THESIS

INVESTIGATING THE EFFECTS OF AN ENDOGENOUS AND AN EXOGENOUS OXIDANT STRESSOR ON PROTEIN SYNTHESIS IN C2C12 MYOBLASTS

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ABSTRACT

INVESTIGATING THE EFFECTS OF AN ENDOGENOUS AND AN EXOGENOUS OXIDANT STRESSOR ON PROTEIN SYNTHESIS IN C2C12 MYOBLASTS

Reactive oxygen species (ROS) related signaling is important for stress adaptation. Specifically, ROS may alter protein turnover in a duration dependent manner with acute ROS exposure increasing protein synthesis for cellular adaptation. *In vitro* experimentation remains a valuable tool investigate the mechanisms underlying ROS-mediated protein synthesis. This study investigates paraquat (PQ), a mitochondrial complex I inhibitor that induces superoxide production, as a potential candidate to induce endogenous ROS production *in vitro* to simulate an oxidative stress similar to *in vivo* conditions. We hypothesized that acute PQ treatment would induce protein synthesis in cells similar to exogenously added hydrogen peroxide (H_2O_2). We treated C2C12 myoblasts with 0, 1, or 2mM PQ or 0, 50, 100, or 500uM H_2O_2 for 4, 8, or 12 h while using 4% deuterium oxide enriched media to measure protein synthesis of mixed, cytosolic, and mitochondrial (mito) fractions. 100uM $H_2O_2$and 2mM PQ transiently increased mito protein synthesis at 4h compared to control (H_2O_2: 0mM: 1.89%/hr +/- 0.068, 100uM: 2.75%/hr +/- 0.098 $p<0.05$; PQ: 0mM: 4.30%/hr +/- 0.33, 2mM: 6.86%/hr +/- 0.552, $p<0.05$). We verified that PQ induced oxidative stress by demonstrating that 2mM PQ 2mM PQ augmented HO-1 content compared to control at 12h (0mM: 0.452a.u. +/- 0.133, 2mM: 1.434a.u. +/- 0.487 $p<0.05$). Thus, we have developed an *in vitro* model that induces endogenous production of ROS to

induce oxidative stress and demonstrates a ROS-mediated increase in mito protein synthesis.

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INTRODUCTION

Oxidative stress is the molecular damage of lipids, proteins, and/or DNA caused by oxidants and plays a causative role in numerous diseases such as cardiovascular disease (Cai & Harrison, 2000) and diabetes (Jeong-a Kim, Wei, & Sowers, 2008) as well as promotes aging (Finkel & Holbrook, 2000). Oxidants, such as reactive oxygen species (ROS), modify macromolecules through redox reactions resulting in structural and, therefore, functional alteration. While ROS-induced redox reactions are always occurring in all individuals, oxidative stress is the pathologic, irreversible oxidation of macromolecules that results in impaired cellular signaling and organelle function (Jones, 2006), DNA damage (Imlay & Linn, 1988), and/or lipid peroxidation (B. Halliwell, 1987). These oxidative stress-induced cellular modifications manifest themselves as physiological impairments involved with disease and aging.

As the prevalence of age-related disease, such as cardiovascular disease and sarcopenia, and the aged population, grows, interventions targeted towards preventing age-related diseases are increasingly important to public health. Thus far, interventions focused on attenuating oxidative stress to slow aging and reduce disease through antioxidant supplementation have failed (Bjelakovic, Nikolova, Gluud, Simonetti, & Gluud, 2007; M. C. Gomez-Cabrera, Ristow, & Viña, 2012). However, recent studies have shown promise for interventions involved in upregulating endogenous antioxidant enzymes. Unlike traditional antioxidant supplementation, which attempt to increase the amount of oxidant scavengers, endogenous antioxidant upregulation augments cellular oxidant defense by increasing the content of enzymatic antioxidants such as superoxide dismutase

(SOD), NAD(P)H oxidoreductase 1 (NQO1), and heme oxygenase-1 (HO-1). Both Donovan and colleagues and Reuland and colleagues have recently demonstrated the efficacy of protecting endothelial cells and cardiomyocytes from oxidative challenges by augmenting endogenous antioxidant enzymes (Donovan, McCord, Reuland, Miller, & Hamilton, 2012; Reuland et al., 2013). However, there is an absence of evidence in human trials supporting the efficacy of this strategy in mitigating disease and promoting healthy aging. Thus, researchers are currently pursuing to answer whether the benefits of upregulating antioxidant enzymes observed in cells translates to benefits in humans.

A characteristic of aging is the accumulation of dysfunctional proteins (Finkel & Holbrook, 2000). Oxidation of proteins often led to their damage and dysfunction. Declines in protein turnover, the process of breaking down damaged proteins and synthesizing new proteins, is a hallmark of aging (Balagopal, Rooyackers, Adey, Ades, & Nair, 1997; Ryazanov & Nefsky, 2002). Consequently, oxidized proteins accumulate and lead to dysfunction and aging (Rebrin & Sohal, 2008). Oxidative stress has a role in the decline in protein synthesis (Powers, Smuder, & Criswell, 2011) as well as the oxidation of proteins (B. Halliwell, 1987). Thus, antioxidant supplementation use to maintain protein turnover and mitigate oxidative damage is a forthcoming and promising approach to slow aging.

Given the recent successes of endogenous antioxidant upregulation in protecting cardiomyocytes from oxidative challenges, it is plausible that increasing antioxidant enzyme content and thus reducing oxidative stress can assist in maintaining protein turnover. A review has highlighted studies demonstrating the suppression of protein synthesis through oxidative stress *in vitro,* though no studies confirm this concept *in vivo* (Powers et al., 2011). However, cell culture studies remain a valuable tool to understand

the underlying mechanisms of an intervention or physiological relationship. Thus, investigating the effects of oxidative stress in cell cultures on protein synthesis and breakdown may provide further insight on the relation between oxidative stress and aging and create a model with which to test interventions (e.g. supplement-mediated upregulation of antioxidant enzymes) targeted towards improving protein turnover.

One chief problem involved in successfully utilizing cell cultures is the ability to replicate *in vivo* conditions in an *in vitro* environment. Research groups focused on oxidative stress employ a variety of oxidants in cell culture experiments including hydrogen peroxide, paraquat, cadmium, and UV light to induce oxidative stress (Akiyama et al., 2011a; Reuland et al., 2013; Salmon et al., 2005). With regard to inducing oxidative stress *in vitro*, recent evidence suggests that the source of oxidant may have an effect on the cellular response to the oxidant challenge. Choi and colleagues observed that exogenously added hydrogen peroxide was less effective in inducing downstream signaling than endogenous hydrogen peroxide in fibroblasts (Choi et al., 2005). Thus, prior to measuring the effects of an intervention aimed to decrease oxidative stress and maintain protein synthesis and breakdown, it is important to ensure that oxidative stress induced *in vitro* reflects oxidative stress *in vivo,* or at least that the source or type of oxidant does not have a moderating effect on protein metabolism.

STATEMENT OF THE PROBLEM

This study aims to compare the effects of an endogenous oxidant stress (paraquat) to an exogenous oxidant (hydrogen peroxide) on protein synthesis rates in C2C12 myoblasts.

HYPOTHESIS

We hypothesize that the acute application of paraquat will result in an increase in mitochondrial protein synthesis as a result of oxidative stress in C2C12 myoblasts.

DELIMITATIONS, LIMITATIONS, AND ASSUMPTIONS

Measuring only protein synthesis inherently presents a limitation when measuring protein turnover, which is a product of both protein synthesis and breakdown. Thus, by measuring only protein synthesis, there is an incomplete scope of how two different oxidants differently affect protein turnover. However, by measuring protein synthesis in three cellular fractions of the cells, this study can provide observations on protein synthetic responses to each oxidant stress in each subcellular fraction.

Additionally, acute bouts of oxidative stress do not typically cause aging. Rather, the chronic exposure to oxidative stress contributes more to aging. In this study, we exposed cells to each oxidant and measured the acute effects of protein synthesis at four and eight hours. Therefore, we potentially missed the immediate responses to oxidative stress as well as long-term adaptations to or determinants of the oxidative stress. However, with both time points, this experiment can provide insight on the effects of acute oxidative stress, similar to that of bouts of exercise, on protein synthesis.

LITERATURE REVIEW

Oxidative stress is a contributor to both disease and aging. Though there is not a consensus on the definition of oxidative stress, it is clear that reactive oxygen species (ROS), with other oxidants, cause oxidative stress. While past studies have demonstrated the role ROS and oxidative stress have in certain diseases and aging, few studies have measured oxidative stress and protein turnover. Thus, this review aims to 1. Introduce fundamental concepts of oxidative stress and redox chemistry, while highlighting the role ROS have both in physiology and pathophysiology; 2. Review the successes and failures of interventions aimed at mitigating oxidative stress in an attempt to attenuate diseases in which oxidative stress has a causative factor; 3. Describe the decline in protein turnover with aging and the potential mechanisms controlling protein synthesis; and finally 4. Discuss the experimental possibilities aimed at establishing a link between oxidative stress and protein synthesis.

REDOX BIOLOGY, CHEMISTRY, AND PHYSIOLOGY

Reactive oxygen species (ROS) are a type of oxidant that serve as signaling molecules and are the underlying contributor to oxidative stress. Incomplete reduction of oxygen atoms generates ROS, which are unstable and highly reactive because of the incomplete reduction. Thus, ROS readily react with other molecules through redox reactions to reestablish stability. ROS react at random with molecules, such as proteins, which then use ROS to serve as signaling molecules (Barry Halliwell, 1999). It is when ROS irreversibly modify and damage cellular machinery such as proteins, lipid, and DNA structures that they cause oxidative stress.

ROS are categorically referred to as either radical or non-radical. Radical ROS, such as superoxide and hydroxyl, have one or more unpaired electrons and are therefore more reactive than non-radical ROS (B. Halliwell, 1987). Some radicals can extract hydrogen atoms from hydrocarbons, which yield a carbon with an unpaired electron. Non-radical ROS, including hydrogen peroxide, are strong oxidizing agents and can generate radicals by reacting with other ROS or metals such as iron and copper(B Halliwell & Gutteridge, 1984). Both types of ROS predominantly react with other molecules through redox reactions serving as either a reducing or oxidizing agent, donating or accepting an electron to a molecule respectively(Barry Halliwell, 2006).

Reactive oxygen species as signaling molecules

While researchers were first focused on the deleterious effects of ROS, Mittal and Murad first described cyclic GMP (cGMP) activation via hydroxyl radicals in the late 1970s (Mittal & Murad, 1977). cGMP is a protein involved in signal transduction acting as a secondary messenger, thus attention shifted towards the role ROS as a signaling molecule (Suzuki, Forman, & Sevanian, 1997). Researchers have further elucidated the role of ROS as a signaling molecule in key cellular functions such as cell cycle regulation (Janssen-Heininger et al., 2008), activation of transcription factors involved in stress adaptation (Kobayashi & Yamamoto, 2006), and mitochondrial biogenesis (Stone & Yang, 2006). ROS serve as important signaling molecules that assist in cellular physiology involved in adaptation and survival by reacting with proteins.

ROS initiate and transduce signals by undergoing redox reactions with redox-sensitive amino groups, such as cysteine residues, of proteins to modify the structure of proteins (Jones et al., 2004). Oxidation of cysteine residues results in conformational and

functional changes of proteins, which can stimulate or perpetuate signal transduction and elicit changes in cellular function. Further, oxidation status of amino acid residues are not binary meaning ROS can induce an array of effects through the oxidation of the same protein (Ray, Huang, & Tsuji, 2012). Importantly, oxidation of cysteine residues is reversible; allowing the protein to stop the signal transduction once the ROS-stimulus has disappeared (Holmström & Finkel, 2014). By affecting the structure and function of proteins, ROS have a significant role in cellular and organismal function and health (Barry Halliwell, 2006).

Biological sources of reactive oxygen species

Organisms have evolved to use oxygen as a necessary component of metabolism, thus the generation of oxygen-derived radicals closely relates to cellular metabolism itself. Over one billion years ago, environmental levels of oxygen began increasing. At that time, oxygen was a poisonous gas. Organisms evolved to adapt to the increasing environmental levels of oxygen by integrating oxygen into metabolic pathways through the development or engulfment of a prokaryote that has now become known as the mitochondria. Now, oxygen is necessary for aerobic respiration and cellular function. Because aerobic respiration does not occur entirely without occasional incomplete reduction of oxygen, ROS are common elements in intra and extracellular systems (Barry Halliwell, 1999).

Because of its role in metabolism, mitochondria generate nearly 90% of all endogenous ROS (Balaban, Nemoto, & Finkel, 2005). Oxidative phosphorylation is the oxygen-driven, progressive oxidation of NADH and/or $FADH_2$ and their intermediates through a series of redox reactions involving protein complexes that occur along the electron transport system. Ultimately, oxidation of NADH and $FADH_2$ generates potential

energy via electrochemical gradient of protons in the intermembrane space that is used by ATP synthase to generate ATP (Brooks, 2005). However, there are instances in which, instead of an electron reducing its respective oxidizing agent, the electron incompletely reduces an oxygen molecule yielding the generation of superoxide (Grivennikova & Vinogradov, 2006). While mitochondrial complexes perpetually produce low levels of ROS, fluctuations in oxidative phosphorylation increase the production of ROS. Enzymatic antioxidants are responsible for dismutating free radical ROS, such as superoxide, into non-radical ROS, such as hydrogen peroxide (Balaban et al., 2005).

Other cellular components generate ROS as a means to respond to external stimuli and send intracellular signals as well as protect the cells. For example, NADPH oxidase (NOx), transmembrane proteins located on the plasma membrane of cells, generates ROS in response to external signals such as growth factors and cytokines. Extracellular ligands bind to and activate NOx, which converts exogenous oxygen into superoxide and imports the superoxide to the cytoplasm (DeYulia, Cárcamo, Bórquez-Ojeda, Shelton, & Golde, 2005). The endogenous superoxide, typically dismutated into non-radical ROS, is capable of initiating signal transduction pathways (Veal, Day, & Morgan, 2007). ROS generated by NOx family enzymes are involved in a wide array of cell functions including gene expression, cell death and senescence, cell cycle regulation, cell immunity response, and ion channel regulation (Bedard & Krause, 2007).

To summarize, ROS are integral components of normal physiology. Particularly, hydrogen peroxide molecules, generated from radical ROS in both the mitochondria and cytoplasm, serve integral roles in cellular function and signaling (Stone & Yang, 2006; Veal et al., 2007). However, because of their reactivity and volatility, ROS can also oxidize

molecules, such as lipids (Barry Halliwell, 2006), proteins (H. Zhang et al., 2012), and DNA (Imlay & Linn, 1988), in a manner that irreversibly alters and damages these structures. In the following section, this review will highlight the role ROS have in the promotion of oxidative stress and how oxidative stress promotes disease and aging.

OXIDANT DAMAGE AND OXIDATIVE STRESS

While radical ROS are more volatile, non-radical ROS can still oxidize molecules like any other oxidant through redox reactions. To establish stability, ROS release or take an electron from neighboring molecules to complete electron pairs or act as strong oxidizing agents. Hydrocarbons of lipids are oxidative targets of ROS. Oxidation of hydrocarbons can lead to a series of hydrocarbon reactions which result in lipid peroxidation, which contributes to a decrease in membrane fluidity, increased membrane leakiness, and damage to membrane receptors, enzymes, and ion channels (Barry Halliwell, 2006; Minotti & Aust, 1989). ROS also react with nucleic acids in DNA. For example, hydroxyl radicals can react with guanine creating the radical 8-hydroxyguanine in DNA which can generate mutagenic transformations and even break DNA strands (Aruoma, Halliwell, Gajewski, & Dizdaroglu, 1991). ROS also oxidize and damage proteins, disabling protein-binding sites critical for catalyzing reactions, impairing ion-gradient membrane proteins, and denaturing key enzymes essential for cell function. As discussed though, this occurs routinely as a mediating pathway for cellular signaling. However, as will be discussed, over oxidation of proteins can lead to damage and oxidative stress.

A previous, prevailing notion of oxidative stress described oxidative stress as an imbalance between oxidants and antioxidants (Finkel & Holbrook, 2000). Under the notion, oxidative stress is related to the sum of all oxidants, including ROS, subtracted from the

sum of all antioxidants. If oxidants exceed antioxidants, then oxidants damage lipids, proteins, and DNA. However, this concept is not wholly accurate as it does not take into consideration the fact that, as discussed, ROS have an important role in signaling (Stone & Yang, 2006). Further, summing of all oxidants in a cell does not consider the fact that the compartmentalization of ROS (Jones & Go, 2010) which limits their area of effect spatially and temporally, which this review will expand upon later. For the reasons and consideration described, oxidative stress causes disease and aging though cellular damage, but is not simply an imbalance.

Oxidative stress

A newer construct of oxidative stress accounts for the role ROS have in signaling. Redox reactions serve as a mode to induce and perpetuate cell signaling involved with normal physiologic function. Therefore, ROS are not toxic entities in a cellular system, rather ROS are integral to cell function and must exist for healthy function. However, ROS still oxidize and damage proteins by causing irreversible conformational changes, rendering the proteins insensitive to ROS and incapable of signal transduction. This new construct of oxidative stress emphasizes that, in addition to DNA and lipid oxidation, ROS disrupt redox signaling and control which results in ROS-related signaling pathways (Jones, 2006).

To understand how ROS disrupt redox signaling and control pathways, it is important to understand how redox-signaling pathways function normally. There are multiple, discrete redox signaling pathways that create a redox circuit. Within each signaling pathway, there is an event that generates a redox signal, such as superoxide or hydrogen peroxide, that a redox signal sensor, such as the thiol group within a cysteine

residue of a protein, detects (Jones, 2006). The signal, ROS, oxidizes the thiol group, which reversibly forms sulfenic acids and disulfide bonds causing a conformational change to the protein triggering downstream signaling events (Brandes, Schmitt, & Jakob, 2009; Cumming et al., 2004). Importantly, conformational changes are reversible through enzymatic systems, such as thioredoxin and glutaredoxin, which reduce the sulfenic acids and disulfide restoring the thiol group in the protein. The previously oxidized protein, now reduced, returns to the pool of redox-sensitive proteins maintaining the function of redox-signaling pathways.

Oxidative stress disrupts multiple signaling pathways by eliminating the pool of redox-sensitive proteins. While ROS form reversible oxidized products, further oxidation of those productions yield irreversible, redox-insensitive products. For example, oxidation of sulfenic acids and disulfide bonds produce sulfinic and sulfonic acids, which are non-reactive and are not reducible by enzymatic systems, decreasing the pool of redox-sensitive proteins in that redox signaling pathway (Brandes et al., 2009). However, excessive oxidation of one protein is not sufficient to cause oxidative stress. As mentioned, redox-signaling pathways are discrete and independent so that oxidative damage to one redox pathway does not affect other parallel and independent pathways (Halvey et al., 2005; Jones et al., 2004). Explicitly, oxidation of the cysteine/cysteine couplet does not affect other redox couplets such as glutathione/glutathione disulfide, NADPH/NAD+, and NADH/NAD+ (Rajindar S. Sohal & Orr, 2012a). Oxidative stress occurs when oxidants disrupt multiple pathways by eliminating redox sensitive proteins such that the redox signaling pathways affected cannot properly transmit signals.

Depleting the reducing redox-sensitive enzymatic systems renders redox proteins incapable of scavenging ROS and transducing redox signals. As excessive oxidation occurs, redox insensitive amino groups, such as sulfenic, sulfonic, and sulfinic acids, render redox switch proteins non-responsive. Oxidative stress results from the accumulation of irreversible redox products of redox-sensitive amino acid groups of redox proteins and ROS(Rebrin, Bayne, Mockett, Orr, & Sohal, 2004). Thus, oxidative stress leads to the depletion of redox-sensitive proteins capable of initiating redox signals, disrupting redox circuitry, and ultimately interfering with normal physiologic cell signaling resulting in deleterious effects such as aging and disease.

Oxidative stress and aging

There is no consensus on the exact mechanisms in which oxidative stress promotes aging. In 1957, Denham Harman proposed that cellular components reacting with free radicals are a driving mechanism behind aging and disease and further suggested that large majority of free radicals arose from reactions that took place within the cell(Harman, 1955). It seems logical that a way to prevent free radical damage is to use antioxidants to prevent reactions between free radicals and cellular machinery. Indeed, early research corroborated the premise that antioxidant supplementation extends lifespan in cell culture (Packer & Smith, 1974), C. elegans (Harrington & Harley, 1988), and mice (Harman, 1961). However, a meta-analysis of randomized control trials involving antioxidant supplementation in humans reveals that antioxidant supplementation increases mortality(Bjelakovic et al., 2007). Further, a comparative study of lung tissues of various organisms found that antioxidant concentration, both enzymatic and non-enzymatic, is inversely related to lifespan (Pérez-Campo, López-Torres, Rojas, Cadenas, & Barja, 1994).

However, this does not mean that ROS do not play a role in aging. Studies conducted in an array of animals of varying lifespans showed that mitochondrial ROS, superoxide and hydrogen peroxide, production is inversely related with maximal lifespan (R. S. Sohal, Svensson, & Brunk, 1990; R. S. Sohal, Svensson, Sohal, & Brunk, 1989). Further, oxidative damage of mitochondrial DNA in mammalian brain and heart is also inversely related to maximum lifespan (Barja & Herrero, 2000). Evidence supports Harman's position that ROS are involved in promoting aging; however, extending lifespan or delaying the aging phenotype is not as simple as adding antioxidants to counterbalance oxidants.

In contrast, the concept that redox-signaling disruption causes aging recognizes that ROS have a significant role in signaling. As discussed, the pool of redox-sensitive proteins shrinks with oxidative stress. To prevent the loss of redox-sensitive proteins, reducing enzymatic systems such as glutaredoxin and thioredoxin must reduce oxidized proteins prior to their irreversible oxidation by ROS (Brandes et al., 2009). The balance between reduction potentials and reducing capacities of redox couplets determines the cellular redox state (Rajindar S. Sohal & Orr, 2012a). Evidence suggests that as an organism ages, the cellular redox state becomes more pro-oxidizing (Rebrin & Sohal, 2008). As the shift towards a more oxidized state occurs, the redox system becomes less efficient as an increasing amount of oxidized proteins accumulate. Greater concentrations of ROS, particularly hydrogen peroxide can lead to detrimental structural and consequently physiological changes within the cell (B Halliwell & Gutteridge, 1984). Oxidized proteins are 10-100 times less reactive toward hydrogen peroxide than their non-oxidized proteins (Stone & Yang, 2006). Lessened sensitivity to ROS reflects the accumulation of oxidized proteins and shrinking the pool of potential redox-sensitive proteins capable of reacting

with ROS. Insufficient amounts of redox-sensitive proteins leads to delayed and/or inadequate responses to stimuli, both indicators of oxidative stress in a redox circuit (Rajindar S. Sohal & Orr, 2012a). Desensitized redox switches causes dysfunction in cell signaling which in turn may promote aging. In a healthy individual, cells could turnover these less redox sensitive proteins, however, in aged individuals, protein turnover is impaired (Young, Steffee, Pencharz, Winterer, & Scrimshaw, 1975). Thus, damaged proteins accumulate (Starke-Reed & Oliver, 1989).

In young mice, which have more robust antioxidant defenses than aged counterparts (Rebrin, Kamzalov, & Sohal, 2003), the amount of non-scavenged ROS does not typically lead to oxidative damage. As organisms transition past the post-reproductive phase of life, ROS production and oxidation of macromolecules increases causing an emergence of the general symptoms of aging (Rebrin et al., 2004; Rebrin & Sohal, 2008). Significant loss in functional capacity of redox circuitry disrupts normal physiology resulting in the increased prevalence of oxidized macromolecules, senescence, and functional losses. Interestingly, maintenance of redox circuitry leads to longer lifespan. A study comparing long-lived breeds or modifying breeds to live longer (i.e. caloric restriction) found that the maintenance of the thiol and glutathione redox states led to longer lifespan (Rebrin, Forster, & Sohal, 2011; Rebrin et al., 2003). Delayed aging through the maintenance of redox states further provides evidence that oxidative stress is a causative factor in aging.

Oxidative stress, exercise, antioxidant supplement use, and the concept of hormesis

Exercise provides a host of beneficial adaptations to humans including decreasing the risk of disease, improving the endurance capacity, and promoting healthy aging. While

some of these benefits are attributed to the energy expenditure associated with exercise, the influx of ROS caused by exercise has a role in the adaptations. With exercise, oxidative phosphorylation increases in response to the energetic demands of the bout, which produces more ROS within the mitochondria. So long as the influx of ROS does not induce oxidative stress, the influx of ROS provides beneficial adaptation. ROS mediate exercise beneficial adaptations such as upregulation of enzymatic antioxidants, regulation of gene expression, and stimulates mitochondrial biogenesis.

As early as 1983, researchers began demonstrating how ROS mediate the health promoting effects of exercise. Salminen and colleagues found exercised individuals were more resistant to exhaustive-exercise-induced oxidative damage when compared to sedentary counterparts. The exercised individuals had greater concentrations of endogenous antioxidant enzymes including SOD2 (the mitochondrial manganese-dependent superoxide dismutase), glutathione peroxidase, and y-glutamylcysteine synthetase (Salminen & Vihko, 1983). Further research corroborated that a single bout of exercise upregulates the transcription of SOD via the NF-κB signaling pathway (Hollander et al., 1999, 2001). Inhibiting the production of ROS with drugs such as allopurinol, which inhibits xanthine oxidase, an enzyme responsible for producing ROS, abolished the exercise-induced upregulation of antioxidants (M.-C. Gomez-Cabrera et al., 2005). Further, exercise upregulates the expression of antioxidants through multiple signaling pathways including the MAPKs, ERK 1/2 and p38 (M.-C. Gomez-Cabrera et al., 2005). Adaptation to exercise-induced oxidative stress protects the cell from future oxidative insults through upregulation of enzymatic antioxidants.

ROS-mediated exercise adaptations prevent disease and promote healthy aging by upregulating antioxidant enzymes and preventing age-associated muscle decline. Research has shown that acute exercise activates Nrf2, a transcription regulator responsible for inducing the antioxidant response element, which augments the antioxidant defense network (Muthusamy et al., 2012). Further, moderate exercise training improves increases Nrf2 in aged myocardium (Gounder et al., 2012) and improves resistance to oxidative stress-related disease in general (M.-C. Gomez-Cabrera, Domenech, & Viña, 2008) . Given that oxidative stress is a causative factor in many diseases including cardiovascular disease (D. Harrison, Griendling, Landmesser, Hornig, & Drexler, 2003), augmentation of antioxidant enzymes provide greater protection against disease. Recently, Zampieri and colleagues demonstrated that aged (> 70 years old) individuals who were physically active were more likely to have greater lower leg muscle mass, strength, and function and greater mitochondrial volume and less likely to have age-related muscle atrophy (Zampieri et al., 2015). Research has yet to elucidate each mediating pathway involved in these exercise-related benefits. However, one potential pathway is that ROS mediate exercise induced mitochondrial biogenesis have determined that ROS play a role in mitochondrial biogenesis (Ventura-Clapier, Garnier, & Veksler, 2008). Given the suspected role mitochondrial dysfunction has in aging (Trifunovic et al., 2004), exercise could help delay aging through transiently increasing ROS that cause the cell to adapt, a concept termed mitohormesis (Yun & Finkel, 2014), which will be addressed later.

While researchers have validated the role exercise has in health, research supporting the use of antioxidant supplements to promote health and lifespan and decrease the risk of disease is equivocal and controversial (M. C. Gomez-Cabrera et al.,

2012). Since ROS causes oxidative stress, it is logical to assume that supplemental antioxidants would yield positive health benefits. In fact, in the mid-1990s, nearly half of all competitive athletes including elite, college, and high school athletes, took antioxidant supplements (Sobal & Marquart, 1994). However, supplemental vitamin C, a known antioxidant, suppresses commonly observed adaptations to exercise reducing the expression of transcription factors (Nrf1, PGC-1α, and TFAM) that promote mitochondrial biogenesis, antioxidants essential to oxidant defense, as well as markers of overall mitochondrial content (M.-C. Gomez-Cabrera, Domenech, Romagnoli, et al., 2008). Blunting exercise-associated adaptations make antioxidant supplementation a counterintuitive practice in the athletic community given that many of these beneficial adaptations lead to improvements in exercise capacity (Davies, Packer, & Brooks, 1981).

Antioxidant supplementation also blunts the remedial effects of exercise in disease populations. Antioxidant supplementation can prevent the ROS-mediated exercise-induced improvement in insulin sensitivity (Ristow et al., 2009). In this case, it is possible antioxidant supplementation scavenged the influx of ROS and blunted the ROS-induced signaling associated with exercise adaptation. Other studies have shown that antioxidant supplementation eliminates ROS and blunts critical physiological adaptation (M.-C. Gomez-Cabrera, Domenech, Romagnoli, et al., 2008; M. C. Gomez-Cabrera et al., 2012). Further, meta-analyses on the efficacy antioxidant supplementation have further demonstrated the lack of effectiveness of antioxidant supplement use. Analysis of 68 randomized trials totaling over 232,606 participants found that supplementing individuals with β carotene, vitamin A, and vitamin E actually increased mortality and that there was neither a benefit nor detriment to supplementing with vitamin C or selenium (Bjelakovic et al., 2007).

Exogenous antioxidant supplementation (i.e. increasing the content of non-enzymatic oxidant scavengers) is detrimental to health likely by blunting ROS-related signaling.

The negligible and negative effects of antioxidant supplementation provide insight on the importance of ROS signaling and perspective on the hormetic behavior of ROS (Ristow, 2014). Hormesis suggests that biological systems respond to chemicals, toxins, and radiation in a bell-shaped manner and that there is an ideal range of exposure to a chemical that can induce healthy adaptation; outside of that range, though, that chemical can have deleterious effects. While excess ROS causes oxidative stress, the excess presence of antioxidants has deleterious effects on health and physiology. The abundance of reduced-state proteins disrupts the overall pool of thiol/disulfide couples, such as cysteine and cystine amino groups on a protein, by shrinking the pool of proteins capable of acting as redox sensing switches (Jones, 2006). In one study, researchers demonstrated that a reduced state, in this case a smaller NAD+/NADH ratio, was associated with decreased presence of PGC-1α and decreased AMPK activation (Teodoro, Rolo, & Palmeira, 2013). The theory of hormesis explains how too many ROS or too little ROS cause deleterious effects. Applied to describe the hormetic relation between ROS and mitochondria, mitohormesis explains how acute influxes of ROS can induce beneficial adaptations and promote healthy aging (Radak, Chung, & Goto, 2005) and how antioxidant supplementation can cause pathophysiologic effect and inhibit pathways involved in adaptation to stress (Ristow, 2014).

Despite the failures of classical antioxidant supplementation, there are new approaches that involve diminishing age- and disease-related oxidative stress through supplementation. One of these approaches involves upregulating enzymatic antioxidants.

Protandim is a supplement that activates Nrf2, the transcriptional regulator of the antioxidant response element involved in upregulating endogenous antioxidants (Reuland et al., 2013, p. 2). A study demonstrated that Protandim pretreatment of endothelial cells reduced hydrogen peroxide-induced apoptosis from 34% to 6% (Donovan et al., 2012). In a study involving human subjects, Protandim treatment increased SOD and catalase content by 30% and 54%, respectively, after 120 days of supplementation in erythrocytes (Nelson, Bose, Grunwald, Myhill, & McCord, 2006). Due to its novelty, researchers have not had the opportunity to validate the efficacy of endogenous upregulation as a mode of preventing oxidative stress-related disease and aging in humans. However, these initial data indicating the protective effects of upregulating enzymatic antioxidants through supplementation provide a new avenue of preventing disease and promoting healthy aging.

PROTEIN TURNOVER AND AGING

The world population over 60 years of age will triple with 50 years. Because of greater longevity, those over the age of 80 years are the fastest growing population ("WHO | Ageing," n.d.). Thus, the need for improving age-related disease and disability outcomes and prevalence is expanding. A significant factor in the decline in health of elderly is the accumulation of damaged proteins mediated by declines in protein turnover (Ryazanov & Nefsky, 2002). Protein turnover, the balance between synthesis and breakdown, involves breaking down damaged proteins and synthesize new proteins to retain functional capacity. Several studies have demonstrated age-related declines in protein synthesis rates (Koopman & Loon, 2009). Accordingly, interventions have focused on augmenting protein synthesis rates in the elderly. Resistance and aerobic exercise interventions generally shown to increase muscle protein synthesis in young adults are less effective in older

adults (Balagopal et al., 1997; Koopman & Loon, 2009). Likewise, feeding has a blunted augmentation of muscle protein synthesis in older adults compared to younger counterparts (Cuthbertson et al., 2004). Researchers now believe this blunted response in protein synthesis is due to impaired anabolic signaling(Cuthbertson et al., 2004; Guillet et al., 2004). Thus, understanding the underlying mechanisms of protein turnover regulation may provide a key to maintaining functional proteins and slowing aging.

The mTOR pathway, protein synthesis, and aging

The signaling pathway of the protein kinase mammalian (mechanistic) target of rapamycin (mTOR) is central to both protein synthesis and degradation. mTOR regulates cell growth, proliferation, survival, and protein transcription (Cornu, Albert, & Hall, 2013). mTOR monitors and responds to nutrient and energy status, stress, and hormones. In an anabolic state, such as in the presence of growth hormone, positive energy status, and/or amino acids, complex 1 of mTOR (mTORC1) aids in translation initiation of proteins involved in cell growth and proliferation through phosphorylation the protein 4EBP1. Phosphorylation of 4EBP1 initiates the recruitment of translation cofactors that have primary roles in protein synthesis (Ma & Blenis, 2009). Simultaneously, mTORC1 increases proteasome content and activity to supply substrate requisite for sustaining protein synthesis (Y. Zhang et al., 2014). mTORC2 also responds to growth factors and regulates metabolic pathways through the phosphorylation of protein Akt (Cornu et al., 2013). Both complexes of mTOR have critical roles in protein synthesis and degradation that may have a central role in protein turnover.

Because of its central role in protein synthesis, evidence supports that mTOR is central to aging (Johnson, Rabinovitch, & Kaeberlein, 2013). While mTOR influences cell

growth and proliferation as well as protein turnover promoting the aging phenotype, the mechanisms with which mTOR inhibition leads to prolonged lifespan are not entirely understood. Studies in the past five years have shown that the mTOR inhibitor rapamycin extends lifespan (Bjedov et al., 2010; D. E. Harrison et al., 2009; R. A. Miller et al., 2011). Our lab has demonstrated that chronic rapamycin feeding in mice inhibits mTOR and slows DNA synthesis in skeletal but maintains mitochondrial biogenesis (Drake et al., 2013). Reductions in DNA synthesis indicate decreased cellular proliferation may indicate an energetic switch towards maintenance of existing cellular structures particularly mitochondria instead of proliferation. Given that mTOR negatively regulates autophagy (Joungmok Kim, Kundu, Viollet, & Guan, 2011), rapamycin may promote autophagy to recycle damaged organelles and proteins, which are some underlying mechanisms of aging (Bürkle, 2001).

Oxidative stress, mTOR, and aging

As already addressed, oxidative stress promotes aging (Finkel & Holbrook, 2000), however recent research supports the notion that mTOR may have an independent role in aging (Cornu et al., 2013). There is little consensus or evidence on which of the two is the driving factor of aging (Blagosklonny, 2008), though it is unlikely that these TOR signaling and oxidative stress are entirely unrelated. Studies of long-lived species have demonstrated the shared characteristic of simultaneous mTOR inhibition and maintenance of mitochondrial biogenesis (Drake et al., 2013; B. F. Miller, Robinson, Bruss, Hellerstein, & Hamilton, 2012). Mitochondrial biogenesis is associated with increased PGC-1α transcription, which is responsible for upregulation of proteins involved in oxidative stress resistance (St-Pierre et al., 2006). mTOR inhibition through pharmacological intervention

promotes lifespan (D. E. Harrison et al., 2009; R. A. Miller et al., 2011). Additionally, mTORC1 inhibition increases mitochondrial respiration, which possibly reflects increases in mitochondrial translation (supported by results in our lab (Drake et al., 2013)), which could increase content of complexes involved in oxidative phosphorylation decreasing ROS-induced oxidative stress (Lambert & Merry, 2004) as well as mitochondrial biogenesis itself (Ventura-Clapier et al., 2008, p. 1).

While mTOR inhibition can have an impact on ROS-related pathways, ROS can also influence mTOR activation as well. Hydrogen peroxide activates the protein translation regulating S6k pathway, which is downstream of mTOR, suggesting that ROS could modulate mTOR signaling (Bae et al., 1999). Radisavlijevic and colleagues also have demonstrated that hydrogen peroxide activates PI3K and Akt, which are upstream of and activate mTOR (Radisavljevic & González-Flecha, 2004). Further, growth factors such as insulin stimulate NOx which generates ROS activating mTOR in the process (Ceolotto et al., 2004). Amino acids, which stimulate mTOR activation (Ma & Blenis, 2009), also stimulate ROS production likely mediated by NOx (Pérez de Obanos et al., 2007). Thus, mitigation of ROS through mitochondrial biogenesis could affect mTOR signaling to slow aging.

Regardless of whether ROS induce TOR or vice versa, their known interaction provides interesting avenues with which to approach treatment of aging. Based on the literature, mTOR inhibition and maintenance mitochondrial biogenesis seem to be keys to slowed aging. Understanding ways in which we can replicate this phenotype in humans may provide possible ways to improve the aging process in humans. As reviewed, few treatments have been effective in attenuating oxidative stress and its outcomes such as disease and aging (Bjelakovic et al., 2007; M. C. Gomez-Cabrera et al., 2012). However,

newer strategies to attenuate oxidative stress through supplement intake designed to upregulated antioxidant enzymes have shown promise (Nelson et al., 2006). Establishing an *in vitro* model that demonstrates mitochondrial biogenesis induced through an oxidative challenge may provide insight into the mechanisms that promote mitochondrial biogenesis and are characteristic of the long-lived phenotype. Further, such a model would provide the opportunity to test therapies designed to manage oxidative stress without interfering with ROS-related signaling involved in beneficial cellular adaptations.

INVESTIGATING OXIDATIVE STRESS AND PROTEIN SYNTHESIS

As our lab, and others, investigates the potential role of oxidative stress in the regulation of protein synthesis, we rely on cell culture models to elucidate the underlying mechanisms and relations between oxidative stress and protein synthesis. However, prior to determining the possibility that upregulation of antioxidant enzymes can prevent oxidative stress-related decrements in protein synthesis, we must develop an *in vitro* model that best simulates *in vivo* conditions of oxidative stress and measure the protein synthesis response.

In vitro methods of inducing oxidative stress

Currently, our lab uses hydrogen peroxide to induce oxidative stress *in vitro*. Considering its role as a signaling molecule and abundance *in vivo*, it is intuitive to use hydrogen peroxide *in vitro* to examine oxidative stress. However, recent research has led researchers to question the efficacy of using hydrogen peroxide *in vitro* to induce oxidative stress (Forman, 2007). Recent studies have shown that hydrogen peroxide does not freely diffuse across cell membranes (Bienert, Schjoerring, & Jahn, 2006), exogenous hydrogen

peroxide is less effective in inducing signal transduction (Choi et al., 2005), and hydrogen peroxide functions spatially (Kaludercic, Deshwal, & Di Lisa, 2014).

Cell membrane lipid composition affects the permeability of hydrogen peroxide serving as a protective mechanism against oxidative stress (Sousa-Lopes, Antunes, Cyrne, & Marinho, 2004). Further, hydrogen peroxide relies upon aquaporins, membrane channels, to pass through membranes. Aquaporins function predominantly to assist in the export of mitochondrial ROS in the cytoplasm to aid in scavenging increased production of ROS (Calamita et al., 2005). Regulation of hydrogen peroxide permeability and diffusion becomes an important aspect in stress regulation given the volatility of ROS. Recent reviews detail the compartmentalization of redox reactions citing the evidence that subcellular compartments have varying thiol:disulfide ratios (Halvey et al., 2005; Jones & Go, 2010). Researchers speculate compartmentalization is a means of controlling signaling, protein trafficking, and regulation of enzyme and gene expression (Kaludercic et al., 2014). By adding spatial barriers, temporal barriers become limiting factors of the area of ROS effect. The half-life for hydrogen peroxide is 1ms and only 1μs for superoxide, which limits the ability for exogenous ROS to diffuse through semi-permeable membranes and affect endogenous cellular components (Bienert et al., 2006). Choi and colleagues speculate that exogenous hydrogen peroxide has a different effect in signaling because of its spatial constraints (Choi et al., 2005).

While not fully understood, the source of ROS used in *in vitro* experiments seems to have an impact on the resulting effects. Currently, there are a variety of ROS used *in vitro* experiments which makes it difficult to draw conclusions from and compare studies. Researchers have used UV light, hydrogen peroxide, cadmium, and paraquat to induce

oxidative stress. One group have demonstrated within the same experiment that cells are resistant to oxidative stress induced by one chemical yet are not to others suggesting there are different mechanisms of action and that the oxidative stress generated by each chemical is different (Salmon et al., 2005). Further, interventions designed to augment resistance against oxidative stress, such as long-lived dwarf mice, have demonstrated that the improvement in tolerance towards an oxidative stressor varies depending on the type. In one study, long-lived mice had greater improved resistance to hydrogen peroxide (147%) compared to their control counterparts. However, their improved resistance to paraquat was only 53% (Murakami, Salmon, & Miller, 2003). Despite the fact that using either stressor (hydrogen peroxide, paraquat) demonstrated the improved resistance to oxidative stress in long-lived mice, the results do raise the issue of selecting a proper stressor for experimental use.

Paraquat

Paraquat (1, 1'-dimethyl-4, 4'-bipyridylium dichloride) is a widely used herbicide that affects a broad spectrum of plants. Paraquat is also a highly toxic substance that is the cause of 300,000 deaths per year. Paraquat causes multi-organ – liver, kidney, heart, and the central nervous system - failure, however the predominant cause of death is irreversible lung damage from pulmonary fibrosis. Paraquat is highly deadly with over a 50% case fatality rate. Pulmonary fibrosis typically sets in about five days after ingestion (Gawarammana & Buckley, 2011; Morán, Ortiz-Ortiz, Ruiz-Mesa, & Fuentes, 2010).

Paraquat is a potent redox cycling agent, which is central to the toxic properties. Paraquat in dication form oxidizes reducing agents to form its radical monocation. In turn, the monocation form of paraquat oxidizes an oxygen molecule to produce superoxide and

returns to its dication form. Because of its reduction potential of -446mV, paraquat is very effective as a redox cycling agent and only reacts with only a few reductants that are capable of donating an electron to the dication form of paraquat (Cochemé & Murphy, 2009). In yeast, the NADPH-cytochrome P450 reductase, xanthine oxidase, NADPH-ubiquinone oxidoreductase, and nitric oxide synthase enzyme systems are the only systems that can metabolize paraquat using NADPH to reduce the dication form of paraquat. However, once paraquat monocation reacts with oxygen to form superoxide, it returns to its dication form (Gawarammana & Buckley, 2011), creating a cycle that is ultimately capable of depleting NADPH, disrupting other pathways that rely on NADPH (Morán et al., 2010). Once NADPH is depleted, remaining paraquat is capable of oxidizing oxygen to form superoxide.

In mammals, the source of reduction of the paraquat dication to monocation is complex I of the mitochondria (Cochemé & Murphy, 2008a). Paraquat inhibits complex I from functioning and impairs the electron transport system. Particularly, by oxidizing complex I, paraquat limits the pool of reducing agents in the electron transport system. Without a sufficient number of available reducing agents, free electrons of the electron transport system are likely to react with oxygen to form oxygen instead of completing the electron transport system to form water. Thus, paraquat is associated with a depletion of NADPH, inhibition of complex I, and increased production of mitochondrial ROS, the most common kind of ROS *in vivo*.

While paraquat causes increased production of ROS, it also causes other detrimental damage to the cell. Paraquat further disrupts mitochondrial function by causing calcium dependent permeability increase of the inner mitochondrial membrane making the

mitochondria more susceptible to depolarization, uncoupling, and matrix swelling (Gawarammana & Buckley, 2011). Because osmotic pressure influences hydrogen peroxide permeability (Bienert et al., 2006), paraquat could potentially affect the diffusion of hydrogen peroxide across the mitochondrial membrane. These additional effects of paraquat on cell function and structure may confound results of studies using paraquat to observe the effects of oxidative stress.

Researchers have used paraquat in experiments involving C2C12 myoblasts to measure differentiation and formation of myotubes. Okabe and colleagues demonstrated that paraquat, not exogenously added hydrogen peroxide, induced differentiation in C2C12 myoblasts. They also found that paraquat did not have an impact on TBAR levels which can be used to measure ROS levels, but did produce enough ROS to induce differentiation (Masaaki Okabe, 2010). The same lab reported that paraquat, in the presence of differentiation media – media in which the concentration of FBS 2% instead of 10% - inhibits myotube formation, a hallmark of differentiation (Akiyama et al., 2011b). The induction and inhibition of myotube formation caused by ROS in the two experiments seems contradictory. The theory of hormesis could explain the phenomenon. Differentiation media may cause the production of ROS or at least induce an energetic stress that signals for differentiation. However the additional ROS generated by paraquat could overwhelm the myoblasts and induce oxidative stress inhibiting myotube formation and even signaling for apoptosis.

Researchers have also compared adaptations to exogenously added hydrogen peroxide and paraquat and found that, while both elicited a similar response, the responses were not identical. The investigators found that NF-κB was responsible for inducing the

upregulation of antioxidant enzymes, glutathione peroxidase and catalase, in response to both paraquat and hydrogen peroxide. However, Zhou and colleagues found that inhibiting NF-κB reduced the response of inducing glutathione peroxidase by 80% in response to hydrogen peroxide whereas it only reduced the response by 60% in response to paraquat. Inhibiting NF-κB reduced the upregulation of catalase by 40% and 60% in response to hydrogen peroxide and paraquat respectively. Inhibiting p50 eliminated the upregulation of glutathione peroxidase in response to hydrogen peroxide but only reduced the response to paraquat by 70%. For catalase, though, inhibiting p50 reduced the response to hydrogen peroxide for catalase by 70% and only 50% for paraquat (Zhou, Johnson, & Rando, 2001). While this and other studies have demonstrated that there are similar responses to hydrogen peroxide and paraquat, they are not identical (Murakami et al., 2003; Salmon et al., 2005). Such data leaves us to question whether there are unique pathways restricted to either endogenous or exogenous stressors and if exogenous and endogenous stressors have the same effect on biological systems. The current study aims to compare the differential effect of hydrogen peroxide and paraquat on the protein synthesis in C2C12 myoblasts to establish a model with which to test antioxidant treatment to maintain protein turnover as a means of mitigating oxidative stress-related sarcopenia.

METHODS AND PROCEDURES

STUDY DESIGN

Experiment 1: Investigating the effects of hydrogen peroxide-induced oxidative stress on protein synthesis in C2C12 myoblasts over 8h.

For the first experiment, we measured the effects of hydrogen peroxide on protein synthesis in myofibrillar, cytosolic, and mitochondrial fractions. For confirmation that we induced an oxidative stress, we also measured the content of heme oxygenase-1 (HO-1) relative to actin expression. We cultured and treated C2C12 myoblasts with 0, 50, 100, and 500uM in triplicate for 4 and 8h and used Western blotting to measure protein content and gas chromatography – mass spectroscopy (GC-MS) to measure protein synthesis.

Experiment 2: C2C12 myoblast viability after 24h exposure to varying concentrations of paraquat

In the second experiment, we determined cell viability over a range of paraquat concentrations. Based on several studies that chronically treated C2C12 myoblasts with paraquat(Akiyama et al., 2011a; Masaaki Okabe, 2010; Masaaki OKABE, n.d.) and several studies that acutely treated fibroblasts with paraquat(Elbourkadi, Austad, & Miller, 2014; Salmon et al., 2005) we chose nine concentrations of paraquat and a control. These concentrations were: 0, 2, 5, 7, 1, 15, 25, 50, 75, 100mM. We cultured and treated C2C12 cells in duplicate for 24 hours. Qualitative assessments of confluence, morphology, and adherence were made at 1, 2, 4, 6, 9, 12, 14, 21, 24, and 30h using microscopy. Because the purpose of this experiment was to verify a concentration of paraquat that C2C12 cells could

sustain for 24h, we measured the cell viability for 30h to ensure any treatments in subsequent 24h experiments would not kill the cells.

Experiment 3: The effect of two concentrations of paraquat on protein synthesis over 12h in C2C12 myoblasts

In the following experiment, we measured the protein synthesis rate of C2C12 myoblasts in response to 0, 1, and 2mM of paraquat compared to control over 4, 8, and 12h. C2C12 myoblasts were cultured and treated in triplicate. To determine the portion of synthesized proteins, we used GCMS to measure mitochondrial, cytosolic, and myofibrillar protein synthesis for each treatment and time point.

Experiment 4: Verification of paraquat-induced oxidative stress

In the fourth experiment, we used treatment concentrations from experiment 1 to determine the relative oxidative stress induced at 8, 12, and 24h with 0 and 2mM of paraquat treatment. Cells were cultured and treated in duplicate. Oxidative stress induced by paraquat was measured by quantifying protein content of heme oxygenase-1 (HO-1). Responses were compared over time points and between treatments measuring whether each concentration of paraquat induced an oxidative stress.

Experiment 5: Comparing metabolic activity of C2C12 myoblasts after exposure of varying concentrations of paraquat and hydrogen peroxide using the WST-1 assay

In the fourth experiment of the series, we sought to compare metabolic activity of C2C12 myoblasts in response to treatments of hydrogen peroxide and paraquat at 24h. Using a WST-1 assay, which directly measures metabolic activity and allows for indirect comparisons of cell viability, we compared various concentrations of hydrogen peroxide and paraquat. We cultured and treated cells with hydrogen peroxide at 5, 10, 25, 50, 100,

125, 150, 250, 500μM, and 1mM and paraquat at 50, 100, 150, 250, 500μM, and 1, 1.25, 1.5, 2, 5mM. We ran eight replicates of each concentration except for control for which we ran 16 replicates. We compared the metabolic activity of the averaged values for each treatment concentration.

STUDY MATERIALS AND METHODS

Cell culture and model

All experiments used C2C12 myoblasts (passages 4-8) on 60mm culture dishes (P60) or 96-well assay plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a 5% CO_2 humidified incubator at 37°C. For synthesis experiments, cells were cultured in P60 plates with media until they were approximately 75% confluent. We then applied D_2O-enriched media concurrently with hydrogen peroxide or paraquat treatments. We enriched media with D_2O by adding sterilized 99% D_2O (Sigma-Aldrich, St. Louis, MO, USA) to the supplemented DMEM to yield approximately 4% D_2O-enriched media. Cells were harvested in 100μL for experiment 2 and 1mL for synthesis experiments of isolation buffer #1 (100mM KCl, 40mM Tris HCl. 10mM Tris Base, 5mM MgCl2, 1mM EDTA, 1mM ATP, pH 7.5) with phosphatase and protease inhibitors (HALT, Thermo Scientific, Rockford, IL, USA). For synthesis experiments, 1mL samples of media were taken from every plate to measure media D_2O-enrichment and then rinsed with 1mL of non-sterile PBS immediately before scraping.

Fractionation

In experiments only using Western blot analysis, we sonicated harvested cells and then spun them at 10,000g for 10 min at 4°C. We extracted the supernatant and used it for

Western blot analysis. For synthesis experiments, we prepared mitochondrial enriched (Mito), cytoplasmic (Cyto), and mixed (Mix) fractions. The initial cell extract was centrifuged at 800g for 10 min at 4°C. We transferred the supernatant to another tube and labeled the pellet as the Mix protein fraction. The Mix pellet was carefully washed with 1ml buffer #1. We centrifuged the supernatant at 9000g for 30 min at 4°C to pellet the Mito fraction. We removed 400μL of the supernatant for the Cyto fraction. Equal volume (400μL) of 14% sulfosalicyclic acid (SSA) was added to the 400μL Cyto-labeled supernatant. We vortexed the Cyto tube incubated it on ice for 1 hr. In the meantime, we washed the Mito pellet with 200μL buffer #2 (100mM KCl, 10mM Tris-HCl, 10mM Tris base, 1mM $MgSO_4$, 0.1mM EDTA, 0.02mM ATP, and 1.5% BSA, pH 7.4) and then centrifuged it at 8000g for 10 min at 4°C. The supernatant was removed and the pellet was washed a second time with 100μL buffer #2. We then centrifuged the resuspended Mito pellet at 6000g for 10 min at 4°C. The supernatant was removed and the Mito pellet was carefully washed with 1 ml ddH_2O. After the 1h incubation, we centrifuged the Cyto tube at 16000g for 10 min at 4°C yielding a protein pellet. We washed both the Cyto and Mix pellets with 500μL 100% ethanol, centrifuged at 1000g for 4 min at 4°C, washed with 500μL ddH_2O and centrifuged at 1000g for 4 min at 4°C. This washing process with ethanol and ddH_2O was repeated once. Mito, Cyto, and Mix pellets were then solubilized in 250μL 1N NaOH for 15 min at 50°C and hydrolyzed in 1.5mL 6N HCl for 24h at 120°C.

Cation Exchange and Derivitization

We ion-exchanged the hydrolysates from the fractionation process, dried them under vacuum, and then resuspended them in 1mL molecular biology grade H_2O. 500μL of sample were derivitized by addition of 500μL acetonitrile, 50mM K_2HPO_4, pH 11, and 20μL

of pentafluorobenzyl bromide. Derivatives were sealed and incubated at 100°C for 1h. Ethyl acetate was added and the organic layer was removed to a GC vial and dried under N_2. Samples were reconstituted in ethyl acetate then analyzed. To prepare cell culture media for analysis, 125µL of sample were pipetted into the inner well of an o-ring screw cap and the tubes were placed on the heat block overnight. 2µL of 10M NaOH and 20µL of acetone were added to each sample. The tubes were capped immediately after addition of acetone, vortexed at a low speed, and then sat overnight at room temperature. Samples were extracted by addition of 200µL hexane and anhydrous Na_2SO_4 and the organic layer was transferred to GC vials to be analyzed.

Gas Chromotography Mass Spectroscopy (GCMS)

Derivatized amino acids were analyzed on an Agilent 7890A GC coupled to an Agilent 5975C MS. GC separation used an Agilent DB-5MS GC column (30m × 0.25mm × 0.25µm) while mass spectrometry was performed with negative chemical ionization (NCI). Samples were injected in 1µL volumes using splitless mode (inlet temperature 220°C). We used helium as the carrier and methane as the reagent gas. We monitored the mass-to-charge ratios of 448, 449, and 450 for the pentafluorobenzyl-N,Ndi(pentafluorobenzyl)alaninate derivative. In all cases, these mass-to-charge ratios represented the primary daughter ions that included all of the original hydrocarbon bonds from the given amino acid. ^2H enrichment was calculated as the M+1 divided by the sum of the abundance of M+0 and M+1. We calculated the newly synthesized fraction of proteins from the ^2H enrichment of alanine in the hydrolyzed protein divided by the precursor enrichment estimated from media enrichment as determined from the media enrichment of water with subsequent adjustment using mass isotopomer distribution analysis (MIDA).

Western Blotting

We measured protein concentration with a bicinchoninic acid assay (Thermo Fisher, Rockford, IL, USA). Diluted samples containing equal amounts of protein were prepared in Laemmli Sample Buffer and 2-mercaptoethanol and heat denatured for 5 minutes at 98°C. Proteins were resolved on a 10% - 15% Tris/glycine SDS-polyacrylamide gel in running buffer containing 25mM Tris, 192mM glycine, pH 8.3. Proteins were transferred at 100V for 75 minutes in 20 % w/v methanol, 0.02% w/v SDS, 25mM Tris Base, 192mM glycine, pH 8.3 to nitrocellulose paper. Non-specific proteins were blocked by incubation of membrane in 5% nonfat dry milk (Kroger) in TBST (20mM Tris, 150mM NaCl, 0.1% Tween 20, pH 7.5) at 4°C overnight. Antibodies were purchased from Abcam (Cambridge, MA, USA; NAD(P)H dehydrogenase quinone 1 (NQO1) #34173, heme oxygenase-1 (HO-1) #13248),or Santa Cruz Biotechnology (Santa Cruz, CA, USA; β-tubulin sc-5274, actin sc-1616). Blots were incubated overnight with primary antibodies diluted 1:1000 (HO-1, NQO1) or 1:500 (actin). Blots were washed in TBST and incubated with HRP- conjugated secondary antibody diluted 1:5000 in 5% nonfat dry milk (Kroger) in TBST with subsequent chemiluminescence detection (West Dura; Pierce, Rockford, IL, USA). Images were captured and densitometry analyzed using a UVP Bioimaging system (Upland, CA, USA). Membranes were probed for actin (1:500) to verify equal loading of protein.

WST-1 Assay

C2C12 myoblasts were cultured normally and plated onto 96-well assay plates with a concentration of 25,000 cells/100μL. Density was determined by suspending cells in media and placing 10μL of the suspension into each chamber of a hemocytometer. Cells were counted in at least three $0.1mm^3$ areas of a hemocytometer and averaged to

determine the density of the suspension. Additional media was added to dilute the suspension to 25,000 cells/100µL of media and then 100µL of the suspension was transferred to each well of the 96 well assay plate. After an hour of allowing the myoblasts to adhere, media was replaced with 100uL of respective treatments (eight replicate samples). After 24 hours, the treatments were removed and replaced with 100µL of fresh media. After replacing the media, we applied 10µL of WST-1 (Roche, Mannheim, Germany), a tetrazolium salt reagent, to each well and allowed them to incubate for 2h. After 2h, we analyzed WST-1 reagent activity using a microplate (ELISA) reader to measure the difference of absorbance at 440nm and 600nm. Greater differences between the two absorbance levels indicated greater metabolic activity.

Statistical Analysis

We performed statistical analyses using SPSS Version 22 (SPSS Inc., Chicago, IL) and Prism Version 6 (GraphPad Software, La Jolla, CA). We assessed the main outcome variables between groups using two-way ANOVA and Tukey's post hoc tests where appropriate. We set statistical significance *a priori* at $p \leq 0.05$.

RESULTS

Experiment 1: Measurement of the transient effect of hydrogen peroxide-induced oxidative on protein synthesis in C2C12 myoblasts

Cells treated with 100uM hydrogen peroxide for 4h had greater mitochondrial protein synthesis rates (CON: 1.89%/hr +/- 0.068, 100uM: 2.75%/hr +/- 0.098 $p<0.05$) whereas 500uM significant decreased mitochondrial protein synthesis rates after 4h (CON: 1.89%/hr +/- 0.068, 500uM: 1.338%/hr +/- 0.226 $p<0.05$) (Figure 1). Mitochondrial protein synthesis was still increased relative to control at 8 hr after when treated with 100uM hydrogen peroxide treatment (CON: 1.075%/hr +/- 0.109, 100uM: 1.419%/hr +/- 0.069 $p<0.05$) (Figure 2).

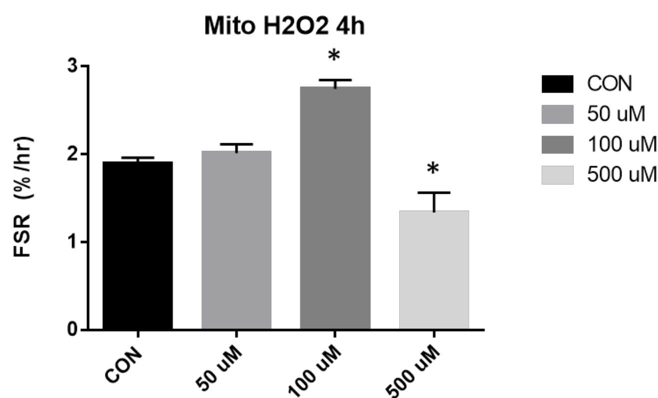

Figure 1. Hydrogen peroxide exposure increases mitochondrial (mito) protein synthesis after 4h of exposure. * indicates $p<0.05$ compared to control at 4h.

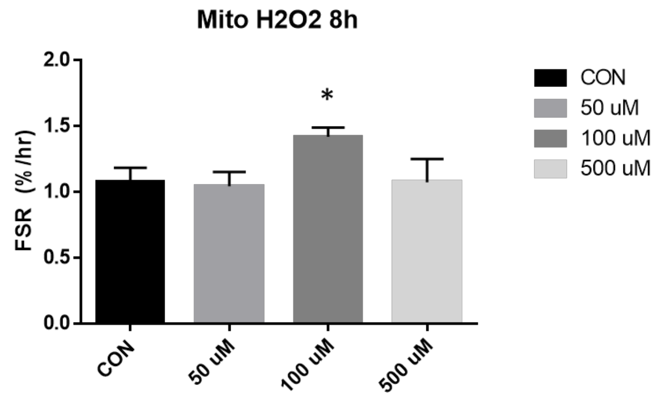

Figure 2. Hydrogen peroxide exposure increases mitochondrial (mito) protein synthesis after 8h of exposure. * indicates $p<0.05$ compared to control at 8h.

50uM hydrogen peroxide decreased cytosolic protein synthesis rates after 4h of exposure, though no other concentrations had significant effects (CON: 2.379%/hr +/- 0.296, 50uM: 1.318%/hr +/- 0.235 $p<0.05$) (Figure 3). After 8h of exposure, hydrogen peroxide had no effect on cytosolic protein synthesis (Figure 4).

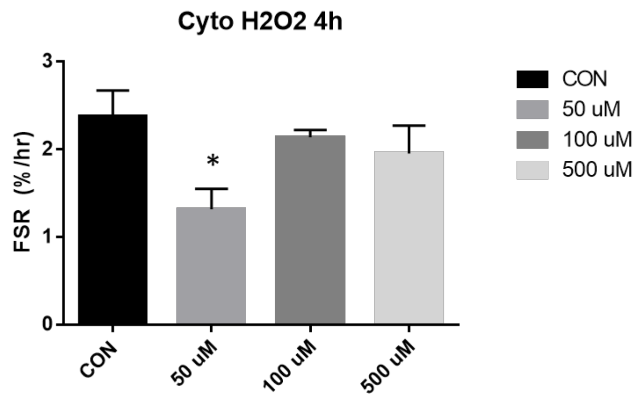

Figure 3. Hydrogen peroxide exposure decreases cytosolic (cyto) protein synthesis after 4h of exposure. * indicates $p<0.05$ compared to control at 4h.

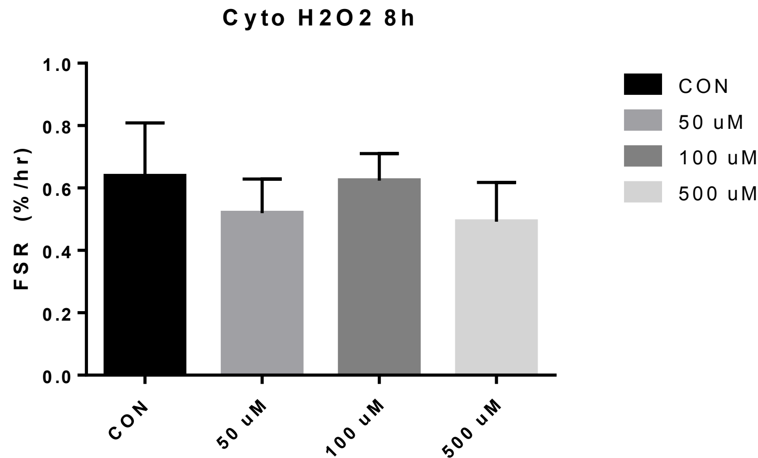

Figure 4. Hydrogen peroxide exposure has no effect on cytosolic (cyto) protein synthesis after 8h of exposure.

We investigated whether treatment with hydrogen peroxide induced an oxidative stress compared to control after 4h and 8h of exposure in C2C12 myoblasts. None of the concentrations of hydrogen peroxide increased heme oxygenase-1 (HO-1) content when assessed at 4 hrs or 8hrs compared to control (Figures 5 and 6).

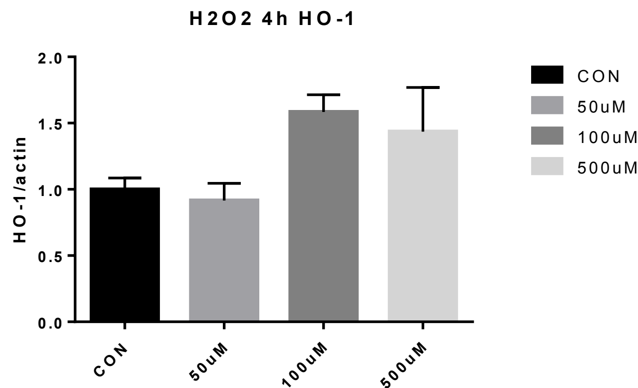

Figure 5. HO-1 content relative to actin after 4h of exposure to various levels of hydrogen peroxide. Arbitrary units are relative to control at 4h.

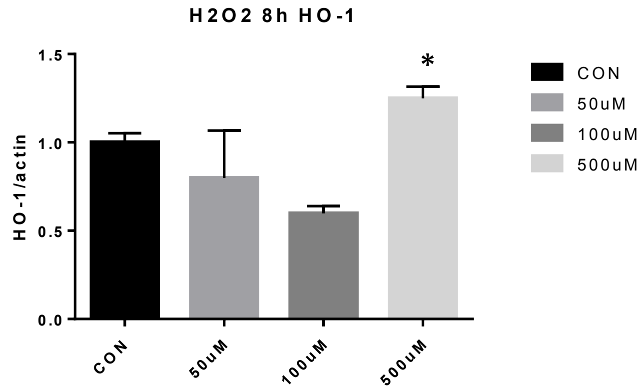

Figure 6. HO-1 content relative to actin after 8h of exposure to various levels of hydrogen peroxide. Arbitrary units are relative to control at 8h. * indicates $p<0.05$ compared to 100uM.

Experiment 2: 30h of C2C12 myoblast viability in varying concentrations of paraquat

After 12h of exposure, concentrations of 25mM and above killed the myoblasts (Table 1). C2C12 myoblasts responded in a concentration dependent manner to paraquat. As paraquat concentration increased, qualitative characteristics indicated greater stress and time to entire cell death decreased. A full report on cell morphology and death is provided in Table 1. Based on these results, the maximum concentration of paraquat that C2C12 myoblasts can tolerate after 30h of exposure is 2mM.

Table 1. Qualitative assessments of C2C12 myoblast morphology and apoptosis in response to varying concentrations of paraquat over 30h.

Concentration	1h	2h	4h	6h	9h	12h	14h	21h	24h	30h
100mM	Nothing remarkable	Signs of stress (less robust)	Shriveling	Shriveled, near dead, floating	Dead	Dead	Dead	Dead	Dead	Dead
75mM	Nothing remarkable	No different	Signs of shriveling starting	Shriveled	Mostly dead	Dead	Dead	Dead	Dead	Dead
50mM	Nothing remarkable	No different	Small difference	Signs of shriveling	Close to death	Dead	Dead	Dead	Dead	Dead
25mM	Nothing remarkable	No different	No difference	Some stress, little difference	Shriveled	Dead	Dead	Dead	Dead	Dead
15mM	Nothing remarkable	No different	No difference	Some stress, little difference	Signs of stress	Shriveled	Mostly dead	Dead	Dead	Dead
10mM	Nothing remarkable	No different	No difference	Some stress, little difference	Little difference	Signs of stress, some shriveling	Shriveled	Dead	Dead	Dead
7mM	Nothing remarkable	No different	No difference	Some stress, little difference	Little difference	Some shriveling started	Shriveling	Dead	Dead	Dead
5mM	Nothing remarkable	No different	No difference	Some stress, little difference	Little difference	Some shriveling started	Shriveling	Dead	Dead	Dead
2mM	Nothing remarkable	No different	No difference	Some stress, little difference	Little difference	Some shriveling started	Shriveling	Shriveling	Stressed	Some death
0mM	Nothing remarkable	No different	No different	No different	No different	No different	No different	No different	Crowded	Crowded

Experiment 3: The effect of two concentrations of paraquat on protein synthesis over 12h in C2C12 myoblasts

Mitochondrial fractional synthesis rate (FSR) remained constant throughout each time point in control. 2mM paraquat transiently increased mitochondrial FSR in cells treated with 2mM paraquat at 4h compared to control and 1mM paraquat treated cells (0mM: 4.30%/hr +/- 0.33, 1mM: 5.74%/hr +/- 1.191, 2mM: 6.86%/hr +/- 0.552, $p<0.05$ comparing 2mM to 0mM and 1mM). Cells treated with both concentrations of paraquat experienced declines mitochondrial FSR over time after 8h of exposure (1mM: 5.74%/hr +/- 1.191, 3.42%/hr +/- 0.032; 2mM: 6.86%/hr +/- 0.552, 4.15%/hr +/- 1.336, 4h and 8h

respectively, p<0.05 between time points). However, there were no differences between groups at 12h (Figure 7).

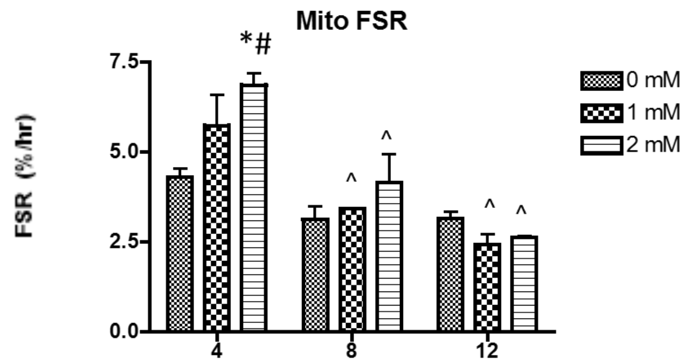

Figure 7. The fractional synthesis rate (FSR) of the mitochondrial (mito) fraction of C2C12 myoblasts treated with 0, 1, 2mM paraquat over 4, 8, 12h. * indicates p<0.05 compared to 0mM, # indicates p<0.05 compared to 1mM, ^ indicates p<0.05 compared to the respective 4hr protein synthesis rate.

In the myofibrillar fraction, both 1mM and 2mM paraquat treatments transiently increased FSR compared to control. At 4h, FSR was higher in paraquat treated cells compared to control (0mM: 1.77%/hr +/- 0.811, 1mM 3.22%/hr +/- 0.57, 2mM 3.46% +/- 0.113 p<0.05), though by 8h there was no difference in FSR between treatments. By 12h, FSR in 2mM paraquat was significantly lower than control (0mM: 2.15%/hr +/- 0.085, 2mM: 1.33%/hr +/- 0.038 p<0.05). FSR in 2mM paraquat treated cells progressively decreased over time as well (4h: 3.46%/hr +/- 0.113, 8h: 2.44%/hr +/- 0.474, 12h: 1.33%/hr +/- 0.398, p<0.05 between each time point) (Figure 8).

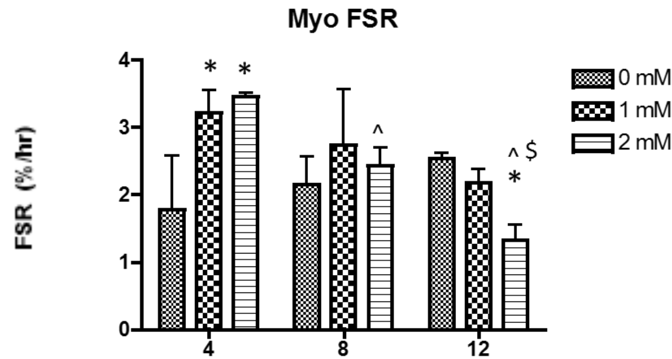

Figure 8. The fractional synthesis rate (FSR) of the myofibrillar (mixed) fraction of C2C12 myoblasts treated with 0, 1, 2mM paraquat over 4, 8, 12h. * indicates p<0.05 compared to 0mM, ^ indicates p<0.05 compared to the respective 4hr protein synthesis rate, $ indicates p<0.05 to the respective 8h protein synthesis rate.

1mM paraquat increased cytosolic FSR in C2C12 myoblasts at 4h, though there was no difference between control and 2mM (0mM: 1.952%/hr +/- 0.125, 1mM: 2.48%/hr +/- 0.247 p<0.05). However, this increase was only transient as cytosolic FSR decreased in 1mM and 2mM paraquat treated cells from 4h to 8h (4h: 2.478%/hr +/- 0.247, 2.34%/hr +/- 0.181, 8h: 1.82%/hr +/- 0.242, 1.27%/hr +/- 0.181 respectively, p<0.05 between time points). 2mM paraquat cells had a lower FSR than control by 8h and 12h (0mM: 1.88%/hr +/- 0.338, 1.96%/hr +/- 0.031; 2mM: 1.27%/hr +/- 0.284, 1.26%/hr +/- 0.109, p<0.05 between concentrations). Cytosolic FSR of 1mM paraquat treated was also significantly lower than control at 12h (0mM: 1.96%/hr +/- 0.031, 1mM: 1.64%/hr +/- 0.027, p<0.05) (Figure 9).

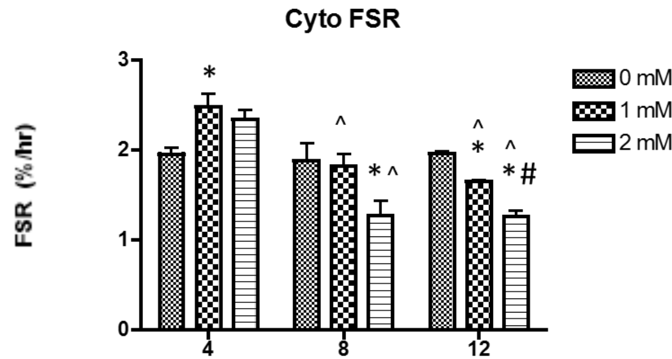

Figure 9. The fractional synthesis rate (FSR) of the cytosolic (cyto) fraction of C2C12 myoblasts treated with 0, 1, 2mM paraquat over 4, 8, 12h. * indicates p<0.05 compared to 0mM, # indicates p<0.05 compared to 1mM, ^ indicates p<0.05 compared to the respective 4hr protein synthesis rate.

Experiment 4: Verification of paraquat-induced oxidative stress

In response to 2mM paraquat, C2C12 myoblasts transiently increased the content of HO-1. At 12h, 2mM paraquat increased HO-1 content relative actin compared to control (0mM: 0.452 +/- 0.133 vs. 2mM: 1.434 +/- 0.487 p<0.05. By 24h, there was no significant difference between paraquat treatment and control (Figure 10).

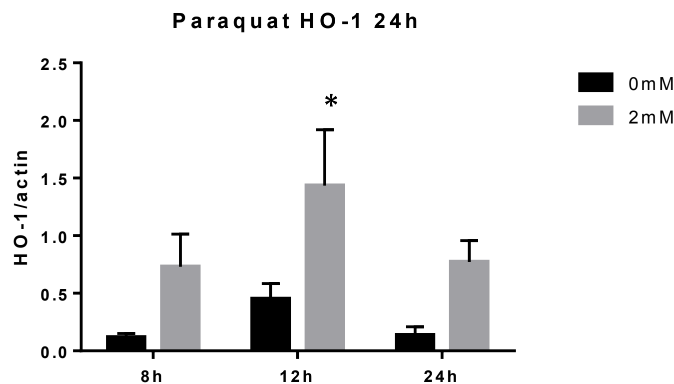

Figure 10. HO-1 content relative to actin content in response to various concentrations of paraquat. Values expressed are arbitrary units indicating degree fold change relative to 0mM 8h. * indicates p<0.05 compared to 0mM.

Experiment 4: Comparing metabolic activity of C2C12 myoblasts after exposure of various concentrations of paraquat and hydrogen peroxide using the WST-1 assay

Metabolic activity was measured through a color change induced by the WST-1 reagent using a spectrophotometer. Activity was measured as the absorbance at 440nM subtracted by the absorbance at 690nM. Larger differences indicate greater levels of metabolic activity. Compared to control, there was no difference in metabolic activity until cells were treated with 500uM of paraquat at which point metabolic activity significantly decreased by 24% (2.55 vs. 3.37, $p<0.05$). Metabolic activity was no different with the application of any concentration below 1mM hydrogen peroxide, which had 81% less absorbance difference than control (0.64 vs. 3.37 $p<0.05$) (Figure 8).

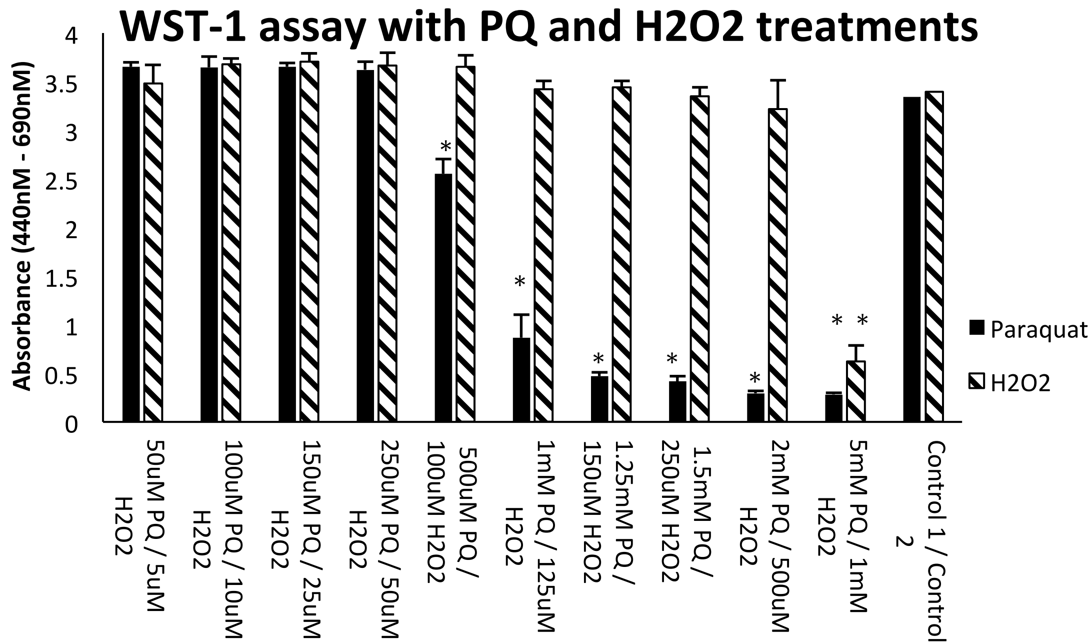

Figure 11. WST-1 Assay results. Shows the difference in absorbance at 440nM and 690nM. X-axis labels indicate paraquat concentration/hydrogen peroxide concentration. * indicates $p<0.05$ compared to control.

DISCUSSION

The main finding in our study is that similar to hydrogen peroxide treatment, 2mM paraquat was able to stimulate mitochondrial protein synthesis in C2C12 myoblasts. Our study demonstrates that paraquat induces oxidative stress in C2C12 myoblasts based on the observed upregulation in HO-1 content in cells treated with 2mM paraquat. Our model demonstrates that paraquat caused transient and mild oxidative stress and induces mitochondrial biogenesis. We observed that cells treated with hydrogen peroxide induces mitochondrial biogenesis as well. With this established model of the effects of a mild oxidative stress on mitochondrial biogenesis, we can further investigate the theory of mitohormesis and the role of ROS in cellular adaptation to stress. In summary, our results indicate we have established a model with which to examine the theory of mitohormesis by showing that an acute oxidative stress induces positive adaptations that may lead to the greater stress resistance.

ROS and mitochondrial biogenesis

ROS are signaling molecules required for positive adaptations that promote stress resistance (Ristow et al., 2009; Ristow & Schmeisser, 2014). ROS increases expression of PGC-1α (Silveira, Pilegaard, Kusuhara, Curi, & Hellsten, 2006) through AMPk activation (Irrcher, Ljubicic, & Hood, 2009). PGC-1α is considered the "master regulator" of mitochondrial biogenesis. Mitochondrial biogenesis is associated with extending the healthspan and slowing aging (López-Lluch, Irusta, Navas, & de Cabo, 2008). We demonstrate that application of hydrogen peroxide and paraquat generated increased ROS concentrations and mitochondrial protein synthesis, mitochondrial biogenesis. It is likely

that the correlation between increased concentrations of ROS and increased mitochondrial protein synthesis is causally linked.

The literature on antioxidant supplementation and its effect on adaptation to exercise is divided. Gomez-Cabrera and colleagues reported that vitamin C blunted improvements in endurance capacity and mitochondrial biogenesis citing decreased expression of PGC-1α and TFAM transcripts (M.-C. Gomez-Cabrera, Domenech, Romagnoli, et al., 2008). Further, Ristow and colleagues found that antioxidant supplementation blunted the 4-week long exercise regimen mediated improvement in insulin sensitivity in humans. Further, antioxidant supplementation blunted increases in PGC-1α expression as well as SOD1 and SOD2 expression levels. Ristow and colleagues suggest the lack of transient oxidative stress indicated by lower levels of TBARS formation, a marker of oxidative stress, explains the antioxidant mediated abolishment of adaptations to exercise (Ristow et al., 2009). However, Wadley and McConell found that vitamin C supplementation did not blunt increases in AMPk phosphorylation, PGC-1α, NRF-2, or NRF-1 mRNA expression after a 60-minute bout of running in rats (Wadley & McConell, 2010). Further, Higashida and colleagues demonstrated that vitamin C supplementation did not blunt exercise adaptations, both acute and chronic, demonstrating that GLUT4 and SOD2 content were no different between exercised individuals supplemented with vitamin C and placebo. They also compared markers of mitochondria content and function and found no difference between the groups (Higashida, Kim, Higuchi, Holloszy, & Han, 2011). Their results contradict the studies conducted by Ristow and Gomez-Cabrera and argue that antioxidant supplementation does not have an effect on mitochondrial biogenesis.

Studies debate whether antioxidants blunt ROS-mediated mitochondrial biogenesis using transcriptional levels of PGC-1α and other surrogate measures as evidence for mitochondrial biogenesis. A measurement such as PGC-1α transcription levels is an incomplete measurement of the multifactorial process as it does not account for downstream processes such as translation regulation and post-translational modification. Instead, direct measurement of mitochondrial protein synthesis using stable isotopic tracers is a more appropriate method of measuring biogenesis (B. F. Miller & Hamilton, 2012). Thus, our series of experiments presented are important because they demonstrate using isotopic tracers (deuterium oxide enrichment of alanine) that ROS, either from extracellular application (hydrogen peroxide) or induction of intracellular production (paraquat), increase mitochondrial protein synthesis.

While previous data in our lab supported that exogenously added hydrogen peroxide increased mitochondrial protein synthesis, there were concerns about the utilization of exogenous hydrogen peroxide. As reviewed, hydrogen peroxide does not freely diffuse across membranes and the half-life of hydrogen peroxide is reportedly 1ms (Bienert et al., 2006). Even then, one study highlighted that hydrogen peroxide was undetectable four hours after applying 250μM hydrogen peroxide *in vitro*(Fan, Hussien, & Brooks, 2010). Further, even within the cell, ROS are compartmentalized and limited to redox signaling proteins within that compartment (Jones & Go, 2010). Thus, it is questionable whether exogenous application of hydrogen peroxide has similar effects of endogenously produced hydrogen peroxide. Thus, finding that paraquat, which induces mitochondrial ROS production, has a stimulating effect on mitochondrial protein synthesis

similar to exogenously added hydrogen peroxide provides further evidence that ROS have a role in mitochondrial biogenesis.

Mitohormesis

Mitohormesis is the notion that low levels of ROS may induce an adaptive response, both in the cytosol and in the nucleus, that leads to greater stress resistance (Yun & Finkel, 2014). Our study found that paraquat stimulated ROS production and mitochondrial biogenesis, which is related to greater stress defenses. Mitochondrial biogenesis is associated with increased levels of PGC-1α transcription does mediate the increase in mitochondrial protein synthesis (Silveira et al., 2006). St-Pierre and colleagues demonstrated that induction of PGC-1α transcription is also associated with key enzymes such as superoxide dismutase which convert the free radical superoxide into the non-radical hydrogen peroxide (St-Pierre et al., 2006). Given the fact that 2mM paraquat was a sustainable treatment for our myoblasts over 30h, our data demonstrate that the mild dose of paraquat acutely induced mitochondrial biogenesis, which could be associated with improved resistance to future oxidative challenges, though further investigation is necessary. Overall, our study establishes a model that could support and allow for further investigation of mitohormesis: exposure to ROS promotes greater stress resistance and mitochondrial protein synthesis that could promote a longer healthspan.

As discussed, ROS are associated with aging and cause disease by damaging key cellular components (Madamanchi, 2004; Rajindar S. Sohal & Orr, 2012b), however, ROS are also necessary for physiological function. The acute increased concentrations of ROS generated by exercise causes a temporary stress that induces positive adaptations such as stress resistance (M.-C. Gomez-Cabrera, Domenech, & Viña, 2008). Without ROS or as some

studies have demonstrated, with antioxidant supplementation, some exercise-related adaptations to stress do not occur (M.-C. Gomez-Cabrera et al., 2005) signifying the importance of ROS in cellular adaptation. These studies considered together describe the hormetic manner in which ROS behave: ROS are necessary for signaling and adaptation, but, ROS in excess cause disease. No study to date, though, has completely demonstrated this hormetic relationship.

Our experiments establish a model with which we can begin directly testing the theory of mitohormesis. In our experiments, we demonstrated increased HO-1 content, a marker of oxidative stress, in response to 2mM paraquat. Further both hydrogen peroxide and paraquat also increased mitochondrial protein synthesis. We also demonstrate that doses above 2mM paraquat caused cell death. Thus, we demonstrate that a treatment, which causes ROS production and causes oxidative stress, has both positive and negative effect on the cell depending on the concentration.

However, further work is necessary to demonstrate that exposure to either stress (paraquat or hydrogen peroxide) was mediated by ROS. If the stimulation of mitochondrial protein synthesis by paraquat is mediated by ROS, concurrent treatment of paraquat and an antioxidant, such as vitamin C, should blunt the increase in mitochondrial protein synthesis and assist in confirming mitohormesis. Data indicating greater resistance to oxidative stress as a result of ROS-induced mitochondrial biogenesis would also assist in confirming the theory of mitohormesis. These points considered, these experiments help establish and provide a model to examine these questions in future studies.

Limitations

It is important to point out that, in the first experiment, our study did not demonstrate that hydrogen peroxide induced an oxidative stress. After 8 hours of hydrogen peroxide exposure, HO-1 content did not increase in response to the treatment compared to control even though paraquat exposure did. It is important to note that this does not mean hydrogen peroxide did not induce an oxidative challenge. A previous study from our lab also demonstrated that the known oxidative stressor *tert*-butylhydroperoxide failed to upregulate HO-1. It is possible that more time for exposure to hydrogen peroxide was necessary for the myoblasts to increase HO-1 content(Reuland et al., 2013). Our results may highlight the importance of using a stressor such as paraquat that induces the production of ROS from within the cell to measure the effects of oxidative stress that best reflects *in vivo* oxidative stress.

We did not verify the potential pathway in which oxidative stress affects protein synthesis even though we demonstrated that oxidative stress changes protein synthesis through direct measurement using stable isotope tracers. Investigating the pathways involved in paraquat-induced mitochondrial biogenesis and overall protein synthesis modification would provide further understanding how paraquat-produced ROS induce mitochondrial biogenesis. However, hydrogen peroxide activates mTOR (Radisavljevic & González-Flecha, 2004), which has a central role in protein synthesis (Johnson et al., 2013), as well as PGC-1α (Irrcher et al., 2009), which has a role in mitochondrial biogenesis (Ventura-Clapier et al., 2008). Thus, we hypothesize that paraquat-induced ROS activate or inhibit proteins involved in these pathways to mediate our observations.

While it is not a limitation, the results of the final experiment examining the cell viability in response to the various concentrations of hydrogen peroxide and paraquat were perplexing. The difference in percentage of lethal dose required to change metabolic activity was 25% of the paraquat lethal 30h dose and 100% of the 30h lethal dose of hydrogen peroxide (based on previous lab observations). However, WST-1 requires $NADH_2$ to form into a formazen salt (Roche, Mannheim, Germany). Paraquat blocks complex I and consumes $NADH_2$ as cycles redox reactions depleting any $NADH_2$ for the electron transport system(Cochemé & Murphy, 2008b) and leaving little $NADH_2$ available to react with the tetrazolium salt. Thus, the results observed in this experiment may not be truly indicative of the toxicity of paraquat, but rather more indicative of paraquat-induced inhibition of complex I and depletion of NADH (Cochemé & Murphy, 2008b).

Implications and future directions

The primary outcome of this study was to measure mitochondrial biogenesis. Mitophagy, mitochondrial breakdown and autophagy, is important to consider. Just as general protein turnover is critical to preventing the accumulation of damaged proteins (Starke-Reed & Oliver, 1989; Young et al., 1975), mitochondrial biogenesis and mitophagy may play a similar role in preventing the accumulation of damaged mitochondrial proteins and DNA to maintain mitochondrial function (Lemasters, 2005). Given the nature of cell culture models, myoblasts may be versatile enough to serve as a model to measure protein breakdown in response to an oxidative stress. In this study, we used a deuterium oxide alanine labeling method to measure protein synthesis, which we can also use to measure protein breakdown.

Another potential use of the model we developed is to address the issue of antioxidant supplementation. Given its wide use (Sobal & Marquart, 1994), understanding how antioxidant supplementation interacts with exercise and ROS-related signaling is an important health matter. Our model could provide the opportunity to test the effects of various, common antioxidant supplements on the directly observed oxidative-stress induced mitochondrial biogenesis. We could also use the model to test new strategies to improve cellular oxidative stress resistance through upregulation endogenous antioxidants with phytochemical supplementation (Donovan et al., 2012; Reuland et al., 2013).

To summarize, we have developed an *in vitro* model of oxidative stress using an endogenous oxidant stressor that causes similar effects to fractional protein synthesis as our established model of oxidative stress using an exogenous oxidant stressor. Specifically, 2mM paraquat induces oxidative stress that increases mitochondrial protein synthesis just as 100μM hydrogen peroxide does. Our established *in vitro* model will enable us to investigate the effects of oxidative stress, of varying intensities and duration, on mitochondrial protein synthesis. This model has the potential to assist in elucidating the effects of transient ROS on cell function and adaptation. Particularly, we can investigate mechanisms that improve cellular resistance to stress as a means to identify potential therapies to slow aging and improve health in humans.

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