ABSTRACT

Title of Document: THE EFFECT OF METABOLIC SUBSTRATE ON ROS PRODUCTION DURING MUSCLE CONTRACTION

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Many different disease states are characterized by mitochondrial dysfunction, which results in excessive reactive oxygen species (ROS) production. In contrast muscle contraction induces ROS generation suggesting there is an optimal range of ROS production necessary for proper cell function. It is unclear if ROS production is influenced by metabolic substrate flux as a result of the energetic demand of contraction. The purpose of this study was to determine the rate and source of ROS production in contracting single muscle fibers (SMF) cultured with different metabolic substrates. ROS production was assessed in SMF isolated from adult male mice exposed to different stimulation conditions and/or different sources of metabolic substrate. Mitochondrial membrane potential was also assessed in SMF under similar conditions. The results of this study demonstrate ROS generation is significantly influenced by metabolic substrate and larges increases in ROS do not affect mitochondrial membrane potential in intact SMF.

THE EFFECT OF METABOLIC SUBSTRATE ON ROS PRODUCTION DURING MUSCLE CONTRACTION

By

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Acknowledgements

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List of Abbreviations

2-OH-E^+ - 2-hydroxyethidium

AD - Alzheimer's disease

Akt - Protein kinase B

AMPK - AMP-activated protein kinase

AP-1 - Activator protein-1

ASK1 - Apoptosis signal-regulated kinase 1

ATP - Adenosine triphosphate

COPD - Chronic obstructive pulmonary disease

CTCF - Corrected total cell fluorescence

CYP - Cytochrome P450 monooxygenase

DCF – Dichlorofluorescein

DPI – Diphenyleneiodonium

DTT – Dithiothreitol

E^+ - Ethidium

eNOS – Endothelial nitric oxide synthase

ER - Endoplasmic reticulum

ERK1/2 - Extracellular signal-regulated kinases

ERO1 - ER oxioreductin

ETC –Electron transport chain

FCCP - carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone

FDB - Flexor digitorum brevis

GLP-1 - Glucagon-like peptide-1

GLUT4 - Glucose transporter type 4

GPx - Glutathione peroxidase

GSH - Glutathione

H_2O_2 – Hydrogen peroxide

HE – Hydroethidine

HSF - Heat shock factor

HSP - Heat shock protein

IGF-I - Insulin-like growth factor I

IL-6 – Interleukin 6

IRE - Iron-responsive element

IRP - Iron regulatory protein

JNK - c-Jun N-terminal kinase

KRB - Krebs-Ringer solution

MAPK - Mitogen-activated protein kinase

MI – Myocardial infarction

MnSOD - Manganese superoxide dismutase

NADPH - Nicotinamide adenine dinucleotide phosphate

NO – Nitric oxide

NOS - Nitric oxide synthase

NRG-1 - Neuregulin-1

NRF-1 - Nuclear respiratory factor 1

O_2^- - Superoxide

OH• - Hydroxyl radical

PD - Parkinson's disease

PDI - Protein disulfide isomerase

PGC-1α - Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PI3K - Phosphoinositide 3-kinase

PLA_2 - Phospholipase A_2

PoIG - Polymerase gamma mutator

PPARγ - Peroxisome proliferator-activated receptor γ

PTEN - Phosphatase and tensin homolog

PUFA - Polyunsaturated fatty acid

RER – Respiratory exchange ratio

RNS – Reactive nitrogen species

ROS – Reactive oxygen species

RyR - Ryanodine receptor

SAS - Statistical Analysis System

SERCA - SR calcium ATPase

SOD – Superoxide dismutase

SR - Sarcoplasmic reticulum

T2D - Type 2 diabetes

TAG – Triglycerides

TLR4 - Toll like receptor 4

TMRE - Tetramethylrhodamine, ethyl ester

TMRM - Tetramethylrhodamine, methyl ester

VEGF - Vascular endothelial grown factor

XO - Xanthine oxidase

Introduction

Regular physical activity prevents non-communicable diseases, such as cardiovascular disease, obesity, and type 2 diabetes (T2D). During physical activity or muscle contraction, there is a high demand for energy in the form of adenosine triphosphate (ATP), especially from the skeletal muscle. To meet this demand, skeletal muscle converts macromolecules, such as glucose and fatty acids, into usable ATP by using two processes, glycolysis and oxidative phosphorylation. The process of glycolysis occurs in the cytosol of the cell, where using a series of enzymatic reactions, glucose is converted into pyruvate resulting in ATP production. In contrast, oxidative phosphorylation, which is localized to the mitochondria, utilizes both fatty acids and glucose to produce ATP. In skeletal muscle, the mitochondria are responsible for the majority of ATP produced in the resting state (i.e. no contraction), with aerobic metabolism being responsible for nearly 100% of ATP production (Powers and Howley 2009).

Due to the importance of bioenergetics to overall cell function, proper mitochondrial function is essential to the muscle cell. Many disease states demonstrate mitochondrial dysfunction, including T2D, neuromuscular diseases, cardiomyopathies, and obesity, among others (Davis and Williams, 2012). Regular exercise training has been shown to improve mitochondrial quality and quantity, and thus may be beneficial in treating these disease states. For example, Taivassalo and Haller (2005) found that individuals with mitochondrial disease who are exposed to endurance training exhibit enhanced mitochondrial function, thus improving

functional capacity. Therefore, understanding mitochondrial function is critical for understanding skeletal muscle function and possible dysfunction in a variety of disease states. Mitochondrial function is largely based on the ability to utilize metabolic substrates and oxygen to produce critical ATP. However mitochondria are capable of producing reactive oxygen species (ROS) when substrate flux into mitochondria exceeds the demand for ATP production. Mitochondrial ROS production is largely the result of low ATP demand coupled with increased flux of NADH and/or FADH into complex I or complex II of the electron transport chain, respectively (Murphy 2009). Although in most cases ROS production is needed for appropriate signaling responses, in certain disease conditions, ROS production exceeds optimal production, leading to cellular dysfunction.

Previous research has suggested that onset of diseases such as T2D and various neuromuscular diseases is thought to be in part due to excessive ROS production or a failure to adequately buffer the ROS accumulation. ROS are considered a form of free radical, which is defined as an atom or molecule in which there is one or more unpaired electron (Powers and Jackson 2008). Two types of free radicals that are formed are reactive nitrogen species (RNS) and ROS. Superoxide ($O_2\bullet^-$) and nitric oxide (NO) are the primary free radicals generated in cells, and due to their reactivity, lead to the formation of other ROS and RNS. These include hydrogen peroxide (H_2O_2), hydroxyl radicals (OH•), singlet oxygen, peroxynitrite, and hyperchlorite (Powers and Jackson 2008).

Recent evidence has surprisingly found that acute bouts of exercise or muscle contraction induce free radical or ROS generation in muscle (Davies et al 1982;

Jackson et al 1985; Reid et al 1992). It is thought that ROS generation is required for contraction-induced physiological adaptation; however excessive ROS production during contraction can also lead to oxidative damage to different cellular organelles (Davies et al 1982; Jackson et al 1985; Reid et al 1992). This suggests that there is an optimal range of ROS concentrations that contribute to the function of skeletal muscle, while excess ROS exposure leads to dysfunction of the muscle cell. Thus, understanding the mechanisms that regulate ROS production and buffering in skeletal muscle will be critical in developing a more comprehensive understanding of skeletal muscle function.

With excessive ROS and RNS and production, normal cell function can be hindered by the disruption of signaling pathways and damaging of organelles due to oxidative stress. The first definition of oxidative stress was "a disturbance in the pro-oxidant-antioxidant balance in favor of the former" (Sies 1985). Parameters used to characterize oxidative stress include increased formation of radicals and other oxidants, a decrease in small molecular weight or lipid-soluble anti-oxidants, disruptions in cellular redox balance, and oxidative damage to cellular constituents (Powers and Jackson 2008). Excessive oxidative stress has a role in the development of muscle fatigue, with many studies showing that the use of ROS antioxidants and scavengers delay the onset of muscular fatigue during submaximal contractions (Anzueto et al 1992; Khawli et al 1994; Reid et al 1992, Supinski et al 1997). Oxidative stress can also impact skeletal muscle force production by disrupting various pathways and mechanisms. For example, inhibition of SR calcium ATPase (SERCA) as a result of high ROS content induces interference with the ATP binding

site and uncoupling of calcium uptake from ATP hydrolysis (Xu et al 1997). Further, high levels of NO reduces SERCA activity through thiol oxidation (Viner et al 1997) and the nitration of tyrosine residues within protein (Viner et al 2000). Moreover, ROS accumulation has negative effects on myofilaments. For example, excessive ROS can alter myofilament structure (Callahan et al 2001), decrease calcium sensitivity (Andrade et al 2001), alter cross-bridge kinetics (Andrade et al 2001), and increase the likelihood of muscle fatigue (Moopanar and Allen 2005).

Although excessive ROS can have negative effects on muscle function, ROS have also been shown to be an important part of different cell signaling pathways. Over a short period of time, small increases in ROS production can lead to the activation of cellular signaling pathways that result in cell adaptation and protection against future stresses (Powers et al 2009). In skeletal muscle, exogenous H_2O_2 has been shown to increase glucose transport (Cartee and Holloszy 1990; Sorensen et al 1980). Although the exact mechanism of this ROS-mediated increase is unknown, possible explanations are increases in AMP-activated protein kinase (AMPK) or protein kinase B (Akt) activity. For example, H_2O_2 increases Akt phosphorylation, which is a critical mediator for glucose transporter type 4 (GLUT4) translocation (Higaki et al 2008; Jensen et al 2008). Oxidative stress also activates various mitogen-activated protein kinases (MAPK), which contribute to cellular regulation. These MAPK include activation of extracellular signal-regulated kinases (ERK1/2) which are necessary for cell survival (Chen et al 2001). In addition, activation of p38 is critical for the cellular response to osmotic stress and endotoxins (Chen et al 2001), and c-Jun N-terminal kinase (JNK) activation which regulates the dynamic activation

of apoptosis (Shen and Liu 2006). One other signaling pathway activated by increased ROS is the NF-κB pathway (Kabe et al 2005), which is thought to influence interleukin 6 (IL-6) production by skeletal muscle during exercise (Spangenburg et al 2006). Thus, understanding the mechanism and localization that mediates ROS production is critical for understanding skeletal muscle adaptability and susceptibility to disease.

For many years, many believed that the largest producer of ROS in skeletal muscle was the mitochondria. However, a number of recent experiments found that skeletal muscle produces superoxide via multiple intracellular sources, leading to the formation of other secondary ROS molecules (Reid et al 1992; McArdle et al 2001). Initial studies reported that between 2% and 5% of the total oxygen consumption by mitochondria resulted in a one-electron reduction of oxygen, leading to the generation of high amounts of superoxide (Boveris and Chance 1973; Loschen et al 1974). This finding by Britton Chance indicated that it was possible to induce a 50 to 100 fold increase in superoxide generation by skeletal muscle during aerobic contractions as a result of increased oxygen consumption by the mitochondria (Kanter 1994; Urso and Clarkson 2003). In fact, recent evidence suggests that ROS production during contraction is mostly derived from cytosolic sources and not the mitochondria (Xia et al 2003; Gong et al 2006), with the studies showing that the amount of superoxide created within the mitochondria may not be as large as once believed. For example, at least one study has suggested that the proportion of the electron flow giving rise to ROS during contraction was overestimated (St-Pierre et al 2002). In support of this finding, Michaelson and colleagues (2010) showed no change in mitochondrial redox

potential during contraction of muscle fibers, indicating a lack of mitochondrial ROS production. Studies using specific mitochondrial complex and channel inhibitors have also supported this result by showing no effect on the contraction induced increases in cytosolic superoxide (Sakellariou et al 2013). Through the use of MitoSOX red, a fluorescent dye specific to mitochondrial superoxide production (Aydin et al 2009; Grundtman et al 2010) investigators have found no increase in mitochondrial ROS production after bouts of tetanic stimulation. Thus, at this point it is unclear if the mitochondria are significant contributors to ROS production during repetitive muscle contraction. Much of the recent evidence utilized ex vivo imaging approaches with isolated single skeletal muscle fibers placed in culture dishes. However, most of these approaches utilize conditions that would likely encourage minimal activation of the mitochondria. For example, high stimulation frequencies coupled with media that does not contain metabolic substrates that would flux into the mitochondria. Thus, at this time the role of ROS production from the mitochondria during contraction is unclear.

Besides mitochondria, another potential source of ROS production is from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity. NADPH oxidase is an enzyme complex that can induce ROS production from molecular oxygen by using NADPH as an electron donor (Chan et al 2009). NADPH oxidase is found in various cell types, and is critical to processes such as cell proliferation and tissue repair (Chan et al 2009). In skeletal muscle, NADPH oxidase is localized to the sarcolemma, sarcoplasmic reticulum, T-tubules, and the mitochondria in skeletal muscle (Jackson 2013). Using dichlorofluorescein (DCF), a fluorescent ROS

indicator, coupled with repeated bouts of contraction, Michaelson and colleagues found that contraction increases ROS production in single muscle fibers through increases in NADPH oxidase activity (Michaelson et al 2010). In electrically stimulated myotubes, treatment with a nonspecific NADPH oxidase inhibitor, diphenyleneiodonium (DPI) was able to reduce the release of extracellular superoxide (Pattwell et al 2004). Phospholipase A_2, an enzyme which can induce ROS generation (Gong et al 2006; Zuo et al 2004), has also been shown to stimulate NADPH oxidase (Zhao et al 2002). Current thinking has suggested that during contraction, the transfer of electrons from cytosolic NADPH to the plasma membrane via different pathways leads to the generation of superoxide at the cell surface (Powers and Jackson 2008).

Recent advances in the ability to isolate, culture, and stimulate single muscle fibers have afforded investigators an improved ability to assess ROS production and localization under a variety of conditions. As previously stated, the majority of experiments conducted have examined ROS content in optimized buffered conditions that provide the single muscle fibers only with glucose or no exogenous metabolic substrate source. A weakness in this method is that cells will primarily use glycolysis for ATP production when only glucose is available; thus, mitochondrial activation potential would remain low. However, it is unclear if the source of ROS would still be largely cytosolic if the cultured single muscle fibers were provided a source of fatty acids during the contractile activity, since fatty acids must be channeled through the mitochondria for ATP synthesis. It has been shown for many years that during contraction or exercise, fatty acids are a critical source of ATP replenishment for

meeting the energetic demand of muscle (Hurley et al 1986). Thus, the purpose of these experiments is to determine the source of ROS production in single muscle fibers cultured with different metabolic substrates.

Specific aim 1: To determine if exposure to extracellular metabolic substrate affects intracellular ROS production during repetitive contraction in isolated single muscle fibers

Hypothesis 1a: In contracting single muscle fibers, the presence of physiologically relevant levels of extracellular glucose will result in increased ROS production compared to single muscle fibers exposed to no glucose

Hypothesis 1b: In contracting single muscle fibers, the presence of physiologically relevant levels of extracellular free fatty acids will result in increased ROS production compared to single muscle fibers exposed to no free fatty acids

-Note: During the course of data collection, it was determined that the originally proposed methodological approaches in Specific Aim 2 were not feasible for multiple reasons. A portion of the data demonstrating the issues encountered with the original approaches will be presented. Thus, Specific Aim 2 and corresponding hypotheses were adjusted to address the original goal of the Specific Aim with a different methodological approach. The revised Aim with the new methodological approach is consistent with my overall research question.

Original Specific aim 2: Originally, Specific Aim 2 sought to determine the intracellular source of ROS production in contracting single muscle fibers in the presence of different metabolic substrates.

Original Hypothesis 2a: Inhibition of NADPH oxidase will attenuate ROS production in contracting single muscle fibers only in the presence of glucose and not in the presence of extracellular free fatty acids

Original Hypothesis 2b: Exposure of single muscle fibers to a mitochondrial specific antioxidant will attenuate ROS accumulation in single muscle fibers in the presence of physiologically relevant levels of free fatty acids, but not in the presence of glucose

Revised Specific Aim 2: To determine if repetitive contraction in isolated single muscle fibers affects mitochondrial membrane potential in the presence of a metabolic substrate that contributes to ROS production.

Revised Hypothesis 2: Single muscle fibers exposed to physiologically relevant levels of extracellular palmitate will exhibit a collapse of the mitochondrial membrane potential without exposure to repetitive contraction.

Methods

Fiber Isolation: Adult male C57Bl/6 mice between 8 to 10 weeks in age were used for fiber isolation (n=21). Mice were placed in an anesthetizing box with 4% isoflurane. After the mouse was anesthetized, it was removed from the box and placed in a

rodent face mask to maintain anesthesia. The hind limbs were removed and placed in chilled Krebs-Ringer solution (KRB) (pH 7.2, 115mM NaCl, 2.5mM KCl, 1.8mM $CaCl_2$, 2.15mM Na_2HPO_4, 0.85mM NaH_2PO_4)., and the mouse was euthanized. The hind limb was then pinned down to cork board, and the flexor digitorum brevis muscle (FDB) was surgically removed. The muscle was placed in a 6-well plate of 2ml of dissociation media containing DMEM (ATCC, Manassas, VA) and collagenase A (Roche Diagnostics, Mannheim, Germany) (4mg/ml), and was incubated at 37°C, 5% CO_2 for 2 hours. The muscle was then triturated with a 3ml transfer pipette until it had dissociated, yielding single muscle fibers, and the isolated fibers were placed in media with no collagenase overnight at 37°C, 5% CO_2. After overnight incubation, the muscle fibers were moved to a subsequent well in the 6-well plate containing 2ml of one of the following: KRB, 5mM glucose in KRB, 30μM palmitate (prepared as bovine serum albumin conjugated palmitate) in KRB, or 100 μM Mito-TEMPO (Enzo Life Sciences, Inc, Farmingdale, NY), a mitochondria-targeted antioxidant, in KRB. Fibers were incubated at room temperature for 2 hours. For the Mito-TEMPO condition, fibers were first incubated in KRB for 1 hour and 30 minutes, and then incubated with 100μM Mito-TEMPO for 30 minutes, for a total incubation time of 2 hours.

Fiber Stimulation:

DCF: Two-hundred and fifty microliters of solution containing single muscle fibers was transferred to a custom-built perfusion/electrical stimulation chamber (Fig. 1) (Four Hour Day Foundation, Towson, Maryland; thanks to Dr. R. Lovering for use of the chamber) with 250μl of KRB at room temperature. One microliter of 10mM DCF

(Life Technologies) was added to the stimulation chamber to yield a DCF concentration of 10µM, and fibers were incubated in the dark for 10 minutes. Fibers were stimulated using the S48 Stimulator (Grass Technologies) with pulses at a frequency of 80Hz, 400ms train duration of 2ms pulses, as previously described (Michaelson et al 2010). Prior to capturing images, fibers were observed to ensure that they were responding to stimulation. Once contraction in the fiber had been seen, image capturing began. In total, images were captured for 12 fibers per condition.

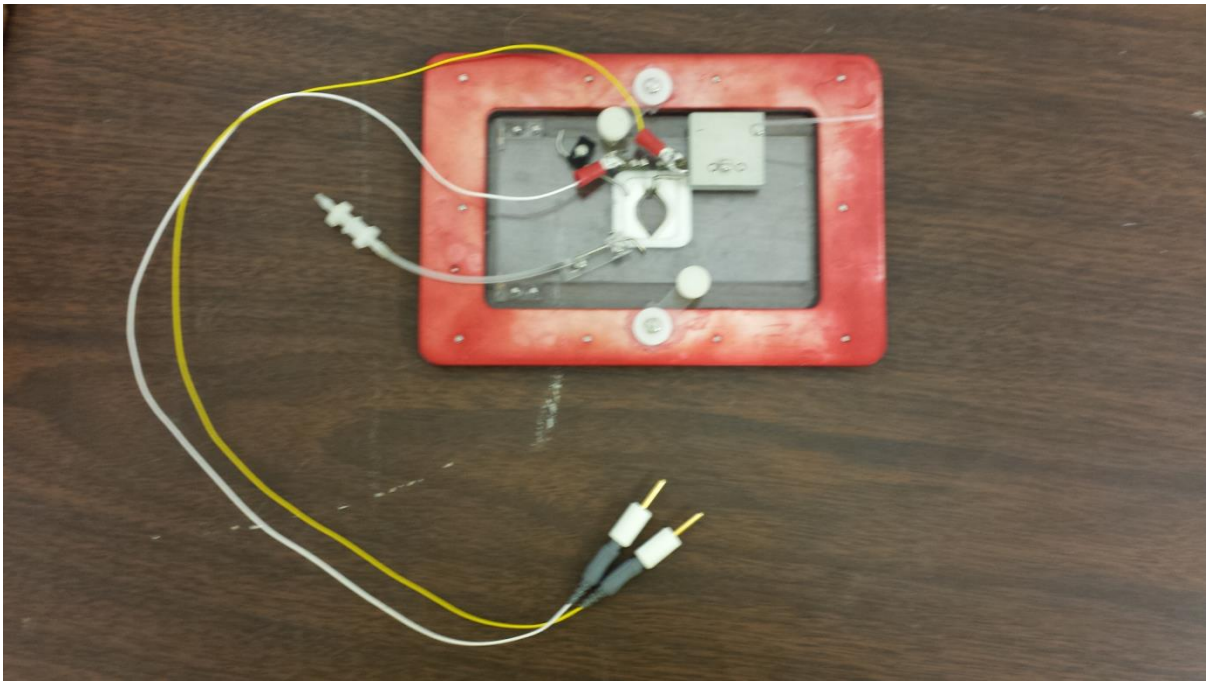

Figure 1: Custom-built perfusion/electrical stimulation chamber (Four Hour Day Foundation, Towson, Maryland)

MitoSOX Red: Four microliters of 5mM MitoSOX Red (Invitrogen, Life Technologies) was added to the 6 well plate containing single muscle fibers to yield a MitoSOX Red concentration of 5µM, and fibers were incubated in the dark for 10 minutes. Two-hundred and fifty microliters of solution containing single muscle fibers was transferred to the stimulation chamber with 250µl of KRB at room

temperature. Fibers were stimulated using the S48 Stimulator with pulses at a frequency of 80Hz, 400ms train duration of 2ms pulses. Prior to capturing images, fibers were observed to ensure that they were responding to stimulation. Once contraction in the fiber had been seen, image capturing began. Images were captured for 8 fibers per condition.

TMRE: Ten microliters of 10μM tetramethylrhodamine, ethyl ester (TMRE) was added to the 6 well plate containing single muscle fibers to yield a TMRE concentration of 50nM, and fibers were incubated in the dark for 10 minutes. Two-hundred and fifty microliters of solution containing single muscle fibers was transferred to the stimulation chamber with 250μl of KRB at room temperature. Fibers were stimulated using the S48 Stimulator with pulses at a frequency of 80Hz, 400ms train duration of 2ms pulses. Prior to capturing images, fibers were observed to ensure that they were responding to stimulation. Once contraction in the fiber had been seen, image capturing began. Images were captured for 17 fibers per condition.

As positive control, for TMRE function, single muscle fibers were exposed to carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (Enzo Life Sciences, Inc, Farmingdale, NY). FCCP is known to induce uncoupling of the mitochondria and loss of mitochondrial membrane potential. Ten microliters of 10μM TMRE was added to the 6 well plate containing single muscle fibers to yield a TMRE concentration of 50nM, and fibers were incubated in the dark for 10 minutes. Two-hundred and fifty microliters of solution containing single muscle fibers was transferred to the stimulation chamber with 250μl of KRB at room temperature. Fibers were imaged at baseline, incubated in 200nm FCCP for 10 minutes, and

imaged again. Prior to capturing images, fibers were observed to ensure that they were responding to stimulation. Once contraction in the fiber had been seen, image capturing began. Images were captured for 7 fibers per condition.

Fiber Imaging and Analysis: Fibers were observed using the Axio Observer Z.1 epifluorescence microscope (Zeiss). Fluorescence was captured at baseline and every 2 minutes during contractile activity for 10 minutes. Minimum and maximum fluorescence was established by loading fibers with 10mM dithiothreitol (DTT) for a fully reduced condition, and 1mM H_2O_2 for a fully oxidized condition (n=3 fibers per condition). The intensity of fluorescence was measured using ImageJ, and compared to the baseline conditions. The total area of the muscle fiber was selected free hand and both area and integrated density were measured. This was repeated twice for each fiber. The same measurements were then made for the background of the image. Intensity of fluorescence was determined by calculating the corrected total cell fluorescence (CTCF) for each image. CTCF is calculated in the following way: integrated density – (area of fiber x mean fluorescence of background reading). CTCF at each time point was then compared to CTCF at baseline.

Statistics: Statistical Analysis System (SAS) software was used to for data analysis. For both Specific Aim 1 and Specific Aim 2, statistical significance was determined using a two-way repeated measures ANOVA. When an interaction was found, the test was followed by a Student Neuman-Keuls test for post-hoc analysis. A p value of <0.05 was considered significant.

Results

Krebs-Ringer:

DCF fluorescence was measured in single muscle fibers after incubation in KRB. DCF fluorescence is presented as percent increase from baseline, with and without electrical field stimulation. Significant increases in DCF fluorescence from the 0 time point was observed at 6 and 10 minutes after the initiation of the electrical stimulation (Fig. 3). No significant differences were found in the unstimulated conditions at any time point (Fig. 3). However, significant differences were detected between the stimulated and unstimulated conditions at the 10 minute time point.

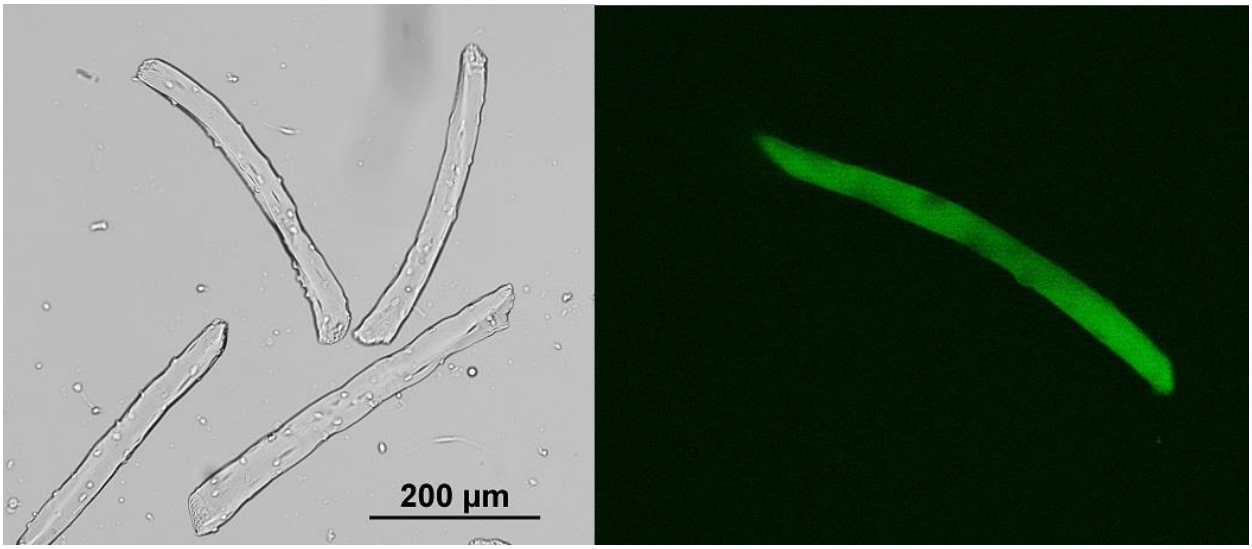

Figure 2: Single muscle fibers isolated from the FDB muscle imaged using brightfield microscopy and the fluorescent probe DCF

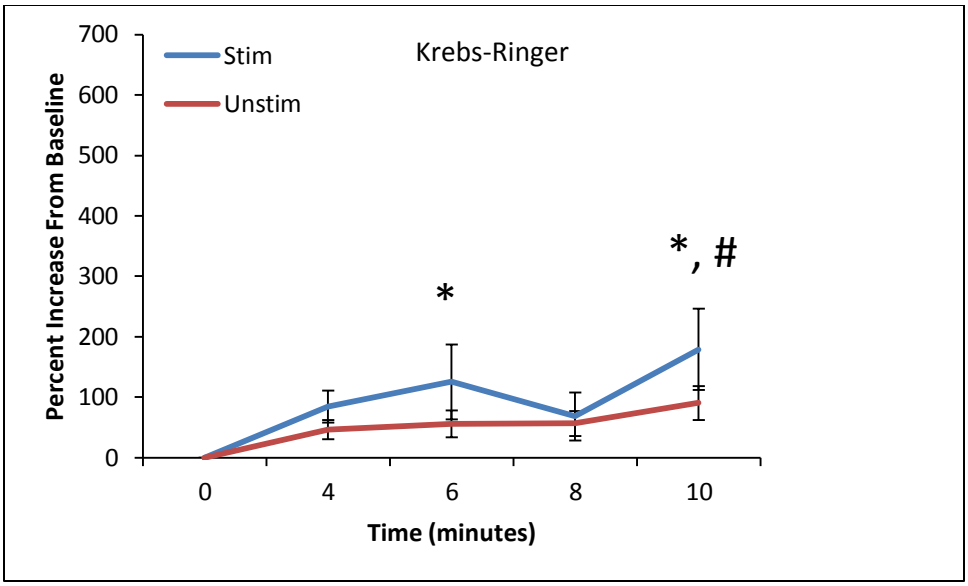

Figure 3: DCF fluorescence measured in single muscle fibers after incubation in Krebs-Ringer. No significant differences in percent increase from baseline were observed at any time point between the stimulated (n=10) or unstimulated (n=9) groups. Bars represent means ± SEM. * indicates significantly different from 0 time point for Stim group only ($p<0.05$), # indicates significantly difference between Stim and Unstim groups ($p<0.05$).

Glucose:

DCF fluorescence was measured in single muscle fibers after incubation in KRB supplemented with 5mM glucose, with and without electrical field stimulation. Significant differences in DCF were detected between the 0 min time point and the 10 min time point in the unstimulated condition (Fig. 4). No significant differences over time were detected in the stimulated condition. No significant differences were seen between the stimulated and unstimulated conditions.

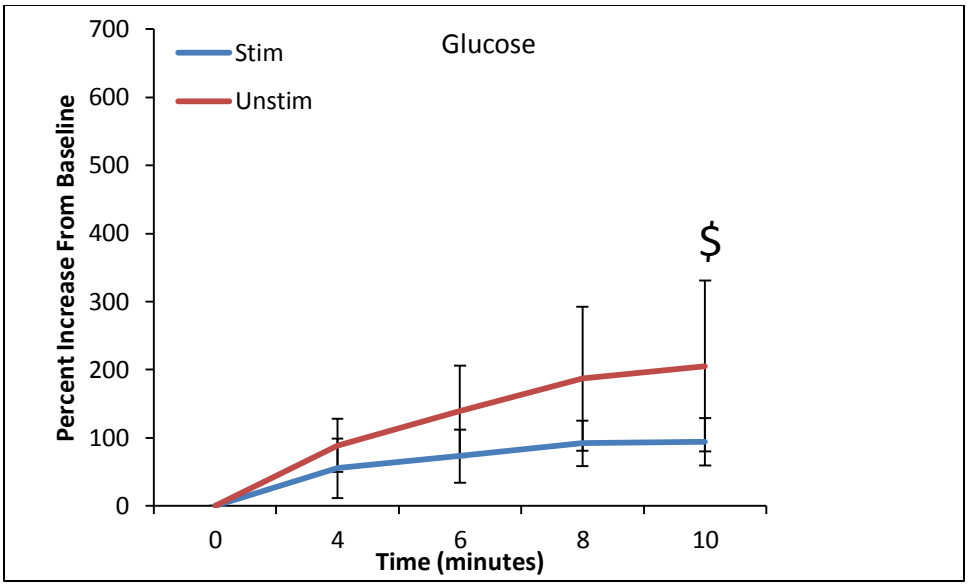

Figure 4: DCF fluorescence measured in single muscle fibers after incubation in glucose. No significant differences in percent increase from baseline were observed at any time point between the stimulated (n=11) or unstimulated (n=12) groups. Bars represent means ± SEM. $ indicates significantly different from 0 time point for Unstim group only ($p<0.05$).

Palmitate:

DCF fluorescence was measured in single muscle fibers after incubation in KRB supplemented with 30µM palmitate with or without electrical field stimulation. Significant differences in DCF fluorescence from the 0 time point were observed at 6, 8, and 10 minutes in both the stimulated and unstimulated conditions (Fig. 5). No significant differences were seen between the stimulated and unstimulated conditions.

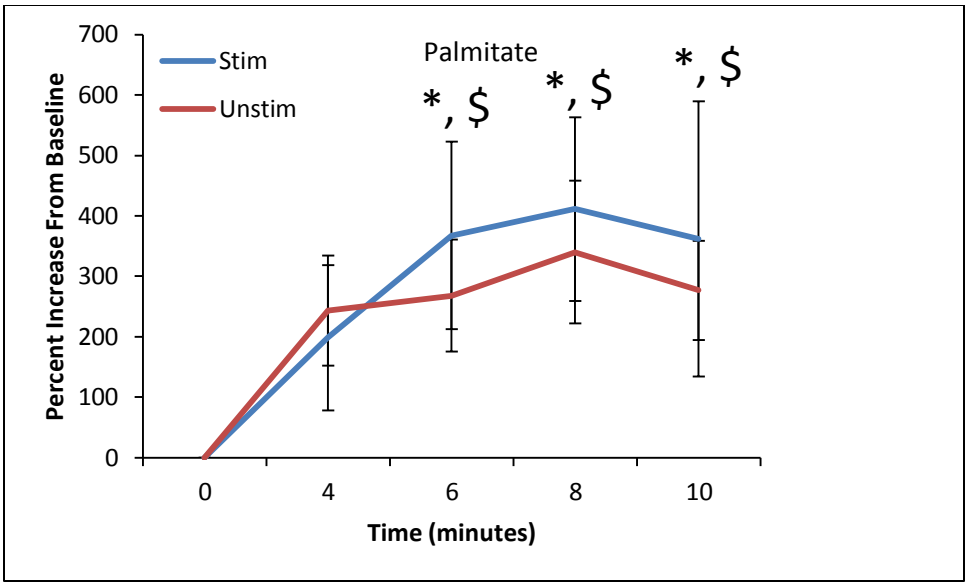

Figure 5: DCF fluorescence measured in single muscle fibers after incubation in palmitate. No significant differences in percent increase from baseline were observed at any time point between the stimulated (n=10) or unstimulated (n=10) groups. Bars represent means ± SEM. . * indicates significantly different from 0 time point for Stim group only ($p<0.05$), $ indicates significantly different from 0 time point for Unstim group only ($p<0.05$).

Mito-TEMPO:

In order to assess the origin of ROS production during contraction, DCF fluorescence was measured in single muscle fibers after incubation in KRB supplemented with the mitochondria-targeted antioxidant Mito-TEMPO with or without electrical field stimulation. Surprisingly, DCF fluorescence increased with the Mito-TEMPO treatment (Fig. 6) at 4 and 6 minutes in the stimulated and at 4, 6, 8, and 10 minutes in the unstimulated conditions. The increase induced by the Mito-TEMPO exposure was not a result of the dilution vehicle (data not shown). No significant differences were observed between the stimulated or unstimulated conditions (Fig. 6). In addition, a surprisingly high number of fibers treated with Mito-TEMPO and exposed

to electrical stimulation died during experiment (4 out of 8). In contrast, no fibers in the unstimulated condition died. No significant differences were seen between the stimulated and unstimulated conditions at any time point.

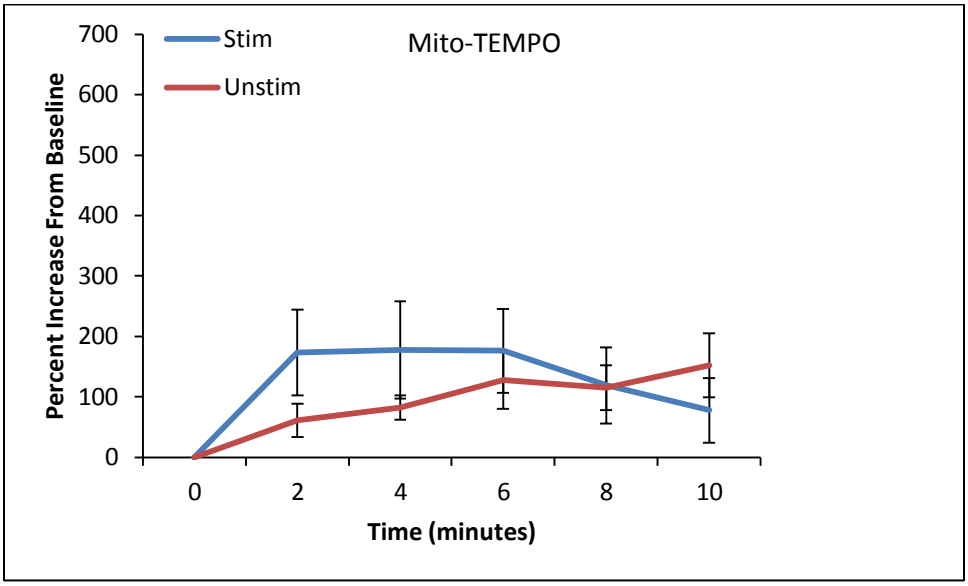

Figure 6: DCF fluorescence measured in single muscle fibers after incubation in Krebs-Ringer and Mito-TEMPO. No significant differences in percent increase from baseline were observed at any time point between the stimulated (n=8) or unstimulated (n=8) groups. Bars represent means ± SEM.

MitoSOX Red: To assess mitochondria-specific ROS production, MitoSOX red fluorescence was measured in singe muscle fibers after incubation in KRB supplemented with the mitochondria-targeted antioxidant Mito-TEMPO, with or without electrical field stimulation. Interestingly, MitoSOX fluorescence was found to be localized in the nuclei. Upon further research, it was discovered that the use of a filter with excitation at 396nm and emission greater than 560nm was needed to observe mitochondria-specific fluorescence with MitoSOX red (Pearson et al 2014). The microscope used for our experiment only had a filter wit excitation at 525nm, resulting in non-specific fluorescence.

TMRE:

To determine if the increased ROS production leads to an alteration in mitochondrial membrane potential, single muscle fibers were loaded with TMRE. Numerous pieces of literature have hypothesized that excess mitochondrial ROS production results in mitochondrial uncoupling and leads to membrane potential collapse. Our results demonstrated that exposure of the single muscle fibers to palmitate led to high ROS production, thus these experiments were completed with palmitate exposure. TMRE fluorescence was measured in single muscle fibers after incubation in KRB supplemented with 30μmM palmitate, as percent change from baseline, with and without electrical field stimulation. No significant differences were observed between the stimulated or unstimulated conditions (Fig. 7). No significant differences were seen with time in both the stimulated and unstimulated conditions. As a positive control, TMRE loaded single muscle fibers were treated with FCCP, a mitochondrial uncoupler, which resulted in a significant 25% decrease in TMRE fluorescence (data not shown).

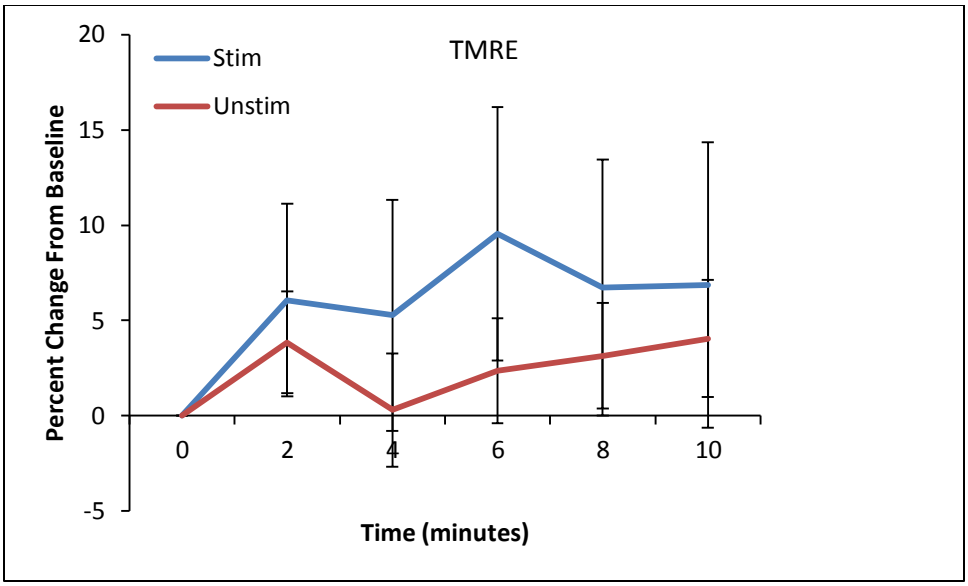

Figure 7: TMRE fluorescence measured in single muscle fibers after incubation in palmitate. No significant differences in percent change from baseline were observed at any time point between the stimulated (n=17) or unstimulated (n=17) groups. Bars represent means ± SEM.

Discussion

In this study, the results demonstrate that ROS production was influenced by alterations in extracellular metabolic substrate exposure. The largest increases in ROS generation were seen when muscle fibers were cultured with physiological concentrations of extracellular palmitate, with significant increases found over time with and without contraction. However, no significant differences were seen over 10 minutes between contracting and non-contracting fibers. Modest increases in ROS generation were found when the muscle cells were exposed to glucose, suggesting that extracellular substrate exposure influences ROS production. Mitochondrial membrane potential was determined with TMRE exposure. The results found no changes in TMRE, indicating the large increases in ROS production do not always result in alterations to mitochondrial membrane potential. Further, the results

demonstrate that increasing energetic demand on the cell through active muscle contraction does not result in reduced ROS production when single muscle fibers are exposed to extracellular palmitate. Likewise, no significant differences in mitochondrial membrane potential were observed between the contracting and non-contracting muscle fibers. The results provide novel insight into the importance of considering metabolic flux when assessing ROS dynamics in skeletal muscle cells.

Many studies have shown that with contraction, ROS production is increased in single muscle fibers (Sakellariou et al 2013, Michaelson et al 2010, Pearson et al 2014). A number of these studies have implicated NADPH oxidase as a major source of ROS production during contraction, and downplayed the contributions of the mitochondria (Sakellariou et al 2013, Michaelson et al 2010). However, in these experiments, single muscle fibers were cultured and exposed to electrical stimulation in the presence of glucose or no metabolic substrate at all. The fact that muscle fibers were in the presence of glucose or no metabolic substrate is an important factor to consider in the interpretation of the results, as ATP production would be primarily derived from glycolysis due to lower activation of the mitochondria. Thus, it is not surprising that low levels of ROS are generated from the mitochondria during contraction under these conditions. Mitochondrial activation is largely driven by metabolic flux and in order to ensure activation of the mitochondria, the culture conditions would likely need to provide substrates that are specifically catabolized by the mitochondria (i.e. fatty acids).

To my knowledge, this is the first study in which ROS production was measured in contracting single muscle fibers in the presence of a fatty acid

(palmitate). In previous studies, incubation of resting (i.e. non-contracting) skeletal muscle with physiological concentrations of palmitate resulted in increased ROS production attributed to the mitochondria and NADPH oxidase. (Lambertucci et al 2008). Indeed I found high levels of ROS production measured by DCF fluorescence with palmitate incubation, regardless if the fibers were exposed to contraction. Surprisingly, the introduction of contraction induced by electric field stimulation did not reduce the amount of ROS produced when the fibers were exposed to palmitate. Anderson and colleagues have hypothesized that an excess flux of lipid into skeletal muscle will cause a surplus of reducing agents and an elevation of the redox state of complex I of the mitochondria, because the energetic demand of the muscle does not match the rate of metabolic flux into the mitochondria (Anderson et al 2009). Specifically, it was proposed that at rest, the rate of electron leak from complex I is highly sensitive to the redox state, meaning the excess of reducing agents would lead to an increase in ROS production from the mitochondria in muscle (Anderson et al 2009). However in contrast, with contraction, the increase in energetic demand would be expected to mitigate the oxidative stress on the mitochondria in the presence of palmitate due to enhanced coupling of mitochondria. Mitochondria uncoupling occurs when substrate flux exceeds energetic demand; thus, mitochondria must release pressure on the membrane potential by allowing for ROS production. However, our results show the same increase in ROS production occurred with repetitive contraction in the presence of palmitate. Further, no decreases in TMRE signal was detected under either condition, suggesting the mitochondrial membrane potential was maintained in the face of high ROS production. It should be noted that

treatment of the fibers with FCCP (a mitochondrial uncoupler) resulted in a significant loss in TMRE signal, confirming that TMRE conditions were appropriate. Current dogma suggests that repetitive contraction should decrease the surplus of reducing agents due to increases in energetic demand; however, our data are in conflict with this interpretation. My results would suggest that a direct relationship between energetic demand and the generation of ROS production might not exist in skeletal muscle.

Our data likely conflicts with the current dogma because the dogma was established by inducing state 4 respiration in the muscle cells. State 4 respiration is a direct measure of mitochondrial oxygen under conditions of zero to low ATP demand. State 4 respiration is typically achieved by exposing the mitochondria or cells to oligomycin, a potent ATP synthase inhibitor. State 4 respiration is a non-physiological state of the cell that investigators use to assess minimal oxygen respiration kinetics of the mitochondria. In previous studies using oligomycin to induce state 4 respiration, there have been substantial increases in ROS generation by the mitochondria (Tan et al 1998, Roy et al 2008). In most studies of skeletal muscle, state 4 respiration was induced by oligomycin in permeabilized muscle fibers (Anderson et al 2009, Larsen et al 2012) or isolated mitochondria (Hoeks et al 2012, Adjeitey et al 2013). In my study, single muscle fibers were kept intact and cultured; thus, the fibers would have a basal energetic demand and would not exist in state 4 respiration by definition. My results indicate that increasing energetic demand with contraction does not significantly affect ROS generation in the presence of palmitate. Thus, my data do not collectively support the concepts of enhanced ROS production

due to increased pressure on the mitochondria as a result of increased substrate flux. This is likely due to investigators use of an artificial state 4 respiration approach.

Another aim of this study was to determine the primary source of ROS generation under rest and contracting conditions. Based on previous literature, both the mitochondria and NADPH oxidase have been implicated in ROS generation during contraction (Muller et al 2004, Barja 1999, Michaelson et al 2010, Pearson et al 2014). To determine which of these sources contributed to ROS generation during contraction, I pre-treated my cells with Mito-TEMPO, a mitochondrial-targeted antioxidant. However, during the course of data collection with Mito-TEMPO, I continually found large increases in ROS generation when using DCF. Although initially surprising to us, the finding is actually quite important. Mito-TEMPO is a mitochondrial specific chemical superoxide dismutase (SOD) mimetic that converts mitochondrial O_2^- into H_2O_2, and because DCF fluorescence is a non-specific ROS indicator, the rise in DCF signal likely means increased ROS generation from the mitochondria. Specifically, it appears that mitochondria are in fact producing O_2^- in response to muscle contraction. Another interesting observation, but one that we did not assess statistically, was that fibers exposed to Mito-TEMPO and contraction often died, suggesting that enhanced O_2^- buffering in the mitochondria is harmful to the muscle cell. Because of the non-specificity of DCF, a new approach was taken to determine the source of ROS generation. Instead of DCF, MitoSOX Red was used, which is described as a specific indicator of mitochondrial O_2^- production. In previous studies of contracting skeletal muscle, MitoSOX Red has been used successfully as probe for mitochondrial ROS generation (Pouvreau 2010, Pearson et

al 2014). However, when used in this study, fluorescence was not observed in the mitochondria, but instead the signal was prominently found in the myonuclei. MitoSOX Red is described as a specific indicator of O_2^- produced by the mitochondria (Mukhopadhyay et al 2007); however, using a detailed literature search, I have found literature that suggests MitoSOX Red does not always specifically react to mitochondrial O_2^-.

MitoSOX Red, also known as Mito-HE, is supposed have increased localization to the mitochondria compared to its counterpart hydroethidine, or HE. However, if a cellular compartment has a more negative membrane potential, MitoSOX Red can co-localize in this location as well (Kalyanaraman et al 2014). In my experiments, fluorescence from MitoSOX Red was strongest in the nuclei (data not shown). Also, when using MitoSOX Red fluorescence, multiple products can be formed that have similar emission spectra. When HE and Mito-HE react with O_2^-, a red fluorescent product, 2-hydroxyethidium (2-OH-E^+) is formed (Zielonka and Kalyanaraman 2010). In a system where only O_2^- is being generated, this would be the only expected product of the reaction, but inside of the skeletal muscle fiber, there are other compounds and metals that are present that can react with MitoSOX Red to produce ethidium (E^+) (Kalyanaraman et al 2014). Unfortunately, E^+ also is a red fluorescent product with an overlapping spectra to 2-OH-E^+, with previous studies showing about a ten-fold higher level of E^+ formation compared to 2-OH-E^+ formation (Zhao et al 2005, Zielonka and Kalyanaraman 2010). This means that any changes in 2-OH-E^+ levels due to the reaction between Mito-HE and O_2^- would likely be masked by the fluorescence of E^+, making it difficult to properly interpret the

results of my study. It was recently discovered that MitoSOX Red can be specific to mitochondrial O_2^-, but only when excited at a wavelength between 385-405nm, which is not consistent with the product insert. Unfortunately, due to limitations of our microscope, it is not possible to excite at this wavelength and emit in the red wavelength. Thus, we concluded that data derived from the use of MitoSOX Red was likely non-specific to mitochondrial O_2^-, and we did not continue with the MitoSOX Red work as planned.

Due to the ineffectiveness of MitoSOX Red as a fluorescent probe, the approach for Specific Aim 2 was changed to address the response of the mitochondrial membrane potential in contracting and non-contracting single muscle fibers under conditions of increased ROS production. Based off of the results of palmitate incubation in Specific Aim 1, it was hypothesized that there would be a significant decrease in mitochondrial membrane potential in non-contracting single muscle fibers incubated in palmitate; however, no changes would be detected in contracting single muscle fibers incubated in palmitate. Surprisingly, no significant changes in mitochondrial membrane potential were observed in either contracting or non-contracting single muscle fibers. The fact that no change in mitochondrial membrane potential was seen in non-contracting fibers is important because it demonstrates that mitochondrial function is not significantly altered in muscle cells *ex vivo*. These results also suggest that while short term exposure to fatty acid may induce an increase in ROS generation in skeletal muscle, this increase in ROS production is not enough to compromise mitochondrial function. To date, there has been little research done on assessing mitochondrial membrane potential in

contracting single muscle fibers. In a study of contracting single muscle fibers monitoring mitochondrial Ca^{2+} uptake, it was shown that Ca^{2+} localization was co-localized with tetramethylrhodamine, methyl ester (TMRM), which is also used to assess mitochondrial membrane potential (Rudolf et al 2004). However, only the change in Ca^{2+} uptake was assessed with contraction, with TMRM fluorescence only being assessed at baseline. One study has been done by Gandra and colleagues monitoring mitochondrial membrane potential in contracting single muscle fibers from *Xenopus laevis*. This study found that with repetitive contraction, mitochondrial membrane potential initially decreases, but is quickly restored within the first minute of contractions to levels that are not significantly different from baseline (Gandra et al 2012). The results of my study are in agreement with these findings, with no significant difference seen in mitochondrial membrane potential over a 10 minute period of repetitive contraction. One possible approach to take in future studies would be perform a similar experiment with TMRE using skeletal muscle fibers from obese mice on a high fat diet. In a study of mitochondria isolated from human skeletal muscle of lean and obese individuals, mitochondrial H_2O_2 emission was significantly higher in obese subjects than the lean subjects (Anderson et al 2009). It was also found that mitochondria in permeabilized muscle fibers from lean individuals had increased mitochondrial H_2O_2 emission during state 4 respiration four hours after a high fat meal; however, it is unclear if this finding would be confirmed under more physiological conditions. It would be interesting to use the same approach from Specific Aim 2 to see if there is also any difference in mitochondrial membrane potential between mitochondria of lean and obese individuals, while also examining if

there any differences in ROS generation. Using the approach from Specific Aim 2 would allow for an *ex vivo* analysis of mitochondrial function in intact skeletal muscle fibers, which would likely be a more accurate representation of *in vivo* mitochondrial function.

The results of this study show that ROS generation in both contracting and non-contracting single muscle fibers is influenced by extracellular metabolic substrate exposure. In addition, the findings confirm that contraction does result in significant elevations in ROS; however, our data do indicate that that ROS generation is occurring in the mitochondria, which contradicts recent results (Sakellariou et al 2013). The largest increases in ROS generation were seen in muscle fibers incubated with palmitate. However, the increases in ROS generation under these conditions were not enough to induce a decrease or collapse in mitochondrial membrane potential as one might predict. This suggests that the acute increases in ROS generation and fatty acid exposure are not enough to cause mitochondrial dysfunction. In the future, it will be necessary to assess the effects of chronic lipid exposure to better understand their long-term effects on ROS generation and mitochondrial function, and their contributions to different diseases states. Overall, the data collected in this thesis demonstrate the importance of considering metabolic substrate conditions in the media, and also provide preliminary evidence that the use of state 4 respiration approaches may be providing misleading results to the field concerning the induction of mitochondrial pressure by nutrient overload.

Review of Literature

Skeletal muscle is a multi-function tissue

Skeletal muscle is a striated tissue composed of interconnected sarcomeres that contain organized bundles of myofibrils. Skeletal muscle is required for movement, breathing, posture, heat production, and metabolic substrate storage.

Skeletal muscle contraction. For movement to occur, skeletal muscle requires input from the nervous system via motor nerve. Activation of the motor neuron induces acetylcholine release into neuromuscular junction resulting in the generation of an action potential on the sarcolemma of the skeletal muscle fiber. The depolarization of the membrane signals Ca^{2+} to be released from the sarcoplasmic reticulum (SR), resulting in calcium binding to troponin and inducing a conformational shift in tropomyosin. This shift encourages strong binding of the myosin and actin filaments, resulting in force production by the muscle cell. In order for force development to occur, all components of a motor unit (motor neurons and muscle fibers) need to be properly functioning. For example, contraction of the diaphragm ensures adequate respiration, with dysfunction of this muscle contributing to respiratory failure (Moxham et al 1981). In degenerative diseases such as ALS and muscular dystrophies, dysfunction or weakness of the respiratory muscles can occur and potentially be fatal (Boillee et al 2006, Laghi and Tobin 2003). Thus, understanding mechanisms that influence force production is important.

When skeletal muscles contract, heat is produced as a result of ATP being hydrolyzed. For example, it has been estimated that up to 85% of heat produced from

shivering comes from muscular contraction (Bell et al 1992), so it is important for skeletal muscle to be functioning properly to regulate body temperature.

Metabolic substrate storage. Skeletal muscle is also used to store metabolic substrates such as glucose or free fatty acids in the form of glycogen or triglycerides, respectively. Due to the volume of skeletal muscle in the body, the majority of glycogen is stored in skeletal muscle (approximately 500g). Glycogen is a preferred substrate during repetitive contraction and can quickly be broken down into glucose and used to produce ATP during times of energetic stress (Jensen et al 2011). Triglycerides (TAG) are predominantly stored in lipid droplets that are found intramuscular and intermuscular regions contributing to energy production via the mitochondria during bouts of prolonged exercise (Gorski 1992). With endurance exercise training, both glycogen storage (Greiwe et al 1999) and TAG storage (van Loon and Goodpaster 2006) increase demonstrating their importance for times of energetic demand.

Secretory potential of skeletal muscle. Skeletal muscle has the ability to release cytokines, known as myokines, into circulation. These myokines include myostatin, IL-6, and insulin-like growth factor I (IGF-I), and can have autocrine, paracrine, and endocrine effects (Pedersen and Febbraio 2012). Myostatin knockout mice exhibit significant muscle hypertrophy, showing that it plays a role in regulating skeletal muscle growth (McPherron et al 1997). IL-6 released from skeletal muscle was shown to have an effect on the pancreas, increasing glucagon-like peptide-1 (GLP-1) secretion, and thus insulin secretion (Ellingsgaard et al 2011). IGF-I is produced in skeletal muscle in response to mechanical loading (Turner et al 1988). Receptors for

IGF-1 are localized to the periosteum at the muscle-bone interface, suggesting that muscle-derived IGF-1 contributes to bone growth and formation (Hamrick et al 2010).

Energy derived from ATP is critical for muscle function

Energy is required for many cellular processes in skeletal muscle, and this energy is released from the hydrolysis of ATP. Force production by skeletal muscle is a result of active cross bridge cycling. The cross-bridge cycle is an ATP dependent process. The myosin ATPase localized in the S-1 head of myosin hydrolyzes ATP during the cycle (Sugi 1993) allowing for the detachment of myosin and actin (Huxley 1957). ATP is also required for pumping Ca^{2+} ions back into the SR via SERCA located in the membrane of the SR. SERCA activation has two important functions: lowering cytosolic Ca^{2+} concentration to cause muscle relaxation and restoration of the SR Ca^{2+} concentration to allow for a subsequent contraction to occur (Periasamy and Kalyanasundaram 2007). For every one molecule of ATP that is hydrolyzed, two Ca^{2+} ions can be transported by SERCA back into the SR (Periasamy and Kalyanasundaram 2007). Another cellular function that ATP is required for is the pumping of Na^{+} and K^{+} ions across the sarcolemma to allow for restoration of the membrane potential after the completion of an action potential. Na-K-ATPase is a transport protein expressed in many different cell types that hydrolyses ATP to transport Na^{+} and K^{+} ions across cell membranes. Na-K-ATPase is critical for the maintenance of osmotic balance, resting membrane potential, and the excitable properties of muscle cells (Blanco and Mercer 1998). One last cellular process that

requires ATP is glycolysis. Glycolysis is metabolic pathway that allows catabolizes a glucose molecule to produce four molecules of ATP. However, in order to completely catabolize glucose to pyruvate two molecules of ATP are required to convert glucose into glucose-6-phosphate and fructose-6-phosphate into fructose-1,6-biphosphate (Kim and Dang 2005). Thus, although four ATP molecules are yielded from glycolysis, two ATP molecules are first required to complete the pathway.

Mitochondria are an important organelle for proper skeletal muscle function

In skeletal muscle, mitochondria are necessary for aerobic respiration, and produce a majority of the ATP needed for various cellular processes. Mitochondrial dysfunction is often associated with reduced skeletal muscle function since the mitochondria is a critical buffer to various types of energetic stress. Patients with T2D exhibit decreased peripheral insulin sensitivity (Pirola et al 2004), which is often associated with decreased mitochondrial function. In patients with T2D, genes associated with oxidative phosphorylation, such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and nuclear respiratory factor 1 (NRF-1) have been shown to be significantly decreased compared to healthy controls (Mootha et al 2003, Patti et al 2003). Studies examining the offspring of people with T2D have seen reduced mitochondrial density, ATP production, and mitochondrial mRNA levels coupled with insulin resistance (Peterson et al 2004, Morino et al 2005, Morino et al 2012). Another case where mitochondrial dysfunction may contribute to reduced skeletal muscle function is aging. Although muscle mass and strength both decline with age, studies have shown that loss in strength precedes loss in muscle

mass (Metter et al 1999, Goodpaster et al 2006). With aging, changes in mitochondria such as decreased total volume, oxidative capacity, and biogenesis are thought to contribute to decreased muscle quality (Peterson et al 2012). Elderly adults with reduced mitochondrial activity have increased lipid accumulation and reduced glucose tolerance, supporting the idea of a reduction of muscle quality with aging (Johannsen et al 2012). Another piece of evidence that mitochondrial dysfunction plays a role in skeletal muscle aging is the polymerase gamma mutator (PoIG) mouse model. In this model, there is an accumulation in somatic mtDNA mutations and the development of symptoms of accelerated aging including alopecia, graying hair, weight loss, poor body conditions, and impaired mobility (Safdar et al 2011). These mice also exhibit increase oxidative damage to mitochondria protein and DNA and a reduction in skeletal muscle mass (Kolesar et al 2014) with a subsequent increase in muscle glycolysis and liver (Saleem et al 2015). Interestingly endurance exercise training protected the mice from the age-related phenotypes (Safdar et al 2011).

Mitochondrial function is necessary for exercise

With high intensity exercise, there is up to a 400 fold increase in energy turnover in skeletal muscle, and up to a 100 fold increase in muscle oxygen consumption (Tonkonogi and Sahlin 2002). Although a portion of the ATP produced in skeletal muscle can come from the phosphocreatine or glycolytic pathways, up to 90% comes from aerobic respiration in the mitochondria (Bo et al 2010). Therefore, it is important that the mitochondria are functioning properly to maintain normal

muscle function, exercise capacity, and peripheral metabolic function. Negative effects on exercise capacity have been shown with varying severity of mitochondrial dysfunction. Taivassalo and colleagues found that subjects with mitochondrial dysfunction due to differing mutations in mtDNA had a significantly lower work capacity, VO_2, and peak systemic a-vO_2 difference during exercise compared to healthy control subjects. Within the subjects with mitochondrial dysfunction, exercise capacity varied widely due to varying levels of oxidative capacity. Interestingly, while at rest, VO_2, ventilation, and respiratory exchange ratio (RER) did not differ between groups. Another study by Conley and colleagues (2007) examined the oxidative capacity of skeletal muscle from elderly and young adults. It was found that elderly subjects had a reduced oxidative capacity, mitochondrial content, and lower oxidative phosphorylation capacity per mitochondrial content compared to younger adults. This can have an effect on activities of daily living, like walking, which has been shown to have a 30% higher energetic cost in elderly subjects than in young subjects (Malatesta et al 2003). These studies demonstrate the importance of proper mitochondrial function in different populations, and that mitochondrial dysfunction can be detrimental at a physiological level.

Mitochondrial dysfunction leads to reactive oxygen species production

Although regularly produced in cells, excess ROS production can occur due uncoupling of mitochondrial flux compared to the energetic demand. To generate ATP, mitochondria utilize electron transport chain (ETC). The ETC is complex of proteins that work in unison to move electrons derived from donor molecules (NADH

or FADH) through the complexes. The movement of electrons allows for the development of a protein gradient within the inner membrane space of the mitochondria that creates the necessary potential energy necessary to drive ATP synthesis. The transfer of the electrons through the complex requires oxygen to accept the electrons. However, if the flux through the complexes is not met by an equal energetic demand it can lead to the production of ROS. Mitochondria have scavenging mechanisms to buffer ROS production; however under certain conditions ROS production can occur at a faster rate than the mitochondria scavenging capacity can buffer. It has been estimated that this can result in about one to three percent of consumed oxygen being reduced prematurely resulting in ROS production (Boveris and Chance 1973). The ROS molecules produced from this include O_2^-, H_2O_2, and OH•. O_2^- is mostly produced by electron chain transport (ETC) complexes I and III, which is then scavenged by manganese superoxide dismutase (MnSOD) to produce H_2O_2 (Turrens and Bovaris 1980, Turrens et al 1982). The enzyme glutathione peroxidase (GPx) is then able to convert H_2O_2 to water, which detoxifies the ROS (Kirkinezos and Moraes 2001). Excessive production of ROS is associated with the onset and/or exacerbation of a number of different chronic health conditions including insulin resistance, T2D, obesity, chronic inflammation, cardiovascular disease, hypertension, Alzheimer's disease, and Parkinson's disease (Alfaada and Sallam 2012, Brieger et al 2012).

Non-mitochondrial enzymes can increase reactive oxygen species production

Besides mitochondria, there are other organelles and cell processes responsible for producing ROS. NADPH oxidase is known to produce ROS in a variety of cells and tissues. There are various homologues of NADPH oxidase that have been identified, but they all are transmembrane proteins that transport electrons across membranes to reduce O_2 to O_2^- (Bedard and Krause 2007). Other ROS such as H_2O_2 can be produced from O_2^-, and this ROS generation can then be used functionally for processes such as the induction of host defense genes, phosphorylation of kinases, activation of transcription factors, and the mobilization of ion transport systems (Touyz and Schiffrin 2004). The NADPH oxidase complex has been observed in adipose tissue (Krieger-Brauer and Kather 1992), the testes, the spleen, lymph nodes (Banfi et al 2001), the prostate (Kikuchi et al 2000), the heart (Oudot et al 2003), the pancreas (Tsubouchi et al 2005), neurons (Tammariello et al 2000, Vallet et al 2005), among many other tissues and organs. Unlike other biological processes where ROS production occurs as a byproduct, ROS generation appears to be the primary function of the NADPH oxidase system. Because of the ubiquitous nature of NADPH oxidase, its roles in specific cells and tissues, such as skeletal muscle, are still being examined.

Xanthine oxidase (XO) is another source of ROS production found throughout the body. XO catalyzes the conversion of hypoxanthine into xanthine, and xanthine into uric acid, producing H_2O_2 in the process. It is also able to produce O_2^- (Kelley et al 2010). Inhibition of XO with drugs like allopurinol are often used to treat gout (Dawson and Walters 2006), but may also be helpful in decreasing ROS production.

In hypoxia-reoxygenation-injured rat cardiomyocytes, XO inhibition using allopurinol was shown to decrease ROS production, along with decreased intracellular Ca^{2+} overload (Kang et al 2006). In mice with diastolic dysfunction, treatment with allopurinol led to decreased myocardial oxidative stress accompanied with the prevention of pathological remodeling of the heart (Jia et al 2012). These studies suggest targeting XO to help with treatment in cardiovascular patients may be viable.

Nitric oxide synthases (NOS) are another family of enzymes that are able to generate ROS. NOS are responsible for producing NO, which is critical in the regulation of systemic blood pressure, intracellular Ca^{2+} levels, and vasorelaxation (Vasquez-Vivar et al 1998). Endothelial NOS (eNOS) and neuronal NOS (nNOS) have both been shown to produce O_2^- (Pou et al 1992, Vasquez-Vivar et al 1998). Although NO production is beneficial, eNOS dysfunction due to uncoupling can have negative physiological effects. In this case, uncoupling of eNOS causes O_2^- production to increase, with decreased NO production (Yang et al 2009). The increase in ROS due to eNOS uncoupling has been implicated in the contribution of multiple diseases states including hypertension, diabetes, atherosclerosis, ischemic heart disease, acute lung injury, and smoking related illnesses (Gielis et al 2011).

Cytochrome P450 monooxygenases (CYPs) are a family of enzymes that oxidize, peroxidize, or reduce many different types of chemical compounds throughout the body, including the liver, heart, vasculature, and lungs (Gottlieb 2003). As a byproduct of its reactions, CYPs produce both O_2^- and H_2O_2. Normally, these ROS react with the substrate of the reaction, but uncoupling of CYP can occur,

leading to excess ROS production (Lewis 2002). CYPs have been shown to generate ROS in both the coronary arteries (Fleming et al 2001) and the heart (Granville et al 2004), demonstrating their possible role in cardiovascular injury.

One other source of intracellular ROS is via endoplasmic reticulum (ER) stress. In the ER, proteins are folded and assembled appropriately, and then transferred throughout the cell. However, proteins can be misfolded or unfolded, and when accumulated, can cause ER stress (Oslowski and Urano 2011). There are many consequences of ER stress, including excess ROS production. It is estimated that approximately 25% of ROS produced by a cell originates from the formation of disulfide bonds in the ER (Tu and Weissman 2004). During the formation of these disulfide bonds, ER oxioreductin (ERO1) and protein disulfide isomerase (PDI) catalyze these reactions, and generate ROS as a byproduct. If proteins are misfolded, glutathione (GSH) is needed to reduce unstable disulfide bonds so they can again react with ERO1 and PDI. It has been theorized that this cycle can lead to excess ROS due to both generating ROS and depleting GSH levels in the ER (Malhotra and Kaufman 2007)

ROS are produced during contraction in skeletal muscle

During the late 1970's, the first evidence that contracting muscle produces ROS was reported. Dillard et al (1978) and Brady et al (1979) reported that during exercise in humans and rats, lipid peroxidation was increased. Soon after, it was discovered that free radical concentrations were two to three times higher in skeletal muscle following exercise to exhaustion, along with markers of muscle tissue damage

(Davies et al 1982). Jackson and colleagues (1982) found that after 30 minutes of contractile activity, free radical production in mammalian skeletal muscle was increased. In diaphragm bundles from rats, repetitive contractions were also shown to induce both O_2^- and H_2O_2 production and contribute to fatigue (Reid et al 1992). Treatment with antioxidants were then able to protect against fatigue at low frequencies, suggesting that contraction induced ROS production can contribute to muscular fatigue (Reid et al 1992).

Source of ROS during contraction is likely multi-factoral

During contraction, ROS may be generated by different organelles and mitochondrial complexes in skeletal muscle. In the mitochondria, O_2^- is generated by both complex I and complex III (Muller et al 2004, Barja 1999). Because contractile activity causes elevated oxygen consumption and increased mitochondrial activity, it has been assumed that there is a 50 to 100 fold increase in O_2^- generation by skeletal muscle (Powers and Jackson 2008). However, recent research suggests that the mitochondria may not play as large a role as previously believed. In myotubes, a four-fold increase was seen in ROS generation during contraction, which is much lower than the 50 to 100 fold estimate (McArdle et al 2005). In single muscle fibers, ROS generation was induced by contraction without a change in mitochondrial redox potential (Michaelson et al 2010), suggesting that ROS is being predominantly produced by another source during contraction. More recently, contracting single muscle fibers were shown to have increased acute cystolic O_2^- production with slower changes in mitochondrial O_2^- production (Pearson et al 2014). In these recent studies,

experiments were done without the presence of metabolic substrates since there is minimal substrate flux into the mitochondria under these condtions. So, more research needs to be done before ruling out mitochondria as a source of increased ROS generation during contraction. NADPH oxidase is another possible source of ROS generation in contracting skeletal muscle. In myotubes, ROS generation during contraction was significantly lowered with the administration of the NADPH oxidase inhibitor apocynin (Espinosa et al 2006). Likewise, the use of apocynin on single muscle fibers produced similar results, with decreased ROS generation with contraction (Michaelson et al 2010). The NADPH oxidase regulatory protein $p40^{phox}$ was shown to translocate from the cytosol to the sarcolemma in concurrence with ROS generation in contracting single muscle fibers, suggesting a link between $p40^{phox}$ and NADPH oxidase activation (Sakellariou et al 2013). Other possible sources of ROS generation during contraction include phospholipase A_2 (PLA_2) and XO. Ca^{2+}-independent PLA_2 has been shown to modulate ROS generation in the cytosol of skeletal muscle (Gong et al 2006), while Ca^{2+}-dependent PLA_2 in the mitochondria has been shown to induce intracellular ROS generation during contraction (Nethery et al 1999). XO has been shown to induce ROS generation in the cytosol of contracting rat skeletal muscle (Gomez-Cabrera et al 2005). However, humans contain much less XO in their skeletal muscle compared to rats, so it's unclear of XO is significantly contributing to ROS generation during contraction (Gomes-Cabrera et al 2003). Although both PLA_2 and XO have been shown to induce ROS generation in skeletal muscle, more research needs to be done to fully understand how much of a role they actu

ROS are necessary signaling molecules

Under normal physiological conditions, ROS are needed as cell signaling molecules throughout the body for processes including cell proliferation, cell survival, and cell differentiation (Ray et al 2012). ROS have been shown to activate many MAPK pathways in different cell types. For example, in mouse dendritic cells, ROS have been shown to activate the MAPK kinase kinase apoptosis signal-regulated kinase 1 (ASK1), which is needed for toll like receptor 4 (TLR4) mediated innate immunity (Matsuzawa et al 2005). H_2O_2 has been shown to increase ASK1 levels in cardiac myoblasts, in turn stimulating p38 MAPK and inducing cell differentiation (Choi et al 2011). Protein kinase A, which among other processes, plays a role in MAPK signaling, was shown to be activated by H_2O_2 in cardiomyocytes (Brennan et al 2006). Another signaling pathway that can be regulated by ROS is the phosphoinositide 3-kinase (PI3K) pathway, which is involved in both cell proliferation and survival. In cells overexpressing NADPH oxidase, H_2O_2 produced by the cells showed inactivation of phosphatase and tensin homolog (PTEN), a negative regulator of the PI3K pathway (Kwon et al 2004). When treated with insulin, human neuroblastoma cells had increased PI3K activation due to PTEN oxidation from increased ROS production (Seo et al 2005). ROS may also play a role in the regulation of cellular iron homeostasis through the iron-responsive element (IRE) iron regulatory protein (IRP) pathway. With treatment with H_2O_2 in both human cells and mouse fibroblasts, IRP2 displayed increased binding to IRE, reducing its degradation (Hausmann et al 2011). This suggests a protective effect of H_2O_2 for IRP2 with high intracellular iron levels.

In skeletal muscle, ROS produced from NADPH oxidase was shown to be coupled with Ca^{2+} release from the SR (Xia et al 2003). Also with isometric contraction, increased activation of transcription factors such as nuclear factor κB (NFκB), activator protein-1 (AP-1) and heat shock factor (HSF) was seen with increased ROS generation (Vasilaki et al 2006). The increase in these transcription factors then resulted in increased content of heat shock proteins (HSP), SOD, and catalase, which can all act as antioxidants.

ROS signaling is also involved in multiple cardiovascular pathways. In endothelial cells, H_2O_2 has been shown to induce the release of neuregulin-1 (NRG-1), which has anti-apoptotic effects and is necessary for proper development of the heart (Kuramochi et al 2004). During chronic load-induced stress, NADPH oxidase 4 was shown to enhance angiogenesis in mouse hearts. Vascular endothelial grown factor (VEGF) signaling was increased in a gain of function mouse model, and was decreased in a loss of function model, suggesting a protective effect of NADPH oxidase 4 induced ROS generation (Zhang et al 2010). Likewise, in NADPH oxidase 4 knockout mice, NO formation and eNOS expression were both significantly reduced when compared to controls (Schroder et al 2012). The fact that ROS are expressed and utilized as signaling molecules in a variety of different tissues and cell types shows their importance under normal physiological conditions. However, this wide expression means that if unusually high levels of ROS are generated, these molecules can be implicated in contributing to disease or dysfunction.

Excessive ROS production can lead to oxidative stress

Although ROS act as signaling molecules in a plethora of cell types, excessive or uncontrolled production can lead to oxidative stress. Oxidative stress can be defined as a disturbance in the balance between the production of pro-oxidants and antioxidants (Jones 2006). When oxidative stress occurs, it can lead to damage such as lipid peroxidation, DNA damage, and protein peroxidation. Lipid peroxidation is initiated by ROS, where a polyunsaturated fatty acid (PUFA) has a hydrogen atom stripped from the molecule resulting in the formation of a lipid radical. This lipid radical can then react with O_2 to form a lipid peroxyl radical, which can then react with another PUFA to form lipid peroxide. Lipid peroxides can then undergo more reactions and become things like reactive aldehydes, alkanes, and isoprostanes (Miwa et al 2008). Excessive lipid peroxidation results in cell toxicity. Damaging effects of lipid peroxidation include decreased membrane fluidity and increased ionic permeability, which can cause depolarization of membrane potential and toxically high levels of intracellular Ca^{2+} (Stark 2005). DNA oxidation events have been estimated to occur around 20,000 times daily in every cell of the human body (Foray et al 2003), with a significant portion of these due to ROS. Hydroxyl radicals react with double bonds of DNA bases by addition, and the removal of a hydrogen atom from the methyl group of thymine and each C-H bond of 2'-deoxyribose (Cooke et al 2005). Damage to DNA can lead to genomic instability and mutations, contributing to carcinogenesis or cell death (Miwa et al 2008). In single cell models, knockouts of antioxidants lead to mutagenesis (Gralla and Valentine 1991, Wong et al 2004). However, when these cells were grown in anaerobic conditions where ROS could not

be generated, mutagenesis was completely reversed (Gralla and Valentine 1991, Ragu et al 2007). Proteins can be oxidized by ROS in numerous ways. Initially, ROS remove a hydrogen atom from an amino acid residue, forming a carbon-centered radical and resulting in a chain reaction where side reactions occur with other amino acid residues to form new radicals (Berlett and Stadtman 1997). Oxidative damage to proteins can cause inactivation of enzymatic function. Examples of this include aconitase, GAPDH, carbonic anhydrase, and glutamine synthase (Gardner and Fridovich 1991, Woo et al 2005, Cabiscol and Levine 1995, Levine 1983).

Skeletal muscle can be damaged by oxidative stress

Oxidative stress in skeletal muscle has been shown to have damaging effects on both insulin signaling and glucose transport. In a study using rat soleus muscles, two hour exposure to approximately 90μM H_2O_2 caused a decrease in insulin stimulation of signaling elements like Akt and of glucose transport (Dokken et al 2008). Likewise, four hours exposure of the same concentration of H_2O_2 of the soleus led to loss of insulin receptor substrate 1 and 2 proteins (Archuleta et al 2009). Possible pathways in which oxidative stress may impair insulin signaling include MAPK pathway and JNK pathway. ROS induced activation of these pathways has been shown to impair insulin signaling and glucose transport in skeletal muscle (Diamond-Stanic et al 2011, Santos et al 2012). Oxidative stress may also contribute to muscle wasting in certain conditions. In studies of both myotonic and Duchenne muscle dystrophy, levels of skeletal muscle ROS were shown to be elevated, while antioxidant levels were decreased (Toscano et al 2005, Rodriguez and Tarnopolsky

2003). When lacking SOD1, chronic oxidative stress has been shown to contribute to muscle atrophy in aging mice, with decreases in myonuclei number and fiber diameter (Jang et al 2010). Oxidative stress has also been shown to contribute to intracellular Ca^{2+} leak and muscle weakness associated with aging. When oxidized, the ryanodine receptor (RyR) becomes leaky, causing reduced tetanic Ca^{2+}, decreased muscle specific force, and decreased exercise capacity (Andersson et al 2011).

Oxidative stress is present in many disease states

Because ROS are produced in various cells and tissues, oxidative stress contributes to several disease states. In the lung, oxidative stress has been shown to contribute to the progression of chronic obstructive pulmonary disease (COPD). Sources of ROS in the lung include the mitochondria (van der Toorn et al 2009), NADPH oxidase, and xanthine oxidase, which have both been shown to be elevated in the bronchoalveolar lavage fluid of chronic obstructive pulmonary disease (COPD) patients (Pinamonti et al 1998, Aaron et al 2001). Other negative effects of oxidative stress in COPD patients include airflow limitation via upregulation of NFκB (DiStefano et al 2002), and a reduced ability of corticosteroids to repress proinflammatory gene expression (Barnes et al 2005).

Damage from oxidative stress is present in neurodegenerative diseases. In Alzheimer's disease (AD) oxidative stress is seen early on in its pathogenesis. Damage to neurons from AD patients is seen at its onset, with reductions in oxidative damage as the disease progresses (Nunomura et al 2001). Increased markers of oxidative damage have been seen in cerebrospinal fluid, plasma, and urine of AD

patients, implying there may be oxidative damage of the brain prior to the development of dementia symptoms (Pratico et al 2002). Parkinson's disease (PD) is another neurodegenerative disease in which oxidative stress plays a role. With PD, there is progressive loss of dopaminergic neurons in the substantia nigra in the brain, with aggregation of the protein α-synuclein (Gandhi and Abramov 2012). Dopaminergic neurons have been shown to have oxidative damage produced via NADPH oxidase in a PD rat model. When NADPH oxidase was inhibited in these cells, cell death was reduced (Choi et al 2012). Significantly higher levels of oxidative damage markers have been seen in PD patents compared to controls, suggesting that elevated oxidative stress in the periphery may contribute to the progression of the disease (Seet et al 2010).

Oxidative stress has also been implicated in the progression of chronic kidney diseases. In podocytes, ROS-induced oxidative stress led to activation of NFκB, which is involved in renal fibrosis (Greiber et al 2002). p66shc is an adapter protein that is phosphorylated under conditions of oxidative stress, and translocates to the mitochondria. In renal proximal tubule cells, it has been shown to cause Ca^{2+} mediated mitochondrial damage and apoptosis (Arany et al 2010). When the gene encoding for p66shc has been knocked out in mice, the animals were protected against oxidative damage to the glomerulus (Menini et al 2007).

Although there are many variables in the progression of cardiovascular disease, oxidative stress is likely a major contributor. In heart failure resulting from myocardial infarction (MI), oxidative stress was increased along with decreases in antioxidants (Hill and singal 1997). Antioxidants like SOD and GPx have decreased

activity in patients with MI, suggesting there is the possibility of oxidative damage after injury (Scott et al 1991, Pasupathi et al 2009). In the vasculature, O_2^- production from NADPH oxidase was shown to be increased in people with systemic risk factors for atherosclerosis (Guzik et al 2000). Hypertensive rats have been shown to have increased O_2^-, resulting in reduced levels of NO (Tanito et al 2004). When this model is treated with antioxidants, vascular O_2^- levels are reduced along with the slowing of hypertension development (Rodriguez-Iturbe et al 2003, Park et al 2002). Administration of angiotensin II has been shown to induce hypertension in rats, with increases in vascular O_2^- (Rajagopalan et al 1996). This increase in ROS activates various signaling molecules like JNK, Akt, and NFκB, which can induce vascular injury by way of expression of proinflammatory genes, production of extracellular matrix proteins, and contraction (Montezano et al 2014).

Antioxidants can attenuate the effects of oxidative stress

To maintain redox balance and prevent oxidative stress, the body uses both enzymatic and non-enzymatic antioxidants. Common enzymatic antioxidants include SOD, catalase, GPx, thioredoxin, peroxiredoxin, and glutathione transferase. Extracellular SOD was shown to reduce oxidative stress in mice injected with streptozotocin, which is used to induce type 1 diabetes mellitus (Call et al 2015). As a result, these mice were protected against such things as cardiac hypertrophy, fibrosis, and dysfunction. In rats overexpressing MnSOD, redox state was improved in skeletal muscle, and the animals were protected from insulin resistance induced by a high fat diet (Boden et al 2012). GPx3 has been shown to mediate the antioxidant

effects of peroxisome proliferator-activated receptor γ (PPARγ) in skeletal muscle. With overexpression of GPx3, H_2O_2 levels were decreased and when PPARγ was knocked down, GPx3 expression was decreased with increased in H_2O_2 (Chung et al 2009). Mitochondrial GPx4 has been shown to reduce oxidative stress related cardiac injury. Diabetic mice overexpressing GPx4 had attenuated ischemia/reperfusion associated cardiac dysfunction, along with significantly lower markers of oxidative stress (Dabowski et al 2008). In addition to these enzymatic antioxidants, non-enzymatic antioxidants are also used to reduce oxidative stress. Examples of these include vitamin C, vitamin E, and β-carotene. Vitamin C acts as an antioxidant by donating electrons and preventing other compounds from being oxidized and also acts as a reducing agent for ROS and RNS (Padayatty et al 2003). Vitamin E functions as a scavenger of peroxyl radicals that terminate chain reactions (Traber et al 2007), while β-carotene can scavenge peroxyl, hydroxyl, and O_2^- radicals (El-Agamey et al 2004).

Conclusions

Based on the current literature, it is apparent that maintaining proper cellular redox balance is critical in preventing disease and dysfunction. Under normal physiological conditions, ROS can act as important signaling molecules that function throughout the body. When ROS levels are increased or not properly buffered, it can lead to oxidative stress. Chronic oxidative stress can contribute to a variety of disease states, so it is worth exploring ways to reduce oxidative stress and improve cellular redox balance. By understanding the underlying mechanisms of ROS generation,

redox balance, and oxidative stress, it will hopefully allow for further improvement in treatment of diseases and dysfunction in which oxidative stress is a contributing factor.

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