Doctorate in Biomedical Sciences
Graduate School
Faculty of Medicine
University of Chile.

NADPH OXIDASE 2 - A NOVEL REGULATOR OF MOLECULAR RESPONSES TO EXERCISE.

Thesis to obtain the Doctoral degree in Biomedical Sciences at the University of Chile.

Carlos Henríquez Olguín

Supervisors:
Enrique Jaimovich
Thomas E. Jensen

July 2018

UNIVERSIDAD DE CHILE
FACULTAD DE MEDICINA
ESCUELA DE POSTGRADO

INFORME DE APROBACION TESIS DE DOCTORADO EN CIENCIAS BIOMEDICAS

Se informa a la Comisión de Grados Académicos de la Facultad de Medicina, que la Tesis de Doctorado en Ciencias Biomédicas presentada por el candidato

Carlos Henríquez Olguín

ha sido aprobada por la Comisión Informante de Tesis como requisito para optar al Grado de **Doctor en Ciencias Biomédicas** en Examen de Defensa de Tesis rendido el día ……. de ……. de ……..

Prof. Enrique Jaimovich
Director de Tesis
Programa de Biología Molecular y Celular, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile

COMISION INFORMANTE DE TESIS

Dr. Mario Chiong ***Dr. José Galgani***

Dr. Hugo Olguín **Dr. Manuel estrada**
Presidente Comisión de Examen

Table of contents

TABLE OF CONTENTS **3**

ACKNOWLEDGEMENTS **5**

LIST OF MANUSCRIPTS **6**

ABSTRACT **7**

RESUMEN **8**

CHAPTER 1: LITERATURE BACKGROUND **9**

1.1 INTRODUCTION 9

1.2 RESEARCH QUESTION AND WORKING HYPOTHESIS 11

CHAPTER 2: IMPORTANCE OF REDOX SIGNALING IN SKELETAL MUSCLE – AN OVERVIEW **12**

2.1 REDOX SIGNALING IN MUSCLE 12

2.2 ROS PRODUCTION DURING MUSCULAR ACTIVITY 13

2.3 MITOCHONDRIA AS A ROS SOURCE IN SKELETAL MUSCLE 14

2.4 NOX EXPRESSION IN SKELETAL MUSCLE. 14

CHAPTER 3: EXERCISE-STIMULATED ROS PRODUCTION: ROLE OF NOX2. ..... **16**

CHAPTER 4. ROLE OF NOX2 IN EXERCISE SIGNALING **19**

4.1 MITOGEN-ACTIVATED PROTEIN KINASES (MAPKS) ACTIVATION 19

4.2 GENE EXPRESSION OF ANTIOXIDANT DEFENSE PROTEINS 20

4.3 MITOCHONDRIAL BIOGENESIS 20

4.4 REDOX CONTROL OF MYOKINE EXPRESSION. 21

5. CHAPTER FIVE: NOX2 IS A NOVEL REGULATOR OF EXERCISE-STIMULATED GLUCOSE UPTAKE. **23**

5.1 REGULATION OF EXERCISE-STIMULATED GLUCOSE UPTAKE 23

5.2 ROLE OF ROS IN REGULATING GLUCOSE TRANSPORT IN SKELETAL MUSCLE 23

5.5 WHICH DOWNSTREAM MECHANISMS MIGHT MEDIATE NOX2-DEPENDENT GLUCOSE UPTAKE? 24

6. CHAPTER SIX: METHODOLOGICAL CONSIDERATIONS **25**

6.1 APOCYNIN AS A NOX2 INHIBITOR. 25

6.2 ASSESSING ROS PRODUCTION DURING *IN VIVO* EXERCISE 25

6.2.1 Generically-encoded redox biosensors. *26*
6.2.2 In vivo redox histology *26*
6.3 GLUT4 TRANSLOCATION 28
7. CHAPTER SEVEN: CONCLUSIONS AND PERSPECTIVES 29
7.1 CONCLUSIONS 29
7.2 FUTURE PERSPECTIVES 29
8. LIST OF REFERENCES. 30
9. MANUSCRIPTS 37

Acknowledgements

The current thesis is based on studies performed in the Section of Molecular Physiology at Department of Nutrition, Exercise and Sport at University of Copenhagen and the Laboratory of Cellular Muscle Physiology at University of Chile in the period from 2014 to 2018.

I wish to thank my supervisor associate professor Thomas E. Jensen for being an inspiring scientific advisor and mentor, and for his huge support and patience. I would also like to thank professor Enrique Jaimovich for the past 8 years being my supervisor and for allowing me to study the exercise from a molecular perspective.

I would like to thank all members of the Section of Molecular Physiology for making the section a fantastic place to learn, work hard, and have fun. In particular, I would like to acknowledge:

-The senior PIs Bente Kiens, Jørgen F.P. Wojtaszewski, and Erik A. Richter for inspiring me to pursue a scientific career, create a world-class scientific environment and for nice scientific discussions.

-Jonas RK, Cheng, and Agnete for their huge contribution to my experiments, for allowing me to help with and learn from their experiments. Also, I really appreciated the nice scientific and non-scientific discussion during the long hours in the lab.

-Steffen and Lykke for helping me with the experiments and their great intellectual contributions to the study II.

-Betina, Nicoline, Irene and Jesper for their really skilled technical assistance and for always making the lab a nice place to work.

-The former members of EJ's lab for their huge contribution to study I.

Last but not least, I owe a huge and loving gratitude to my family and friends. In particular, I wish to thank my wife Pía for her unconditional love, patience, and understanding during this long and difficult process.

List of Manuscripts

The current PhD thesis includes the following manuscripts, located at the back of the thesis.

Study 1

Carlos Henríquez-Olguín, Alexis Díaz-Vegas, Yirldy Utreras-Mendoza, Cristian Campos, Manuel Arias-Calderón, Paola Llanos, Ariel Contreras-Ferrat, Alejandra Espinosa, Francisco Altamirano, Enrique Jaimovich, Denise Valladares. NOX2 Inhibition Impairs Early Muscle Gene Expression Induced by a Single Exercise Bout. Front Physiol. 2016 Jul 14;7:282.

Study 2

Carlos Henríquez-Olguín, Jonas R. Knudsen, Steffen H. Raun, Zhencheng Li, Lykke Sylow, Emilie Dalbram, Jonas T. Treebak, Erik A. Richter, Enrique Jaimovich, and Thomas E. Jensen. Exercise-stimulated reactive oxygen species by NOX2 is a major regulator of muscle glucose uptake. Manuscript in preparation for Nature Communications.

Abstract

Physical activity plays a protective role in the development of chronic non-communicable diseases. Molecular adaptations explain the beneficial effects of exercise in diverse tissues such as skeletal muscle, adipose tissue, and heart. One of the multiple signals involved in the benefits of exercise are the oxidation-reduction reactions called *redox* signaling. Reversible and non-reversible posttranslational modifications of cysteine residues are capable of changing the function, localization, or stability of diverse proteins. In skeletal muscle, reactive oxygen species (ROS) are continuously produced and cleared during resting and contracting conditions. There is substantial evidence indicating that *redox* signaling plays a role in some of the health-benefits elicited by endurance training, however, the precise mechanism has been long unknown.

The aim of the current Ph.D. thesis was therefore to study the involvement of NOX2 and redox signals in the regulation of exercise-stimulated glucose transport and adaptive gene expression in mature skeletal muscle. A combination of pharmacological inhibitors and murine NOX2-deficient models were used to address the necessity of NOX2 for glucose transport and adaptive signals induced by acute exercise.

The current Ph.D. thesis demonstrated for the first time that NOX2 is activated during moderate-intensity endurance exercise in skeletal muscle and it is a major source of ROS under those conditions. Furthermore, the analyses of genetic mouse models lacking the regulatory NOX2 subunits p47phox and Rac1 revealed striking phenotypic similarities, including severely impaired exercise-stimulated glucose uptake and GLUT4 translocation, indicating that NOX2 is a requirement for this classic acute myocellular adaptation to exercise.

Overall, NOX2 is thus a major ROS source regulating adaptive responses to exercise in skeletal muscle.

Resumen

La actividad física juega un papel protector en el desarrollo de enfermedades crónicas no transmisibles. Las respuestas moleculares explican los efectos beneficiosos del ejercicio en diversos tejidos como el músculo esquelético, el tejido adiposo y el corazón. Una de las múltiples señales involucradas en los beneficios del ejercicio son las reacciones de oxidación-reducción llamadas señalización redox. Las modificaciones postraduccionales reversibles e irreversibles de residuos de cisteína son capaces de cambiar la función, localización o estabilidad de diversas proteínas. En el músculo esquelético, las especies de oxígeno reactivo (ROS) se producen y eliminan continuamente durante las condiciones de reposo y contracción. Existe evidencia sustancial que indica que la señalización redox juega un papel en algunos de los beneficios para la salud provocados por el entrenamiento de resistencia, sin embargo, el mecanismo preciso ha sido desconocido durante mucho tiempo.

El objetivo de la presente la tesis fue estudiar la participación de NOX2 en la regulación del transporte de glucosa durante el ejercicio y la expresión de genes adaptativos en el músculo esquelético adulto. Se utilizó una combinación de inhibidores farmacológicos y modelos deficientes en NOX2 ratón para abordar la necesidad de NOX2 para el transporte de glucosa y las señales adaptativas inducidas por el ejercicio agudo.

Esta tesis demostró por primera vez que el NOX2 se activa durante el ejercicio de resistencia de intensidad moderada en el músculo esquelético y es una fuente importante de ROS en esa condición. Además, los análisis de modelos de ratones genéticos que carecen de las subunidades reguladoras NOX2 p47phox y Rac1 revelaron sorprendentes similitudes fenotípicas, incluida la captación de glucosa estimulada por el ejercicio y la translocación de GLUT4, lo que indica que NOX2 necesaria para esta respuesta fisiológica durante el ejercicio.

En resumen, NOX2 es, por lo tanto, una importante fuente de ROS que regula las respuestas de adaptación al ejercicio en el músculo esquelético.

Chapter 1: Literature Background

1.1 Introduction

Physical inactivity is associated with increased morbidity and all-cause mortality. A sedentary lifestyle and obesity pose a substantial social and economic burden worldwide (Ding *et al.* 2016, Tremmel *et al.* 2017). Regular exercise impacts dramatically on the function of many tissues affecting whole-body metabolic homeostasis.

Skeletal muscle is particularly important for explaining the metabolic benefits of the exercise. Literally thousands of post-translational intracellular signaling events are altered by a single bout of exercise in human skeletal muscle (Hoffman *et al.* 2015), acutely controlling *e.g*. metabolism, ion fluxes, protein trafficking, mRNA transcription and protein translation. The integration of successive acute molecular exercise bouts is furthermore translated into the chronic phenotypic changes produced by long-term training (Perry *et al.* 2010).

Reactive oxygen species (ROS) are a diverse group of chemically reactive chemical species containing oxygen able to modify biological molecules (Espinosa *et al.* 2016). In skeletal muscle ROS are generated in several subcellular compartments under both resting and contracting conditions (Jackson *et al.* 2016). The increase of ROS production during exercise has traditionally been viewed as a by-product of the increased oxidative metabolism in mitochondria (Lawler *et al.* 2016). Alternatively, other ROS-generating enzymes such as xanthine oxidase or NADPH oxidase (NOX) have been proposed to contribute to oxidant generation during high-intensity exercise. Despite the increased attention on *redox* signaling in skeletal muscle during the last 20 years (Reid 2016), the main ROS source during exercise has remained uncertain.

Dietary ROS scavengers disrupt acute exercise-stimulated signaling in humans (Paulsen *et al.* 2014) and murine (Gomez-Cabrera *et al.* 2008) skeletal muscle. Indeed, the long-term health benefits induced by regular training are blunted by exogenous antioxidants in untrained and trained subjects (Ristow *et al.* 2009). However, the molecular mechanisms involved in ROS-mediated muscle adaptation in exercising context are not well understood.

Exercise stimulates glucose uptake via translocation of Glucose transporter 4 (GLUT4) translocation from intracellular stores to the muscle cell-surface by an insulin-independent mechanism (Richter and Hargreaves 2013). Exogenous and endogenous ROS enhance glucose transport in skeletal muscle (Cartee and Holloszy 1990, Higaki *et al.* 2008). Interestingly, ROS scavengers reduce *ex vivo* glucose transport stimulated by contraction (Sandstrom *et al.* 2006) and passive stretch (Chambers *et al.*

2009), suggesting that ROS are both sufficient and necessary for changes in glucose transport in response to muscle contraction.

The aim of the current introduction is to review the literature and discuss the knowledge gaps and research questions investigated in the studies. Selected data from study I and study II as well as additional unpublished data not included in the manuscripts will be introduced where appropriate and discussed in the context of relevant literature. Lastly, the introduction will briefly touch upon methodological considerations associated with Study I and Study II, followed by concluding remarks and perspectives for future research.

1.2 Research Question and Working hypothesis

The overall aim this PhD thesis was to answer the following research question:

Does exercise activate NOX2 and control redox-dependent biological processes in skeletal muscle?

Aim study 1: To investigate the role of NOX2 in exercise-stimulated ROS production and adaptive changes in gene expression induced by endurance exercise in murine muscle.

Hypothesis: I hypothesized that pharmacological NOX2 inhibition would blunt the upregulation of genes related to exercise training adaptation.

Aim study 2: To determine the need of exercise-stimulated NOX2 activation in regulating glucose uptake in murine skeletal muscle.

Hypothesis: I hypothesized that NOX2 would be necessary for exercise-stimulated glucose uptake by regulating GLUT4 translocation in skeletal muscle.

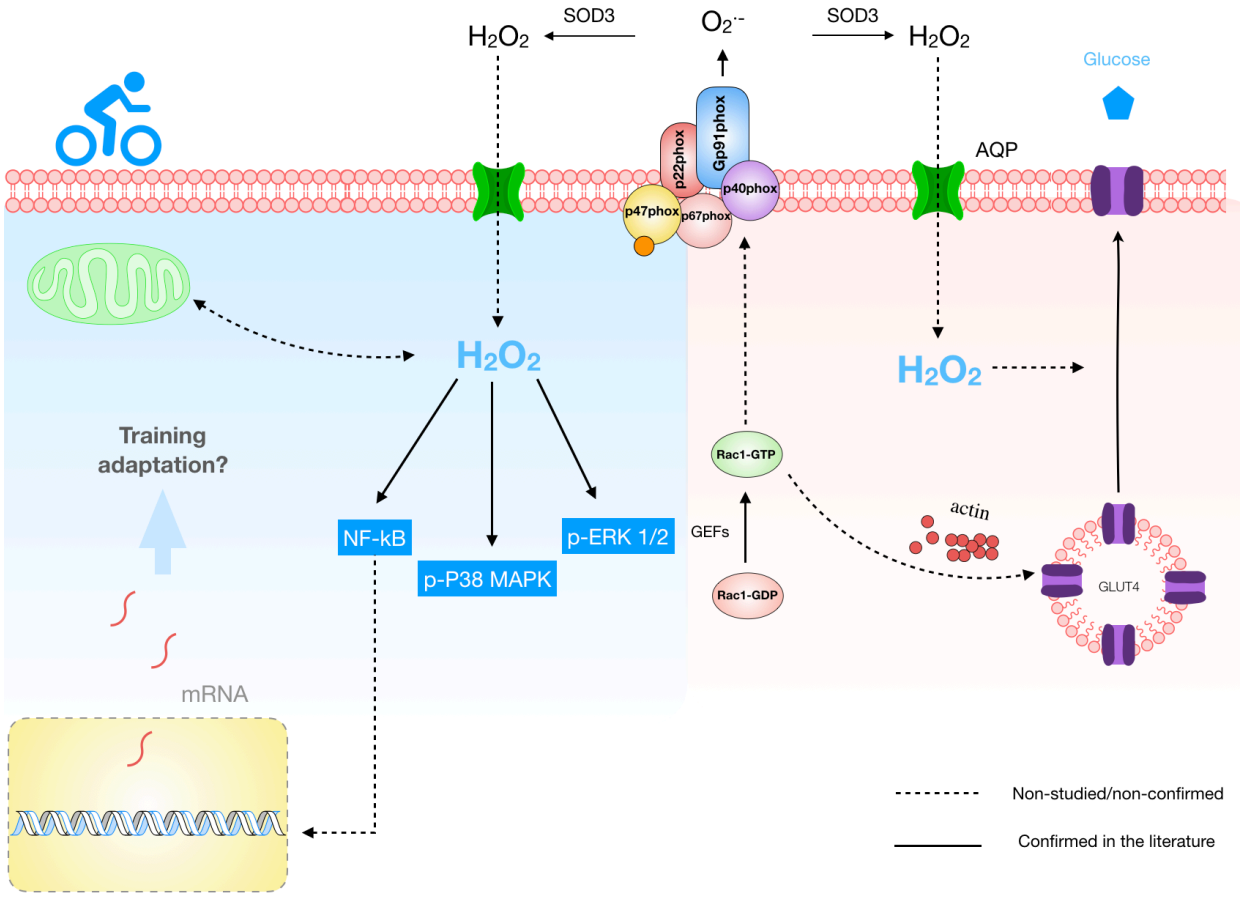

Figure 1. Schematic illustration of the working hypothesis addressed in the current PhD thesis. The activation of NOX2 by acute exercise leads to glucose uptake and post-exercise upregulation of adaptive genes. AQP: Aquaporin, SOD3: Superoxide dismutase 3, NF-κB: Nuclear transcription factor kappa B, GEFs: Guanine nucleotide exchange factors.

Chapter 2: Importance of redox signaling in skeletal muscle – an overview

2.1 Redox signaling in muscle

ROS are part of a complex system of signal transduction involved in a large number of functions in cellular physiology (Sies 2017). Reversible and non-reversible posttranslational modifications of cysteine residues are capable of changing function, localization, or stability of diverse proteins (Azzi *et al.* 2004). Collectively, this signal transduction mechanism is called redox signaling and is emerging as a key regulator of cellular biology in many tissues including skeletal muscle.

The first evidence describing the presence of free oxygen radicals in skeletal muscle was reported in 1954 (Commoner *et al.* 1954). After more than 50 years of research, a complex and dynamic oxidant and antioxidant machinery has now been described in skeletal muscle. For a detailed description of the antioxidant system and ROS production sites, the reader is referred to previous excellent reviews (Powers and Jackson 2008, Powers *et al.* 2011).

Changes in the redox environment of the myofibers dramatically affects the metabolism and function under a diverse set of physiological and pathophysiological conditions. Thus, altered redox signaling has been implicated in several muscle dysfunctions such as Duchenne muscular dystrophy (Henriquez-Olguin *et al.* 2015), insulin resistance (Furukawa *et al.* 2004), and sarcopenia (Kelley *et al.* 2018). ROS has also been associated with normal physiological muscle remodeling in response to environmental factors such hypoxia, caloric restriction and exercise (Gomez-Cabrera *et al.* 2008).

A well-accepted conceptual framework for understanding the paradoxical dual role of ROS as both beneficial and detrimental to cellular function is called hormesis. The hormesis-concept posits a biphasic dose-response to an environmental agent characterized by a low dose eliciting beneficial effects and a high dose being toxic (Mattson 2008). A described example of hormesis in skeletal muscle is the redox control of force production, where modest increases of ROS enhance force generation and this force generation is blunted by ROS scavengers. Conversely, the opposite effect is observed in response to supraphysiological levels of ROS, where force production is decreased (Reid *et al.* 1993).

Despite the great attention that redox signaling in skeletal muscle has received, there are still substantial literature gaps in the understanding of ROS sources, redox targets, propagation of the signal and biological endpoints.

2.2 ROS production during muscular activity

The first evidence suggesting increments of ROS during exercise was obtained by measuring increased lipid peroxidation by-products in expired air from exercising human subjects (Dillard *et al.* 1978). In a subsequent study, Davis and colleagues demonstrated an increase in free radicals elicited by running using electron spin resonance spectroscopy in rat muscle lysates (Davies *et al.* 1982). It is now widely accepted that myofibers can generate ROS at several cellular locations, some of which could increase their production during muscle contraction.

The primary source of ROS during *in vivo* exercise has been a topic of controversy during the last years (Goncalves *et al.* 2015, Powers *et al.* 2016, Wong *et al.* 2017). Technical shortcomings in measuring ROS production during exercise has been the main limitation (Jackson 2016). Biochemical analysis of oxidative markers in whole-muscle lysates has traditionally been used to study changes in ROS generation in response to exercise (Jackson 2016). However, mature myofibers are highly compartmentalized and densely packed cells (Prats *et al.* 2011). Therefore, the global redox status of cells and tissues may not reflect the changes in subcellular location-specific redox modifications which elicit a given cellular adaptation.

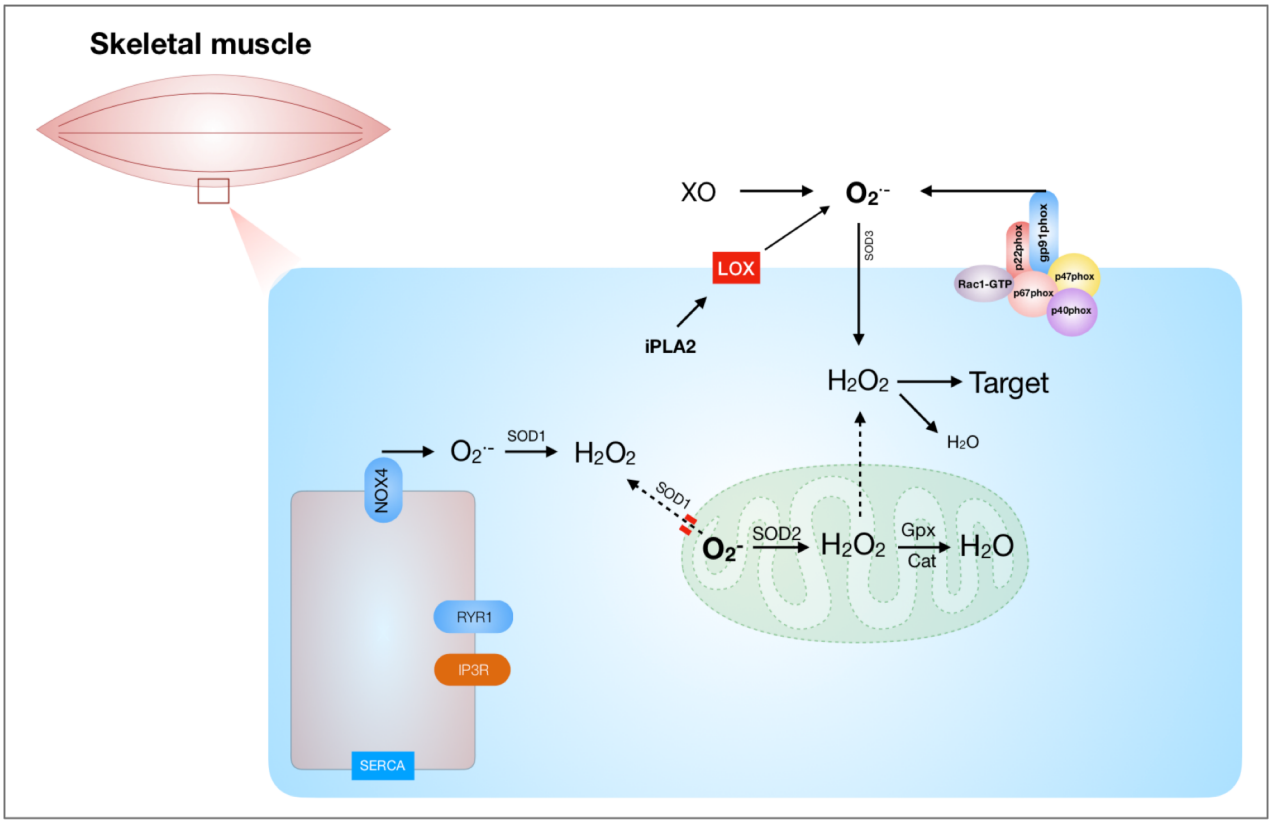

Figure 2. Illustration of potential ROS sources in skeletal muscle. Superoxide anion (O2) is produced by NOX2, NOX4, xanthine oxidase (XO), and phospholipase A-regulated lipoxygenases (PLA). RyR: ryanodine receptor, IP3R: Inositol trisphosphate receptor, SOD1-3: Superoxide dismutase 1-3, Gpx: Glutathione peroxidase, Cat: catalase.

Elucidation of the mechanism governing ROS production in skeletal muscle under resting and exercising conditions could significantly improve our understanding of oxidative stress-related diseases as well as optimization of muscle performance in athletes.

2.3 Mitochondria as a ROS source in skeletal muscle

Over 40 years ago, it was reported that mitochondria can produce ROS (Jensen 1966, Loschen *et al.* 1971). Since then, a substantial amount of research has been done to elucidate the role of mitochondrial ROS in cell biology (Friedman and Nunnari 2014). Electron transport chain complex I and complex III are believed to be the major sites of mitochondrial superoxide production (Munro and Treberg 2017).

Since oxygen consumption is increased during exercise, several authors have assumed that ROS production during exercise is a consequence of the elevated oxygen consumption to meet the increased myocellular energy demand (Koren *et al.* 1983, Kanter 1998, Katz 2016). Early reports estimated superoxide production to account for 1-2% of consumed oxygen by isolated mitochondria under particular experimental conditions (Boveris and Chance 1973). In contrast, more recent studies with improved techniques have shown that only approximately 0.12-0.15% of mitochondrial respiration is converted to H_2O_2 (St-Pierre *et al.* 2002, Kudin *et al.* 2004, Tahara *et al.* 2009).

In mature skeletal muscle, a few studies have addressed the changes in mitochondrial ROS in response to increased muscular activity. Michaelson *et al.* studied the changes in mitochondria redox potential using a mitochondria-targeted redox-sensitive GFP (roGFP) in cultured single muscle fibers (Michaelson *et al.* 2010). After fifteen minutes of repeated tetanic stimulation of cultured single FDB fibers, there was no measurable change in mitochondrial ROS production. Similar results were reported using the superoxide-specific MitoSox probe (Aydin *et al.* 2009, Sakellariou *et al.* 2013). Moreover, a recent study reported no changes in 4-hydroxynonenal levels in mitochondrial fractions immediately after exercise but an increase after 3 and 6 hours post-exercise (Laker *et al.* 2017). These studies clearly suggest, at least under *in vitro* and *ex vivo* conditions, that mitochondria are not the primary source of ROS during muscle contraction.

2.4 NOX expression in skeletal muscle.

In mature murine skeletal muscle, NOX2, NOX4, DUOX1 mRNA are expressed whereas data on the expression of DUOX2 are conflicting (Sun *et al.* 2011, Loureiro *et al.* 2016). NOX2 and NOX4 proteins exhibit a fiber type-dependent mRNA expression, being greater in slow-twitch oxidative compared to fast-twitch glycolytic muscles (Loureiro *et al.* 2016). At the protein level, the expression

of all NOX2 subunits and NOX4 has been confirmed by immunoblotting in isolated mouse FDB muscle fibers (Sakellariou *et al.* 2013). In human muscle biopsies, p67phox (Nyberg *et al.* 2014) and Rac1 (Sylow *et al.* 2013) protein expression were detected by immunoblotting, suggesting conserved expressed of NOX2 in human muscle but this requires further investigation.

At the subcellular level, NOX2 in phagocytes is known to reside in phagosomes, secretory vesicles, specific granules, and the surface membrane. Upon activation, NOX2 translocates to the plasma membrane via exocytosis to produce ROS extracellularly and inside endocytosed phagosomes (Nguyen *et al.* 2017). In skeletal muscle, NOX2 proteins have been reported to be predominantly localized to T-tubules based on immunofluorescence microscopy and biochemical fractionation studies (Hidalgo *et al.* 2006, Barrientos *et al.* 2015) but also close to or within the sarcolemma for gp91phox, p67phox, p47phox and p22phox in transverse cryosections (Javeshghani *et al.* 2002, Sakellariou *et al.* 2013). p40phox has been shown by immunofluorescence microscopy of single mouse FDB fibers to translocate to the sarcolemma whereas no movement of p67phox could be detected (Sakellariou *et al.* 2013). Whether this reflects a bona-fide non-canonical movement pattern of some NOX2 regulatory subunits in skeletal muscle or a limitation of confocal microscopy to resolve movement in the molecularly packed muscle fiber is currently unknown. NOX4 was found in the surface membranes and in mitochondrial fractions (Sakellariou *et al.* 2013) co-localizing with RyR1 in SR-enriched fractions and by immunofluorescence microscopy (Sun *et al.* 2011).

In summary, in murine muscle, gp91phox and p22phox subunits are expressed in the skeletal muscle membrane and t-tubules system, while regulatory subunits are expressed in cytosolic compartments. The expression and activation patterns of NOX2 in human muscle require further investigation.

Chapter 3: Exercise-stimulated ROS production: role of NOX2.

Several studies have addressed the question of whether NOX isoforms play a role in contraction-stimulated ROS production (Table 1). In rat myotubes, diphenyleneiodonium (DPI), a non-specific NOX2 inhibitor, decreased superoxide production elicited by electrical stimulation by ~50% (Pattwell *et al.* 2004). Espinosa *et al.* demonstrated that apocynin markedly reduced the oxidation of the ROS-sensitive fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate DCFH-DA in electrically stimulated rat myotubes (Espinosa *et al.* 2006). More recently, studies using the NOX2 competitive inhibitor gp91ds-TAT have corroborated these finding (Pal *et al.* 2013, Sakellariou *et al.* 2013, Diaz-Vegas *et al.* 2015, Henriquez-Olguin *et al.* 2015)

Liu *et al*. (Liu *et al.* 2012) reported that electrical field stimulation increased DCFH-DA oxidation in WT fibers but not in gp91phox-deficient fibers. To further characterize the activation of NOX2 in skeletal muscle, George G. Rodney's group developed a biosensor to measure NOX2 activity in living cells by fusing p47phox to the ROS-sensitive roGFP protein (Pal *et al.* 2013). Interestingly, p47phox-roGFP oxidation was increased by muscle contraction in WT muscles but not in the gp91phox-deficient fibers (Pal *et al.* 2013).

Muscle model	**Stimulation model**	**Inhibition approach**	**Inhibition effect**	**Reference**
Rat myotubes	Field ES	DPI	↓ Cytochrome c. reduction	(Pattwell *et al.* 2004)
Rat myotubes	Field ES	Apocynin	↓ DCFH-DA oxidation	(Espinosa *et al.* 2006)
FDB fibers	Field ES	DPI	↓ DCFH-DA oxidation	(Michaelson *et al.* 2010)
FDB fibers	Field ES	DPI Apocynin gp91ds-TAT	↓ DHE Oxidation	(Sakellariou *et al.* 2013)
FDB fibers	Field ES Stretch	gp91ds-TAT gp91phox KO P47phox KO	↓ DHE Oxidation ↓ Oxidation p47-roGFP	(Pal *et al.* 2013)
FDB fibers	Field ES	NOX2 KO mice.	↓ DCFH-DA oxidation	(Liu *et al.* 2012)

Mouse myotubes	Field ES	gp91ds-TAT Apocynin	↓ DCFH-DA oxidation	(Henriquez-Olguin *et al.* 2015)
FDB fibers	Field ES	gp91ds-TAT Apocynin	↓ DCFH-DA oxidation	(Diaz-Vegas *et al.* 2015)

Table 1. Studies addressing the contribution of NOX2 in ROS production in skeletal muscle. FDB: flexor digitorum brevis, ES: electrical stimulation, DPI: Diphenyleneiodonium, DHE: Dihydroethidium, DCFH-DA: 2',7'-dichlorodihydrofluorescein diacetate.

The evidence presented above clearly showed that NOX2 is required for contraction and stretch-stimulated ROS production skeletal muscle. However, *ex vivo* models have previously been shown in the glucose uptake-field to have a limited predictive value when it comes to in vivo moderate intensity exercise (Jensen *et al.* 2014, Sylow *et al.* 2015, Sylow *et al.* 2017b). My current studies have therefore focused on the in vivo condition. Specifically, **study I** demonstrated using a proximity ligation assay that endurance exercise increased the p47phox interaction with gp91phox in FDB muscle compared to rest, suggesting NOX2 activation. These results were corroborated by **study II**, where oxidation of the p47roGFP biosensor was upregulated by endurance exercise in WT but not in Rac1-deficient tibialis anterior muscles. Additionally, DCFH-DA oxidation induced by exercise was prevented in muscles lacking functional p47phox protein (ncf1*) (**Study II**). Together, these results strongly suggest that NOX2 is activated and necessary for ROS production during endurance exercise in skeletal muscle.

I further characterized the exercise-stimulated NOX2 activation during the post-exercise period in tibialis anterior muscle (unpublished data). As shown in figure 3, the p47roGFP sensor was oxidized immediately after treadmill exercise but not at 1 or 4 hours post-exercise. This suggests that exercise transiently activates NOX2 and that the effect is rapidly reversed, likely by NOX2 inactivation and/or the action of the antioxidant system.

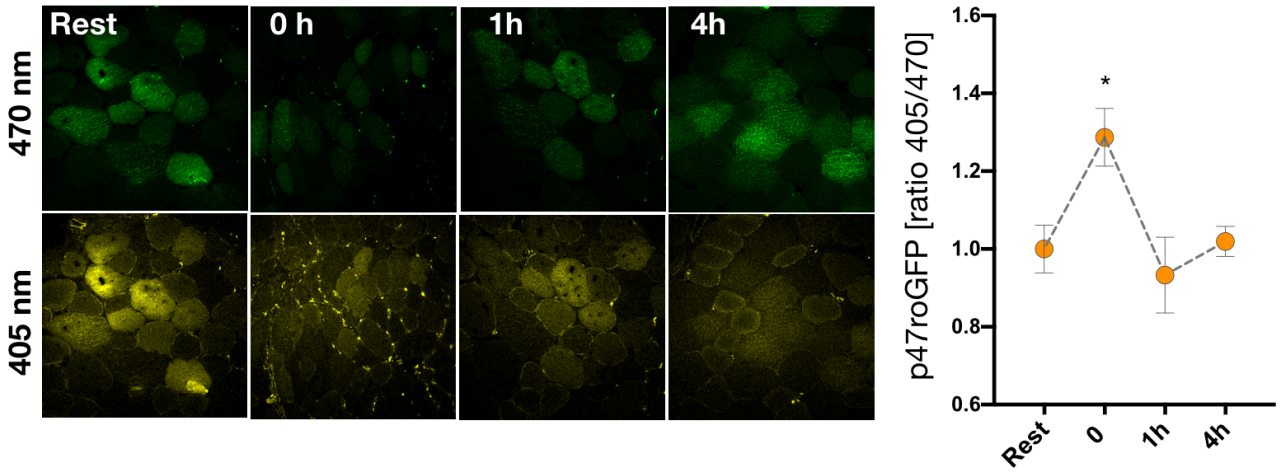

Figure 3. Exercise-stimulated ROS production by NOX2 is transiently upregulated in skeletal muscle. A p47roGFP biosensor was transfected *in vivo* by electroporation of tibialis anterior muscle. Mice performed a high-intensity interval running exercise bout for 1 hour with tissue dissection at the indicated times. Redox histology was conducted as described for **study II.** * Denotes significant difference vs. resting condition (n=4). Data are shown as mean ± SEM.

Chapter 4. Role of NOX2 in exercise signaling.

Exercise training is characterized by repeated transient exposure to a metabolic, mechanical, and hypoxic stress, among other stimuli (Peake *et al.* 2015). These signals are translated into stress-protective adaptive responses, collectively referred to as training-induced hormesis (Ji *et al.* 2016). Skeletal muscle training adaptations include increases in mitochondrial biogenesis, endogenous antioxidant defense, and insulin sensitivity, among others. This chapter will summarize the evidence linking ROS to exercise training-mediated muscle adaptations.

4.1 Mitogen-activated protein kinases (MAPKs) activation

MAPKs comprise a family of serine-threonine proteins kinases that transduce extracellular signals from the cell membrane to the nucleus via a cascade of phosphorylation events (Son *et al.* 2011). There are several subfamilies of MAPKs: extracellular regulated kinases (ERKs), the c-Jun N-terminal kinases (JNK) and p38 MAPKs, all of which are expressed in skeletal muscle. Different modalities of exercise activate MAPK signaling in mouse and human muscle (Kramer and Goodyear 2007). However, the mechanisms behind their activation are presently unclear. ROS are candidates to mediate exercise-stimulated MAPK activation. Indeed, ROS scavengers such as vitamin C or N-acetyl cysteine (NAC) reduced p38 MAPK and ERK 1/2 phosphorylation in response to endurance exercise (Gomez-Cabrera *et al.* 2008, Petersen *et al.* 2012), suggesting that exercise-stimulated ROS production is involved in the activation of these signaling cascades.

In order to determine the role of NOX2 in exercise-induced MAPK activation, we presently used pharmacological and genetic approaches. In **study I**, WT were intraperitoneally injected for 3 days with either the NOX2 inhibitor apocynin (3mg/kg) or vehicle, as previously described (Khairallah et al., 2012). Mice were then exercised for 1 hour using swimming exercise as an endurance exercise model. Interestingly, apocynin treatment attenuated the exercise-induced ERK 1/2 and p38 MAPK phosphorylation in FDB muscle. In **study II**, the dependence of exercise-stimulated MAPK activation on NOX2 was examined in treadmill-exercised WT and *ncf1** mice. Consistent with the apocynin-data in **study I**, the running-induced p38 MAPK phosphorylation was attenuated in quadriceps muscle from *ncf1** mice compared to WT mice. Interestingly, however, this genotype-difference was not detected in soleus and tibialis anterior muscle.

Although not entirely consistent, this indicates that NOX2 may regulate the activity of certain MAPK isoforms in response to in vivo exercise. More studies are required to address the effects of muscle-type, exercise mode, time and intensity on this dependency.

4.2 Gene expression of antioxidant defense proteins

It is well known that the gene expression of antioxidant defense proteins is increased in response to exercise training in mice and humans. ROS seem to be necessary for induction of both MnSOD and GPx gene expression in rodents (Gomez-Cabrera *et al.* 2008) and human skeletal muscle (Ristow *et al.* 2009, Petersen *et al.* 2012) but the source(s) of ROS are again unclear. In **Study 1,** we found that skeletal muscle responded to a single bout of exercise or electrical stimulation with an increased gene transcription of both MnSOD and GPx. Moreover, NOX2 inhibition by apocynin or gp91ds-TAT prevented the exercise-induced mRNA upregulation. This indicates that NOX2 is required to increase the expression of antioxidant-defense proteins in response to endurance exercise.

4.3 Mitochondrial biogenesis

The increases in mitochondrial content, function, and turnover are some of the most well-described effects of endurance training on skeletal muscle (Vainshtein and Hood 2016). Muscle mitochondrial content is a product of the relationship between synthesis (biogenesis) and degradation (mitophagy) (Hood *et al.* 2015).

In murine skeletal muscle, exercise training-induced mitochondrial biogenesis markers such as PGC-1α, COX4 and citrate synthase activity are attenuated after oral supplementation with ROS scavengers (Gomez-Cabrera *et al.* 2008, Strobel *et al.* 2011, Meier *et al.* 2013, Venditti *et al.* 2014). In humans, the evidence is less clear. Hence, Ristow and colleagues showed that oral supplementation of vitamin C and E decreased PPARγ, PGC-1α, and PGC-1β mRNA after 6 weeks of training in both previously untrained and trained individuals (Ristow *et al.* 2009). In contrast, a subsequent study reported no effect of vitamin C and E supplementation on CS and A-HAD activities in muscle after 12 weeks of endurance training in moderately trained subjects (Yfanti *et al.* 2010). Recently, a double-blinded, placebo-controlled randomized control trial reported that Vitamin C and E supplementation did not affect VO2max, CS activity or COX4 activity (Paulsen *et al.* 2014).

Recently, ROS have been suggested to be important exercise signals able to regulate training adaptation. Margaritelis and colleagues demonstrated that elevated systemic oxidative stress markers were associated with greater adaptations after 6-weeks of training in human (Margaritelis *et al.* 2018). They proposed that larger oxidative signals would translate into greater training adaptions. However, the causality in this relationship remains to be tested.

Presently, we hypothesized that ROS production by NOX2 would upregulate mitochondrial biogenesis in response to exercise in muscle. **In study I**, we measured transcription factor A (Tfam),

a transcription factor necessary and sufficient to enhance mitochondrial biogenesis (Campbell *et al.* 2012, Picca and Lezza 2015). Apocynin-treated mice showed attenuated exercise-induced Tfam mRNA levels in FDB muscle. A similar tendency was observed for citrate synthase mRNA.

It has been proposed that a given exercise training adaptation is the cumulative effect of the transient increases in mRNA transcripts that encode for various proteins after each successive exercise bout (Perry *et al.* 2010). However, acute mRNA transcriptional responses are not necessarily predictive of long-term training effects (Fentz *et al.* 2015, Miller *et al.* 2016). Therefore, we also exercise-trained WT vs. *ncf1** mice using a high-intensity interval training protocol for 6 weeks and determined the protein levels of mitochondrial electron chain complexes in quadriceps muscle lysates (unpublished data). As shown in Figure 4, complex I, II and IV were upregulated by exercise training to a similar extent in WT vs. *ncf1** quadriceps muscle. These data suggest that NOX2 may be required for acute gene expression but not for long-term training changes in mitochondrial content. Worth noting, this does not exclude that exercise training-induced mitochondrial function could be influenced by redox signaling.

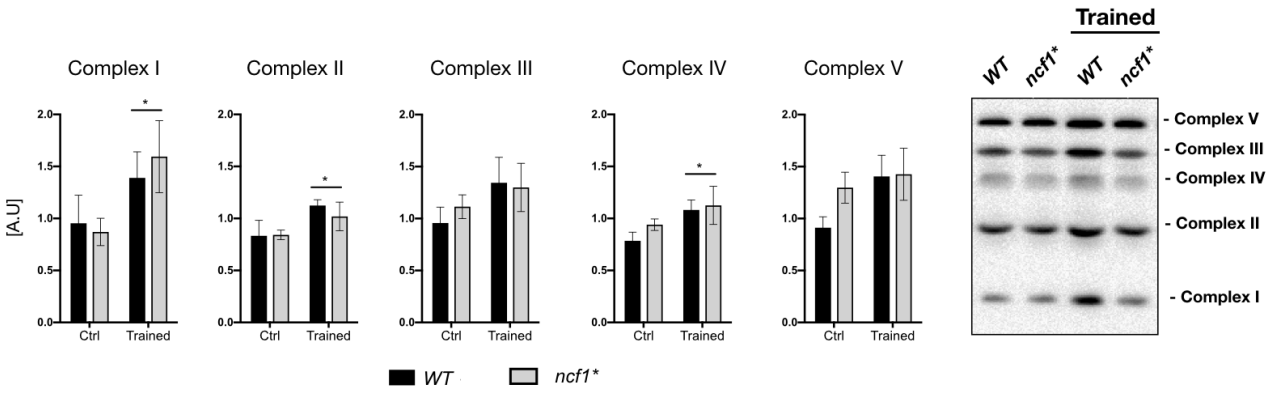

Figure 4. Mitochondrial electron chain complexes (ETC) levels increase similarly in WT and *ncf1 mice.** Both WT and *ncf1** mice were exercised 3 times per week for 6 weeks on a motorized treadmill. ETC were determined by western blotting in quadriceps muscle lysates from WT and *ncf1** mice. Data are shown as mean ± SEM (n=6-8).

4.4 Redox control of myokine expression.

In 1961, Goldstein suggested the possibility that muscle contraction stimulates the release of "factors" from muscles which communicate with other organs (Goldstein 1961). Later, skeletal muscle was indeed discovered to be an endocrine organ releasing peptides (Pedersen and Febbraio 2008, Parker *et al.* 2017), metabolites, and more recently, small vesicles in response to exercise (Whitham *et al.* 2018).

Interleukin 6 (IL-6) is one of the most studied myokines, produced and released by skeletal muscle in response to diverse exercise modalities. The molecular mechanisms involved in IL-6 expression and release in skeletal muscle are still incompletely described. ROS has been implicated in the expression of IL-6 in skeletal muscle cells (Kosmidou *et al.* 2002, Luo *et al.* 2003, Henriquez-Olguin *et al.* 2015). Interestingly, antioxidant supplementation was reported to lower the exercise-stimulated IL-6 expression in human subjects (Vassilakopoulos *et al.* 2003, Fischer *et al.* 2004, Yfanti *et al.* 2011). These studies strongly suggest that redox signaling regulates exercise-mediated IL-6 expression in muscle.

In **study I**, we addressed the role of NOX2 in exercise-stimulated IL-6 expression in skeletal muscle from saline and apocynin-treated mice. Apocynin treatment inhibited the upregulation of IL-6 mRNA induced by endurance exercise in FDB muscle. Interestingly, this inhibition was accompanied by decreased IL-6 plasma levels in the mice treated with apocynin compared with saline. Similar results were obtained in isolated FDB muscle fibers where a decreased IL-6 mRNA response to electrical stimulation was observed in the presence of gp91ds-TAT or apocynin.

Together, these studies demonstrated that pharmacological inhibition of NOX2 abolished exercise-stimulated IL-6 expression in skeletal muscle, suggesting that NOX2 is an important regulator of the IL-6 response to exercise. Future studies should confirm these results using NOX2-deficient genetic models.

5. Chapter Five: NOX2 is a novel regulator of exercise-stimulated glucose uptake.

5.1 Regulation of exercise-stimulated glucose uptake

Muscle contraction increases the demand for energy substrate metabolism and is well-known to increase skeletal muscle glucose uptake by insulin-independent mechanisms (Sylow *et al.* 2017a). Key steps regulating glucose uptake during exercise are glucose delivery, glucose transport, and glucose metabolism (Richter and Hargreaves 2013).

Exercise-stimulated glucose transport requires the translocation of glucose transporter 4 (GLUT 4) to the plasma membrane and transverse tubular system (Zisman *et al.* 2000, Lauritzen *et al.* 2006). Although a substantial amount of research has been done, no single intracellular pathway has been shown to account for the entire exercise-stimulated glucose uptake response and this process is likely regulated by multiple independent signaling pathways (Sylow *et al.* 2017a).

5.2 Role of ROS in regulating glucose transport in skeletal muscle

Cartee & Holloszy were the first to report that exogenous H_2O_2 (3 mM) could increase glucose transport into rat epitrochlearis muscles (Cartee and Holloszy 1990). Later, an inverted U-shaped dose-response relationship between H_2O_2 stimulation and glucose transport was reported in EDL muscles (Higaki *et al.* 2008), in support of both the sufficiency of ROS to stimulate muscle glucose transport and the aforementioned hormesis-concept.

Conversely, glucose transport elicited by *ex vivo* contraction and passive stretch is partially blocked by preincubating with ROS scavengers (Sandstrom *et al.* 2006, Chambers *et al.* 2009, Merry *et al.* 2010b), suggesting that ROS are necessary for the glucose transport-response. Nevertheless, the infusion of NAC in rats or human did not affect *in situ* contraction- or exercise-mediated glucose transport in skeletal muscle (Merry *et al.* 2010a, Merry *et al.* 2010c). Methodological differences might explain these divergent results regarding the necessity of ROS for glucose transport in muscle, including “exercise models”, ROS scavenger properties, and supplementation dynamics.

Rac1 has emerged as a regulator of glucose transport by diverse stimulus in muscle cells (Chiu *et al.* 2011) and mature skeletal muscle (Sylow *et al.* 2014). Thus, both pharmacological inhibition of Rac1 and the actin depolymerizing agent latrunculin B blunted passive stretch- and contraction-stimulated glucose transport in mouse soleus and EDL muscles ex vivo. Besides, incubated muscles from inducible muscle-specific Rac1 KO (Rac1 mKO) mice glucose had impaired glucose transport responses to passive stretch (Sylow *et al.* 2015) and contraction ex vivo (Sylow *et al.* 2013).

Strikingly, Rac1 seemed even more critical for the increases in glucose uptake and GLUT4 translocation in WT vs. Rac1 mKO mice during physiological treadmill exercise *in vivo* (Sylow *et al.* 2016, Sylow *et al.* 2017b).

Since Rac1 is necessary for exercise-stimulated ROS production in mature skeletal muscle (**Study II**), we tested whether a functional NOX2 complex was required for the muscle glucose uptake response to *in vivo* exercise. In **Study II**, we found that *ncf1** mice showed reduced exercise-stimulated glucose uptake in quadriceps, tibialis anterior and a tendency towards a reduction in soleus muscle.

Since no differences were seen in hexokinase II, endogenous GLUT4 or mitochondrial ECT complexes in *ncf1** muscles and because GLUT4 translocation was previously reported to be reduced in the NOX2-deficient Rac1 mKO model (Sylow *et al.* 2016) (**Study II**), we assessed *in vivo* GLUT4 translocation in tibialis anterior muscle using a GLUT4-myc-GFP construct. Consistent with the Sylow et al. study, *ncf1** mice displayed reduced GLUT4 translocation to the sarcolemma in response to exercise, suggesting that NOX2 is an important mediator of exercise-induced GLUT4 translocation.

5.5 Which downstream mechanisms might mediate NOX2-dependent glucose uptake?

An obvious candidate is p38 MAPK, since ROS have been suggested to mediate stretch-stimulated glucose transport by a p38 MAPK-dependent process ex vivo (Chambers *et al.* 2009). Consistent with this scenario, the defect in glucose transport in *ncf1** was accompanied by a decrease in exercise-stimulated p38 MAPK phosphorylation in quadriceps muscle (**Study II**). However, similar exercise-induced p38 MAPK phosphorylation levels were seen in WT vs. *ncf1** tibialis anterior and soleus, despite decreased glucose uptake into these muscles, suggesting that other mechanisms are probably involved. Moreover, Rac1 mKO mice do not show defects in p38 MAPK activation during exercise (Sylow *et al.* 2015), despite the similarly reduced glucose uptake and GLUT4 translocation in Rac1 mKO and *ncf1** mice compared to WT.

Future studies should aim to identify the redox-responsive targets modfied by exercise that could be involved in the regulation of GLUT4 translocation in mature skeletal muscle.

6. Chapter Six: Methodological considerations

6.1 Apocynin as a NOX2 inhibitor.

Apocynin has been used as an efficient inhibitor of the NOX2 complex in many experimental models representing phagocytic and non-phagocytic cells. The mechanism of action involves blocking the membrane translocation of the cytosolic component p47phox and hence its ability to activate the NOX2 complex (Stefanska and Pawliczak 2008)

Some *in vitro* studies have shown that apocynin can has antioxidant properties or may even induce ROS generation (Vejrazka *et al.* 2005). However, the efficacy of apocynin to block ROS production during *in vivo* conditions has been consistently reported (Khairallah *et al.* 2012, Espinosa *et al.* 2013, Henriquez-Olguin *et al.* 2015, Cheng *et al.* 2016). A potential explanation is that apocynin requires activation by myeloperoxidase (MPO) which is differentially expressed in different cell types (Simons *et al.* 1990). It is assumed that apocynin is activated by H_2O_2 and MPO to form an apocynin radical (Stefanska and Pawliczak 2008). The discrepancies observed in the effect of apocynin in diverse tissues could therefore be mediated by a differential MPO expression (de Almeida *et al.* 2012). Interestingly, MPO activity is enhanced during exercise in cardiac and skeletal muscle (Belcastro *et al.* 1996). Regardless of its known limitations, apocynin remains a useful tool for a time- and cost-effective preliminary investigation of the involvement of NOX2 in the regulation of a given biological outcome.

In **study I**, we injected apocynin intraperitoneally for 3 days, a protocol previously described to be effective at blocking NOX2 activity in skeletal muscle (Khairallah *et al.* 2012). The results showed that apocynin disrupted the assembly of the NOX2 complex during *in vivo* exercise in FDB muscle. Indeed, similar results were found using either apocynin or gp91ds-TAT peptide to block NOX2 in cultured muscle fibers. Even though this evidence supports that the apocynin-effect is due to blocked NOX2 assembly and ROS production, we cannot exclude side effects of our treatment. Therefore, in **study II**, we used genetic NOX2 loss-of–function models instead.

6.2 Assessing ROS production during *in vivo* exercise

A number of direct or indirect analytical methods are currently available for monitoring and quantification of ROS generation in biological samples (Dikalov and Harrison 2014). This thesis aimed to study ROS production by NOX2 during in vivo exercise conditions. Assessing NOX2 activity during *in vivo* conditions is methodologically difficult. Customary redox measurements of

NOX2 activity use colorimetric or luminescent probes that require cell lysis and addition of NADPH, FAD^+, and recombinant cytosolic factors, making measurements prone to artifacts such unspecific signals (Rezende *et al.* 2016). For example, results from NOX1-NOX2-NOX4 triple KO mice have clearly demonstrated that the NADPH-stimulated chemiluminescence-based membrane ROS generation assay, does not reflect the NOX activity it is intended to measure (Rezende *et al.* 2016)

6.2.1 Generically-encoded redox biosensors.

The discovery of fluorescent proteins (FPs) has substantially expanded the possibility to study biological processes in living organisms (Shimomura 2005). Based on modifications of FPs, genetically-encoded biosensors allow real-time imaging of a wide range of cellular events in an organelle-specific manner. Specifically relevant to this thesis, the development of genetically-encoded sensors based on redox-sensitive yellow fluorescent protein (rxYFP) (Dardalhon *et al.* 2012) and green fluorescent proteins (roGFPs) (Ostergaard *et al.* 2001) now allow in vivo monitoring of thiol redox dynamics. In the presence of oxidants, roGFP changes its chromophore protonation state, increasing excitation wavelength of the protonated form (405 nm) and decreasing the excitation band of the anionic form (470 nm) (Schwarzlander *et al.* 2016)

George G. Rodney´s group developed a roGFP-based construct to estimate NOX2 activity in living cells. They reasoned that linking roGFP2 to the NOX2 subunit $p47^{phox}$ would allow for subcellular targeting and oxidation of the redox-sensitive fluorescent protein reporter directly to NOX2 in an activity-dependent manner (Pal *et al.* 2013). As presented in table 1, the p47roGFP construct was able to detect stretch- and contraction-stimulated ROS production in a NOX2 dependent manner.

In **study II**, we detected an increase in ROS production after electrical stimulation in living muscle fibers. Interestingly, this electrical stimulation-mediated p47roGFP oxidation was completely absent in the Rac1-deficient fibers. Furthermore, this construct in combination with redox histology allowed for estimation of NOX2 activity after in vivo exercise as outlined below.

6.2.2 In vivo redox histology

In **study II**, the current thesis aimed to study the exercise-stimulated ROS production induced by *in vivo* exercise. We adapted a recently described redox histology method for this purpose (Fujikawa *et al.* 2016). This procedure enabled the preservation and visualization of the redox state of an expressed oxidation-sensitive biosensor in mouse muscle sections. In brief, the muscle cryosections were immediately incubated in 50 mM N-ethylmaleimide (NEM), a fast-acting membrane permeable alkylating agent, to preserve the redox state of roGFP2 within the section. NEM incubation worked by rapidly and irreversibly trapping reduced biosensor molecules in the reduced state, preventing *ex vivo* oxidation by atmospheric oxygen or paraformaldehyde (PFA) treatment.

We first tested the ability of this approach to preserve redox modifications of p47roGFP biosensor during ex- and *in vivo* conditions. Whole-muscles were incubated for 5 min in dPBS control, 0.5 mM H_2O_2, or 0.5 mM DTT at 37º C. The muscles were snap-frozen in liquid nitrogen-cooled isopentane and stored at -80º C until further analysis. As is shown in figure 5, H_2O_2 (0.5 mM) increased the p47roGFP ratio by ~5-fold compared to dPBS incubated muscles. On the other hand, incubation on DTT (0.05) reduced the p47roGFP fluorescence-ratio by 60% compared to the control (figure). Following this ex vivo validation, mice were running-exercised at a relative intensity of 60% of their individual maximal running capacity for 30 min. Exercise increased p47roGFP oxidation by to ~2-fold in tibialis anterior muscle compared to non-exercised controls.

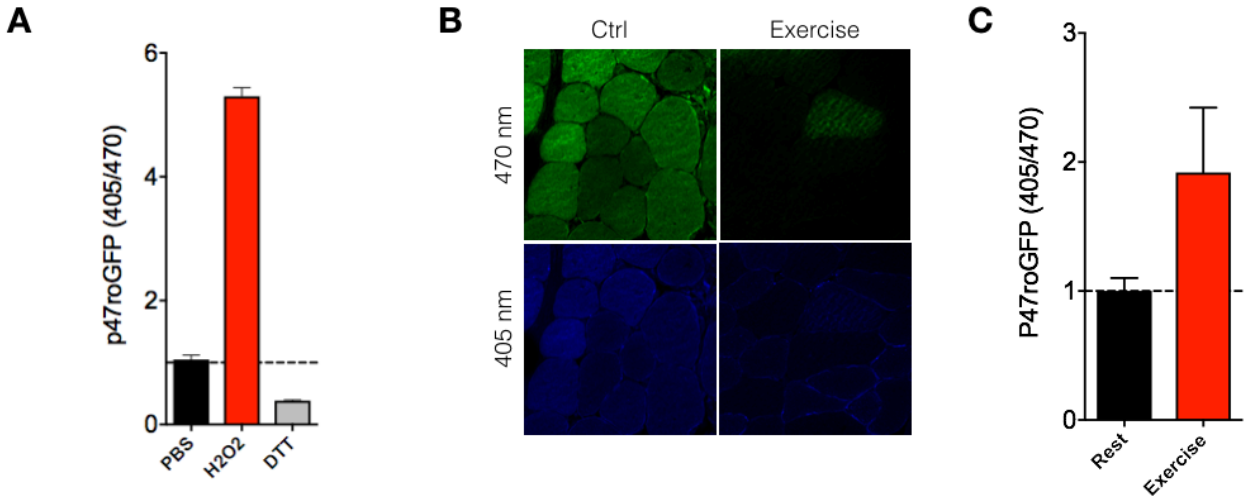

Figure 5. Redox histology preserves changes during ex vivo and in vivo stimulation. p47roGFP biosensor modifications in a) tibialis anterior muscles treated ex vivo with dPBS, H_2O_2 (0.5 mM), and DTT (0.5 mM) during 5 min, and b) tibialis anterior muscle from exercised mice after 30 min at moderate intensity. Data are shown as mean ± SEM (n=3).

This methodology for preserving the endogenous state of genetically-encoded redox probes of histological tissue sections has the intriguing perspective of allowing us to investigate the occurrence of muscle redox differences and changes in vivo under various physiological and pathological conditions in mouse and human muscle. Indeed, my work supports the utility of this method to preserve and detect redox-changes in exercising human muscle (**study II**).

6.3 GLUT4 translocation

GLUT 4 is required for both insulin- and contraction stimulated glucose uptake in skeletal muscle (Zisman *et al.* 2000). Several methods have been used to quantify exercise-stimulated GLUT4 translocation in mature skeletal muscle, including membrane fractionation, exo-facial photolabeling, confocal and electron microscopy of endogenous or exogenous tagged GLUT4 in a variety of muscle preparations (Lauritzen and Schertzer 2010). Technical limitations have hampered a thorough analysis of GLUT4 translocation under exercising conditions in mature muscle.

Muscle cell culture lines expressing a GLUT4 construct with either an exofacial tag or fluorescent GFP reporter protein have been valuable tools to study GLUT4 traffic events (Antonescu *et al.* 2008). However, muscle cells in culture, even upon induction of myotubes, lack many characteristics of mature skeletal muscle, such as transverse-tubule development and although some contractility can be obtained with chronic electrical pulse stimulation in cell lines such as mouse C2C12 (Fujita *et al.* 2007), but not in others such as rat L6 (T.E. Jensen, personal communication), this contractility remains low compared to adult muscle.

GFP-tagged GLUT4 is useful to visualize intracellular GLUT vesicles, but standard confocal microscopy resolution does not allow one to discern the GLUT4 inserted in the plasma membrane from GLUT4 vesicles located 1–2 μm away from the cell surface (Lauritzen and Schertzer 2010). To unequivocally measure GLUT4 insertion in the PM by confocal microscopy, one therefore needs to detect the exposure of an extracellularly exposed GLUT4 epitope.

In **study II**, we used a previously described plasmid encoding a GLUT4-myc-GFP reporter (Bogan *et al.* 2001) which was electroporated into TA muscle. Right after exercise, TA muscles were fixed by immersion in ice-cold 4% in paraformaldehyde in PBS for 4 h. Non-permeabilized individual fibers were teased from fixed muscle with fine forceps under a dissection microscope. GLUT4myc was then detected by indirect immunofluorescence of the exofacial *myc*-epitope in non-permeabilized muscle fibers. The ratio myc/GFP signal was calculated to normalize the *myc* signal to the transfection efficiency. The exercise-stimulated GLUT4 translocation was attenuated in *Ncf1** mice compared to exercised WT mice. These results are in agreement with reports in L6 cells, where insulin-stimulated GLUT 4 translocation was reduced with knockdown of p47phox (Contreras-Ferrat *et al.* 2014) and exercise-stimulated GLUT 4 translocation in Rac1 mKO mice (Sylow *et al.* 2016).

7. Chapter Seven: Conclusions and perspectives

7.1 Conclusions

The main finding of this thesis is that endurance exercise acutely activates NOX2 in mature skeletal muscle. The major ROS source during exercise has been largely undetermined for 35 years; the results of the current thesis demonstrated that NOX2 subunits are required for exercise-stimulated ROS production and is likely a, if not the, major source of ROS during moderate intensity exercise.

We identified two biological endpoints depending on NOX2 activation induced by acute exercise. Firstly, the current data support that NOX2 regulates glucose uptake thought GLUT4 translocation in exercising muscle. Secondly, NOX2-dependent processes are required for the upregulation of genes related to antioxidant defense and mitochondrial biogenesis as well the upregulation of the myokine IL-6.

7.2 Future perspectives

Despite the finding from the current PhD thesis providing new molecular insights into how exercise-stimulated redox signaling regulates adaptations to endurance exercise, it also raises many further questions. It would be sensible for futures studies to determine upstream regulators of NOX2 activation during exercising conditions, as well as downstream redox targets. Furthermore, the mechanisms by which NOX2 regulates GLUT4 translocation and glucose uptake during exercise need to be further determined.

Although the current thesis shows that NOX2 is required for some exercise adaptations, NOX2 upregulation and hyperactivation has also been implicated in inflammation and whole-body insulin resistance. It is unknown how the oxidative stress-induced insulin resistance interacts with exercise-stimulated ROS production in skeletal muscle. Future studies should address the importance of NOX2 in the regulation of insulin sensitivity in the context of diet-induced obesity and the protective effect of exercise. Resolving these mechanisms would likely improve our molecular understanding of the development of pre-diabetic insulin resistance in skeletal muscle.

8. List of References.

Antonescu, C. N., V. K. Randhawa and A. Klip (2008). "Dissecting GLUT4 traffic components in L6 myocytes by fluorescence-based, single-cell assays." Methods Mol Biol **457**: 367-378.

Aydin, J., D. C. Andersson, S. L. Hanninen, A. Wredenberg, P. Tavi, C. B. Park, N. G. Larsson, J. D. Bruton and H. Westerblad (2009). "Increased mitochondrial Ca2+ and decreased sarcoplasmic reticulum Ca2+ in mitochondrial myopathy." Hum Mol Genet **18**(2): 278-288.

Azzi, A., K. J. Davies and F. Kelly (2004). "Free radical biology - terminology and critical thinking." FEBS Lett **558**(1-3): 3-6.

Barrientos, G., P. Llanos, J. Hidalgo, P. Bolanos, C. Caputo, A. Riquelme, G. Sanchez, A. F. Quest and C. Hidalgo (2015). "Cholesterol removal from adult skeletal muscle impairs excitation-contraction coupling and aging reduces caveolin-3 and alters the expression of other triadic proteins." Front Physiol **6**: 105.

Belcastro, A. N., G. D. Arthur, T. A. Albisser and D. A. Raj (1996). "Heart, liver, and skeletal muscle myeloperoxidase activity during exercise." J Appl Physiol (1985) **80**(4): 1331-1335.

Bogan, J. S., A. E. McKee and H. F. Lodish (2001). "Insulin-responsive compartments containing GLUT4 in 3T3-L1 and CHO cells: regulation by amino acid concentrations." Mol Cell Biol **21**(14): 4785-4806.

Boveris, A. and B. Chance (1973). "The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen." Biochem J **134**(3): 707-716.

Campbell, C. T., J. E. Kolesar and B. A. Kaufman (2012). "Mitochondrial transcription factor A regulates mitochondrial transcription initiation, DNA packaging, and genome copy number." Biochim Biophys Acta **1819**(9-10): 921-929.

Cartee, G. D. and J. O. Holloszy (1990). "Exercise increases susceptibility of muscle glucose transport to activation by various stimuli." Am J Physiol **258**(2 Pt 1): E390-393.

Chambers, M. A., J. S. Moylan, J. D. Smith, L. J. Goodyear and M. B. Reid (2009). "Stretch-stimulated glucose uptake in skeletal muscle is mediated by reactive oxygen species and p38 MAP-kinase." J Physiol **587**(Pt 13): 3363-3373.

Cheng, X., X. Zheng, Y. Song, L. Qu, J. Tang, L. Meng and Y. Wang (2016). "Apocynin attenuates renal fibrosis via inhibition of NOXs-ROS-ERK-myofibroblast accumulation in UUO rats." Free Radic Res **50**(8): 840-852.

Chiu, T. T., T. E. Jensen, L. Sylow, E. A. Richter and A. Klip (2011). "Rac1 signalling towards GLUT4/glucose uptake in skeletal muscle." Cell Signal **23**(10): 1546-1554.

Commoner, B., J. Townsend and G. E. Pake (1954). "Free radicals in biological materials." Nature **174**(4432): 689-691.

Contreras-Ferrat, A., P. Llanos, C. Vasquez, A. Espinosa, C. Osorio-Fuentealba, M. Arias-Calderon, S. Lavandero, A. Klip, C. Hidalgo and E. Jaimovich (2014). "Insulin elicits a ROS-activated and an IP(3)-dependent Ca(2)(+) release, which both impinge on GLUT4 translocation." J Cell Sci **127**(Pt 9): 1911-1923.

Dardalhon, M., C. Kumar, I. Iraqui, L. Vernis, G. Kienda, A. Banach-Latapy, T. He, R. Chanet, G. Faye, C. E. Outten and M. E. Huang (2012). "Redox-sensitive YFP sensors monitor dynamic nuclear and cytosolic glutathione redox changes." Free Radic Biol Med **52**(11-12): 2254-2265.

Davies, K. J., A. T. Quintanilha, G. A. Brooks and L. Packer (1982). "Free radicals and tissue damage produced by exercise." Biochem Biophys Res Commun **107**(4): 1198-1205.

de Almeida, A. C., M. M. Dos Santos Vilela, A. Condino-Neto and V. F. Ximenes (2012). "The importance of myeloperoxidase in apocynin-mediated NADPH oxidase inhibition." ISRN Inflamm **2012**: 260453.

Diaz-Vegas, A., C. A. Campos, A. Contreras-Ferrat, M. Casas, S. Buvinic, E. Jaimovich and A. Espinosa (2015). "ROS Production via P2Y1-PKC-NOX2 Is Triggered by Extracellular ATP after Electrical Stimulation of Skeletal Muscle Cells." PLoS One **10**(6): e0129882.

Dikalov, S. I. and D. G. Harrison (2014). "Methods for detection of mitochondrial and cellular reactive oxygen species." Antioxid Redox Signal **20**(2): 372-382.
Dillard, C. J., R. E. Litov, W. M. Savin, E. E. Dumelin and A. L. Tappel (1978). "Effects of exercise, vitamin E, and ozone on pulmonary function and lipid peroxidation." J Appl Physiol Respir Environ Exerc Physiol **45**(6): 927-932.
Ding, D., K. D. Lawson, T. L. Kolbe-Alexander, E. A. Finkelstein, P. T. Katzmarzyk, W. van Mechelen, M. Pratt and C. Lancet Physical Activity Series 2 Executive (2016). "The economic burden of physical inactivity: a global analysis of major non-communicable diseases." Lancet **388**(10051): 1311-1324.
Espinosa, A., C. Campos, A. Diaz-Vegas, J. E. Galgani, N. Juretic, C. Osorio-Fuentealba, J. L. Bucarey, G. Tapia, R. Valenzuela, A. Contreras-Ferrat, P. Llanos and E. Jaimovich (2013). "Insulin-dependent H2O2 production is higher in muscle fibers of mice fed with a high-fat diet." Int J Mol Sci **14**(8): 15740-15754.
Espinosa, A., C. Henriquez-Olguin and E. Jaimovich (2016). "Reactive oxygen species and calcium signals in skeletal muscle: A crosstalk involved in both normal signaling and disease." Cell Calcium **60**(3): 172-179.
Espinosa, A., A. Leiva, M. Pena, M. Muller, A. Debandi, C. Hidalgo, M. A. Carrasco and E. Jaimovich (2006). "Myotube depolarization generates reactive oxygen species through NAD(P)H oxidase; ROS-elicited Ca2+ stimulates ERK, CREB, early genes." J Cell Physiol **209**(2): 379-388.
Fentz, J., R. Kjobsted, C. M. Kristensen, J. R. Hingst, J. B. Birk, A. Gudiksen, M. Foretz, P. Schjerling, B. Viollet, H. Pilegaard and J. F. Wojtaszewski (2015). "AMPKalpha is essential for acute exercise-induced gene responses but not for exercise training-induced adaptations in mouse skeletal muscle." Am J Physiol Endocrinol Metab **309**(11): E900-914.
Fischer, C. P., N. J. Hiscock, M. Penkowa, S. Basu, B. Vessby, A. Kallner, L. B. Sjoberg and B. K. Pedersen (2004). "Supplementation with vitamins C and E inhibits the release of interleukin-6 from contracting human skeletal muscle." J Physiol **558**(Pt 2): 633-645.
Friedman, J. R. and J. Nunnari (2014). "Mitochondrial form and function." Nature **505**(7483): 335-343.
Fujikawa, Y., L. P. Roma, M. C. Sobotta, A. J. Rose, M. B. Diaz, G. Locatelli, M. O. Breckwoldt, T. Misgeld, M. Kerschensteiner, S. Herzig, K. Muller-Decker and T. P. Dick (2016). "Mouse redox histology using genetically encoded probes." Sci Signal **9**(419): rs1.
Fujita, H., T. Nedachi and M. Kanzaki (2007). "Accelerated de novo sarcomere assembly by electric pulse stimulation in C2C12 myotubes." Exp Cell Res **313**(9): 1853-1865.
Furukawa, S., T. Fujita, M. Shimabukuro, M. Iwaki, Y. Yamada, Y. Nakajima, O. Nakayama, M. Makishima, M. Matsuda and I. Shimomura (2004). "Increased oxidative stress in obesity and its impact on metabolic syndrome." J Clin Invest **114**(12): 1752-1761.
Goldstein, M. S. (1961). "Humoral nature of the hypoglycemic factor of muscular work." Diabetes **10**: 232-234.
Gomez-Cabrera, M. C., E. Domenech, M. Romagnoli, A. Arduini, C. Borras, F. V. Pallardo, J. Sastre and J. Vina (2008). "Oral administration of vitamin C decreases muscle mitochondrial biogenesis and hampers training-induced adaptations in endurance performance." Am J Clin Nutr **87**(1): 142-149.
Goncalves, R. L., C. L. Quinlan, I. V. Perevoshchikova, M. Hey-Mogensen and M. D. Brand (2015). "Sites of superoxide and hydrogen peroxide production by muscle mitochondria assessed ex vivo under conditions mimicking rest and exercise." J Biol Chem **290**(1): 209-227.
Henriquez-Olguin, C., F. Altamirano, D. Valladares, J. R. Lopez, P. D. Allen and E. Jaimovich (2015). "Altered ROS production, NF-kappaB activation and interleukin-6 gene expression induced by electrical stimulation in dystrophic mdx skeletal muscle cells." Biochim Biophys Acta **1852**(7): 1410-1419.

Hidalgo, C., G. Sanchez, G. Barrientos and P. Aracena-Parks (2006). "A transverse tubule NADPH oxidase activity stimulates calcium release from isolated triads via ryanodine receptor type 1 S -glutathionylation." J Biol Chem **281**(36): 26473-26482.
Higaki, Y., T. Mikami, N. Fujii, M. F. Hirshman, K. Koyama, T. Seino, K. Tanaka and L. J. Goodyear (2008). "Oxidative stress stimulates skeletal muscle glucose uptake through a phosphatidylinositol 3-kinase-dependent pathway." Am J Physiol Endocrinol Metab **294**(5): E889-897.
Hoffman, N. J., B. L. Parker, R. Chaudhuri, K. H. Fisher-Wellman, M. Kleinert, S. J. Humphrey, P. Yang, M. Holliday, S. Trefely, D. J. Fazakerley, J. Stockli, J. G. Burchfield, T. E. Jensen, R. Jothi, B. Kiens, J. F. Wojtaszewski, E. A. Richter and D. E. James (2015). "Global Phosphoproteomic Analysis of Human Skeletal Muscle Reveals a Network of Exercise-Regulated Kinases and AMPK Substrates." Cell Metab **22**(5): 922-935.
Hood, D. A., L. D. Tryon, A. Vainshtein, J. Memme, C. Chen, M. Pauly, M. J. Crilly and H. Carter (2015). "Exercise and the Regulation of Mitochondrial Turnover." Prog Mol Biol Transl Sci **135**: 99-127.
Jackson, M. J. (2016). "Recent advances and long-standing problems in detecting oxidative damage and reactive oxygen species in skeletal muscle." J Physiol **594**(18): 5185-5193.
Jackson, M. J., A. Vasilaki and A. McArdle (2016). "Cellular mechanisms underlying oxidative stress in human exercise." Free Radic Biol Med **98**: 13-17.
Javeshghani, D., S. A. Magder, E. Barreiro, M. T. Quinn and S. N. Hussain (2002). "Molecular characterization of a superoxide-generating NAD(P)H oxidase in the ventilatory muscles." Am J Respir Crit Care Med **165**(3): 412-418.
Jensen, P. K. (1966). "Antimycin-insensitive oxidation of succinate and reduced nicotinamide-adenine dinucleotide in electron-transport particles. I. pH dependency and hydrogen peroxide formation." Biochim Biophys Acta **122**(2): 157-166.
Jensen, T. E., L. Sylow, A. J. Rose, A. B. Madsen, Y. Angin, S. J. Maarbjerg and E. A. Richter (2014). "Contraction-stimulated glucose transport in muscle is controlled by AMPK and mechanical stress but not sarcoplasmatic reticulum Ca(2+) release." Mol Metab **3**(7): 742-753.
Ji, L. L., C. Kang and Y. Zhang (2016). "Exercise-induced hormesis and skeletal muscle health." Free Radic Biol Med **98**: 113-122.
Kanter, M. (1998). "Free radicals, exercise and antioxidant supplementation." Proc Nutr Soc **57**(1): 9-13.
Katz, A. (2016). "Role of reactive oxygen species in regulation of glucose transport in skeletal muscle during exercise." J Physiol **594**(11): 2787-2794.
Kelley, R. C., B. McDonagh and L. F. Ferreira (2018). "Advanced aging causes diaphragm functional abnormalities, global proteome remodeling, and loss of mitochondrial cysteine redox flexibility in mice." Exp Gerontol **103**: 69-79.
Khairallah, R. J., G. Shi, F. Sbrana, B. L. Prosser, C. Borroto, M. J. Mazaitis, E. P. Hoffman, A. Mahurkar, F. Sachs, Y. Sun, Y. W. Chen, R. Raiteri, W. J. Lederer, S. G. Dorsey and C. W. Ward (2012). "Microtubules underlie dysfunction in duchenne muscular dystrophy." Sci Signal **5**(236): ra56.
Koren, A., C. Sauber, M. Sentjurc and M. Schara (1983). "Free radicals in tetanic activity of isolated skeletal muscle." Comp Biochem Physiol B **74**(3): 633-635.
Kosmidou, I., T. Vassilakopoulos, A. Xagorari, S. Zakynthinos, A. Papapetropoulos and C. Roussos (2002). "Production of interleukin-6 by skeletal myotubes: role of reactive oxygen species." Am J Respir Cell Mol Biol **26**(5): 587-593.
Kramer, H. F. and L. J. Goodyear (2007). "Exercise, MAPK, and NF-kappaB signaling in skeletal muscle." J Appl Physiol (1985) **103**(1): 388-395.
Kudin, A. P., N. Y. Bimpong-Buta, S. Vielhaber, C. E. Elger and W. S. Kunz (2004). "Characterization of superoxide-producing sites in isolated brain mitochondria." J Biol Chem **279**(6): 4127-4135.

Laker, R. C., J. C. Drake, R. J. Wilson, V. A. Lira, B. M. Lewellen, K. A. Ryall, C. C. Fisher, M. Zhang, J. J. Saucerman, L. J. Goodyear, M. Kundu and Z. Yan (2017). "Ampk phosphorylation of Ulk1 is required for targeting of mitochondria to lysosomes in exercise-induced mitophagy." Nat Commun **8**(1): 548.
Lauritzen, H. P., T. Ploug, C. Prats, J. M. Tavare and H. Galbo (2006). "Imaging of insulin signaling in skeletal muscle of living mice shows major role of T-tubules." Diabetes **55**(5): 1300-1306.
Lauritzen, H. P. and J. D. Schertzer (2010). "Measuring GLUT4 translocation in mature muscle fibers." Am J Physiol Endocrinol Metab **299**(2): E169-179.
Lawler, J. M., D. A. Rodriguez and J. M. Hord (2016). "Mitochondria in the middle: exercise preconditioning protection of striated muscle." J Physiol **594**(18): 5161-5183.
Liu, Y., E. O. Hernandez-Ochoa, W. R. Randall and M. F. Schneider (2012). "NOX2-dependent ROS is required for HDAC5 nuclear efflux and contributes to HDAC4 nuclear efflux during intense repetitive activity of fast skeletal muscle fibers." Am J Physiol Cell Physiol **303**(3): C334-347.
Loschen, G., L. Flohe and B. Chance (1971). "Respiratory chain linked H(2)O(2) production in pigeon heart mitochondria." FEBS Lett **18**(2): 261-264.
Loureiro, A. C., I. C. do Rego-Monteiro, R. A. Louzada, V. H. Ortenzi, A. P. de Aguiar, E. S. de Abreu, J. P. Cavalcanti-de-Albuquerque, F. Hecht, A. C. de Oliveira, V. M. Ceccatto, R. S. Fortunato and D. P. Carvalho (2016). "Differential Expression of NADPH Oxidases Depends on Skeletal Muscle Fiber Type in Rats." Oxid Med Cell Longev **2016**: 6738701.
Luo, G., D. D. Hershko, B. W. Robb, C. J. Wray and P. O. Hasselgren (2003). "IL-1beta stimulates IL-6 production in cultured skeletal muscle cells through activation of MAP kinase signaling pathway and NF-kappa B." Am J Physiol Regul Integr Comp Physiol **284**(5): R1249-1254.
Margaritelis, N. V., A. A. Theodorou, V. Paschalis, A. S. Veskoukis, K. Dipla, A. Zafeiridis, G. Panayiotou, I. S. Vrabas, A. Kyparos and M. G. Nikolaidis (2018). "Adaptations to endurance training depend on exercise-induced oxidative stress: exploiting redox interindividual variability." Acta Physiol (Oxf) **222**(2).
Mattson, M. P. (2008). "Hormesis defined." Ageing Res Rev **7**(1): 1-7.
Meier, P., M. Renga, H. Hoppeler and O. Baum (2013). "The impact of antioxidant supplements and endurance exercise on genes of the carbohydrate and lipid metabolism in skeletal muscle of mice." Cell Biochem Funct **31**(1): 51-59.
Merry, T. L., R. M. Dywer, E. A. Bradley, S. Rattigan and G. K. McConell (2010a). "Local hindlimb antioxidant infusion does not affect muscle glucose uptake during in situ contractions in rat." J Appl Physiol (1985) **108**(5): 1275-1283.
Merry, T. L., G. R. Steinberg, G. S. Lynch and G. K. McConell (2010b). "Skeletal muscle glucose uptake during contraction is regulated by nitric oxide and ROS independently of AMPK." Am J Physiol Endocrinol Metab **298**(3): E577-585.
Merry, T. L., G. D. Wadley, C. G. Stathis, A. P. Garnham, S. Rattigan, M. Hargreaves and G. K. McConell (2010c). "N-Acetylcysteine infusion does not affect glucose disposal during prolonged moderate-intensity exercise in humans." J Physiol **588**(Pt 9): 1623-1634.
Michaelson, L. P., G. Shi, C. W. Ward and G. G. Rodney (2010). "Mitochondrial redox potential during contraction in single intact muscle fibers." Muscle Nerve **42**(4): 522-529.
Miller, B. F., A. R. Konopka and K. L. Hamilton (2016). "The rigorous study of exercise adaptations: why mRNA might not be enough." J Appl Physiol (1985) **121**(2): 594-596.
Munro, D. and J. R. Treberg (2017). "A radical shift in perspective: mitochondria as regulators of reactive oxygen species." J Exp Biol **220**(Pt 7): 1170-1180.
Nguyen, G. T., E. R. Green and J. Mecsas (2017). "Neutrophils to the ROScue: Mechanisms of NADPH Oxidase Activation and Bacterial Resistance." Front Cell Infect Microbiol **7**: 373.

Nyberg, M., S. P. Mortensen, H. Cabo, M. C. Gomez-Cabrera, J. Vina and Y. Hellsten (2014). "Roles of sedentary aging and lifelong physical activity in exchange of glutathione across exercising human skeletal muscle." Free Radic Biol Med **73**: 166-173.
Ostergaard, H., A. Henriksen, F. G. Hansen and J. R. Winther (2001). "Shedding light on disulfide bond formation: engineering a redox switch in green fluorescent protein." EMBO J **20**(21): 5853-5862.
Pal, R., P. Basu Thakur, S. Li, C. Minard and G. G. Rodney (2013). "Real-time imaging of NADPH oxidase activity in living cells using a novel fluorescent protein reporter." PLoS One **8**(5): e63989.
Parker, B. L., J. G. Burchfield, D. Clayton, T. A. Geddes, R. J. Payne, B. Kiens, J. F. P. Wojtaszewski, E. A. Richter and D. E. James (2017). "Multiplexed Temporal Quantification of the Exercise-regulated Plasma Peptidome." Mol Cell Proteomics **16**(12): 2055-2068.
Pattwell, D. M., A. McArdle, J. E. Morgan, T. A. Patridge and M. J. Jackson (2004). "Release of reactive oxygen and nitrogen species from contracting skeletal muscle cells." Free Radic Biol Med **37**(7): 1064-1072.
Paulsen, G., K. T. Cumming, G. Holden, J. Hallen, B. R. Ronnestad, O. Sveen, A. Skaug, I. Paur, N. E. Bastani, H. N. Ostgaard, C. Buer, M. Midttun, F. Freuchen, H. Wiig, E. T. Ulseth, I. Garthe, R. Blomhoff, H. B. Benestad and T. Raastad (2014). "Vitamin C and E supplementation hampers cellular adaptation to endurance training in humans: a double-blind, randomised, controlled trial." J Physiol **592**(8): 1887-1901.
Peake, J. M., J. F. Markworth, K. Nosaka, T. Raastad, G. D. Wadley and V. G. Coffey (2015). "Modulating exercise-induced hormesis: Does less equal more?" J Appl Physiol (1985) **119**(3): 172-189.
Pedersen, B. K. and M. A. Febbraio (2008). "Muscle as an endocrine organ: focus on muscle-derived interleukin-6." Physiol Rev **88**(4): 1379-1406.
Perry, C. G., J. Lally, G. P. Holloway, G. J. Heigenhauser, A. Bonen and L. L. Spriet (2010). "Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle." J Physiol **588**(Pt 23): 4795-4810.
Petersen, A. C., M. J. McKenna, I. Medved, K. T. Murphy, M. J. Brown, P. Della Gatta and D. Cameron-Smith (2012). "Infusion with the antioxidant N-acetylcysteine attenuates early adaptive responses to exercise in human skeletal muscle." Acta Physiol (Oxf) **204**(3): 382-392.
Picca, A. and A. M. Lezza (2015). "Regulation of mitochondrial biogenesis through TFAM-mitochondrial DNA interactions: Useful insights from aging and calorie restriction studies." Mitochondrion **25**: 67-75.
Powers, S. K. and M. J. Jackson (2008). "Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production." Physiol Rev **88**(4): 1243-1276.
Powers, S. K., L. L. Ji, A. N. Kavazis and M. J. Jackson (2011). "Reactive oxygen species: impact on skeletal muscle." Compr Physiol **1**(2): 941-969.
Powers, S. K., Z. Radak and L. L. Ji (2016). "Exercise-induced oxidative stress: past, present and future." J Physiol **594**(18): 5081-5092.
Prats, C., A. Gomez-Cabello and A. V. Hansen (2011). "Intracellular compartmentalization of skeletal muscle glycogen metabolism and insulin signalling." Exp Physiol **96**(4): 385-390.
Reid, M. B. (2016). "Redox interventions to increase exercise performance." J Physiol **594**(18): 5125-5133.
Reid, M. B., F. A. Khawli and M. R. Moody (1993). "Reactive oxygen in skeletal muscle. III. Contractility of unfatigued muscle." J Appl Physiol (1985) **75**(3): 1081-1087.
Rezende, F., O. Lowe, V. Helfinger, K. K. Prior, M. Walter, S. Zukunft, I. Fleming, N. Weissmann, R. P. Brandes and K. Schroder (2016). "Unchanged NADPH Oxidase Activity in Nox1-Nox2-Nox4 Triple Knockout Mice: What Do NADPH-Stimulated Chemiluminescence Assays Really Detect?" Antioxid Redox Signal **24**(7): 392-399.
Richter, E. A. and M. Hargreaves (2013). "Exercise, GLUT4, and skeletal muscle glucose uptake." Physiol Rev **93**(3): 993-1017.

Ristow, M., K. Zarse, A. Oberbach, N. Kloting, M. Birringer, M. Kiehntopf, M. Stumvoll, C. R. Kahn and M. Bluher (2009). "Antioxidants prevent health-promoting effects of physical exercise in humans." Proc Natl Acad Sci U S A **106**(21): 8665-8670.
Sakellariou, G. K., A. Vasilaki, J. Palomero, A. Kayani, L. Zibrik, A. McArdle and M. J. Jackson (2013). "Studies of mitochondrial and nonmitochondrial sources implicate nicotinamide adenine dinucleotide phosphate oxidase(s) in the increased skeletal muscle superoxide generation that occurs during contractile activity." Antioxid Redox Signal **18**(6): 603-621.
Sandstrom, M. E., S. J. Zhang, J. Bruton, J. P. Silva, M. B. Reid, H. Westerblad and A. Katz (2006). "Role of reactive oxygen species in contraction-mediated glucose transport in mouse skeletal muscle." J Physiol **575**(Pt 1): 251-262.
Schwarzlander, M., T. P. Dick, A. J. Meyer and B. Morgan (2016). "Dissecting Redox Biology Using Fluorescent Protein Sensors." Antioxid Redox Signal **24**(13): 680-712.
Shimomura, O. (2005). "The discovery of aequorin and green fluorescent protein." J Microsc **217**(Pt 1): 1-15.
Sies, H. (2017). "Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: Oxidative eustress." Redox Biol **11**: 613-619.
Simons, J. M., B. A. Hart, T. R. Ip Vai Ching, H. Van Dijk and R. P. Labadie (1990). "Metabolic activation of natural phenols into selective oxidative burst agonists by activated human neutrophils." Free Radic Biol Med **8**(3): 251-258.
Son, Y., Y. K. Cheong, N. H. Kim, H. T. Chung, D. G. Kang and H. O. Pae (2011). "Mitogen-Activated Protein Kinases and Reactive Oxygen Species: How Can ROS Activate MAPK Pathways?" J Signal Transduct **2011**: 792639.
St-Pierre, J., J. A. Buckingham, S. J. Roebuck and M. D. Brand (2002). "Topology of superoxide production from different sites in the mitochondrial electron transport chain." J Biol Chem **277**(47): 44784-44790.
Stefanska, J. and R. Pawliczak (2008). "Apocynin: molecular aptitudes." Mediators Inflamm **2008**: 106507.
Strobel, N. A., J. M. Peake, A. Matsumoto, S. A. Marsh, J. S. Coombes and G. D. Wadley (2011). "Antioxidant supplementation reduces skeletal muscle mitochondrial biogenesis." Med Sci Sports Exerc **43**(6): 1017-1024.
Sun, Q. A., D. T. Hess, L. Nogueira, S. Yong, D. E. Bowles, J. Eu, K. R. Laurita, G. Meissner and J. S. Stamler (2011). "Oxygen-coupled redox regulation of the skeletal muscle ryanodine receptor-Ca2+ release channel by NADPH oxidase 4." Proc Natl Acad Sci U S A **108**(38): 16098-16103.
Sylow, L., T. E. Jensen, M. Kleinert, J. R. Mouatt, S. J. Maarbjerg, J. Jeppesen, C. Prats, T. T. Chiu, S. Boguslavsky, A. Klip, P. Schjerling and E. A. Richter (2013). "Rac1 is a novel regulator of contraction-stimulated glucose uptake in skeletal muscle." Diabetes **62**(4): 1139-1151.
Sylow, L., M. Kleinert, E. A. Richter and T. E. Jensen (2017a). "Exercise-stimulated glucose uptake - regulation and implications for glycaemic control." Nat Rev Endocrinol **13**(3): 133-148.
Sylow, L., L. L. Moller, M. Kleinert, E. A. Richter and T. E. Jensen (2014). "Rac1--a novel regulator of contraction-stimulated glucose uptake in skeletal muscle." Exp Physiol **99**(12): 1574-1580.
Sylow, L., L. L. Moller, M. Kleinert, E. A. Richter and T. E. Jensen (2015). "Stretch-stimulated glucose transport in skeletal muscle is regulated by Rac1." J Physiol **593**(3): 645-656.
Sylow, L., L. L. V. Moller, M. Kleinert, G. D'Hulst, E. De Groote, P. Schjerling, G. R. Steinberg, T. E. Jensen and E. A. Richter (2017b). "Rac1 and AMPK Account for the Majority of Muscle Glucose Uptake Stimulated by Ex Vivo Contraction but Not In Vivo Exercise." Diabetes **66**(6): 1548-1559.
Sylow, L., I. L. Nielsen, M. Kleinert, L. L. Moller, T. Ploug, P. Schjerling, P. J. Bilan, A. Klip, T. E. Jensen and E. A. Richter (2016). "Rac1 governs exercise-stimulated glucose uptake in skeletal muscle through regulation of GLUT4 translocation in mice." J Physiol **594**(17): 4997-5008.

Tahara, E. B., F. D. Navarete and A. J. Kowaltowski (2009). "Tissue-, substrate-, and site-specific characteristics of mitochondrial reactive oxygen species generation." Free Radic Biol Med **46**(9): 1283-1297.
Tremmel, M., U. G. Gerdtham, P. M. Nilsson and S. Saha (2017). "Economic Burden of Obesity: A Systematic Literature Review." Int J Environ Res Public Health **14**(4).
Vainshtein, A. and D. A. Hood (2016). "The regulation of autophagy during exercise in skeletal muscle." J Appl Physiol (1985) **120**(6): 664-673.
Vassilakopoulos, T., M. H. Karatza, P. Katsaounou, A. Kollintza, S. Zakynthinos and C. Roussos (2003). "Antioxidants attenuate the plasma cytokine response to exercise in humans." J Appl Physiol (1985) **94**(3): 1025-1032.
Vejrazka, M., R. Micek and S. Stipek (2005). "Apocynin inhibits NADPH oxidase in phagocytes but stimulates ROS production in non-phagocytic cells." Biochim Biophys Acta **1722**(2): 143-147.
Venditti, P., G. Napolitano, D. Barone and S. Di Meo (2014). "Vitamin E supplementation modifies adaptive responses to training in rat skeletal muscle." Free Radic Res **48**(10): 1179-1189.
Whitham, M., B. L. Parker, M. Friedrichsen, J. R. Hingst, M. Hjorth, W. E. Hughes, C. L. Egan, L. Cron, K. I. Watt, R. P. Kuchel, N. Jayasooriah, E. Estevez, T. Petzold, C. M. Suter, P. Gregorevic, B. Kiens, E. A. Richter, D. E. James, J. F. P. Wojtaszewski and M. A. Febbraio (2018). "Extracellular Vesicles Provide a Means for Tissue Crosstalk during Exercise." Cell Metab **27**(1): 237-251 e234.
Wong, H. S., P. A. Dighe, V. Mezera, P. A. Monternier and M. D. Brand (2017). "Production of superoxide and hydrogen peroxide from specific mitochondrial sites under different bioenergetic conditions." J Biol Chem **292**(41): 16804-16809.
Yfanti, C., T. Akerstrom, S. Nielsen, A. R. Nielsen, R. Mounier, O. H. Mortensen, J. Lykkesfeldt, A. J. Rose, C. P. Fischer and B. K. Pedersen (2010). "Antioxidant supplementation does not alter endurance training adaptation." Med Sci Sports Exerc **42**(7): 1388-1395.
Yfanti, C., A. R. Nielsen, T. Akerstrom, S. Nielsen, A. J. Rose, E. A. Richter, J. Lykkesfeldt, C. P. Fischer and B. K. Pedersen (2011). "Effect of antioxidant supplementation on insulin sensitivity in response to endurance exercise training." Am J Physiol Endocrinol Metab **300**(5): E761-770.
Zisman, A., O. D. Peroni, E. D. Abel, M. D. Michael, F. Mauvais-Jarvis, B. B. Lowell, J. F. Wojtaszewski, M. F. Hirshman, A. Virkamaki, L. J. Goodyear, C. R. Kahn and B. B. Kahn (2000). "Targeted disruption of the glucose transporter 4 selectively in muscle causes insulin resistance and glucose intolerance." Nat Med **6**(8): 924-928.

9. Manuscripts