at pre- and post-training, (Figure 6.2). No difference was observed in body mass (p>0.1, Figure 6.2A), fat mass (p>0.1, Figure 6.2B), lean mass (p>0.1, Figure 6.2C), average of left and right leg mass (p>0.1, Figure 6.2D) and average of left and right arms mass (p>0.1, Figure 6.2E) after 8 weeks training in any groups.

6.3.2. Effect of SIT training combined with/without ribose on the change of VO_2 and muscle power

Our data showed that VO_2 max had a trend to increase after 8 weeks SIT training (p=0.056, Figure 6.3A). Individually, a male participant treated with ribose had 30% increase in VO_2 max (from 250 to 325 $ml.kg^{-1}.min^{-1}$, Figure 6.3A), which is the highest among all participants. We also found that HIT training had a positive trend for mean power (p=0.098, Figure 6.3B). Interestingly, a male participant, without ribose treatment, had a better improvement in mean power output (17.45% increase from 523.86 to 615.24 W, Figure 6.3B) and peak power output (17.97% increase from 682.20 to 804.78 W, Figure 6.3C) than other participants after 8 weeks SIT training. During 30 s sprint, average of power output at the 30 s endpoint decreased 35.54% in untrained participants and 31.77% in trained participants compared with their initial levels (Figure 6.3D).

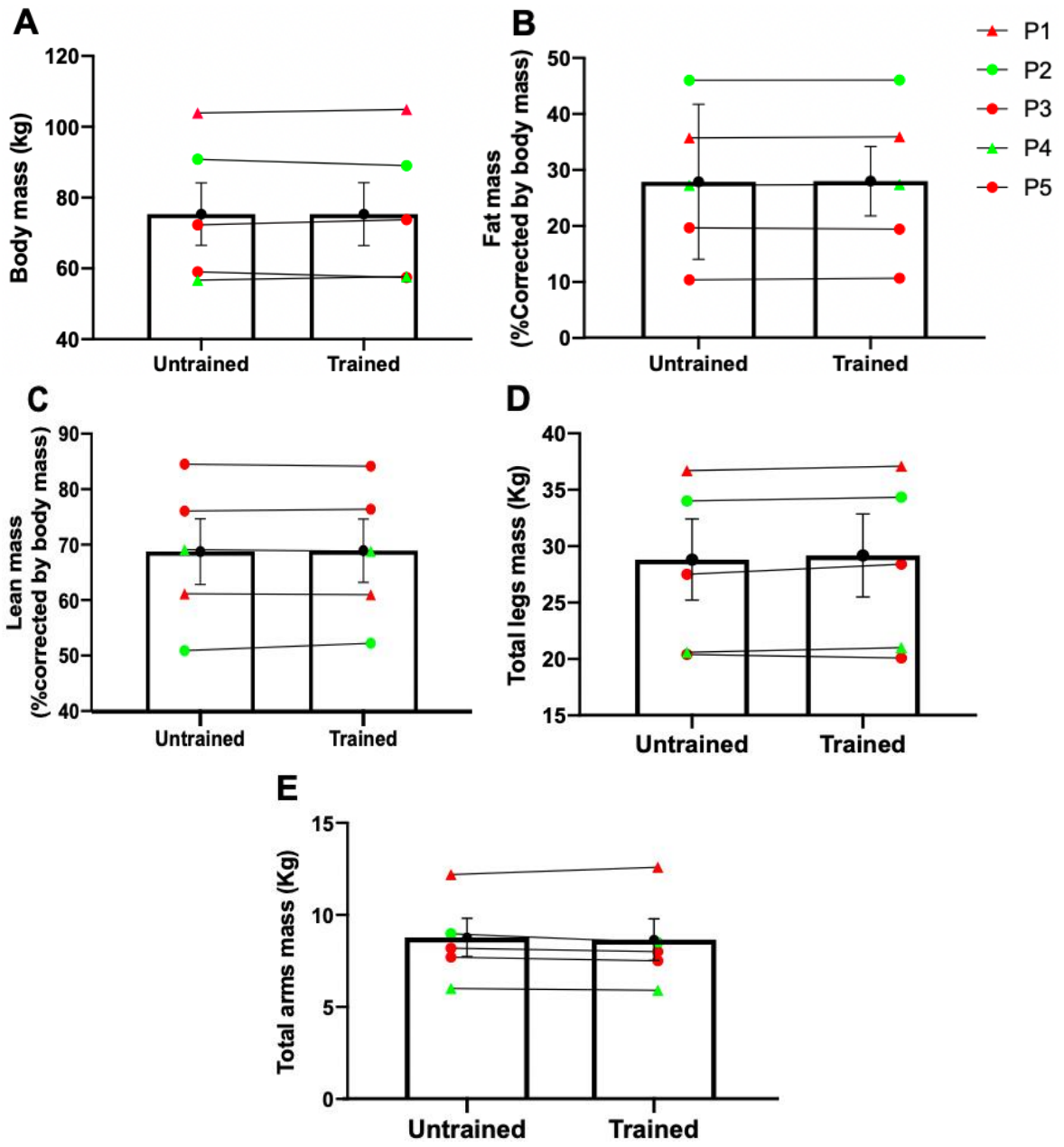


Figure 6-2 Effect of SIT on the alteration of body mass and body compositions. Body mass (A), fat mass corrected body mass (B), lean mass corrected body mass (C), total legs mass (D) and total arms mass were present. Green symbols represent female subject data. Red symbols represent male subject data. Tri-angle symbols represent ribose treatment. Dot symbols represent placebo treatment. Error bars represent the standard error of the mean (SEM).

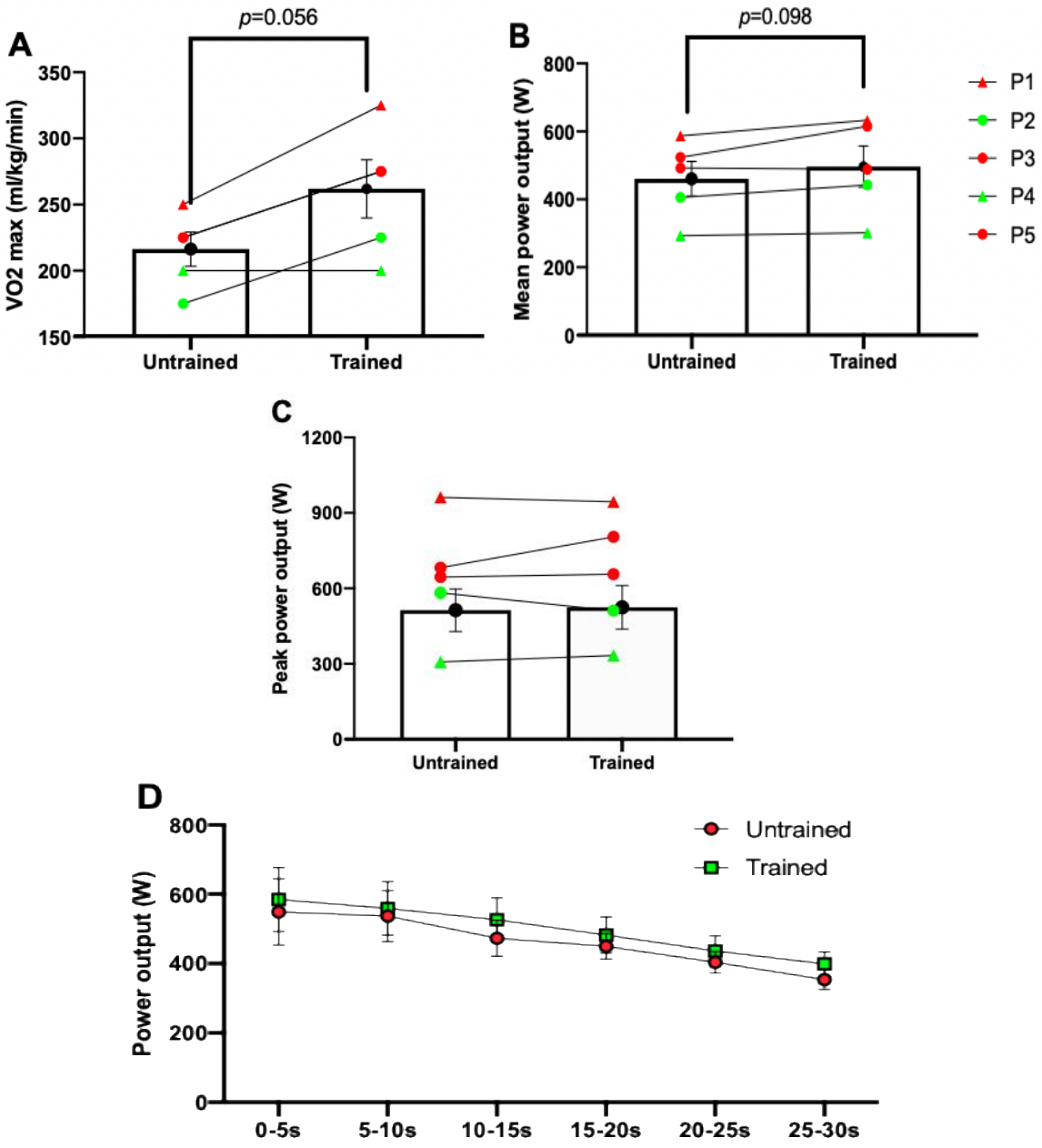


Figure 6-3 SIT training had a strongly positive trend in VO_2 max (A) and impact the muscle power (B and C). Our data shows that there was a positive relationship between SIT training and mean power output (B), whereas there is no change in peak power output (C) after 8 weeks SIT training. Power output in 30 s sprint exercise were present. $p<0.1$ there was trend in SIT trained compared to untrained.

6.3.3. Effect of SIT training on muscle metabolites

Muscles biopsies were collected in pre, post, 24 and 72 hr of a 30 s “all out” sprint cycle bout before and after training (Figure 6.4). We found that ATP significantly reduced after 30 s sprint exercise both in untrained (p<0.05) and trained statuses (p<0.01, Figure 6.4A). There was no difference in ATP at rest between untrained and trained status and the levels were not different following a 30 s sprint. Interestingly, ATP contents was higher at 72 hr in trained participants compared to untrained status (p<0.05, Figure 6.4A). PCr content reduced immediately post exercise compared to pre exercise after 8 weeks training exercise (p<0.05) and incrementally increasing 24 hr post (p<0.05, Figure 6.4B). In contrast, Creatine levels elevated immediately post exercise compared to pre exercise after 8 weeks training (p<0.01, Figure 6.4C) and gradually decreasing 24 hr (p<0.05) and 72 hr post exercise (p<0.05, Figure 6.4C). No significant increase was observed in muscle ATP (p>0.1, Figure 6.4A) and TCr levels in any group (p>0.1, Figure 6.4D). Muscle lactate immediately increased after 30 s sprint (p<0.05, Figure 4.6E) and return to baseline levels at 24 hr and 72 hr post exercise both in untrained and trained participants (p<0.1, Figure 4.6E and p<0.05, Figure 4.6E, respectively). We also summarized the calculated ATP turnover (ΔATP+ ΔCP+ 3/2 ΔLactate + 1/2 ATP) following a 30 s sprint between trained and untrained statuses (p>0.1, Figure 4.6F).

6.3.4. Effect of SIT training on purine metabolites, lactate and glucose

Plasma metabolites measured at pre, post, resting 10, 20, 30, 60 min, 24 and 72 hr after a single bout of 30 s sprint are summarized in untrained and trained (Figure 6.5). There were no differences in plasma inosine (p>0.1, Figure 6.5A), Hx (p>0.1, Figure 6.5B), xanthine (p>0.1, Figure 6.5C), uric acid (p>0.1, Figure 6.5D) between untrained and trained groups in response to HIT training. There were no differences in plasma lactate (p>0.1, Figure 6.5E) and glucose levels (p>0.1, Figure 6.5F) between the two groups.

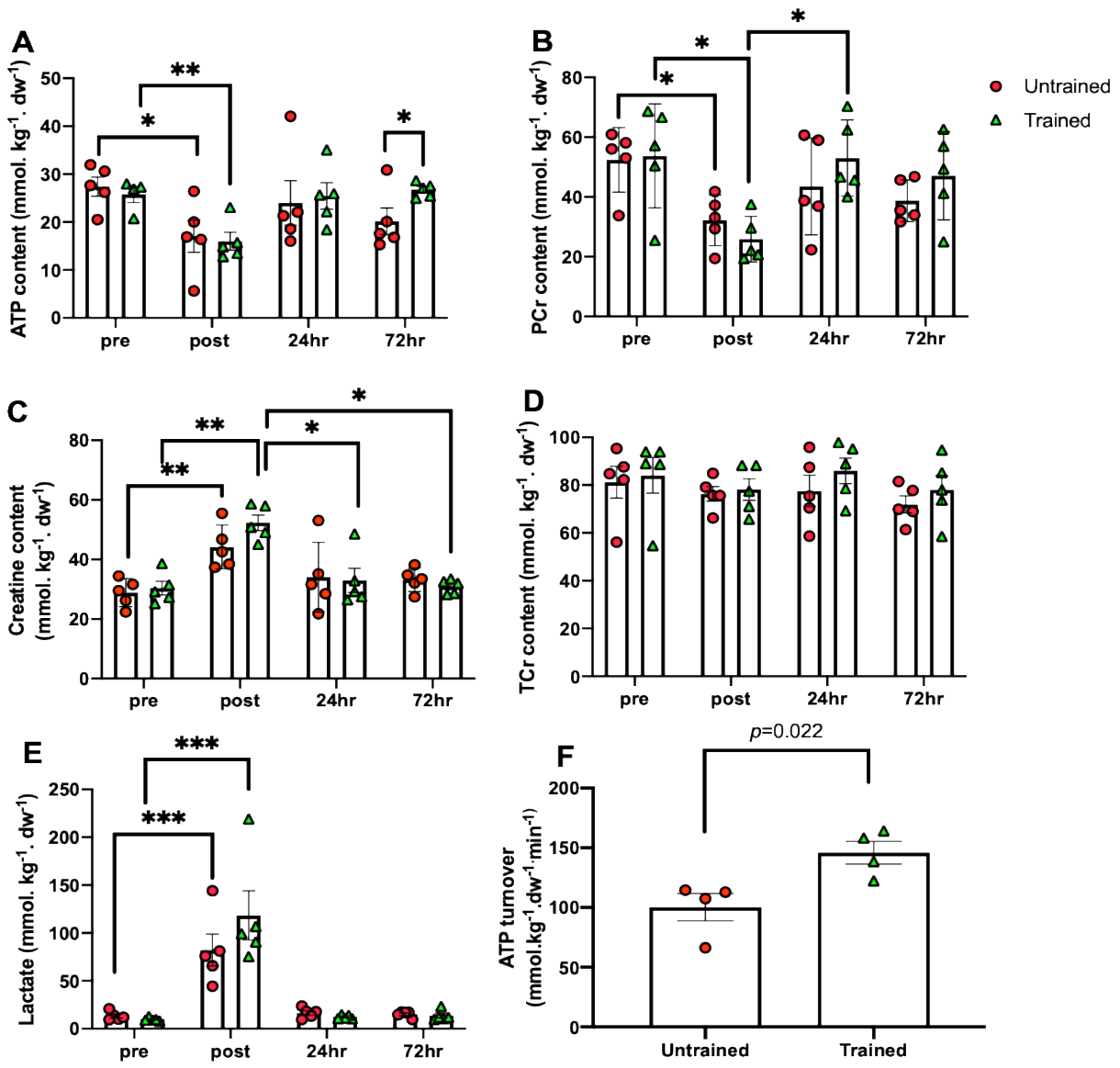
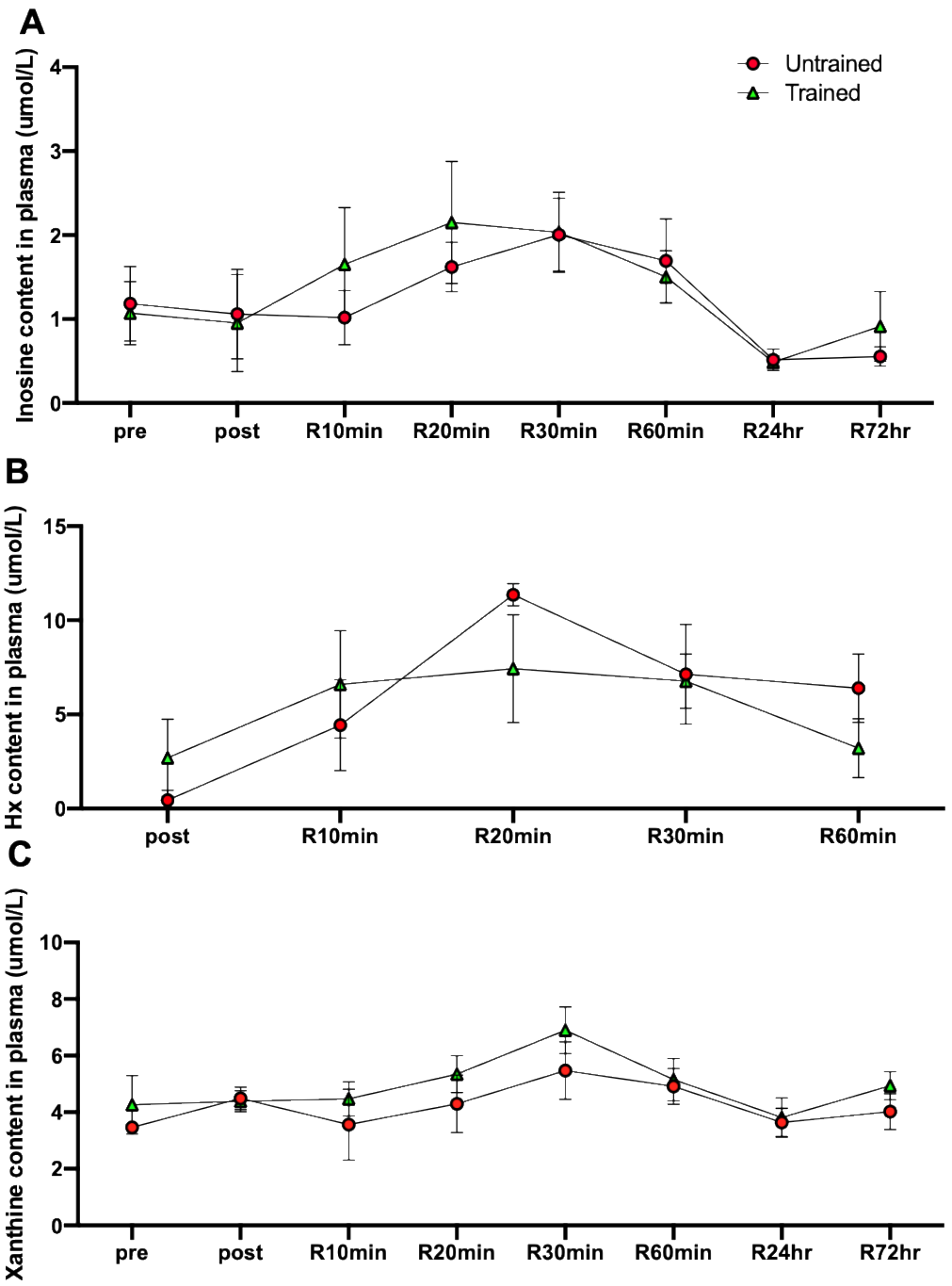


Figure 6-4 Alteration of ATP, PCr, creatine, TCr and lactate contents in human muscle in response to SIT training. ATP levels (A) significantly decreased after a single bout of 30 s sprint exercise both in trained and untrained status. PCr levels (B) decreased immediately, whereas creatine levels (C) increased immediately post exercise after a 30 s sprint exercise and both return to baseline at 24hr both in trained and untrained status. A single bout of 30 s exercise caused lactate accumulation (E) in muscle regardless of untrained or trained participants. No significant differences in the level of TCr (D) was detected in any groups. ATP turnover (ΔATP+ ΔCP+ 3/2 ΔLactate + 1/2 ΔADP, F) significantly increased following a single bout of 30 s sprint in trained participants compared to untrained status. ΔADP was assumed approximately 0 that take from Stathis work (Stathis et al., 1994). *p<0.05, **p<0.01, ***p<0.001 significant differences between individual groups.


A
Untrained
Trained
Inosine content in plasma (umol/L)
0
1
2
3
4
pre
post
R10min
R20min
R30min
R60min
R24hr
R72hr
B
Hx content in plasma (umol/L)
0
5
10
15
post
R10min
R20min
R30min
R60min
C
Xanthine content in plasma (umol/L)
0
2
4
6
8
10
pre
post
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R20min
R30min
R60min
R24hr
R72hr

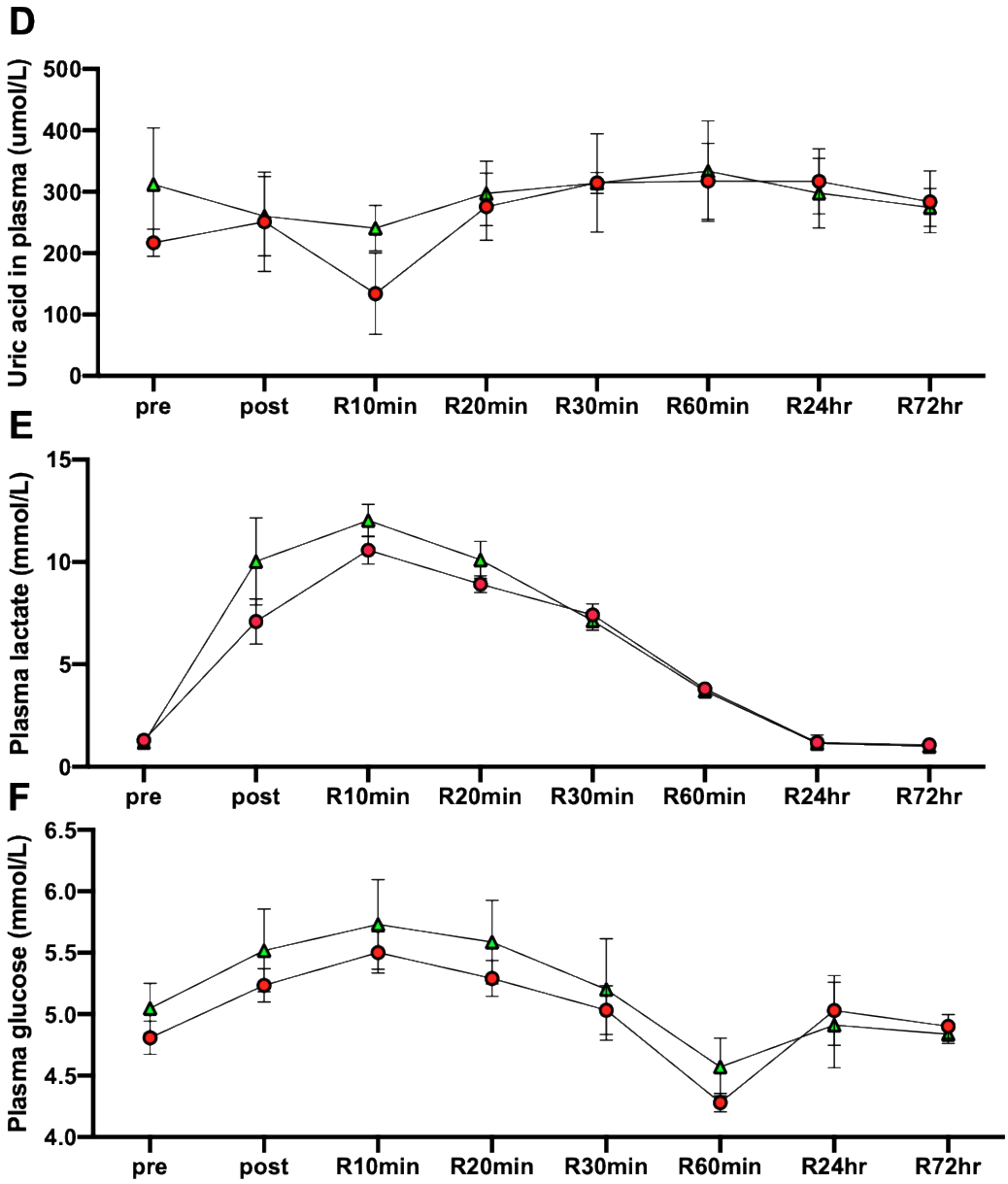

Figure 6-5 Plasma Inosine (A), Hx (B), xanthine (C), uric acid (D), lactate (E) and glucose concentrations (F) were presented. We didn't observe any SIT training effect in plasma metabolites, lactate and glucose except at post timepoint where Hx content was greater in trained status when compared to untrained status. *p<0.05 significant difference between trained and untrained groups.

6.3.5. Effect of HIT on stress signaling markers

6.3.5.1. Oxidative stress markers

In a bid to explore the effect of HIT on oxidative stress, we assessed the Nrf2 mediated proteins and antioxidant enzymes. We found Nrf2 expression significantly reduced at 24 hr post exercise in trained compared to untrained (p<0.01, Figure 6.6A). Keap1 expression increased immediately after 30 s “all out” exercise in trained participants compared to untrained. At 72 hr, Keap1 expression was higher than post and 24 hr in untrained participants (p<0.05, Figure 6.6B). NQO1 expression, a main antioxidant enzyme regulated by Nrf2 pathway, was higher at rest after 8 weeks training compared to resting NQO1 content in untrained participants (p<0.05, Figure 6.6D). No notable differences were observed in the expression of HO1 in any group (p>0.1, Figure 6.6C).

6.3.5.2. ER stress markers

We assess the expression of ER stress markers: ATF4, Sestrin1 and Sestrin2. We found Sestrin2 levels significantly increased immediately after a single bout of 30 s sprint exercise in trained participants (p<0.05, Figure 6.7C), which is a potential HIT training effect, because there was no change in the expression of Sestrin2 in untrained participants. The expression of ATF4 (p>0.1, Figure 6.7A) and Sestrin1 (p>0.01, Figure 6.7B) were unchanged in response to HIT training.

6.3.5.3. Hypoxia stress markers

The protein markers (HIF1α, Sirt1 and PGC-1α) related to hypoxia induced by exercise were also determined and presented in Figure 6.8. We found HIF1α levels, a main regulator in response to hypoxia condition, was elevated immediately after a single bout of 30 s sprint exercise and return to initial levels at 24 hr in untrained participants (p<0.01, Figure 6.8A), which means HIF1α is a transient and active protein within 24 hr after exercise in human muscle. Resting Sirt1 level was higher in trained

status than that in untrained status (p<0.01, Figure 6.8B). Interestingly, expression of PGC-1α is slightly elevated, not significant at post (p>0.1, Figure 6.8C), but it was significantly increased at 24 hr and 72 hr after a 30 s sprint exercise in trained status (p<0.05, Figure 6.8C), however not in the untrained status, which means HIT training might contribute to promote mitochondrial biogenesis via increased PGC-1α levels.

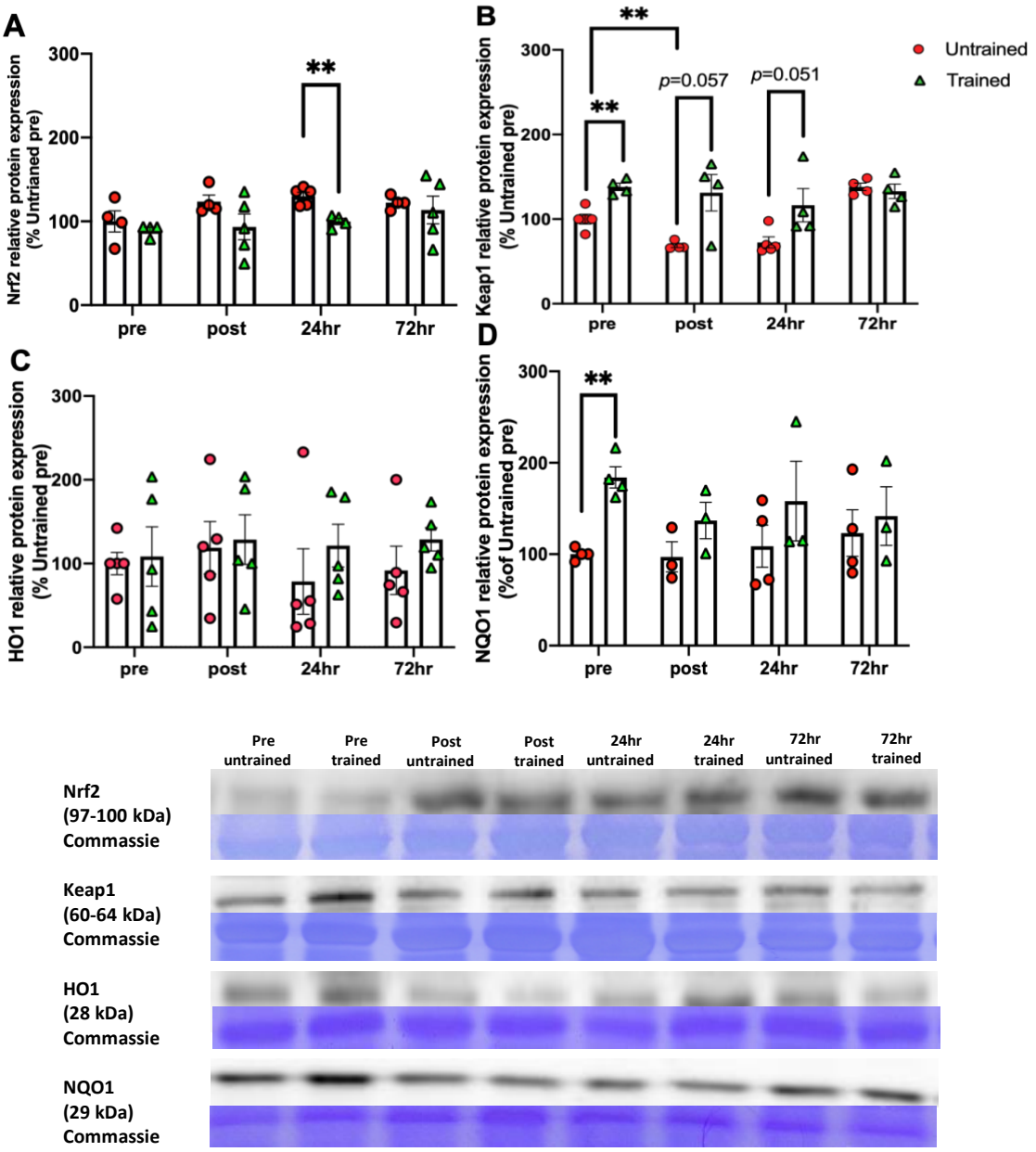

Figure 6-6 The expression of Nrf2 related proteins in response to SIT training were presented. SIT training decreased the Nrf2 expression (A) at 24 hr post-exercise, significantly increased keap1 expression immediately at post exercise and NQO1 resting levels (D) in trained participants compared to untrained participants. Antioxidant enzyme HO1 expression (C) was unchanged in any group. *p<0.05, **p<0.01 significant difference between individual groups and p<0.1 there was a trend between individual groups.

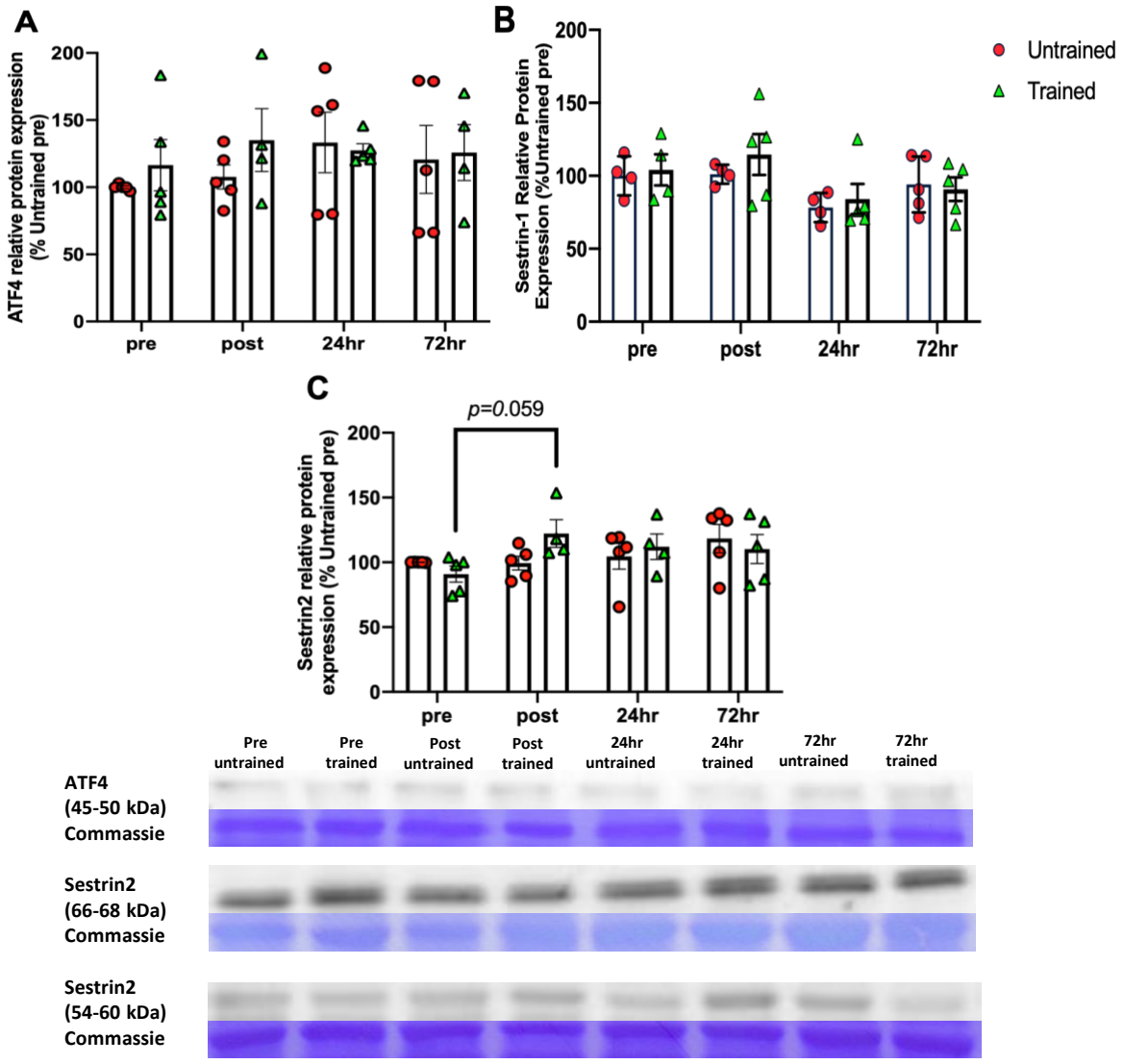

Figure 6-7 The expression of ATF4 (A), Sestrin1 (B) and Sestrin2 (C) in response to SIT training were presented. HIT training increased Sestrin2 levels after performing a 30 s sprint exercise. No notable differences were observed in the expression of ATF4 and Sestrin1. $p<0.1$ there was a trend between individual groups.

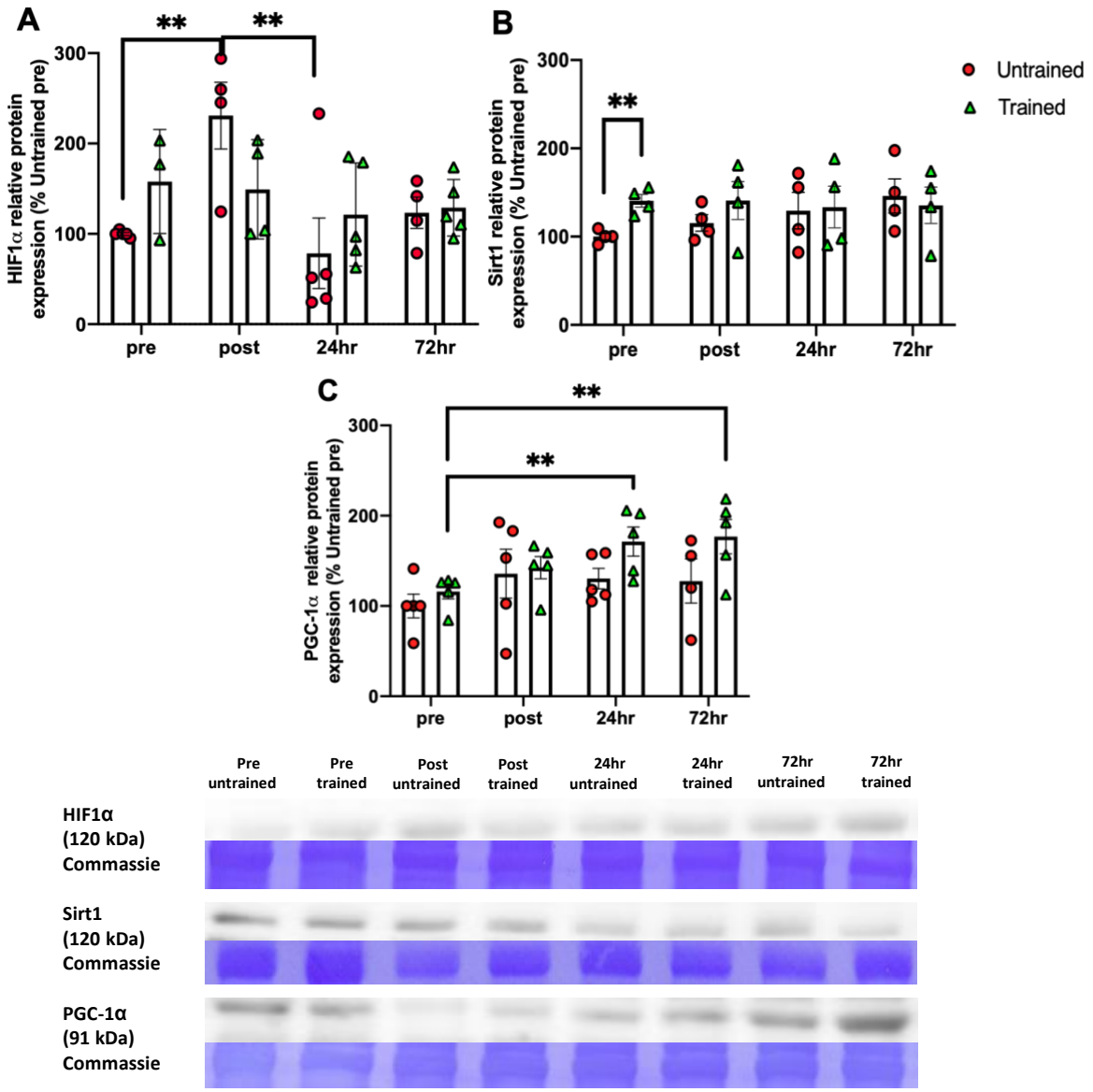

Figure 6-8 Alteration of hypoxia stress markers in response to HIT trained in human muscle. The expression of HIF1α (A) was increased immediately after exercise and went back to baseline at 24 hr. After 8 weeks SIT training, resting Sirt1 levels increased (B) and PGC-1α levels (C) went up after 30 s sprint exercise. ** $p<0.01$ differences between individual groups.

6.4. Discussion

The major findings of this study were that 1) 8 weeks SIT training had a strong trend to improve VO_2 max and increased 30 s sprint mean power output in human. G-Power software (Version 3.1, Heinrich-Heine university Düsseldorf, Düsseldorf, Germany) was used to analyse VO_2 max (combined data from five participants) in current study, we calculated conventional effect value is 1.06 at α value = 0.05, which is large relevant effect size that defined by Cohen. 2) Our combined data showed that SIT training (with/without ribose supplementation) activated the Nrf2-mediated protein and antioxidants response, stimulate the ER and hypoxia pathway, which combined to make a positive potential adaptation in response to SIT induced metabolic stress.

Improvement in aerobic fitness with SIT and HIT training has been reported previously (Trapp et al., 2008, Astorino et al., 2012, Larsen et al., 2014, Macpherson and Weston, 2015). Twenty-three participants (aged 25 ± 4 years) with two weeks SIT training, including 4-6 maximal 30 s sprints, one session a week had a small beneficial on VO_2 max compared to control group (Macpherson and Weston, 2015). Another study demonstrated that eight active men (aged 27 ± 3.4 years) performed 6 sessions Wingate tests within 2 weeks, VO_2 max increased 9.7% in trained group compared to control group (Larsen et al., 2014), which is similar result with Astorino study, which demonstrated that 6x3 weeks Wingate test training increased VO_2 max by 6.3 ± 5.4% compared to control group (Astorino et al., 2012). Furthermore, SIT significantly increased VO_2 max by 24% after 15 weeks SIT training, which contains 20 min of alternating 8 s sprint and 12 s of light pedaling in female group (Trapp et al., 2008). This result is consistent with our current data that showed 8 weeks of SIT training (combined data) effect enhanced 20.1% of VO_2 max. Interestingly, although a very limited sample size, SIT training with ribose supplementation increase 30% VO_2 max compared to untrained, indicating (anecdotally) SIT training with ribose supplementation might have a positive relationship in improving the aerobic capacity.

During the period of SIT, muscle ATP supply and utilisation are increased (Burgomaster et al., 2006), therefore, increasing capacity of muscle ATP turnover might contribute to improve the ability to

train at the highest level. SIT depletes more ATP in active muscle to a greater extent than moderate or lower intensity exercise due to a large temporary difference between ATP hydrolysis and resynthesis. Ribose has been reported to increase the rate of resting ATP synthesis and restore the ATP more quickly after intense exercise (Hellsten et al., 2004). During the intense exercise, there is an increase in ATP turnover supplying contractile demand, our data showed that ATP turnover during a 30 s sprint increased 45.5% from (200.57 to 291.82 $mmol.kg^{-1}.dw^{-1}.min^{-1}$) after 8 weeks HIT training. When consumption of ATP exceeds resynthesis, changes occur rapidly to purine metabolites, Hx, a naturally occurring purine derivative, is increased in plasma in our study. Stathis had demonstrated that a single bout of 30 s sprint exercise on a cycle ergometer cause the decreased in PCr and ATP Levels (Stathis et al., 1994), which is the consistent with our study that 30 s sprint reduced muscle ATP content by 38.25% and PCr levels by 51.87% of its initial levels. There was a 30-40% reduction in ATP levels in mixed muscle fibres (Stathis et al., 1994). Another study revealed that PCr levels quickly recovered to 80% of resting levels in 4 min after a single bout of 30 s sprint (Bogdanis et al., 1995). Although we didn't collect any muscle samples between post-exercise and 24 hr, our data showed that PCr contents returned to initial levels at 24 hr after a 30 s "all out" exercise. Fatigue rate during the sprint cycle bout might be due to the increased muscle lactate and decreased glycolytic ATP regeneration (Bogdanis et al., 1998). Our data showed that the fatigue indices (power output) was 35.53% (from 548.74 to 353.73 W) in untrained participants and 31.17% (from 585.26 to 399.31 W) in trained status during 30 s Wingate test.

In addition, we also evaluated the proteins expression involved in response to exercise induced stress. Oxidative stress occurs during SIT and HIT training, which may lead to damage muscle and disrupted cellular process. Once antioxidant defences are overwhelmed, the increased ROS stimulate a single cascade to develop an enhanced response to protect the muscle cells from oxidative damage. Nrf2/keap1 system is the primary intracellular defence mechanism to counteract oxidative stress, In our study, we found SIT training increase the expression of keap1 (a Nrf2 repressor) immediately after acute sprint exercise, but we didn't observe any changes in the expression of Nrf2. However, activation of Nrf2 signaling pathway can be impacted by alteration of Nrf2 content or Nrf2

downstream target genes. Our data showed that resting NQO1 level increased after 8 weeks training, but there is no change in the expression of HO1. NQO1, a key factor that is upregulated by Nrf2, has been reported to reduce ROS by supressing antioxidant activity (such as vitamin E). In addition, higher level of NQO1 might make better adaptations in response to cellular stress induced by intense exercise. For example, some muscle cells (breast, ovary and lungs) have high level of NQO1, which might provide protection against oxidative damaged (Siegel and Ross, 2000, Schlager and Powis, 1990). Therefore, the molecular profile of the oxidative stress markers following a 30 s sprint indicate that activation of Nrf2 signaling pathway could trigger the expression of Nrf2 downstream target gene NQO1 and consequently contribute to alleviate the oxidative stress in future exercise challenges.

HIT (SIT) causes the accumulation of misfolded proteins, leading to ER stress (Hong et al., 2021, Pinto et al., 2019). Our data shows that the expression of Sestrin2, not Sestrin1, increased immediately at post exercise after 8 weeks training. Upregulation of Sestrin2, an essential regulator in ER status, could ameliorate ER stress through modulation of mTORC-dependent protein translation (Lee et al., 2013, Kim et al., 2020). Additionally, Sestrin2 promotes catabolic pathway response by activating the AMPK pathway, resulting in the ATP production. Muscle ATP was increased at 72 hr after a single bout of 30 s sprint in trained status compared to untrained status in our study. Sestrin2 also might contributes to promote the aerobic fitness via AMPK pathway. For instance, It is reported that Sestrin2 might prevent muscle fibre shift (from slow to fast) via AMPK pathway (Yang et al., 2022). Studies demonstrated that ATF4 had positive effects in ameliorating the ER stress. For instance, activation of ATF4 induced transcriptional factor CHOP, which play a vital role in the regulation of autophagic process (Kong et al., 2018, Ogbechi et al., 2018). however, there is no difference in the expression of ATF4 after 8 weeks HIT training.

Our data indicates that HIT training increased the resting Sirt1 levels compared to the untrained state which is consistent with a previous study, which demonstrated that 2 weeks of SIT (8–12 × 60 s intervals at 100% VO_2 peak, 75 s recovery, 3x per week) and a 56% increase in Sirt1 expression (Little et al., 2010). Upregulation of Sirt1 has been reported to protect muscle cell from hypoxia stress via activation of redox sensitive transcription factors (such as FOXO3a and p53) to

inhibit or scavenge the ROS (induced by exercise) in muscle. For example, FOXO3a regulates autophagy (Fitzwalter and Thorburn, 2018) and also inhibits the apoptosis by activating the protein cAMP response element-binding protein, which leads to the reduction of proapoptotic target genes (Du et al., 2017, Fitzwalter and Thorburn, 2018). Gurd and colleagues demonstrated that 6 weeks of HIIT (10 x 4 min intervals at 90% VO_2 max, 2 min recovery) lead to a 16% increase in skeletal muscle PGC-1α protein content (Gurd et al., 2010), which is consistent to our study, which indicated HIT training increased the PGC-1α content compared to pre-exercise in trained participants. Increased PGC-1α contributes to the improvements of oxygen maximum uptake, which increase in this study. Increased in PGC-1α could induced mitochondrial biogenesis, which might protect muscle cell against from exercised induced stress.

6.5. Conclusion

Sprint interval training improved the VO_2 max and increased the peak power output and mean power output. Our data shows ribose might have positive interaction with SIT training in the improvement of exercise performance. However, our sample size is too small, therefore this awaits further study. HIT training also increase the Nrf2-mediated antioxidant enzyme NQO1, which might protect muscle cells against exercised induced oxidative damages. Resting Sirt1 levels elevated after 8 weeks SIT training, which might assist with alleviating the exercise-induced stress.

Chapter 7

Conclusions, limitations and future directions

7.1. Conclusion

The overall aim of this thesis was to investigate the molecular response to metabolic stress in skeletal muscle induced by exercise and disease and the influence of RIB supplementation. Chapter 4 probed the molecular stress signaling that occurs in response to HIT in male and female mice with tissue samples collected from a previously published study (Wilson et al., 2018). HIT results in a rapid depletion of purine nucleotides in response to extreme stress on the metabolic systems, which can also further stress antioxidant systems. Chapter 5 investigated the involvement of metabolic stress-induced signaling on skeletal muscle adaptations to stressful moderate intensity exercise training, and the ability of RIB supplementation to circumvent this stress signaling in healthy WT and metabolically-stressed *mdx* mice. Moderate intensity exercise on the metabolically stressed *mdx* background was hypothesised to exacerbate purine degradation and the molecular stress signature in skeletal muscle. Chapter 6 focussed on the role of stress signaling in the adaptations induced by SIT training on exercise performance in humans. We conducted a small pilot study, which was intended to be a full study, but was unfortunately impacted by the COVID-19 pandemic and curtained. The details of these studies are summarised below.

7.1.1. Effect of HIT training on skeletal muscle stress signaling in female and male mice

HIT impacts the body composition and drives molecular adaptations to exercise induced stress. To our knowledge, this is the first study to explore the relationship between protein biomarkers of different cellular stress responses in response to HIT and the impact of sex on these responses. We found the expression of Keap1, SOD and PGC1-α were higher in female skeletal muscle following HIT, yet the expression of HO-1, Sirt1 and PGC-1α, which can all be induced by hypoxic stress protein, HIF-1, were upregulated in male skeletal muscle. Our data suggest that female muscle may be more

responsive to oxidative stress signals, while male muscle may be more responsive to hypoxia stress signals.

7.1.2. Effects of moderate aerobic training and RIB supplementation on skeletal muscle stress signaling in healthy and *mdx* mice

This is the first study to explore the potential of RIB supplementation to mitigate muscle dysfunction in dystrophic *mdx*, and particularly, metabolically stressed exercised mice. Our data show that RIB treatment increased fatigue resistance and might have potential benefits on improved muscle function via upregulation of Sirt1 in *mdx* mice. These affects appear to be fibre type specific since only SOL mass and forelimb grip strength were positively impacted by RIB, although the reasons are presently unclear. Perhaps, more importantly, this study revealed that hypoxia stress signaling was already rampant in *mdx* mice and the application of EX, and then again RIB, exacerbated it in a graded fashion. Concomitantly, Keap1 expression also increased, but appeared complexed with Nrf2 and to exert a repressor function on the expression of some Nrf2-mediated antioxidant proteins (Catalase). For the first time, out data suggest Keap1 acts as a vital switch to selectively mediate either hypoxia or oxidative stress responses, but not together at once. Our data also suggests that ER stress signaling adaptations compensate for the failed activation of Nrf2 to take over oxidant scavenging and that increased purine degradation is linked to this response. RIB attenuated purine degradation resulting in reduced ER stress signaling in particular. How Keap1 mediates this stress switch is currently unclear and requires further study. RIB could be a therapeutic adjunct to effectively manage muscle fatigue in DMD patients and female carriers of the dystrophin gene mutation.

7.1.3. Effects of combined SIT and RIB supplementation on skeletal muscle stress signaling and purine metabolism in humans: A pilot study

This study aimed to investigate the metabolic and molecular remodelling of skeletal muscle with sprint interval training (SIT). Our data shows that SIT improved the VO_2 max and increased the

peak power output and mean power output. RIB might have positive effects in addition to SIT training to improve exercise performance. SIT training increased the Nrf2-mediated antioxidant enzyme NQO1, which might protect muscle cells against exercised induced oxidative damage. Resting Sirt1 levels were elevated after 8 weeks SIT training, which might assist with alleviating the exercise-induced stress.

7.2. Limitations

The data we provide in this thesis demonstrates that EX training and RIB supplementation had potential benefit on muscle function in dystrophic muscle in animals and improve the exercise performance in human. However, there are limitations that exist in the thesis.

In Chapter 4, we found that female and male skeletal muscle respond differently to HIT training at the molecular level. It was originally intended that my PhD studies would investigate RIB's effect on exercise capacity and on training adaptations in response to HIT. However, after getting a subset of subjects through the first planned study, the COVID-19 pandemic arrived in Australia and medical human research was suspended over the course of 2020-2022. To circumvent this complication and to progress my PhD studies, I was able to access some cryo-stored muscle samples from a previous study in our laboratory. This study investigated the effects of HIT in obese male and female mice (induced via a high fat diet). Because the samples were already collected and we were not able to manipulate the experimental design, there were several limitations:

1. All mice in the study were high fat fed, which may induce different molecular stress signaling response compared to normally nourished mice. For example, lipotoxic stress is known to impede the ER stress induction of Sestrin 2 (Kim et al., 2021), leaving tissues exposed to oxidative stress To purely understand the impact of metabolic stress involving extreme purine degradation, it would be ideal to normalise the diet and pair feed mice to ensure that the caloric intake was consistent across male and female, and trained and untrained groups.

2. Because the study was already completed and published, there was insufficient sample to complete the extensive analyses we would have liked to perform. For example, there was insufficient sample to analyse urine metabolites to further explore sex effects. We saw that plasma uric acid levels, the end-product of purine metabolism and flux from muscles, were different between female and male mice. These data suggest that estrogen appears to have a protective effect on metabolism in general, or on purine degradation specifically, resulting in less uric acid excretion (Sumino et al., 1999).
3. We assessed only a small subset of possible oxidative, ER and hypoxia stress related proteins and we used quantitative western blot to do this. There are limitations to this approach. Firstly, our data only indicate that certain pathways were activated, they do not provide a holistic portrayal of integrative stress signaling or the inducers involved. Using alternative methodological approaches, such as integrative measures, i.e. metabolomics/transcriptomics/proteomics would enable more extensive and precise signaling pathways to be elucidated while muscle is under metabolic stress and confirm whether purine degradation is a driver of the stress signaling that leads to cellular adaptations.

In Chapter 5, we investigated the same stress signaling pathways, in additional to in vivo and in vitro measures of muscle function and fatigue following moderate intensity exercise training to induce metabolic stress in healthy WT and metabolically-stressed *mdx* mice. Although this study was well-controlled and took a holistic approach by assessing various in vivo and in vitro functional measures (e.g., grip strength, fatigue run, ex vivo muscle function) and a multitude of indicators of metabolic stress (extracellular flux, muscle and plasma metabolites, molecular stress signaling), there remained some limitations:

1. It was sometimes difficult to apply the EX regimen to *mdx* mice. Especially at the commencement of EX training, *mdx* mice regularly stopped running, hitting the back of the treadmill chamber before running forward again. WT mice were much better at

continuously running over the training period. While we did apply bursts of air to encourage the mice to run when they stopped, this could have led to differential degrees of EX stressor being applied to WT and *mdx* mice.

2. Further from point 1, we did not monitor normal cage activity in SED or EX mice. It is possible that mice from either group participated in more or less cage activity during the nocturnal period which could have been influential on baseline stress responses.
3. Further from point 2, we applied EX training during the diurnal period, when mice are typically sleeping and their hormonal and metabolic systems are geared toward energy restoration rather than utilisation. this may limit the transferability of our data between mice and humans, since in the human study, we applied exercise during the diurnal period (equivalent to the nocturnal period in mice).
4. The dosage of ribose used in our study was set as an intermediate between the dosage used in Hellsten et al's 2004 study using ribose to improve recovery following exercise-induced ATP depletion (600 mg/kg/day spread across x3 dosages daily) and Griffith et al's 1985 study investigating ribose in DMD patients where no beneficial effects on ATP levels were observed (500 mg/day, subject weight unfactored). FDA guidelines for drug conversion between humans and animals stipulate a standard human weight of 60kg (which needed to be factored into the Griffith's et al. ribose dosage) and a 12.3x conversion factor to account for the faster metabolic rate of mice (both studies). The mouse converted daily ribose dosages from the Hellsten et al. and Griffiths et al. studies were, therefore, 7380mg/kg/day and103mg/kg/day, respectively. We settled on 1600mg/kg/day, which was 16x higher than the human comparative dose administered in Griffith et al's DMD study and ~5x lower than the human comparative dose administered in Hellsten et al's exercise study since we were unsure how mice (particularly juvenile mice) would respond. The dose administered in our study was sufficient to induce HIF1, Sirt 1 and Keap1 expression, however there was only a trend toward reduced urinary uric acid levels, for example.

Optimising dosage to effect statistically significant recovery of purine nucleotide levels should be pursued to enable the proper testing of our hypotheses.

5. Further from point 4, ribose was shown to be beneficial for exercise-related ATP depletion and recovery when administered 3x daily in the post-exercise period. We administered ribose via once daily a.m. gavage only. Sustained blood levels of ribose via frequent (or continuous) administration may induce very different effects than periodical administration with longer durations of time between dosages. For example, in Griffiths 1985 trial that used only twice daily treatment with very low dose (200 mg) ribose showed no significant impact on ATP or other metabolite levels in skeletal muscle of DMD patients.
6. Further to points 4 & 5, RIB treatment very specifically influenced SOL muscles, which infers it either (1) has fibre type specific effects since SOL is predominantly composed of type II fibres in mice; or (2) that SOL muscles were subject to more pathology in *mdx* mice due to their location and compartmentalisation, and therefore were under more metabolic stress to benefit from RIB treatment. Indeed, current findings in our lab (Timpani et al., 2022) show extensive damage to SOL muscles from juvenile *mdx* mice that is not apparent in EDL muscles. Traditionally, Type II fibres are more prone to metabolic stress than type I fibres given their low mitochondrial number and propensity for anaerobic metabolism, and RIB is likely to benefit the recovery of energy homeostasis in these fibres more (Hellsten et al., 2004). Since human muscle is mixed fibres (40-60%) but mice are comprised primarily of type I or type II fibres, dose could be escalated in mice to account for the higher demand on ribose-mediated PRPP energy recovery. The *mdx* pathology adds a layer of complexity to this idea though, since their well-documented mitochondrial dysfunction also puts strain on type I fibres (perhaps even moreso than type II fibres as is suggested in our current work).

In Chapter 6, we completed a small pilot study in male and female humans investigating the effect of a SIT bout, and SIT training, on molecular stress signaling in skeletal muscle. Due to

the COVID-19 pandemic, this study was suspended for 2 years and couldn't be completed within the scope of this thesis.

1. The insufficient sample size is a consequential limitation of this COVID shortened study rendering it underpowered for most physical parameters and group comparisons, especially male/female differences and the influence of ribose supplementation. However, we were able to utilise this as a pilot, which allowed us to investigate molecular signaling in response to intense sprint exercise before and after extreme intensity SIT to some extent. Obviously, a larger sample size would be required to enable us to test the hypothesis.
2. In human study, there is no food limitations for the participants, we cannot monitor the foods and drinks during the entire experiment (for example, some energy drinks contain ribose), which might impact our experiments.
3. The participants we recruited have different lifestyle. One male participant in our trial has soccer training twice a week, which can increase the VO_2 max (Milanovic, 2015). Therefore, we cannot sure the RIB has any benefit in this situation.

7.3. Future directions

The results of this thesis raised several questions that needed to be further explored.

1. Chapter 4 showed that female and male mice adapt differently in response to HIT and our data. Based on this data, we can expand our human study and recruit more female and male participants to explore this hypothesis that female and male people might makes different adaptations through the different signaling pathways.
2. Further from point 1, various studies demonstrated high-intensity exercise (HIT) in hypoxia improved the VO_2 max and exercise performance than HIT in normoxia (Scott et al., 2016, Smith et al., 2022). Based on the Chapter 4 & 5, further studies can be targeted to explore the effect of HIT in hypoxia condition on stress signaling pathway in female and male participants.

3. Type II muscle fibres are more prone to stress, and to the adaptations that result from that stress, than type I fibres – especially in the context of exercise training – in humans (Lievens et al., 2020) and mice (Plotkin et al., 2021). Moderate exercise aggravates muscle wasting in *mdx* mice (De Luca et al., 2003), however, whether ATP recovery is more efficient in type II fibres is still unknown. It would be interesting to explore ATP recovery in different muscle fibre types in response to metabolic stress induced by moderate exercise in both WT and metabolically-stressed *mdx* mice to determine whether fibre-type specific signaling pathways are activated.
4. Our data show that hypoxia induced signaling was predominant in *mdx* muscles (and was exacerbated by EX and RIB). ER signaling and oxidative stress signaling were also stimulated in our *mdx* study. Some biomarkers can be activated by multiple pathways. For instance, Keap1 may act as a stress switch between oxidative stress and hypoxia stress signaling (Liu et al., 2017a, Zhan et al., 2021). Both RIB and EX appeared to exacerbate this stress response. However, the mechanisms remain unclear and should be elucidated. There is growing evidence that metabolites can induce “pseudo-hypoxia” and our data infer that RIB can induce this effect. As such, it could be useful as an exercise mimetic, especially in diseases, such as DMD, where exercise adaptations might be beneficial, but exercise itself is impossible once patients lose the ability to ambulate.
5. We did observe the RIB treatment had benefits on contractile properties- specifically, there appeared to be more contractile proteins leading to increased muscle mass and force output in the SOL muscles. Our data suggest that type I muscle fibres may express more RIB transporter or carriers than fast-twitch fibres. It would be interesting to determine the expression of these transporters and elucidate whether their expression is modulated by RIB therapy or exercise and training.
6. Both SIT training in healthy models and moderate intensity exercise in metabolically-stressed *mdx* mice induce the purine degradation and activate the multiply stresses. Xanthine oxidase (XO) is an enzyme that regulates the rate at which uric acid is produced

from xanthine and hypoxanthine with the accompanying production of ROS (Doehner and Landmesser, 2011, Day et al., 2016). Uric acid, final product of purine metabolism and along with ROS production, has been considered as a biomarker of metabolic stress in disease (Lytvyn et al., 2015, Packer, 2020). Our data shows that RIB has a tendency to decrease the plasma uric acid concentration in *mdx* mice and has potential benefit to alleviate metabolic stress through activation of the multiple signaling in dystrophic *mdx* mice. Collectively, the mechanism of how stress signaling pathway impact purine metabolism in response to metabolic stress is still unknown.

Chapter 8
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