DISSERTATION

CHANGING MYOGLOBIN'S PARADIGM: CHARACTERIZING THE ROLE BETWEEN LIPIDS AND MYOGLOBIN EXPRESSION

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ABSTRACT

CHANGING MYOGLOBIN'S PARADIGM: CHARACTERIZING THE ROLE BETWEEN LIPIDS AND MYOGLOBIN EXPRESSION

Myoglobin (Mb) is a muscular heme protein generally localized to oxidative muscle, where it functions to store and transport oxygen, as well as scavenge nitric oxide and reactive oxygen species (ROS). While the former role of Mb in oxygen storage/transport is undisputed in diving mammals and other hypoxia-adapted species, this function appears to be far more ambiguous in terrestrial, non-hypoxia-adapted species. During endurance exercise, terrestrial mammals rely on erythrocytic oxygen to fuel aerobic metabolism in working muscle. Physiological changes associated with endurance training elicit responses that increase muscular blood flow and subsequent oxygen delivery. Intramuscular oxygen stores, alternatively, appear to bear little significance in sustaining aerobic metabolism during endurance exercise, as evident by the inability to appreciably release intra-muscular stored oxygen during normoxic exercise; yet, terrestrial endurance athletes who tend to have a higher reliance on lipid-fueled metabolism have more Mb than their sedentary counterparts. Accordingly, Mb's traditional functional paradigm pertaining to oxygen storage and transport does not appear to be fully applicable to terrestrial mammals *in vivo*. Here, a series of datasets are provided offering alternative paradigm, where increases in Mb expression are associated with increases in lipid supplementation. C_2C_{12} cells cultured in normoxic and hypoxic (0.5% oxygen) environments show increased Mb when supplemented with a 5% lipid mixture compared to glucose controls. While Mb regulatory pathways have been shown to involve Ca^{2+} signaling pathways via calcineurin (CN), this lipid-induced Mb stimulation is *not* affiliated with an increase in CN expression, suggestive of a

regulatory pathway for Mb independent of Ca^{2+}. Moreover, lipid-induced Mb stimulation parallels oxidative stress marker augmentation concomitant with Mb augmentation. Addition of antioxidant to lipid-supplemented cells reverses Mb increases, and acute exposure to H_2O_2 during hypoxic differentiation showed an increase in Mb relative to control cells, collectively suggesting a Mb regulatory pathway through redox signaling. Furthermore, comparison of two commonly used Mb assay techniques revealed that normoxic lipid-induced Mb increases are nearly explicitly oxidized, thus bearing important functional implications on Mb increases consequent of lipid stimulation. In light of these novel data and in conjunction with the inability of terrestrial mammals to appreciably utilize Mb oxygen stores during exercise, an alternative paradigm for Mb is proposed. I propose that the role of Mb as an antioxidant defense during terrestrial exercise, which increases lipid-based aerobic metabolism and ROS production, is more relevant and applicable than a role relevant to storage and transport of oxygen.

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DEDICATION

This dissertation is dedicated to my family, especially Sam, Henry, and Eden.

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INTRODUCTION

Background and Broader Impact

As a monomeric heme protein, myoglobin is widely recognized as having physiological importance in both oxidative striated and smooth muscle (Rayner et al., 2009; Wittenberg, 1989). Regarding aerobic metabolism, myoglobin has the ability to reversibly bind oxygen and thus act as a muscular oxygen reservoir. This role in oxygen storage and transport was characterized in 1939 by Glenn Millikan, a physiologist who originally described this "muscle hemoglobin" as having "beautiful simplicity"; yet, research over the past 75 years has elucidated information in support of perhaps a much more complex protein than Millikan originally postulated (Millikan, 1939). In addition to its ability to store and transport oxygen, myoglobin has been shown to aid aerobic cells in that it has the ability to scavenge harmful byproducts of aerobic metabolism; specifically, myoglobin has been proposed as a scavenger of nitric oxide (NO) and reactive oxygen species (ROS) (Flögel et al., 2001; Garry and Mammen, 2007; Hendgen-Cotta et al, 2010; Merx et al., 2001; Wittenberg, 1970). Interestingly, it can be deduced from an oxygen dissociation curve that mammalian myoglobin has a strong affinity for oxygen, and can consequently only release oxygen under a low partial oxygen pressure (Antonini, 1971; Garry and Mammen, 2007; Gros et al., 2010; Nichols and Weber, 1989). Thus, the muscle must necessarily be in a hypoxic state in order to release myoglobin-bound oxygen to be utilized in aerobic metabolism under standard conditions. This presents an interesting perspective of myoglobin in that it theoretically assists cellular respiration solely in a time of potential stress.

Physiological examples of hypoxic stress can occur in high altitude environments or in cardiovascular and pulmonary disease states. Because each of the aforementioned conditions causes low oxygen at the cellular level, they can ultimately manifest into pathological conditions

at the tissue level; yet, myoglobin is capable of providing cytoplasmic oxygen reserves that can be readily used when blood-borne oxygen becomes insufficient. As such, myoglobin presents a potential target for pharmacological intervention and treatment of human hypoxic disease, or more specifically, prevention and/or treatment of consequent tissue necrosis.

Furthermore, given its role as an oxygen store in addition to its ability to scavenge NO and ROS, myoglobin has more recently been proposed as a target in the treatment of tumor metastasis (Galluzzo et al., 2009; Hendgen-Cotta et al, 2010). Rationale for this possibility largely lies within the ability of myoglobin to prevent the stabilization of hypoxia-inducible factor 1α (HIF-1α); namely, myoglobin can provide cellular oxygen and scavenge NO and ROS, thus preventing HIF-1α from inducing angiogenesis and promoting tumor metastasis (Harris et al., 2002; Quintero et al., 2006; Simon, 2006). A recent study by Galluzzo et al., 2009 incorporated the myoglobin gene into a line of human lung carcinoma cells and subsequently injected the transduced cells into mice; their results demonstrated that mice injected with myoglobin transduced cells had suppressed tumor growth and invasion (Galluzzo et al., 2009). Tumor cells with myoglobin were presumed to maintain aerobic respiration while concomitantly mitigating nitrosative and oxidative stress, thus more effectively degrading HIF-1α and subsequently suppressing angiogenesis, in addition to forcing the cell out of the growth phase and into terminal differentiation (Flögel et al. 2004; Harris et al., 2002; Quintero et al., 2006; Simon, 2006). Thus, considering the ability for myoglobin to store oxygen *and* act as an antioxidant, it presents a potentially important target in combating a range of human pathologies, and understanding regulation and stimulation of myoglobin can bridge the gap between pharmacological potential and actual pharmacological practice.

Myoglobin Regulation and Stimulation

The regulation of myoglobin is not yet fully understood; however, our understanding of myoglobin regulation and stimulation is perpetually growing. The myoglobin gene itself is transcriptionally activated via CCAC box, A/T element, NFAT response element, E box, and myocyte enhancing factor-2 (MEF-2) motifs (Bassel-Duby et al., 1993; Grayson et al., 1998; Yan et al., 2001). Beyond the scope of transcription, hypoxia had classically been implicated as a stimulator of myoglobin given its upregulation in high-altitude-adapted native populations (Reynafarje, 1962); however, recent work from Kanatous et al. 2009 has shown that calcium-signaling associated with exercise *in conjunction* with hypoxia is necessary to upregulate myoglobin. Specifically, calcium signaling concomitant with hypoxic exercise releases calcium from the sarcoplasmic reticulum under low partial pressures of oxygen and causes increased translocation of nuclear factor of activated t-cells (NFAT) to the nucleus, thus stimulating an increase in myoglobin gene expression (Kanatous et al., 2009). In addition to hypoxic exercise, myoglobin has been shown to be stimulated through the accumulation of cellular NO in vascular smooth muscle. Addition of NO to vascular smooth muscle cells has a positive correlation with myoglobin gene and protein expression (Rayner et al., 2009). The interpretation of this response is that the smooth muscle cell is responding to an increase in potentially dangerous reactive nitrogen species by increasing its ability to scavenge and subsequently protect itself from NO.

Similarly, if an increase in oxygen-demanding activity in an oxygen-limited environment (i.e. hypoxic exercise) and an increase in NO can stimulate myoglobin expression, then perhaps an alternative stimuli may explain and pertain to myoglobin's third proposed role. Although current knowledge of this third function is limited to in vitro data, Mb has also been proposed to

function as a scavenger of ROS (Flogel et al., 2004). Increasing a ROS scavenger in response to elevated levels of cellular ROS, therefore, would seem adaptive/beneficial to the cell.

ROS are most commonly generated in skeletal muscle from superoxide radicals and subsequent H_2O_2 production. These can be produced in several different sites throughout muscle cells, including the mitochondria, sites of NADPH oxidases (e.g. sarcoplasmic reticulum, transverse tubules and sarcolemma), and sites of xanthine oxidase (e.g. the cytosol) (Powers et al., 2011; Quinlan et al., 2013, Zepeda et al., 2013). Because data on the latter two sources of superoxide are highly limited, this project will focus on the mitochondria as a source of superoxide, which is a byproduct of aerobic metabolism (specifically, the electron transport chain). Because basal mitochondria have been shown to produce more ROS than exercising mitochondria, inactive C_2C_{12} cells will therefore maintain their status as the cell's primary source of ROS generation (St-Pierre et al., 2002; Powers et al., 2011). Moreover, beta-oxidation of fatty acids has been shown to increase mitochondrial ROS and subsequent H_2O_2 generation (St-Pierre et al., 2002); thus, increasing aerobic metabolism through a rise in lipid availability in skeletal muscle cells presumably will increase ROS generation, which may, in turn, stimulate an increase in myoglobin expression.

Interestingly, recent data from our lab shows a positive correlation between lipid supplementation and myoglobin expression. Because our lab focuses primarily on diving mammal physiology, work went into establishing a line of primary Weddell seal (*Leptonychotes weddelli*) skeletal muscle cells (specifically from the primary swimming muscle, *M. longissimus dorsi*). In adjusting a protocol for these primary seal cells to include media more similar to the diet of a pinniped, it was discovered that through increasing the amount of lipid in the differentiation media, cells increased their myoglobin expression (De Miranda et al., 2012).

These novel data imply that lipid may perhaps stimulate myoglobin's expression, thus playing a role in myoglobin regulation.

In the context of the life history of a seal, this lipid-induced Mb stimulation makes sense. Seals and other Pinnipeds (sea lions, Otariids; walrus, Odobenid) endure chronic bouts of hypoxic exercise, as they are air-breathing vertebrates that actively hunt marine fishes and invertebrates that reside in the water. One of the hallmark adaptations that allows for these diving Pinnipeds to endure such extreme bouts of breath-hold exercise is the tremendous abundance of stored internal oxygen, which of in the skeletal muscle translates as increased Mb expression (Davis and Kanatous, 1999; Guyton et al., 1995; Kanatous et al., 2002; Kanatous et al., 2008; Kooyman, 1975; Kooyman and Ponganis, 1998; Ponganis et al., 1993). Development of these Mb stores has been characterized across the different age classes of seals, and while non-diving seal pups have the least amount of Mb across age classes, they still have drastically more Mb expressed in their swimming muscles as compared to primary locomotor muscles of terrestrial species (e.g. dog hindlimb) (Kanatous et al., 2008). This finding was initially somewhat surprising, considering the newly established role of exercise in the stimulation of Mb (Kanatous et al., 2009); however, reflecting on this newly characterized relationship between lipids and Mb in seal cells, high Mb expression in pup muscles can now better be explained. Seals are known to consume some of the most lipid-rich milk across all mammalian species, thus providing a source of Mb stimulation prior to the commencement of exercise (Costa, 1991; Crocker et al., 2001; Crocker et al., 2014; Debier et al., 1999; Fowler et al., 2014; Oftedal et al., 1988). Moreover, seals continue to consume relatively lipid-rich prey species as they transition into adults (Burns et al., 1998; Trumble et al., 2010; Trumble and Kanatous 2012; Williams et al., 2004).

Direct Lipid Signaling in Terrestrial Vertebrates

Given these life history ties between Pinnipeds and Mb stimulators (specifically, hypoxia and lipid), it is unclear whether lipid-induced Mb stimulation might work similarly in terrestrial species, though lipids have been shown to promote adaptive changes in other terrestrial vertebrates. For example, recent work describing the role of fatty acids in promoting beneficial cardiac growth in the postprandial Burmese python (*Python molurus*) showed protection from lipid deposition during a state of increased triglyceride circulation due to an increase in both oxidative capacity and ROS scavenging (Riquelme et al., 2011). Thus, in this uniquely-adapted system, an influx in lipids is associated with adaptive physiological changes.

Beyond the extreme physiology of the python, fatty acid signaling is known to confer important metabolic adaptations across a wide range of terrestrial species. Of the more studied aspects of fatty acid signaling are their interactions with peroxisome proliferator-activated receptors (PPARs), a family of nuclear receptor transcription factors. Fatty acids are an important natural ligand for PPARs, whereby agonistic binding of fatty acid to PPARs can induce a host of cellular adaptation relating to energy metabolism. PPAR isoforms exist largely in a tissue-specific manner, with PPARγ having adipocyte specificity, PPARα having hepatocyte specificity, and PPARδ having myocyte specificity (Chawla et al., 2001; Nakamura et al., 2014). In the skeletal muscle, activation of PPARδ by lipid agonists has been shown to have influence on a suite of metabolic genes, including fatty acid transport protein, hormone sensitive lipase, uncoupling proteins 2 & 3 (UCP2&3), and PPARγ coactivator 1α (PGC1α). Various studies in human, mouse, and horse models, for example, show that activation of PPARδ and its subsequent downstream gene targets has been implicated as a vital component in skeletal muscle adaptation to fasting and endurance exercise (Eivers et al., 2012; Holst et al., 2003; Wang et al.,

2012). Accordingly, this kind of adaptive fatty acid signaling may provide a basis for lipids and their potential to adaptively influence Mb stimulation in non-hypoxia adapted, terrestrial species.

In addition to PPARs and the ability for fatty acid signaling to influence various metabolic adaptations, fatty acid signaling has also been implicated in both pro and anti-inflammatory pathways. Whether fatty acids will confer pro-inflammation or anti-inflammation is contingent upon the specific species of fatty acid (i.e. saturated [SFA], monounsaturated [MUFA], and polyunsaturated [PUFA]). SFAs and n-6 PUFAs are thought to increase pro-inflammatory pathways through activation of toll-like receptor (TLRs), protein kinase C (PKC), and nuclear factor κB (NFκB) signaling pathways, thus promoting expression of inflammatory genes (Kennedy et al., 2009). MUFAs and n-3 PUFAs, alternatively, are thought to confer anti-inflammation through their ability to increase anti-inflammatory adipokines such as adiponectin, possibly through PPAR pathways (Jeckel et al., 2011; Neschen et al., 2006; Salvadó et al., 2013; Siriwardhana et al., 2013). Thus, in addition to further highlighting important physiological examples of lipid signaling, this also introduces the heterogeneity amongst differing fatty acid classes and how lipid quality may dictate adaptive or maladaptive fatty acid-mediated signaling. Taken together, lipids are unambiguously essential signaling molecules in a variety of physiological systems and tissues and may therefore demonstrate influence over an even broader range of adaptive responses than already established, including a possible role in stimulating Mb in non-hypoxia-adapted species. Because there are no known binding sites on the Mb promoter for PPARs or lipids, it is likely that this kind of signaling pathway may stimulate Mb through activation of transcription factors known to have Mb promoter binding sites, such as NFAT or MEF2.

An alternative explanation for the stimulation of Mb production consequent of increased lipid availability may be through an increase in amount of available substrate for oxidative metabolism in these aerobic muscle cells. Specifically, by increasing the availability of lipids and thus removing substrate limitation, it is possible that the cells are responding by increasing their ability to opportunistically metabolize the lipids by increasing necessary oxygen stores in an oxygen-limited environment. If lipids are then stimulating myoglobin expression as a means of increasing metabolism, then aerobic output would be expected to increase as well.

Preliminary observations of high-fat cells show no obvious stores of lipid droplets, suggesting that the lipids are being metabolized. Moreover, it has been elucidated that myoglobin is capable of binding fatty acids, thus increasing the solubility of hydrophobic fatty acids within the aqueous cytosol (Sriram et al., 2008). Myoglobin's binding affinity for fatty acids is lower than that of fatty acid binding protein, suggesting a less complex release mechanism, potentially increasing the diffusion rate of fatty acids to the mitochondria (Gros et al., 2010).

Indirect Lipid Signaling in Terrestrial Vertebrates: A Role for ROS?

In addition to the aforementioned methods of direct lipid signaling, increasing bioavailability of lipids may also confer cellular adaptation consequent of an influx in byproducts from beta-oxidation, an aerobic process that metabolically breaks down fatty acids. This metabolism of fatty acids has been shown to increase cellular production of ROS as a byproduct (St. Pierre et al., 2002). Given the ability for myoglobin to scavenge ROS (Flögel et al., 2004; Garry and Mammen, 2007; Hendgen-Cotta et al., 2010), lipid-supplementation may

prompt cells to increase Mb as an adaptive mechanism to protect cells from a subsequent surplus of ROS.

Although chronically elevated ROS production can lead to cellular aging, muscle disuse atrophy, and related pathologies (Gomez-Cabrera, 2012; Hamilton, 2007; Powers et al., 2005; Powers et al., 2007; Wang et al., 2013), acute redox signaling has been established as a highly important avenue for skeletal muscle adaptation. This latter phenomenon is of particular importance in regard to cellular adaptation to endurance exercise, whereby administration of exogenous antioxidants during endurance training can actually dampen mitochondrial biogenesis and overall training adaptations through suppression of key transcription factors, such as PGC1α and nuclear respiratory factor 1 (Nrf1), in addition to suppression of various antioxidants (Gomez-Cabrera et al., 2008; Powers et a., 2011). Acute redox signaling, therefore, is of paramount importance in the facilitation of cellular adaptation, especially in the skeletal muscle.

Examples of redox-sensitive signaling pathways include NF-κB, mitogen-activated protein kinases (MAPK), and PGC1α-associated signaling; these pathways respond to acute bouts of ROS, such as those associated with endurance exercise, in a broad adaptive fashion to increase antioxidant expression, glucose transport, angiogenesis, and mitochondrial biogenesis (Gomez-Cabrera et al., 2005; Irrcher et al., 2008; Irrcher et al., 2009; Ji and Zhang, 2014; Powers et al., 2011). Thus, given these important roles for redox signaling in adaptation, and given the adaptive properties of myoglobin (e.g. oxygen storage, ROS scavenging), it may be possible that myoglobin is responsive to redox signaling.

Specific Aims

Myoglobin is an oxygen-binding hemoprotein generally localized to muscle tissue, specifically including cardiac muscle, oxidative skeletal muscle, and smooth muscle, where it functions as an oxygen store, scavenger of NO, and scavenger of ROS (Garry and Mammen, 2007; Kanatous and Mammen; 2010; Wittenberg and Wittenberg, 1989). The oxygen storage capacity of this protein has become of particular interest due to its potential pharmacological implications in human hypoxic diseases, such as cardiovascular and pulmonary disease, and thus research necessarily is moving into the direction of describing its regulatory mechanisms. Diving marine vertebrates are known for having an extreme abundance of myoglobin in their skeletal muscles and cardiac tissue, and as such, comparative physiology has become a targeted field in understanding myoglobin regulation (Castellini and Somero 1981; Guyton et al., 1995; Kanatous et al., 2008; Snyder, 1983).

Regulation of Mb has been shown to occur through a calcium-dependent signaling pathway involving calcineurin and nuclear factor of activated t-cells (NFAT) (Chin et al., 1998; Kanatous et al., 2009; Kanatous and Mammen, 2010; Oh et al., 2005). Increases in Mb expression have been attributed to factors such as hypoxia (Reynafarje, 1962), exercise (Duteil et al., 2004; Garry et al., 1996; Kanatous and Garry, 2006; Kanatous et al., 2009), and NO (Rayner et al., 2009). Fundamentally, these stimulators of Mb expression are intuitive and sensible because they are direct reflections of Mb functionality. In other words, it makes sense that the lack of oxygen associated with hypoxia or the increased oxygen demand associated with exercise would amplify the expression of an oxygen store, just as it makes sense that an increase in potentially dangerous NO production would causally increase a cell's method of NO scavenging. Going along that same trend of thinking, it should seem that an elevation in aerobic metabolic

substrate or influx of ROS, should result in the same outcome: increased Mb. One particular substrate that has the potential to play both of these roles is lipid. In addition to providing a fuel for oxidative metabolism and thus prospectively increasing a demand for oxygen in a system, lipids also have the potential to increase the presence of ROS in muscle cells as a metabolic byproduct, thus increasing the demand for ROS scavenging. It seems probable, then, that a stimulatory relationship between myoglobin and lipids should exist.

To test whether or not lipids play a role in Mb stimulation, and specifically whether or not lipids stimulate Mb expression to directly provide fuel for metabolism in the form of oxygen or indirectly to protect the cells from harmful metabolic byproducts, the following specific aims will be explored:

Specific Aim I: To test the hypothesis that lipids alone stimulate myoglobin expression in terrestrial, non-hypoxia-adapted model species.

- Culture line of C_2C_{12} cells with addition of 5% lipid mixture into differentiation media and culture a line of control C_2C_{12} cells with a high glucose differentiation media.
- Isolate protein for myoglobin assay, myoglobin and calcineurin western blots, and metabolic marker enzyme assays (citrate synthase) for both cell lines.
- Isolate RNA and identify RNA transcripts differentially expressed between both cell lines.
- Compare lipid-supplemented C_2C_{12} cell line with whole tissue from the soleus of rats fed high fat diets.

Specific Aim II: To test the hypothesis that fat and hypoxia increase myoglobin expression in a terrestrial, non-hypoxia-adapted model species.

- Culture line of C_2C_{12} cells with addition of 5% lipid mixture into differentiation media and differentiate cells in both normoxic (21% O_2) and hypoxic conditions (0.5% O_2).
- Isolate protein for myoglobin assay, myoglobin & calcineurin western blots, and metabolic marker enzyme assays (citrate synthase) for both cell lines.
- Isolate RNA and identify RNA transcripts differentially expressed between both cell lines.

Specific Aim III: To test the hypothesis that lipid-induced myoglobin stimulation occurs through an increased production of reactive oxygen species (ROS), and that this type of stimulation should incur functional protein modification.

- Culture C_2C_{12} cells with addition of H_2O_2 (rather than fat) *or* ROS-scavenger (with fat) into differentiation media; differentiate both cell lines in both normoxic (21% O_2) and hypoxic conditions (0.5% O_2).
- Isolate protein for myoglobin assays for all experimental cohorts.
- Compare myoglobin assay data across lipid cohorts, both normoxic and hypoxic, using two different methodologies, either with or without the addition of a reducing agent, to characterize differences in oxidized versus reduced cellular myoglobin.

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CHAPTER 1

Changing Myoglobin's Paradigm: a Novel Link between Lipids and Myoglobin[1]

Summary

Myoglobin (Mb) is an oxygen-binding muscular hemeprotein regulated via Ca^{2+}signaling pathways involving calcineurin (CN), with Mb increases attributed to hypoxia, exercise, and nitric oxide. Here, we show a link between lipid supplementation and increased Mb in skeletal muscle. C_2C_{12} cells were cultured in normoxia or hypoxia with glucose or 5% lipid. Mb assays and western blots revealed lipid cohorts had higher Mb than glucose cohorts in both normoxia and hypoxia. Normoxic cells were compared to soleus tissue from normoxic rats fed high fat diets; while tissue sample cohorts showed no difference in CO-binding Mb, fat-fed rats showed increases in total Mb protein (similar to cells), suggesting increases in modified Mb. Moreover, Mb increases did not parallel CN increases, but did, however, parallel oxidative stress marker augmentation concomitant with Mb augmentation. Addition of antioxidant to lipid-supplemented cells reversed Mb increases, suggesting a Mb regulatory pathway through redox signaling independent of CN.

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Introduction

During endurance exercise, terrestrial mammals rely primarily on erythrocytic oxygen stores bound to hemoglobin to fuel aerobic metabolism in working muscle. Physiological changes associated with endurance training elicit responses that increase muscular blood flow and subsequent oxygen delivery (e.g. increasing capillary density) (Breen et al., 1996; Fluck et al., 2006; Olfert et al., 2009). Muscle oxygen stores, alternatively, appear to bear little significance in sustaining aerobic metabolism during endurance exercise, evident by the inability to appreciably release intra-muscular stored oxygen during exercise (Mole et al., 1999); yet, terrestrial endurance athletes have more myoglobin (Mb) than their sedentary counterparts (Duteil et al., 2004; Mielnik et al., 2011). Accordingly, Millikan's coined alias "muscle hemoglobin" (Millikan, 1939), in which Mb's functional paradigm pertains to oxygen storage and transport, does not appear to be fully applicable to terrestrial mammals in vivo. Here, we provide data that offer an alternative paradigm for Mb increases associated with aerobic metabolism in the skeletal muscle of terrestrial mammals.

Mb is an oxygen-binding hemeprotein generally localized to oxidative muscle and functions as an oxygen store, nitric oxide (NO) scavenger, and reactive oxygen species (ROS) scavenger (Flogel et al., 2001; Flogel et al., 2004; Garry and Mammen, 2007; Godecke, 2010; Gros et al., 2010; Kanatous and Mammen, 2010; Merx et al., 2001; Millikan, 1939; Rayner et al., 2009; Wittenberg and Wittenberg, 1989). Interestingly, as deduced from the low p50 of its oxygen dissociation curve (p50=2.39 mmHg in equine Mb), Mb has a strong affinity for oxygen and only releases oxygen under a very low partial oxygen pressure (Antonini, 1971; Garry and Mammen, 2007; Gros et al., 2010; Nichols et al. 1989; Schenkman et al., 1997). Thus, under standard conditions muscle must necessarily be hypoxic in order to utilize Mb-bound oxygen for

aerobic metabolism. This presents an interesting theoretical perspective of Mb as it assists cellular respiration via oxygen supply solely in a time of potential stress. Interestingly, oxymyoglobin measured in submaximally exercising adult humans desaturates only during the first 20-40 seconds of exercise; moreover, during its brief desaturation period, Mb never desaturates beyond 50% (Mole et al., 1999). Many terrestrial, athletic mammals, however, have relatively high levels of Mb within their skeletal muscle, despite never being truly oxygen-limited due to higher muscular capillary densities (Duteil et al., 2004; Mielnik et al., 2011).

Regarding a role in oxygen transport, muscular oxygen flux is attributed to competing contributions of Mb-facilitated oxygen diffusion and free oxygen. This relationship is described by the equipoise diffusion pO_2, which is the pO_2 that allows Mb and oxygen to contribute equally to oxygen transport. Declines in the equipoise diffusion pO_2 indicate lower contributions of Mb to oxygen flux and can vary with changes in p50. Specifically, as the p50 of Mb increases, the equipoise diffusion pO_2 decreases. Temperature change has been characterized as a modifier of the p50 of Mb, such that temperature increases will increase the Mb p50. Taken together, this means that an increase in cellular temperature is concomitant with a decreased cellular dependence on Mb-facilitated oxygen diffusion. So, during exercise, when cellular oxygen consumption rises and contractile activity increases temperature, Mb contribution to oxygen transport is actually *decreased* (Chung et al., 2005; Lin et al., 2007; Schenkman et al., 1997). Collectively, in light of its low p50, minimal oxygen desaturation, and decreased contribution to oxygen transport during exercise, high Mb levels in healthy terrestrial animals that do not experience routine hypoxic stress is nonsensical in the context of increasing oxygen storage and transport.

Physiological hypoxic stress can occur in high altitude environments or in cardiovascular and/or pulmonary disease states. Because the aforementioned conditions cause low oxygen at the cellular level, they can ultimately manifest into pathological conditions at the tissue level over time; yet, Mb is capable of providing cytoplasmic oxygen reserves that can be readily used in pathological states when erythrocytic oxygen becomes insufficient. As such, Mb presents a potential target for pharmacological intervention and treatment of human hypoxic disease, or more specifically, treatment of consequent tissue ischemia. Understanding regulation of Mb and potential stimuli thus bridges the gap between pharmacological potential and actual practice.

Although Mb regulation is not yet fully understood, understanding of Mb stimulation is escalating. Transcriptionally, the Mb gene is activated via CCAC box, A/T element, nuclear factor of activated t-cells (NFAT), E box, and myocyte enhancing factor-2 (MEF-2) motifs (Grayson et al., 1998; Kanatous and Mammen, 2010; Yan et al., 2001). Transcriptional regulation is sensitive to increases in intracellular calcium, which activate calcineurin (CN), a calcium-activated phosphatase. Activated CN, in turn, dephosphorylates NFAT, thus enabling NFAT translocation to the nucleus and subsequent stimulation of Mb expression (Chin et al., 1998; Kanatous et al., 2009; Kanatous and Mammen, 2010; Oh et al., 2005). This pathway, therefore, demonstrates a component of calcium-dependency in Mb regulation. Beyond transcription, Mb increases are attributable to environmental factors including hypoxia (Reynefarje, 1962), exercise (Kanatous et al., 2009), and NO (Rayner et al., 2005). Recently, Mb has also been shown to increase in response to lipid supplementation in seal cells (De Miranda et al., 2012). Seals and other diving vertebrates have an extreme abundance of Mb in their skeletal muscles as an adaptation to chronic severe hypoxia (Guyton et al., 1995; Kanatous et al., 2008; Castellini and Somero, 1981; Snyder, 1983). Moreover, diving marine vertebrates

have diets rich in lipids and protein, from the extremely lipid-rich milk consumed early in life (Burns et al., 2010) to consumption of fish and invertebrates as adults (Burns et al., 1998; Ponganis et al., 2003). As such, it is unknown whether lipid-induced Mb increases observed in seal cells are relevant to Mb regulation across all vertebrates, both marine and terrestrial alike, or if it is specific to hypoxia-adapted marine mammals.

Here, we aimed to determine the role of lipids in Mb regulation within the skeletal muscle of terrestrial model species. C_2C_{12} cells, immortalized mouse skeletal muscle cells, were differentiated in normoxic (21% O_2) or hypoxic (0.5% O_2) conditions with standard, glucose or 5% lipid-supplemented differentiation media. Also, a subset of normoxic and hypoxic C_2C_{12} lipid-supplemented cells were differentiated with the addition of a ROS scavenger (i.e. antioxidant), phenyl-alpha-tert-butyl nitrone (PBN), starting on day 3 of differentiation. Between treatment groups, Mb levels were determined via functional CO-binding Mb assays and immunoblots, CN levels were determined via immunoblots, citrate synthase (CS) levels were determined via CS assays, and oxidative stress was inferred via PCR transcript analysis. Also, normoxic cells were compared to normoxic whole tissue using soleus muscle from Sprague-Dawley rats fed high fat diets (40% saturated fatty acids [SAT]).

Results

Myoglobin

Cellular Mb assays showed Mb increased in normoxic 5% lipid C_2C_{12} cells as compared to normoxic glucose C_2C_{12} cells (0.158967 ± 0.00145 versus 0.055978 ± 0.00169 mg mg^{-1} protein, respectively, $p<0.001$, n=6). Similarly, cells differentiated in hypoxia also showed Mb increases in 5% lipid C_2C_{12} cells as compared to normoxic glucose C_2C_{12} cells (0.116773 ± 0.00238 versus 0.027788 ± 0.00111 mg mg^{-1} protein, respectively, $p<0.001$, n=6) (Table 1.3,

Figure 1.1). Western blots for normoxic cells indicated a trend for an increase in Mb protein in 5% lipid cells (0.875 ± 0.0472 relative units [R.U.] in lipid cells compared to 0.636 ± 0.100 R.U. in control cells, p=0.142) (Figure 1.2A), and western blots for hypoxic cells showed an increase in Mb protein (0.0809 ± 0.0029 R.U. in lipid cells compared to 0.0420 ± 0.0089 R.U. in control hypoxic cells, p=0.014) (Figure 1.2C).

Subsets of all lipid-supplemented cells were cultured with PBN, a ROS scavenger. Normoxic cells showed that addition of PBN *in conjunction* with 5% lipid caused a complete reversal of previously seen lipid-induced Mb increase. Specifically, 5% lipid + PBN cells showed no difference in Mb as compared to glucose control cells (0.052323 ± 0.00132 versus 0.055978 ± 0.00169, mg mg^{-1} protein, respectively) (Table 1.3, Figure 1.1). In regard to the hypoxic cohorts, 5% lipid + PBN cells showed a decrease in Mb relative to 5% lipid cells (0.09634 ± 0.00165 versus 0.116773 ± 0.00238 mg mg^{-1} protein, respectively, $p<0.001$, n=6); interestingly, the hypoxic 5% lipid + PBN cells *still* had more Mb than hypoxic control cells (0.09634 ± 0.00165 versus 0.027788 ± 0.00111 mg mg^{-1} protein, respectively, $p<0.001$, n=6) (Figure 1.1).

At the tissue level, Mb assays showed no difference in functional CO-binding Mb between control (CON) versus 40% saturated fat (SAT) diets in rat soleus muscle (1.460 ± 0.0298 verses 1.501 ± 0.033 mg mg^{-1}wet mass muscle, respectively, p=0.467, n=3) (Table 1.3). Interestingly, western blots for tissue indicated an increase in Mb protein in the SAT rat soleus as compared to the CON (1.312 ± 0.109 R.U. in SAT rats compared to 0.596 ± 0.0906 in CON rats, p=0.013) (Figure 1.2B).

Calcium Regulatory Protein

Unlike previous studies that show increases in CN with increases in Mb, our study found an increase in Mb with either no change or a decrease in CN. Western blots comparing CN expression showed a decrease in CN protein in normoxic high fat cells as compared normoxic control cells (0.529 ± 0.077 in lipid cells compared to 0.795 ± 0.0166 R.U. in control cells, p=0.028), while hypoxic cells showed no difference in CN between glucose and high fat cells (0.663 ± 0.0269 R.U. in lipid cells compared to 0.555 ± 0.0488 R.U. in control hypoxic cells) (Figure 1.3A).

At the tissue level, there was a trend toward decreased CN protein in soleus muscle of rats fed high fat diets relative to control rats fed high starch diets (2.526 ± 0.442 R.U. in SAT rats compared to 3.463 ± 0.213 R.U. in CON rats, p=0.200) (Figure 1.3B).

Aerobic Capacity

CS, the rate-limiting enzyme of the citric acid cycle, provides a measure of aerobic capacity in cells; CS activity measured in normoxic cells showed a decrease in CS activity in 5% lipid as compared with glucose cells (2.555 ± 0.006 versus 0.211 ± 0.006 units mg^{-1} protein, respectively, p=0.002). CS activity measured in hypoxic cells also showed a decrease in CS activity in 5% lipid as compared to glucose cells (0.130 ± 0.008 verses 0.175 ± 0.006 units mg^{-1} protein, p=0.001). Moreover, CS activity in normoxic glucose conditions was higher than both hypoxic conditions (p<0.001) (Figure 1.4A).

At the tissue level, CS activity showed no difference between SAT versus CON rat soleus muscle tissue (2.543 ± 0.450 versus 2.244 ± 0.214 units mg^{-1} protein, respectively, p=0.710) (Figure1.4B).

RNA Transcript Expression

Data reported from PCR arrays in this chapter are limited to transcripts exhibiting ≥2-fold differences between experimental cohorts, with a p-value ≤ 0.05; for the full list of PCR array data, please refer the appendix (Tables A.S1-A.S4). PCR array analysis in normoxic cells showed lipid cells as being more oxidatively stressed than control cells. Lipid cells had an increase (>2-fold difference) in nine transcripts of antioxidant genes, ten transcripts of genes involved in ROS metabolism, and five transcripts of oxygen transporter genes (Figure 1.5). Mb transcript showed no difference between glucose and lipid normoxic cells. Glucose cells, alternatively, showed greater expression of only two antioxidant transcripts relative to lipid cells.

Interestingly, transcript analysis in hypoxic cells suggests glucose hypoxic cells are more oxidatively stressed than lipid hypoxic cells. Transcripts of five genes involved in ROS metabolism, one antioxidant gene, and two oxygen transporter genes were up-regulated in glucose hypoxic cells relative to lipid hypoxic cells. Lipid hypoxic cells, conversely, only showed three transcripts of genes involved in superoxide metabolism as being up-regulated (Figure 1.5).

Analysis of both lipid-supplemented cell groups suggests lipid normoxic cells are more oxidatively stressed than lipid hypoxic cells. Transcripts of two antioxidant genes, thirteen genes involved in ROS metabolism, and four oxygen transporter genes were up-regulated in normoxic lipid cells, whereas transcripts of one antioxidant gene and two genes involved in ROS metabolism were up-regulated in hypoxic lipid cells (Figure 1.5).

Lastly, results from rat soleus PCR array analysis suggests SAT rats were more oxidatively stressed than CON rats. Specifically, transcripts of two antioxidant genes and three

genes involved in ROS metabolism were up-regulated in soleus tissue from SAT rats relative to CON rats (Table A.S4).

Discussion

As previously seen in diving mammals, here, for the first time in terrestrial models, we show a scenario whereby lipid increases Mb in the skeletal muscle without elevated CN, suggesting a pathway for Mb gene regulation *independent* of calcium signaling. On the cellular level, supplementation of a 5% lipid mixture into differentiation media increased functional CO-binding Mb in both normoxic and hypoxic C_2C_{12} cells. Addition of PBN, a ROS scavenger, to lipid-supplemented cohorts caused a complete reversal of lipid-induced Mb increases in normoxic cells, suggesting a connection between oxidative stress and Mb stimulation. Hypoxic cells also showed an inhibitory effect on lipid-induced Mb increases upon the addition of PBN to lipid-supplemented cohorts; however, hypoxic lipid + PBN cells still had more Mb than hypoxic controls, suggesting that Mb stimulation in hypoxic conditions may be in response to a secondary stimulus beyond oxidative stress and in relation to lower oxygen availability. Moreover, immunoblot analysis showed total Mb protein increased in hypoxic lipid cells, with a trend of increasing in normoxic lipid-supplemented cells. On the tissue level, normoxic SAT rats showed no difference in functional CO-binding Mb as compared to CON rats; however, immunoblot analysis showed an increase in total tissue Mb protein in SAT rats. This latter tissue Mb dataset parallels, in part, normoxic cellular Mb data, whereby increasing exogenous fat accompanies an increase in Mb protein. Taken together, these tissue data suggest increasing lipid availability in normoxic environments increases a modified, non-CO-binding Mb in whole muscle. Moderate lipid signaling, thus, may initially be adaptive and beneficial in the skeletal

muscle, while excessive, long term lipid signaling may become maladaptive and lead to pathologies.

Another interesting perspective from these data is that high fat conditions that produced an increase in Mb did not show the predicted increase in CN protein, which is reflective of CN activity (Diedrichs et al., 2007). Normoxic, lipid-supplemented cells showing a trend for increased Mb showed a *decrease* in CN, with normoxic tissue showing similar trends, while hypoxic, lipid-supplemented cells showing an increase in Mb showed no change in CN. CN, a calcium-calmodulin activiated phosphatase, has previously been established as a transcriptional regulator of whereby calcium released from the sarcoplasmic reticulum during contraction activates CN, which then dephosphorylates NFAT, allowing NFAT to translocate into the nucleus and subsequently bind the Mb promoter (Chin et al., 1998; Kanatous et al., 2009; Kanatous and Mammen, 2010). Here, for the first time, we show Mb being stimulated in mammalian skeletal muscle in the absence of altered CN protein expression.

If an increase in Mb observed in lipid-supplemented skeletal muscle is occurring independent of calcium-signaling, then how is Mb being stimulated? Lipid metabolism is known to accompany increased ROS production (St. Pierre et al., 2002). Skeletal muscle ROS are most commonly generated from superoxide radicals and subsequent H_2O_2 production. These are spawned in several sites throughout sarcomeres, including the mitochondria, sites of NADPH oxidases, and sites of xanthine oxidase (Powers et al., 2011). Regarding the former, mitochondria generate ROS as a byproduct of aerobic metabolism (specifically, the electron transport system, ETS). Because β-oxidation increases ROS production through the ETS, perhaps increasing lipid availability in skeletal muscle is stimulating Mb expression via ROS signaling. Suggested Mb functions include oxygen reservoir, NO scavenger, and ROS scavenger

(Floget et al., 2001; Flogel et al., 2004; Garry and Mammen, 2007; Godecke, 2010; Merx et al., 2001). Previous research has proposed roles of the former two functions in Mb regulation. Regarding oxygen storage, Mb increases in response to hypoxic exercise, which increases oxygen demand in an oxygen-limited environment (Kanatous et al., 2008). Alternatively, regarding the role of Mb as an NO scavenger, NO stimulates smooth muscle Mb, whereby addition of NO to vascular smooth muscle cells positively correlates with Mb gene and protein expression (Rayner et al., 2009). The interpretation of this response is that the smooth muscle responds to increases in potentially dangerous reactive nitrogen species (RNS) by enhancing scavenging and subsequent protection from RNS. Building upon this same rationale, if increases in oxygen demand in an oxygen-limited environment (i.e. hypoxic exercise) and increases in NO can stimulate Mb, then perhaps an alternative stimulus should pertain to Mb's third proposed role as a ROS scavenger. Although current knowledge of this function is limited to *in vitro* studies (Flogel et al., 2004), increases in cellular ROS production could be capable of stimulating Mb in oxidative muscle.

In support of this theory, our data show an inverse relationship between Mb and CS; increased lipid availability in cells produced Mb increases coupled with CS activity decreases. In tissue, alternatively, there was no change in CS activity. Because Mb has traditionally been regarded for its role as an oxygen reservoir (Gros et al., 2010; Millikan, 1939), it has a direct connection to aerobic metabolism. Thus, if Mb were increasing solely as means of increasing cellular oxygen reservoirs, then we would not have expected a biomarker of aerobic metabolic activity to decrease. This result, therefore, suggests that measured increases in functional CO-binding Mb and Mb protein expression may actually reflect an increased cellular demand pertaining to one of Mb's other functional roles, specifically as a ROS scavenger; thus, increased

Mb *without* a simultaneous increase in CS activity may be the cell's response to increased ROS production from β-oxidation of lipids (Figure 1.6).

Beta-oxidation of fatty acids has been shown to increase mitochondrial ROS and subsequent H_2O_2 generation (St. Pierre et al., 2002); thus, increasing aerobic metabolism through lipid oxidation in muscle will increase ROS generation, which may, in turn, stimulate Mb. Muscle antioxidants have been found to correlate positively with lipid supplementation; feeding mice high fat diets increases activity and protein expression of catalase, an H_2O_2-metabolizing enzyme (Rindler et al., 2013). Accordingly, this trend of lipid-stimulated increases in muscular oxidative defense holds true in regard to Mb as well. Moreover, this theory may better explain observed elevation in Mb in healthy, athletic, terrestrial mammals that are not oxygen-limited. Terrestrial endurance athletes preferentially burn polyunsaturated fatty acids (PUFAs) in their skeletal muscle, which conserves oxygen, thus lowering oxygen consumption (and demand) in their working muscles. Increases in internal oxygen stores, therefore, does not appear physiologically necessary given this lower oxygen demand, whereas oxidative scavenging appears to better match physiologically. PUFAs generate more ROS than saturated fatty acids; consequently, increased ROS production attributable to β-oxidation of PUFAs may account for Mb increases Mb in terrestrial endurance athletes (Trumble and Kanatous, 2012). Moreover, this information may better explain the somewhat disparate results between lipid normoxic cells, which were given a heterogeneous mixture of fatty acids, versus fat-fed rats, which were fed exclusively SFAs

Support of ROS-induced Mb stimulation is further evident through addition of an antioxidant to lipid-supplemented cell culture cohorts. Mb assay data from normoxic cells cultured with 5% lipid + 1mM PBN, a scavenger of ROS, showed a complete reversal of

previously seen lipid-induced Mb increases (Figure 1.1). In other words, removing the predicted influx of ROS associated with augmented lipid metabolism prevented increases in Mb stimulation. These data imply that lipid-induced Mb increases occur through elevated ROS production consequent of amplified beta-oxidation. Mb assay data from hypoxic cells cultured with 5% lipid + 1mM PBN also showed a decrease in Mb as compared to hypoxic lipid cells, but not to the same degree as observed in normoxia (Figure 1.1). This observation may be consequent of cellular Mb increasing via lipid *and* a secondary stimulus, hypoxia. While hypoxia alone actually decreases cellular Mb, it has previously been established as an important secondary stimulus for increasing expression of Mb, particularly in the context of exercise (Kanatous et al., 2009). Accordingly, the presence of this secondary stimulus may account for the damped decrease of Mb in hypoxic lipid + PBN cells as compared to the normoxic PBN response.

RNA transcript analysis of oxidative stress markers supports differential stress levels between experimental conditions. Of the normoxic cells, lipid-supplemented cells showed increases in 39 transcripts of oxidative stress markers (antioxidants, genes involved in ROS metabolism, and oxygen transporters). These data, suggest lipid-supplemented cells experience more oxidative stress than control cells. Moreover, substantial increases (24.6-fold) of the lipid transporter (Zhang et al., 2011) apolipoprotein E in lipid-supplemented cells suggests lipids are being metabolized, thus supporting lipid metabolism byproducts as Mb stimulators. According to soleus data, SAT rats were more oxidatively stressed than the CON rats. While five transcripts indicative of oxidative stress were up-regulated in SAT rats, no transcripts were up-regulated from CON rats. Thus, these tissue data support our cellular data, showing an increase in oxidative stress with lipid supplementation in normoxic environments. Oxidative stress in

normoxic conditions may explain observed increases in Mb protein without increases in functional CO-binding Mb; perhaps Mb scavenging ROS has a conformational change inhibiting ligand binding of CO.

Of the hypoxic cells, glucose hypoxic cells were more oxidatively stressed than lipid-supplemented hypoxic cells. Eight transcripts of genes involved in ROS metabolism and oxygen transportation were up-regulated in glucose hypoxic cells (ranging from 2.9-fold increase to a 13.1-fold increase), while only three transcripts of genes involved in ROS metabolism were up-regulated (ranging from 2.4-2.7-fold increases) in lipid-supplemented cells. These somewhat surprising data may be a result of Mb differences, and specifically, differences in Mb ROS scavenging, as Mb was significantly higher in lipid hypoxic cells relative to glucose hypoxic cells. Interestingly, Mb transcript was down 3.79-fold in lipid hypoxic cells relative to glucose. While contradictory to protein and functional assay data, these data are likely due to early Mb increases during myotube differentiation, such that by the time cells were harvested, cellular demand for Mb was already being met, reflected as decreased transcript. Protein abundance in some human cell lines, for example, only partially correlate with relative mRNA abundances, thus illustrating the importance of protein abundance regulation and the effect on protein-to-mRNA ratios (Vogel and Marcotte, 2012).

Of the lipid-supplemented cells, lipid normoxic cells appear to be *more* oxidatively stressed than their hypoxic counterparts. Nineteen gene transcripts were up-regulated in lipid normoxic relative to lipid hypoxic cells (ranging from 2.4-fold increase to 24.4-fold increase), while lipid hypoxic cells show transcripts of only three genes being up-regulated relative to normoxic lipid cells. These data initially seem paradoxical, since hypoxic lipid cells have two confounding factors contributing to oxidative stress (increased lipid *and* insufficient oxygen);

however, lipids associated with the hypoxic cells are clearly providing a beneficial physiological change that allows the cells to better adapt to ameliorating oxidative stress. The ability of cells to adaptively increase Mb early in differentiation is likely mitigating physiological stress and compensating for the necessity to increase oxidative stress-related transcripts that would otherwise be prominent. A disparity between Mb protein and transcript expression, where transcript is down in experimental conditions where protein is up, mirrors Mb protein and transcript disparities in hypoxic cells. Again, these seemingly contradictory data are likely due to early Mb transcript and subsequent protein increases in the lipid hypoxic cells, whereby cellular Mb demands are being sufficiently met upon cellular harvesting.

An alternative theory to ROS stimulating Mb is that cells may be responding to increased insoluble lipid by increasing means of lipid transportation. Proton nuclear magnetic resonance (H NMR) data indicate that Mb can bind fatty acids; this trait is speculated to relate to a role for Mb in fatty acid transport, that is, the transport of an insoluble macromolecule in the aqueous cellular environment (Sriram et al., 2008). In this light, Mb increases here may be a response to increased exogenous lipids working in concert with fatty acid binding proteins, thus making lipids accessible to the mitochondria for β-oxidation (Figure 1.2). Despite the known ability of Mb to bind fatty acids, lipid has never been shown to stimulate Mb in a terrestrial species.

In summary, we show that lipid supplementation is associated with increased Mb expression in both C_2C_{12} mouse muscle cells and Sprague-Dawley rat soleus muscle independent of CN. This overarching pattern shows similar responses between normoxia and hypoxia, whereby lipids increase total Mb protein in addition to functional CO-binding Mb. Interestingly, addition of PBN, a ROS scavenger, inhibited lipid-induced Mb increases, though this response differed between normoxic and hypoxic cohorts. In normoxia, addition of PBN to lipid-

supplemented cells completely reversed lipid-induced Mb increases, while in hypoxia, addition of PBN to lipid-supplemented cells decreased Mb relative to hypoxic lipid cells, but did not decrease Mb down to hypoxic control cell levels, suggesting that in hypoxia, Mb increases are in response to lipids *and* a secondary stimulus (i.e. lower oxygen availability). Moreover, because all experimental conditions in which Mb increases concomitantly show unchanged CS activity, and because normoxic lipid-supplemented experimental conditions show increases in RNA transcripts associated with oxidative stress, we propose that lipid-stimulated Mb increases are consequent of redox signaling associated with increased ROS production via β-oxidation. Thus, in light of these novel data and in conjunction with the inability of terrestrial mammals to appreciably utilize Mb oxygen stores during exercise, we propose an alternative paradigm for Mb, whereby the role of Mb as an antioxidant defense during terrestrial exercise, which increases aerobic metabolism and ROS production, is more relevant and applicable than the role relevant to storage and transport of oxygen in healthy animals.

Materials and Methods

Cell Culture

Normoxic C_2C_{12} cells were grown and differentiated in a 37°C humidified incubator with 5% CO_2. Hypoxic cells differentiated in a humidified hypoxic chamber (Coy Laboratories, Grass Lake MI, USA) at 37°C, 5% carbon dioxide, 0.5% oxygen, and 94.5% nitrogen. Standard growth media was used for myoblast proliferation (Dulbecco's modified Eagle's media high glucose [DMEM], 20% fetal bovine serum [FBS], 1% sodium pyruvate, 1% penicillin/streptomycin antibiotic). At 90% confluency, myotube differentiation was induced with either glucose control media (high glucose DMEM, 5% equine serum, 10μg ml^{-1} insulin,

10μg ml^{-1} transferrin) or 5% lipid-supplemented media (2μg/ml arachadonic acid; 10μg/ml each of linoleic, linolenic, myristic, oleic, palmitic, and stearic fatty acids, Sigma Aldrich [Milwaukee, WI, USA]). A subset of normoxic and hypoxic lipid-supplemented cells were differentiated with the addition of 1mM PBN (Sigma Aldrich, Milwaukee, WI, USA). At differentiation day 7, cells were harvested for protein or RNA with a standard homogenization buffer (20% glycerol, 1% Tween20, 0.001M DTT in PBS with a protease inhibitor table) or TriPure (Roche, Indianapolis, IN, USA), respectively.

High Fat Rats

Soleus muscle came from rats fed control starch diets (CON) or high fat diets (40% saturated fatty acid [SAT]) as previously described (Jeckel et al., 2011). Briefly, adult male Sprague-Dawley rats (CD® IDS Rats, Charles River Laboratories, Wilmington, MA, USA) were maintained in a temperature and humidity controlled environment at Colorado State University Laboratory Animal Resource Center under a normal 12 hour dark/12 hour light cycle. Rats were housed in pairs under regulations of the Animal Welfare Act, the Guide for Care and Use of Laboratory Animals and the Guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching. Prior to commencing dietary treatments, rats were acclimated to the facilities for two weeks.

Diet. At 6 weeks of age, rats were fed either control (CON) or 40% saturated fat diets (SAT) for a total of 32 weeks. Diets were supplied by Harlan Teklad (Madison, WI, USA) and are detailed in Tables 1.1 and 1.2. Body weight was measured weekly. Prior to terminal sample collection, rats were fasted overnight.

Tissue Collection. Rats were placed in a commercial rodent anesthesia chamber for anesthetic induction using 4% isofluorane in a 95% O_2/5% CO_2 gas mixture, and anesthesia was maintained at identical gas concentrations administered via nosecone. Animals were euthanized by exsanguinations and removal of the heart. The soleus muscle was excised at 0°C and stored at $^{-}$80°C until used.

Tissue Homogenization. Tissue was mechanically homogenized at 0°C in lysis buffer (79% PBS, 20% glycerol, 1% Tween20, 0.001M DTT with a protease inhibitor tablet). Samples were centrifuged at 10,000 x g at 4°C for 5 minutes and the supernatant was frozen at $^{-}$80°C until samples were used. Protein concentrations were determined using a Coomassie Plus^{TM} Protein assay (Thermo Scientific, Rockford, IL, USA)

Protein Assays

Assays were performed using a BioTek Synergy HT Multi-Detection microplate reader. Protein concentrations were determined using Coomassie Plus^{TM} (Thermo Scientific, Rockford, IL, USA). CS assays were performed as previously described (Kanatous et al., 2008) to determine aerobic capacity of cells. CS assay buffers included: 50 mmol l^{-1} imidazole, 0.25 mmol l^{-1} 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), 0.4 mmol l^{-1} acetyl-CoA and 0.5 mmol l^{-1} oxaloacetate, pH 7.5; ΔA_{412}, ε_{412}=13.6

Mb assays were performed as adapted from Reynafarje 1963 and Kanatous et al., 2002. Briefly, protein homogenates were diluted with phosphate buffer (0.04 mol^{-1}, pH 6.6) and subsequently centrifuged at 28,000 x g at 4°C for 50 minutes. The resultant supernatant was then bubbled with 99.9% carbon monoxide (CO), which converts myoglobin to carboxymyoglobin. After three minutes of bubbling, samples were combined with 0.01g sodium

dithionite, a reducing agent, and then bubbled again for two minutes; this was done to account for Mb that may have been oxidized and thus not accounted for in the assay otherwise. The absorbance of the supernatant at 538 and 568 nm was measured using a Bio-Tek PowerWave 340x microplate reader (Winooski, VT, USA). A Mb standard (horse Mb, Sigma-Aldrich, St Louis, MO, USA) was included with each set of samples. The Mb concentrations were calculated as described previously (Reynefarge, 1963) and expressed in mg mg^{-1} protein. All assays were performed in triplicate.

Western Blots

Changes in protein expression were determined using Western blots as previously described (Kanatous et al., 2008). Briefly, samples were mixed in a 1:1 ratio with SDS and 0.05% bromophenol blue, boiled for 5 minutes, and spun through glass wool spin columns. Then, 20µg of protein were loaded into wells of pre-cast, 4-20% polyacrylamide gels and gel electrophoresis was run out in standard running buffer (1X tris-glycine SDS) at 150V for ~40 minutes, until dye front reached the bottom of the gel. Gels were dry transferred onto nitrocellulose membranes using iBlot® gel transfer stacks (Invitrogen, Grant Island, NY, USA) and membranes were subsequently probed with primary antibodies. Polyclonal rabbit, anti-human myoglobin (1:3,000) (DakoCytomation, Carpinteria, CA, USA), polyclonal rabbit anti-actin (1:5,000) (Thermo Scientific, Rockford, IL, USA), and anti-mouse CN (1:250) (BD Transduction Laboratories, San Diego, CA, USA) were the primary antibodies used; each primary antibody was detected with a horseradish peroxidase-conjugated secondary anti-serum. Resultant protein bands were visualized using the Supersignal West Dura Luminol

chemiluminscent agent (Thermo Scientific, Rockford, IL, USA). Band intensity was quantified using Bio-Rad's Image Lab 3.0 software (Hercules, CA, USA).

RNA Transcript Analysis

RNA was isolated using TriPure (Roche, Indianapolis, IN, USA) and cleaned using RNeasy (Qiagen); yield was determined via optical density measurements on a DU580 spectrophotometer (Beckman Coulter, Indianapolis, IN, USA). cDNA was synthesized from 500 ng RNA via first-strand synthesis kit (Qiagen, Valencia, CA, USA) and thermocycler (MJ Research, St. Bruno, Quebec, Canada). Polymerase chain reaction (PCR)was performed with RT^2 profiler PCR array PAMM-065ZG-4 (Mouse Oxidative Stress and Antioxidant Defense superarray) with RT^2 Real-Time SYBR Green Mastermix on the Roche 480 Light Cycler (Indianapolis, IN, USA) for 10 minutes at 95°C, then 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. Gene expression changes were calculated using the Second Derivative Maximum analysis method, which uses cross point analysis of the PCR reaction to obtain relative fold changes. Samples were run in replicates of three; difference in transcript expression was defined at < 2-fold or > 2-fold change.

Statistical Analysis

Student's t-test or one-way analysis of variance (ANOVA) with a Tukey *post-hoc* test was used for statistical analyses with SigmaStat version 2.0 (Ashburn, VA, USA). Significance was considered at $p \leq 0.05$; all data are presented as means ± SEM.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Author Contributions

A.S. and S.K. designed the research; A.S., M.D., and S.K. conducted the research; A.S. and S.K. analyzed the data; M.F. provided tissue samples; S.T. donated experimental supplies; A.S. wrote the manuscript and A.S., M.D., M.F., S.T., and S.K. edited the manuscript.

Table 1.1. Macronutrient composition and caloric density of rat diets.

	CON	**SAT**
Protein % kcal	20	20
Carbohydrate % kcal	69	38
Fat % kcal	11	42
Saturated Fat % total FA	43	92
Monounsaturated fat % total FA	8	4
Polyunsaturated fat % total FA	49	4
Kcal/g	3.6	4.5

CON, Control; SAT, 40% saturated fat.

Table 1.2. Fatty acid composition of diets (% of total diet).

Fatty Acid	**CON**	**SAT**
8:0	0.14	1.6
10:0	0.09	1.1
12:0	0.72	8.6
14:0	0.24	2.9
16:0	0.28	2.0
18:0	0.24	2.9
18:1 n-9	0.33	0.8
18:2 (LA)	1.95	0.8
n-6	1.95	0.84

LA, Linoleic acid; CON, Control; SAT, 40% saturated fat.

Table 1.3. Mb assay data from C2C12 cells (normoxic and hypoxic, glucose versus 5% lipid) and rat soleus (CON versus SAT).

Sample	N	Mean Mb (mg/mg protein)	S.E.M.
Normoxic C_2C_{12}, glucose control	6	0.05599	0.00169
Normoxic C_2C_{12}, 5% lipid	5	0.1590 [#, $]	0.00145
Normoxic C_2C_{12}, 5% lipid + PBN	6	0.05232	0.00132
Hypoxic C_2C_{12}, glucose control	6	0.02779	0.00111
Hypoxic C_2C_{12}, 5% lipid	6	0.11678 [*, ‡]	0.00238
Hypoxic C_2C_{12}, 5% lipid + PBN	6	0.09634 [*]	0.00165
CON Rat Soleus	3	1.460	0.0298
SAT Rat Soleus	6	1.501	0.0330

 # data significantly different from normoxic control, p < 0.001, n=6; $ data significantly different from normoxic 5% Lipid + PBN cells, p < 0.001, n=6; * data significantly different from hypoxic control, p < 0.001, n=6; ‡ data significantly different from hypoxic 5% Lipid + PBN cells, p < 0.001, n=6 ; CON = control; SAT = 40% saturated fat; PBN = phenyl-alpha-tert-butyl nitrone (ROS scavenger)

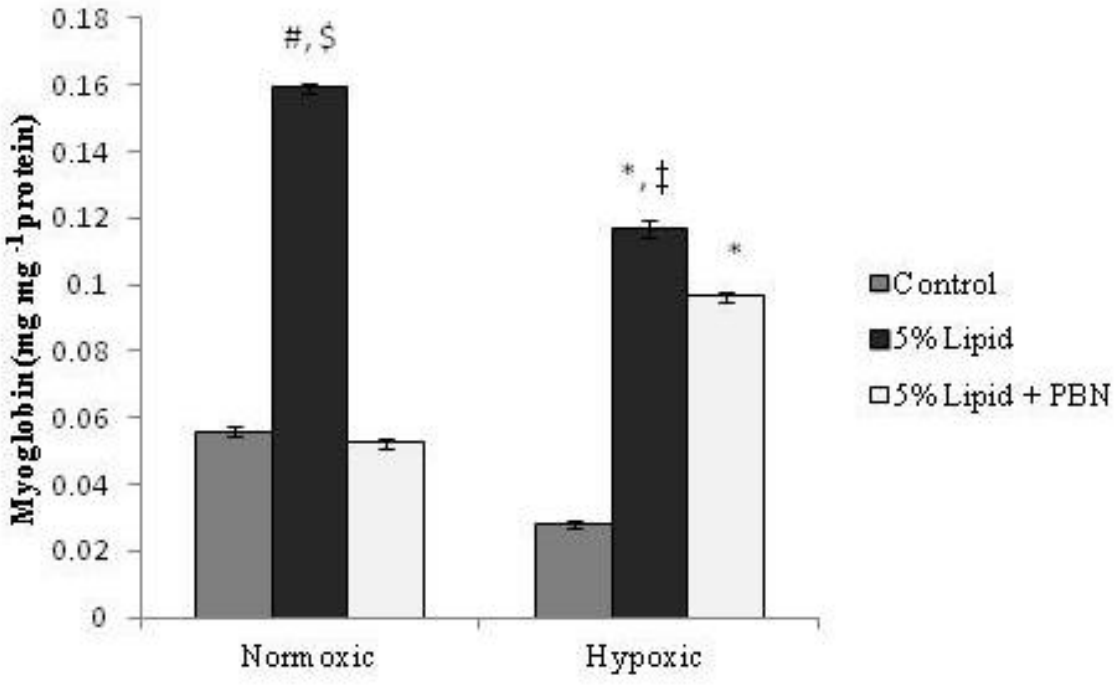

Figure 1.1. Mb measured in C2C12 cells. In normoxic cells, 5% lipid conditions increased Mb relative to control cells; interestingly, addition of a ROS scavenger, PBN, reversed this lipid-induced Mb increase in normoxic cells. In hypoxic cells, 5% lipid-supplemented cells showed an increase in Mb. Hypoxic, 5% lipid-supplemented cells supplemented with PBN showed a reduction in hypoxic lipid-induced Mb increase, but these cells still showed a Mb increase relative to hypoxic control cells (# data significantly different from normoxic control, $p < 0.001$, n=6) ($ data significantly different from normoxic 5% Lipid + PBN cells, $p < 0.001$, n=6) (* data significantly different from hypoxic control, $p < 0.001$, n=6) (‡ data significantly different from hypoxic 5% Lipid + PBN cells, $p < 0.001$, n=6).

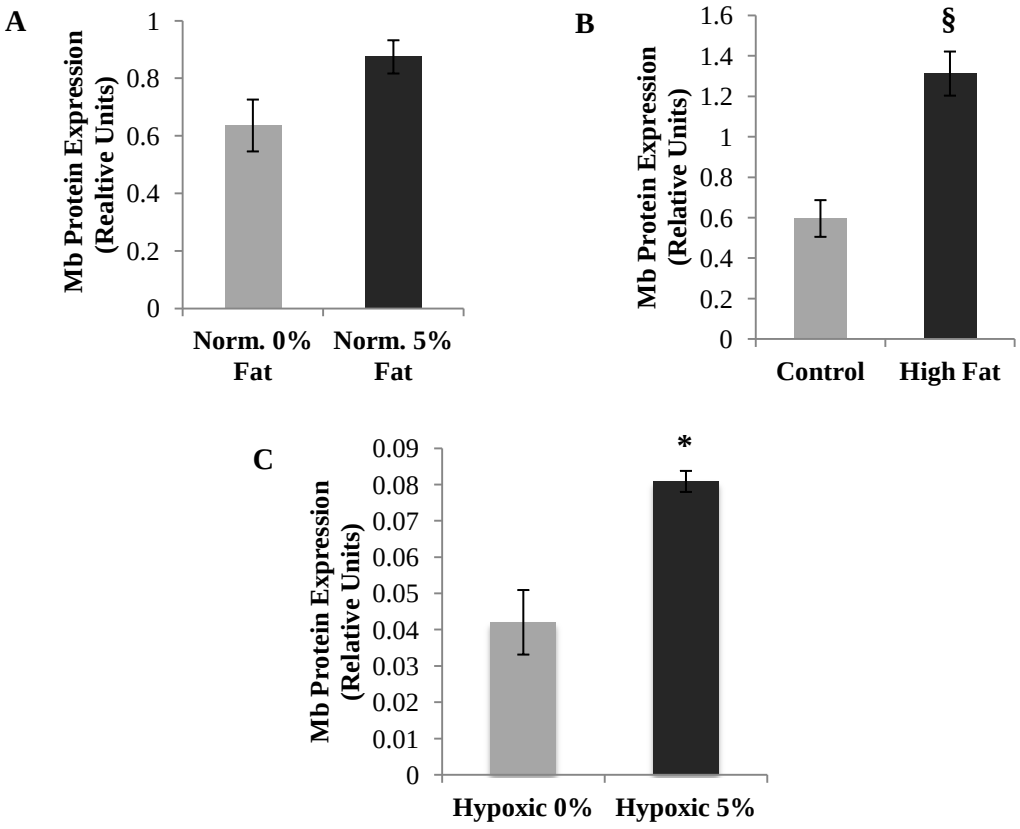

Figure 1.2. Mb Protein Expression in C2C12 Cells and Rat Soleus. Total Mb protein expression, normalized to alpha-actin as determined by Western blot analysis showed A) C2C12 cells have a trend toward increased Mb protein expression (n = 6, p=0.09), B) soleus muscle from fat-fed rats has more total Mb protein expression (n = 8, p = §0.013), and C) hypoxic C2C12 cells have a significant increase in total Mb protein when supplemented 5% lipid (n = 3, *p = 0.014).

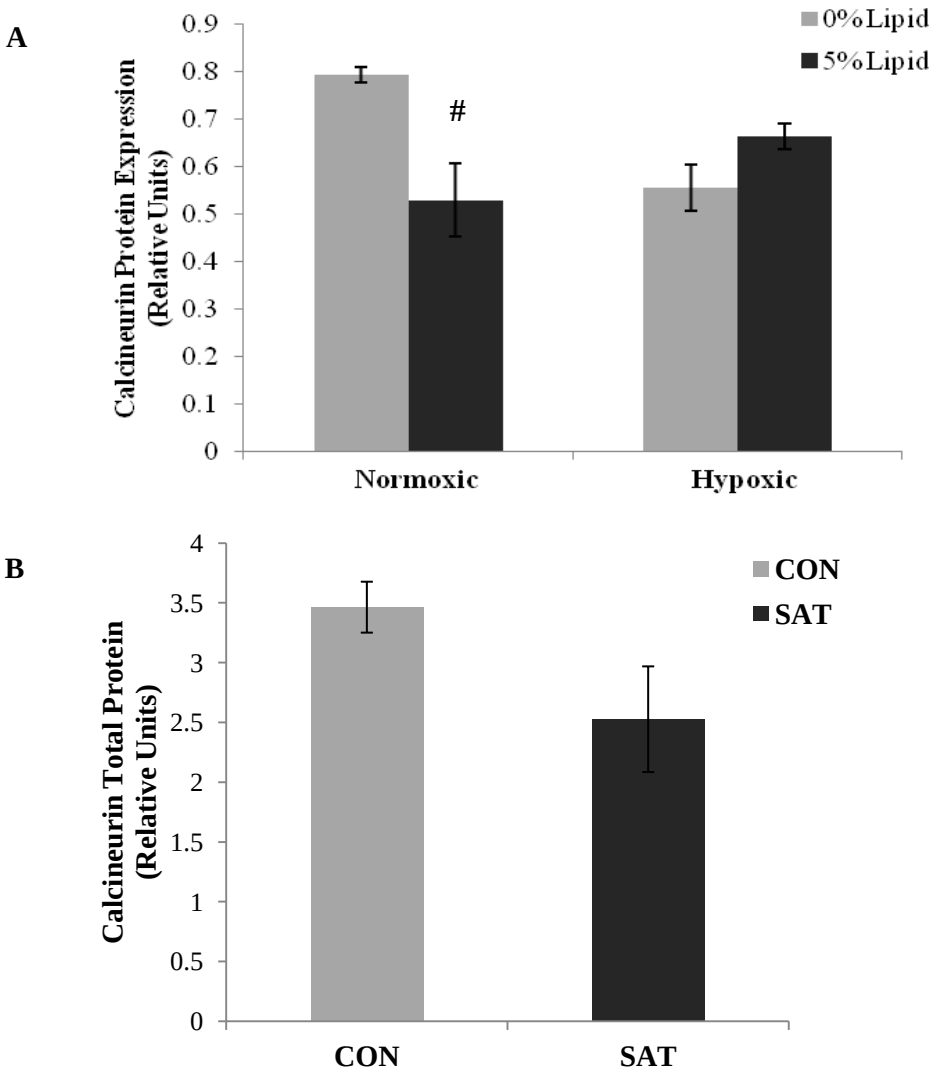

Figure 1.3. CN Total Protein in Hypoxic and Normoxic C2C12 Cells. Total CN protein expression, normalized to alpha-actin, as determined by western blot analysis showed A) a decrease in CN expression in normoxic high fat cells as compared to normoxic control cells (n = 3, #p = 0.028), with no difference in CN expression between control and high fat cells (n = 3) and B) a trend toward decreased CN expression in SAT vs. CON rats (n = 3, p = 2.000).

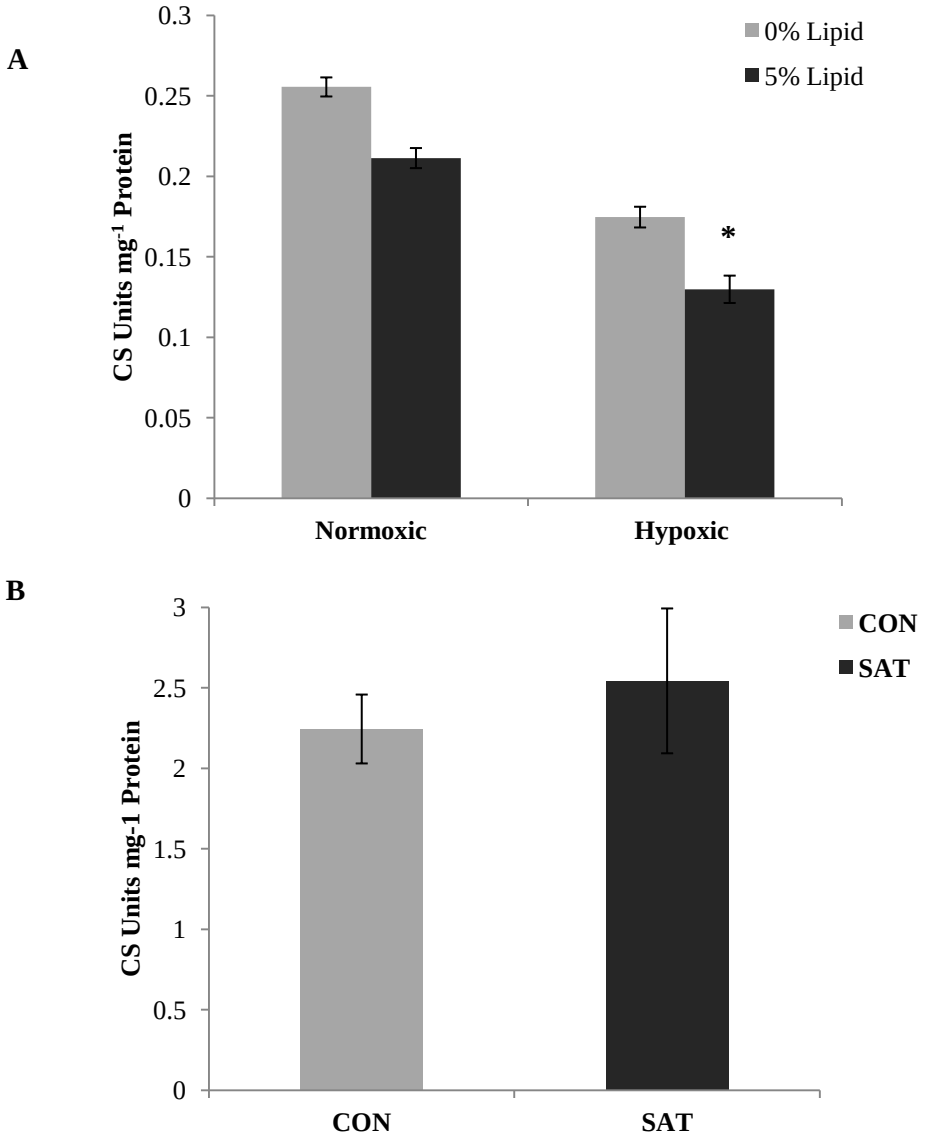

Figure 1.4. CS Activity in C2C12 Cells and Rat Soleus. CS activity determined by CS assay showed in A) normoxic cells that CS decreased in 5% lipid conditions as compared to control conditions (n = 9 , #p = 0.002). Similarly, CS assay showed in hypoxic cells that CS also decreased in 5% lipid conditions as compared to control conditions (n = 9 , *p = 0.001); B) Whole rat soleus tissue shows no difference in CS activity in SAT versus CON rats (n = 27, p=0.71).

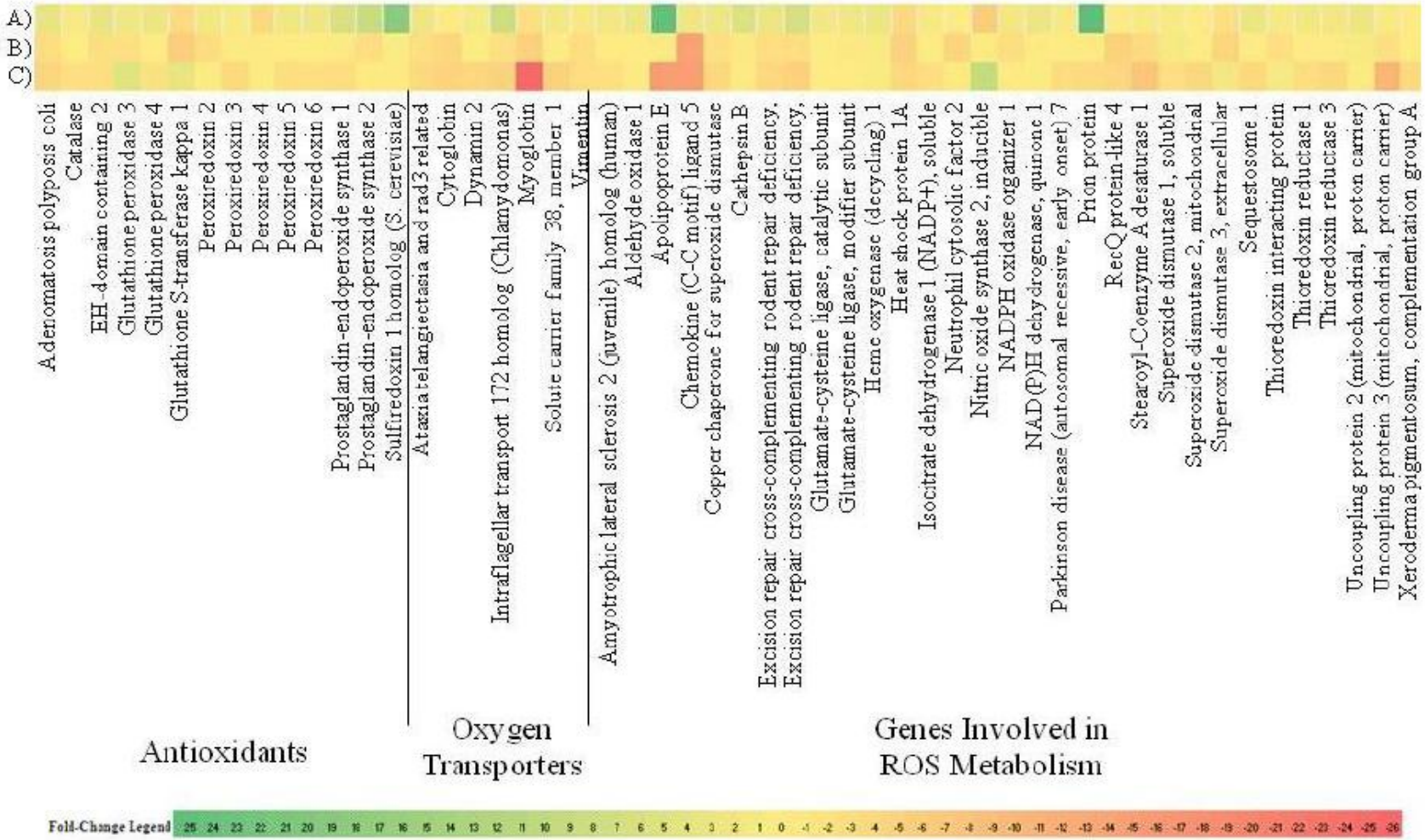

Figure 1.5. Heat map of oxidative stress transcripts within cell culture treatments. Transcripts of genes related to oxidative stress with at least a 2-fold change difference between treatment groups ($p \leq 0.05$, n=3) show in A) lipid normoxic versus glucose normoxic cells, lipid cells appear to be more oxidatively stressed, while in B) lipid hypoxic versus glucose hypoxic cells, the lipid hypoxic cells are less oxidatively stressed than glucose hypoxic, and C) lipid hypoxic cells are less oxidatively stressed than lipid normoxic cells.

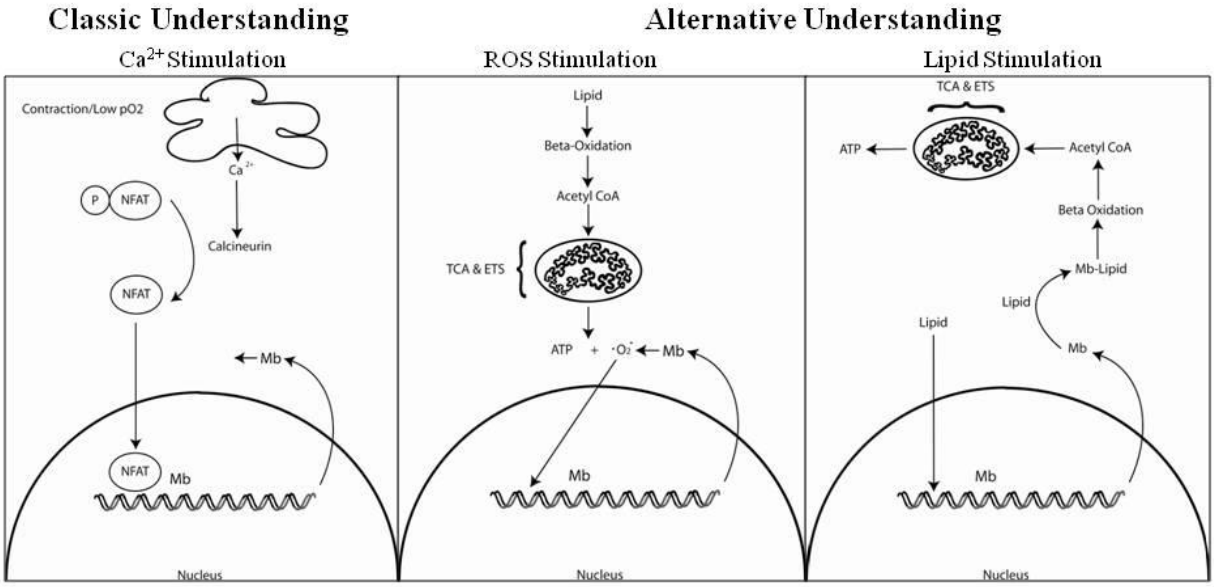

Figure 1.6. Mb Stimulation: Classic vs. Alternative Understanding. Classically, Mb stimulation relates to muscle contraction releasing calcium from the sacroplasmic reticulum, activating CN, and dephosphorylating and translocating NFAT to the nucleus (left panel). Alternatively, redox signaling may involve increased ROS associated with β-oxidation affecting the Mb gene to increase transcription, providing more Mb to prevent accumulation of potentially harmful metabolic byproducts (middle panel). Or, if directly lipid-stimulated, then lipid increases Mb gene transcription within the nucleus, which then provides more Mb to aide in the movement of free fatty acids to the mitochondria to be used for ATP production (right panel).

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CHAPTER 2

Implications of oxidative stress on myoglobin stimulation and function[2]

Summary

Myoglobin (Mb) is a small, monomeric hemeprotein that functions to store oxygen and scavenge nitric oxide & reactive oxygen species (ROS); recently, Mb stimulation has been affiliated with lipids, thus prompting a newly hypothesized role for Mb in lipid metabolism. While the mechanism through which lipids stimulate Mb is unclear, the data point to a role for ROS signaling, consequent of lipid metabolism. Accordingly, this paper aims to establish a role for ROS in Mb stimulation. C_2C_{12} cells were differentiated in normoxic or hypoxic environments and incubated with 25μM or 50μM H_2O_2 for 15 or 30 minutes per day; mature myotubes were harvested after seven days. In normoxia, acute ROS exposure actually decreased Mb values relative to controls, whereas in hypoxia, acute ROS exposure yielded an increase in Mb as compared to control cells. Thus, ROS appear to increase Mb in the presence of a secondary stimulus, hypoxia; this may, in part, account for recently established lipid-induced Mb stimulation. Also, this paper addresses current disparities in Mb assay methodology, highlights both advantages and limitations of these disparities, and suggests amalgamating techniques for attaining the most insightful Mb data.

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Introduction

Myoglobin (Mb) is a small, monomeric heme protein associated with a suite of important physiological functions within the muscle. Although conventionally only regarded for its role in oxygen storage and transport, Mb function now also extends out to cellular protection as a scavenger of reactive nitrogen species (RNS) and reactive oxygen species (ROS) (Flögel et al., 2001; Flögel et al., 2004; Garry and Mammen, 2007; Gödecke, 2010; Gros et al.; 2010, Kanatous and Mammen, 2010; Merx et al., 2001; Millikan, 1939; Rayner et al., 2009; Wittenberg and Wittenberg, 1989). Generally, these functions of Mb are tied to the stimulation of cellular Mb production. Increased oxygen demand in oxygen-limited environments, i.e. hypoxic exercise, increases Mb, and increased cellular production of RNS increases Mb (Kanatous et al., 2009; Rayner et al., 2009). Recently, increased availability of exogenous lipids has also been shown to stimulate Mb (Schlater et al., In Revision).

Lipid-induced Mb stimulation may be consequent of direct lipid stimulation, or alternatively indirect stimulation, whereby lipids are being metabolized and subsequently byproducts of beta-oxidation are stimulating Mb production. With regard to the former, lipids have influence in regulating various metabolic processes through direct interaction with peroxisome proliferator-activated receptors (PPARs) (Chawla et al., 2001; Nakamura et al., 2004; Jump, 2008; Nakamura et al., 2014). Skeletal muscle benefits broadly from lipid-PPAR interactions, including via muscular ability to adapt to fasting, exercise, and diet through altered gene expression. One of the more notorious genes induced by PPAR activity is PPARγ coactivator 1α (PGC1α), which acts as a coactivator to enhance various transcription factors; in addition to coactivating or enhancing PPAR transcriptional activity, PGC1α also coactivates myocyte enhancement factor 2 (MEF2), a Mb transcription factor, thus providing a possible

avenue for lipid-induced Mb stimulation (Figure 2.1A) (Michael et al., 2001; Lin et al., 2002; Lin et al., 2005; Kanatous et al., 2010; Nakamura et al., 2014).

Though the pathway through which lipids induce Mb stimulation has an apparent direct connection to a known Mb transcription factor, recent data are suggestive of an indirect role for lipid-induced Mb stimulation. Meaning, increasing lipid bioavailability may be increasing Mb through byproducts of beta-oxidation, an aerobic process that metabolically breaks down fatty acids. This metabolism of fatty acids has also been shown to increase cellular production of ROS as a byproduct (St. Pierre et al., 2002). Previous data show that lipid-induced Mb increases can be completely inhibited in the presence of exogenous ROS scavengers in normoxic cell culture, with a slightly damped effect in hypoxic cell culture, suggesting a role for redox signaling in lipid-induced Mb stimulation (Schlater et al., In Revision). Although chronically elevated ROS production can lead to cellular aging and related pathologies, acute redox signaling has been established as a highly important avenue for skeletal muscle adaptation. Examples of redox-sensitive signaling pathways include nuclear factor κB (NF-κB), mitogen-activated protein kinases (MAPK), and PGC1α; these pathways respond to acute bouts of ROS, such as those associated with endurance exercise, in a broad adaptive fashion, including increasing antioxidant expression, glucose transport, angiogenesis, and mitochondrial biogenesis (Gomez-Cabrera et al., 2005; Irrcher et al., 2008; Irrcher et al., 2009; Powers et al., 2011; Ji and Zhang, 2014). Furthermore, as previously mentioned, PGC1α is a known coactivator of MEF2, a Mb transcription factor. Thus, given these important roles for redox signaling in adaptation, in addition to a direct connection to a known Mb transcription factor via redox-sensitive PGC1α, it may be possible that Mb is responsive to redox signaling (Figure 2.1B).

In addition to the possibility for a redox-sensitive regulatory pathway and its proposed function as a scavenger of ROS, the structural makeup of this heme protein makes Mb sensitive to oxidation. The heme prosthetic group at the center of Mb contains iron, and in addition to being an important physical factor in Mb functionality, it can also act as an electron source or sink. While the reduced form of iron, ferrous (Fe^{2+}), is conducive to proper Mb function and ligand binding, in the presence of oxidants, the iron in Mb can be oxidized into ferric iron (Fe^{3+}) (Richards, 2012). To compensate for this physical property when experimentally calculating Mb values, some researchers began adding a reducing agent, dithionite, to samples during CO bubbling to adequately account for all Mb, both reduced and oxidized. This step, however, is somewhat debated, and while it provides a more global assessment of Mb in samples, it also provides a misrepresentation of Mb in vivo. That is, addition of a reducing agent reflects a greater pool of "functional" Mb that may, in actuality, be largely oxidized in vivo and therefore not necessarily functionally accessible in living cells.

The purpose of this paper is to further explore lipid-induced Mb stimulation, particularly through the increased production of ROS associated with increased beta-oxidation. Hydrogen peroxide, H_2O_2, was added in varying concentrations and for varying time intervals to differentiating normoxic and hypoxic cells. Between treatment groups, Mb levels were determined via functional CO-binding Mb assays. Previously published manuscripts using Mb assays show disparate methodology, such that some researchers add reducing agents to account for oxidized Mb, while many others bypass this step. Accordingly, this paper also aims to address differences in Mb assay methodology, how the addition of a reducing agent to samples is both advantageous and limiting, and functional implications these differing methodologies can have for in vitro and/or in vivo environments.

Results

H_2O_2 Assaults

In normoxic conditions, Mb assays showed that H_2O_2 assaults caused a decrease in Mb for all concentrations and lengths of time relative to control normoxic cells ($p<0.001$, n=6) (Figure 2.2a). Cells differentiated in hypoxic conditions, alternatively, revealed that H_2O_2 assaults increased Mb relative to hypoxic controls. Specifically, cells exposed to 25μM H_2O_2 for 15 minutes/day, 50μM H_2O_2 for 15 minutes/day, and 25μM H_2O_2 for 30 minutes/day all had greater Mb values as compared to hypoxic control cells (0.037452 ± 0.00215, 0.026534 ± 0, 0.036725 ± 0.00459 versus 0.0209 ± 0.00102 mg mg^{-1} protein, respectively, $p<0.05$, n=6) (Figure 2.2b). Mb values for hypoxic cells exposed to 50μM H_2O_2 for 30 minutes/day did not differ from hypoxic controls (0.023814 ± 0.00301 versus 0.0209 ± 0.00102 mg mg^{-1} protein, respectively, $p<0.05$, n=6).

Impact of Reducing Agent on Mb Assays

In order to determine the impact of a common addition of dithionite, a reducing agent, on Mb values, Mb assays were run on a subset of normoxic and hypoxic cells cultured with 5% lipid *without* a dithionite addition, and subsequently compared to Mb assays from the same samples run *with* dithionite. In normoxia, Mb assays run without dithionite (i.e. no reducing agent added) on cells supplemented with 5% lipid showed no difference in Mb values as compared to control cells (0.0792 ± 0.00278 versus 0.0777 ± 0.00477 mg mg^{-1} protein, respectively, n=6) (Figure 2.3). In hypoxia, alternatively, Mb assays run without dithionite on cells supplemented with 5% lipid showed an increase in Mb values as compared to hypoxic control cells (0.157 ± 0.0243 versus 0.0792 ± 0.00393 mg mg^{-1} protein, respectively, $p<0.001$, n=6) (Figure 2.3).

Further Mb assays were run with an addition of dithionite. These assays, interestingly, showed a different trend in normoxia, whereby lipid addition appears to increase Mb as compared to glucose controls (0.158967± 0.00145 versus 0.055978 ± 0.00447 mg mg^{-1} protein, respectively, $p<0.001$, $n=6$). This indicates a large presence of oxidized Mb (ferric Mb, Fe^{3+}) in lipid-supplemented normoxic samples. Mb assays run with the addition of dithionite in hypoxia showed trends similar to those from assays run without dithionite, whereby addition of lipid similarly increased Mb values as compared to hypoxic controls (0.116773 ± 0.00238 versus 0.027788 ± 0.00111 mg mg^{-1} protein, respectively, $p<0.001$, $n=6$), thus indicating a decreased prevalence of Mb oxidation in hypoxic cells.

In regard to individual cell culture cohorts, addition of sodium dithionite actually yielded a lowered calculated Mb value in three of the four cohorts. Normoxic control cells had lower calculated Mb values when run with dithionite as compared to calculated Mb values run without dithionite (0.055978 ± 0.00447 versus 0.0777 ± 0.00169 mg mg^{-1} protein, respectively, $p<0.001$, $n=6$), hypoxic control cells had lower calculated Mb values when run with dithionite as compared to those run without dithionite (0.027788 ± 0.00111 versus 0.0792 ± 0.00393 mg mg^{-1} protein, respectively, $p<0.001$, $n=6$), and hypoxic lipid cells had lower calculated Mb values when run with dithionite as compared to those hypoxic lipid samples run without (0.116773 ± 0.00238 versus 0.157 ± 0.00243 mg mg^{-1} protein, respectively, $p<0.001$, $n=6$). The only cell cohort in which a dithionite addition increased calculated Mb values as compared to those run without dithionite was the normoxic lipid cell group (0.158967± 0.00145 versus 0.0792 ± 0.00278 mg mg^{-1} protein, respectively, $p<0.001$, $n=6$).

Discussion

Lipids have recently been established as stimulators of Mb (De Miranda et al., 2012; Schlater et al., In Revision); here, we show data that suggests this lipid-induced stimulation may, in part, be consequent of a surplus of ROS. C_2C_{12} cells differentiated in a hypoxic environment showed that acute daily exposure to H_2O_2 caused an increase in Mb as compared to hypoxic control cells (Figure 2.2B). Normoxic cells, alternatively, showed a decrease in Mb expression concomitant with H_2O_2 exposure (Figure 2.2A).

The disparity between normoxic and hypoxic data may be due to hypoxia acting as a secondary stimulus for regulating Mb expression. Previous research has shown hypoxia to be an important secondary stimulus in Mb expression, particularly in its ability to reprogram Ca^{2+} signaling. Hypoxia alone, for example, targets Ca^{2+} release from the endoplasmic reticulum and can actually inhibit Mb expression, whereas hypoxia in combination with exercise (i.e. a secondary stimulus) will enhance the release of Ca^{2+} from the sarcoplasmic reticulum (SR), causing an increase in Mb expression through a calcineurin/nuclear factor of activated t cells (NFAT) pathway (Kanatous et al., 2009). Though a molecular basis for this requires further analysis, perhaps hypoxia is also capable of working in combination with ROS to reprogram Ca^{2+} signaling. ROS have recently been shown to modify Ca^{2+} signaling in muscle cells, whereby ROS derived from NADPH oxidase (Nox) work to increase the sensitivity of ryanodine receptors (RyR) in cardiomyocytes and transient receptor potential (TRP) channels in skeletal muscle (Ward et al., 2014). Given this connection between ROS and release of Ca^{2+} from the SR, we can speculate that hypoxia may enhance this Ca^{2+} release, which in turn may activate the Ca^{2+} sensitive calcineurin/NFAT and MEF2 pathways to increase Mb, thus providing a possible explanation for the Mb disparity between normoxic and hypoxic cells.

Recent studies in the hypoxia-adapted northern elephant seal pups (*Mirounga angustirostris*) demonstrated an adaptive increase in antioxidant defense systems following bouts of apnea, proposing a role for acute apnea-induced ROS in governing this response (Vázquez-Medina et al., 2011). Concomitant with the increase in antioxidant defenses was an increase in Mb. This Mb increase was attributed to ROS-related stabilization of hypoxia-inducible factor-1α (HIF1α), but HIF1α has no binding site on the Mb promoter, and as previously mentioned, hypoxia alone actually decreases Mb in mammalian skeletal muscle (Kanatous et al., 2009). Collectively and in support of the present study, an alternative interpretation of this result is that perhaps ROS and hypoxia as a secondary stimulus are responsible for apnea-induced Mb increases in northern elephant seal pups.

This rationale, however, maintains some ambiguity. The present study's investigation of ROS involvement in Mb stimulation was prompted by a search for explanation in the recently described lipid-induced Mb stimulation; in that study, hypoxic lipid-supplemented cells showed an increase in Mb concomitant with no change in calcineurin (Schlater et al., In Revision); however, because calcineurin is ubiquitously present and must therefore be activated (e.g. via Ca^{2+}) in order to confer phosphatase activity (Molkentin et al., 1998; Molkentin, 2006; Kanatous et al., 2009, Heineke and Ritter, 2012), perhaps then protein levels need not change in order to reflect enzyme activity differences. Furthermore, ROS have been shown to decrease calcineurin activity through proteolysis (Lee et al., 2007). It may be, however, that the previously described enhancement of MEF2 through PGC1α accounts for Mb stimulation independent of calcineurin. Taken together, this potentially synergistic interaction between ROS and hypoxia, and its relation to SR Ca^{2+} release and calcineurin activity, warrant further investigation.

Despite the apparent ability for acute ROS exposure to increase Mb in hypoxia, H_2O_2 assaults appeared to exhibit the opposite effect in normoxia; namely, acute exposure to H_2O_2 yielded a Mb decrease in normoxic cells. Although these data do not necessarily support a role for beta-oxidation byproducts (i.e. ROS) in lipid-induced Mb stimulation, they do not necessarily refute this hypothesis, either. In lipid supplementation experiments, lipids were present in the cell culture media throughout the *entire* duration of myotube differentiation. Following suit, those lipids were likely being metabolized throughout the entire duration of myotube differentiation (and thus producing ROS chronically), while this experiment was just a very brief period exposure, ostensibly too short to cause an adaptive change (i.e. Mb increases). Moreover, considering the Mb assay/dithionite experiments included in the present study, all normoxic cellular data yielded lipid-induced Mb increases that were effectively exclusively oxidatively modified. Meaning, an interaction between Mb and ROS in a lipid-supplemented environment cannot be overlooked. With this rationale, future cell culture studies examining ROS in the context of Mb regulation should look to multiple daily ROS exposures to more effectively capture the cellular environment during chronic lipid exposure.

In addition to H_2O_2 assaults, the implications of ROS on Mb were further explored via experimental modifications of the Mb assay. Specifically, Mb assay values were compared across samples with and without the addition of sodium dithionite, a reducing agent commonly used during CO-bubbling. These methodological comparisons were done in lipid-supplemented samples, which are presumably more oxidatively stressed than glucose control cells due to increased ROS associated with increased beta-oxidation (St-Pierre et al 2002). Mb assay values calculated without the dithionite addition showed no difference in Mb values for lipid-supplemented normoxic versus control normoxic cells, while lipid-supplemented hypoxic cells

had greater Mb values as compared to hypoxic control cells (Figure 2.3). Interestingly, this data trend differs from Mb assay trends that were calculated *with* dithionite in the same cell cohorts. Specifically, lipid-supplemented normoxic C_2C_{12} cells appeared to have greater Mb as compared to glucose controls when Mb assay values were calculated with dithionite (Figure 2.3). Lipid supplementation in normoxic environments, therefore, appears to significantly increase Mb as previously established (Schlater et al., In Review), but this trend can only be detected upon the incorporation of a reducing agent. In other words, detection of Mb differences with a functional assay in these samples necessitates pharmacological modification of the protein. Measured values for Mb, therefore, do not necessarily reflect the functional state of Mb in the living cell. So, while lipid supplementation in a normoxic environment significantly increases Mb expression, it appears to be largely oxidized in vitro, which warrants important functional considerations (Richards, 2012). Furthermore, the aforementioned positive relationship between cellular ROS production and beta-oxidation provide a physiological basis for this increased occurrence of what appears to be ferric, or oxidized, Mb. Trends in hypoxic cell cohorts, alternatively, were the same between dithionite and non-dithionite assays, whereby lipid supplementation in hypoxic environments yielded higher calculated Mb values as compared to hypoxic controls with both Mb assay methodologies.

Within individual experimental cohorts, addition of dithionite yielded further surprising results for calculated Mb assay values. To begin with, three of the four experimental cohorts (normoxic control, hypoxic control, and hypoxic 5% lipid) yielded calculated Mb values that were actually lower with the addition of dithionite than those calculated without the addition of dithionite. These data were surprising because dithionite was originally incorporated into Mb assays to account for additional Mb pools that had been oxidized, which theoretically should

increase calculated Mb values. Alternatively, the only experimental cohort in which dithionite did increase calculated Mb values was the normoxic 5% lipid group, which, as mentioned previously, completely altered the perception of lipid-induced Mb stimulation and can have important implications regarding Mb oxidation and ability to function in the presence of lipids.

Collectively, these comparisons between Mb assay methodologies highlight both the advantages and limitations associated with adding a reducing agent during CO bubbling. Specifically, in contrast to previous assumptions, addition of dithionite does not always capture an increased representation of homogenate Mb content; rather, addition of this reducing agent can occasionally decrease calculated Mb values. Moreover, dithionite additions may also largely misrepresent functional Mb pools in vivo, while excluding dithionite additions may, alternately, misrepresent total Mb protein content. Given the role of Mb in scavenging ROS, which is arguably more important than its role in O2 storage/transport in terrestrial/non-hypoxia-adapted species (Schlater et al., In Revision), and given the potential role for ROS in Mb stimulation, we suggest incorporation of *both* Mb assay techniques for future Mb surveys. In this fashion, Mb values can provide more meaningful and insightful information regarding in vivo function rather than simple measures of protein content.

In summary, the present study proposes a role for ROS in the stimulation of Mb in hypoxic environments. This observation is proposed to account, in part, for previously seen lipid-induced Mb stimulation, whereby increased cellular ROS attributable to increased beta-oxidation stimulate Mb. While the present study presents data whereby acute ROS exposure appear to inhibit Mb expression in normoxia, we cannot rule out the possibility for a similar ROS affiliation with normoxic lipid-induced Mb stimulation, reasoning that these acute ROS incubations are likely a misrepresentation of chronic ROS exposure concomitant with continual

lipid exposure. Moreover, the present study offers data that challenges current popular methodology for Mb assays, suggesting that although addition of a reducing agent can skew and largely misrepresent functional Mb pools in vivo, it may still be advantageous to gain insight regarding Mb oxidation and overall oxidative stress in vivo. Given the role of Mb in protecting the cell from oxidative stress, the significance of this functional component should not be overlooked. Thus, an amalgamation of both methodologies is recommended for the most meaningful and accurate Mb measurements, which in turn will provide finer functional insight.

Materials and Methods

Cell Culture

Normoxic C_2C_{12} cells were grown and differentiated in a 37°C humidified incubator with 5% CO_2. Hypoxic cells differentiated in a humidified hypoxic chamber (Coy Laboratories, Grass Lake MI, USA) at 37°C, 5% carbon dioxide, 0.5% oxygen, and 94.5% nitrogen. Standard growth media was used for myoblast proliferation (Dulbecco's modified Eagle's media high glucose [DMEM], 20% fetal bovine serum [FBS], 1% sodium pyruvate, 1% penicillin/streptomycin antibiotic). At 90% confluency, myotube differentiation was induced with a glucose media (high glucose DMEM, 5% equine serum, 10μg ml^{-1} insulin, 10μg ml^{-1} transferrin). Normoxic and hypoxic cells were assaulted with varying concentrations of H_2O_2 (25μM or 50μM) (Sigma Aldrich, Milwaukee, WI, USA) added to differentiation media for 15 or 30 minutes once a day for the seven days of differentiation; additionally, normoxic and hypoxic control cells were differentiated for seven days without H_2O_2 assaults. A subset of normoxic and hypoxic cells were differentiated in media supplemented with a 5% lipid mixture (2μg/ml arachadonic acid; 10μg/ml each of linoleic, linolenic, myristic, oleic, palmitic, and stearic fatty

acids, Sigma Aldrich [Milwaukee, WI, USA]). At differentiation day 7, cells were harvested for protein with a standard homogenization buffer (20% glycerol, 1% Tween20, 0.001M DTT in PBS with a protease inhibitor table).

Protein Assays

Assays were performed using a BioTek Synergy HT Multi-Detection microplate reader. Protein concentrations were determined using Coomassie Plus^{TM} (Thermo Scientific, Rockford, IL, USA). Mb assays were performed as adapted from Reynafarje 1963 and Kanatous et al 2002. Briefly, protein homogenates were diluted with phosphate buffer (0.04 mol^{-1}, pH 6.6) and subsequently centrifuged at 28,000 x g at 4°C for 50 minutes. The resultant supernatant was then bubbled with 99.9% carbon monoxide (CO), which converts myoglobin to carboxymyoglobin. After three minutes of bubbling, a subset of samples were combined with 0.01g sodium dithionite, a reducing agent, and then bubbled again for two minutes; this was done to account for Mb that may have been oxidized and thus not accounted for in the assay otherwise. The absorbance of the supernatant at 538 and 568 nm was measured using a Bio-Tek PowerWave 340x microplate reader (Winooski, VT, USA). A Mb standard (horse Mb, Sigma-Aldrich, St Louis, MO, USA) was included with each set of samples. The Mb concentrations were calculated as described previously (Reynefarge, 1963) and expressed in mg mg^{-1} protein. All assays were performed in triplicate.

Statistical Analysis

Student's t-test or one-way analysis of variance (ANOVA) with a Tukey *post-hoc* test were used for statistical analyses with SigmaStat version 2.0 (Ashburn, VA, USA). Significance was considered at $p \leq 0.05$; all data are presented as means ± SEM.

Competing Interests

The authors declare no competing financial interests.

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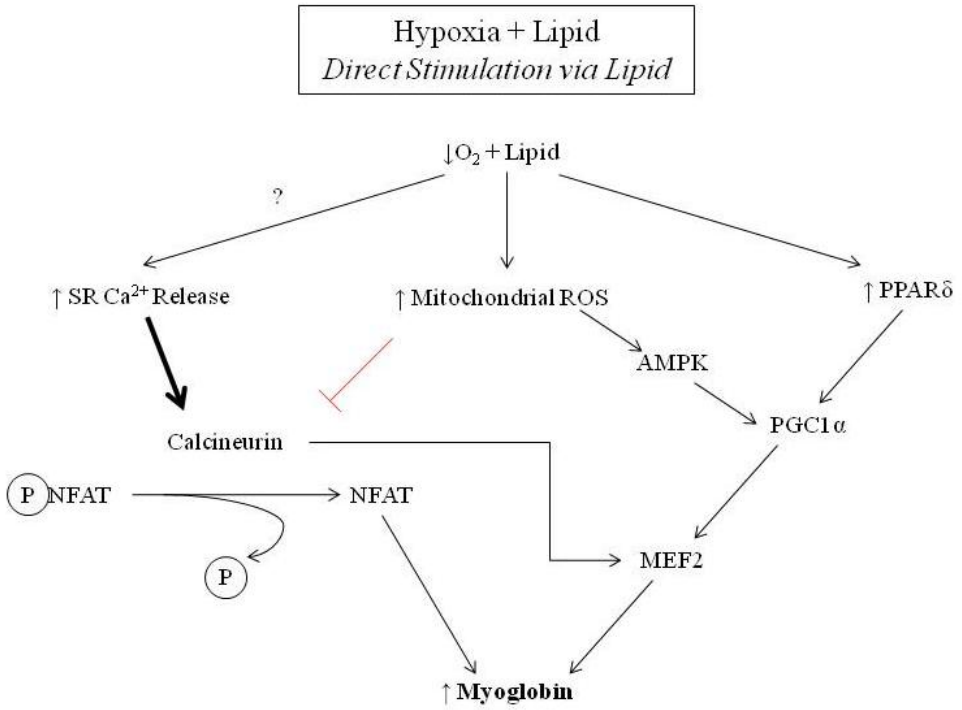
Author Contributions

A.S. and S.K. designed the research; A.S. conducted the research; A.S. and S.K. analyzed the data; A.S. wrote the manuscript and A.S. and S.K. edited the manuscript.

A


Hypoxia + Lipid
Direct Stimulation via Lipid
↓O2 + Lipid
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↑ SR Ca2+ Release
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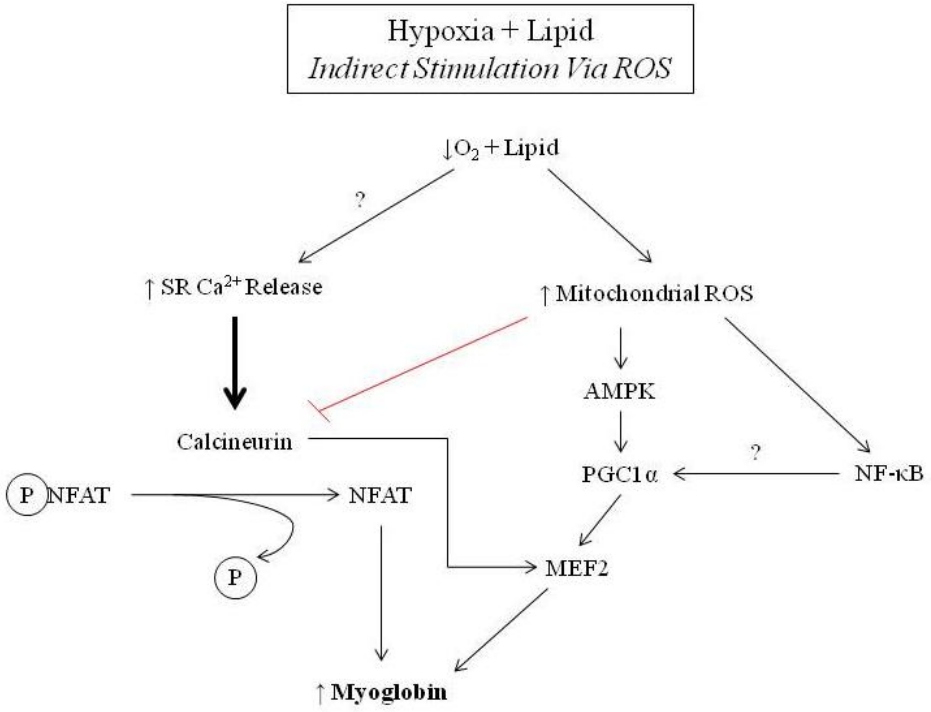
Hypoxia + Lipid
Indirect Stimulation Via ROS
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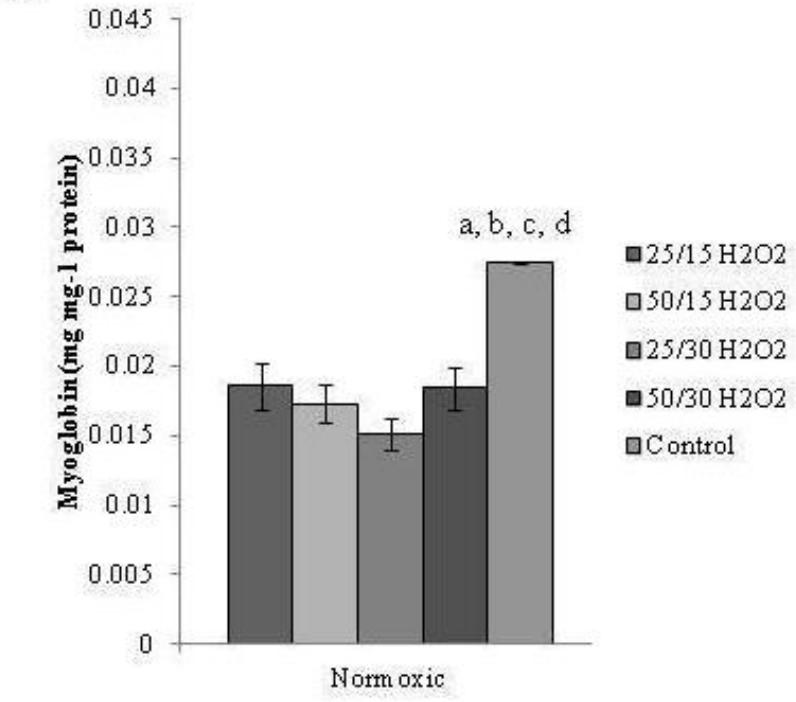
MEF2
↑ Myoglobin

Figure 2.1. Hypothesized Mechanisms for Lipid-Induced Mb Stimulation Hypoxia. Hypoxia may reprogram Ca^{2+} release in the presence of ROS generated from lipid metabolism to enhance sarcoplasmic reticulum (SR) stores of Ca^{2+}, thus activating the calcineurin-NFAT pathway stimulating Mb. This may be overpowering the ability for ROS to otherwise inhibit calcineurin. This influx in ROS may also induce PGC1α through an AMP-activated protein kinase (AMPK) pathway, thus stimulating MEF2 to increase Mb. Furthermore, A) , lipids may be activating PPARδ, which is known to induce PGC1α in skeletal muscle, and PGC1α, in turn, has been implicated as a coactivator of MEF2. Alternatively, B) ROS may be activating NF-κB pathways, which has recently been shown to have a binding site on the PGC1α promoter and thus may also be stimulating Mb via MEF2.

A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Myoglobin(mg mg-1 protein)
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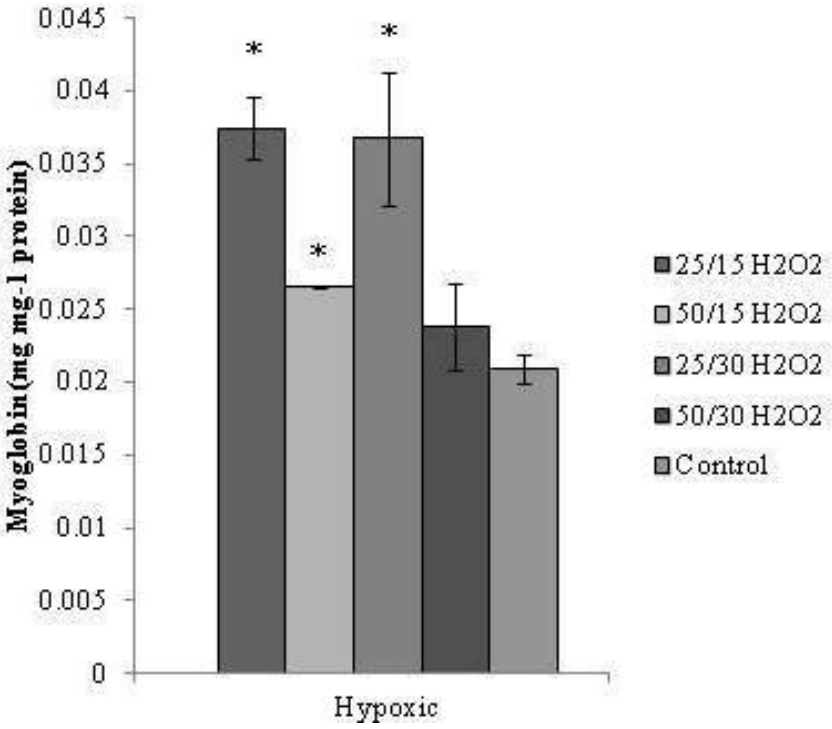
50/15 H2O2
25/30 H2O2
50/30 H2O2
Control
Normoxic

B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Myoglobin(mg mg-1 protein)
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25/15 H2O2
50/15 H2O2
25/30 H2O2
50/30 H2O2
Control
Hypoxic

Figure 2.2. H_2O_2 Assaults on Normoxic and Hypoxic C_2C_{12} Cells. In A) normoxia, H_2O_2 assaults in all concentrations and for all lengths of time had less Mb as compared to normoxic controls ("a" data significantly greater than 25μM H_2O_2 for 15 minutes/day, $p<0.001$, n=6) ("b" data significantly greater than 50μM H_2O_2 for 15 minutes/day, $p<0.001$, n=6) ("c" data significantly greater than 25μM H_2O_2 for 30 minutes/day, $p<0.001$, n=6) ("d" data significantly greater than 50μM H_2O_2 for 30 minutes/day, $p<0.001$, n=6). Cells differentiated in B) hypoxia showed cells exposed to 25μM H_2O_2 for 15 minutes/day, 50μM H_2O_2 for 15 minutes/day, and 25μM H_2O_2 for 30 minutes/day had more Mb as compared to hypoxic controls (* data significantly greater than hypoxic control, $p<0.05$, n=6).

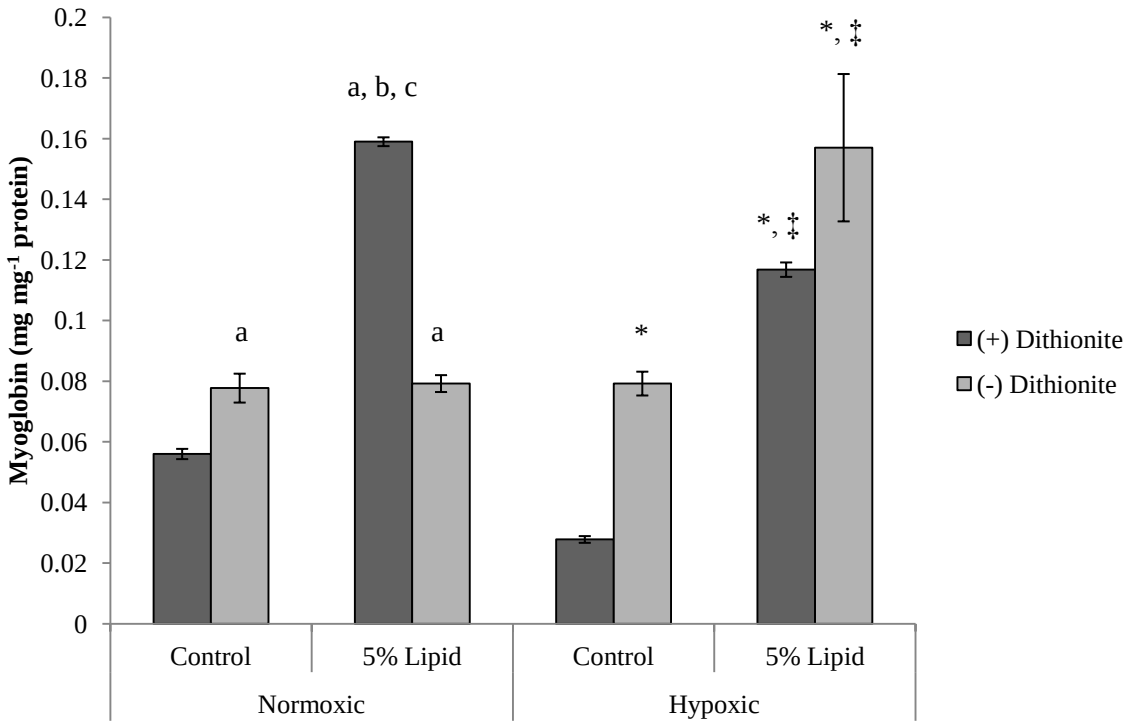

Figure 2.3. Mb Assay Data without Dithionite Addition. Mb assay values *without* an addition of dithionite, a reducing agent, were compared in normoxic and hypoxic C_2C_{12} cells cultured with a 5% lipid supplementation or a glucose control. Normoxic cells showed no difference in Mb values between 5% lipid versus control cells, while hypoxic cells supplemented with 5% lipid had higher Mb values as compared to hypoxic control cells ("a" data significantly greater than normoxic control (+) dithionite, $p<0.001$, $n=6$) ("b" data significantly greater than normoxic control (-) dithionite, $p<0.001$, $n=6$) ("c" data significantly greater than normoxic 5% lipid (-) dithionite, $p<0.001$, $n=6$) (*data significantly greater than hypoxic control (+) dithionite, $p<0.001$, $n=6$) (‡data significantly greater than hypoxic control (-) dithionite, $p<0.001$, $n=6$).

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CONCLUSION

The purpose of this dissertation was to explore regulation of myoglobin (Mb), one of the most widely studied proteins, using a novel approach that links a dietary substrate to capability for hypoxia adaptation. Here, for the first time, a scenario is shown whereby lipids are capable of inducing Mb expression in the skeletal muscle of terrestrial mammals. Implications of this relationship, as well as hypotheses addressing *how* lipids stimulate Mb, are surveyed. In conjunction with these novel data, evidence is presented that challenges the traditional function paradigm of Mb, and instead offers an alternative paradigm for how Mb functions in the skeletal muscle of terrestrial, non-hypoxia-adapted mammals.

Primary Findings

The primary finding of this research uses rodent models to demonstrate a role for lipids in the stimulation of Mb. This relationship has never before been described in a terrestrial mammal, though recent evidence has shown this trend in hypoxia-adapted, diving mammals. Primary muscle cells isolated from the swimming muscle (Longissimus dorsi) of a Weddell seal (*Leptonychotes weddellii*) showed that incorporation of a heterogeneous lipid mixture into cell differentiation media produced an increase in Mb (De Miranda et al., 2012). Given the large amounts of lipids inherent in seal diets (Costa, 1991; Debier et al., 1999; Fowler et al., 2014; Oftedal et al., 1988; Trumble and Kanatous, 2012), in addition to their extreme physiological capabilities to endure chronic bouts of hypoxic exercise (Davis and Kanatous, 1999; Guyton et al., 1995; Kooyman, 1975; Ponganis et al., 1993), it was unclear whether this trend could be duplicated in a non-hypoxia-adapted, terrestrial species. Ergo, a line of immortalized mouse skeletal muscle cells, C_2C_{12} cells, was cultured for seven days in the presence of a similar

heterogeneous lipid mixture in both normoxic and hypoxic environments and Mb was subsequently measured in each respective cohort. Given that information from preliminary data showed the most appreciable and viable lipid-induced Mb stimulation with a 5% lipid supplementation in C_2C_{12} cells (data not shown), the present study exclusively tested 5% lipid supplementation and did not further examine dose responsiveness. As previously established in seal cells, C_2C_{12} cells increased Mb in response to 5% lipid supplementation in both normoxic and hypoxic environments. In contrast to seal cells, however, normoxic lipid-induced Mb stimulation only produced Mb detectable with a reducing agent, implying that C_2C_{12} cells, normoxic lipid-induced Mb stimulation produces Mb that is exclusively oxidized.

In addition to the C_2C_{12} cells, lipid/Mb data were characterized in soleus muscle from Sprague-Dawley rats fed high fat diets, which offered perspective from the whole tissue level. Interestingly, while Mb assay values showed no difference in Mb, western blots yielded an increase in fat-fed rats relative to control rats. These data suggest a slight difference from cell to tissue regarding the ability of lipids to stimulate Mb expression. Whereas normoxic, lipid-supplemented cells had higher Mb assay values and higher western blot quantifications as compared to normoxic control cells, fat fed rats only shared the latter results. This may, in part, be attributable to differences in quality of fat between these two groups; while normoxic cells had a mixture of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs), the rats were fed a fat chow composed of SFAs exclusively. Given the known differences in lipid signaling action amongst varying lipid classes, whereby SFA signaling generally has maladaptive physiological consequences (e.g. inflammation) and MUFA and n-3 PUFA signaling generally have more beneficial physiological outcomes (e.g. anti-inflammation) (Jeckel et al., 2011; Kennedy et al., 2009; Neschen et al.,

2006; Siriwardhana et al., 2013), specific lipid class may also be differentially affecting Mb stimulation. Moreover, given the Mb differences in functional CO-binding assays versus total protein (as quantified via western blot), these data imply that Mb in these fat-fed rats may be increasing, but some sort of post-translational modification to the protein, such as phosphorylation, may be occurring that is preventing the binding of CO. Although phosphorylation has never been described in Mb from terrestrial mammals, proteomic data from Weddell seals suggests these post-translational modifications may, in fact, exist.

Following the characterization of lipid-induced Mb stimulation in a terrestrial model, this research aimed to elucidate possible mechanisms through which lipids work to increase Mb. Because of its ability to store and transport oxygen, in addition to its extreme prevalence in hypoxia-adapted animals (Kanatous et al., 2002; Kanatous et al., 2008; Kooyman and Ponganis, 1998; Noren and Williams, 2000), hypoxia has historically been implicated in the regulation of Mb. More recently, however, hypoxia has been carefully re-evaluated in its role as a Mb stimulator. New data suggests that hypoxia alone actually *decreases* Mb in terrestrial mammal skeletal muscle; instead, the role of hypoxia has been redefined as an important secondary stimulus, such that hypoxia and calcium-signaling (in association with muscle contraction/exercise) stimulate Mb (Kanatous et al., 2009).

The specific pathway through which hypoxic exercise works to stimulate Mb is through a protein kinase, calcineurin (CN). Upon contraction, calcium released from the sarcoplasmic reticulum activates CN, which then dephosphorylates nuclear factor of activated t-cells (NFAT), which is a transcription factor. Following this dephosphorylation, NFAT can then translocate into the nucleus, where it binds to the Mb promoter and subsequently stimulates its expression (Grayson et al., 1998; Kanatous and Mammen, 2010; Yan et al., 2001).

In accordance with this established role in Mb stimulation, CN protein was quantified in cell cohorts that showed Mb increasing in response to lipid supplementation to determine if, perhaps, lipid-induced Mb stimulation worked through a CN-NFAT pathway. Interestingly, all experimental cohorts (including both cell and tissue samples) in which Mb increased, CN protein expression was either not different from controls, or less than controls. These data, thus, suggested that lipids may increase Mb through a calcium-independent pathway; other Mb stimulation pathways, therefore, were necessarily hypothesized and tested. Recent research has established that in addition to hypoxic exercise, Mb increases are also attributable to exogenous nitric oxide (NO) (Rayner et al., 2009). Given the clear link between these two stimulators and Mb function (i.e. cells increase oxygen stores in response to an increased oxygen demand in a low oxygen environment, or cells increase their ability to scavenge potentially harmful NO when NO is present in excess), this newly characterized lipid-induced Mb increase should, too, pertain to Mb functionality. As such, redox signaling and the role of reactive oxygen species (ROS) were explored as a possible avenue through which lipids work, due to the ability of Mb to scavenge ROS (Flögel et al., 2004), in conjunction with the characterized influx of ROS associated with increased lipid metabolism (St-Pierre et al., 2002).

In order to assess oxidative stress levels and whether that may correlate with lipid-induced Mb stimulation, RNA was isolated from experimental cohorts for qRT-PCR. Subsequent data from PCR array analysis gave a comprehensive assessment of oxidative stress in each cohort, based on transcript analysis from a wide range of oxidative stress markers. In hypoxia, lipid-supplemented C_2C_{12} cells showed a downregulation of oxidative stress markers as compared to glucose cells. Normoxic cohorts, surprisingly, showed lipid-supplemented cells as having an upregulation of oxidative stress markers as compared to glucose cells, implying more

oxidative stress. Moreover, lipid hypoxic cells showed a downregulation of oxidative stress markers as compared to lipid normoxic cells, suggesting hypoxic lipid cells were *less* oxidatively stressed than normoxic lipid cells. This disparate trend in amongst lipid-supplemented groups may be attributed to the oxidative state of Mb consequent of increased oxidative stress. Meaning, the presumed influx of ROS production associated with increased lipid metabolism may be oxidating the iron at the center of Mb, changing its oxidation state from Fe^{2+} (ferrous) to Fe^{3+} (ferric) (Richards, 2012). A reducing agent added during the Mb assay would have acted to reverse this oxidation state, which is why these lipid cell cohorts would appear to have an increase in Mb, but in vitro, these lipid cohorts may have a surplus of ferric Mb exclusively, which would theoretically prevent Mb from scavenging ROS, thus causing the cells to ramp up expression of other antioxidants as a compensatory mechanism. In hypoxic lipid cells, hypoxia as a secondary stimulus in Mb stimulation may be acting to maintain the reduced state of Mb in vitro, thus preserving Mb function to scavenge a surplus of ROS, resulting in the observed decrease in oxidative stress markers relative to hypoxic glucose cells and normoxic lipid cells.

Further exploration of a lipid-induced Mb stimulation involving redox signaling was studied in the context of an antioxidant; specifically, normoxic and hypoxic C_2C_{12} cells were again cultured with lipid, but now with the addition of a ROS-scavenger, phenyl-alpha-tert-butyl nitrone (PBN). If, in fact, lipids work to stimulate Mb through increased ROS associated with beta-oxidation, then removal of said ROS through the incorporation of a ROS-scavenger, PBN, should prevent Mb increases affiliated with lipid. Indeed, lipid-supplemented cells cultured with PBN showed a decrease in the predicted Mb response, but these data were once again disparate between normoxic and hypoxic cells. While lipid-supplemented normoxic cells measured having the same Mb values as normoxic control cells (i.e. complete inhibition of lipid-induced

Mb stimulation), lipid-supplemented hypoxic cells had less Mb in the presence of PBN, but still significantly more Mb than hypoxic control cells. This latter dataset is indicative of an involvement of a secondary Mb stimulus, i.e. hypoxia.

Following experimental inhibition of ROS in lipid-supplemented cells, this dissertation explored the possibility of direct application of oxidative stress in the stimulation of Mb. C_2C_{12} cells were cultured in normoxic and hypoxic environments, but instead of supplementing cells with lipid, these experimental cohorts were assaulted with varying treatments of hydrogen peroxide, H_2O_2, a strong oxidizer. In both normoxia and hypoxia, cell groups incubated in differentiation media supplemented with 25μM or 50μM H_2O_2 for 15 or 30 minutes during the seven days of differentiation. All normoxic cell cohorts that underwent H_2O_2 assaults had less Mb than normoxic controls, while most hypoxic cell cohorts that underwent H_2O_2 assaults had more Mb than hypoxic control cells (specifically, 25μM treatments for 15 and 30 minutes as well as 50μM treatment for 15 minutes had more Mb than hypoxic controls). Hypoxic groups likely had more Mb with H_2O_2 assaults due to being differentiated in hypoxia, which, as mentioned earlier, is an important secondary regulator of Mb due to its ability to reprogram calcium signaling (Kanatous et al., 2009). Given recent data describing the ability for ROS to stimulate sarcoplasmic reticulum calcium release, it was postulated that perhaps then hypoxia is enhancing this stimulation, thus promoting Mb through calcineurin and MEF2 activation (Kanatous et al., 2010; Ward et al.; 2014). As for the normoxic cells, while these may data superficially imply a negative effect of ROS on Mb stimulation, they do not necessarily refute ROS-induced Mb stimulation, either. In the previous experiment, lipids were present in the media throughout the *entire* duration of myotube differentiation. Following suit, those lipids were likely being metabolized throughout the entire duration of myotube differentiation (and thus producing ROS

chronically), while this experiment was just a very brief period exposure, too short to cause an adaptive change (i.e. Mb increases).

In addition potential roles for ROS in Mb regulation, ROS also maintain important connotations regarding Mb protein structure and subsequent function. A heme prosthetic group is centrally located within the Mb protein; herein lays iron, a charged metal capable of acting as either an electron source or sink. Consequent of this function is the potential for iron to be oxidatively modified. Conducive to Mb function and ligand binding is ferrous iron (Fe^{2+}), or iron in the reduced state; yet, this iron can be modified to ferric iron (Fe^{3+}) in the presence of oxdiants, thus changing Mb ability to effectively function (Richards, 2012). Given this known physical character, some researchers began implementing a new step into traditional Mb assays to compensate for Mb that may have been oxidatively modified. The conventional method to quantify Mb is to bubble clarified protein homogenates with CO and measure absorbance of carboxy-Mb; this technique necessitates reduced, or ferrous, iron to ensure complete protein quantification. In accordance with this, some researchers began adding a reducing agent half way through CO-bubbling as to adequately account for all Mb in the sample, both reduced and oxidized (Etnier et al., 2004; Noren and Williams, 2000). This method, however, may not be providing an accurate representation of Mb within the sample. While the calculated quantity of Mb protein may be correct, the implications regarding its ability to function in vivo warrant reconsideration; that is to say, in the absence of experimental addition of reducing agents, a theoretical pool of this calculated Mb may not be functional in a living cell.

Given this functional oversight, and in light of the newly elucidated potential for ROS regulating Mb expression, differences in Mb assay methodologies were compared. Normoxic and hypoxic C_2C_{12} cells were differentiated with and without 5% lipid supplementation.

Following protein harvest, Mb was quantified with Mb assays, either with or without the addition of dithionite, a commonly used reducing agent. These two methodologies revealed disparate results, particularly in regard to normoxic cells. That is, Mb assays run with dithionite showed a drastic increase in Mb concomitant with lipid supplementation in normoxic cells, while Mb assays run *without* dithionite showed no difference between lipid-supplemented and control normoxic cells. In other words, while lipids are capable of significantly increasing cellular Mb, this protein may be effectively nonfunctional in the living cell as indicated by the apparent magnitude of protein oxidation. These data, therefore, highlight the importance of considering the powers and limitations inherent of both experimental assay conditions. Rather than existing as contending methodologies, these data warrant an amalgamation both techniques.

Additional Interpretations

While the aforementioned series of data largely supports a role for lipid-induced Mb stimulation through a ROS-mediated pathway, there still remains potential for direct lipid stimulation, too. This notion is of particular relevance in hypoxia, where lipid-supplemented cells given PBN, a ROS-scavenger, did not show full inhibition of lipid-induced Mb stimulation. Fatty acids have been shown to be important intracellular signaling molecules, demonstrating a high affinity for a family of nuclear receptor transcription factors, peroxisome proliferator-activated receptors (PPARs) (Nakamura et al 2014). A connection, then, was made between Mb and lipid-activated PPARs through PPARγ-coactivator 1α (PGC1α), a known coactivator of Mb transcription factor MEF2, which is induced by PPARs (Kanatous and Mammen, 2010; Lin et al., 2002; Lin et al., 2005; Michael et al., 2001; Nakamura et al., 2014). Thus, given the

unanticipated decrease in CN in cellular lipid cohorts, this interpretation offers an alternative palpable explanation independent of CN that accounts for lipid-induced Mb stimulation.

Interestingly, proposed pathways for both direct and indirect lipid interaction in Mb regulation have overlap. In addition to the aforementioned connection between direct fatty acid signaling and MEF2, a Mb transcription factor, ROS also have a pathway connection to MEF2. Mitochondrial ROS have been shown to stimulate PGC1α via AMP-kinase (AMPK) activation, which is presumably consequent of a drop in cellular ATP levels (Irrcher et al 2009, Powers et al 2011). This upregulation of PGC1α, therefore, allows for MEF2 stimulation. Given this overlap between pathways, it may be that direct and indirect hypotheses for lipid-induced Mb stimulation are not mutually exclusive. This may be particularly true in hypoxic environments where incorporation of ROS scavengers only partially inhibited lipid-induced Mb stimulation and thus suggested a more complex interplay between lipids and Mb.

Paradigm Switch

In light of this newly established link between lipids and Mb, a reconsideration of the functional paradigm of Mb may be warranted. The first functional character of Mb to be described was its ability to reversibly bind oxygen, prompting Glenn Millikan to assign the nickname "muscle hemoglobin" to this small, muscular heme protein 75 years ago (Millikan 1939). For decades, Mb research focused on this sole function. Bolstering the notion of its importance in oxygen storage/transport were systems that pushed physiological thresholds of hypoxia tolerance to extreme. Diving, air-breathing vertebrates, such as penguins, seals, and whales, have significantly more Mb in their swimming muscles, some even with fold-change magnitudes of differences, as compared non-hypoxia-adapted, terrestrial controls (Davis and

Kanatous, 1999; Guyton et al., 1995; Kanatous et al., 2002; Kanatous et al., 2008; Kooyman, 1975; Kooyman and Ponganis, 1998; Noren et al., 2001; Ponganis et al., 1993); this skeletal muscle feature is considered to be a hallmark adaptation in maintaining frequent and prolonged breath-hold dives. Moreover, increased Mb is a characterized adaptation concomitant with high altitude hypoxia experienced by terrestrial vertebrates (Bailey et al., 2003; Gelfi et al., 2004; Mathieu-Costello, 2001; Reynafarje, 1962). Thus, as an adaptation to chronic hypoxia, as experienced by divers and high altitude residents, Mb as an important oxygen store and transporter coincides with the physiology.

Elevated Mb has additionally been described in terrestrial endurance athletes (Duteil et al., 2007; Mielnick et al., 2011); yet, during endurance exercise, terrestrial mammals rely primarily on erythorcytic oxygen stores bound to hemoglobin to fuel aerobic metabolism in working muscle. Endurance training, accordingly, elicits physiological changes that increase muscular blood flow and subsequent oxygen delivery (e.g. increases in capillary density) (Breen et al., 1996; Fluck, 2006; Olfert et al., 2009), thus reasoning Mb-bound oxygen stores to be of little significance. Moreover, Mb has a very strong affinity for oxygen and only releases oxygen under a very low partial oxygen pressure, as indicated by the low p50 of its oxygen dissociation curve (p50=2.39 mmHg in equine Mb) (Antonini, 1971; Garry and Mammen, 2007; Gros et al., 2010; Nichols and Weber, 1989; Schenkman et al., 1997). In other words, under standard conditions the muscle must necessarily be hypoxic in order to utilize Mb-bound oxygen in aerobic metabolic processes.

In vivo Mb saturation data on submaximally exercising adult humans show that oxymyoglobin only actually desaturates during the first 20-40 seconds of exercise, with total muscular Mb stores never desaturating beyond 50% (Molé et al., 1999). Furthermore,

measurements of oxymyoglobin's contribution to oxygen flux suggest that Mb contributes minimally, and during exercise, this contribution is even lower, suggesting that Mb bears little significance to oxygen transport (Chung et al., 1998; Lin et al., 2007; Schenkman et al., 1997). Thus, given its low p50, minimal oxygen desaturation, and decreased contribution to oxygen transport during exercise, high Mb levels in healthy terrestrial animals that do not experience routine hypoxic stress is nonsensical in the context of increasing oxygen storage and transport.

Taken together with data presented this dissertation, the traditional functional paradigm of Mb warrants some reconsideration. Rather than highlight Mb as the "muscle hemoglobin", perhaps more emphasis should be placed on other functions of Mb in order to gain more meaningful insight into skeletal muscle physiology. Specifically, the role of Mb as an antioxidant defense during endurance exercise, which increases lipid-based aerobic metabolism and ROS production, appears to be more relevant and applicable than the role relevant to storage and transport of oxygen in healthy, non-hypoxia-adapted animals.

Future Direction

While data from this dissertation unambiguously support a connection between lipids and Mb stimulation, the finer details of this relationship are ostensibly more complex; nearly every answer prompted new questions. Perhaps one of the more glaring ambiguities with this newly characterized lipid-induced Mb stimulation pertains to disparities between normoxia and hypoxia. In both cell culture environments, lipid supplementation induced a Mb increase. Subsequent ROS scavenger additions, however, differentially inhibited this response and suggested that introduction of hypoxia, a confounding factor, relies on additional signaling pathways. Future research elucidating these pathways is thus warranted. Lipids in combination

with hypoxia may be stimulating Mb through a reprogramming of calcium release and subsequent activation of CN; while lipid normoxic cells and rat tissue showed an actual drop in CN protein as compared to their respective controls, lipid supplementation in hypoxia did not CN protein. As such, a meaningful experiment in determining a role for CN in lipid-induced Mb stimulation could be to incorporate a CN-inhibitor (e.g. cyclosporine A) into lipid-supplemented cohorts. If lipid-induced Mb stimulation were truly working independent of CN, then Mb increases in response to lipid should not change. If, however, CN still contributes to Mb regulation in the presence of lipids, cyclosporine A should prevent lipid-induced Mb stimulation.

Considering the common connection between direct and indirect lipid signaling pathways via MEF2, another worthwhile experiment would be to silence MEF2 or its proposed upstream coactivator, PGC1α, in lipid-supplemented cells. If lipids are stimulating Mb through an alternate MEF2 pathway as hypothesized (i.e. other than calcium-stimulated MEF2 activation), then removing MEF2 and/or PGC1α should subsequently prohibit lipid-induced Mb stimulation. Although this experiment would not discriminate between direct versus indirect lipid signaling, it would offer insight into overall lipid influence over Mb transcription factors.

Another complexity within these data is in reference to disparities in Mb transcripts across cell cohorts. Hypoxic lipid cells had far less Mb transcript as compared to hypoxic control and normoxic groups, yet it had increased levels of Mb protein, as determined by functional assays and western blots. The hypothesized rationale for this disparity was that this cell cohort upregulated Mb expression earlier in the differentiation period. Accordingly, future studies may consider the time course of Mb development in response to these environmental stimuli and harvest cellular protein and RNA over multiple days of differentiation.

Future studies further examining lipid-induced Mb stimulation would additionally benefit from characterizing the importance of lipid quality in this phenomenon. As previously mentioned, lipid quality may account for slight differences between normoxic lipid-supplemented cells versus normoxic fat-fed rats, of which the former had access to a heterogeneous mixture of lipids, while the latter was exclusively supplemented SFAs. Different lipid classes demonstrate drastically different physiological ramifications in vivo, most notably in regard to pro versus anti-inflammatory capacities; collectively, differing lipid classes should confer differing capacities to stimulate Mb.

In addition to the further research questions prompted by the lipid-supplemented cell cohorts, data generated from the H_2O_2 assaulted cells raised further questions. While acute exposure to H_2O_2 in hypoxia provoked a cellular increase in Mb, the same was not true of normoxia. This was unprecedented, particularly in light of the complete inhibition of normoxic lipid-induced Mb stimulation upon the incorporation of a ROS scavenger. As previously postulated, however, these data do not necessarily rule out the possibility of a role for ROS in Mb stimulation. A key differing feature between lipid-supplemented and H_2O_2-assaulted cells was length of exposure to the respective stimuli. With regard to the former, cells were exposed to lipid throughout the entire duration of the differentiation period, while with the latter, cells were only exposed to the H_2O_2 stimulus for a very brief period of time each day, which thus may not be an accurate representation of ROS exposure consequent of lipid metabolism. As such, future experiments might entail multiple H_2O_2 assaults each day to more effectively mimic ROS exposure consequent of beta-oxidation.

Summary

In summary, this dissertation applied a variety of molecular techniques to rodent models to elucidate a relationship between lipids and the stimulation of a muscular heme protein, Mb. This novel relationship was originally established in a line of primary muscle cells, isolated from the extreme hypoxia-adapted Weddell seal, and was recapitulated in non-hypoxia adapted, terrestrial control species. While data, in part, support lipid-induced Mb stimulation through intermediary ROS-signaling, the degree to which this may be true appears to be contingent upon environmental oxygen availability, as demonstrated by a complete reversal of Mb increases in lipid + antioxidant normoxic cell cohorts, and only a partial reversal of Mb increases in lipid + antioxidant hypoxic cell cohorts. Unlike seal cells and hypoxic lipid-supplemented C_2C_{12} cells, lipid-induced Mb stimulation in normoxic C_2C_{12} cells appears to increase Mb nearly entirely in an oxidative state, thus maintaining functional implications about Mb in the cellular environment. It is likely that this increased presence of an exclusively oxidated/ferric Mb in normoxia also accounts for PCR array results from normoxic lipid cohorts, which largely appeared to be more oxidatively stressed than hypoxic lipid-supplemented cohorts, despite both groups having similar Mb levels. Moreover, while direct application of H_2O_2 to hypoxic cells increased Mb as compared to hypoxic controls, direct application of H_2O_2 to normoxic cells actually decreased Mb; these data, in conjunction with previously published data on hypoxia acting as a secondary Mb stimulus, suggest that hypoxia may be working in conjunction with ROS to reprogram calcium signaling. In turn, this may account for observed Mb increases in hypoxic H_2O_2–assaulted cells and not normoxic H_2O_2–assaulted cells. Lipids, thus, appear to play a role in the initiation of Mb stimulation in terrestrial, non-hypoxia-adapted mammals; this

relationship, in part, may be attributable to indirect effects of ROS, and demonstrates differences in the presence of hypoxia.

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SUPPLEMENTAL RESEARCH 1

Preface

In addition to research directly addressing the specific aims of this dissertation, which characterized a role for lipids in hypoxia adaptation via myoglobin, a significant portion of research completed during this time studied cellular and subcellular adaptations to hypoxia in a hypoxia-adapted species, the northern elephant seal (*Mirounga angustirostris*). Accordingly, data from "Supplemental Research 1 & 2" are included as a complement to the primary focus of the dissertation.

Culturing primary cells from the extreme, hypoxia-adapted northern elephant seal (*Mirounga angustirostris*): a comprehensive method for shipping muscle biopsies and isolating satellite cells[3]

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Summary

Cell culture is an important tool in elucidating basic aspects of cell and molecular physiology by offering a controlled environment, free of confounding systematic influences and open to experimental manipulation. While commercially-available, immortalized cell lines are accessible and easy to culture, generations can lose physiological traits over time, and having all been derived from the same individual, do not represent a true sample size. Primary cells, alternatively, exist without these limitations; moreover, primary cell culture presents a minimally-invasive technique that has potential to provide years of data and thus maintains appeal in the study of limited-access samples. Success in isolation of primary cells, however, can prove difficult, and has conventionally been avoided in wild animal populations due to logistical concerns, including sterility and lack of cell culture facilities at field sites. Moreover, culturing primary cells from uniquely adapted, non-model species necessarily deviates from standardized techniques and can entail a considerable optimization period, thus further deterring its usage. The present study provides a comprehensive method for isolating satellite cells and subsequent myoblasts from the primary swimming muscle in a wild population of northern elephant seals (*Mirounga angustirostris*). Due to the remote location of the field site, skeletal muscle biopsies had to be shipped out of state to our cell culture facilities; interestingly, we were able to keep the tissue viable and successfully isolate cells up to 72 hours post-biopsy. Thus, the present study also addresses shipping logistics for successfully isolating cells from remotely-located samples.

Introduction

Cell culture is a valuable experimental tool in determining basic cellular and molecular phenomena. In addition to offering opportunity for a vast scope of experimental manipulations,

individual parameters can be studied independent of the suite of confounding parameters associated with integrated systems, thus providing the most basic answers to physiological questions. Moreover, cell culture presents a minimally-invasive technique that has the potential to generate years' worth of data. This latter aspect of cell culture can be particularly appealing when working with limited-access samples, such as exceptional human subjects and wild animals. This methods paper, therefore, provides a novel technique for cell culture in a wild animal, the northern elephant seal (*Mirounga angustirostris*), remotely located from the cell culture site.

A common practice in cell culturing is the use of immortalized cell lines. While these commercially-available cells have proved to be of paramount importance in elucidating countless cellular and molecular data, their accessibility and ability to rapidly proliferate maintain some limitations. Particularly, immortalized lines are effectively identical, as they come from one individual, and thus fail to represent a true sample size (i.e. "n"); also, generations can lose certain physiological traits over time, which may broadly affect experimental outcomes (Fuller and Insel, 2014; Miller, 2012; Shah et al., 2014). Primary cell culture, alternatively, lacks the aforementioned limitations inherent of immortalized cells. Additionally, usage of primary cell isolation techniques can facilitate cell culturing in many comparative, non-model species in which immortalized cell lines are not available.

Phocid seals (i.e. "true" seals) are remarkable aerobic powerhouses capable of pushing physiological thresholds to the extreme when faced with hypoxia. Members of this family exhibit unique metabolic features in that they primarily rely on aerobic metabolism in the working muscle during prolonged bouts of breath-hold exercise, which can average around 20 minutes (Davis and Kanatous, 1998; Kanatous et al., 2002; Kooyman, 1980; Ponganis et al.,

1993). Decades of research has characterized a host of adaptive traits that account for this otherwise paradoxical physiology, with a hallmark adaptation being a pronounced increase in internal oxygen stores (Kanatous et al., 2002; Kanatous et al. 2008). While descriptive physiological traits and correlations have been largely characterized in Phocids, molecular and causative mechanisms through which these adaptations arise remain generally elusive, as Phocids are non-model species. This kind of knowledge, however, may prove to be very important, both for long-term management implications for these populations and for potential health implications for human hypoxic diseases; primary cell culture, thus, could prove an important tool in furthering our knowledge of these unique physiological systems.

Of the Phocid seals, one of the more extreme divers is the northern elephant seal. Individuals of this highly migratory species have been documented reaching depths over 2,000 meters, occasionally approaching two hours between surface intervals (Robinson et al., 2012). Elephant seals are capable of such great feats, in part, by having large stores of internal oxygen, a high hypoxemic tolerance, and tight mitochondrial management of these stores during aerobic metabolism (Chicco et al., In Prep; Meir et al., 2009; Ponganis et al., 2003; Thorson and Le Boeuf, 1994). While more cutting-edge techniques are being utilized for characterizing unique elephant seal adaptations to hypoxia, such as pO_2 electrodes, H-NMR, high resolution respirometry, and metabolomics (Champagne et al., 2013; Chicco et al., In Prep; Meir et al., 2009; Ponganis et al., 2008), the finite mechanisms through which these adaptations arise remain unclear. Cell culture, alternatively, offers promise in actually elucidating the unique regulatory mechanisms responsible for the development of various adaptations to hypoxic exercise.

The purpose of this study was to establish a technique for the isolation, proliferation, and differentiation of primary skeletal muscle cells from the uniquely adapted northern elephant seal.

Unlike our previous efforts in isolating Weddell seal (*Leptonychotes weddellii*) muscle cells, during which cell culture facilities were available at the field site, this study had the added challenge of shipping samples out of state due to a lack of accessible cell culture facilities at the field site. Thus, in addition to establishing a technique, this project largely surveyed the ability to isolate satellite cells from muscle tissue 1-3 days following muscle biopsy procedures. Moreover, previous efforts in isolating Weddell seal primary cells only yielded success in one individual, therefore warranting methodological optimization for further success in primary cell culture of non-traditional species.

Materials and Methods

Study Site and Subjects

All elephant seal sampling was conducted under the NMFS Marine Mammal Permit #786-1463, and all procedures were approved by the Colorado State University Institutional Animal Care and Use Committee (IACUC # 11-3085A). Adult male elephant seals (*Mirounga angustirostris*) were sampled pre- and post-breeding season, (January and July, respectively), juvenile male elephant seals were sampled during the end of summer haul-out (September) and mid-molt (April-May), and four pups were sampled early in the post-weaning fast (PWF; pup weaning dates established when their mothers departed for sea); all sampling occurred at Año Nuevo State Reserve, CA. Animals were captured as previously described (Boaz et al., 2012); briefly, an initial dose of Telazol was administered via intramuscular (IM) injection and immobilization was maintained through subsequent ketamine injections into the extradural vein. Skeletal muscle biopsy sampling was performed using a local anesthetic and samples were taken from the mid-belly of the *longissimus dorsi* muscle using an established procedure (described

below). Following the biopsy procedure, animals were monitored until full voluntary locomotion was regained.

Muscle Biopsies

Following sterilization of the biopsy area with betadine and subsequent subcutaneous administration of local anesthesia (1% lidocaine), a small incision was made and muscle biopsies were taken. Two muscle samples, weighing approximately 50mg each, were collected from the primary swimming muscle, *m. longissimus dorsi*, using a 6-mm biopsy cannula (Depuy, Warsaw, IN, USA). Biopsies were quickly dipped in 100% ethanol for sterilization and subsequently placed into aerated tubes (to ensure adequate air circulation), where they were fully bathed in transfer cell culture media (detailed description below). In order to promote muscle (and satellite cells) viablity for the duration of the transit period (24-72 hours), tubes were placed in shipping coolers filled with thermal beads and ceramic beads (Bioexpress Kaysville, UT, USA) supplemented with ice packs, so as to keep tissue cool but also prevent freezing and subsequent fracture of cellular membranes.

Cell Culture Transfer Media

Due to the nature of our cell culture facility location relative to the field site, windy conditions on a sandy beach, and the overall unique physiology of these wild animals, several cell culture media mixtures were tested over the duration of the field season. Regarding shipping logistics, muscle biopsies needed to be kept alive over the duration of shipping (24-72 hours post-biopsy); accordingly, biopsies were placed in a nutrient mixture, Ham's F-10 (Life Technologies, Grand Island, NY, USA). Given the high reliance on a lipid-based metabolism in

elephant seals, half of the transfer media was supplemented with 0.5% lipid mixture, which contained the following fatty acids: 2mg/L arachidonic acid and 10mg/L each of linoleic, linolenic, myristic, oleic, palmitic, palmitoleic, and stearic acids (Life Technologies). While our initial field season included 1% antibiotic (penicillin/streptomycin, Sigma-Aldrich, St. Louis, MO, USA) into the transfer media, it did not incorporate antimycotics; all subsequent field trips, alternatively, incorporated 1% antibiotic/antimycotic mixture, which contained 25 µg/mL of Fungizone and 10,000 units/mL each of penicillin and streptomycin (Life Technologies).

Satellite Cell Isolation

Upon arrival to our cell culture facilities at Colorado State University, biopsies underwent one of two tested procedures for isolating satellite cells: enzyme digestion ("enzymatic method") or enzyme digestion plus mechanical teasing ("fiber separation method"). Biopsies being processed via the enzymatic method were treated with 250 µl of an enzyme cocktail containing collagenase-D, dispase, $CaCl_2$ (Boehringer Mannheim Corp., Indianapolis, IN, USA) on a 60mm dish, and placed in an incubator (37°C, 5% CO_2), where they were gently agitated every 15 minutes for an hour. Cells were then centrifuged at 400 x g at 4°C. Cells were then re-suspended in the F-10 based growth media (20% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA, USA) , 1% antibiotic/antimycotic mixture, 0.5% chicken embryo extract (GemBio, Sacramento, CA, USA), and 0.5% lipid mixture, brought up in Ham's F-10), and triturated twice before the suspension was filtered through a 40 µm filter. Cells were then centrifuged at 1000 x g at 4°C for 10 minutes, re-suspended in the aforementioned F-10 based growth media, and plated on Matrigel-coated (BD Biosciences, Bedford, MA, USA) six-well plates in an incubator (37°C, 5% CO_2) overnight.

Biopsies being processed via the fiber separation method were treated with 250 µl of an enzyme cocktail containing collagenase-D, dispase, $CaCl_2$ on a 60mm dish, where fiber bundles were teased apart with straight-edged forceps for up to 3 minutes. Ham's F-10 based growth media was added to the samples (same as used in "enzymatic protocol"), which were then placed in an incubator (37°C, 5% CO_2) for 24 hours. Samples were then placed on a shaking table in an incubator (37°C) for 15 minutes, and filtered through a 40 µm filter. Lastly, samples were centrifuged at 1000 x g for 10 minutes, re-suspended in the abovementioned Ham's F-10 based growth media, and plated in Matrigel-coated six-well plates. Cells were visualized using a Zeiss Invertoskop 40C brightfield microscope (Carl Zeiss, Oberkochen, Germany).

Myoblast Proliferation, Differentiation, and Freezing Down

Cells derived from biopsies were proliferated on p150 plates coated in 1% gelatin (Sigma-Alrich). Growth media was adjusted from standard myoblast growth medias to better match seal diet in vivo (20% FBS , 1% antibiotic/antimycotic, 2.5% lipid, 1% sodium pyruvate (Life Technologies), and 0.5% chicken embryo extract (GemBio) brought up in low-glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich)) and passaged onto new plates at 40-60% confluence. Cells were not passaged with trypsin; instead, PBS was used and cells were gently scraped off the plate with a rubber policeman.

Following proliferation, most cells were frozen down in DMEM + 10% dimethyl sulfoxide (DMSO, a cryoprotectant to prevent ice formation and subsequent cell death) for future cell culture experiments. These cells were initially placed into -80°C in isopropanol storage containers, conducive to a slow freeze without ice crystal formation, and then after 24hr were moved into -150°C liquid nitrogen storage for future use. A small subset of the primary cells,

however, was differentiated into myotubes for muscle cell verification. Six plates of cells derived from adult males were differentiated at 80-99% confluence in HIT media (10% equine serum, 10 μg insulin, 10 μg transferrin in low glucose DMEM) for seven days. Three of the plates from each animal were differentiated in normoxic conditions, and three were differentiated in hypoxic (0.5% oxygen) conditions in a humidified hypoxic environmental chamber (Coy Laboratory Products Inc., Grass Lake, MI, USA). DAPI (1 μg/ml, incubated in PBS) was used to stain nuclei and obtain live-cell images from hypoxic differentiated multinucleated myotubes.

Results and Discussion

Satellite Cell Viability post Transport

Due to the remote location of the field site without immediate access to cell culture facilities, muscle biopsies had to be shipped overnight to our cell culture facilities at Colorado State University. Depending on shipping logistics (e.g. shipping versus non-shipping days), samples took anywhere from 24-72 hours to arrive to our lab in Colorado; we necessarily had to provide a temporary environment for the tissue samples that was conducive to keeping the satellite cells alive during that time. Accordingly, we bathed the biopsies in a nutrient-rich media contained within aerated tubes and, to the best of our ability using thermal beads and ice packs, maintained the samples in a cool environment, thus avoiding heat-induced necrosis or alternatively ice crystal-induced membrane-rupture. Shipped samples across all field trips yielded viable satellite cells and exhibited no visible signs of necrosis or freeze fracture (Figure S1.1), even biopsies that were not processed for 72 hours post-biopsy, demonstrating our success in this biopsy transportation method.

Despite the fact that all field trips yielded viable satellite cells, our pilot field trip did not produce myoblasts due to contamination issues. To reiterate, in addition to being collected from wild animals, these samples were collected on windy beach, whereby sand frequently integrated itself into the muscle biopsies, which posed additional challenges to sterility. Thus, all subsequent trips incorporated both antibiotics and antimycotics, consequently eliminating virtually all contamination issues associated with sample collection and shipment.

Proliferation of Myoblasts

After overcoming contamination issues from our pilot field study, all subsequent field trips yielded myoblasts in cell culture. At the commencement of breeding season, we sampled 9 adult animals (8 males and 1 female). From these animals, we isolated viable myoblasts from each of the individuals and subsequently froze down cells for future cell culturing experiments. With no appreciable difference in cell viability between F10 and F10 + 0.5% lipid medias, we decided to transfer cells exclusively in F10 (with antibiotics/antimycotics) for all subsequent field trips. By the end of our field season, we were able to successfully isolate myoblasts from 21 pups, 18 juveniles, and 21 adults (see Figure S1.2 for representative myoblast images). Over the duration of the field season, we started exclusively using the enzymatic method, as it generally resulted in greater myoblast yield in comparison to the fiber separation method.

Despite having success in myoblast proliferation in the vast majority of these wild animals, a significant portion of cell proliferation was spent troubleshooting. Specifically, all samples had an abundance of fibroblasts (Figure S1.3). In an effort to purify cultures for myoblasts and thus eliminate fibroblasts, various modifications of preplating techniques were employed when splitting cell culture plates. Briefly, passaged cells were plated onto gelatin-

coated plates with our lipid-supplemented growth media and allowed to settle for varying time increments (8 minutes, 15 minutes, or 30 minutes). Then, non-adherent cells in the suspension, presumed to be myoblasts, were removed and plated onto new plates (Jankowski et al., 2001). This preplating process, however, did not improve the myoblast purity of our cultures as we had hoped. As such, we are currently employing magnetic beads and muscle-specific antibodies (i.e. anti-desmin) to eliminate fibroblasts.

Re-growing Primary Cells from Frozen Stock

In addition to isolating and proliferating these unique primary cells, another important apsect of the present study was to grow and subsequently differntiate primary cells from frozen stock. The ability to freeze cells without compromising cell integrity and subsequently killing the cell is an important feature in maintaining cell culture experiments over time, particularly when working with rare samples, such as those collected from protected wild animals. Accordingly, primary myoblasts frozen down from an adult male were unfrozen and re-plated after three months of cryostorage. These cells were not only successfully re-grown, but successfully differentiated into multinucleated myotubes (Figure S1.4); thus, in addition to verifying that we had primary muscle cells capable of being induced into terminal differentiation, we have also verified our ability to successfully freeze down and subsequently re-grow our primary cells.

Finalized Method for Elephant Seal Primary Cell Isolation

Given our overall success in northern elephant seal cell culture over a one year field season, we have devised a finalized protocol for isolating unique satellite cells from a remote field location as follows:

- Using forceps, quickly dip freshly excised muscle into 100% ethanol and then move biopsy to aerated tube filled with enough transfer media (Ham's F-10 with 1% antibiotic/antimycotic) to completely bathe the sample.
- Store biopsy tubes upright in shipping cooler filled with thermal beads and ice packs. To avoid freezing, be sure to keep tubes out of direct contact with ice pack; ship overnight to cell culture facility (cells can remain viable up to 72 hours in this setting).
- Upon arrival to cell culture facility, place muscle biopsy on culture dish.
- Add enough F10-based growth media to keep sample moist (250-500 microliters).
- Under a dissecting microscope, remove as much tendon, fat, vessels, and connective tissue as possible.
- Cut biopsy with forceps in culture dish (do not mince – fragments that are too small have less effective results with mechanical trituration that follows digestion).
- Move dish and sample into the cell culture hood (to maintain sterility).
- Add 250µl of collagenase/dispase/$CaCl_2$ to tissue
- Transfer minced tissue to sterile tube and incubate at 37^oC for 60 min (until mixture is a fine slurry).
- During those 60 mins, gently swirl tube every 15 min.
- In the hood, transfer the tissue and media to a 15 ml conical tube.

- Spin down via low-speed centrifugation at 400x g for 5 minutes.
- Remove supernatant and resuspend pellet (in same tube) in 5 ml of F10-based growth media.
- First trituration: pass muscle fragments through 10ml glass pipette until tissue bits pass easily through tip.
- Allow suspension to settle in 15ml conical tube so that remaining larger bits separate from the supernatant that contains the released cells.
- Collect supernatant and transfer to new 15ml conical tube.
- Second Trituration: add 5ml of F10-based growth media to 15 ml conical tube containing the remaining muscle pieces and replace the muscle trituration process
- Allow second trituration to settle and collect supernatant in same 15ml conical tube with first trituration's supernatant.
- Place a 40micrometer cell strainer onto a 50ml conical tube
- Using a 10ml pipette, transfer the pooled supernatants from the two triturations to the 40 micrometer cell strainer (*Carefully tap the side to reduce residual large debris from cell suspension)
- Add an additional 1ml F10-based growth media to drip through strainer to recover residual cells trapped by debris in the strainer
- Centrifuge strained cells at 1000x g for 10 min
- Carefully discard supernatant and resuspend pellet in 2 ml of F-10 based growth media + 0.5% lipid (pre-warmed to 37^{o}C)
- Plate cells on matrigel-coated plates.
- Check on cells in 24 hours.

- Following initial appearance of myoblasts, cells should be proliferated and differentiated in a low glucose-based growth media (20% FBS , 1% antibiotic/antimycotic, 2.5% lipid, and 1% sodium pyruvate brought up in low glucose DMEM) or differentiation media (10% equine serum, 2.5% lipid mixture, 10 μg insulin, 10 μg transferrin in low glucose DMEM), respectively.
- When splitting cells during proliferation phase, avoid use of trypsin, which may compromise these delicate primary cells; instead, use PBS concomitant with gentle rubber policeman scraping to remove cells from plates.

In summary, the present study offers a verified technique on shipping and subsequently isolating primary cells from a unique and remotely-located wild animal population. Unlike our lab's previous attempt to isolate primary myoblasts from Weddell seals, which only yielded success from one individual, here we were able to successfully isolate primary myoblasts from multiple elephant seals over all age classes and both sexes.

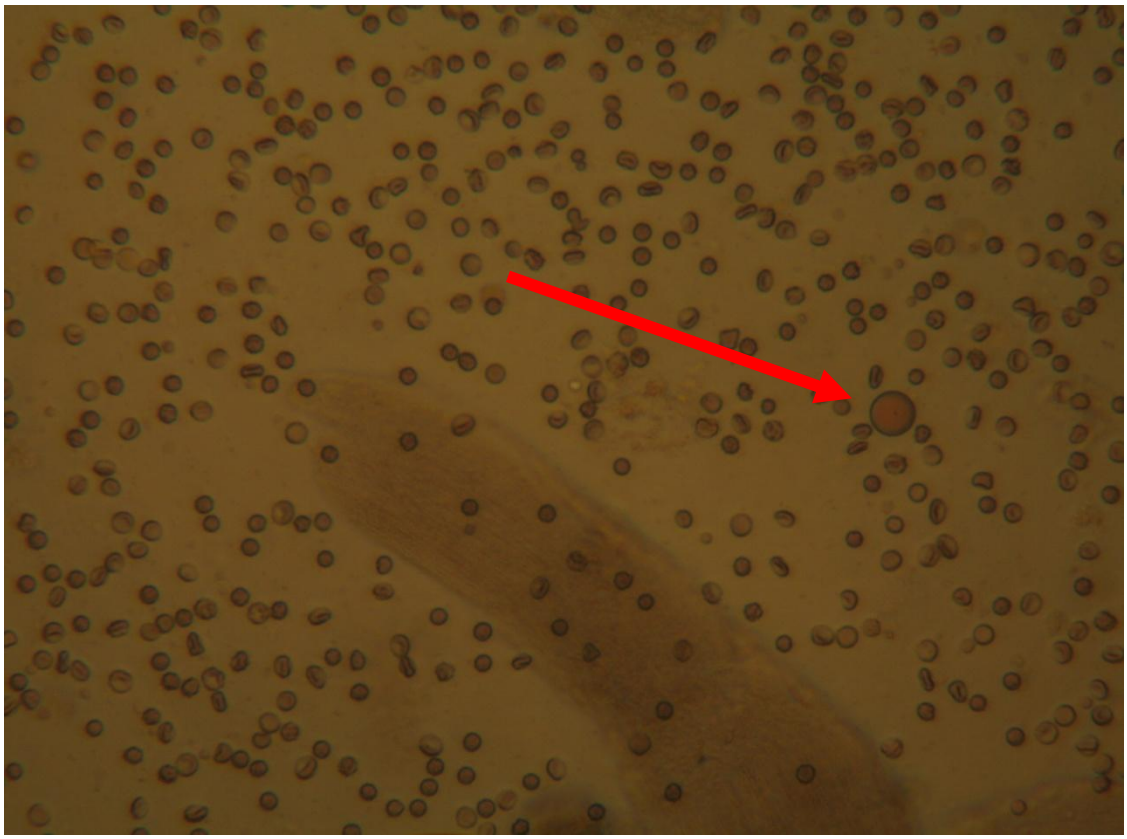

Figure S1.1. Successfully isolated northern elephant seal satellite cell. High magnification (320x) image of a juvenile male elephant seal's satellite cell (as denoted by the arrow) isolated from a biopsy of the primary swimming muscle, longissimus dorsi. This image was taken from our pilot field trip, demonstrating our initial success in shipping viable cells overnight (note: small cells abundantly present are red blood cells and the large fragment in the background is a piece of skeletal muscle).

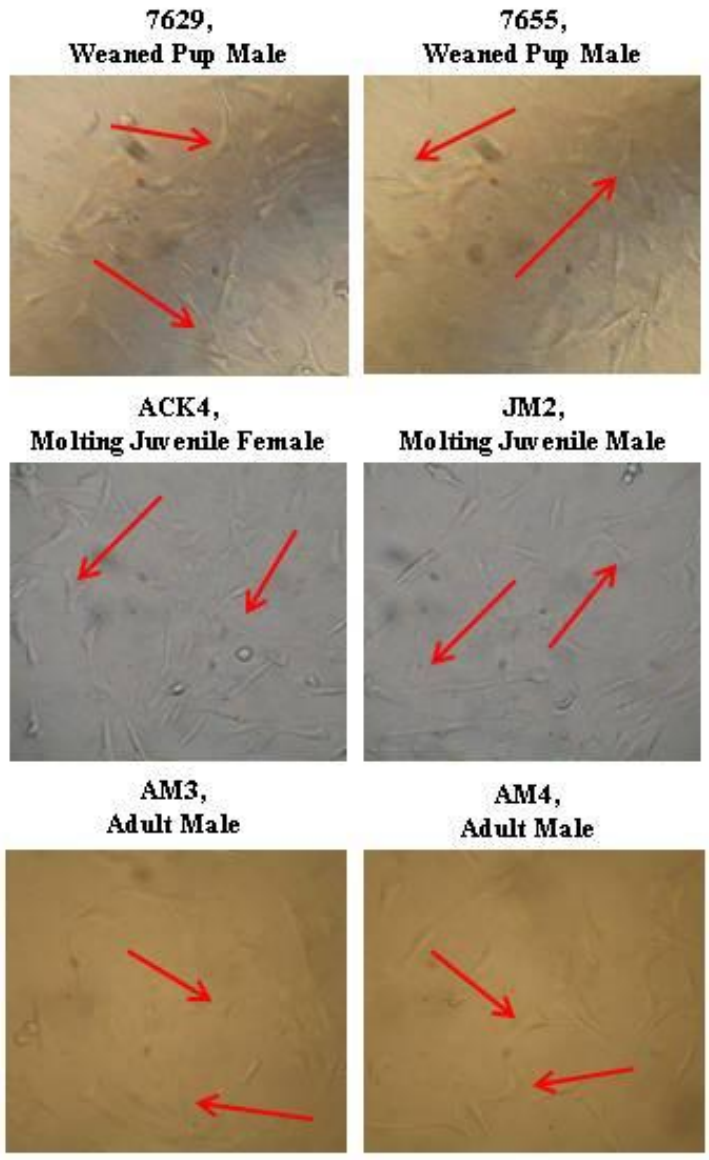

Figure S1.2. Images of primary myoblasts obtained from biopsies of the swimming muscles of northern elephant seals. Select myoblasts isolated from each age class are identified in each image by red arrows. The myoblasts can be distinguished from the fibroblasts by their distinct trianugular shape. After our first attempt in September 2012, we have been able to successfully isolate myoblasts from every animal and subsequently have frozen stock from all age classes and both sexes.

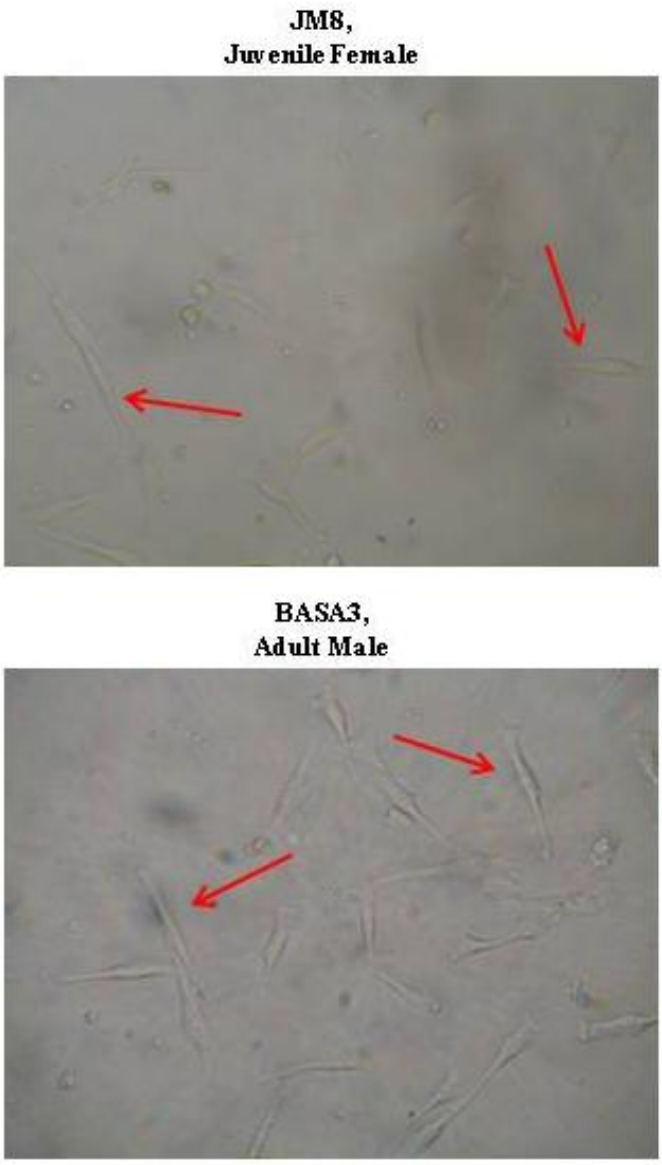

Figure S1.3. Images of fibroblasts persisting amongst myoblasts. Select fibroblasts are identified by red arrows (distinct from triangular-shaped myoblasts). Despite pre-plating techniques, fibroblasts continue to persist in culture; currently a magnetic bead system is being employed to immunoprecipitate myoblasts exclusively away from fibroblasts.

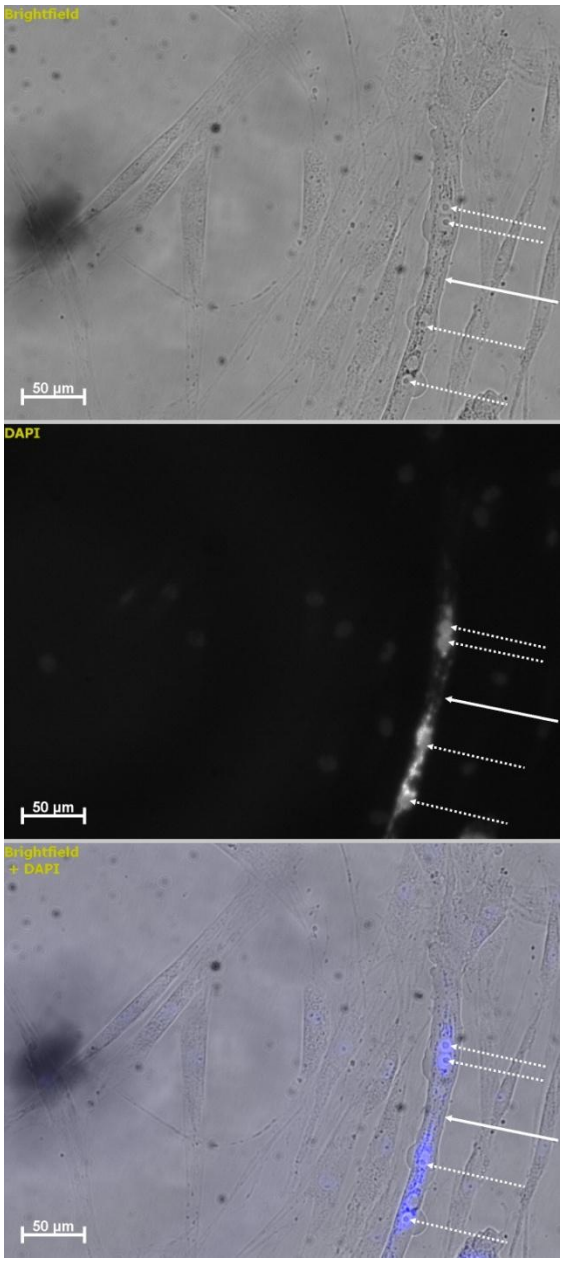

Figure S1.4. Composite image of a 7-day myotube from the nothern elephant seal. This composite image shows northern elephant seal primary cells following seven days of differentiation into a multinucleated myotube. The solid arrow points out one of the multinucleated myotubes in the frame, while dashed arrows point to the nuclei. The top panel is a brightfield image, the middle panel is the DAPI-stained nuclei, and the lower panel is an overlay of the DAPI stained nuclei on the brightfield image.

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SUPPLEMENTAL RESEARCH 2

High fatty acid oxidation capacity and phosphorylation control despite elevated leak and reduced respiratory capacity in northern elephant seal muscle mitochondria[4]

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Summary

Northern elephant seals (*Mirounga angustirostris*) are extreme, hypoxia-adapted endotherms that largely rely on aerobic metabolism during extended breath-hold dives in water temperatures near 2°C. While many aspects of their physiology have been characterized to account for this seemingly paradoxical phenomenon, information pertaining to aerobic respiration within the actual aerobic powerhouse of the cell, the mitochondria, remains elusive. The present study, therefore, investigated the ontogeny and comparative physiology of elephant seal muscle mitochondria using high-resolution respirometry. Intact mitochondrial networks were isolated from primary swimming muscle biopsies taken across pup, juvenile, and adult age classes and compared to human leg muscle mitochondria. Using a variety of substrate conditions and respiratory states, we demonstrated that adult elephant seal mitochondria maintain a high capacity for fatty acid oxidation and enhanced respiratory control, despite having a lower overall respiratory capacity, as compared to pups and humans. Interestingly, adult and juvenile elephant seal mitochondria exhibited ~50% greater capacity for respiratory leak. To our knowledge, this is the first report of a seemingly paradoxical elevation in both phosphorylation efficiency *and* respiratory leak capacity in mammalian muscle mitochondria, two phenomena previously believed to be mutually exclusive. Given the known reliance on aerobic respiration during bouts of prolonged breath-hold exercise, in addition to exposure to highly variable ambient temperatures, this unique mitochondrial functional phenotype matches the unique life history of elephant seals. Collectively, these data highlight the plasticity of mitochondrial respirometry function and reflect adaptations conducive to efficient aerobic ATP production and endothermy maintenance in an extreme, oxygen-limited environment.

Introduction

Muscle mitochondria are functionally dynamic organelles capable of responding to bioenergetic demands by regulating the capacity and efficiency of oxidative phosphorylation and metabolic substrate utilization. In addition, imperfect coupling of mitochondrial electron transport to ADP phosphorylation results in oxygen consumption in the absence of ATP production, reflecting a dissipation of mitochondrial membrane potential independent of proton flux through the ATP synthase. In endotherms, this respiratory "leak" is thought to contribute significantly to metabolic heat production at the expense of phosphorylation efficiency (van den Berg et al. 2011). However, the extent to which modulation of these processes contributes to phenotypic adjustments of mammalian muscle at the organismal and evolutionary levels remains largely speculative due to a paucity of studies evaluating mitochondrial physiology in species uniquely adapted to extreme bioenergetic stress and environmental conditions.

Diving mammals exhibit the remarkable capacity for extended periods of exercise (foraging) under apneic conditions at a variety of depths and water temperatures. Prominent among these impressive feats is that of the Northern Elephant Seal (*Mirounga angustirostris;* E-seal), which can dive for up to 2 hours without resurfacing for air, reaching depths of over 2,000 meters in water temperatures near 2°C (Robinson et al., 2012). Remarkably, E-seals are thought to rely almost exclusively on aerobic metabolism of lipids to meet energy demands during dives, despite the steady decline in O_2 availability (Hindell et al., 1992, Le Boeuf et al., 1988; Le Boeuf et al., 1996; Meir et al., 2009). This is possible due to a number of physiological adjustments that maintain supply of substrate and O_2 to muscle mitochondria in the absence of exogenous O2 supply (Meir et al., 2009; Ponganis et al., 2003; Thorson and Le Boeuf, 1994), and high biomechanical efficiency ("gliding") to reduce energy demands during dives (Williams et al.,

2000). Paradoxically, muscle mitochondrial content in diving mammals, assessed by electron microscopy and marker enzyme content in Weddell seals, actually decreases as seals reach maturity despite evidence for improvements in aerobic exercise capacity to sustain longer and deeper dives in adulthood (Kanatous et al., 2008). The mechanism of this response and potential involvement of adaptive changes in mitochondrial respiratory function in diving mammal phenotype are entirely unknown.

The purpose of this investigation was to evaluate the ontogeny and comparative physiology of mitochondrial respiration in E-seal skeletal muscle. We have employed high-resolution respiromety methods under a variety of substrate conditions and respiratory states to examine differences in respiratory capacity, leak, substrate utilization, and phosphorylation control between adult E-seal and human muscle mitochondria, and adaptations that occur as E-seals reach maturity. These studies are the first to comprehensively investigate muscle mitochondrial respiratory function in a diving mammal, and reveal novel adaptive changes that compliment known aspects of the E-seal phenotype and highlight the remarkable plasticity of mitochondrial respiratory function to bioenergetic and thermoregulatory stress.

Results

Comparison of adult human and E-seal muscle mitochondrial function

Mitochondrial respiratory function was determined in permeabilized muscle fiber bundles obtained from the latissimus dorsi of adult male Northern Elephant Seals (E-seals, n = 7) and vastus laterals of adult male humans (aged 26 ± 3 years; n = 4) by high resolution respirometry using an Oxygraph-O2k high resolution respirometer (Oroboros Instruments, Innsbruck, Austria). Human and animal subject characteristics are presented in Table 1. Two respiration

protocols were run in duplicate to evaluate mass-specific oxygen (O_2) flux rates in response to sequential (additive) administration of various respiratory substrates, inhibitors of complex 1 (rotenone; Rot) and/or ATP synthase (oligomycin; Omy), or the uncoupling protonophore carbonylcyanide p-trifluoromethoxy-phyenylhydrazone (FCCP). These protocols provide a comprehensive evaluation of muscle respiratory capacity and substrate control during oxidative phosphorylation (OXPHOS; P state), as well as the extent of uncoupled "leak" respiration (L state) and the enzymatic capacity of the electron transport system (ETS; E state). A detailed description of the respiration protocols and associated respiratory states generated by the sequential titration of each constituent are provided in Table S2.1.

Protocol 1 - *Adult Human vs. E-seal comparison*

Protocol 1 examined various respiratory states of muscle mitochondria from humans (n = 4) and adult E-seals (n = 7) in the presence of fatty acid (palmitoyl-carnitine; PalM). Fig. 1A illustrates the mass-corrected O_2 flux rates from human and E-seal muscle following the sequential addition of each protocol constituent corresponding to the respiratory states described in Table S2.1. Across all respiratory states, E-seal muscle exhibited O_2 flux rates that were 25-40% lower than values obtained from human vastus lateralis, indicating a lower respiratory capacity of E-seal vs. human muscle per mg wet weight. The greatest relative difference in flux between humans and seals was during "leak" respiration following addition of PalM in the absence of adenylates (L_N), which was approximately 40% lower in E-seals vs. human muscle ($P = 0.04$). As expected, adding a saturating concentration of ADP increased respiratory flux 5-6 fold in both species, reflecting the maximal OXPHOS capacity using fatty acids as the source of electrons for the respiratory system (P_{ETF}). The relative difference in flux between humans and

seals decreased to 25% in the P_{ETF} state, which corresponded to a 26% higher PalM respiratory control ratio (RCR) in seals vs. humans (P_{ETF}/L_N; Fig. S2.1B; $P < 0.05$). This indicates a greater control of fatty acid oxidation capacity by ADP (the phosphorylation system) in E-seal vs. human muscle.

Upon adding pyruvate, respiratory flux increased 82% in humans and 50% in seals, demonstrating an expected additive effect of pyruvate oxidation on OXPHOS capacity in the presence of fatty acids in both species. The greater relative stimulation of respiration by pyruvate in humans restored the relative difference in O_2 flux between species to ~40% ($P < 0.001$), indicating a much greater responsiveness of human mitochondria to pyruvate. This also equated to a 21% greater capacity to utilize fatty acids over pyruvate in seal vs. human mitochondria when provided both substrates at saturating concentrations (Pal/Pyr OXPHOS flux ratio, Fig. S2.1C; $P = 0.04$). Subsequent addition of glutamate increased respiratory flux to similar relative extents in both species, indicating a comparable limitation of maximal respiratory flux through complex 1 by pyruvate and lipid oxidation pathways in both humans and seal muscle (Gnaiger 2009).

Respiratory responses to the addition of cytochrome c (Cyt *c*) were negligible in both human and seal fibers, confirming the preservation of mitochondrial outer membrane integrity in the samples used in our studies. Subsequent addition of the protonophore FCCP generates the fully uncoupled respiratory rate (E_{CI}), which increased flux ~27% in both human and seal fibers, demonstrating similar restraints of maximal respiratory capacity by the phosphorylation system in both species. Expressing the maximal CI-supported OXPHOS rate (P_{CI}) relative to the fully uncoupled ETS rate (E_{CI}) reveals the extent to which maximal OXPHOS utilizes the full

enzymatic capacity of the ETS (P/E_{CI}, Fig. 1D). This was nearly identical seals and humans, indicating both species operate at ~80% of ETS capacity during maximal OXPHOS.

Protocol 2- ***Adult Human vs. E-seal comparison***

Protocol 2 provides an assessment of L_N and OXPHOS rates using pyruvate (+ malate) alone (P_{Pyr}), maximal OXPHOS rates through respiratory complex I (P_{CI}), complex II (P_{CII}) and combined CI+CII substrates ("total" OXPHOS capacity; P), as well as CII-linked Omy-induced "leak" respiration in the presence of high adenylates (L_{Omy}). Similar to data from Protocol 1, E-seal muscle exhibited mass-corrected O_2 flux rates that were 30-58% lower than those from human muscle in the presence of complex 1 and complex II substrates (Fig. S2.2A). Relative differences in OXPHOS flux rates were generally consistent (52-58%) and highly significant ($P < 0.001$) under all substrate conditions, confirming the lower respiratory capacity of seal vs. human muscle indicated by Protocol 1. The respiratory control ratio (RCR) using pyruvate + malate as substrates (P/L_{Pyr}; Fig. S2.2B) was similar in seals vs. humans (12% higher in seals; P = NS), indicating similar control pyruvate oxidation capacity by ADP. The addition of glutamate increased respiratory flux to similar extents in both species, as seen in Protocol 1. As expected, flux increased further and to similar extents in both species upon adding succinate, supplying additional electrons to the ETS through complex II, thereby fully reconstituting the supply of reducing equivalents from the tricarboxylic acid (TCA) cycle to the ETS (P) (Gnaiger 2009, Pesta and Gnaiger 2012). As in Protocol 1, responses to the subsequent addition of Cyt c were negligible in both human and seal fibers, indicating structurally sound mitochondria in our experiments.

To evaluate the relative contribution of complex II to total respiratory flux, the complex I inhibitor rotenone was added next, generating the maximal complex II-linked OXPHOS rate (P_{CII}). Expression of this rate as a percent of the total complex I+II OXPHOS rate (P_{CII} /P) revealed an 8% higher relative contribution of complex II in seals vs. humans (P = 0.02; Fig. 2C). Subsequent addition of oligomycin (Omy) blocks proton flux through the ATP synthase, thereby revealing the maximal rate of respiratory leak in the presence of high concentrations of adenylates (L_{Omy}) (Pesta and Gnaiger 2012). This state is contrasted with the respiratory leak state obtained in the absence of adenylates at the beginning of the respiration protocols (L_N). The addition of Omy inhibited flux in humans by 63%, indicating that respiratory "leak" represents ~36% of respiratory flux seen during maximal CII-linked OXPHOS (L_{Omy}/P_{CII}; Fig. S2.2D). Interestingly, the inhibitory effect of Omy was only 46% in seals, which equates to a 50% higher relative leak rate in seals vs. humans in the presence of adenylates (L_{Omy}/P_{CII}; Fig. 2D) (P < 0.001). Subsequent addition of FCCP releases the restraint of respiration by the high mitochondrial membrane potential ($\Delta\Psi_M$), generating the maximal CII-linked ETS capacity, which restored O_2 flux to near the previous P_{CII} rate.

Ontogeny of E-Seal muscle mitochondrial function

To evaluate the effect of ontogeny on the mitochondrial phenotype of E-seals, we compared mitochondrial respiration data from adult male E-seals (n = 7) to those obtained from male seal pups (n = 4) and juveniles (n = 6) using the same HRR protocols (Fig. S2.3 and S2.4).

Protocol 1- *E-seal ontogeny*

When comparing mass-corrected O_2 flux rates across the three stages of development, no statistically significant differences were found for any of the respiratory states assessed in

protocol 1 (Figure S2.3A), indicating generally similar muscle mitochondrial functional capacity in the presence of saturating concentrations of fatty acid. However, trends for a progressively lower "leak" (L_N) respiration and higher OXPHOS (P_{ETF}) rates as animals develop from pups to adulthood equated to highly significant increases in the lipid RCR (P_{ETF}/L_N), indicating a progressively greater control of fatty acid oxidation by ADP as animals develop from pups to diving adults (Fig. S2.3B; $P < 0.01$ for all group comparisons). The addition of pyruvate tended to increase flux to a greater extent in pups vs. juveniles and adults, which equated to a much higher relative contribution of fatty acid vs. pyruvate oxidation to OXPHOS flux in the juveniles and adults vs. pups (Fig. S2.3C; $P < 0.01$). The calculated P/E_{CI} was significantly higher in adults compared to juveniles and pups (Fig. S2.3D; $P < 0.05$) indicating a greater utilization of ETS capacity during maximal OXPHOS in adults vs. juveniles and pups. Respiratory responses to the addition of cytochrome c (Cyt c) were <10% in all samples used in our analyses, confirming the preservation of mitochondrial outer membrane integrity in the samples used in our studies.

Protocol 2- *E-seal ontogeny*

In contrast to data obtained in the presence of fatty acid in Protocol 1, mass-corrected O_2 flux rates were significantly lower in adult seals compared to both pups and juveniles under all respiratory states evaluated in Protocol 2 using complex I and complex II substrates in the absence of fatty acid (Fig. S2.4A). No significant differences were seen in the pyruvate RCR (P_{Pyr}/L_N), indicating similar control of pyruvate oxidation by ADP across ontogeny (Fig. 4S2.B). Differences among groups became more apparent with the sequential addition of glutamate and succinate, leading to a strong trend for a progressive reduction in mass-corrected OXPHOS

capacity as seals develop from pups to adulthood. Group differences in P_{CII} rates were congruent with the preceding complex I+II rates, which equated to a nearly identical contribution of CII to total OXPHOS capacity across ontogeny (P_{CII}/P; Fig. S2.4C). However, L_{Omy} normalized to the OXPHOS or ETS capacities were markedly higher in juveniles and adults compared to pups (Fig. S2.4D; $P < 0.01$), with no significant difference between juveniles and adults. As in Protocol 1, respiratory responses to the addition of cytochrome c (Cyt c) were negligible in all samples used in our analyses, confirming the integrity of the samples used in our studies.

Discussion

Northern elephant seals possess the remarkable ability to maintain aerobic metabolism for up to two hours of apnea while foraging in cold water at depths of over 500 meters (Robinson et al., 2012). Several biochemical, physiological and biomechanical adaptations are known to facilitate these impressive feats by enhancing muscle O_2 storage, diffusion and metabolic efficiency (see (Davis 2013) for review), but the present study is the first to comprehensively evaluate muscle mitochondrial respiratory function in a diving mammal. Our studies indicate that adult E-seal muscle maintains a high capacity for fatty acid oxidation and enhanced respiratory control despite a lower mass-corrected respiratory capacity compared to humans and seal pups naïve to diving. The ontogeny of this phenotype indicates that it is an adaptive response to the diving lifestyle rather than an intrinsic property of E-seal muscle mitochondria. Interestingly, adult and juvenile seal mitochondria also exhibit a ~50% greater capacity for respiratory leak in the presence of high substrate and adenylate concentrations, which also appears to be an adaptive response to the diving lifestyle. To our knowledge, this is the first report of a seemingly paradoxical elevation in both phosphorylation efficiency and respiratory

leak capacity in mammalian muscle mitochondria, highlighting the plasticity of mitochondrial respiratory function to meet the unique bioenergetic and thermoregulatory demands of the diving mammal phenotype.

Mass-corrected OXPHOS rates from adult E-seal muscle were 25-58% lower than rates obtained from human vastus lateralis depending on the substrates used. This is consistent with a lower respiratory capacity of adult E-seal vs. human muscle per unit mass, as would be predicted by the well-established inverse relationship between muscle mitochondrial volume density and body mass across mammalian species (Dobson and Headrick, 1995; Mathieu et al., 1981). However, the highly variable relative differences in respiratory flux between E-seals and humans highlights the importance of considering substrate utilization and multiple respiratory states when evaluating adaptive changes in muscle aerobic capacity. Previous studies indicate that diving mammals rely almost exclusively on lipid metabolism for muscle ATP production (Crocker et al., 2014; Houser et al., 2013; Kanatous et al., 2008; Le Boeuf and Laws, 1994; Trumble et al., 2010; Trumble and Kanatous, 2012). Therefore, it was not surprising that compared to humans, adult E-seal mitochondria exhibited a greater capacity to oxidize lipid versus carbohydrate substrates (Fig S2.1C). Moreover, maximal OXPHOS capacity of E-seal muscle with palmitoylcarnitine + malate (P_{ETF}) was 75% of rates seen in human muscle, whereas "total" OXPHOS capacity supported by maximal delivery of reducing equivalents from the TCA cycle (P) was only 43% of rates seen in human muscle. Fatty acid-supported respiratory flux is limited by electron delivery by the ETF, which obtains reducing equivalents from flavin adenine nucleotide in the acyl-CoA dehydrogenase reaction of the beta-oxidation cycle (Pesta and Gnaiger 2012). Therefore, E-seal mitochondria likely maintain comparatively high lipid

OXPHOS capacity by a selective increase in the transport, oxidation and/or delivery of reducing equivalents derived from fatty acids to the respiratory system.

A previous study in Weddell seals demonstrated a paradoxical decrease in muscle mitochondrial volume density as seals reached maturity despite evidence for improved aerobic exercise capacity to sustain longer and deeper dives in adulthood (Kanatous, Hawke et al. 2008). Consistent with this finding, we observed a progressive decrease in total mass-corrected OXPHOS capacity (P) as E-seals developed from pups to adults in the present study (Fig S2.4A). However, despite this apparent decline in overall muscle respiratory capacity, mass-specific rates of fatty acid-supported OXPHOS (P_{ETF}) tended to increase in juveniles and adults, along with highly significant increases in respiratory control (RCR) suggestive of greater OXPHOS coupling efficiency (Fig S2.3B). Improvements in the PalM RCR resulted primarily from increased OXPHOS flux rather than decreases in respiratory leak (L_N), suggesting an improvement in the control and/or capacity of the ADP phosphorylation system on respiratory flux in the presence of fatty acids. Consistent with this interpretation, a significantly higher CI-linked OXPHOS capacity was observed in adult e-seals compared to pups and juveniles when expressed as a percent of maximal (non-coupled) ETS capacity (P/E_{CI}) in Protocol 1 (Fig S2.3D). Taken together, these findings suggest that E-seal mitochondria become more effective at generating ATP from fatty acids as they mature, which overcomes a decline in total mass-corrected respiratory capacity as body mass increases, ultimately improving muscle aerobic capacity for longer and deeper dives in adulthood. The molecular basis of this adaptation will require further investigation, but might involve a selective increase in content or coupling of phosphorylation system components (e.g., ATP synthase and the adenine nucleotide translocase;

ANT) in conjunction with an upregulation of fatty acid oxidation enzymes and/or ETF in muscle mitochondria.

Enhanced OXPHOS efficiency is typically defined by reduced rates of leak respiration, improved phosphorylation control of respiratory flux (RCR), and/or a decrease in the amount of O_2 required to phosphorylate a given amount of ADP during classic State 3 respiration (ADP/O ratio) (Gnaiger et al. 2000, Jacobs et al. 2012, Pesta and Gnaiger 2012). Accurate assessment of ADP/O is not feasible in permeabilized muscle fibers due to the presence of residual ATPases, therefore respiratory leak normalized to OXPHOS or ETS capacities under common substrate conditions is routinely used to assess respiratory coupling in permeabilized fibers (Gnaiger 2009, Pesta and Gnaiger 2012). As discussed above, RCR values based on OXPHOS and L_N rates obtained in the presence of PalM or pyruvate + malate were similar or higher in adult seals compared to humans and seal pups in this study. This is indicative of similar or improved respiratory control (P/L_N) and a preservation or reduction of its reciprocal index of respiratory leak (L_N/P) in adult seals. However, indices of respiratory leak based on L_{Omy} flux normalized to OXPHOS and ETS capacity were nearly 50% higher in juvenile and adult seals compared to pups and humans (Fig S2.2D and S2.4D). This seemingly paradoxical finding is perhaps the most intriguing aspect of the mature E-seal mitochondrial phenotype, the discussion of which highlights both the plasticity of mitochondrial respiratory function in response to bioenergetic and/or thermal stress and the key role these adaptations may play in the development of the diving mammal phenotype.

In both L_N and L_{Omy} leak states, mitochondrial membrane potential is high due to saturating concentrations of respiratory substrates in the absence of proton flux through the ATP synthase (Pesta and Gnaiger 2012). Therefore, respiration results from dissipation of the inner

membrane chemiosmotic gradient due to proton leak, slip and/or cation cycling across in the inner mitochondrial membrane. A primary mechanism of respiratory leak in skeletal muscle is increased proton conductance by uncoupling proteins (UCPs) and the adenine nucleotide translocase (ANT) (Parker et al. 2008), both of which may be activated by fatty acids, reactive oxygen species (ROS) or membrane lipid peroxidation products (Parker et al. 2008, Jastroch et al. 2010). Given the demonstrated preference of mature e-seal mitochondria for palmitoylcarnitine (versus pyruvate) compared to pups and humans (Fig S2.1C and S2.3C) and known reliance of E-seal on lipids for energy production (Crocker et al., 2014; Houser et al., 2013; Le Boeuf and Laws, 1994), activation of UCPs or the ANT by fatty acids might be expected to drive respiratory leak in the juvenile and adult seals. However, L_N in presence of PalM was not increased with ontogeny; rather, it decreased relative to OXPHOS rates, indicated by higher PalM RCR values in mature seals compared to pups and humans. Moreover, E-seal pups subsist exclusively on milk from lactating cows, which is even richer in lipid than the diet of foraging juvenile and adult seals (Crocker et al., 2014; Fowler et al., 2014). This argues strongly against a specific role for fatty acids in inducing the enhanced respiratory leak seen in mature e-seal muscle mitochondria. Indeed, elevated indices of respiratory leak in juveniles and adults were obtained during CII-linked L_{Omy} in the absence of fatty acids, suggesting the involvement of complex II, a higher rate electron delivery to ETS, and/or the presence of high adenylate concentrations.

Interestingly, non-shivering thermogenesis in cold-acclimatized ducklings (Cairina moschata) is associated with an upregulation of avian UCP and increased CII-linked oligomycin-induced respiratory leak (L_{Omy}) in skeletal muscle mitochondria, despite no change in the CII-linked RCR or efficiency of ATP production (Teulier et al. 2010). The striking similarity of

these findings to the respirometry data in the present study suggests that cold-acclimatization may play a role in enhanced leak that develops in juvenile and adult e-seals; perhaps via UCP activation. A thermogenic role of mitochondrial uncoupling in seal locomotor muscle was proposed by Grav et al. almost 35 years ago (Grav and Blix 1979), however the extent to which mature E-seals experience cold stress during dives given their thick insulating blubber layer is unclear. Notably, enhanced proton conductance by both UCP and ANT was linked to adaptive thermogenesis in skeletal muscle of cold-adapted penguins, but by different mechanisms of activation of UCPs (by ROS) and ANT (by fatty acids) (Talbot et al. 2004). Basal capacity of skeletal muscle to produce ROS is higher in diving vs. terrestrial mammals *in vitro* (Zenteno-Savin et al. 2002, Zenteno-Savin et al. 2010); however, this does not result in greater indices of oxidative damage *in vivo*, suggesting that ROS release might may a physiological role in adaptive responses to the diving mammal lifestyle (Zenteno-Savin, Clayton-Hernandez et al. 2002). Mitochondrial ROS release was not investigated in present study, but is it plausible that conditions encountered by E-seals during deep foraging dives promotes a physiological ROS release that facilitates an adaptive increase in the respiratory leak capacity of muscle mitochondria under conditions of high mitochondrial membrane potential.

Mitochondrial ROS generation is driven primarily by the reduced state of the respiratory complexes and the PO_2 of the local environment (Korshunov et al. 1997, Barja 2007), both of which are near maximal in the CII-linked L_{Omy} state. Indeed, CII substrates (succinate + rotenone) in combination with Omy are routinely employed to assess maximal ROS emission from isolated mitochondria (Starkov 2010). While CII-linked L_{Omy} is not a physiological respiratory state, it reflects the concomitantly high reducing pressure (high NADH/NAD+) and low phosphorylation pressure (high ATP/ADP) that occurs in skeletal muscle mitochondria with

chronic overnutrition (e.g.., a high fat diet) combined with a sedentary lifestyle. In humans and rodents, this drives a persistent elevation in mitochondrial ROS that induces UCP3 expression and respiratory uncoupling, but ultimately leads to excessive oxidative stress and the development of muscle insulin resistance (Hesselink et al. 2003, Fisher-Wellman and Neufer 2012). Conversely, a similar oversupply of fatty acid-derived reducing equivalents unmatched by ATP demand occurs transiently during recovery from aerobic exercise, leading to brief periods of mitochondrial ROS release (Anderson et al. 2007). This also triggers UCP3 activity/expression and induces respiratory uncoupling, which limits ROS emission during fatty-acid supported respiration and enhances fatty acid OXPHOS capacity in muscle mitochondria (Pilegaard et al. 2000, Anderson, Yamazaki et al. 2007, Fernstrom et al. 2007). During deep foraging dives, E-seals engage in brief bouts of stroking "exercise" to acquire prey separated by longer periods of "gliding" that reduce energy costs and facilitate longer and deeper dives (Davis et al. 2001, Aoki et al. 2011). Both activities are supported almost entirely by aerobic metabolism of fatty acids, made possible by high cellular stores of lipid and myoglobin-bound oxygen (Guyton et al., 1995; Kanatous et al., 2002; Noren et al., 2001; Ponganis et al., 1993; Snyder, 1983; Trumble et al., 2010; Williams et al. 2004). Such conditions would be expected to favor elevated ROS release and oxidative stress in the absence of adaptive increases in uncoupling and/or antioxidant defenses. Indeed, hypoxia-adapted seals have been characterized as having a suite of elevated antioxidant defenses as compared to non-hypoxia adapted, terrestrial controls (Murphy and Hochachka, 1981; Vázquez-Medina et al., 2006; Vázquez-Medina et al., 2010; Vázquez-Medina et al. 2011; Wilhelm Filho et al., 2002; Zenteno-Savín et al., 2002). Moreover, in addition to serving as an oxygen store, the abundance of myoglobin in the skeletal muscle can also function as an antioxidant defense by scavenging ROS (Flögel et al.

2004; Garry and Mammen, 2007). It is, nonetheless, intriguing to speculate that the greater leak rates of mature E-seal mitochondria observed in the CII-lined L_{Omy} state herein reflects an adaptive increase in the respiratory leak capacity that limits excessive ROS release and contributes to non-shivering thermogenesis during the gliding phases of deep dives through activation of UCPs and perhaps the ANT.

In summary, we provide the first comprehensive comparative and ontongenic evaluation of muscle mitochondrial function of a diving mammal. Our studies demonstrate maintenance of fatty acid oxidation capacity and improved OXPHOS efficiency in E-seal muscle despite a reduced overall mass-corrected respiratory capacity that develops as animals mature from pups to adults. A seemingly paradoxical increase in respiratory leak capacity under conditions of high membrane potential, substrate and adenylate concentration may serve to increase muscle thermogenesis and reduce generation of ROS during deep foraging dives. Future studies investigating the molecular underpinning of this remarkable E-seal mitochondrial phenotype may reveal novel insights into how mitochondrial plasticity contributes to diving mammal physiology, with potential relevance to the study of oxidative stress and metabolic disease in humans.

Materials and Methods

Animal Subjects.

All e-seal sampling was conducted under the NMFS Marine Mammal Permit #786-1463, and all procedures were approved by the Colorado State University Institutional Animal Care and Use Committee (IACUC # 11-3085A). Seven adult male e-seals (*Mirounga angustirostris*) were sampled post-breeding season, May-July, six juvenile male e-seals were sampled mid-molt,

April-May, and four pups were sampled early in the post-weaning fast (PWF; pup weaning dates established when their mothers departed for sea); all sampling occurred at Año Neuvo State Reserve, CA. Animals were captured as previously described (Boaz et al., 2012); briefly, an initial dose of Telazol was administered via intramuscular (IM) injection and immobilization was maintained through subsequent ketamine injections into the extradural vein. Muscle biopsy sampling was performed using a local anesthetic and samples were taken from the mid-belly of the muscle using an established procedure (described below). Following the biopsy procedure, animals were monitored until full voluntary locomotion was regained.

Human Subjects

Human subjects (n = 4) were sedentary or recreationally active males aged 26 ± 2 years without a history of regular tobacco use products or medications. Assessments of body mass index (kg/m^2), body composition (dual-energy x-ray absorptiometry using a DXA-IQ; Lunar Radiation Corp., Madison, WI), and peak oxygen consumption assessed during a graded exercise test (using a Parvo Medics Metabolic Cart as previously described (8)), were typical of young sedentary adults. Muscle biopsy sampling was performed by a trained technician using an established procedure (described below) that conformed to the standards set by the Declaration of Helsinki of 1975, as revised in 1983, and was approved by the Institutional Review Board at Colorado State University. The nature, purpose and risks of the procedure were explained to each subject before written informed consent was obtained.

Skeletal Muscle Sampling and Handling.

Human Subjects: Upon completion of health screening, research participants reported to the laboratory following a 12-hour fast and 24-hour abstention from vigorous exercise to provide skeletal muscle samples. The medial vastus lateralis muscle was sampled using the Bergström

technique under local anesthesia (1% lidocaine s.c.) and immediately placed into ice-cold biopsy preservation medium medium (BIOPS) containing (in mM) 10 Ca^{2+}-EGTA, 20 imidazole, 50 potassium-4-morpholinoethanesulfonic acid, 0.5 dithiothreitol, 6.56 $MgCl_2$, 5.77 ATP and 15 phosphocreatine at pH 7.1 (6) until processing for mitochondrial respiration experiments.

Elephant Seals: Following sterilization of the biopsy area with betadine and subsequent subcutaneous administration of local anesthesia (1% lidocaine), a small incision was made and muscle biopsies were taken. Three muscle samples, weighing approximately 50mg each, were collected from the primary swimming muscle, m. longissimus dorsi, using a 6-mm biopsy cannula (Depuy, Warsaw, IN, USA). Biopsies were immediately placed into ice-cold biopsy preservation medium medium (BIOPS) containing (in mM) 10 Ca^{2+}-EGTA, 20 imidazole, 50 potassium-4-morpholinoethanesulfonic acid, 0.5 dithiothreitol, 6.56 $MgCl_2$, 5.77 ATP and 15 phosphocreatine at pH 7.1 (6) until processing for mitochondrial respiration experiments. Due to location logistics, samples in BIOPS were shipped on ice back to Colorado State University in Fort Collins, CO, for mitochondrial respiratory analysis. To evaluate the potential effect of cold storage on mitochondrial function in E-seal biopsies, pilot studies were performed on a subset of seal biopsies within 3-4 hours of sampling in Santa Cruz, then again in Colorado following 24-36 hours on ice, which showed only minor changes in respiratory parameters and cytochrome c response (data not shown).

Preparation of Permeabilzed Muscle Fibers.

Structurally sound fiber bundles were selected from biopsies maintained in ice-cold BIOPS, and mechanically separated, removing any visible adipose and connective tissue using fine forceps under a dissecting microscope. Teased fiber bundles (2-6 mg) were then transfered to BIOPS containing 50 µg/mL saponin for twenty minutes of permeablization of the

sarcolemma while leaving the mitochondria and intracellular structures intact (1), followed by 3 x 10 min washes in ice-cold MiR06 respiration buffer containing (in mM) 0.5 EGTA, 3 $MgCl_2$, 60 K-lactobionate, 20 taurine, 10 KH_2PO_4, 20 HEPES, 110 sucrose, with 1 g/L fatty-acid free BSA and 2,800 U/mL catalase. Permeabilized fibers were carefully blotted on Whatman filter paper for 2-3 seconds to remove excess buffer, weighed and immediately placed in the Oxygraph chamber containing MiR06 at 37˚C for stabilization prior to respiration experiments described below.

Mitochondrial Respiration.

Mitochondrial respiratory function was determined in permeabilized muscle fiber bundles obtained from latissimus dorsi (seals) and vastus laterals (humans) by high resolution respirometry using an Oxygraph-O2k high resolution respirometer (Oroboros Instruments, Innsbruck, Austria). Oxygen flux was monitored in real-time by resolving changes in the negative time derivative of the chamber oxygen concentration signal following standardized instrumental and chemical background calibrations using Datlab software (Oroboros, Innsbruck) (Pesta and Gnaiger 2012). All respirometry data were collected at 37˚C in a hyperpoxygenated environment (275-400 μM) to avoid potential limitations in oxygen diffusion in permeabilized fiber bundles (6). A detailed description of the respiration protocols and associated respiratory states generated by the sequential titration of each constituent are provided in Table S2.1. Any fiber preparations exhibiting a greater than 10% increase in flux with Cyt c were excluded from analyses. Selected flux control ratios were calculated for determination of respiratory coupling/leak, substrate control, and the relative contribution of fatty acid vs. pyruvate as described in Results and Discussion.

Statistical Analyses.

All data are presented as group means ± standard error, with the number of samples per group noted in the Results and figure legends. Data from respiration studies comparing adult seals and humans (Fig. S2.1 and S2.2) were compared by independent sample t-tests. Data from seal pups, juveniles and adults (Fig. S2.3 and S2.4) were compared by one-way ANOVA, with Tukey tests post hoc to reveal individual group differences. The level of statistical significance was set at $P < 0.05$ for all analyses.

Table S2.1. High resolution respirometry protocols and associated respiratory flux states assessed in mitochondrial respiration experiments.

Protocol constituents (listed in order of titration)	**Abbreviation**	**Respiratory Flux State; Explanation**
Protocol 1		
Palmitoylcarnitine + Malate (0.2 mM + 2 mM)	PalM	L_N; "Leak" respiration in the presence of high lipid concentration, but no ADP
ADP (5 mM)	ADP	P_{ETF}; Lipid OXPHOS capacity, limited by maximal electron flux through ETF
Pyruvate (5 mM)	Pyr	$P_{ETF+Pyr}$; Lipid + pyruvate OXPHOS capacity, limited by pyruvate oxidation capacity
Glutamate (10 mM)	Glut	P_{CI} ; Maximal CI-supported OXPHOS capacity; limited by ADP phosphorylation capacity ($\Delta\Psi_m$)
Cytochrome *c* (10 μM)	Cyt c	Test of outer mitochondrial membrane integrity (fully intact mitochondria give minimal response)
FCCP (1μM followed by 0.5μM titrations)	FCCP	E_{CI}; Maximal non-coupled CI-supported respiratory rate; limited by CI-III-IV enzymatic capacity
Protocol 2		
Pyruvate + Malate (5 mM + 2 mM)	PyrM	L_N; "Leak" respiration in the presence of high substrate concentration, but no ADP
ADP (5 mM)	ADP	P_{Pyr}; Pyruvate OXPHOS capacity, limited by pyruvate oxidation capacity
Glutamate (10 mM)	Glut	P_{CI} ; Maximal CI-supported OXPHOS capacity; limited by ADP phosphorylation capacity ($\Delta\Psi_m$)
Succinate (10 mM)	Succ	P_{CI+II} ;Maximal CI+II-supported OXPHOS capacity; limited by ADP phosphorylation capacity ($\Delta\Psi_m$)
Cytochrome *c* (10 μM)	Cyt c	Test of outer mitochondrial membrane integrity (fully intact mitochondria give minimal response)

Rotenone (0.5 μM)	Rot	P_{CII} ; Maximal CII-supported OXPHOS capacity; limited by ADP phosphorylation capacity ($\Delta\Psi_m$)
Oligomycin A (0.5 μM)	Omy	Maximal CII-supported "Leak" respiration in the presence of high adenylate concentration

Protocol constituents are listed in the order they are added in the respiration experiment, generating the cumulative respiratory states described in the right column. See text and ref. (Pesta and Gnaiger 2012) for additional explanation and interpretation of respiratory states. Abbreviations not listed above: ETF, electron transferring Flavoprotein; OXPHOS, oxidative phosphorylation; CI- IV, respiratory complexes I- IV; $\Delta\Psi_m$; mitochondrial membrane potential.

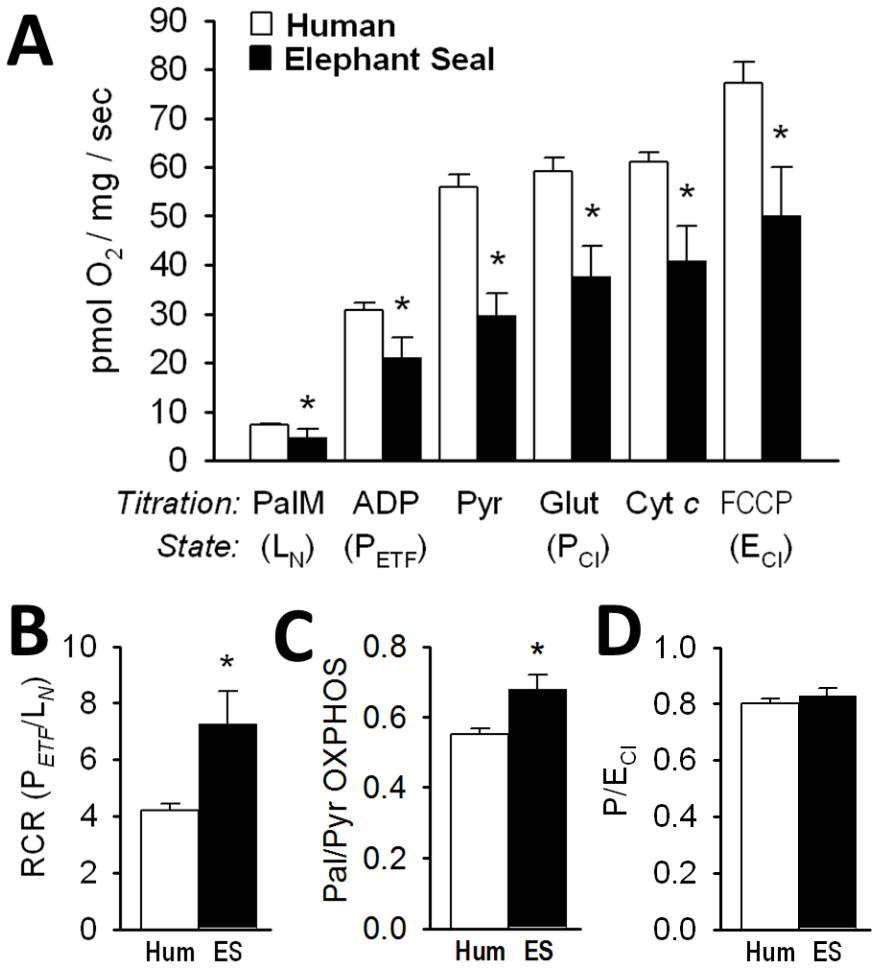

Figure S2.1. Respirometry data from adult human and E-seal muscle mitochondria in the presence of fatty acids in Protocol 1. A) Mass-corrected respiratory flux rates of permeabilized muscle fibers from humans (Hum; n = 4) and E-seals (ES; n = 7) during each of the respiratory states examined in Protocol 1 (see text and Table 2 for details and abbreviations). B) The respiratory control ratio (RCR) for fatty acid-supported OXPHOS oxidation, calculated as the fatty acid OXPHOS rate (P_{ETF}) divided by the preceding leak rate without ADP (L_N) in A (an index of OXPHOS "control"). C) The relative contribution of fatty acid to combined lipid+carbohydrate-supported OXPHOS rate. D) The combined lipid+complex 1 (CI) OXPHOS capacity expressed relative to total ETS capacity (an index of OXPHOS "efficiency"). Data are means ± SEM. *P < 0.05 versus human.

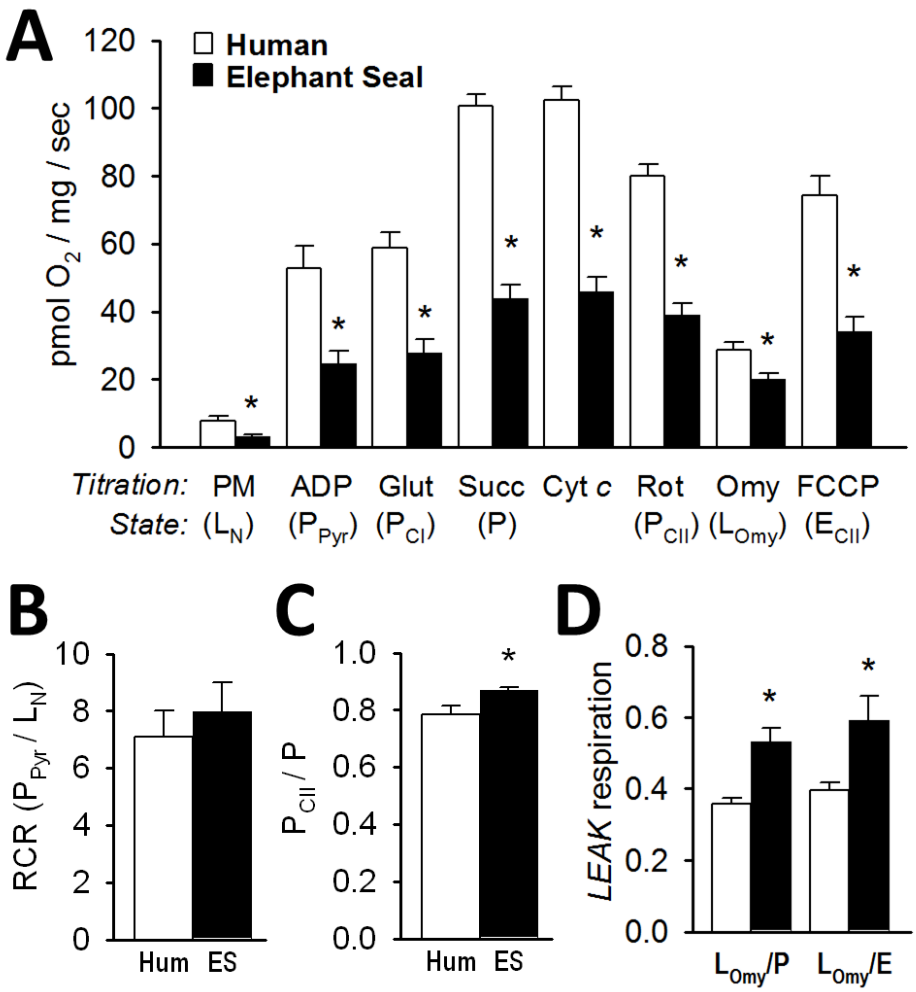

Figure S2.2. Respirometry data from adult human and E-seal muscle mitochondria in the presence of complex 1 and complex II substrates by Protocol 2. A) Mass-corrected respiratory flux rates of permeabilized muscle fibers from human (Hum; n = 4) and E-seals (ES; n = 7) during each of the respiratory states examined in Protocol 2 (see text and Table 2 for details and abbreviations). B) The respiratory control ratio (RCR) for pyruvate oxidation, calculated as the pyruvate+malate OXPHOS rate (P_{Pyr}) divided by the preceding leak rate without ADP (L_N) in A. C) The relative contribution of complex II (CII) to total OXPHOS capacity, calculated as the CI+II OXPHOS rate in the presence of rotenone (a CI inhibitor; P_{CII}) divided by the preceding uninhibited OXPHOS rate (P) in A. D) Indices of respiratory leak during CII-supported respiration, calculated as CII-linked respiration in the presence of ATP synthase inhibition by oligomycin (L_{Omy}) divided by the preceding uninhibited OXPHOS rate (P_{CII}) or subsequent non-coupled ETS capacity (E_{CII}) in A. Data are means ± SEM. *P < 0.05 versus human.

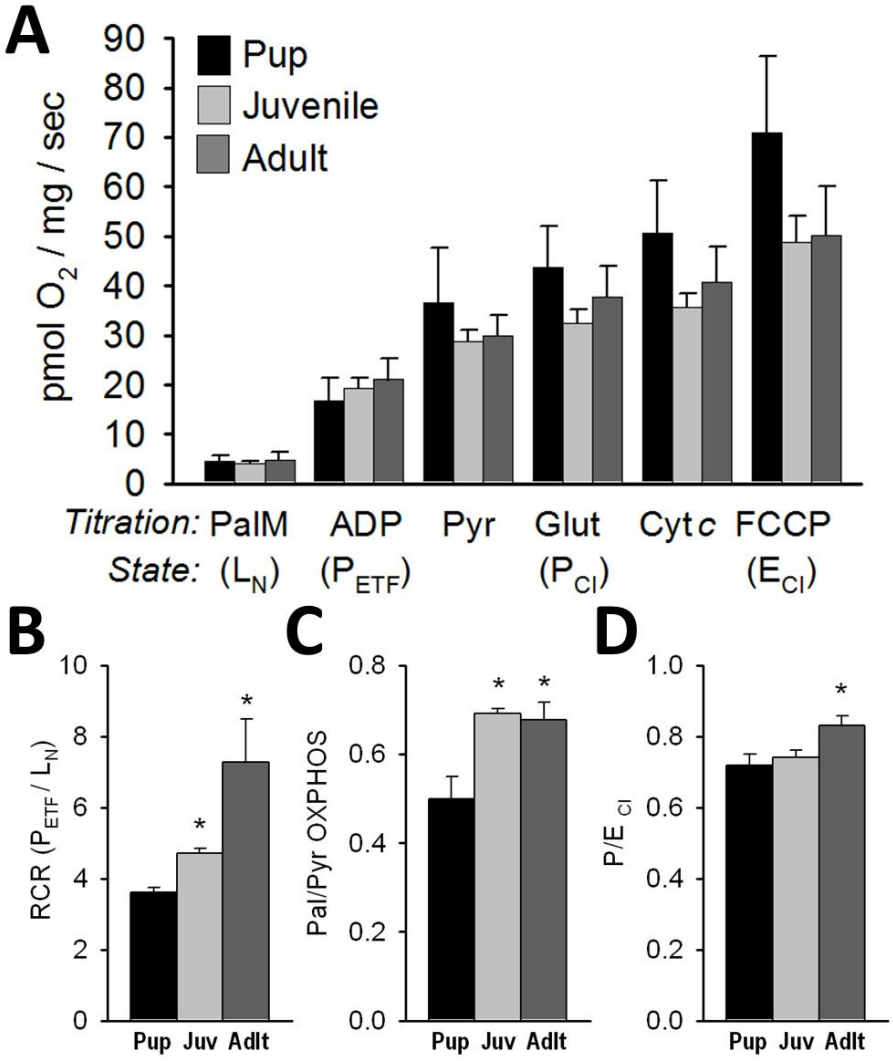

Figure S2.3. Ontogeny of mitochondrial respiratory function in E-seal muscle assessed in the presence of fatty acids by Protocol 1. A) Mass-corrected respiratory flux rates of permeabilized muscle fibers from E-seal pups (n = 4), juveniles (n = 6) and adults (n = 7) for each of the respiratory states examined in Protocol 1 (see text and Table 2 for details and abbreviations). B) The respiratory control ratio (RCR) for fatty acid-supported OXPHOS oxidation, calculated as the fatty acid OXPHOS rate (P_{ETF}) divided by the preceding leak rate without ADP (L_N) in A (an index of OXPHOS "control"). C) The relative contribution of fatty acid to combined lipid+carbohydrate-supported OXPHOS flux with PalM. D) The combined lipid+complex 1 (CI) OXPHOS capacity expressed relative to total ETS capacity (an index of OXPHOS "efficiency"). Data are means ± SEM. *P < 0.05 versus pups.

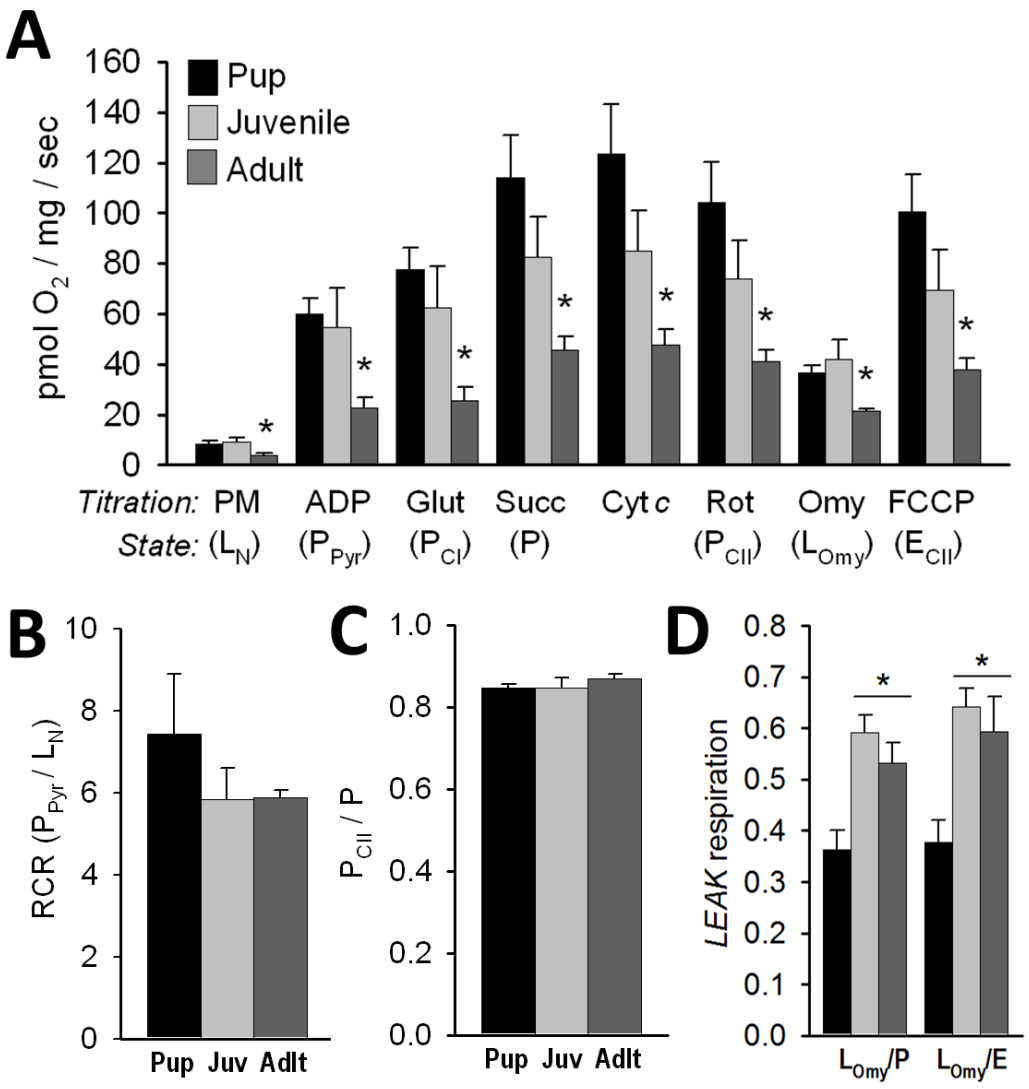

Figure S2.4. Ontogeny of mitochondrial respiratory function in E-seal muscle assessed in the presence of complex 1 and complex II substrates by Protocol 2. A) Mass-corrected respiratory flux rates of permeabilized muscle fibers from E-seal pups (n = 4), juveniles (n = 6) and adults (n = 7) for each of the respiratory states examined in Protocol 2 (see text and Table 2 for details and abbreviations). B) The respiratory control ratio (RCR) for pyruvate oxidation, calculated as the pyruvate+malate OXPHOS rate (P_{Pyr}) divided by the preceding leak rate without ADP (L_N) in A. C) The relative contribution of complex II (CII) to total OXPHOS capacity, calculated as CI+II OXPHOS in the presence of rotenone (a CI inhibitor; P_{CII}) divided by the preceding uninhibited OXPHOS rate (P) in A. D) Indices of respiratory leak during CII-supported respiration, calculated as CII-linked respiration in the presence of ATP synthase

inhibition by oligomycin (L_{Omy}) divided by the preceding uninhibited OXPHOS rate (P_{CII}) or subsequent non-coupled ETS capacity (E_{CII}) in A. Data are means ± SEM. *P < 0.05 versus pups.

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APPENDIX

Table A.S1. Transcripts differences from oxidative stress PCR array between lipid vs. glucose C_2C_{12} normoxic cells.

Symbol	Well	AVG ΔC_t (Ct(GOI) - Ave Ct (HKG))		2^-ΔC_t		T-TEST	Fold Up- or Down-Regulation
		Lipid Normoxic	Glucose Normoxic	Lipid Normoxic	Glucose Normoxic	p value	Lipid Normoxic /Glucose Normoxic
Symbol	A01	11.55	9.92	3.3E-04	1.0E-03	0.072866	-3.10
Alb	A02	4.18	5.81	5.5E-02	1.8E-02	0.013862	3.10
Als2	A03	4.96	7.02	3.2E-02	7.7E-03	0.002496	4.19
Aox1	A04	3.93	6.07	6.6E-02	1.5E-02	0.007864	4.41
Apc	A05	2.71	7.33	1.5E-01	6.2E-03	0.001119	24.59
Apoe	A06	4.17	5.98	5.6E-02	1.6E-02	0.009199	3.52
Atr	A07	1.87	3.09	2.7E-01	1.2E-01	0.003867	2.33
Cat	A08	7.43	9.25	5.8E-03	1.6E-03	0.000894	3.54
Ccl5	A09	2.62	4.44	1.6E-01	4.6E-02	0.006912	3.53
Ccs	A10	-0.92	1.76	1.9E+00	3.0E-01	0.002747	6.40
Ctsb	A11	11.55	9.59	3.3E-04	1.3E-03	0.078593	-3.89
Cyba	A12	8.65	9.09	2.5E-03	1.8E-03	0.182791	1.35
Cygb	B01	1.53	2.85	3.5E-01	1.4E-01	0.006893	2.49
Dnm2	B02	7.16	8.37	7.0E-03	3.0E-03	0.057855	2.31
Duox1	B03	1.31	3.45	4.0E-01	9.2E-02	0.012294	4.39
Ehd2	B04	11.50	9.97	3.5E-04	1.0E-03	0.088351	-2.89
Epx	B05	5.74	7.75	1.9E-02	4.6E-03	0.008005	4.02
Ercc2	B06	4.55	7.46	4.3E-02	5.7E-03	0.002578	7.52
Ercc6	B07	6.96	8.03	8.0E-03	3.8E-03	0.086408	2.10
Fancc	B08	11.06	9.97	4.7E-04	1.0E-03	0.158463	-2.14
Fmo2	B09	-6.22	-6.35	7.4E+01	8.1E+01	0.694877	-1.09
Fth1	B10	2.66	4.14	1.6E-01	5.7E-02	0.004617	2.78
Gclc	B11	1.22	2.98	4.3E-01	1.3E-01	0.000225	3.39
Gclm	B12	-2.72	-3.79	6.6E+00	1.4E+01	0.126701	-2.10
Gpx1	C01	11.55	9.97	3.3E-04	1.0E-03	0.082199	-3.00
Gpx2	C02	3.80	5.54	7.2E-02	2.1E-02	0.005093	3.33
Gpx3	C03	-2.75	-0.77	6.7E+00	1.7E+00	0.007523	3.94
Gpx4	C04	11.55	9.97	3.3E-04	1.0E-03	0.082199	-3.00
Gpx5	C05	11.55	9.97	3.3E-04	1.0E-03	0.082199	-3.00
Gpx6	C06	11.35	9.97	3.8E-04	1.0E-03	0.101460	-2.62
Gpx7	C07	2.76	5.86	1.5E-01	1.7E-02	0.004178	8.59
Gsr	C08	1.55	3.80	3.4E-01	7.2E-02	0.001160	4.76
Gss	C09	10.78	9.88	5.7E-04	1.1E-03	0.145739	-1.86
Gstk1	C10	-1.20	0.12	2.3E+00	9.2E-01	0.006976	2.48

Gstp1	C11	1.60	3.65	3.3E-01	8.0E-02	0.004881	4.15
Hmox1	C12	6.14	5.01	1.4E-02	3.1E-02	0.018709	-2.19
Hspa1a	D01	0.46	1.72	7.3E-01	3.0E-01	0.005462	2.38
Idh1	D02	3.09	6.16	1.2E-01	1.4E-02	0.005396	8.38
Ift172	D03	11.37	9.93	3.8E-04	1.0E-03	0.083146	-2.73
Il19	D04	11.55	9.65	3.3E-04	1.2E-03	0.193803	-3.73
Il22	D05	11.55	9.97	3.3E-04	1.0E-03	0.082199	-3.00
Krt1	D06	11.55	9.78	3.3E-04	1.1E-03	0.090312	-3.41
Lpo	D07	-1.44	-1.66	2.7E+00	3.1E+00	0.497288	-1.16
Mb	D08	11.55	9.97	3.3E-04	1.0E-03	0.082199	-3.00
Mpo	D09	10.98	9.95	4.9E-04	1.0E-03	0.165441	-2.05
Ncf1	D10	9.21	9.57	1.7E-03	1.3E-03	0.602722	1.28
Ncf2	D11	11.42	9.97	3.7E-04	1.0E-03	0.089124	-2.74
Ngb	D12	8.61	6.38	2.6E-03	1.2E-02	0.001218	-4.68
Nos2	E01	11.55	9.96	3.3E-04	1.0E-03	0.084845	-3.02
Nox1	E02	6.77	7.94	9.2E-03	4.1E-03	0.059242	2.25
Nox4	E03	11.55	9.97	3.3E-04	1.0E-03	0.082199	-3.00
Noxa1	E04	7.60	9.22	5.2E-03	1.7E-03	0.016454	3.07
Noxo1	E05	3.30	5.38	1.0E-01	2.4E-02	0.001409	4.22
Nqo1	E06	-0.58	0.75	1.5E+00	6.0E-01	0.002357	2.51
Park7	E07	3.00	3.61	1.3E-01	8.2E-02	0.168773	1.53
Prdx1	E08	-0.83	0.82	1.8E+00	5.7E-01	0.008631	3.15
Prdx2	E09	0.57	1.71	6.8E-01	3.1E-01	0.024529	2.20
Prdx3	E10	-0.39	-1.61	1.3E+00	3.0E+00	0.092225	-2.32
Prdx4	E11	0.04	1.82	9.7E-01	2.8E-01	0.006540	3.42
Prdx5	E12	0.92	2.76	5.3E-01	1.5E-01	0.007255	3.57
Prdx6	F01	1.39	6.04	3.8E-01	1.5E-02	0.001855	25.06
Prnp	F02	-1.40	-0.96	2.6E+00	1.9E+00	0.072780	1.36
Psmb5	F03	0.20	2.94	8.7E-01	1.3E-01	0.004375	6.68
Ptgs1	F04	1.83	5.08	2.8E-01	3.0E-02	0.000353	9.55
Ptgs2	F05	11.36	9.91	3.8E-04	1.0E-03	0.118987	-2.72
Rag2	F06	7.22	7.62	6.7E-03	5.1E-03	0.469981	1.32
Recql4	F07	-4.29	-3.30	2.0E+01	9.8E+00	0.003160	1.99
Scd1	F08	4.26	3.58	5.2E-02	8.4E-02	0.210228	-1.60
Serpinb1b	F09	1.42	3.01	3.7E-01	1.2E-01	0.000874	3.01
Slc38a1	F10	-1.38	-0.28	2.6E+00	1.2E+00	0.156194	2.14
Sod1	F11	-0.46	0.20	1.4E+00	8.7E-01	0.130710	1.58
Sod2	F12	5.68	5.59	2.0E-02	2.1E-02	0.882913	-1.06
Sod3	G01	-1.62	0.32	3.1E+00	8.0E-01	0.003761	3.84
Sqstm1	G02	2.30	6.51	2.0E-01	1.1E-02	0.001028	18.60
Srxn1	G03	11.55	9.97	3.3E-04	1.0E-03	0.082199	-3.00

Tpo	G04	8.29	8.68	3.2E-03	2.4E-03	0.933474	1.31
Txn1	G05	2.61	3.78	1.6E-01	7.3E-02	0.004148	2.25
Txnip	G06	1.08	2.73	4.7E-01	1.5E-01	0.006744	3.13
Txnrd1	G07	6.16	6.69	1.4E-02	9.7E-03	0.105639	1.44
Txnrd2	G08	4.47	5.95	4.5E-02	1.6E-02	0.005150	2.78
Txnrd3	G09	1.81	3.75	2.9E-01	7.5E-02	0.011328	3.84
Ucp2	G10	6.80	8.10	9.0E-03	3.6E-03	0.002834	2.46
Ucp3	G11	-2.96	-1.80	7.8E+00	3.5E+00	0.050058	2.23
Vim	G12	1.29	1.55	4.1E-01	3.4E-01	0.783221	1.20
Xpa	H01	-2.02	-0.58	4.0E+00	1.5E+00	0.145745	2.71
Actb	H02	-4.54	-4.28	2.3E+01	1.9E+01	0.382220	1.19
B2m	H03	-4.37	-3.83	2.1E+01	1.4E+01	0.738788	1.46
Gapdh	H04	2.95	3.48	1.3E-01	9.0E-02	0.224266	1.44
Gusb	H05	-2.65	-1.32	6.3E+00	2.5E+00	0.022417	2.51

Table A.S2. Transcripts differences from oxidative stress PCR array between lipid vs. glucose hypoxic C_2C_{12} cells.

Symbol	AVG ΔC_t (Ct(GOI) - Ave Ct (HKG))		2^-ΔC_t		T-TEST	Fold Up- or Down-Regulation
	Hypoxic Lipid	Hypoxic Glucose	Hypoxic Lipid	Hypoxic Glucose	p value	Hypoxic Lipid /Hypoxic Glucose
Alb	13.67	12.16	7.7E-05	2.2E-04	0.077530	-2.85
Als2	6.50	6.91	1.1E-02	8.3E-03	**0.038860**	1.33
Aox1	6.34	7.02	1.2E-02	7.7E-03	0.208173	1.60
Apc	7.61	7.17	5.1E-03	6.9E-03	0.102959	-1.35
Apoe	8.65	9.36	2.5E-03	1.5E-03	N/A	1.63
Atr	7.47	7.00	5.7E-03	7.8E-03	0.208836	-1.38
Cat	4.18	4.79	5.5E-02	3.6E-02	**0.041512**	1.53
Ccl5	13.35	9.64	9.6E-05	1.3E-03	**0.001264**	**-13.12**
Ccs	6.01	5.93	1.5E-02	1.6E-02	0.681959	-1.06
Ctsb	1.19	2.11	4.4E-01	2.3E-01	**0.028280**	1.90
Cyba	13.64	11.45	7.8E-05	3.6E-04	0.061335	**-4.56**
Cygb	12.59	11.04	1.6E-04	4.7E-04	**0.015004**	-2.91
Dnm2	5.12	5.46	2.9E-02	2.3E-02	0.329183	1.27
Duox1	9.46	9.26	1.4E-03	1.6E-03	0.504345	-1.14
Ehd2	3.68	3.39	7.8E-02	9.5E-02	0.435921	-1.23
Epx	13.83	12.07	6.9E-05	2.3E-04	0.125229	**-3.39**
Ercc2	9.17	8.83	1.7E-03	2.2E-03	0.379007	-1.26
Ercc6	8.61	8.09	2.6E-03	3.7E-03	**0.004873**	-1.43
Fancc	9.03	8.37	1.9E-03	3.0E-03	**0.021596**	-1.58
Fmo2	13.50	11.75	8.6E-05	2.9E-04	0.314897	**-3.36**
Fth1	-4.36	-3.54	2.0E+01	1.2E+01	**0.002128**	1.77
Gclc	4.52	5.21	4.4E-02	2.7E-02	**0.004008**	1.61
Gclm	2.93	3.78	1.3E-01	7.3E-02	**0.000081**	1.80
Gpx1	-0.63	-0.50	1.5E+00	1.4E+00	0.436256	1.09
Gpx2	13.63	13.26	7.9E-05	1.0E-04	0.362695	-1.30
Gpx3	3.42	3.17	9.3E-02	1.1E-01	0.436536	-1.19
Gpx4	-0.55	-0.16	1.5E+00	1.1E+00	**0.010222**	1.31
Gpx5	13.92	13.46	6.5E-05	8.8E-05	0.481719	-1.37
Gpx6	13.92	11.98	6.5E-05	2.5E-04	0.081876	**-3.84**
Gpx7	13.92	11.75	6.5E-05	2.9E-04	0.174668	**-4.48**
Gsr	5.36	5.98	2.4E-02	1.6E-02	0.050713	1.53
Gss	3.85	4.15	6.9E-02	5.6E-02	0.088881	1.23
Gstk1	10.72	8.58	5.9E-04	2.6E-03	**0.025144**	**-4.41**
Gstp1	0.33	1.11	7.9E-01	4.6E-01	**0.004219**	1.71

Hmox1	2.73	4.01	1.5E-01	6.2E-02	**0.013853**	2.42
Hspa1a	9.51	7.55	1.4E-03	5.3E-03	**0.001762**	**-3.89**
Idh1	2.67	3.07	1.6E-01	1.2E-01	**0.020924**	1.32
Ift172	6.97	6.76	8.0E-03	9.2E-03	0.662484	-1.16
Il19	13.58	11.77	8.2E-05	2.9E-04	0.165854	**-3.51**
Il22	13.78	11.05	7.1E-05	4.7E-04	0.100634	**-6.67**
Krt1	13.06	9.78	1.2E-04	1.1E-03	0.094775	**-9.69**
Lpo	13.62	12.01	7.9E-05	2.4E-04	0.088318	**-3.06**
Mb	5.29	3.37	2.6E-02	9.7E-02	**0.000611**	**-3.79**
Mpo	13.92	11.38	6.5E-05	3.8E-04	0.170874	**-5.82**
Ncf1	13.72	12.86	7.4E-05	1.3E-04	0.256393	-1.82
Ncf2	11.19	9.65	4.3E-04	1.2E-03	**0.032178**	-2.90
Ngb	13.61	11.79	8.0E-05	2.8E-04	0.239354	**-3.52**
Nos2	7.45	7.52	5.7E-03	5.4E-03	0.718191	1.05
Nox1	13.21	11.16	1.1E-04	4.4E-04	0.181237	**-4.15**
Nox4	7.01	7.56	7.8E-03	5.3E-03	0.072467	1.47
Noxa1	13.92	13.33	6.5E-05	9.7E-05	0.306625	-1.51
Noxo1	9.62	9.26	1.3E-03	1.6E-03	0.246365	-1.28
Nqo1	5.29	5.49	2.6E-02	2.2E-02	0.150653	1.15
Park7	2.09	2.31	2.4E-01	2.0E-01	0.105028	1.16
Prdx1	5.20	6.17	2.7E-02	1.4E-02	**0.009184**	1.95
Prdx2	1.27	0.84	4.1E-01	5.6E-01	**0.004195**	-1.35
Prdx3	3.92	4.07	6.6E-02	6.0E-02	**0.049563**	1.10
Prdx4	1.54	1.56	3.4E-01	3.4E-01	0.938949	1.02
Prdx5	2.60	2.89	1.7E-01	1.4E-01	0.124042	1.22
Prdx6	3.29	3.97	1.0E-01	6.4E-02	**0.018376**	1.61
Prnp	4.38	4.48	4.8E-02	4.5E-02	0.685876	1.07
Psmb5	1.07	1.63	4.8E-01	3.2E-01	**0.008272**	1.47
Ptgs1	2.85	2.95	1.4E-01	1.3E-01	0.604036	1.07
Ptgs2	3.82	3.51	7.1E-02	8.8E-02	**0.003204**	-1.24
Rag2	12.53	11.40	1.7E-04	3.7E-04	0.265398	-2.18
Recql4	10.67	8.80	6.1E-04	2.2E-03	**0.001727**	**-3.65**
Scd1	0.43	-0.51	7.4E-01	1.4E+00	**0.006272**	-1.92
Serpinb1b	5.75	5.47	1.9E-02	2.2E-02	0.452535	-1.21
Slc38a1	5.33	5.59	2.5E-02	2.1E-02	0.173889	1.20
Sod1	0.78	2.22	5.8E-01	2.1E-01	**0.018396**	2.71
Sod2	2.93	3.57	1.3E-01	8.4E-02	0.065784	1.55
Sod3	6.24	4.59	1.3E-02	4.2E-02	**0.005543**	**-3.15**
Sqstm1	1.68	2.15	3.1E-01	2.3E-01	0.276916	1.39
Srxn1	4.33	5.06	5.0E-02	3.0E-02	**0.044243**	1.66
Tpo	13.92	13.07	6.5E-05	1.2E-04	0.236637	-1.80

Txn1	10.85	10.35	5.4E-04	7.7E-04	0.386746	-1.41
Txnip	3.44	3.20	9.2E-02	1.1E-01	0.179719	-1.18
Txnrd1	3.61	3.91	8.2E-02	6.6E-02	0.057571	1.24
Txnrd2	8.65	8.58	2.5E-03	2.6E-03	0.715359	-1.05
Txnrd3	7.95	8.43	4.0E-03	2.9E-03	0.202259	1.39
Ucp2	3.92	5.20	6.6E-02	2.7E-02	**0.013992**	2.43
Ucp3	12.11	11.91	2.3E-04	2.6E-04	0.657025	-1.14
Vim	-0.05	-0.65	1.0E+00	1.6E+00	0.058810	-1.52
Xpa	4.79	5.20	3.6E-02	2.7E-02	0.061138	1.33
Actb	-0.95	-1.54	1.9E+00	2.9E+00	**0.007075**	-1.51
B2m	-1.76	-1.34	3.4E+00	2.5E+00	0.270925	1.33
Gapdh	-1.92	-2.06	3.8E+00	4.2E+00	0.465782	-1.10
Gusb	4.47	4.95	4.5E-02	3.2E-02	**0.001762**	1.40
Hsp90ab1	0.15	-0.01	9.0E-01	1.0E+00	0.335454	-1.12

Table A.S3. Transcripts differences from oxidative stress PCR array between hypoxic vs. normoxic lipid-supplemented C_2C_{12} cells.

Symbol	AVG ΔC_t (Ct(GOI) - Ave Ct (HKG))		$2^{-\Delta C_t}$		T-TEST	Fold Up- or Down-Regulation
	Lipid Hypoxic	Lipid Normoxic	Lipid Hypoxic	Lipid Normoxic	p value	Lipid Hypoxic /Lipid Normoxic
Alb	13.67	13.67	7.7E-05	7.7E-05	0.972044	-1.00
Als2	6.50	6.30	1.1E-02	1.3E-02	0.366736	-1.15
Aox1	6.34	7.08	1.2E-02	7.4E-03	0.136505	1.66
Apc	7.61	6.05	5.1E-03	1.5E-02	0.007692	-2.93
Apoe	8.65	4.83	2.5E-03	3.5E-02	N/A	-14.15
Atr	7.47	6.29	5.7E-03	1.3E-02	0.014171	-2.26
Cat	4.18	3.99	5.5E-02	6.3E-02	0.276067	-1.14
Ccl5	13.35	9.55	9.6E-05	1.3E-03	0.010474	-13.94
Ccs	6.01	4.74	1.5E-02	3.7E-02	0.000280	-2.41
Ctsb	1.19	1.20	4.4E-01	4.3E-01	0.889271	1.01
Cyba	13.64	13.67	7.8E-05	7.7E-05	0.993598	1.02
Cygb	12.59	10.78	1.6E-04	5.7E-04	0.062713	-3.51
Dnm2	5.12	3.66	2.9E-02	7.9E-02	0.000035	-2.77
Duox1	9.46	9.28	1.4E-03	1.6E-03	0.562855	-1.13
Ehd2	3.68	3.44	7.8E-02	9.2E-02	0.502193	-1.19
Epx	13.83	13.62	6.9E-05	7.9E-05	0.691178	-1.16
Ercc2	9.17	7.87	1.7E-03	4.3E-03	0.008656	-2.46
Ercc6	8.61	6.68	2.6E-03	9.8E-03	0.000250	-3.82
Fancc	9.03	9.08	1.9E-03	1.8E-03	0.916995	1.04
Fmo2	13.50	13.19	8.6E-05	1.1E-04	0.591933	-1.24
Fth1	-4.36	-4.09	2.0E+01	1.7E+01	0.607875	1.20
Gclc	4.52	4.78	4.4E-02	3.6E-02	0.061561	1.20
Gclm	2.93	3.34	1.3E-01	9.9E-02	0.002619	1.33
Gpx1	-0.63	-0.59	1.5E+00	1.5E+00	0.920997	1.02
Gpx2	13.63	13.67	7.9E-05	7.7E-05	0.972775	1.03
Gpx3	3.42	5.93	9.3E-02	1.6E-02	0.001619	5.68
Gpx4	-0.55	-0.63	1.5E+00	1.5E+00	0.622859	-1.06
Gpx5	13.92	13.67	6.5E-05	7.7E-05	0.706906	-1.18
Gpx6	13.92	13.67	6.5E-05	7.7E-05	0.706906	-1.18
Gpx7	13.92	13.48	6.5E-05	8.8E-05	0.439775	-1.36
Gsr	5.36	4.88	2.4E-02	3.4E-02	0.070673	-1.40
Gss	3.85	3.67	6.9E-02	7.8E-02	0.235934	-1.13
Gstk1	10.72	12.90	5.9E-04	1.3E-04	0.002001	4.53
Gstp1	0.33	0.93	7.9E-01	5.3E-01	0.003759	1.51
Hmox1	2.73	3.72	1.5E-01	7.6E-02	0.008398	1.98

Hspa1a	9.51	8.27	1.4E-03	3.2E-03	0.025150	-2.36
Idh1	2.67	2.59	1.6E-01	1.7E-01	0.598566	-1.06
Ift172	6.97	5.21	8.0E-03	2.7E-02	0.007883	-3.39
Il19	13.58	13.50	8.2E-05	8.7E-05	0.909354	-1.06
Il22	13.78	13.67	7.1E-05	7.7E-05	0.827560	-1.08
Krt1	13.06	13.67	1.2E-04	7.7E-05	0.158876	1.53
Lpo	13.62	13.67	7.9E-05	7.7E-05	0.930934	1.04
Mb	5.29	0.68	2.6E-02	6.2E-01	0.005422	-24.41
Mpo	13.92	13.67	6.5E-05	7.7E-05	0.706906	-1.18
Ncf1	13.72	13.11	7.4E-05	1.1E-04	0.275201	-1.53
Ncf2	11.19	11.34	4.3E-04	3.9E-04	0.903841	1.11
Ngb	13.61	13.54	8.0E-05	8.4E-05	0.987916	-1.05
Nos2	7.45	10.73	5.7E-03	5.9E-04	0.000089	9.76
Nox1	13.21	13.67	1.1E-04	7.7E-05	0.364933	1.38
Nox4	7.01	8.89	7.8E-03	2.1E-03	0.000152	3.69
Noxa1	13.92	13.67	6.5E-05	7.7E-05	0.706906	-1.18
Noxo1	9.62	9.72	1.3E-03	1.2E-03	0.752573	1.07
Nqo1	5.29	5.43	2.6E-02	2.3E-02	0.488747	1.10
Park7	2.09	1.54	2.4E-01	3.4E-01	0.031952	-1.46
Prdx1	5.20	5.12	2.7E-02	2.9E-02	0.621667	-1.06
Prdx2	1.27	1.29	4.1E-01	4.1E-01	0.821845	1.01
Prdx3	3.92	2.69	6.6E-02	1.6E-01	0.000756	-2.35
Prdx4	1.54	1.73	3.4E-01	3.0E-01	0.845436	1.14
Prdx5	2.60	2.17	1.7E-01	2.2E-01	0.012185	-1.35
Prdx6	3.29	3.05	1.0E-01	1.2E-01	0.283933	-1.18
Prnp	4.38	3.51	4.8E-02	8.8E-02	0.052358	-1.83
Psmb5	1.07	0.72	4.8E-01	6.1E-01	0.254768	-1.28
Ptgs1	2.85	2.32	1.4E-01	2.0E-01	0.085525	-1.44
Ptgs2	3.82	3.95	7.1E-02	6.5E-02	0.184318	1.10
Rag2	12.53	13.48	1.7E-04	8.8E-05	0.183363	1.93
Recql4	10.67	9.30	6.1E-04	1.6E-03	0.046892	-2.59
Scd1	0.43	-2.17	7.4E-01	4.5E+00	0.000000	-6.05
Serpinb1b	5.75	6.38	1.9E-02	1.2E-02	0.073588	1.55
Slc38a1	5.33	3.45	2.5E-02	9.2E-02	0.000019	-3.69
Sod1	0.78	0.65	5.8E-01	6.4E-01	0.630695	-1.10
Sod2	2.93	1.67	1.3E-01	3.2E-01	0.010082	-2.41
Sod3	6.24	7.80	1.3E-02	4.5E-03	0.004158	2.94
Sqstm1	1.68	0.50	3.1E-01	7.1E-01	0.009470	-2.26
Srxn1	4.33	4.42	5.0E-02	4.7E-02	0.769762	1.07
Tpo	13.92	13.67	6.5E-05	7.7E-05	0.706906	-1.18
Txn1	10.85	10.42	5.4E-04	7.3E-04	0.177978	-1.35

Txnip	3.44	4.64	9.2E-02	4.0E-02	0.001382	2.30
Txnrd1	3.61	3.11	8.2E-02	1.2E-01	0.063298	-1.41
Txnrd2	8.65	8.29	2.5E-03	3.2E-03	0.169171	-1.29
Txnrd3	7.95	6.59	4.0E-03	1.0E-02	0.000967	-2.57
Ucp2	3.92	3.93	6.6E-02	6.6E-02	0.891249	1.01
Ucp3	12.11	8.92	2.3E-04	2.1E-03	0.000289	-9.08
Vim	-0.05	-0.83	1.0E+00	1.8E+00	0.006685	-1.72
Xpa	4.79	3.63	3.6E-02	8.1E-02	0.001339	-2.23
Actb	-0.95	0.11	1.9E+00	9.3E-01	0.006207	2.08
B2m	-1.76	-2.41	3.4E+00	5.3E+00	0.115660	-1.57
Gapdh	-1.92	-2.25	3.8E+00	4.7E+00	0.293720	-1.25
Gusb	4.47	5.07	4.5E-02	3.0E-02	0.020614	1.52
Hsp90ab1	0.15	-0.52	9.0E-01	1.4E+00	0.028338	-1.60

Table A.S4. Transcripts differences from oxidative stress PCR array between CON vs. SAT rat soleus tissue.

Symbol	AVG ΔC_t (Ct(GOI) - Ave Ct (HKG))		$2^{-\Delta C_t}$		T-TEST	Fold Up- or Down-Regulation
	Fat Rat Sol	Control Rat Sol	Fat Rat Sol	Control Rat Sol	p value	Fat Rat Sol /Control Rat Sol
Alb	13.98	13.13	6.2E-05	1.1E-04	0.435729	-1.80
Als2	7.83	8.20	4.4E-03	3.4E-03	N/A	1.29
Aox1	7.80	7.71	4.5E-03	4.8E-03	0.592479	-1.07
Apc	6.02	6.87	1.5E-02	8.5E-03	0.005415	1.81
Apoe	4.98	6.50	3.2E-02	1.1E-02	0.082871	2.87
Cat	2.29	3.00	2.0E-01	1.3E-01	0.273239	1.63
Ccl5	6.01	6.89	1.5E-02	8.4E-03	0.158588	1.84
Ccs	4.57	5.57	4.2E-02	2.1E-02	0.051412	2.01
Ctsb	3.83	4.43	7.0E-02	4.6E-02	0.038765	1.51
Cyba	6.59	7.78	1.0E-02	4.5E-03	0.010014	2.28
Cygb	7.63	8.25	5.0E-03	3.3E-03	0.310093	1.53
Dhcr24	9.77	11.20	1.1E-03	4.2E-04	0.030669	2.71
Dnm2	9.78	10.02	1.1E-03	9.6E-04	0.994436	1.19
Duox1	12.42	13.53	1.8E-04	8.5E-05	0.017630	2.16
Duox2	12.69	12.98	1.5E-04	1.2E-04	0.714964	1.22
Ehd2	6.09	6.89	1.5E-02	8.4E-03	0.213453	1.75
Epx	10.77	10.69	5.7E-04	6.0E-04	N/A	-1.06
Ercc2	8.44	8.86	2.9E-03	2.2E-03	0.365186	1.33
Ercc6	9.18	9.18	1.7E-03	1.7E-03	0.929473	1.01
Fancc	9.31	9.91	1.6E-03	1.0E-03	0.640725	1.52
Fmo2	7.02	7.34	7.7E-03	6.2E-03	0.532897	1.24
Fth1	-3.76	-3.74	1.4E+01	1.3E+01	0.744112	1.01
Gclc	5.07	5.24	3.0E-02	2.6E-02	0.562602	1.13
Gclm	4.31	4.33	5.0E-02	5.0E-02	0.730782	1.01
Gpx1	-0.73	-1.56	1.7E+00	3.0E+00	0.820868	-1.79
Gpx2	12.88	13.33	1.3E-04	9.7E-05	0.273409	1.37
Gpx3	5.00	5.02	3.1E-02	3.1E-02	0.814098	1.01
Gpx4	0.47	1.16	7.2E-01	4.5E-01	0.269916	1.61
Gpx5	13.46	13.38	8.9E-05	9.4E-05	0.944879	-1.06
Gpx6	13.60	13.43	8.1E-05	9.0E-05	0.891313	-1.12
Gpx7	7.06	7.36	7.5E-03	6.1E-03	0.513915	1.23
Gsr	4.69	4.54	3.9E-02	4.3E-02	0.998077	-1.11
Gstk1	3.63	4.26	8.1E-02	5.2E-02	N/A	1.55
Gstp1	3.73	4.04	7.6E-02	6.1E-02	N/A	1.24
Hba-a2	0.10	0.79	9.3E-01	5.8E-01	N/A	1.61

Hmox1	6.69	5.68	9.7E-03	1.9E-02	0.127263	-2.01
Hspa1a	13.98	13.74	6.2E-05	7.3E-05	0.837567	-1.18
Idh1	4.82	4.03	3.6E-02	6.1E-02	0.000371	-1.73
Ift172	7.37	8.09	6.0E-03	3.7E-03	N/A	1.64
Krt1	13.33	13.81	9.7E-05	6.9E-05	0.339960	1.40
LOC367198	13.60	13.81	8.1E-05	7.0E-05	0.451960	1.16
Lpo	13.60	13.48	8.1E-05	8.8E-05	0.982436	-1.09
Mb	-4.53	-3.78	2.3E+01	1.4E+01	N/A	1.68
Mpo	13.60	13.74	8.1E-05	7.3E-05	0.578926	1.10
Ncf1	4.03	4.45	6.1E-02	4.6E-02	N/A	1.34
Ncf2	10.82	10.32	5.5E-04	7.8E-04	N/A	-1.42
Ngb	12.58	12.11	1.6E-04	2.3E-04	N/A	-1.39
Nos2	11.67	13.53	3.1E-04	8.5E-05	0.079008	3.62
Nox4	8.58	8.77	2.6E-03	2.3E-03	0.721481	1.13
Noxa1	13.60	13.55	8.1E-05	8.3E-05	0.849273	-1.03
Noxo1	13.57	13.82	8.2E-05	6.9E-05	N/A	1.19
Nqo1	6.20	6.49	1.4E-02	1.1E-02	N/A	1.22
Nudt1	6.77	7.14	9.1E-03	7.1E-03	N/A	1.29
Park7	0.28	0.56	8.2E-01	6.8E-01	0.460581	1.21
Prdx1	-0.35	-0.42	1.3E+00	1.3E+00	0.943917	-1.05
Prdx2	1.71	2.26	3.1E-01	2.1E-01	0.373922	1.46
Prdx3	1.73	2.16	3.0E-01	2.2E-01	0.257751	1.35
Prdx4	5.36	5.93	2.4E-02	1.6E-02	0.071080	1.48
Prdx5	1.25	2.42	4.2E-01	1.9E-01	0.007458	2.25
Prdx6	3.26	3.94	1.0E-01	6.5E-02	0.038484	1.60
Prnp	3.92	4.18	6.6E-02	5.5E-02	0.263801	1.20
Psmb5	-0.46	0.26	1.4E+00	8.4E-01	0.091930	1.65
Ptgs1	10.68	10.76	6.1E-04	5.8E-04	0.782714	1.06
Ptgs2	12.24	10.76	2.1E-04	5.8E-04	0.147918	-2.80
Rag2	13.60	13.27	8.1E-05	1.0E-04	0.629906	-1.25
Scd1	10.34	11.21	7.7E-04	4.2E-04	0.242080	1.83
Sels	3.37	3.09	9.7E-02	1.2E-01	0.301969	-1.21
Sepp1	1.96	1.27	2.6E-01	4.1E-01	0.147109	-1.61
Serpinb1b	13.10	10.85	1.1E-04	5.4E-04	N/A	-4.76
Slc38a1	4.39	5.21	4.8E-02	2.7E-02	N/A	1.76
Slc38a5	13.60	13.81	8.1E-05	7.0E-05	0.451960	1.16
Sod1	-0.98	-0.87	2.0E+00	1.8E+00	0.956361	1.08
Sod2	0.08	0.28	9.5E-01	8.3E-01	0.588428	1.15
Sod3	6.55	6.50	1.1E-02	1.1E-02	0.700306	-1.04
Sqstm1	3.58	3.94	8.4E-02	6.5E-02	0.316164	1.29
Srxn1	6.66	4.95	9.9E-03	3.2E-02	0.078498	-3.27

Tpo	13.60	13.81	8.1E-05	7.0E-05	0.451960	1.16
Txn1	0.57	0.24	6.7E-01	8.5E-01	0.297379	-1.26
Txnip	2.64	3.22	1.6E-01	1.1E-01	0.262367	1.49
Txnrd1	8.19	8.34	3.4E-03	3.1E-03	0.764959	1.11
Txnrd2	5.92	6.70	1.7E-02	9.6E-03	N/A	1.72
Ucp2	8.78	8.93	2.3E-03	2.0E-03	0.814286	1.12
Ucp3	3.10	4.45	1.2E-01	4.6E-02	0.806989	2.56
Vim	3.02	3.91	1.2E-01	6.7E-02	0.180013	1.85
Actb	1.01	1.83	5.0E-01	2.8E-01	0.164387	1.77
B2m	-1.27	-0.84	2.4E+00	1.8E+00	0.291040	1.35
Hprt1	1.64	0.46	3.2E-01	7.3E-01	0.054520	-2.27
Ldha	1.17	1.30	4.4E-01	4.1E-01	0.703027	1.09
Rplp1	-2.55	-2.75	5.8E+00	6.7E+00	0.134472	-1.15