Nox4 mediates metabolic stress responses

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ABSTRACT

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Deficits in skeletal muscle mitochondrial metabolism are associated with a wide variety of chronic skeletal muscle and metabolic-related diseases, including diabetes and sarcopenia. Even in patients with advanced skeletal muscle-related diseases, exercise is a well-established method to improve skeletal muscle mitochondrial metabolism, culminating in enhanced whole-body metabolism and decreased disease severity. In response to exercise, there is an increase in reactive oxygen species (ROS) production. Historically, ROS were solely considered to drive disease development. However, ROS are also required for physiological adaptation and many questions still remain regarding their downstream pathways. One significant producer of skeletal muscle ROS with exercise is Nadph oxidase 4 (Nox4). Nox4 is unique compared to other Nox members as it predominantly produces hydrogen peroxide (H_2O_2), an effective signaling molecule. Here we demonstrate an essential role for Nox4 in mediating the beneficial effects of exercise. This work will contribute to our understanding of physiological ROS and their downstream targets by identifying a novel role for Nox4 in exercise adaptation. Further defining the molecular events that promote exercise adaptation will be essential for formulating new treatment strategies for patients with chronic metabolic diseases.

GENERAL AUDIENCE ABSTRACT

Exercise is a widely effective tool for both preventing and reversing disease. Even patients with advanced skeletal muscle and metabolic-related diseases can benefit from continual and repeated exercise training. While decades of work have supported the effectiveness of exercise as a therapeutic intervention, the mechanistic understanding of what occurs at the cellular level remains incomplete. Here, we elucidate a novel pathway mediating important metabolic adaptations to exercise. In response to exercise stress, reactive oxygen species (ROS) are produced in skeletal muscle. ROS facilitate metabolic adaptations to meet the body's need for increased energy. One significant source of ROS comes from Nadph oxidase 4 (Nox4) which plays an essential role in metabolic regulation. The skeletal muscle metabolic response to stress is largely dependent on adaptations that include changes in gene expression, substrate oxidation, and mitochondrial metabolic adaptations. These mitochondrial adaptations include mitochondrial recycling after exercise in skeletal muscle (referred to as mitophagy). We have shown that Nox4 increases the expression of a subset of metabolic genes, is required for substrate oxidation after exercise, and is important for exercise-induced mitophagy.

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List of Abbreviations

ACC: Acetyl-CoA carboxylase	NADH: Nicotinamide adenine dinucleotide
Ad-Cat Adenoviral catalase	*Nf-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells*
Ad-GFP Adenoviral green fluorescent protein	Nox: Nicotinamine diadenine dinucleotide phosphate oxidase
AMPK: AMP-activated protein kinase	*Nox4-/-: Global Nox4 deletion*
Ap-1: Activator protein 1	Nox4: Nicotinamine diadenine dinucleotide phosphate oxidase 4
ATP: Adenosine triphosphate	*$Nox4^{ECKO}$: Endothelial Nox4 specific deletion*
BHAD: Beta-hydroxyacyl-CoA-dehydrogenase	*Nr4a3: Nuclear receptor subfamily 4 group A member 3*
CPT1: Carnitine palmitoyl transferase 1	*Nrf2: Nuclear factor erythroid 2-related factor 2*
Creb: CAMP-responsive element-binding protein 1	OXPHOS: Oxidative phosphorylation
CS: Citrate Synthase	PCr: Phosphocreatine
EDL: Extensor Digitorum Longus	PDH: Pyruvate dehydrogenase
eNOS: Endothelial nitric oxide synthase	*Pdk4: Pyruvate dehydrogenase kinase 4*
EPOC: Post exercise oxygen consumption	*Pgc-1a: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha*
ETC: Electron transport chain	Pink1: PTEN-putative kinase
$FADH_2$: Flavin adenine dinucleotide	RER: Respiratory exchange ratio
GC: Gastrocnemius	ROS: Reactive oxygen species
GLUT4: Glucose transporter 4	*Sik1: Salt-inducible kinase 1*
H_2O_2: Hydrogen Peroxide	*Slc25a25: Calcium-binding mitochondrial carrier protein*
Hif-1: Hypoxia-inducible factor-1	TCA cycle: Tricarboxylic acid cycle
HK: Hexokinase	UCP3: Uncoupling protein 3

Chapter 1: Introduction

Decreases in skeletal muscle mitochondrial energy production are a hallmark of numerous skeletal muscle-related diseases, including diabetes[1] and sarcopenia[2]. Skeletal muscle is an extraordinarily plastic and metabolic organ[3] that is an essential mediator of whole-body energy homeostasis. During conditions of energetic stress (e.g., exercise), there is an increased energy demand. To meet this metabolic demand, skeletal muscle mitochondrial energy production increases through activation of the tricarboxylic acid cycle (TCA) and oxidative phosphorylation (OXPHOS). Perturbations in these metabolic responses (either delayed or inefficient energy production) may exacerbate disease prognosis[4] and increase the risk of morbidity and mortality[5].

However, repeated and consistent bouts of exercise improve disease prognosis for skeletal muscle-related diseases[6]. Exercise training improves skeletal muscle mitochondrial function and efficiency[7], culminating in improved health outcomes. While it is well accepted that exercise is an effective intervention for patients with a host of skeletal muscle, and other metabolic-related diseases, the molecular signals that promote these beneficial adaptations are not fully known[6,8,9].

At the onset of exercise, there is an increase in reactive oxygen species (ROS) production. However, ROS were initially thought to drive the aging process by causing damage to macromolecules such as proteins, lipids, and nucleic acids[10], referred to as *oxidative stress*[11], where there is a cellular ROS imbalance[12]. However, decades of research have demonstrated a physiological role for ROS and their efficacy as signaling agents[13,14]. In fact, ROS may be required for promoting adaptive responses to exercise[15].

While it is clear there is a role for ROS in both physiological and pathological processes, there are several methodological limitations to assessing ROS production *in vivo* and *in vitro*. Highly specific ROS probes bind to one form of ROS (H_2O_2 versus $O_2^{\bullet-}$). In addition, other

fluorescent probes are non-specific[16], which lends little insight into sources of ROS during stress, and may be limited by cell or tissue type. Furthermore, for *in vivo* studies, it may be challenging to distinguish cell type-specific sources of ROS. For example, skeletal muscle is a mixed tissue consisting of multiple cell types including myocytes, immune cells, and endothelial cells. With current ROS probes, there are challenges associated with distinguishing endothelial-derived ROS from skeletal muscle cell-derived ROS in response to exercise. Thus, indirect measures of ROS with the use of genetic modeling can provide extensive insight into the downstream targets of ROS and their physiological role in metabolic adaptation.

In Chapter 2, we provide the conceptual framework with a review of the literature on exercise metabolism, Nadph oxidase 4 (Nox4) and mitochondrial quality control in skeletal muscle. Chapter 3 reviews the specific aims for this dissertation. In Chapter 4, we utilize novel genetic models to address the role of Nox4 in mediating skeletal muscle adaptation to acute and chronic exercise. Based on the molecular insight gained from Chapter 4, Chapter 5 addresses the role of Nox4 on a specific facet of mitochondrial function, mitophagy, that is important for both exercise adaptation and skeletal muscle metabolism. These studies utilize a novel tool, *pMitoTimer* via somatic gene transfer[17], to assess mitophagy in response to acute exercise.

In the subsequent chapters, we highlight the essential physiological role of ROS in promoting skeletal muscle metabolic adaptation in response to exercise. As exercise remains the most effective treatment for managing many skeletal muscle-related diseases, dissecting the mechanistic changes that promote these beneficial physiological adaptations is essential for identifying novel therapeutic targets. We have examined *metabolic adaptation* across multiple levels, including changes in gene expression, protein expression, enzyme activity, substrate oxidation, and ex vivo mitochondrial function. In addition, we have assessed these molecular

adaptations after acute and chronic exercise. Taken together, we provide significant evidence that Nox4 is an essential mediator of skeletal muscle metabolic adaptation in response to exercise. These results highlight the physiological significance of ROS in mediating adaptations to exercise and highlight novel downstream targets of Nox4. In conclusion, these studies will help characterize the beneficial effects of exercise and may represent novel targets for skeletal muscle-related diseases.

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Chapter 2: Literature Review

2.1 Introduction

Decreases in skeletal muscle mitochondrial energy production are associated with numerous metabolic diseases, including diabetes[1] and sarcopenia[2]. However, even patients with advanced skeletal muscle metabolic dysfunction have the potential to improve mitochondrial function with consistent and repeated exercise training[3]. Exercises is effective as a preventative and therapeutic tool for patients with a wide range of metabolic diseases[4,5]. However, the mechanistic insight as to how and why exercise is effective remains incomplete[6].

Exercise elicits a robust physiological response in multiple systems and tissues[7]. As global -omics have become more accessible and widely used, the breadth of molecular networks that promote both acute and chronic adaptations to exercise have only begun to become more appreciated[8]. While exercise targets multiple systems, skeletal muscle is integral in supporting human movement and whole-body energy regulation[9,10]. In response to chronic training, skeletal muscle metabolic remodeling is integral for global metabolic health and disease prognosis[11].

Disruptions in skeletal muscle mitochondrial energy production are linked to disease severity[12]. Patients with advanced metabolic disease and skeletal muscle metabolic deficits can exhibit exercise intolerance (e.g., perturbations in skeletal muscle metabolism in response to exercise)[13]. Regular exercise improves skeletal muscle mitochondrial function[14] and enhances glucose and lipid metabolism[15], culminating in improved health outcomes[16–18]. However, there are still unanswered questions regarding the molecular signaling of these necessary adaptive processes.

At the onset of an acute exercise bout, there is an immediate change in the metabolic environment[10,19]. Reactive oxygen species (ROS) produced in contracting skeletal muscle[20] were

thought to be the main cause of muscle fatigue[21,22]. However, decades later it became clear that ROS mediate physiological processes[23] and are effective signaling molecules[24,25]. The roles of ROS are two-fold. While ROS drive oxidative stress, lower ROS concentrations are effective signaling molecules and promote cellular adaptation[25], particularly for skeletal muscle metabolic adaptation to exercise[26,27]. This review chapter will discuss exercise metabolism, the role of ROS in promoting skeletal muscle adaptation, and downstream mitochondrial adaptations are essential for skeletal muscle mitochondrial quality.

2.2 Metabolism overview

Metabolism is a general scientific term that refers to both catabolic and anabolic processes that promote cellular energy production in the form of adenosine triphosphate (ATP). During exercise, there is an increased demand for energy. To meet this increased demand, there is increased ATP hydrolysis, an exothermic reaction that releases free energy to meet the increased demand[10,28]. ATP synthesis is derived from a series of metabolic reactions. These processes include glycolysis (the breakdown of glucose), beta-oxidation (breakdown of fatty acids and subsequent oxidative phosphorylation at the inner mitochondrial membrane), and proteolysis (the breakdown of protein)[29]. However, the focus of this review will remain on exercised mediated changes in mitochondrial metabolism.

Glycolysis is a ten-step oxygen-independent (anaerobic) metabolic pathway[30]. Glucose, a six-carbon sugar, is transported into the cell through a family of glucose transporters[31]. Upon entry into the cell, hexokinase immediately phosphorylates glucose and is a central regulatory point in the glycolysis pathway[32]. Upon complete oxidation of glucose, glycolysis yields energy in the form

of ATP and two pyruvate molecules[30]. Pyruvate is a three-carbon molecule that is converted into acetyl-CoA at the tricarboxylic acid cycle (TCA).

The TCA cycle is an eight-step reaction that includes reduction, oxidation, dehydration, rehydration, and decarboxylation to produce reducing equivalents that are the source of electrons at the mitochondrial electron transport chain (ETC)[33]. In contrast to glycolysis, the TCA is an oxidative pathway that feeds into multiple biochemical pathways, including the breakdown of carbohydrates, fats and amino acids. The TCA oxidizes pyruvate (the product of glycolysis) into reducing equivalents ($FADH_2$ and NADH)[34]. The net yield of the oxidation of 1 pyruvate yields 1 ATP, 2 CO_2, 3 NADH, and 1 $FADH_2$[33,34]. NADH and $FADH_2$ are then oxidized at the mitochondrial electron transport chain. Mitochondrial electron transport is coupled with oxidative phosphorylation, which is essential to drive ATP synthesis.

Beta-oxidation, the breakdown of fatty acids, is an oxygen-dependent (aerobic) ATP generating process[35]. Beta-oxidation also generates acetyl-CoA, which undergoes further oxidation at the TCA[36]. Mitochondrial oxidative phosphorylation is a coupled biochemical process that connects electron transport to ATP synthesis. Electron donors (in the form of NADH and $FADH_2$) undergo oxidation at Complex I[37] and Complex II[38], respectively, of the mitochondrial electron transport chain (ETC). Electrons are passed down the mitochondrial electron transport chain which generates an electrical and proton gradient[39]. This process is coupled with ADP phosphorylation at Complex V of the ETC[40]. These energy-producing biochemical pathways in the mitochondrial matrix are collectively referred to as *mitochondrial bioenergetics*[41]. While the stepwise reactions of central metabolism are well established, the molecular regulation of these pathways, particularly during conditions of energetic stress, is incompletely understood.

2.3 Skeletal muscle exercise metabolism

Exercise metabolism encompasses multiple catabolic processes including the oxidation of glucose and fatty acids to generate ATP. Historically, exercise-mediated molecular pathways have focused on protein expression, enzyme activity, and respiratory capacity in skeletal muscle[15]. However, many molecular pathways are involved in promoting substrate mobilization, delivery, transport and oxidation during and after exercise[10]. These adaptive changes occur at the epigenetic[42], transcriptional[43], translational[44], post-translational[45] levels. Recently, a global phosphoproteomic analysis identified 1,000 exercise-regulated phospho-sites on over 500 different proteins after just a single bout of high-intensity exercise[45]. Large-scale -omics studies unveil new molecular targets that are imperative for exercise adaptation[43], with many new opportunities to understand further how exercise is an effective preventative and therapeutic tool for a wide range of patients.

The respiratory exchange ratio (RER) is a physiological measure for whole-body substrate metabolism by quantifying carbon dioxide output relative to oxygen intake[46]. The respiratory exchange ratio will be approximately 0.85 in a fasted healthy individual and with exercise, the RER can exceed 1.0, indicative of an increased reliance on carbohydrate oxidation[47]. Thus, an RER of 0.70 represents a predominant reliance on fatty acid oxidation[46]. For long duration aerobic activity, an RER will decrease and can return to a value of 0.70, indicating increased reliance on fatty acids for energy when glycogen stores are depleted[15].

Another measure of post-exercise metabolism at the whole-body level is post-exercise oxygen consumption, EPOC. This correlates with an increase in metabolic rate that remains elevated for up to 24 hours[15]. However, while these methods effectively assess global metabolism, minimal information can be gathered at the cellular level using RER and other indirect calorimetry

measurements[48], given the complexity of tissue-specific metabolic function. It is imperative to examine what is occurring at the tissue and cell level to glean insight into the metabolic effects of exercise. While exercise targets many systems at the global and cellular level, the scope of this review will focus on skeletal muscle substrate oxidation post-exercise.

Metabolic adaptation works to quickly and efficiently respond to increased energy demand[10,15]. Exercise intensity and duration are determinants of substrate utilization and oxidation in skeletal muscle[49,50]. Phosphocreatine (PCr) is a predominant source of energy for a short bout of exercise that may only last seconds[51]. Whereas during lower intensity, longer duration aerobic activity, oxidative phosphorylation and beta-oxidation (fatty acid oxidation) are responsible for providing ATP to working skeletal muscle[10].

During aerobic exercise, glucose is mobilized and delivered to contracting skeletal muscle[52]. To increase skeletal muscle glucose uptake, glucose transporter 4 (GLUT4), the predominant glucose transporter in skeletal muscle, is translocated to the plasma membrane[53]. Once glucose has entered the cell, hexokinase will phosphorylate glucose into glucose-6-phosphate, which can undergo glycolysis. This specific phosphorylation event is essential in skeletal muscle as glucose-6-phosphate cannot be transported out of the cell (e.g., glucose is "trapped" in the cell). Hexokinase-2 is a rate-limiting step for glucose oxidation as overexpression of hexokinase will support an increase in exercise-stimulated glucose uptake[52,54]. After a single acute bout of exercise, *Hexokinase* mRNA and activity are both increased with a single acute bout of exercise in skeletal muscle in rats [55] and humans [56]. After a chronic training regimen, Hexokinase 2 content will increase, and transgenic overexpression of HK in mice will enhance endurance capacity during exercise[57]. Recent proteomic analysis has highlighted hundreds of

potential exercise targets, suggesting that exercise-mediated glucose uptake and glucose oxidation regulation are far more complex than what is already known[52].

Glucose and fatty acid oxidation are not independently regulated processes – while each catabolic process is substrate-specific, there are multiple points at which these two pathways converge. Pyruvate dehydrogenase (PDH) is a regulatory point between fatty acid metabolism, glucose metabolism and the TCA as PDH catalyzes the oxidative decarboxylation of pyruvate[58]. The oxidative decarboxylation of pyruvate by PDH acts as the main entry point for carbohydrate derived substrate to enter the mitochondria for further oxidation at the TCA cycle and then thorough mitochondrial electron transport[59]. Pyruvate dehydrogenase kinase (PDK) is a mitochondrial enzyme upstream of PDH and acts as a negative regulator by inhibiting PDH activity[60]. PDK4 is the predominant isoform in skeletal muscle and *PDK4* mRNA is increased in human skeletal muscle after an acute exercise bout[61]. Changes in PDK4 may have functional bioenergetic implications. Increased PDK4 expression correlates with a greater reliance on fatty acids as the predominant fuel source[62].

Fatty acid oxidation is a multi-step process that breaks down fatty-acyls to acetyl-CoA in the mitochondrial matrix. In the context of exercise, beta-oxidation rates are differentially regulated depending on the mode, duration, and intensity of exercise. Maximal fat oxidation typically occurs between 45 and 65% VO_2 max[49]. During exercise, fatty acids are mobilized from other tissues (adipose)[63] and then released into circulation to fuel working skeletal muscle. It was initially postulated that skeletal muscle fatty acid transport occurred through passive diffusion, but in the last 20 years, significant evidence suggests this is a protein-mediated mechanism[64,65]. CD36 is highly expressed in skeletal muscle[66] and will translocate to the plasma membrane approximately one minute after skeletal muscle contractions[65,66]. In fact, with chronic endurance

exercise, CD36 cell membrane expression correlates with fatty acid oxidation rates[66,67]. In addition, fatty acid binding protein (FABP) is also increased after six weeks of exercise training[68], suggesting that fatty acid transport is integral in adaptive changes in exercise-mediated fat oxidation.

Once fatty acids cross the skeletal muscle plasma membrane, fatty-acyls must be transported across the mitochondrial membrane to undergo complete beta-oxidation in the mitochondrial matrix. The carnitine shuttle is the main transport system for mitochondrial fatty acid import into the inner mitochondrial membrane space[69]. Carnitine palmitoyltransferase 1 (CPT1) is an outer mitochondrial membrane bound protein that is integral for fatty acid import[70]. While inhibition of CPT1 with etomoxir during muscular contractions reduced fatty acid oxidation[71], only moderate changes in malonyl-CoA (an inhibitor of CPT1) content are observed in skeletal muscle during exercise[72]. While CPT1 is likely involved in fatty acid important during exercise, its molecular regulation and role in adaptation is not fully resolved.

Acetyl-CoA carboxylase (ACC) catalyzes the conversion of acetyl-CoA to malonyl-CoA, an essential step in fatty acid synthesis. Phosphorylation of ACC turns off fatty acid synthesis and promotes beta-oxidation[73–76]. ACC is an immediate downstream target of AMP-activated protein kinase (AMPK) a central regulator of energy metabolism[77]. Phosphorylation of AMPK and ACC increase progressively during moderate-intensity aerobic exercise[78]. Recently demonstrated, mice deficient in ACC2 had average rates of fatty acid oxidation ex vivo and in vivo, suggesting induction of fatty acid oxidation is independent of ACC2 phosphorylation[79]. While the phosphorylation of ACC is a widely accepted target in the context of acute exercise, the exact role in metabolic adaptation is less clear.

Once fatty acids are transported to the mitochondria matrix, long chain acyl-CoAs will undergo a series of oxidation reactions to generate acetyl-CoA. Very long chain acyl-CoA dehydrogenase (VLCAD) oxidizes acyl-CoA into trans-2-enoyl CoA while generating $FADH_2$[80]. Other members of the acyl-CoA dehydrogenase family include the long-chain acyl-CoA dehydrogenase (LCAD), medium-chain acyl-CoA dehydrogenase (MCAD), and short chain acyl-CoA dehydrogenase (SCAD) that catalyze the reaction based off the size of the fatty acid chain length[81]. VLCAD localizes to the inner mitochondrial membrane[82], whereas MCAD and SCAD localize to the matrix[81]. The second step is the hydration of trans-2-enoyl-CoA into 3-hydroxyacyl-CoA by enoyl-CoA hydratase[83] followed by a dehydrogenation reaction by beta-hydroxyacyl CoA dehydrogenase (BHAD) to produce B-ketoacyl-CoA while generating NADH[84]. BHAD activity increases with chronic training and is integral in mediating changes in beta-oxidation[85].

The final step of beta-oxidation is the thiolytic cleavage by 3-ketyoacyl-CoA thiolase to generate acyl-CoA[80]. Together, these enzymes that catalyze the 2nd through 4th step of beta-oxidation to form a heterotrimer protein referred to as trifunctional protein (TFP)[86]. Little is known regarding *HADHB* encodes for the beta-subunit of hydroxyacyl-CoA dehydrogenase and is significantly upregulated after exercise training in human skeletal muscle[87]. The functional bioenergetics implications of this adaptation as there is much to be learned regarding the exercise mediated molecular regulation of acyl-CoA dehydrogenase and TFP[88]. This is because the majority of the research on the trifunctional protein has been conducted in the context of rare inherited mitochondrial diseases[89,90] that result in cardiomyopathy, muscle breakdown, and death[91]; often the patients are exercise intolerant[92]. While exercise training improves mitochondrial metabolism efficiency, the molecular regulation of beta-oxidation during and after exercise is incompletely understood.

The end product of the breakdown of fatty acids is the generation of multiple acetyl-CoA molecules that will then reenter the TCA. The oxidation of acetyl-CoA at the TCA cycle produces NADH and $FADH_2$. NADH and $FADH_2$ are electron carriers and will undergo oxidation at Complex I and Complex II of the mitochondrial electron transport chain (ETS). Citrate synthase (CS) catalyzes the condensation reaction of acetyl-CoA and oxalacetate to produce citrate[93]. Citrate synthase is a classic marker of mitochondrial adaptation to exercise, and both content and activity are increased after chronic exercise training[94–96].

The mitochondrial electron transport chain consists of multiple protein complexes and over 20 redox couples, all within the inner mitochondria membrane[97]. The flow of electrons starts at Complex I or II and is subsequently passed down the ETC to Complex IV. This is driven by oxygen's high electronegativity as oxygen is the final electron acceptor at Complex IV and the subsequent redox potential of each carrier[98]. The generation of the electrochemical gradient through mitochondrial electron transport creates a proton gradient[99] that allows for phosphorylation of ADP into ATP at Complex V.

Exercise promotes adaptive changes in mitochondrial function and content[100]. Historically, oxidative phosphorylation (OXPHOS) capacity is a predictor of exercise performance[101]. Adaptive changes in mitochondrial bioenergetic capacity have often been attributed to mitochondrial electron transport chain content[102]. However, recent findings demonstrated that changes in electron flow to OXPHOS may be more important than mitochondrial content for efficient ATP production in human skeletal muscle after chronic exercise training[103] further highlighting the complexity of these biochemical processes, particularly in the context of exercise.

For decades, the biochemical mechanism of energy generating processes (glycolysis, TCA cycle, mitochondrial OXPHOS, and beta-oxidation) has been well documented. However, their

molecular orchestration and regulation in different physiological and pathological contexts are not completely understood. Only recent studies using comprehensive omics have shed insight into the vast metabolic response that is initiated by even a single bout of exercise. This has been observed at the phosphoproteomic level[45], transcriptomic[104], epigenetic level [42,105] and metabolomic level[106]. There is much to be learned about the molecular signals that promote exercise mediated metabolic adaptation and how this culminates in beneficial health effects.

ATP hydrolysis

$ATP + H_2O \rightarrow ADP + P_i + H^+$

ATP synthesis

Phosphocreatine

$PCr + ADP + H^+ \rightarrow ATP + creatine$

Glycogen

$glycogen_n^a + 3\ ADP + 3\ P_i \rightarrow glycogen_{n-1} + 2\ lactate + 3\ ATP$

Oxidative phosphorylation

$Glucose + 6\ O_2 + 36\ ADP \rightarrow 6\ CO_2 + 6\ H_2O + 36\ ATP$

$Palmitate + 23\ O_2 + 130\ ADP \rightarrow 16\ CO_2 + 16\ H_2O + 130\ ATP$

Figure 2. Energy metabolism in skeletal muscle

2.4 History of ROS

Reactive oxygen species (ROS), as their name suggests, are highly reactive molecules, that are derived from molecular oxygen and are essential for adaptive metabolic processes[107]. Superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), are particularly important for adaptation, and will be the predominant focus in this section. The reaction starts with an electron transfer to oxygen to form superoxide, which then undergoes further reduction to form hydrogen peroxide (a non-radical oxidant)[108].

Hydrogen peroxide was first recognized by Louis Jacques Thénard in 1818. It wasn't until the 1950's that Plaine et al. indicated ROS could damage both DNA and lipids[109]. In the 1970s, a study by Czech et al. demonstrated exogenous supplementation of H_2O_2 in isolated fat cells similarly stimulated 3-O-methylglucose transport, as would insulin[110], suggesting H_2O_2 may act as an important signaling molecule. Decades later, Sundaresan et al. demonstrated vascular smooth muscle cells stimulated with platelet-derived growth factor (PDGF) increased intracellular concentrations of H_2O_2[111]. Subsequently treatment with antioxidants blunted the downstream response and blocked MAPK activation and DNA synthesis suggesting that H_2O_2 acts as a signal transducing molecule[111].

H_2O_2 is unique due to its ability to modify cysteine residues and subsequently alter protein function[112] and its longer half-life (~1 ms) compared to other forms of ROS[107]. In addition, H_2O_2 can cross cellular membranes[113,114]. While it is now recognized that ROS can play a role in physiologic signaling, ROS were initially thought to drive the aging process by causing damage to macromolecules such as proteins, lipids, and nucleic acids[115] often referred to as *oxidative stress*[116], where there is a cellular ROS imbalance[117]. While *pathological* ROS are critically involved in disease development[118], *physiological* ROS (sometimes denoted as

oxidative eustress) are essential for the activation of multiple cellular pathways[119], including skeletal muscle metabolism[120].

2.5 ROS and skeletal muscle

While it is now recognized that ROS can play a role in skeletal muscle function, ROS were initially thought to drive skeletal muscle fatigue. Studies in the 1980s clearly demonstrated that contracting skeletal muscle produces ROS[21,121,122]. Davies et al. demonstrated that exercise-produced ROS damaged the mitochondria. However, they postulated that with consistent and repeated exercise training, ROS may promote mitochondrial biogenesis[21]. Shortly thereafter, Quintanilha and Packer et al. demonstrated that with consistent and repeated bouts of exercise, antioxidant enzyme content increased in skeletal muscle[123]. In 1987, nitric oxide (NO) was demonstrated to promote vasodilation, demonstrating that reactive nitrogen species were promoting physiological adaptations[124]. Taken together, while most of the studies conducted during this time suggested that ROS were driving fatigue, they laid the groundwork for subsequent exploration of whether this was required for chronic adaptative processes.

The notion that ROS were driving skeletal muscle fatigue was further validated in different studies through the 1990s. In 1990 Novelli et al. demonstrated that radicals promote skeletal muscle fatigue by utilizing spin traps (to scavenge ROS) which delayed fatigue onset (as defined by decrease in contractile force[125,126]. During this time, Reid demonstrated that skeletal muscle releases superoxide into the interstitial space[22,127]. This brought up a unique question; if ROS were able to cross cellular membranes does this mean they can act as signal transductors?[22]. In 1994, follow up study was published showing oxidants contribute to muscle fatigue *in humans* and that the use of a non-specific antioxidant inhibited fatigue onset[128]. Here, Reid and

colleagues proposed a model where there is an optimal balance between the cellular redox state and isometric force production[126,128]. The insight gained here showed that a moderate (more physiological) increase in ROS impacted skeletal muscle isometric force production, supplementing the notion that there was a physiological role for ROS in skeletal muscle contraction and function.

The 2000s were a critical decade that further highlighted a role for ROS in promoting metabolic adaptation in skeletal muscle. As described above, it was ROS promoted skeletal muscle fatigue, which led to the hypothesis that quenching ROS (via antioxidant supplementation) would enhance performance. McKenna et al. demonstrated that intravenous infusion of N-acetylcysteine delayed fatigue during submaximal exercise in humans[129] further demonstrating that ROS promote muscle fatigue, acutely. However, the role for ROS in promoting fatigue during an acute exercise bout does not preclude a role for ROS in chronic exercise training. Shortly thereafter, Ristow et al. demonstrated the notion that ROS may in fact be beneficial and conducted a paradigm shifting clinical study demonstrating that chronic supplementation with exogenous antioxidants actually was shown to *prevent* the beneficial metabolic effects of exercise in skeletal muscle[130], including blunting adaptive changes in PGC1α expression in skeletal muscle, a key regulator of mitochondrial biogenesis[131]. It is now well established that exercise is an effective tool for disease prevention and improves long-term outcomes for patients with cardiometabolic-related diseases. Historically, while ROS were thought to promote muscle fatigue and promote pathological development, recent evidence suggests ROS are effective signaling molecules and are essential for the beneficial effects of exercise, particularly in skeletal muscle.

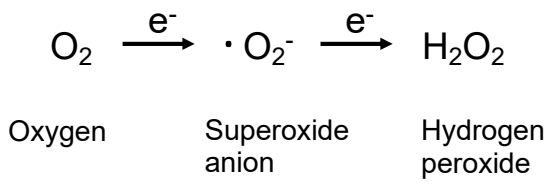

Figure 2. Representative image of the reduction of molecular oxygen into superoxide and hydrogen peroxide.

2.6 Cellular sources of ROS and the Nox family

There are multiple cellular sources of ROS as ROS can come from both endogenous and exogenous sources. The endogenous sources of ROS stem from organelles where there is significant oxygen consumption, including the mitochondria, peroxisomes and endoplasmic reticulum[132,133]. Xanthine oxidase in the cytosol is also a significant ROS producer[134,135]. In addition, ROS are also produced as a metabolic biproduct by the mitochondrial electron transport chain[136]. The respiratory chain is driven by a series of reduction oxidation reactions with the predominant sites of mitochondrial ROS production are at Complex I and Complex III[137]. Recent evidence suggests that Complex II can also produce ROS[137].

Mitochondrial-ROS is produced as a biproduct. In contrast, the sole function of the Nadph oxidase (Nox) family (NOX1-5 and DUOX1-2) is to transport electrons across membranes and to reduce oxygen to superoxide or hydrogen peroxide[138]. The overall primary structure of Nox consists of an NADPH-binding site at the carboxyl terminus, an FAD-binding region, six transmembrane domains, and four highly conserved heme-binding histamines[138]. Only recently

has the crystal structure been identified for select members of the Nox family[139], but the exact crystal structure of specific Nox members, including Nox4, remains incomplete.

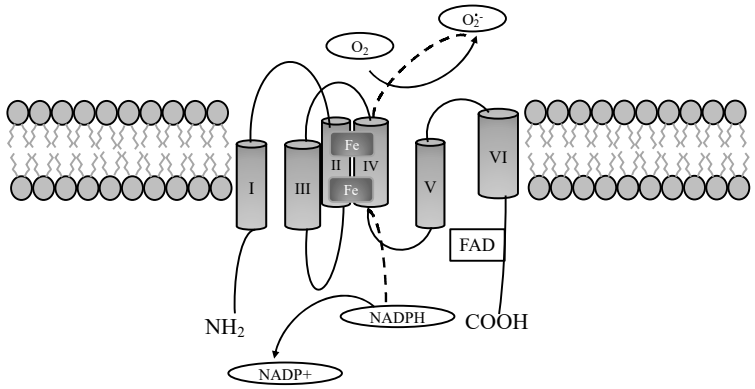

Figure 3. Nadph oxidase family transfer electrons to molecular oxygen to produce ROS.

Nox is a prominent source of ROS in skeletal muscle[140,141]. While it was postulated that mitochondria might be the primary source of ROS in skeletal muscle, several *ex vivo* studies have demonstrated that mitochondrial ROS production is blunted during conditions of low energy (State 3 respiration)[142–144]. These data suggest that during conditions of energetic stress (e.g., exercise) mitochondrial ROS production is halted.

Other work in the literature supports this as inhibition of Nox enzymes block both basal and contraction-stimulated skeletal muscle ROS production[145]. Nox2 and Nox4 are expressed in skeletal muscle[146]. In 2019 Henriquez-Olguin et al. utilized a novel ROS probe to assess the role of Nox2 in promoting cytosolic glucose uptake in skeletal muscle during exercise[147] and found

that Nox2 is the main source for cytosolic ROS production during exercise and is required for GLUT4 translocation. Recent evidence points to Nox4 as an essential mediator of exercise adaptation[148–150].

Nox4 is a unique member of the Nadph oxidase family as 90 percent of the ROS produced by Nox4 is hydrogen peroxide, whereas the other 10% is superoxide[151]. To this date, the manner in which Nox4 produces H_2O_2 remains incompletely understood. Nox4 colocalizes with $p22^{phox}$[152], which may be required for ROS production. It is possible that due to the localization of the enzyme, there is a subsequent electron transfer reaction that results in an immediate reduction of superoxide into hydrogen peroxide[138]. In addition, Nox4 has a third extra cytosolic loop (E-loop) which makes Nox4 28 amino acids longer than Nox1 or Nox2[153], promoting H_2O_2 production over superoxide production. While it was originally thought that Nox4 was constitutively active[154], as changes in Nox levels seem to reflect in intracellular ROS levels, recent work has indicated that Nox4 contains a phosphate-binding loop (Walker-a binding motif). which binds phosphate groups of ribonucleotides[155]. These data suggest Nox4 may be allosterically regulated; when ATP is low (unbound), Nox4-derived H_2O_2 production is increased[156], further signifying an essential role for Nox4 during conditions of energetic stress (low ATP).

2.7 Physiological and pathological role of Nox4

ROS are effective signaling agents in physiological and pathological cascades[25,157]. There are multiple forms of ROS, which may dictate its cellular function. Hydrogen peroxide (H_2O_2) is a particularly effective signaling molecule due to its ability to interact and modify cysteine thiol functional groups[112]. This redox modification can subsequently alter protein function[25]. In addition, H_2O_2 has a longer half-life (~1 ms) compared to other forms of ROS and is able to diffuse

through cellular membranes[107]. The concentrations of ROS are critical in mediating cell homeostasis. In eukaryotic cells during non-pathological conditions, intracellular concentrations of H_2O_2 are typically maintained in the nanomolar range (~1-100nM)[25,158] and concentrations greater than 100nM can damage biomolecules and initiate oxidative destress and even cell death[24].

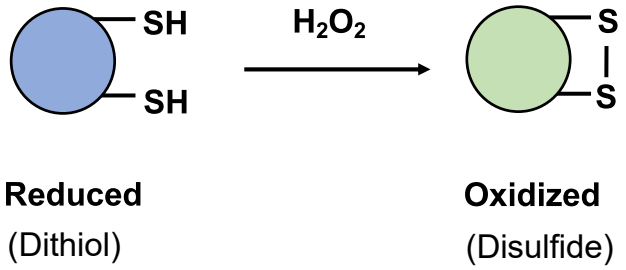

Figure 4. Hydrogen peroxide can modify cysteine thiol functional groups to alter protein function.

As Nox4 is a source of H_2O_2[151], studies modulating Nox4 expression have elucidated Nox4 as both protective and a mediator of oxidative stress in pathological processes, further highlighting the complexity of ROS as a signaling agent. Kuroda et al. demonstrated cardiac-specific Nox4 deletion mice (*c-Nox4-/-*) had decreased superoxide levels and mitochondrial O_2^- production during pressure overload (as seen in hypertensive heart disease). The cardiac-specific Nox4 deletion mice had improved cardiac hypertrophy and interstitial fibrosis compared to WT mice, where overexpression of cardiac Nox4 exacerbated this phenotype with pressure overload[159], suggesting that Nox4 promotes cardiac tissue damage during hypertrophy and drives oxidative stress within the heart. A different study the same year by Zhang et al. highlighted a different Nox4

phenotype by demonstrating overexpression of Nox4 is cardioprotective during chronic overload by targeting hypoxia inducible factor 1 (HIF1a) and the release of vascular endothelial growth factor (VEGF)[160]. Zhang's Nox4-null mice were generated with deletion at exons 1 and 2. In contrast, Kuroda's study deleted the entire exon 9, leading to early termination in exon 10, and truncation of the FAD- and NADPH-binding domain[159]. These divergent phenotypic results could be, in part, explained by the specific exon deletions of the Nox4 model or heart failure model. However, it is also plausible that the use of these models differentially alters the cellular redox state. Other studies demonstrated endothelial Nox4 is important for vascular recovery by targeting endothelial nitric oxide synthase[161,162]. This could be, in part, due to the different pathological stress or model as the overexpression of Nox4 was specific to the endothelium. In addition, recent work has demonstrated that endothelial derived ROS are central signal transductors for a host of vascular adaptations[163].

Nox4 has demonstrated to be both deleterious and beneficial for adaptation. The juxtaposition of the role of Nox4 in mediating these processes seems to be dependent on the type of stress (injury versus exercise) and targeted system (heart, skeletal muscle, or vasculature), or model (exon deletion and tissue specific Nox expression). Taken together, these studies suggest a diverse role for Nox4-derived ROS and indicate exhibiting gaps in understanding the sources of physiological ROS and their downstream metabolic processes.

2.8 Skeletal muscle mitochondrial quality control

Recent work demonstrated that Nox4 acts as a mitochondrial energy sensor and binds ATP[156]. While these studies were conducted in cancer cells, it's possible this mechanistic role of Nox4 in metabolic sensing may apply to other forms of stress and cell types. Nox4 has been

linked to mitochondrial metabolism. Moon et al. suggested Nox4 is required for fatty acid oxidation in macrophages[164] and in cardiomyocytes[165]. Two critical adaptations to chronic exercise, increases in angiogenesis and citrate synthase activity, were shown to be Nox4 dependent[148,149,166]. Recently, it was demonstrated that deficiency of Nox4 in skeletal muscle disrupted adaptive changes in the antioxidant response and mitochondrial biogenesis[150]. Taken together, these findings underscore Nox4 as a mediator of mitochondrial energetic sensing, particularly during conditions of physiological stress (e.g., exercise).

Energetic sensing is, in part, dictated by subcellular localization[167]. Nox4 localizes to different cellular membranes including the nucleus[168], endoplasmic reticulum[169], plasma membrane[170] and mitochondria of cardiac myocytes[171], cortical cells[172], cancer cells[156], and skeletal muscle cells[140]. Nox4 contains a mitochondrial-targeting sequence that is 73 amino acids long at the N-terminal region[173] that is consistent with other mitochondrial localized proteins[171]. Based on the literature, Nox4 may localize to the inner mitochondrial membrane in skeletal muscle and responds to decreases in ATP during exercise by producing hydrogen peroxide in skeletal muscle.

Mitochondrial metabolism is essential for skeletal muscle health and mediating efficient metabolic adaptations to exercise across the lifespan[174]. Skeletal muscle is composed of cells called myofibers[175]. These cells are structurally unique as they are responsible for generating force that supports movement[176] and is critical for the regulation of whole-body metabolic health[9]. During conditions of metabolic stress (e.g., exercise), energy production in skeletal muscle can increase up to 100 fold[177,178]. Mitochondria play a crucial role in supplying energy to meet the increased metabolic demand during exercise[179]. Mitochondria are highly dynamic organelles that constantly undergo changes in both their structure and function depending on the

metabolic demand[180]. This is particularly important during exercise conditions as mitochondria house TCA cycle and are the predominant sites for beta-oxidation – which is the primary fuel source during aerobic physical activity.

The collective term to assess how well mitochondria respond and generate energy has been termed mitochondrial bioenergetic capacity and has been researched for decades within the field of exercise physiology[181,182]. In 1967, John Holloszy made a paradigm shifting observation that mitochondrial oxygen consumption would increase with chronic exercise and postulated this could be facilitated through an increase in mitochondrial number (biogenesis)[183,184]. However, mitochondrial adaptations in response to exercise are vast and are not limited to increased mitochondrial mass alone, but also include other aspects of mitochondrial quality.

Mitochondrial quality includes biogenesis, dynamics (fission and fusion) and mitophagy (the selective degradation of mitochondria)[185]. These adaptive processes work in coordination to support skeletal muscle health[186]. Exercise leads to an increase in mitophagy in skeletal muscle[187,188] and mitophagy has several regulatory points in response to exercise including autophagosome biogenesis, mitochondrial fission (the separation of damaged mitochondria), and fusion of the autophagosome-lysosome[189].

Mitochondria exist in interconnected networks[190] and during conditions of energetic or oxidative stress, mitochondrial morphology is changed through distinct pathways that promote fission or fusion[191]. Mitochondrial fission will separate damaged mitochondrial regions from rest of the mitochondrial reticulum[192]. Regulators of mitochondrial fission include; mitochondrial outer membrane proteins including; fission 1 protein (FIS1), mitochondrial fission factor (MFF), dynamin-like 120 kDa protein (DLP1) and dynamin-related protein 1 (DRP1)[189]. Fis1 has two tetratricopeptide repeat motifs that Drp1 can bind to[193]. However, MFF binds DRP1 with a

higher affinity than Fis1[191,194]. DRP1 has recently been shown to be critical for mitochondrial exercise adaptation to training[195]. Gene expression levels of *Fis1* were increased with a 90 min endurance exercise bout and remained elevated for the following 3 hours after exercise[195]. No changes in DRP1 were observed but there was a significant increase in phosphorylation of Drp1 at Serine-616. This has functional implications as muscle-specific reduction in Drp1 leads to impaired adaptation to exercise[195]. These findings suggest that changes in mitochondrial fission are essential for skeletal muscle metabolic responses to exercise. The precise kinetics of how mitochondrial fission coordinates with mitophagy during exercise is not fully known.

Several studies have investigated exercise-mediated mitophagy in skeletal muscle[187,196]. Mitophagy begins with the formation of an autophagosome that will engulf mitochondria and subsequently fuse with the lysosome for degradation, where lysosomal hydrolyses occur and amino acids are recycled and released[197,198]. Unc-51 autophagy activating kinase (ULK1) is a central mediator of autophagy and is activated by 5'-AMPK-activated protein kinase (AMPK) in skeletal muscle with exercise[187]. Importantly, knocking out skeletal muscle Ulk1 blunted mitophagy in response to exercise and disrupted glucose metabolism to chronic exercise training[187]. These results suggest that mitophagy is integral for metabolic adaptations to exercise.

Pink1/Parkin are important signaling mediators for mitophagy, particularly in neurodegenerative diseases[199]. However, exercise-induced skeletal muscle mitophagy occurs in the absence of Pink stabilization at the mitochondria[200]. In 2021 Hung et al. demonstrates that AMPK-dependent ULK1 phosphorylated the conserved serine108 of Parkin. And this phosphorylation typically occurs within five minutes of mitochondrial damage, whereas activation of Pink1 and ATBK1 is observed 30-60 min after mitochondrial damage[201]. These results potentially highlight a mechanistic link between Ulk1 and Pink/Parkin mediated

mitophagy. It may be that the temporal link between AMPK/Ulk1 and Pink/Parkin with exercise has yet to be fully elucidated. However, the current evidence suggests that exercise-mediated mitophagy occurs independently of Pink/Parkin activation.

2.10 ROS mediated mitophagy

Mitochondrial quality control has only recently been suggested to play a key role in skeletal muscle metabolism and exercise adaptation[189]. During conditions of stress (e.g., exercise or nutrient deprivation) there is an increase in ROS[202], changes in mitochondrial membrane potential, and subsequent mitophagy[203]. However, the underlying mechanisms connecting ROS and mitophagy are not fully understood. There is also evidence to suggest that ROS act as second messengers to induce mitophagy signaling through cysteine modification of autophagy-related proteins[204] or regulation of kinase activity[205]. Another possibility is that ROS-induced mitochondrial damage activates mitophagy[206].

While a correlative relationship between ROS and mitophagy has been established, whether ROS act as second messenger signals or works to uncouple mitochondria to promote mitophagy is unknown. ROS may promote mitophagy through the regulation of the mitochondrial membrane potential[207]. Most of the studies assessing the role of ROS in promoting mitophagy utilize potent mitochondrial uncouplers or depolarizing agents[208] which lead to accumulation of PTEN-putative kinase (PINK1) on the outer mitochondrial membrane of depolarized mitochondrial, subsequently recruiting Parkin, an E3 ubiquitin ligase[209].

In addition to membrane potential mediation, an early study demonstrated that H_2O_2 directly promoted mitophagy through oxidation of a specific cysteine residue on the cysteine

protease, Atg4, leading to enhanced mitophagy during nutrient starvation in mammalian cells[204]. There are multiple other proteins that may be susceptible to direct cysteine oxidation in mitophagy[210]. However, the functional implications of ROS-mediated mitophagy are still being discerned.

There are multiple energy sensing pathways that may contribute to ROS-mediated mitophagy. For instance, AMPK[211], while traditionally thought to be activated by changes in ADP or AMP during conditions of low energy[212], may also be activated by ROS[213–216], particularly hydrogen peroxide[217]. Recent evidence demonstrates that AMPK activates Ulk1 to induce skeletal muscle mitophagy post-exercise[187]. Whether ROS directly activates AMPK to mediate mitophagy or as an intermediate signal is currently unknown.

Other central regulators of mitophagy include the mitogen-activated protein kinase (MAPK), a central metabolic regulator. Blockage of the p38 signaling pathway inhibited ROS-mediated mitophagy highlighting that ROS activates upstream mediators of mitophagy[218]. In addition to the ROS-mitophagy pathways that were previously described, there is also evidence that ROS activates mitophagy through Beclin[219] or NF-κB[220].

Nrf2 is a transcription factor that activates the antioxidant response – a class of genes involved in mitigating oxidative stress[221] and has been implicated to be important for mitochondrial adaptions, including mitophagy[222]. Recently, Nox4 is required for biogenesis through activation of Nrf2 in heart and skeletal muscle after exercise[150,223]. It is possible that Nox4 acts upstream of Nrf2 to initiate mitophagy in skeletal muscle, but this requires further investigation. Another potential mechanism where Nox4 may influence mitophagy is through changes in mitochondrial dynamics, particularly fission. Nox4 is upregulated during cerebral ischemia/reperfusion injury and inhibition of Nox4 attenuated DRP1 activity[224], a regulator of

mitochondrial fission. Taken together, Nox4 may influence multiple aspects of mitophagy. While a correlative relationship between Nox4, ROS and mitophagy has been established, whether ROS damage mitochondrial proteins to signal mitophagy, act as second messengers to impact mitophagy-related proteins[204,210] or uncouple mitochondria to promote mitophagy is an area of active investigation.

2.11 Conclusion

Exercise remains one of the top clinical interventions for patients with skeletal muscle-related diseases. Individuals who participate in regular exercise training have a lower risk for more than 30 different diseases and have an increased life span[225]. Exercise specifically targets skeletal muscle mitochondrial metabolism, leading to improved global metabolic health. Historically, ROS were thought to be deleterious to working skeletal muscle. Recent work highlights an essential role for ROS in mediating exercise adaptation but their downstream mechanistic targets are incompletely understood. In order to formulate new treatment interventions for patients with skeletal muscle and metabolic related-diseases, it is imperative to understand the molecular signaling that promotes the beneficial effects of exercise. The following chapters will demonstrate a novel metabolic role for Nox4 in promoting substrate oxidation and mitochondrial quality in skeletal muscle in response to exercise.

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Chapter 3: Specific Aims

Impairments in skeletal muscle mitochondrial energy production can exacerbate disease pathology and prognosis in multiple chronic diseases[1], including diabetes[2] and sarcopenia[3]. Exercise is an effective treatment for decreasing disease severity[4] by promoting skeletal muscle mitochondrial energy production[5]. During exercise, there is an increased demand for energy which is met by adaptive changes in mitochondrial energy production through activation of the tricarboxylic acid cycle (TCA) and oxidative phosphorylation (OXPHOS)[6]. However, the mechanism as to how exercise promotes these beneficial health changes is not fully known. Therefore, it is critical to further define the molecular events that promote exercise-mediated adaptations to formulate new treatment strategies for chronic skeletal muscle diseases.

There is an immediate change in substrate availability and cellular signals with exercise, including increased reactive oxygen species (ROS)[7]. One significant producer of ROS is NADPH oxidase 4 (Nox4), a membrane-bound protein whose sole function is to produce ROS by transferring electrons from NADPH to molecular oxygen. Nox4 is unique compared to other Nox enzymes as it predominantly produces hydrogen peroxide (H_2O_2), which is able to change protein structure and function through thiol oxidation[8]. In addition, H_2O_2 is the most stable form of ROS[9], making it an effective signaling molecule. Recently, Nox4 was demonstrated to serve as a mitochondrial energy sensor[10], further supporting the role of Nox4 in mediating mitochondrial adaptations to exercise, supporting the central hypothesis that Nox4 modulates **skeletal muscle metabolism** to promote adaptations to exercise. To test this hypothesis, I propose the following aims:

Specific Aim 1: Determine if Nox4-ROS is required for the expression of key metabolic genes in skeletal muscle. The working hypothesis of this aim is that Nox4-H_2O_2 is essential for the expression of genes that promote skeletal muscle metabolic adaptation. *Rationale*: With exercise, there is an increase in ROS in working skeletal muscle. While there are many forms of ROS, hydrogen peroxide (H_2O_2) is the most stable form of ROS, making it an effective signaling molecule[11]. In *Aim 1a*, to determine the role of H_2O_2, I will co-transfect wild-type (WT) mice with catalase, an enzyme that quenches H_2O_2 and green fluorescent protein (GFP). The transfected WT mice will undergo an acute exercise bout, and I will harvest and isolate RNA from skeletal muscle and measure downstream transcripts, including *calcium-binding mitochondrial carrier (Slc25a25), hexokinase 2 (Hk2),* and *mitochondrial uncoupling protein 3 (Ucp3). In Aim 1b,* I will treat skeletal muscle cells with H_2O_2 and assess the expression of metabolic genes *in vitro.* In *Aim 1c*, I will subject global *Nox4-/-* mice and WT mice to an acute exercise bout and harvest skeletal muscle to assess gene expression of these same transcripts. *This aim will define specific metabolic downstream targets of H_2O_2 and Nox4 in skeletal muscle.*

Objective 1: Determine if catalase overexpression impacts exercise-induced changes in metabolic gene expression.

Objective 2: Determine if H_2O_2 is sufficient to induce expression of key metabolic genes *in vitro*?

Objective 3: Investigate the role of Nox4 in mediating gene expression of transcripts involved in substrate oxidation post-acute exercise.

Specific Aim 2: Determine if Nox4 is required for substrate oxidation and mitochondrial enzyme activity. The working hypothesis of this aim is that Nox4 is required for substrate

oxidation post-acute exercise and is important for chronic exercise adaptation. *Rationale:* Our preliminary data indicated that *hydrogen peroxide* is a top-regulated pathway for initiating transcriptional responses during exercise. To assess the downstream metabolic role of Nox4, in *Aim 2a*, global *Nox4-/-* and age-matched WT mice will undergo an acute bout of exercise. Upon completion of the exercise protocol, I will assess glucose and fatty acid oxidation. In *Aim 2b*, as skeletal muscle is a mixed tissue composed of multiple cell types (primarily skeletal muscle and endothelial), we assessed the endothelial contribution of Nox4 in promoting post-exercise changes in substrate oxidation with mice deficient in endothelial Nox4 ($Nox4^{ECKO}$). In *Aim 2c*, I will subject *Nox4-/-* and WT mice to a chronic exercise regimen for 6 weeks and assess exercise capacity and mitochondrial enzyme activity. *These studies will assess the role of Nox4 in promoting skeletal muscle adaptation in a tissue-specific manner after acute exercise and will define a physiological role for Nox4 in chronic exercise adaptation.*

Objective 1: Determine of Nox4 mediates post-exercise changes in substrate oxidation.

Objective 2: Assess the tissue-specific role of Nox4 by using mice deficient in endothelial Nox4 and determine substrate oxidation post-exercise.

Objective 3: Assess the role of Nox4 in mediating chronic adaptations to exercise via exercise capacity and mitochondrial enzyme activity.

Specific Aim 3: Determine if Nox4 is essential for skeletal muscle mitophagy. The working hypothesis of this aim is that Nox4 is essential for post-exercise mitophagy in skeletal muscle. *Rationale*: Nox4 is a predominant producer of skeletal muscle ROS in response to exercise[12]. Mitochondrial oxidation is thought to be an early and crucial step to mark damaged mitochondria for mitophagy[13]. In addition, mitophagy is induced after acute exercise and necessary for metabolic

adaptations to exercise[14]. Therefore, in ***Aim 3a***, to distinguish the role of Nox4 in mitophagy, I will use a tool developed to investigate both mitochondrial oxidation and mitophagy *in vivo*, *pMitoTimer*[14,15] to assess exercise-induced mitophagy *ex vivo* in global *Nox4-/-* and WT mice. In Aim 3b, I will determine metabolic signaling immediately post-exercise, including p-Ampk, p-Ulk1, p-Drp1, and the expression of genes related to mitochondrial dynamics. *Importantly, any information regarding this upstream signaling would be novel regarding the role of Nox4 in initiating mitophagy post-acute exercise.*

Objective 1: Determine if Nox4 is required for *ex vivo* skeletal muscle mitophagy post-acute exercise.

Objective 2: Determine if Nox4 is required for AMPK activation in skeletal muscle post-exercise.

Objective 3: Assess changes in gene expression of transcripts involved in mitochondrial dynamics post-exercise.

These independent yet synergistic aims will address unresolved questions by utilizing cutting-edge techniques, including metabolic assays, gene transfer, and novel mouse lines, to address the role of Nox4 in mediating skeletal muscle metabolic adaptation to exercise.

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Chapter 4: Nox4 mediates skeletal muscle metabolic responses to exercise

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Abstract

Objective: The immediate signals that couple exercise to metabolic adaptations are incompletely understood. Nicotinamide adenine dinucleotide phosphate oxidase 4 (Nox4) is produces reactive oxygen species (ROS) and plays a significant role in both metabolic and vascular adaptation during stress conditions. Our objective was to determine the role of Nox4 in exercise-induced skeletal muscle metabolism.

Methods: Mice were subjected to acute exercise to assess their immediate responses. mRNA and protein expression responses to Nox4 and hydrogen peroxide (H_2O_2) were measured by qPCR and immunoblotting. Functional metabolic flux was measured via ex vivo fatty acid and glucose oxidation assays using ^{14}C-labeled palmitate and glucose, respectively. A chronic exercise regimen was also utilized and time to exhaustion along with key markers of exercise adaptation (skeletal muscle citrate synthase and beta-hydroxyacyl-coA-dehydrogenase activity) were measured. Endothelial-specific Nox4-deficient mice were then subjected to the same acute exercise regimen and subsequent substrate oxidation was measured.

Results: We identified key exercise-responsive metabolic genes that are dependent on H_2O_2 and Nox4 using catalase and Nox4-deficient mice. Nox4 was required for expression of uncoupling protein 3 (*Ucp3*), hexokinase 2 (*Hk2*), and pyruvate dehydrogenase kinase 4 (*Pdk4*), but not expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Pgc-1a*). Global Nox4 deletion resulted in decreased UCP3 protein expression and impaired glucose and fatty acid oxidization in response to acute exercise. Furthermore, Nox4-deficient mice demonstrated impaired adaptation to chronic exercise as measured by time to exhaustion and

activity of skeletal muscle citrate synthase and beta-hydroxyacyl-coA-dehydrogenase. Importantly, mice deficient in endothelial-Nox4 similarly demonstrated attenuated glucose and fatty acid oxidation following acute exercise.

Conclusion: We report that H_2O_2 and Nox4 promote immediate responses to exercise in skeletal muscle. Glucose and fatty acid oxidation were blunted in Nox4-deficient mice post-exercise, potentially through regulation of UCP3 expression. Our data demonstrate that endothelial-Nox4 is required for glucose and fatty acid oxidation, suggesting inter-tissue cross-talk between the endothelium and skeletal muscle in response to exercise.

Introduction

Skeletal muscle is an extraordinarily plastic tissue that quickly responds to repeated contractions in an effort to supply sufficient adenosine triphosphate (ATP) for working muscle. Exercise is a potent strategy to reduce muscle wasting and prevent many chronic vascular and metabolic diseases. A single bout of exercise elicits immediate upregulation of mRNA expression associated with metabolic adaptation, stimulation of glucose and fatty acid transport, and increased substrate oxidation (glucose and fatty acid)[1]. Consistent, repeated bouts of exercise over weeks to months (chronic exercise) increases angiogenesis, mitochondrial biogenesis, and enhances glucose and lipid metabolism[1]. These changes in skeletal muscle metabolism are critically linked to skeletal muscle health which affects systemic metabolism. Therefore, understanding the initiating stimuli that trigger exercise-mediated metabolic adaptation is important to characterize the underlying molecular signaling involved in the health benefits of exercise.

Skeletal muscle produces reactive oxygen species (ROS) during exercise[2]. Likewise, ROS levels are increased in subjects with muscular diseases leading to fatigue and atrophy[3]. Therefore, ROS production during exercise was initially thought to be solely deleterious. However, multiple studies have now demonstrated that, in healthy adults, ROS-responsive signaling pathways are important for improved glucose metabolism and increased efficiency of mitochondrial function in response to exercise[4]. Specifically, human studies have demonstrated that quenching the ROS signal with antioxidant supplementation attenuates increased insulin sensitivity and mitochondrial biogenesis[5]. While studies have documented that exercise-induced ROS promote muscle adaptation to exercise, the source(s) and target(s) of these ROS are largely unknown.

Several lines of evidence suggest that exercise promotes ROS production in the skeletal muscle from multiple sources (Nox2, Nox4, mitochondria, and xanthine oxidase)[6,7,7–11]. However, NADPH oxidase (Nox) enzymes contribute to cytosolic ROS production both at rest and during contraction. The Nox family of enzymes transfer electrons from NADPH to molecular oxygen, producing ROS. Inhibition of Nox enzymes blocked both basal and stretch/contraction-stimulated skeletal muscle ROS production[6,10]. Skeletal muscle is a mixed tissue; its parenchyma consists of myofibers while its stromal composition includes myocytes, endothelial cells, pericytes and immune cells. Thus, Nox's contribution to skeletal muscle ROS production is likely significantly influenced by its expression patterns in these different cell types.

Of the multiple ROS, hydrogen peroxide (H_2O_2) is an adept signaling molecule. H_2O_2 is a relatively stable oxidant that is able to cross membranes and react with protein thiol moieties to produce post-translational modifications, thus altering protein function[12]. Nox4 is unique among the Nox enzymes (Nox 1-5) as it produces primarily produces H_2O_2[13,14] due to a unique third extracytosolic loop (E-loop)[14]. Initially, Nox4 was thought to be constitutively active and regulated at the transcriptional level; however, other reports have demonstrated that post-translational modifications introduce an alternative level of regulation[15]. Furthermore, recent data demonstrate that Nox4 can be regulated by ATP levels as it contains an ATP-binding motif[16]. ATP can directly bind and negatively regulate Nox4 activity, suggesting that Nox4 can serve as a metabolic sensor and become activated with decreased mitochondrial ATP levels, which may be important in the response to exercise. Thus, we hypothesized Nox4 may be responsible for initiating metabolic adaptations to exercise. Our findings here demonstrate that Nox4 is

responsible for initiating transcriptional changes and mediating substrate oxidation in response to acute exercise.

Methods

Animals

Nox4 floxed (*Nox4*$^{fl/fl}$) mice were obtained from Jun Sadoshima[17] and bred with the CMV-Cre line (Jax 006054) to produce a line of global Nox4 deletion (*Nox4-/-*). These mice were back crossed for a minimum of 10 generations to the C57Bl/6J mice and C57Bl/6J mice (Jackson Laboratories, Bar Harbor, ME, USA) were used as controls for the *Nox4-/-* mice. To make the endothelial-specific Nox4 deletion, the Nox4 floxed mice were bred with Ve-Cadherin Cre (Jax 006137). Male mice were used in all groups. All groups were allowed to eat *ad libitum* throughout the duration of the study (except during the time periods of exercise/sedentary). The animals were housed on a 12:12-h light-dark cycle in a temperature-controlled room at 25 °C. The Virginia Tech and University of Massachusetts Medical School Institutional Animal Care and Use Committee approved all procedures.

Exercise Intervention

Exercise was conducted on a motorized treadmill (Columbus Instruments Model #1055-SRM-D58, Columbus, Ohio, Columbus, OH, USA). All mice were acclimated to the treadmill for 3 days prior to the exercise regimens. On Day 1, the mice were allowed to stand on the stationary treadmill for 15 min. On Day 2, the mice walked on the treadmill at 5 m/min for 15 min. On Day 3, the mice began walking at 5 m/min and the treadmill speed was gradually

increased to 15 m/min and the mice ran at this speed for 15 min. To control for any non-exercise effects of treadmill running (handling, novel environment, noise, and vibration), the non-exercised group of mice (sham exercise) were placed on the top of the treadmill apparatus for an identical period of time. The mice were not subjected to electric shock during the treadmill sessions to avoid stress.

The *chronic exercise* training consisted of treadmill running for 60 min/day at 18 m/min 5 days/week for 4 weeks. Tissue was harvested >24 h following the last exercise bout.

The *exhaustive exercise* protocol[18] began at 5 m/min for 15 min followed by gradual increases in speed at 3 min intervals until the treadmill speed reached 24.25 m/min, at which point it was held at this speed for 30 min or until the mice reached exhaustion. The state of exhaustion was established by a mouse remaining in the lower quarter of the treadmill 3 cumulative times despite gentle encouragement.

The *acute exercise* protocol consisted of a 60 min run beginning with a 4 min warm-up period from which the mice progress from 0 to 20 m/min. Mice were then exercise at 20m/min (~85% VO_2 max)[19] for the remainder of the 60 min protocol. Mice were fasted 4 h prior to exercise to eliminate any acute metabolic changes due to food and were assigned to either a non-exercise control group or an exercise group. Previous studies have shown that healthy, young, C57BL/6 mice participating in this exercise protocol complete the 60 min run close to 100% of the time[20].

Glucose Tolerance Test

The mice were fasted for 12 hours and glucose (2 g/kg) was delivered by intraperitoneal injection. Blood samples were harvested from the tail vein at the indicated times, and glycaemia

was determined using a Bayer Breeze 2 glucometer. Data were plotted as milligrams (mg)/deciliters (dl) over time and area under the curve (AUC).

RNA Extraction and Gene Expression

Tissue was harvested and total RNA was extracted using QIAzol (QIAGEN). mRNA was isolated by Qiagen RNeasy Mini Kit (RNeasy Mini Kit, QIAGEN, 74106) and 1 μg of total RNA was used for one step real-time reverse transcription PCR (iScript cDNA Synthesis Kit, BioRad, 1708890). For specific mRNA expression analysis TaqMan and Sybrgreen gene (Table 1 for Sybrgreen primers) expression assays were used. The ΔΔcycle threshold method was used for relative mRNA quantification and the gene expression was normalized to the housekeeping gene (HPRT).

Table 1: Sybr Green Primer Sequences

Gene	Forward Sequence	Reverse Sequence
Hprt	GGACTAATTATGGACAGGACTG	GCTCTTCAGTCTGATAAAATCTAC
Sik1	CTGTAGGCTACCCACCTCCT	GCGAGTCAGAAGGGTTGACA
Mt2	TCACCACGACTTCAACGTCC	GTTGGGGTCCATTCCGAGAT
Nr4a3	TGCTGCAAAGTGTAACCCAGA	ACATCTCAAGCCCTGTCACC
Slc25a25	CACGTGTGTACCACTCTGCT	TGCCGTTCCCTCTGTTTCTG
Pdk4	CCGAAGCTGATGACTGGTGT	CTTCTCCCGGGTCATCCAAC
Pgc-1a	GACAGGTGCCTTCAGTTCAC	CAACCAGAGCAGCACACTCTA
Hk2	GCCACCAGACGAAACTGGAT	TGTCAAAGTCCCCTCTGCG
Ucp3	CCATGATACGCCTGGGAACT	CTGGCGATGGTTCTGTAGGC

Microarray Analysis

RNA was harvested from gastrocnemius (GC) and isolated as described above. The RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only samples with an RNA integrity number above 7.5 and normal 18-s and 28-s fractions on microfluidic electrophoresis were used. Second-strand cDNA was then labeled with the Affymetrix WT terminal labeling kit, and samples were hybridized to Affymetrix Mouse Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA). Pathway analysis of the transcriptomic data was conducted using Ingenuity Pathway Analysis (IPA; Qiagen). Heatmapper (http://www.heatmapper.ca) was used to create the heat maps[21].

Adenoviral Constructs

An adenoviral vector expressing CMV driven catalase (Ad-Cat) was a gift from Dr. Kai Chen[22]. The adenoviral vector expressing the control virus, adenoviral CMV GFP (Ad-GFP) was purchased from Vector Biolabs (Cat 1060). For *in vivo* administration of adenovirus, 5 days before exercise, either Ad-GFP or Ad-Cat ($2x10^8$ plaque-forming units) were injected into the gastrocnemius (GC).

Proteins

Gastrocnemius (GC) muscle was collected and homogenized in lysis buffer (25 mM HEPES, pH 7.0, 0.4% CHAPS) with Calbiochem Protease Inhibitor Cocktail Set III (EMD Biosciences, La Jolla, CA). Proteins (10 µg loaded) were separated by SDS-Page and transferred onto PVDF membranes. Membranes were blocked in 5% nonfat-dry milk or bovine serum albumin (BSA), washed in tris-buffered saline containing 0.1% Tween-20 (TBST) and then

placed in primary antibody: UCP3 (Invitrogen, PA1-055), green fluorescent protein (GFP, Abcam, ab290), and catalase (R&D Systems, MAB3398). The membrane was then incubated in secondary antibody (donkey anti-rabbit; Jackson ImmunoResearch 711-035-152; 1:5000) or goat-anti mouse (LI-COR 1:10,000) and imaged on Bio-Rad ChemiDoc MP or the LI-COR Odyssey CLx. Blots were analyzed using Image J (https://imagej.nih.gov/ij/index.html) for densitometry and normalized to total protein (Control) as quantified using the No-Stain Protein Labeling Reagent (Invitrogen, A44449), Ponceau S (59803S, Cell Signaling Technologies) or pan-actin (mAb 8456, Cell Signaling Technologies).

Substrate Oxidation

GC were excised and washed in cold PBS. Approximately 15-50 mg of muscle were placed in 200 µL of modified sucrose EDTA medium (SET Buffer), containing 250 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl, and 1 mM ATP, pH 7.4. Muscle samples were minced with scissors followed by the addition of SET Buffer to produce a final 20-fold dilution (wt:vol). The samples were then homogenized in a Potter-Elvehjem glass homogenizer at 10 passes across 30 seconds at 150 RPM with a motor-driven Teflon pestle.

Fatty acid oxidation was assessed using radiolabeled fatty acid ([1-^{14}C]- palmitic acid, American Radiolabeled Chemicals, St. Louis, MO.) to quantify $^{14}CO_2$ production and ^{14}C-labeled acid-soluble metabolites. Samples were incubated in 0.5 µCi/mL of [1-^{14}C]-palmitic acid for 1 hour after which the media was acidified with 200 µL 70% perchloric acid for 1 hour to liberate $^{14}CO_2$. The $^{14}CO_2$ was trapped in a tube containing 1 M NaOH, which was then placed into a scintillation vial with 5 mL scintillation fluid. The ^{14}C concentrations within the vials were

measured on a 4500 Beckman Coulter scintillation counter. Acid soluble metabolites were determined by collecting the acidified media and measuring ^{14}C levels as previously described[23].

Glucose oxidation was measured utilizing a similar method to that of fatty acid oxidation with the exception of the substitution of [U-^{14}C]-glucose for [1-^{14}C]- palmitic acid. Total protein content in the skeletal muscle homogenates was measured via a bicinchoninic acid procedure (Thermo Fisher Scientific, Waltham, MA.) and was used to normalize oxidation values.

Metabolic Enzyme Assays

The activity of citrate synthase (CS), a biochemical marker of mitochondrial density and oxidative capacity[24] and beta-hydroxyacyl-coA-dehydrogenase (BHAD), a key regulatory enzyme in the beta oxidation of fatty acids to acetyl-CoA, were determined spectrophotometrically in muscle homogenates[25]. Briefly, CS activity was measured at 37 °C in 0.1 M Tris-HCl (pH 8.3) assay buffer containing 0.12 mM 5,5'-dithio-bis (2-nitrobenzoic acid) and 0.6 mM oxaloacetate. After an initial 2-minute absorbance reading taken at 412 nm, the reaction was initiated with the addition of 3.0 mM acetyl-CoA, and the change in absorbance was measured every 10 seconds for 7 minutes. BHAD activity was measured at 37 °C in assay buffer containing 0.1 M triethanolamine- HCl, 5 mM EDTA, and 0.45 mM NADH (pH 7.0). After an initial 2-minute absorbance reading at 340 nm, the reaction was initiated with the addition of 0.1 mM acetoacetyl-CoA, and the change in absorbance was measured every 10 seconds for 5 minutes. Maximal enzymatic activity is presented in μM/mg of protein/min.

Cell Culture

Mouse lung endothelial cells were prepared by immunoselection with anti-ICAM-2 antibody as previously described[26] and used for experiments during passages 2-6. The mouse skeletal muscle cell line, C2C12, was purchased from ATCC and differentiated after they reached confluence by switching the medium to containing 2% horse serum and 1 μM insulin. Differentiation media was changed, and changed every 24 hours for the next 3 days.

Statistics

Results are expressed as means ± SEM. Data were analyzed by GraphPad Prism 8.0 software (GraphPad Software, La Jolla, CA, USA) using unpaired Student t test, one-way analysis of variance (ANOVA), or two-way ANOVA with Newman-Keuls post hoc test, as appropriate. Differences were considered significant with $p < 0.05$*.

Results

3.1. Acute exercise transcriptional responses were influenced by hydrogen peroxide signaling

After an acute bout of exhaustive exercise, multiple metabolic changes occur in the skeletal muscle, such as increased fatty acid and glucose metabolism[1]. To gain insight into the immediate signals that initiate the change in metabolism after exercise, we utilized a single bout of treadmill exercise on C57Bl/6J wild-type (WT) mice and examined the gastrocnemius (GC) transcriptomic profiles of the exercised (EX) mice compared to sedentary (SED) controls (**Figure 1A**). To elucidate the upstream pathways mediating these early gene changes, we utilized Ingenuity Pathway Analysis (IPA, Qiagen) (**Figure 1B**). Prominent among these

activated pathways was hydrogen peroxide (H_2O_2) signaling. We confirmed our transcriptomic analysis using qPCR. Shown in **Figure 1C** are the top genes from our microarray. **Figure 1D** shows the confirmation of genes involved in metabolic adaptation to acute exercise[27,28]. These genes include uncoupling protein 3 (*Ucp3)*, known to be important for mediating substrate oxidation; hexokinase 2 (*Hk2)*, which is involved in glycolysis; peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Pgc-1a)* which mediates multiple adaptations including mitochondrial biogenesis*;* and pyruvate dehydrogenase kinase 4 (*Pdk4),* which is involved in metabolic flexibility[29–33]. Together, these data demonstrated that acute exercise increased skeletal muscle expression of several genes involved in substrate oxidation, suggesting potential regulation by H_2O_2.

3.2. Hydrogen peroxide was responsible for mediating a subset of genes involved in substrate metabolism after acute exercise

H_2O_2 is responsible for mediating multiple signaling pathways[34]. Our IPA results identified H_2O_2 signaling as an upstream pathway responsible for initiating transcriptional responses to exercise. Thus, we tested whether H_2O_2 signaling was important in the acute exercise response by using catalase which is a potent enzyme that reduces H_2O_2 to water (**Figure 2A**). For this experiment, we injected one hind leg (GC) with adenoviral catalase (Ad-Cat; **Supplementary Figure 1**) in the WT mice. The other hind leg was used as a control and injected with adenoviral GFP (Ad-GFP). We observed robust protein expression of both GFP and catalase (**Supplementary Figure 1B and C**). Gene expression of GFP and catalase was not different due to treatment (SED vs. EX; **Supplementary Figure 1B and C**). We then examined the exercise responsive-genes by comparing the Ad-Cat injected leg to the control Ad-GFP

injected leg. We found that a subset of genes was specifically modified by H_2O_2 after acute exercise (*Slc25a25*, *Hk2*, and *Ucp3*; **Figure 2B**), whereas other genes (*Sik1, Mt2,* and *Nr4a3*), were not altered in expression with the addition of catalase (**Figures 2C**).

3.3. Metabolic adaptation to exercise required Nox4

As Nox4 is a prominent H_2O_2 producer in the skeletal muscle, we utilized mice with global Nox4 deletion (*Nox4-/-*; **Supplementary Figure 2**) and investigated the genes changed with exercise. To identify pathways that were specifically influenced by Nox4 with exercise, we compared the *Nox4-/-* EX/SED gene profile with the control WT EX/SED gene profile and used IPA to determine the top canonical pathways modified by exercise in the WT mice that were not activated in the *Nox4-/-* mice. These data demonstrated that genes involved in oxidative phosphorylation and mitochondrial metabolism were not changed with exercise in mice lacking Nox4 (**Figure 3A-C**). To further investigate the transcriptome-profile of *Nox4-/-* mice after exercise, we examined gene expression using qPCR (**Figure 3D**). We found that Nox4 expression was important for the exercise response of *Ucp3 Pdk4*, and *Hk2*, but not *Pgc-1a*.

As our data suggested Nox4 plays an important role in metabolic adaptation to exercise, we determined if Nox4-deficient mice similarly had a blunted response to chronic exercise. Indeed, the WT mice significantly increased the distance to exhaustion run, but this response was significantly blunted in Nox4-deficient mice (**Supplementary Figure 3A**). We measured enzymes important for metabolic adaptation to exercise. Citrate synthase (CS) is the first enzyme in the tri-carboxylic acid (TCA) cycle and is a known marker of adaptation to chronic exercise[35]. In the red GC, we found that CS enzyme activity was significantly increased in the WT, but not in the Nox4-deficient mice (**Supplementary Figure 3B and C**). We similarly examined beta-

hydroxyacyl-coA-dehydrogenase (BHAD) activity as this enzyme is important for fatty acid oxidation and its enzyme activity is increased with exercise[36]. The WT exercised red GC had a significant increase in BHAD activity that was not seen in the *Nox4-/-* mice (**Supplementary Figure 3D and E**). Together these data suggest the impairment of the immediate responses to exercise in mice lacking Nox4 result in defects in chronic adaptation to exercise.

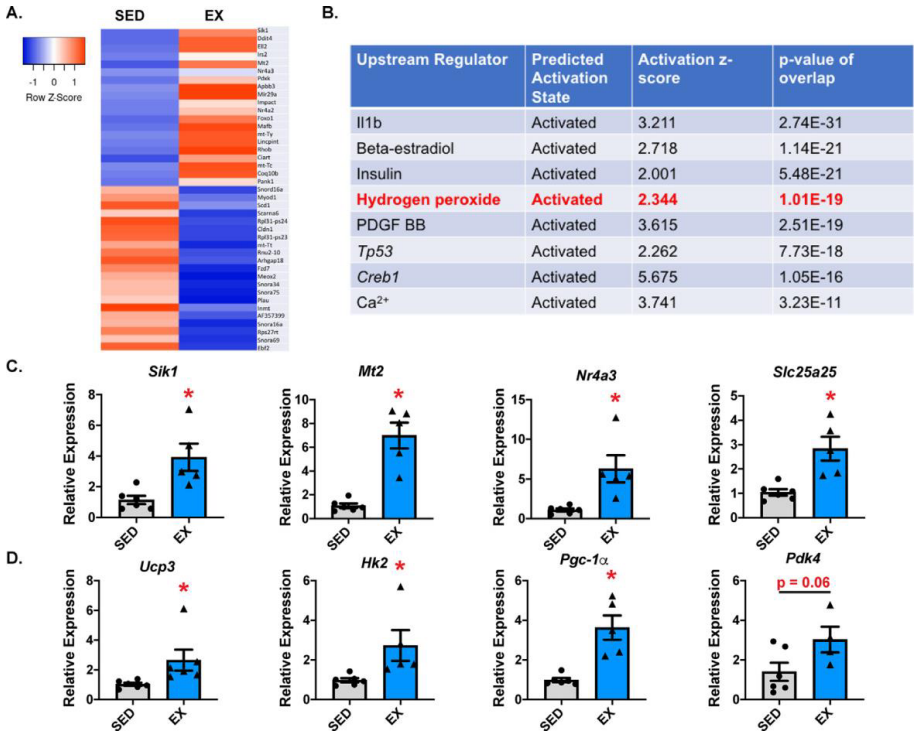
3.4. Endothelial Nox4 was required for substrate oxidation following acute exercise.

Recovery from exercise is a metabolic state involving increased reliance on fatty acid metabolism and the elevated expression of uncoupling protein 3 (UCP3)[37]. H_2O_2 (**Figure 2B**) and Nox4 (**Figure 3D**) are important for the expression of *Ucp3* following exercise. Therefore, we examined UCP3 protein expression and found that WT mice had a significant increase in UCP3 after exercise, which was significantly blunted in mice lacking Nox4 (**Figure 4A and B**).

Changes in UCP3 expression are known to modulate substrate oxidation[38]. Therefore, we investigated both glucose and fatty acid beta oxidation following a single acute bout of exercise. In the WT mice, we found a significant increase in glucose oxidation that was blunted in the *Nox4-/-* mice (**Figure 4C**). Similarly, we found that fatty acid oxidation was significantly increased in WT mice after acute exercise and this response was blunted in the Nox4-deficient mice (**Figure 4D**).

Given the heterogenous cell populations within skeletal muscle, we wanted to understand the cell type-specific mediation of the changes in substrate oxidation. Nox4 is highly expressed in the endothelium (**Supplementary Figure 4A**). Therefore, we hypothesized that it may be endothelial Nox4 principally mediates the response to acute exercise. To investigate this question, we utilized Ve-Cadherin driven Cre mice and crossed them with our Nox4 floxed mice

to generate endothelial cell (EC) specific Nox4 deletion (*Nox4*^{ECKO}). With Nox4 deleted in the endothelium alone, the mixed skeletal muscle expression of Nox4 was decreased by ~80%, indicating that Nox4 is more highly expressed in the endothelial cells than myocytes *in vivo* (**Supplementary Figure 4B**). We also found that in red gastrocnemius, which is highly vascularized compared to the white GC, Nox4 was more highly expressed (**Supplementary Figure 4C**) which also aligns with UCP3 expression[39]. To determine if the endothelial Nox4 deletion drives gene expression similarly to the global deletion, we examined the expression of *Ucp3*, *Pdk4*, and *Hk2*. Following exercise, expression of these genes was blunted in *Nox4*^{ECKO}. Next, to assess the effect of endothelial Nox4 deletion on functional metabolism, we measured glucose and fatty acid oxidation. We found that mice deficient in endothelial Nox4 phenocopied the global Nox4 deletion as both glucose and fatty acid oxidation were similarly blunted after exercise (**Figure 4E-G**), suggesting that endothelial Nox4 may be important for driving the acute increase in substrate oxidation in skeletal muscle after exercise.



Upstream Regulator	Predicted Activation State	Activation z-score	p-value of overlap
Il1b	Activated	3.211	2.74E-31
Beta-estradiol	Activated	2.718	1.14E-21
Insulin	Activated	2.001	5.48E-21
Hydrogen peroxide	**Activated**	**2.344**	**1.01E-19**
PDGF BB	Activated	3.615	2.51E-19
Tp53	Activated	2.262	7.73E-18
Creb1	Activated	5.675	1.05E-16
Ca^{2+}	Activated	3.741	3.23E-11

Figure 1: Acute exercise mediates early transcriptional responses influenced by hydrogen peroxide. (A) Microarray was performed on pooled (N = 3/group) gastrocnemius muscle from WT mice after a bout of exhaustive exercise. A heat map of the top upregulated and downregulated genes is shown. (B) Ingenuity Pathway Analysis (IPA) identified the upstream regulators of the expression pattern after exercise. (C) qPCR was performed to confirm the top exercise responsive genes and (D) metabolic genes responsive to acute exercise (N = 5-7/group; *p < 0.05 compared to SED). Data are presented as mean ± SEM.

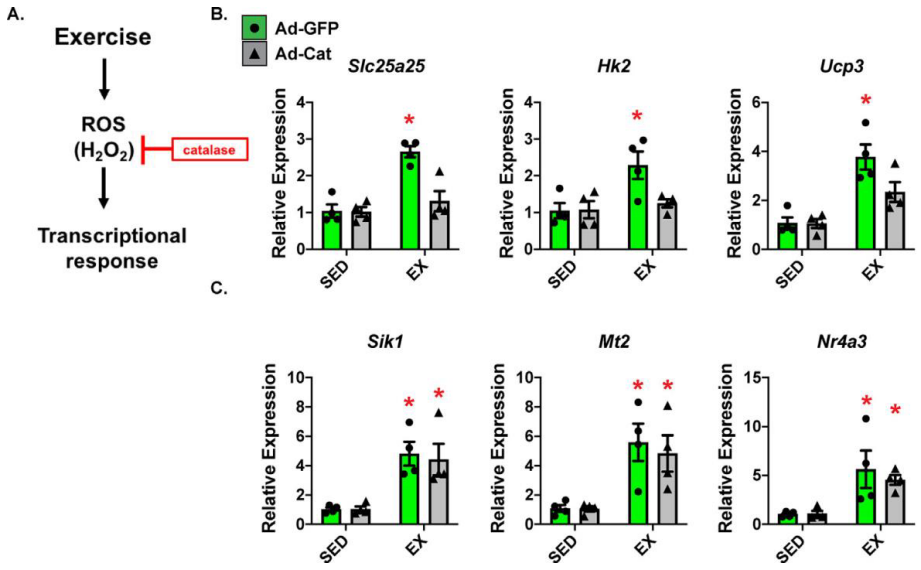


Figure 2: Hydrogen peroxide was responsible for mediating a subset of genes involved in metabolism after acute exercise. (A) We utilized catalase which is a potent enzyme that reduces H_2O_2 to water to investigate the influence of H_2O_2 (shown in the schematic). Adenoviral catalase (Ad-Cat) was injected into one leg and control virus (Adenoviral GFP; Ad-GFP) was injected into the other leg. Five days post-injection, treadmill exercised mice were examined by qPCR for gene expression of (B) H_2O_2-responsive and (C) H_2O_2-unresponsive transcripts. (N = 4/group; *$p < 0.05$ compared to SED or indicated control leg (paired comparison)). Data are presented as mean ± SEM.

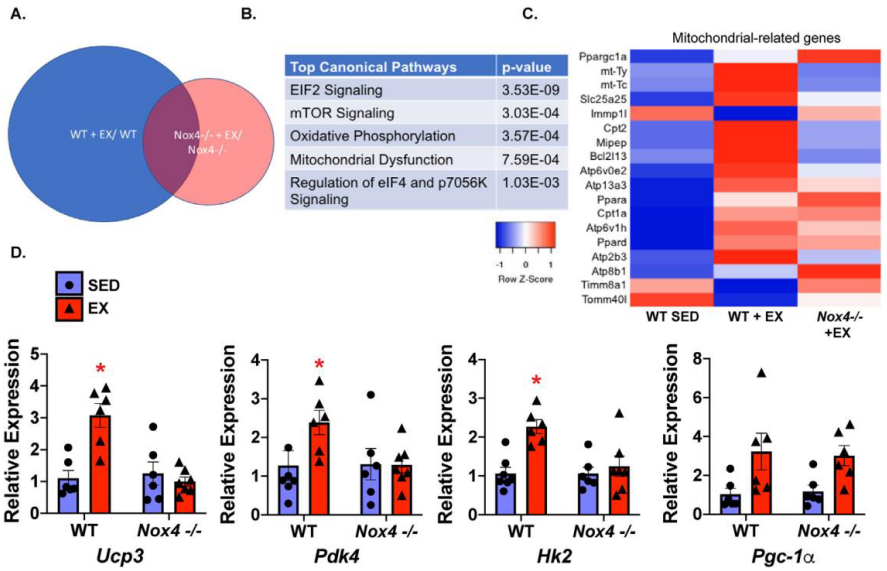


Figure 3: Nox4 mediates early transcriptional responses after acute exercise. (A) Microarray was performed on pooled (N = 3/group) gastrocnemius muscle in each group and pathways activated in WT + EX mice that were not activated by *Nox4-/-* + EX were identified. (B) Using IPA (Qiagen), the top canonical pathways driven by Nox4 (activated only in the WT + EX group and not in the *Nox4-/-* + EX) were identified. (C) Mitochondrial-related genes are shown in the heat map. (D) qPCR confirmed changes in metabolism-related genes: uncoupling protein 3 (*Ucp3*), hexokinase 2 (*Hk2*), pyruvate dehydrogenase kinase 4 (*Pdk4*), and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Pgc-1a*) (N = 6-7/group; *p < 0.05 compared to SED). Data are presented as mean ± SEM.

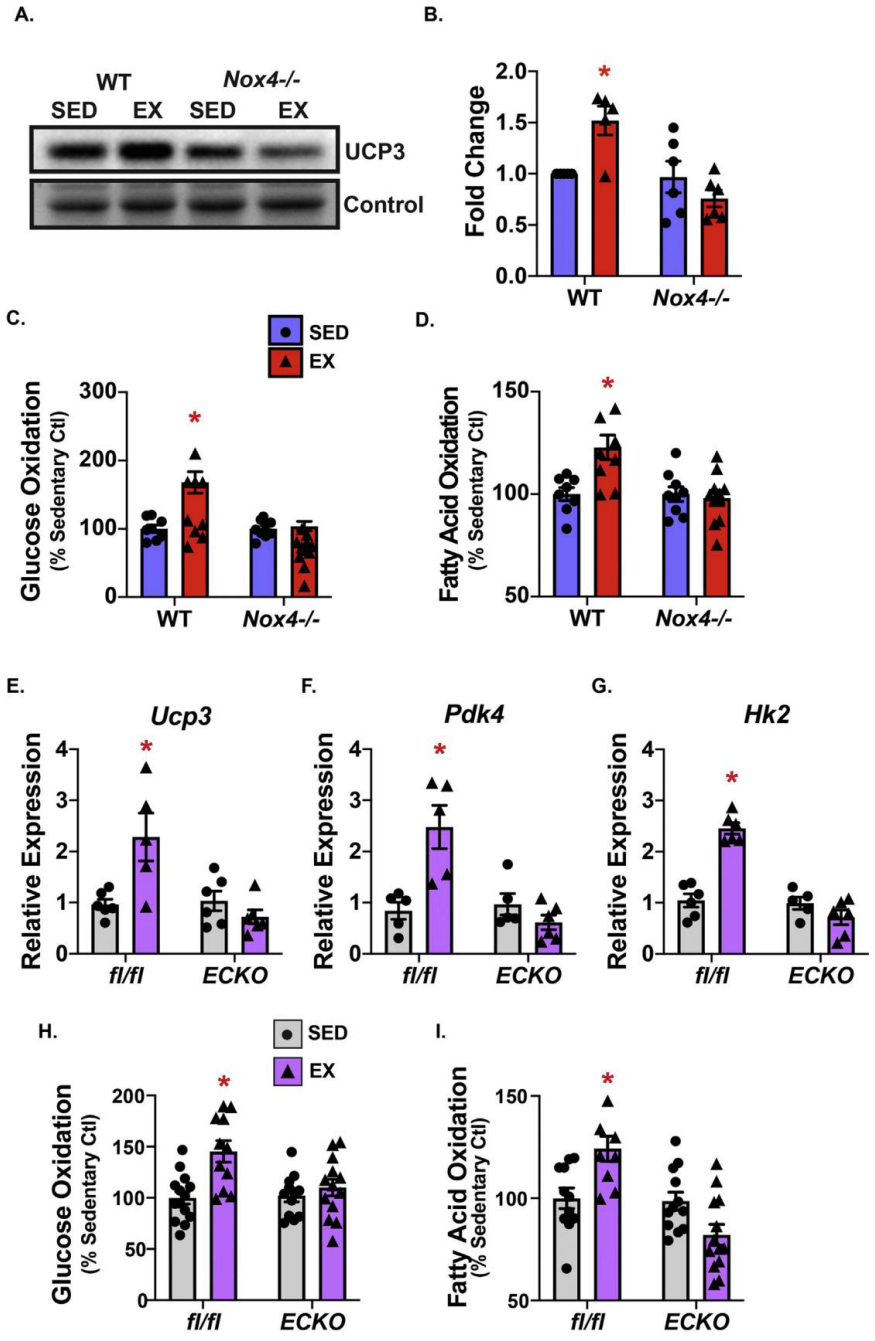
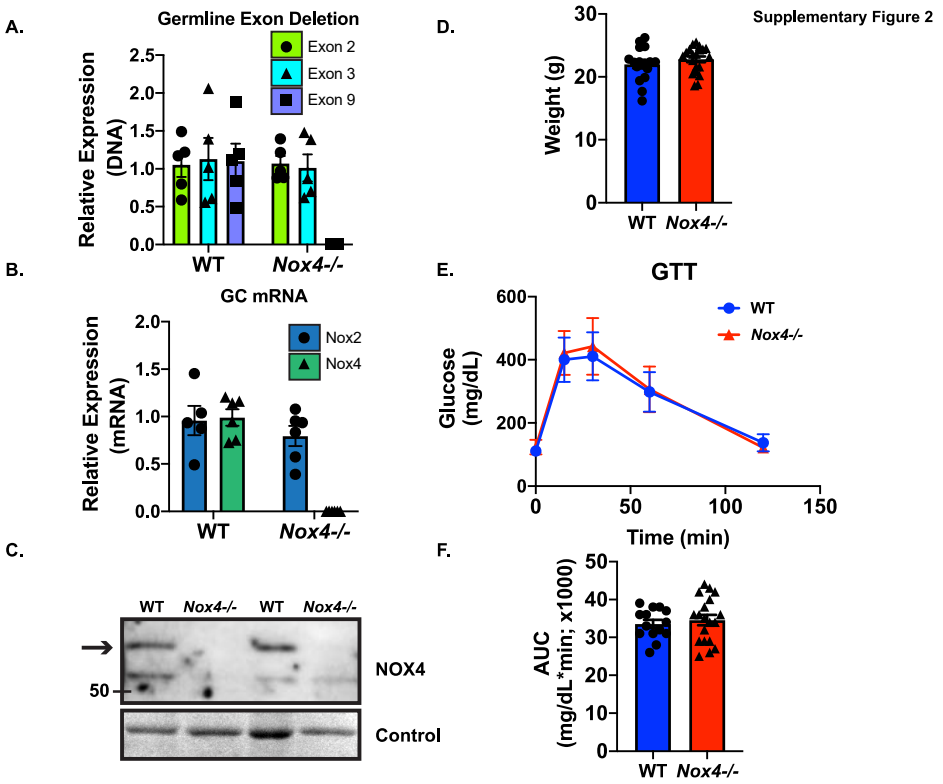
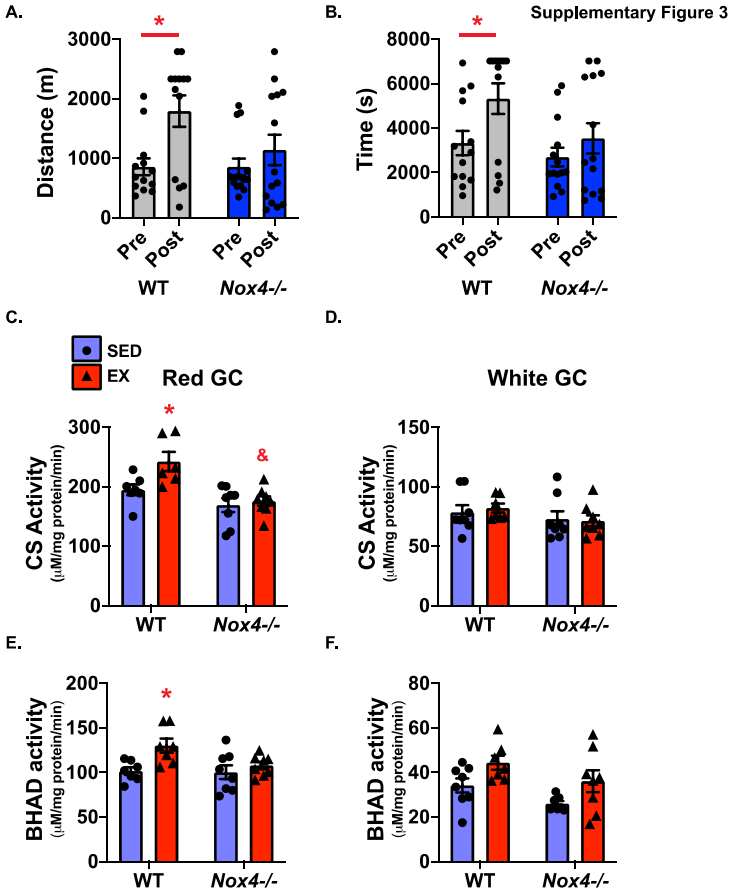

A.
WT
Nox4-/-
SED
EX
SED
EX
UCP3
Control
B.
Fold Change
2.0
1.5
1.0
0.5
0.0
*
WT
Nox4-/-
C.
SED
EX
Glucose Oxidation
(% Sedentary Ctl)
300
200
100
0
*
WT
Nox4-/-
D.
Fatty Acid Oxidation
(% Sedentary Ctl)
150
100
50
*
WT
Nox4-/-
E.
Ucp3
Relative Expression
4
3
2
1
0
*
fl/fl
ECKO
F.
Pdk4
Relative Expression
4
3
2
1
0
*
fl/fl
ECKO
G.
Hk2
Relative Expression
4
3
2
1
0
*
fl/fl
ECKO
H.
SED
EX
Glucose Oxidation
(% Sedentary Ctl)
200
150
100
50
0
*
fl/fl
ECKO
I.
Fatty Acid Oxidation
(% Sedentary Ctl)
150
100
50
*
fl/fl
ECKO

Figure 4: Endothelial Nox4 is required for substrate oxidation in response to acute exercise. Mice were subject to an acute bout of exercise. Red gastrocnemius (GC) was harvested 3h after exercise and immediately analyzed for: (A) UCP3 expression shown by western blot (B) and quantified using image J (N = 5-6; *p < 0.05 compared to SED). (C) Glucose oxidation (D) Fatty acid oxidation; (N = 8-11/group; *p < 0.05 compared to SED). We crossed our Nox4 floxed ($Nox4^{fl/fl}$) mice with our Ve-Cadherin Cre mouse line resulting in endothelial-specific of Nox4 deletion ($Nox4^{ECKO}$). Mice were subject to an acute bout of exercise and GC was harvested as above for (E) qPCR confirmed changes in metabolism-related genes, mitochondrial uncoupling protein 3 (*Ucp3*) (F) pyruvate dehydrogenase 4 (*Pdk4*) (G) hexokinase 2 (*Hk2*) (H) glucose oxidation and (I) fatty acid oxidation; (N = 11-14/group; *p < 0.05 compared to SED). Data are presented as mean ± SEM.

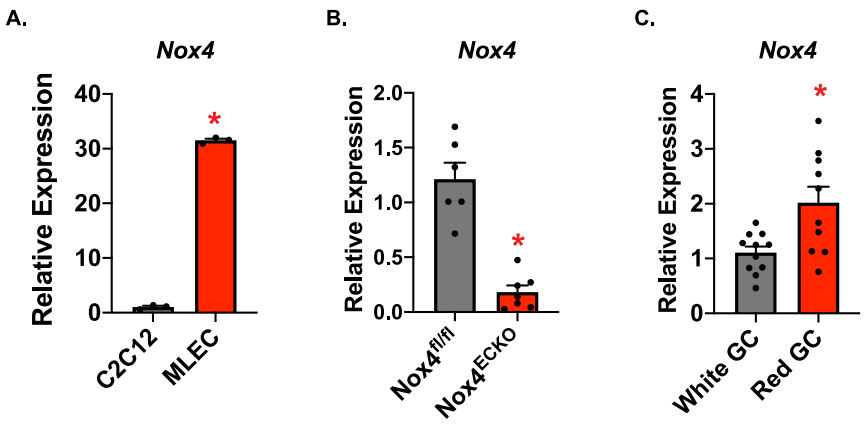
Supplementary Figure 1: Ad-cat expression was not different between sedentary and exercise groups. (A) Adenoviral GFP (Ad-GFP) was injected into the left leg (L) and adenoviral catalase (Ad-Cat) to inhibit H_2O_2 signaling was injected into the right leg (R). (B,C) Five days post-injection, treadmill exercised mice were examined by qPCR and western blotting for gene and protein expression. Data shown for qPCR are the delta Ct (dCt) image j densitometry in comparison to the Ad-GFP control leg for each mouse (N = 4/group). Data are presented as mean ± SEM.



Supplementary Figure 2: Confirmation of Nox4 deletion. (A) Exon expression in WT vs. *Nox4-/-* mice. (B) mRNA expression of Nox4 and Nox2 in WT and *Nox4-/-*. (C) Protein expression of Nox4 in WT and *Nox4-/-* mice. No difference in (D) weight or (E) GTT or (F) GTT AUC was observed in *Nox4-/-* mice (*$p < 0.05$). Data are presented as mean ± SEM.



Supplementary Figure 3: Enzymatic adaptation to chronic exercise was blunted in Nox4-/- mice. We examined the influence of loss of Nox4 on adaptation to chronic exercise (4 weeks treadmill). (A) Distance and (B) time run to exhaustion was measured. (C,D) Skeletal muscle was assessed for activity of citrate synthase and (E,F) beta-hydroxyacyl-coA-dehydrogenase (BHAD) (N = 6-8/group; *$p < 0.05$ compared to WT SED; $^{\&}p < 0.05$ compared to WT + EX). Data are presented as mean ± SEM.



Supplementary Figure 4: Nox4 expression in the endothelium. (A) Cells were harvested and qPCR of Nox4 expression measured. (N = 3; *p < 0.05). (B) mRNA expression of Nox4 from GC. (C) Nox4 expression in red vs. white GC. Data are presented as mean ± SEM.

Discussion

In this study, we investigated the metabolic pathways initiated by exercise and uncovered a novel role for Nox4 in the regulation of skeletal muscle metabolism. Our primary observations demonstrated that Nox4 influenced the acute expression of key metabolic transcripts following exercise. Loss of Nox4 blunted UCP3 expression which resulted in diminished glucose and fatty acid oxidation post-exercise. Endothelial Nox4 deletion similarly blunted substrate oxidation post exercise, suggesting an important role for the endothelium in mediating the early metabolic responses to exercise.

By analyzing the transcriptomic profile of WT mice, we identified H_2O_2 as an important upstream regulator of the skeletal muscle responses to exercise. We then confirmed these observations using single-leg injections of catalase (an H_2O_2 scavenger). Previous studies support our data by showing that the formation of H_2O_2 occurs in contracting skeletal muscle[40] and during acute exercise[41]. In addition, there are multiple H_2O_2-responsive transcription factors such as *Ap-1*, *Nrf2*, *Creb*, *Hif-1*, and *Nf-kB*[42] that are essential for the exercise metabolic response to exercise and subsequent adaptation. We found that Nox4 deletion mediated similar gene expression profiles to catalase expression, indicating Nox4 may be responsible, in part, for the exercise-induced H_2O_2 initiation of metabolic gene expression. Nox4 predominantly produces H_2O_2[13,14] and may serve as a metabolic sensor during acute exercise. Nox4 was previously thought to be constitutively active; however, a recent publication found that Nox4 directly binds ATP[16], and with decreased mitochondrial ATP, Nox4 activity increases. In terms of acute exercise, this mechanism of Nox4 activation would make sense where Nox4-dependent ROS production may be driven by decreased mitochondrial ATP.

UCP3 is a major regulator of ROS homeostasis and mitochondrial metabolism[43]. The *UCP3* gene is highly responsive to changes in the metabolic environment, including diet and exercise and, in both humans and rodents, expression is increased following exercise[31,44]. ROS are known to promote expression of *Ucp3*[45] and specifically H_2O_2 increases UCP3 expression[46]. This is supported by our data which demonstrated that exercise-induced *Ucp3* expression was blunted in the presence of catalase or with deletion of Nox4 following exercise. It has been proposed that UCP3 abundance correlates with the degree of fatty acid oxidation[37] which occurs during the recovery phase following an acute bout of exercise[38]. We observed diminished UCP3 expression and blunted substrate oxidation in Nox4-deficient mice after acute exercise. Taken together, these observations suggest that the induction of UCP3 may be in response to the increased production of ROS which drives the post-exercise increases in substrate oxidation[47].

Our data represent a new avenue of investigation where Nox4 is responsible for initiating immediate metabolic changes in glucose and fatty acid oxidation in skeletal muscle. A previous study demonstrates the importance of Nox4 in acute exercise adaptation in the heart where Nox4-deficient mice exhibit reduced mitochondrial antioxidant capacity, as shown through decreased activation of the redox regulated transcription factor, *nuclear factor erythroid 2 related factor* (*Nrf2*)[48]. While *Nrf2* was not identified as an upstream regulator from our studies here, we cannot rule out the possibility that Nox4 activation of *Nrf2* occurs in the skeletal muscle, which may be important for some of the downstream metabolic effects we see in our models. Our findings regarding the requirement of Nox4 to drive fatty acid oxidation resonate with findings demonstrating that overexpression of Nox4 promotes fatty acid oxidation in heart failure[49]. Interestingly, in contrast to our loss-of-function data, Nox4 overexpression in the heart did not impact glucose oxidation.

Chronic exercise adaptation is the result of multiple acute bouts of exercise; we found that Nox4 was necessary for adaptive responses to chronic exercise. Given ROS are short-acting molecules, our hypothesis was that Nox4 initiated immediate metabolic changes that ultimately resulted in chronic adaptation. With chronic exercise, previous studies have documented that Nox4 is required for increased vascularization[50], which is consistent with studies from our lab and others demonstrating that Nox4 increases angiogenesis in a mouse model of peripheral artery disease[26,51]. Importantly, one study concluded that Nox4 is dispensable for exercise-induced muscle fiber type switching[52]. This observation may appear to contradict the current findings which demonstrated significant adaptations in enzymatic activity (CS and BHAD) to chronic endurance-type exercise in the WT mice that was blunted in the *Nox4-/-* mice. However, it is important to note that the pathways that drive exercise induced fiber-type switching may differ from those driving metabolic adaptation[53]. In support of our findings with chronic exercise, a recent publication similarly reported that Nox4 promoted exercise adaptation in obese mice, demonstrating increased muscle citrate synthase activity in response to chronic exercise[54]. Future studies will be important to address the interaction between physiologic and pathologic ROS production in the skeletal muscle with exercise.

To meet increases in energy demand with exercise, multiple intracellular processes need to occur at different subcellular locations (plasma membrane, cytosol, mitochondria, etc.) in parallel[1]. This suggests that localized and/or compartmentalized ROS production may be important for the spatial and temporal resolution of metabolic adaptation to different modes of exercise (duration, intensity, and type). In this study, our data demonstrated the role of Nox4 in oxidative metabolism, which suggests a deficit in mitochondrial function, although this speculation will require further study. Recent studies have demonstrated skeletal muscle Nox2 as

a major producer of cytosolic ROS which is important for acute glucose uptake[6] metabolic stress-responsive gene expression[55]. One study examining loss of Nox2 in both acute and chronic high intensity exercise[56] suggested that there is crosstalk between Nox2 and mitochondrial ROS, emphasizing the potential coordination between different sites of ROS production in skeletal muscle responses to exercise. It is likely that Nox2 and Nox4 are responsible for mediating specific signaling pathways via ROS production in different subcellular microdomains leading to localized metabolic signaling responses[57]. In the current study, we did not find any differences in Nox2 expression in any of our models; however, as Nox2 is mainly regulated by complex assembly[58], we cannot rule out that a subset of these metabolic effects were due to Nox2. Given the potential crosstalk between Nox4 and Nox2, it will be interesting to further delineate the relative contributions of these Nox members in different modes of exercise and in specific subcellular compartments.

An additional factor that may be important in defining the metabolic role of Nox in exercise adaptation is the relative expression and activity of the Nox members in skeletal muscle. We primarily examined the GC, which is composed of both red (oxidative) and white (glycolytic) muscle. Nox4 mRNA expression (**Supplementary Figure 4**) and activity is higher in the red GC than the white GC[39]. Furthermore, mRNA expression patterns of genes involved in energy metabolism, such as Ucp3, follow a similar pattern to Nox4 expression in red GC compared to white GC[39,59]. It is likely that there are multiple sources of ROS production both during and post-exercise that mediate discrete signaling niches based on their localization and expression profiles.

Importantly, we have found that endothelial Nox4 may initiate substrate oxidation with acute exercise in muscle. Skeletal muscle is a mixed tissue composed of myocytes, endothelial

cells, pericytes and immune cells. Therefore, Nox contribution to skeletal muscle ROS production could reflect expression patterns in these other cell types. Utilizing endothelial-specific Nox4 deletion, the total *Nox4* mRNA level in the skeletal muscle was decreased significantly (~80%), suggesting the endothelium expresses Nox4 much more highly than the myocytes. Indeed, we found that in vitro, Nox4 was expressed much more significantly in the microvascular endothelial cells than in the myocytes. We found a comparable impact on gene expression and substrate oxidation between the $Nox4^{ECKO}$ and the global Nox4 deletion. These data demonstrated that endothelial Nox4 is important for influencing exercise-induced immediate changes to skeletal muscle gene expression and substrate oxidation. Indeed, a significant relationship between the endothelium and skeletal muscle in regard to metabolism has been demonstrated as multiple studies show that the oxidative capacity of muscles correlates with capillary density[60,61]. This is in line with the fact that patients with severe reductions in blood flow reportedly have decreased oxidative capacity and impaired TCA enzyme activity, which is likely involved in the development of muscle atrophy[62,63]. While no basal differences in skeletal muscle capillarization have been observed in mice lacking Nox4[50], there may be paracrine signaling between the two tissues which is responsible for the endothelial Nox4-dependent metabolic adaptation observed here. Furthermore, studies have demonstrated that the skeletal muscle mitochondria closely line the vasculature[64]. This strategically places the skeletal muscle mitochondria adjacent to the endothelium to receive paracrine signals. Additional studies are needed to further define the inter-tissue cross-talk between the endothelium and the muscle.

Conclusion

Our findings here support the hypothesis that Nox4 is responsible for initiating important immediate metabolic signaling in the skeletal muscle response to exercise. Taken together, our results have demonstrated Nox4 serves as a catalyst for altered gene expression and subsequent glucose and fatty acid oxidation after acute exercise. Furthermore, we show that endothelial Nox4 is required for the response in skeletal muscle, suggesting that inter-tissue cross-talk between the endothelium and the muscle is important for the metabolic responses to exercise. This is further supported by our findings that glucose and fatty acid oxidation were blunted after acute exercise in the red GC in both the *Nox4-/-* and $Nox4^{ECKO}$ mice. Interestingly, our chronic exercise studies similarly demonstrated a robust influence of Nox4 expression on enzyme activity in the red GC, but not in the white GC (**Supplementary Figure 3**).

Acknowledgments

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Abbreviations

Nox4	Nicotinamide dinucleotide phosphate (NADPH) oxidase 4
Nox4-/-	Global Nox4 deletion
$Nox4^{ECKO}$	Endothelial specific deletion of Nox4
Ad-Cat	Adenoviral catalase

Ad-GFP	Adenoviral green fluorescent protein
H_2O_2	Hydrogen peroxide
ROS	Reactive oxygen species
TCA cycle	Tri-carboxylic acid cycle
CS	Citrate Synthase
BHAD	Beta-hydroxyacyl-coA-dehydrogenase
GC	Gastrocnemius
EDL	Extensor Digitorum Longus
UCP3	Uncoupling protein 3
Hk2	Hexokinase 2
Pgc-1a	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
Pdk4	Pyruvate dehydrogenase kinase 4
Slc25a25	Calcium-binding mitochondrial carrier protein
Sik1	Salt-inducible kinase 1
Mt2	Metallothionein 1
Nr4a3	Nuclear receptor subfamily 4 group A member 3
eNOS	Endothelial nitric oxide synthase
Ap-1	Activator protein 1
Nrf2	Nuclear factor erythroid 2-related factor 2
Creb	CAMP-responsive element-binding protein 1
Hif-1	Hypoxia-inducible factor-1
Nf-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells

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Addendum

ROS act as intracellular and intercellular signaling agents[1]. Hydrogen peroxide (H_2O_2) is a particularly effective signaling agent due to its long half-life and ability to cross cellular membranes[2]. To explore the role of hydrogen peroxide in an *in vitro* system, we exposed differentiated C2C12 mouse skeletal muscle cells to hydrogen peroxide for 1-hour to assess metabolic genes downstream of H_2O_2 in vitro. Here we demonstrate that genes involved in glucose oxidation (**Fig. i)** and fatty acid oxidation (**Fig. ii**) significantly increased after 1-hour treatment with H_2O_2.

ACC2 is the predominant isoform in skeletal muscle and is important for activating beta-oxidation during exercise[3]. We then assessed phosphorylation of acetyl-CoA carboxylase immediately post-exercise. Here we observed an increase in phosphorylation of ACC in the WT that is blunted in the *Nox4-/-* (**Fig. iii**). While these data were not significant, we did observe a trend ($p = 0.1$). Future studies will further explore the mechanistic link between Nox4 and substrate oxidation post-acute exercise.

Methods

C2C12 Differentiation and RNA isolation

Mouse skeletal muscle cells (C2C12 cells) were cultured in DMEM (GIBCO #11965) with 10% FBS and 1% penicillin/streptomycin (Gibco #15140). When cells reached 60-70% confluency, cells were split and placed in a 6-well plate. Cells were brought to 90-100% confluency and differentiation media (DMEM, 2% donor equine serum, and 1μM insulin) was

added. 24 hours later, differentiation media was changed, and changed every 24 hours for the next 4 days. After cells were fully differentiated, on the day of experimentation, media was changed for 1 hour. Then cells, were dosed with 300 μM hydrogen peroxide (H_2O_2) for 1 hour. RNA was then isolated using the RNeasy Kit (Qiagen, Cat #74104).

Western Blotting

Protein homogenization was conducted as previously described in gastrocnemius collected from *Nox4-/-* and WT mice immediately post-acute exercise[4]. Antibodies for p-ACC (CST 3661) and ACC (CST 3662) were used. Blots were analyzed using Image J (https://imagej.nih.gov/ij/index.html) for densitometry and normalized to the non-phosphorylated (total) protein.

Results

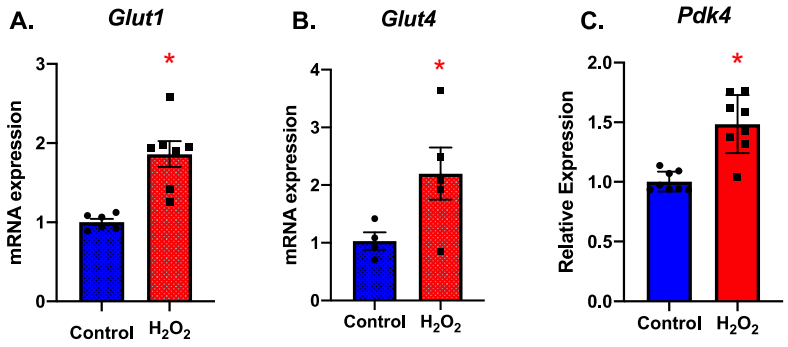

Figure i: Hydrogen peroxide is responsible for mediating changes in glucose oxidation gene expression in skeletal muscle cells in vitro. RNA was isolated from differentiated C2C12 cells after 1 hour treatment with 300 μM H_2O_2. mRNA expression was assessed of (A) glucose transporter 1 (*Glut1*) (B) glucose transporter 4 (*Glut4*) (C) pyruvate dehydrogenase kinase 4 (*Pdk4*) (n=5 – 7/group; *$p < 0.05$ compared to CON). Data are presented as mean ± SEM.

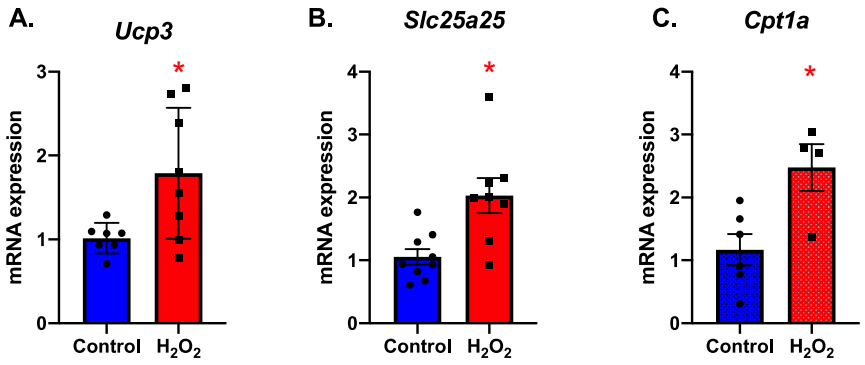

Figure ii: Hydrogen peroxide is responsible for mediating changes in fatty acid oxidation gene expression in skeletal muscle cells in vitro. RNA was isolated from differentiated C2C12 cells after 1 hour treatment with 300 μM H_2O_2. mRNA expression was assessed of (A) mitochondrial uncoupling protein 3 (*Ucp3*) (B) solute carrier family 25 member 25 (*Slc25a25*) and (C) carnitine palmitoyltransferase 1A (*Cpt1a*) (n=6 – 9/group; *$p < 0.05$ compared to CON). Data are presented as mean ± SEM.

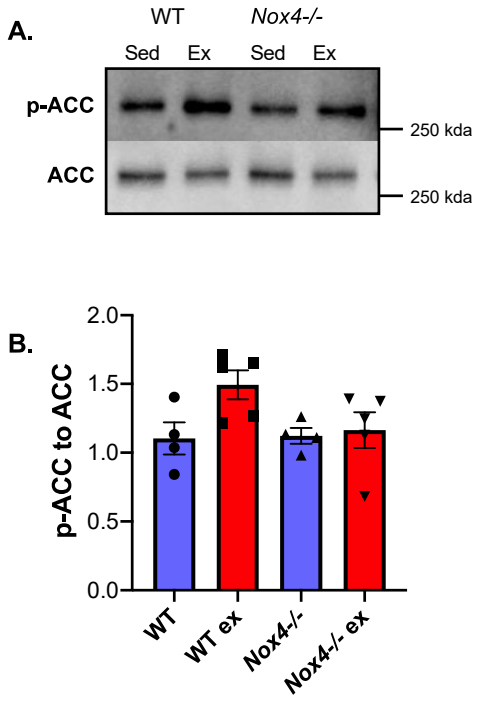

Figure iii. Phosphorylation of acetyl-CoA carboxylase in skeletal muscle post-exercise is independent of Nox4. Gastrocnemius from Nox4-/- and WT mice were collected immediately after an acute bout of exercise. (A) Phosphorylation of ACC was quantified via western blot and normalized to total ACC. Data are presented as mean ± SEM.

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Chapter 5: Nox4 mediates exercise induced skeletal muscle mitophagy

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In preparation

Abstract

In response to exercise, skeletal muscle produces reactive oxygen species (ROS). While initially thought to be drivers of skeletal muscle fatigue, studies using exogenous antioxidant supplementation to quench ROS have been shown to blunt the beneficial effects of exercise in humans and rodents. While these data are suggestive that ROS are required for exercise adaptation, the mechanistic insight into how ROS mediates these beneficial metabolic adaptations is incompletely understood. We recently showed that after acute exercise, there was an increase in mitochondrial oxidative stress in WT mice that was not observed in *Nox4-/-* mice. Mitochondrial oxidation occurs as a prelude to mitophagy. With this in mind, we utilized a novel tool, *pMitoTimer*, to investigate both mitochondrial oxidation and mitophagy. We demonstrated that in the absence of Nox4, both mitochondrial oxidation and mitophagy are significantly blunted. Previous work has demonstrated that skeletal muscle mitophagy is mediated by AMPK. Here we provide evidence that Nox4 mediates skeletal muscle mitophagy independent of AMPK As disruptions in mitochondrial dynamics impact cellular energy homeostasis, we show that *Fis-1*, a mediator of mitochondrial fission, is blunted in skeletal muscle of *Nox4-/-* mice post-acute exercise. These data suggest Nox4 is required for adaptive changes in mitochondrial connectivity prior to the induction of mitophagy, which requires further investigation.

Introduction

Deficits in skeletal muscle mitochondrial metabolism are a common hallmark of many metabolic diseases[1,2]. Exercise is an effective strategy to mitigate disease severity and improve disease prognosis[3]. While the initial observations that regular exercise improves skeletal muscle mitochondrial metabolism were made over 50 years ago[4], much of the mechanistic insight remains limited.

At the onset of exercise, working skeletal muscle produces reactive oxygen species (ROS). ROS include superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2)[5]. While initially thought to be deleterious and a primary contributor to muscle fatigue[6,7], ROS are now recognized as second messenger signals which contribute to adaptative signaling processes[8]. In particular, H_2O_2 is the most stable form of ROS due to its longer half-life (~ 1 ms) and ability to cross cellular membranes and interact with cysteine residues to alter protein function[9–11]. There are multiple endogenous sources of ROS in skeletal muscle, including the mitochondria, xanthine oxidase, and the Nadph oxidase family (Nox)[12]. Mitochondrial-derived ROS were previously thought to be the predominant source of ROS during exercise[13,14]. However, recent observations suggest Nox are more significant contributors to exercise-induced ROS production[15].

In particular, Nox2 and Nox4 are expressed in skeletal muscle but have different roles in regulating metabolic function[15]. Nox4 is a unique member of the Nox family, as it predominantly produces H_2O_2[16]. Recent studies have highlighted the importance of Nox4 in regulating mitochondrial function and metabolism[17–19]. We and others have published that Nox4 is required for adaptations in mitochondrial enzyme activity with chronic training[18,20]. While Nox4 has been documented in a few subcellular locations, several studies demonstrate Nox4 is present in the mitochondria in different tissues[17,21], including skeletal muscle[22,23]. Importantly, Nox4 was shown

to localize at the inner mitochondrial membrane and is activated during conditions of energetic stress (low ATP) in cancer cells[17]. These data suggest that Nox4 acts as a mitochondrial energy sensor, which may be important in conditions such as exercise. However, while sufficient evidence suggests Nox4 mediates metabolic adaptation during conditions of energetic stress, many questions remain as to how Nox4 impacts mitochondrial function resulting in metabolic adaptation.

One component of mitochondrial quality is mitophagy, which has been recently described as an adaptive response to exercise[24–26]. Mitochondria exist in interconnected networks and constantly undergo changes in their connectivity through fission and fusion, coined *mitochondrial dynamics,* in response to metabolic demand[27]. This is particularly important for mitophagy, a selective form of autophagy[28], as it is necessary to separate damaged mitochondria prior to degradation[29]. This catabolic process is integral for cell homeostasis[30] and skeletal muscle function[31]. Disrupted mitophagy flux is associated with numerous pathologies, including aging[32] and several skeletal muscle metabolic disorders[33].

During conditions of metabolic stress, there is an increase in ROS and a subsequent need for cell recycling[34]. Multiple in vitro studies have established a correlative link between ROS and mitophagy[34–36]. Some evidence suggests that H_2O_2 is the predominant ROS species that promotes autophagy during nutrient deprivation[37]. However, little is known regarding the sources and species of ROS that promote mitophagy. We hypothesized Nox4-ROS may mediate skeletal muscle mitochondrial oxidation and mitophagy after exercise. To assess the role of ROS in promoting skeletal muscle mitophagy and the mitochondrial oxidative environment, we utilized a Nox4 global knockout (*Nox4-/-*) and a novel mitochondrial reporter gene, *pMitoTimer*[26,38,39]. Here we identified a new role for Nox4 in mediating mitochondrial quality control in response to acute

exercise. Our findings highlight the importance of Nox4-ROS signaling in promoting essential adaptive responses in skeletal muscle after exercise.

2. Methods

Mice

Nox4-floxed (*Nox4*$^{fl/fl}$) mice were obtained from Jun Sadoshima and bred with the CMV-Cre line (Jackson Laboratories, Bar Harbor, ME; Jax 006054) to produce a line with global Nox4 deletion (*Nox4-/-*). C57Bl/6J mice (Jax 000664) were used as controls for the *Nox4-/-* mice as previously described[18]. All of the groups were allowed to eat ad libitum throughout the duration of the study (except during the time periods of exercise/sedentary). The animals were housed on a 12:12-h light–dark cycle in a temperature-controlled room at 25°C. The Virginia Tech Use Committee approved all of the procedures.

Exercise Protocol

Exercise was conducted on a motorized treadmill (Columbus Instruments Model #1055-SRM-D58, Columbus, OH, USA). All of the mice were acclimated to the treadmill for 3 days prior to the exercise regimens. On the day of acute exercise, mice allocated to perform treadmill running were subject to 5% incline and 10 min at 13 m/min, 10 min at 16 m/min, 50 min at 19 m/min, and then 20 min at 21 m/min[26]. Mice were sacrificed immediately following treadmill running or 6 hours after exercise[26]. A second exercise protocol was utilized and consisted of a 60 min run beginning with a 4 min warm-up period from which the mice progressed from 0 to 20 m/min[18]. The mice were then exercised at 20 m/min for the remainder of the 60 min protocol.

The mice were fasted for 4 h prior to exercise to eliminate any acute metabolic changes due to food and assigned to either a non-exercise control group or an exercise group as we've previously published[18].

Plasmid DNA and transfection

Mice were anesthetized with isoflurane and were injected with hyaluronidase at the FDB (0.36 mg/ml; Sigma). One hour later the mice were anesthetized a second time and the FDB was injected with 20 µg of MitoTimer as previously described[26]. Mice were allowed to recover from anesthesia, and after 10 minutes, two acupuncture needles were inserted at the heel and parallel to the toes. 10 pulses were provided with 20 ms duration at 1 hz and 75 V/cm. Mice were allowed to recover and were stable for 10 days prior to any exercise intervention as previously published.

Tissue preparation and confocal microscopy

Flexor digitorum muscle (FDB) was harvested after making a small incision at the bottom of the foot and utilizing forceps to remove excess skin as previously described[26]. FDB was placed in 1mL of 4% paraformaldehyde for 20 minutes at room temperature without any exposure to light. Sample was then transferred into 1mL of PBS for 5 minutes. Afterward, the tendon was removed from the muscle and placed on a gelatin-coated slide face down. Three small drops of PBS were added to the muscle and allowed to dry away from light. A coverslip was placed and adhered to the slide and allowed to dry. All tissues were imaged on a confocal microscope (Leica TCS SP8) using the green (excitation/emission 488/518 nm) and red (excitation/emission 543/572) channels. Laser intensity was defined and all samples were

confined to these specific parameters. MitoTimer contains a mitochondrial targeting sequence and a modified dsRed protein (Timer) that fluoresces green (Em/Ex 448/518nm) when mitochondria are synthesized but switches its fluorescence to red (Em/Ex 558/583nm) when mitochondria are oxidized[26]. Mitophagy was assessed by quantifying red puncta under confocal microscopy and the mitochondrial oxidative environment was assessed in the ratio of the change of red to green fluorescence[24,26].

Oroboros O2K High Resolution Respirometry

Oxygen consumption rates were determined utilizing the Oroboros O2K High Resolution Respirometer[40]. Quadriceps muscle was collected from age-matched WT and *Nox4-/-* mice and red and white fibers were separated. Red quadriceps fibers were permeabilized with saponin for 30 minutes and washed with wash buffer for 15 minutes. Following an oxygen calibration, 4.0-4.6mg of tissue were placed in the Oroboros O2K[41]. The following substrates were added in succession after oxygen consumption rates stabilized: pyruvate/malate (2 mM), glutamate (10 mM), ADP (5 mM), succinate (10 mM) and cytochrome c (20 μM). Rates of respiration were normalized to mg/mL of skeletal muscle[42,43].

Isolated Mitochondria Fractions

Mitochondrial-enriched lysates were isolated via Percoll gradient fractionation from gastrocnemius as previously described[24,44]. Gastrocnemius muscle was placed in FRAC buffer (20 mM Hepes, 250 mM Sucrose, 0.1 mM EDTA and Sigma phosphatase inhibitors 1 and 2). Samples were then spun at 800 g for 10 minutes. The supernatant was then placed on a 60% percoll gradient and spun for 60 min at 36,000 g. The mitochondrial layer was then collected

from the 20% layer of the percoll gradient and further diluted in FRAC buffer and spun at 17,000 g for 10 min. The mitochondrial fractions were then resuspended in Laemmli buffer with phosphates inhibitor 1 and 2 (Sigma) and boiled for 5 min at 97°C.

Westerns

GC was collected and homogenized in lysis buffer (25 mM of HEPES, pH 7.0, and 0.4% CHAPS) with Calbiochem Protease Inhibitor Cocktail Set III (EMD Biosciences, La Jolla, CA, USA) for immunoblotting. Proteins (10 μg loaded) were separated by SDS-Page and transferred onto PVDF or nitrocellulose membranes. The membranes were blocked in 5% non-fat dry milk and the following antibodies were used: p-AMPK (CST #2535), AMPK (CST #2532) p-Ulk1 (CST #5869), Ulk1 (CST #5869), and p-Drp1 (CST #3455), total Drp1 (CST #8570), Nox4 (ProteinTech #14347), VDAC (CST #4866), alpha-tubulin (CST #2144). All primary antibodies were used at a concentration of 1:1000. Membranes were probed with a goat-anti rabbit IR800 (LICOR) secondary antibody on the Odyssey infrared imaging system (LICOR) with a concentration of 1:10,000. Proteins were analyzed with Image J. Samples were normalized to total protein (Invitrogen #A44449) or the non-phosphorylated form of the protein.

RNA extraction and Gene Expression

Tissue was harvested and total RNA was extracted using Qiagen RNeasy Mini Kit (RNeasy Mini Kit, QIAGEN, 74106) and 500 ng of total RNA was used for one step real-time reverse transcription PCR (iScript cDNA Synthesis Kit, BioRad, 1708890). The ΔΔcycle threshold method was used for relative mRNA quantification and the gene expression was normalized to the housekeeping gene (HPRT) as previously described[18].

Gene	Forward	Reverse
Hprt	GGACTAATTATGGACAGGACTG	GCTCTTCAGTCTGATAAAATCTAC
Mfn1	CCTTGTACATCGATTCCTGGGTTC	CCTGGGCTGCATTATCTGGTG
Mfn2	GATGTCACCACGGAGCTGGA	AGAGACGCTCACTCACTTTG
Fis1	GCACGCAGTTTGAATACGCC	CTGCTCCTCTTTGCTACCTTTGG
Atg7	GCCAGGTACTCCTGAGCTGT	ACTTGACCGGTCTTACCCTG
FoxO1	CACCTTGCTATTCGTTTGC	CTGTCCTGAAGTGTCTGCC
Beclin	CGCAGCTGGATAAGCTGAAGAAAACC	CGACCCAGCCTGAAGTTATTGATTG
Opa1	TCCTGGTGAAGAGCTTCAATG	TTTGCAGAAGACGGTGAGAA
Nrf1	TTA CTC TGC TGT GGC TGA TGG	CCT CTG ATG CTT GCG TCG TCT
Nrf2	CGA GAT ATA CGC AGG AGA GGT AAG A	GCT CGA CAA TGT TCT CCA GCT T

Statistics

Results are expressed as means ± SEM. Data were analyzed by GraphPad Prism 7.0 software (GraphPad Software, La Jolla, CA, USA) using two-way analysis of variance (ANOVA), unpaired Student t-test, or Brown-Forsythe and Welch as appropriate. Differences were considered significant with $p < 0.05$*. To assess data outliers, a Grubb's test was used.

Results

3.1 Nox4 is present in mitochondrial fractions from skeletal muscle.

Nox4 is a transmembrane protein that has been identified to be present in different subcellular compartments including the endoplasmic reticulum[45] and the mitochondrial membrane in different cell types[21,46], including skeletal muscle[23]. To determine if Nox4 is present in mitochondria from skeletal muscle, we utilized a percoll gradient to isolate mitochondria from skeletal muscle of WT mice (**Fig. 1**). Here we found enriched expression of Nox4 in isolated mitochondria fractions of WT mice absent in the *Nox4-/-*. We confirmed the Nox4 band in whole cell lysates from skeletal muscle of *Nox4-/-* and WT mice as previously published[18].

3.2 Nox4 is not required for Complex I and Complex II supported mitochondrial respiration.

Nox is required for initiating a wide range of metabolic processes[47]. We have demonstrated a role for Nox4 in promoting adaptive changes to glucose and fatty acid oxidation post-exercise[18], suggesting that Nox4 may be promoting mitochondrial metabolism as mitochondria are the predominant site for fatty acid oxidation (via beta-oxidation) and glucose oxidation (through pyruvate oxidation in the TCA cycle)[48]. In other models, Nox4 has demonstrated to drive mitochondrial respiration[49], we assessed baseline mitochondrial respiration in quadriceps of mice lacking Nox4. We assessed Complex I and Complex II supported respiration and found no differences between groups (**Fig. 2**). We assessed State 3 (ADP supported) respiration and similarly found no differences in mitochondrial respiration in skeletal muscle fibers between *Nox4-/-* and WT mice.

3.3 Nox4 is required for skeletal muscle mitophagy post-acute exercise.

Previously, we subjected global *Nox4-/-* and WT mice to an exhaustive bout of exercise and utilized ingenuity pathway analysis (IPA) to identify genes that were significantly changed in WT mice, but unchanged in the *Nox4-/-*. When we investigated the top differentially regulated pathways, IPA highlighted "mitochondrial dysfunction"[18] which is defined as occurring, "when the ROS-mediated oxidative stress overpowers the antioxidant defense system." Essentially this demonstrated that the WT exercised mice had increased mitochondrial oxidative stress after exercise whereas mitochondrial oxidative stress was not identified in mice lacking Nox4. This led us to hypothesize that Nox4 may be important for regulating the mitochondrial oxidative environment post-exercise.

With exercise, there is an increase in skeletal muscle ROS production[23]. We and others have demonstrated that Nox4 drives adaptive metabolic response to exercise, and that point to a role for Nox4 in mediating mitochondrial metabolism[18–20]. Mitochondrial homeostasis is hinged on a number of regulatory processes that maintain metabolic function, including mitophagy[50]. As mitophagy is integral for metabolic function, we hypothesized mitophagy may dysregulated in *Nox4-/-* mice after exercise.

To assess skeletal muscle mitophagy and the mitochondrial oxidative environment, we utilized a novel plasmid, *pMitoTimer*. The *pMitoTimer* reporter gene targets a fluorescent Timer protein to the mitochondria by conjugating Timer to a mitochondrial-targeted sequence of the cytochrome c oxidase subunit VIII gene at the N terminus with a constitutive CMV promoter[38]. Timer encodes for a DsRed mutant (DsRed1-E5) that undergoes a fluorescent shift from green to red[51] when there is a change in the oxidative environment. Compared to WT, *Nox4-/-* mice did not undergo a fluorescent shift suggesting Nox4 may be important for regulating the

mitochondrial oxidative environment post-acute exercise (**Fig. 3B**). Following the cessation of an acute exercise bout there is a peak in the accumulation of red puncta, a marker of oxidized mitochondrial fragments that have been transported to the lysosome for subsequent depredation[26]. We assessed red puncta and found that there was a significant increase in red puncta in the WT that was blunted in the *Nox4-/-* (**Fig. 3C**), suggesting Nox4 mediates post-exercise induced skeletal muscle mitophagy.

3.4 Nox4 mediates skeletal muscle mitophagy independent of AMPK activation

It was previously demonstrated that AMPK is required for initiation of skeletal muscle mitophagy after exercise[26]. Thus, we set out to assess phosphorylation of AMPK after acute exercise. Interestingly, we found a significant difference with exercise in both the WT and *Nox4-/-* (**Fig. 4A-B**). In addition, we assessed downstream mediators of mitophagy including phosphorylation of Ulk1 and Drp1 and found no differences with exercise or between genotypes (**Fig. 4C-F**).

3.5 Nox4 is required for expression of *Fission-1* after exercise.

Mitochondria exist in interconnected networks and constantly undergo changes in their connectivity through fission and fusion in response to increased metabolic demand[27]. As changes in mitochondrial dynamics alter mitochondrial responses to exercise, we assessed markers of fusion and fission. Expression of markers of fusion (*Mitofusin-1* and *Mitofusin-2*) in quadriceps of *Nox4-/-* and WT mice were not different between groups (**Fig. 5A-B**). In addition, we assessed expression of *mitochondrial dynamin like GTPase* (*Opa1*) but observed no differences with exercise or between genotypes (**Fig. 5D**) despite Opa1 increasing at the protein level post-

exercise[52]. No significant differences in *Beclin-1*, a marker of autophagosome formation[53], were observed with exercise or between groups (**Fig. 5E**). We then assessed expression of *Autophagy related 7* (*Atg7*), a regulator of mitochondrial quality, and found a significant increase post-exercise in the WT and *Nox4-/-* (**Fig. 5F**). As it is necessary for mitochondria to undergo fission prior to mitophagy, and fission is required for exercise adaptation[52], we assessed expression of Fis1 and observed a significant increase in the WT that was blunted in the *Nox4-/-* (**Fig. 5C**).

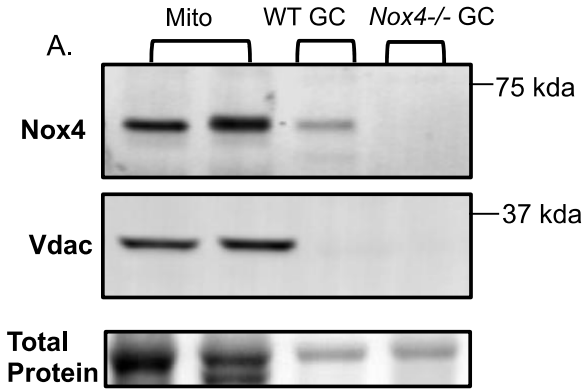


Figure 1: Nox4 is present in isolated mitochondrial from skeletal muscle. Percoll gradients were used to isolate mitochondria from gastrocnemius (GA) of WT mice from Dr. Joshua Drake and Dr. Anna Nichenko. Representative western blot of isolated mitochondria fractions and skeletal muscle homogenates immunoblotted for Nox4, VDAC, and total protein.

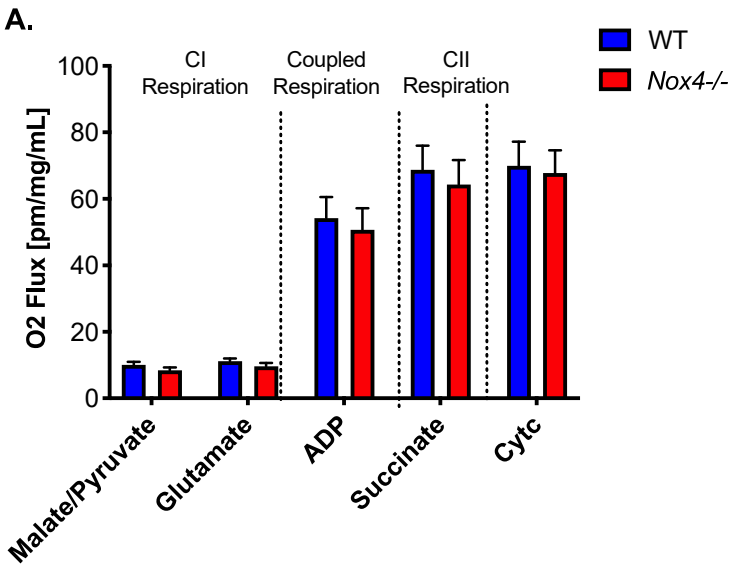


Figure 2: Nox4 is not required for Complex I and Complex II supported respiration.

Skeletal muscle fibers from quadriceps were permeabilized to assess Complex I, Complex II, and State 3 supported respiration. There were no differences in mitochondrial respiration rates from *Nox4-/-* and WT skeletal muscle. (N = 6/group). Data are presented as mean ± SEM.

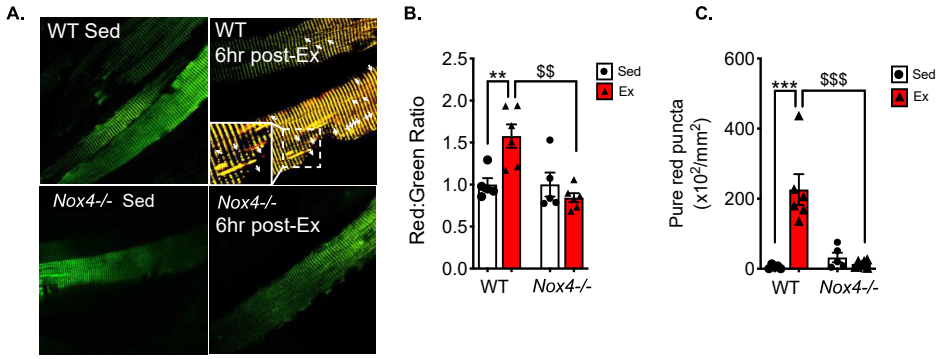


Figure 3: Nox4 is required for skeletal muscle mitophagy post-acute exercise. **A)** Representative images of FDB muscle fibers of *Nox4-/-* mice and wild-type (WT) littermates (10-12 weeks) transfected with *pMitoTimer*. Tissues from mice chosen for acute exercise were harvested 6 hours after the cessation of exercise, corresponding to the peak in post-exercise-induced mitophagy[26]. Images are merged *red* and *green* channels. **B)** Quantification of MitoTimer Red:Green fluorescence intensity and **C)** pure red puncta (indicated by white arrows). Results of two-way ANOVA for **B**: $F = 11.13$, $DF = 1$, $p = 0.004$ and **C**: $F = 22.29$, $DF = 1$, $p = 0.0002$ ($N = 5$-7/group). Tukey multiple comparison tests were used when a significant interaction effect was observed (**$p < 0.01$ and ***$p < 0.001$ compared to sedentary control and \$\$\$$p < 0.001$ for WT exercise vs *Nox4-/-* exercise comparisons). Data are presented as mean ± SEM.

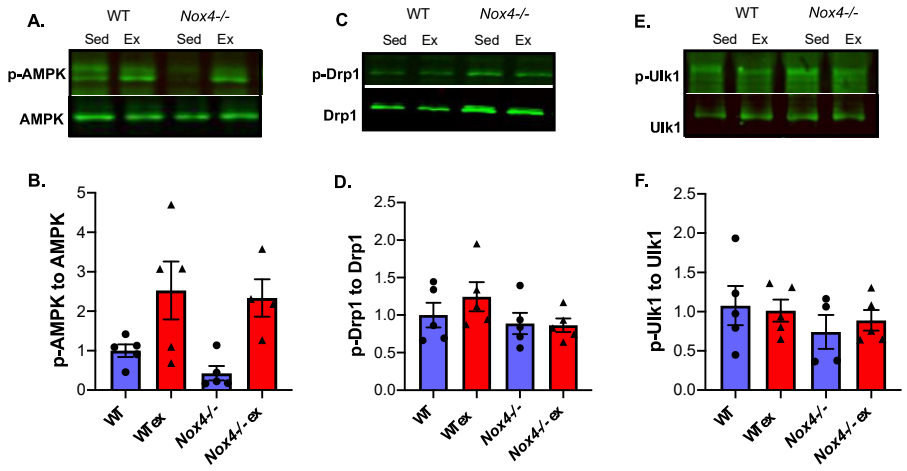


Figure 4: Nox4 mediates skeletal muscle mitophagy independent of AMPK activation. A) Relative expression of p-AMPK to AMPK (A) representative western blot and (B) densitometry quantification. Relative expression of p-Drp1 to Drp1 (C) representative western blot and (D) densitometry quantification. Relative expression of p-Ulk1 to total Ulk1 (E) representative western blot and (F) densitometry quantification. All western blots were quantified via Image J. (N = 5-6/group). Data are presented as mean ± SEM.

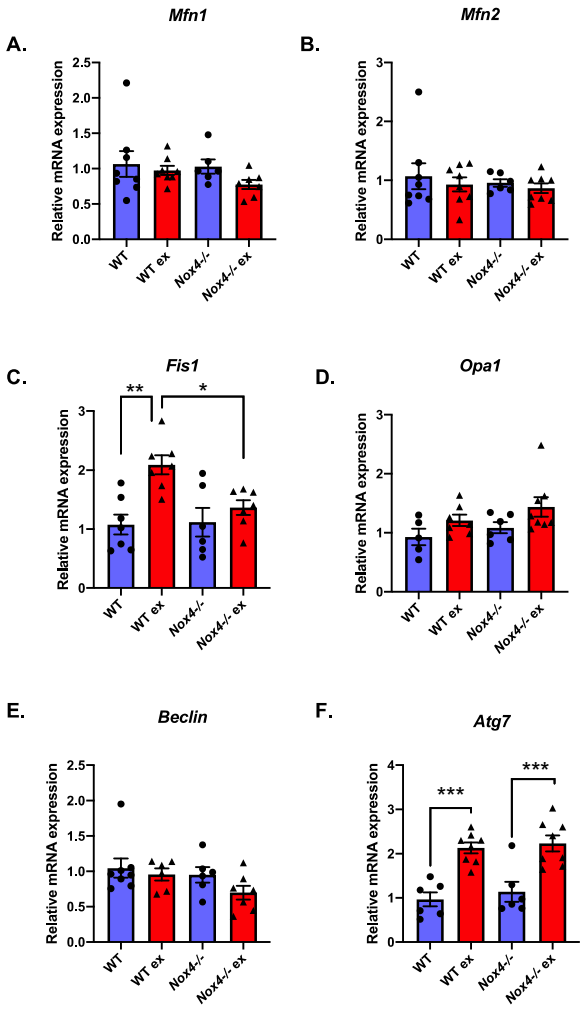
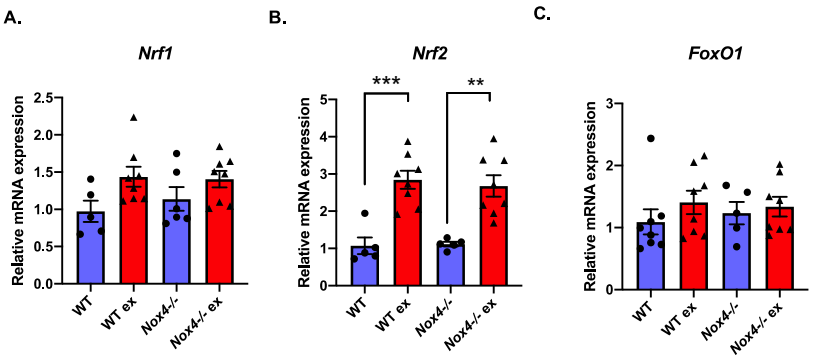


Figure 5: Nox4 is required for expression of *Fission-1* after acute exercise. A) mRNA expression of *Mfn1* in skeletal muscle of WT and *Nox4-/-* mice. **B)** mRNA expression of *Mfn2* in skeletal muscle of WT and *Nox4-/-* mice. **C)** mRNA expression of *Fis1* in quadriceps muscle of WT and *Nox4-/-* mice. **D)** mRNA expression of *Opa1* in quadriceps muscle of WT and *Nox4-/-* mice. **E)** mRNA expression of *Beclin* in quadriceps muscle of WT and *Nox4-/-* mice. **F)**

mRNA expression of *Atg7* in quadriceps muscle of WT and *Nox4-/-* mice. A two-way ANOVA was used (N = 5-7/group; *p < 0.05 compared to sedentary control **p < 0.01 and ***p < 0.001 compared to sedentary control). Data are presented as mean ± SEM.



Supplementary Figure 1: No significant differences in mRNA expression of metabolic transcription factors between *Nox4-/-* and WT mice. **A)** mRNA expression of *Nrf1* in WT and *Nox4-/-* mice. **B**) mRNA expression of *Nrf2* in WT and *Nox4-/-* mice. **C)** mRNA expression of *FoxO1* in WT and *Nox4-/-* mice. A two-way ANOVA was used (N = 5-7/group; **p < 0.01 and ***p < 0.001 compared to sedentary control). Data are presented as mean ± SEM.)

Discussion

Here we investigated the role of Nox4 in mediating skeletal muscle mitophagy in response to acute exercise. Our primary findings utilizing MitoTimer demonstrate that Nox4 promotes skeletal muscle mediates mitochondrial oxidation and mitophagy post-acute exercise. AMPK is a primary regulator of exercise-mediated mitophagy in skeletal muscle[26]. However, our data does not point to AMPK. Our findings demonstrate Nox4 may play a role in mitochondrial fission (a prelude to mitophagy), as we found *Fis-1* significantly increased with exercise and is blunted in the absence of Nox4 post-exercise.

Nox4 is expressed in the membrane of several cell types[54]. Recent evidence suggests Nox4 localizes to the mitochondria[17,21–23]. We confirmed the expression of Nox4 in enriched mitochondria fractions from skeletal muscle. While localization does not establish a clear molecular function, Nox4 does contain an ATP binding domain (Walker-a binding motif) which may allow it to act as an energetic sensor [17]; when ATP is low (unbound), Nox4-H_2O_2 production is increased[17]. While the study documenting that Nox4 contains a Walker-A motif was conducted in cancer cells, this may highlight a novel role for Nox4 as an energetic sensor in skeletal muscle. Thus, when energy is low, Nox4 increases ROS production driving mitochondrial oxidation and mitophagy during conditions of stress.

Our work[18], alongside others[19,20], has demonstrated that Nox4 is required for metabolic adaptations to exercise and suggests Nox4 is required for mediating mitochondrial metabolism. Here we wanted to assess if baseline mitochondrial respiration differed in skeletal muscle of *Nox4-/-* mice. Other work evaluating the role of Nox4 in mitochondrial bioenergetics in macrophages found that mitochondrial respiration was reduced in *Nox4-/-* macrophages compared to WT[49]. While we did not observe differences in State 3 (ADP stimulated) respiration with glutamate and

malate (**Fig. 2**), there are a few reasons this might occur. First, the absence of a difference in State 3 respiration (with glutamate and malate) does not preclude the possibility of a difference in functional adaptation (e.g., exercise). These studies were conducted utilizing saturating substrate[55] as a measure of mitochondrial capacity. Second, under non-stressed conditions, we and others have not observed differences in the baseline metabolic phenotype of *Nox4-/-* mice[18,56].

Here we demonstrate that Nox4 mediates an exercise-induced change in the mitochondrial oxidative environment as there is a blunted shift from the green to red ratio in *Nox4-/-* mice (**Fig. 3B**). Our observations here align with our previous findings that genes involved in promoting mitochondrial oxidative stress were impacted by acute exercise in WT mice, but not *Nox4-/-* mice. The exact biochemical reaction that promotes the DsRed fluorescent shift is not known, but there is evidence to suggest there is a dehydrogenization reaction at the Tyr-67 residue[57,58], potentially from hydrogen peroxide[57,58]. As to how Nox4 promotes changes in the mitochondrial oxidative environment post-exercise is not understood, but these results may suggest the following: 1) Nox4 localizes to the inner mitochondrial membrane[17] and oxidizes mitochondrial matrix proteins[59] or 2) there is crosstalk between Nox4-ROS and mitochondrial-ROS that promotes this oxidative shift[21] post-exercise. Specifically, there is some evidence that activation of Nox promotes mitochondrial ROS production[60] as targeting the mitochondria with MitoTEMPO results in decreased Nadph oxidase activity[61]. These results could be explained, in part, by Nox4 localization to the mitochondria or an unknown mechanism of cross-talk between Nox4-ROS and mitochondrial-ROS.

While MitoTimer can quantify the mitochondrial oxidative environment, it is also an effective tool for quantifying skeletal muscle mitophagy post-exercise[26,39]. Fragments of oxidized mitochondria that have undergone degradation at the lysosome are visualized as pure red puncta

and are a marker of mitophagy as validated by a co-transfection study of MitoTimer with a transmembrane lysosomal protein, Lamp1, that was conjugated to YFP (Lamp1-YFP). Colocalization of pure red MitoTimer puncta and Lamp1-YFP post-exercise suggests that mitochondrial fragments have fused with the lysosome, and that red puncta are oxidized mitochondria fragments that are present in the lysosome[26]. Thus far, there are at least three possible mechanisms as to how ROS mediated mitophagy: 1) Depolarization of the mitochondrial membrane and subsequent activation of Pink/Parkin mediated mitophagy[62] or 2) there is selective autophagic degradation of mitochondria that are producing ROS (e.g., sources of oxidative stress)[63] or 3) ROS act as second messenger signals[10]. While there is an apparent temporal association between increases in ROS and the induction of mitophagy, the mechanism is yet to be fully unveiled. In the WT, we observed a significant increase in the accumulation of red puncta post-exercise, where this was blunted in the *Nox4-/-*, demonstrating Nox4 is required for skeletal muscle mitophagy post-exercise. Our results resonate with other findings in different cell types that Nox4 is required for autophagy during energy deprivation in cardiomyocytes[45] and in cancer through recruitment of LC3B-II[64]. While Nox4 promotes autophagy, it is unclear if there are distinct and separate signaling pathways for autophagy and mitophagy. Therefore, we cannot conclude that the mechanisms that link Nox4 to autophagy are related to the role of Nox4 in mediating mitophagy that we have documented here.

5'-AMPK-activated protein kinase (AMPK) has been found to mediate skeletal muscle mitophagy post-exercise through phosphorylation of Unc-51 Like Autophagy Activating Kinase (Ulk1)[26]. There is some evidence to suggest ROS can activate AMPK[65]. However, we observed Nox4 is not required for phosphorylation of AMPK in gastrocnemius, suggesting an AMPK independent mechanism is promoting Nox4 mediated mitophagy in skeletal muscle. However, we

cannot exclude the possibility that there is temporal regulation of AMPK or that there are localized pools of AMPK[24] that are activated in response to exercise that cannot be detected by looking at total lysates.

We also assessed phosphorylation of Dynamin related protein 1 (Drp1), a key regulator of mitochondrial fission. While we found a trend for an increase in phosphorylation in the WT after exercise ($p = 0.1$), there were no significant differences between genotypes. Previous studies have verified that Drp1 is phosphorylated during exercise in quadriceps[52] and in plantaris[26], but no studies to our knowledge have assessed activation of Drp1 post-acute exercise in gastrocnemius. Similarly, previous work has demonstrated a significant increase in phosphorylation of Ulk1 is observed in plantaris after acute-exercise[26], but we did not see any significant changes in gastrocnemius. Skeletal muscle metabolic responses to exercise differ based on skeletal muscle fiber type. There are three types of muscle fibers, slow-twitch type I (red; oxidative), fast-twitch type IIb (white; glycolytic), and type IIa (intermediate). Nox4 expression profiles differ between fiber types, as Nox4 is more expressed in red muscle[66]. As Nox4 expression and activity was greater in soleus and red GC compared to white GC, it is possible that in oxidative muscles, the difference in the adaptive metabolic phenotype would be exacerbated in *Nox4-/-* muscle compared to WT.

There are multiple regulatory points for mitophagy, including changes in mitochondrial dynamics (fission/fusion), as damaged mitochondria undergo fission to separate from the rest of the mitochondrial reticulum before degradation[29]. We assessed the expression of genes involved in mitochondrial dynamics in quadriceps, a mixed skeletal muscle composed of red (oxidative) and white (glycolytic) fibers. We found no significant differences in *Mitofusin 1* (*Mfn1*) or *Mitofusin 2* (*Mfn2*) mRNA, genes that encode for a class of outer mitochondrial membrane proteins

that promote mitochondrial fusion (connectivity). We assessed the expression of *Fission 1 (Fis1)*, and found a significant increase in the WT that was blunted in the *Nox4-/-* post-exercise. These data suggest that Nox4 promotes changes in mitochondrial connectivity after exercise which may be important to separate damaged parts of the mitochondria prior to mitophagy. This is supported by other work showing that *Fis1* is regulated at the mRNA level after acute exercise and mitochondrial dynamics machinery is essential for exercise adaptation in skeletal muscle[52]. Taken together, these data demonstrate that Nox4 mediates changes in *Fission-1* post-exercise. Thus, Nox4 may mediate changes in mitochondrial fission - as it is necessary to separate mitochondria from the rest of the reticulum prior to the induction of mitophagy[67].

Multiple studies have identified Pink/Parkin as mediators of ROS-induced mitophagy[68,69]. Many *in vitro* studies exploring ROS mediated mitophagy use cyanide m-chlorophenylhydrazone (CCCP), a mitochondrial depolarizing agent[62] and induces the accumulation of PTEN-putative kinase (PINK1) to the outer mitochondrial membrane of depolarized mitochondrial which subsequently recruits Parkin, an E3 ubiquitin ligase[70]. However, current studies suggest skeletal muscle exercise-mediated mitophagy is independent of Pink/Parkin as exercise induced mitophagy occurs in the absence of Pink1 translocation to the mitochondria[71]. Due to these observations, we did not investigate Pink/Parkin signaling here. Moreover, we cannot discount the role of Nox4 mediating Pink/Parkin in other conditions of energetic stress.

Here we demonstrate an essential role for Nox4 in mediating mitophagy in skeletal muscle after exercise, suggesting that Nox4 impacts mitochondrial dynamics signaling prior to mitophagy. Nox4 is a prominent producer of hydrogen peroxide (H_2O_2) in skeletal muscle[23]. Multiple studies have highlighted the effectiveness of both H_2O_2 as a signaling agent[10] and the role of Nox4 in activating second messenger signals, including Nrf2[19,56], FoxO[72,73], NF-κB[74,75], and MAPK[76].

Nox4 is required for Nrf2 activation during exercise in both the heart[56] and skeletal muscle[19]. The nuclear factor erythroid 2 like 2 (Nrf2) is a transcription factor that induces the antioxidant response as it is a master regulator of the cellular redox environment[77]. Interestingly, we found no differences in *Nrf1* or *Nrf2* mRNA expression in skeletal muscle between the *Nox4-/-* and WT mice after exercise (**Supplementary Fig. 1A-B**). The forkhead family of transcriptional regulators are involved in numerous in metabolic pathways. While FoxO1, FoxO3, FoxO4 and FoxO6 are all expressed in skeletal muscle, FoxO1 and FoxO3 mediate mitochondrial metabolism and energy homeostasis[78]. FoxO1 transcriptionally regulates a class of autophagy genes in skeletal muscle[79–82]. We found no differences in *FoxO1* between *Nox4-/-* and WT mice at the transcriptional level (**Supplementary Fig. 1C**). Of the major metabolic transcription factors we assessed, there were no significant differences from skeletal muscle of *Nox4-/-* and WT mice. A recent large-scale analysis of in vivo oxidation in yeast demonstrated that cytosolic translational machinery has a high potential to be regulated by ROS[83], suggesting that ROS may have a greater role in regulating protein translation. Further studies will need to distinguish where Nox4-ROS assists in the synthesis or recruitment of mitophagy machinery in skeletal muscle after exercise.

Our findings are consistent with the concept that ROS are necessary for mitochondrial homeostasis, particularly during exercise[12]. Most notably, Nox4 drives mitochondrial oxidation, an important signaling cascade that promotes mitochondrial quality in skeletal muscle after exercise. This resonates with other findings that note that Nox4 drives mitochondrial oxidative stress in the heart[84].

Much of the work on ROS-mediated mitophagy is in its nascence. While it is clear that mitophagy occurs after periods of oxidative stress, it is unknown whether oxidized mitochondria are preferentially subjected to mitophagy. Our novel observations have shed light on a complex

relationship between Nox4-ROS in mediating the mitochondrial oxidative environment and the subsequent initiation of mitophagy which may be essential for the mitochondrial metabolic adaptations we previously observed[18]. Our results have elucidated an important Nox4-mediated concept, but further work is needed to explore how ROS initiates mitophagy and how this links to mitochondrial metabolism. Future studies should investigate whether Nox4-ROS are the source for mitochondrial oxidation, or whether there is cross-talk between Nox4-ROS and mitochondrial-ROS that promotes this post-exercise oxidative shift and how this leads to the induction of mitophagy.

Abbreviations

H_2O_2	Hydrogen peroxide
Nox4	Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4
Nox4-/-	Global Nox4 deletion
WT	Wild-type
ROS	Reactive oxygen species
Mfn1	Mitofusin 1
Mfn2	Mitofusin 2
Fis1	Fission 1
Opa1	Mitochondrial dynamin like GTPase
Nrf2	Nuclear factor erythroid 2 like 2
Atg7	Autophagy related 7
Drp1	Dynamin related protein 1
AMPK	5'-AMPK-activated protein kinase
Ulk1	Unc-51 Like Autophagy Activating Kinase
Lamp1	Lysosomal associated membrane protein 1
CytC	Cytochrome C

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Chapter 6: Conclusion

Delayed or inefficient skeletal muscle mitochondrial energy production will advance disease pathology[1,2] for many skeletal muscle-related diseases (e.g., sarcopenia[3] or diabetes[4]). With consistent exercise training, patients with skeletal muscle-related diseases see improvements in their mitochondrial function[5], leading to improved health outcomes and disease prognosis[6]. As the impact of mitochondrial metabolism becomes a key target for disease prognosis and treatment, it is imperative to explore the underlying cellular mechanisms that promote these beneficial adaptive processes.

In these studies, we investigated the role of Nox4 in promoting physiological adaptations to exercise. We have identified novel metabolic targets downstream of Nox4 utilizing innovative genetic models and approaches. However, there is much to be unveiled about how Nox4-ROS interacts with mitochondrial metabolism at the transcriptional, translational, and bioenergetic levels.

Chapter 4 defined metabolic targets downstream of H_2O_2 and Nox4 in skeletal muscle after exercise. In particular, we identify novel mRNA and protein targets important in mediating glucose and fatty acid oxidation changes. At the onset of exercise, there is an increased energy demand. There is strategic coordination of multiple catabolic processes to meet this increased energy demand. These biochemical processes include fatty acid oxidation, glycolysis, mitochondrial oxidative phosphorylation (OXPHOS), and tricarboxylic acid cycle (TCA) activity. Here we demonstrate that Nox4 is essential for mediating post-exercise metabolic responses at different time points. Immediately following the cessation of an acute exercise bout, Nox4 is required for changes in metabolic gene expression. Interestingly, three hours after an acute exercise bout, Nox4 is required for glucose and fatty acid oxidation. While Nox4 seems to

be essential for physiological responses to acute exercise, this begs the question as to whether Nox4 is required for chronic adaptations to exercise. Here, we subjected global *Nox4-/-* and WT mice to a chronic exercise protocol and found that *Nox4-/-* mice had blunted adaptations to exercise. Taken together, Nox4 is required for adaptive physiological adaptation to exercise – ranging from the immediate cessation of an acute exercise bout to promoting chronic adaptation after weeks of consistent training.

In addition to the acute exercise studies in the global knockout, we asked whether endothelial Nox4 is required for skeletal muscle metabolic responses to exercise. Nox4 is expressed in both endothelial cells and myocytes. Thus, we deleted Nox4 from the endothelial ($Nox4^{ECKO}$). We observed a profound metabolic phenotype where mice lacking endothelial Nox4 had similar blunted metabolic responses to exercise as the global *Nox4-/-* mice. These data further highlight that ROS may act as intercellular signaling agents where endothelial Nox4 is required for skeletal muscle substrate oxidation post-acute exercise[7].

Mitochondrial metabolism is a key component of skeletal muscle function and health[8]. Mitochondrial quality includes mitophagy, the selective degradation of mitochondria, mitochondrial dynamics (fission/fusion), and mitochondrial biogenesis (the renewal of mitochondria)[9]. Several studies have identified a correlative relationship between ROS and mitophagy[10,11]. There is increased oxidative stress (via ROS signaling) and subsequent autophagy during conditions of energetic stress[12]. However, many questions remain as to how Nox4 may be promoting mitochondrial metabolism in response to exercise. Our published work has identified *mitochondrial oxidative stress* as a top differentially regulated pathway between WT and *Nox4-/-* mice[7]. This led us to the hypothesis that Nox4 mediates the mitochondrial oxidative environment in response to exercise. Thus, to assess the mitochondrial oxidative

environment and skeletal muscle mitophagy, we utilized a novel mitochondrial-targeted plasmid, *pMitoTimer. pMitoTimer* is a mitochondrial-targeted plasmid conjugated to a DsRed sequence that undergoes a fluorescent shift from green to red when there is a change in the oxidative environment. Here, we defined a novel role for Nox4 in mediating mitochondrial adaptations to exercise and shed insight into the role of ROS-mediated mitophagy.

Exercise remains an effective intervention for dozens of skeletal muscle and metabolic-related diseases. Many of these studies cannot be addressed in human or clinical studies. Defining the mechanistic role of reactive oxygen species in skeletal muscle metabolic adaptation is imperative for identifying novel therapeutic targets. While the paradigm has begun to shift, and it has become increasingly apparent that ROS have a physiological role in mediating adaptation, there are still many unanswered questions about their downstream targets. The impact of identifying ROS-mediated signaling pathways could culminate in improving and informing new treatment strategies for patients with skeletal muscle-related diseases.

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