THESIS

ARE YOU WHAT YOU EAT? ASSESSING INTRACELLULAR EFFECTS OF SUPPLEMENTAL LIPID WITHIN MOUSE (C2C12) SKELETAL MUSCLE CELLS

Submitted by

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In partial fulfillments of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Summer 2021

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ABSTRACT

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The expression of myoglobin, an oxygen-binding protein, facilitates aerobically-powered dives by effectively distributing a finite oxygen supply within skeletal muscle; particularly during breath-hold dives in marine mammals (Costa 2004, Kanatous et al. 1999, Wright 2006). Multiple factors have been experimentally shown to contribute to increasing myoglobin expression, contributing to oxygen availability and maintaining aerobic metabolism. Among the known expression pathways, the most recent and least studied involves a link between lipid supplementation and increased myoglobin expression. We are unaware of the specific conditions that facilitate this expression, and whether fatty acids play an intracellular role in upregulating myoglobin. My research elucidates the effects of lipid supplementation on the expression of myoglobin in terrestrial mammals, as well as enhances our understanding of cues that initiate the expression of factors contributing to the positive development of aerobically based exercise in the whole animal. To understand these lipid influences on an aerobically-adapted animal such as a marine mammal, we focused on developing experimental designs and applications geared towards a model mammalian system of C2C12 mouse cells.

Lipids, specifically fatty acids, possess the capacity to influence aerobic ability in mammalian models, whether through marine mammals' reliance on lipid metabolism to power aerobically-based exercise, regulating mitochondrial respiration, fatty acid metabolism that generates increased ATP from oxidative phosphorylation relative to metabolic pathways like

glycolysis, lipids acting as ligands to affect genes that contribute to aerobic capacity, or lipid supplementation that increases myoglobin expression. Multiple studies from our lab have shown that lipid present in the media upregulates myoglobin in C2C12 cells, driving expression of the protein in conjunction with other elements Although we have determined a link between lipid supplementation and myoglobin expression, the particular intracellular effects ranging from uptake, transporter protein regulation, and either storage, metabolism, or specific effects directly affecting myoglobin expression still remain to be elucidated.

The factors and conditions that regulate myoglobin expression are extensive, and lipids as an avenue to myoglobin expression is a relatively new exploration that will have applications for numerous areas of physiological research. This work has applications in multiple mammalian models, including humans, and will serve to enhance our understanding of the factors that contribute to aerobically-based endurance exercise, as well as understanding the cues and stimuli required to increase expression of key factors that regulate aerobic capacity in mammalian systems.

Acknowledgements

I am filled with immense gratitude at the friends, family, colleagues and mentors that have placed their stock and faith in me and my ability to complete this program: in so many immeasurable, unspoken, emotional ways, thank you. Thank you to the Kanatous Lab undergraduates: Mira, Adrianna, and Sami for their tremendous help and patience with cell culture. Thank you to all my little celly cells that perished in the name of science, and the ones that pulled through to contribute to this data. An enormous extension of thanks to my committee members: Drs. Christopher Bell, Gregory Florant, and Terry Engle, of whose expertise, patience, and expectations drove the quality of the work and the development of my scientific ability. Thank you to their respective labs that opened their doors and to complete this work. To the Biology faculty that lent their aid and equipment, to Ben Sharp for his incalculable help and selfless statistics, Dr. Adam Chicco and Dr. Graham Peers for their expertise, Dr. Matthew Hickey for being an approachable professor that makes physiology engaging. Thank you to Alex Adams for his teaching and emotional support. To Julian Cassano for coding and curry nights. To Ale and Arielle for the best two friends anyone could ask for. To Pa for encouraging me to just "eat that elephant one bite at a time" and Gram Gram, it's finally happening! An enormous thank you to Ashley Larsen for teaching me what I could hardly learn, and her unwavering (minus lab dishes), immeasurable support as a lab mate, a colleague, and a friend. To Dr. Shane Kanatous, who has been my mentor and advisor for the better part of a decade, who has always managed to see what I couldn't see in myself, and has always encouraged me to reach my full potential, be it in research or in art or in life, and without whom I would not have developed into the scientist and academic I am today. Finally, an exceptional thank you to my brother Dr. Manuel, without whom graduate school would have seemed unachievable, and who's honest and (sometimes brusque) support was necessary to get me to where I needed to be.

Dedication

For Shawn. This is your second chapter

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Chapter 1: Introduction

Express yourself: Myoglobin's role in skeletal muscle

Myoglobin is a cytoplasmic hemeprotein that plays multiple roles within cardiomyocytes and skeletal muscle cells (Kanatous et al, 2010, Schlater et al. 2012, Cotta et al. 2017). The primary role of myoglobin serves to reversibly bind oxygen, maximizing oxygen delivery by distributing stores of oxygen within working muscles during periods of limited oxygen availability, such as hypoxia or high levels of intracellular aerobic metabolism. It can also serve as a reactive oxygen species (ROS) or nitric oxide (NO) scavenger (Schlater et al. 2012, Schlater et al. 2014, Cotta e al. 2017). Expression of myoglobin in a plethora of mammals, as well as its contributions to aerobic capacity, make it a relevant protein to investigate the primers of oxidative efficiency in mammalian models. Its increased expression in marine mammals of up to ten-fold the amount seen in terrestrial mammals (Kanatous et al. 2010, Wright and Davis, 2015) serves a critical role maintaining the extreme aerobic demands of breath-hold diving. Its increased expression in marine mammals relative to terrestrial mammals is evidenced to be a function of a primarily lipid-based diet, limited oxygen availability, or skeletal muscle activity, with these same conditions serving to upregulate the protein in terrestrial mammals as well.

The factors and conditions that regulate myoglobin expression are extensive and have applications for multiple scientific fields, ranging from cancer research; as the requirements that turn on myoglobin expression can be altered to downregulate myoglobin expression that facilitates the growth of tumors in cancer cells (Flonta et al., 2009) to ischemic reperfusion injuries, to the maintenance of aerobic capacity in mammalian models. This work has applications in multiple mammalian models, including humans, and could enhance our

understanding of the factors that drive aerobically-based endurance exercise, as well as understanding the cues and stimuli required to increase expression of key factors that facilitate aerobic capacity in mammalian systems. Investigating a protein responsible for aiding the endurance of cells under limited oxygen availability, particularly during active states where oxygen consumption enables metabolism, is a critical area of research for multiple applications. As we have explored several pathways to myoglobin expression, the lipid pathway as an alternate and novel avenue holds important implications for the function of diet on the mammalian metabolic scope. Particularly in marine mammals, where myoglobin is expressed tenfold the amount seen in terrestrial mammals (Kanatous et al. 2008, Wittenberg 2007), analyzing the cues that coordinate expression from system to system is key to understanding the physiological implications of the presence or absence of this protein, and the performance, and more importantly, survival aspects that it holds for mammals of all life histories.

The role of diet in performance for humans is an important avenue, and this exploration holds a critical conservation role for marine mammals and other marine life, that have historically faced declining populations. As animals that may be radically affected by any perturbation in a diet heavily in lipid that is demonstrated to affect blubber quality, cognitive ability in young, energy for fasting pups, and now the influence of diet on maintaining dive times to forage and avoid predators, understanding the influence of diet on endangered and declining species is an understudied conservation concept that will hold enormous ecological value (Clarke et al. 1983, Guerrero et al. 2019, McCafferty et al. 1998, Kanatous et al. 2008, Shero et al. 2015, Thiemenn et al. 2009). Particularly as lipid studies in fish display varied lipid types and energetic availability among prey (Anthony et al. 2000), prey-specificity in marine mammal species, and the emergence of the junk food hypothesis, where animals are observably feeding on dietary

elements removed from their standard diet to their detriment (Gremillet et al. 2008, Romano et al. 2006), the larger implications for research assessing quality of diet and its relation to aerobic performance , especially in a changing climate, is critical to the conservation of key sentinel species like marine megafauna.

Marine to murine: Expression patterns in terrestrial and marine mammals

The increased expression of myoglobin in marine mammals relative to terrestrial mammals is the subject of a great deal of study, as marine mammals regularly subject themselves to prime conditions that facilitate myoglobin expression (DeMiranda 2012, Kanatous 1999, Kanatous 2002, Kooyman and Ponganis 1998). Maintenance of a sufficient aerobic capacity that allows marine mammals to sustain their dive times while traveling or foraging is a critical element to their survival (Davis et al. 2004, Kanatous et al. 2002), and a pinniped's lipid-based diet is a key facilitator of its aerobic ability (Kanatous et al. 1999, Kanatous et al. 2008), an ability that is an amalgamation of numerous factors ranging from muscle fiber type, blood volume, mitochondrial density, and expression of myoglobin (Kanatous 2010).

Seals and sea lions display an almost exclusively lipid-based diet that powers oxidative pathways, generating ATP to provide metabolic power and enable working dives (De Miranda et al. 2012, Davis et al. 1990, Kanatous et al. 2002, Trumble and Kanatous 2012). In addition, lipid stores provide a vastly important source of energy for feeding and fasting, providing sufficient nutrition to pups, playing a role in buoyancy and facilitating diving costs, and increasing myoglobin expression in conjunction with hypoxia exposure (De Miranda et al. 2012, Noren et al. 2003, Svard et al. 2009). In effect, the diving capabilities of pinnipeds are strongly influenced

by the fatty acid composition of their prey and its effects on physiology, specifically in marine mammals. The development of aerobic capacity as a consequence or a function of myoglobin expression, in a 'chicken or the egg scenario,' has also been investigated. During investigations of young emperor penguins, the appearance of myoglobin in the skeletal muscle was found to be a precursor to diving, indicating that hypoxia (usually during breath-hold dives) was not singly influencing myoglobin expression, and the protein levels were instead driven by other factors- mainly a high concentration of diet-based lipids regulating myoglobin expression in several marine mammal species (Kanatous et al. 2010, Ponganis et al. 2010).

Studies have demonstrated that there are parallel conditions across marine and terrestrial mammals facilitating the expression of myoglobin, key among them are skeletal muscle activity in conjunction with a secondary stimulus such as hypoxia, or more recently, an alternate pathway reliant on lipid supplementation and completely independent of skeletal muscle activity (Schlater et al. 2014) (Figure 1-1). Myoglobin has been studied extensively due to its role in skeletal muscle and cardiac physiology, and as a consequence, research investigating the pathways of its expression has resulted in multiple proposed pathways for myoglobin.

From NFAT to real fat: Multiple pathways of myoglobin expression

The networks leading to stimulation and expression of myoglobin are extensive. Leading research has established an interplay with calcium release stimulated by muscle contraction. On a cellular level, we have found that the pathways stimulating myoglobin expression rely on the translocation of NFAT into the nucleus through a calcineurin/NFAT pathway that activates specific calcium-signaling pathways and subsequently increases myoglobin expression (Chin et

al. 1998, Kanatous 2009, Kanatous and Pradeep 2010, Schlater 2014). This release of calcium from the sarcoplasmic reticulum activates the calcium-calmodulin activated phosphatase calcineurin, activating a sequence wherein calcineurin dephosphorylates NFAT (Nuclear factor of activated T-cells) and MEF2 (myocyte enhancer factor-2). Following translocation to the nucleus of NFAT, the promoter for myoglobin is bound and subsequent expression follows (DeMiranda et al. 2012).

Calcium release is also dependent on fiber type and research indicates that Type I oxidative fibers generate sufficient release of calcium for activation of calcineurin, in contrast to type II glycolytic fibers (Kanatous et al. 2009, Kanatous et al. 2010). The sustained release of calcium characteristic of slow-twitch (Type I fibers) is more conducive to activating calcineurin, as opposed to the smaller calcium fluxes characteristic of fast twitch (Type II) muscle fibers (Kanatous et al. 2008). The smaller calcium fluxes defined in the latter can also result in a complete removal of NFAT from the nucleus, leading to the downregulation of myoglobin. Indeed, Type I, slow-twitch fibers regularly express more myoglobin than their faster Type II skeletal muscle counterparts. This interplay between myoglobin expression and fiber type further displays the intricacies of aerobic capacity, physiology, and performance, and the role that myoglobin plays in their genesis.

The most prominently studied pathway of myoglobin expression demonstrates its dependency on calcineurin activation; specifying a reliance on skeletal muscle activation, a process that is replicated in cell culture through the addition of caffeine (Rosser et al. 2009, Kolawole et al. 2011). Increased myoglobin expression in absence of an increase in calcineurin expression, suggests that an alternate pathway independent of skeletal muscle activity may be present (Schlater et al. 2014). In conjunction with these findings, was also an ablation of

myoglobin in response to the addition of phenyl-alpha-tert-butyl nitrone (PBN), a known antioxidant. This furthered the potential interaction between myoglobin and reactive oxygen species (ROS), suggesting additional stimulants for myoglobin, allowing myoglobin to fulfill one of its roles as a ROS scavenger (Schlater et al 2014). As myoglobin would require low partial pressures of O2 to facilitate oxygen release, hypoxia was found to be a key facilitator for myoglobin appearance (Figure 1-1).

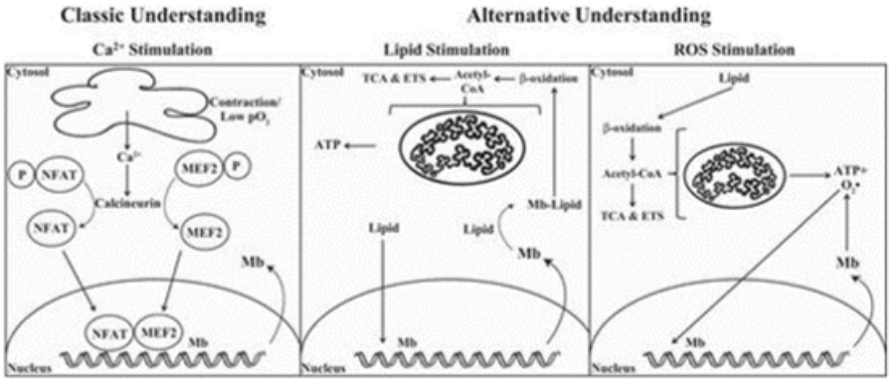

Figure 1-1: Schlater et al's proposal for a new paradigm of myoglobin expression. From left, the classic calcineurin activation pathway from skeletal muscle contraction, intracellular lipid activity at the nucleus promoting myoglobin expression, and lipid activity at the mitochondria generating ROS from beta oxidation to drive myoglobin expression. (Schlater et al., 2014)

Particularly in Weddell seal (*L. weddellii*) muscle cells, where this species routinely undergoes long breath-hold dives. these key conditions of limited oxygen availability within skeletal muscle facilitated an increase in myoglobin. (Kanatous et al. 2008, DeMiranda 2012). While hypoxia in marine mammal cells was enough to sustain myoglobin expression, it was with the addition of a secondary stimulus like muscle contraction that expression was driven exponentially upward to the large levels seen in adult marine mammals . In regards to these findings, two pathways were proposed: one with just skeletal muscle activity, and one with contraction in conjunction with hypoxia. In addition, hypoxia was demonstrated to be enough to

facilitate myoglobin expression in Weddell seal cells, directly opposing the findings observed in terrestrial mammal cells undergoing hypoxia exposure (Kanatous et al. 2008).

Finally, the addition of lipids alone without a secondary stimulus was experimentally determined to promote increased expression of myoglobin independent of skeletal muscle activity. When lipid-treated cells were exposed to hypoxic conditions, once again this secondary stimulus method stimulated the expression of myoglobin. With marine mammals highly reliant on a lipid-based diet and already largely primed for lipid-driven expression relative to terrestrial mammals, the reciprocity of lipids and myoglobin activation in tandem seems a key adaptation for marine mammals, particularly with their reliance on apneic dives with extreme bouts of hypoxia. It followed to further investigate if this stimulation and regulation of myoglobin was a consequence of hypoxic conditions, a unique adaptation in marine mammals, or if the same mobilizers held true for other mammals of a terrestrial nature (Kanatous et al. 2008, DeMiranda et al. 2012).

Particularly within terrestrial mammals, who may not be innately primed for almost exclusive lipid metabolism like marine mammals (as a result of an exclusively lipid-based diet), investigating the lipid-myoglobin link in a preferentially glucose-driven system of C2C12 cells (Abdelmoez et al. 2020), allows for further clarification on the metabolic dynamics of protein regulation. The protein itself shares considerable homologies between terrestrial and marine mammals despite radically different lifestyles (Kanatous et al. 2010). The protein is defined by three exons and two introns, although the mouse myoglobin gene was found to be shorter than other studied myoglobin genes (Blanchetot et al. 1986). Based on past research, terrestrial mammals may not express the same amounts of myoglobin as marine mammals but are still susceptible to the same regulators that promote its expression, albeit in a conditional manner.

Studies demonstrate that unlike marine mammals, hypoxia alone is not enough to stimulate myoglobin increase in terrestrial mammal cells (Kanatous et al. 2008). In terrestrial mammals, the primary recognized facilitators of myoglobin expression in isolation do not significantly affect myoglobin's appearance; it is only in conjunction with one another (contraction + hypoxia) that a significant myoglobin increase can be observed (DeMiranda et al. 2012, Kanatous et al. 2008, Kanatous et al. 2010, Schlater et al. 2012). This varies considerably with observations seen in marine mammal cells, and it is worth noting the same drivers in different combinations results in a myoglobin increase between the two mammalian cell types. However, the addition of lipids seems to be the most ubiquitous promoter of myoglobin expression between both terrestrial and marine mammals that remains minimally explored; as lipids alone in both terrestrial and marine mammals facilitates myoglobin increases, and furthermore when lipids are combined with hypoxia (Schlater et al. 2014). Studies have demonstrated a dependence on lipid concentration as well, with an increase in lipid concentration from 2.5 to 5 percent producing an upwards drive in myoglobin expression in both mammal cell types (and above 5 percent producing lipotoxicity not conducive to cell life) (DeMiranda et al. 2012). Lipids possess a consistent influence on expression in both marine and terrestrial mammals: addition promotes upregulation (as opposed to some other conditions that are required in conjunction with one another such as contraction plus hypoxia) (Figure 1-2).

	Muscle Contraction	Hypoxia	Hypoxia + Contraction	Lipids	Lipids + Hypoxia
Terrestrial Mammal	+	-	+	+	+
Marine Mammal	+	+	++	+	+

Figure 1-2: A summary of known drivers of myoglobin expression in both marine and terrestrial mammals. A + indicates increased expression, a – indicates downregulation, and a ++ indicates a substantial increase from basal conditions.

Overall, these findings present lipids as a mechanism that unlocks myoglobin regulation consistently across terrestrial and marine mammal species. The demonstration of both marine and terrestrial mammals adhering to similar pathways of myoglobin expression implies similar pathways for species possessing radically different life histories. In essence, this conservation of expression pathways in different mammals suggests an underlying mechanism for expression across all mammals: and what specifically occurs downstream in that pathway remains unknown but is critical to uncovering the regulation of a vital oxygen-delivery protein. As such, investigating the cell's handling of lipids in terrestrial mammals could begin to tease apart the metabolic consequences of its addition to skeletal muscle and its overall impact on myoglobin expression: specifically how the cells are using (or not using) lipids to achieve myoglobin upregulation.

Fat chance: Lipid mobilization into skeletal muscle

Knowledge of how lipids are mobilized and metabolized has been the subject of a great deal of study, with lipids useful for everything ranging from storage (Welte and Gould, 2017),

fuel (Alsabeeh et al. 2017, Askew 1984, Cooney et al. 2014, Horowitz et al. 2000, Kanatous et al. 2002), ligands that can promote upregulation of proteins (Hardie 2007, Ravnskjaer et al. 2010, Varga et al. 2011), and facilitators of aerobic capacity (Yamada et al. 2015). The usage or storage of lipids is dependent on metabolic demand, lipid concentration, and the capacity of cells to chaperone their movement. Within standard mammalian skeletal muscle cells, lipids are mobilized with the aid of lipoprotein lipase and bound to albumin for their movement through the blood stream.

Upon arrival at the cell membrane, while there is some evidence that lipids can move based on diffusion (Luiken et al. 2002, Stahl and Kazantizis, 2011), the overall uptake of fatty acids at the membrane level is expedited by fatty acid transport proteins (FATP) or fatty acid binding proteins (FABP) of which there are a significant number of variants and locations (Glatz 2001, Glatz and Luiken 2017, Stahl et al. 2002) (see Figure 1-3). Within skeletal muscle, the key fatty acid transport proteins are plasma-membrane fatty acid binding protein (FABPpm) and fatty acid translocase/cluster of differentiation 36 (FAT/CD36) (Bastie et al. 2004, Luiken et al. 2002). Presence of these transporters is a key index for fatty acid oxidation capacity (Nickerson et al. 2009). Utilization of lipids to be taken into the cell and intracellularly stimulate myoglobin could be achieved through two ways: translocation into the nucleus to directly affect myoglobin expression or metabolism through beta-oxidation to somehow promote myoglobin. Both avenues would have to begin at the uptake site: the plasma membrane and the transport proteins associated with it.

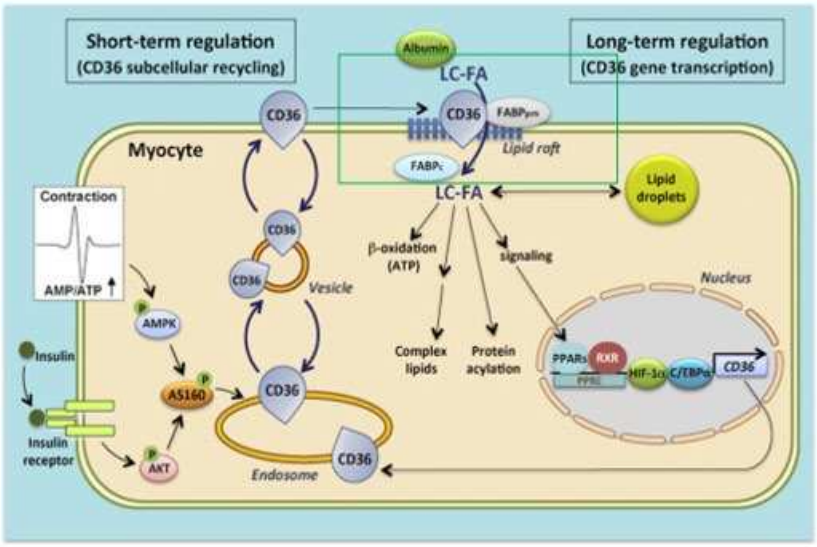

Figure 1-3: The key transporters in mammalian skeleton muscle cells. A long chain fatty acid bound to albumin binds to FABPpm at the plasma membrane (green box to indicate research focus sites), were it is then transported by CD36 (which translocates to the membrane from the nucleus through insulin or skeletal muscle activation), after which the fatty acids are destined for storage as lipid droplets, as a signaling molecule within th emitochondria, or metabolism through beta oxidation into ATP (Glatz and Luiken, 2017).

Studies indicate that supplemental lipids can affect the expression of these key transporters (Roepstorff et al. 2004, Glatz et al. 2010), so in effect, the uptake of lipids would result in an increased expression of fatty acid transporters to account for an increased lipid load within the cell culture media. Following lipid uptake, the lipids undergo numerous fates dictated by several factors. Lipids fated for storage would be sequestered into lipid droplets or introduced to the phospholipid membrane of the cell. If metabolic demand were to initiate beta-oxidation, the entry of lipids into the mitochondria for beta-oxidation and TCA cycle usage could occur. Finally, outside of storage and metabolism, lipids function as ligands to directly promote or deregulate protein expression. On the transcriptional level, fatty acids were repeatedly demonstrated to bind to peroxisome-proliferate activated receptors (PPARs) that actively increase fatty acid oxidation capacity, as well as influence mitochondrial biogenesis, effectively

increasing aerobic capacity and oxygen distribution (Varga et al. 2011, Ravnskjaer et al. 2010). This variance in lipid destinations provides several avenues to trace the specific intracellular effects of lipids on myoglobin expression that have, until this point, remained uninvestigated.

The fat of the matter: Investigating intracellular effects of lipids

It is still unclear if the mere presence of the lipids influences the cell to upregulate myoglobin or if the cell actually metabolizes the lipids, adapting itself to its metabolic environment. This opens the question: *does complete lipid metabolism result in increased myoglobin expression in C2C12 cultured cells?* Studies indicate that intracellular effects can still occur in the absence of lipid metabolism. However, the relationship between ROS (most likely produced during beta-oxidation and aerobic oxidative metabolism) and myoglobin expression cannot be discounted. Myoglobin expression can be ablated with the addition of an antioxidant (Schlater et al. 2014), demonstrating the appearance of myoglobin in response to the appearance of reactive oxygen species (ROS), primarily produced during the beta-oxidation process as fatty acids are fully metabolized. More specifically, if the cell is able to take up particular types of fatty acids more quickly than others or if they can increase expression of fatty acid transport proteins in response to lipids for a variation in its metabolic scope to a more lipid-based metabolism remains to be investigated.

The association between lipids and myoglobin, a component of the development of aerobic capacity suggests that lipid presence can potentially influence the ability of cells to adapt conditionally so as to metabolize lipids effectively. Lipids and myoglobin have been studied intermittently in cardiomyocytes, but work investigating lipids as a stimulant for myoglobin in

skeletal muscle has been lacking, particularly the investigation of the specific pathways or destinations of the lipids that precede myoglobin's appearance. In short: the Interaction ? between lipids added and myoglobin appearing is sorely incomplete and exploring this interaction will better uncover the mechanisms that facilitate not only myoglobin's expression, but also the triggers that nullify its arrival and the overall intracellular interplay between metabolic substrate and a key aerobic protein.

Overall, the purpose of this work is to investigate the specific intracellular effects (or lack thereof) of lipids on the expression of myoglobin. This specificity has been minimally investigated, and the proposal of new paradigms for the protein's appearance in skeletal muscle suggests a necessary investigation of the specific effect lipid supplementation has on myoglobin's expression. This research will continue elucidating the mechanisms that regulate the expression of the oxygen-binding protein myoglobin. We will resolve if increased expression of myoglobin is truly a direct reaction to intracellular lipid effects within the cell and begin to tease out other processes that contribute to the development of aerobic capacity through the expression of myoglobin. Studying its capabilities of oxygen release in low-oxygen conditions provides crucial information that can benefit victims of heart attacks, strokes, or ischemic reperfusion injuries, as well as contribute to our knowledge of physiological effects of diet, maintenance of aerobic capacity, and enrich our knowledge of development of diving ability in marine mammals. In effect, this work will elucidate some of the important cellular mechanisms driving the development of aerobic capabilities. My goal is to eventually extrapolate these data into the study of several aerobically adapted marine mammal species.

While we know C2C12s can take up lipids into the beta-oxidation process (Henique et al. 2010), it is not clear whether the increase in myoglobin expression is a product of this uptake,

metabolism, or more broadly, if the cells are even taking up the supplemental lipids at all. To achieve this goal, three experimental groups were used in cell culture. The control condition consisted of C2C12 skeletal muscle myoblasts grown in a standard media C2C12 consisting of high glucose (4.5 mg/mL) Dulbecco's Modified English Media (DMEM) and no supplemented lipid. The two experimental groups consisted of low glucose (1 mg/mL) DMEM and 2.5% supplemental lipid, and high glucose (4.5mg/mL) DMEM and 2.5% supplemental lipid. The lipids themselves were a cocktail consisting of both unsaturated and saturated fatty acids (2 μg/ml arachidonic acid, 10 μg/ml linoleic, linolenic, myristic, oleic, palmitic and stearic acid, 0.22 mg/ml cholesterol from New Zealand sheep′s wool). The final treatment group consisted of high glucose (4.5 mg/mL) DMEM and 2.5% supplemented lipid.

We utilized crude fat extraction methods on two levels: to determine the lipid concentration within the cells themselves, and on a temporal scale (Day 1 and Day 7) to determine whether or not lipids were being extracted from the media from initiation of differentiation to harvest for eventual fates within the cell. Myoglobin concentrations were determined across all treatment groups to determine the response of the protein to lipid supplementation. Finally, oxygen consumption rates were measured to give an index as to fatty acid oxidation resulting in increased consumption of lipid-supplemented cells from the control condition. Rather than assuming that lipids directly influence myoglobin, this research will elucidate the potential uptake and oxidation of lipids present in the media. The measurement of the cell's capacity to effectively move lipids into the cytosol from the exterior media can be achieved by measuring fatty acid transport proteins, namely Plasma Membrane Fatty Acid Binding Protein (FABPpm), as they can be directly stimulated by a lipid-based diet. Analyzing their concentration before and after lipid supplementation will indicate if the cell is adapting to

the presence of lipids. Fatty acid transport protein expression was measured through the usage of Western Blots.

We hypothesized that all lipid-supplemented groups would have an increase in myoglobin relative to the control condition. The low glucose/lipid treatment was a significant source of intrigue, as we hypothesized that restriction of glucose would force the uptake of lipids and demonstrate a much higher increase in myoglobin as compared to the other two groups. We hypothesized this would also translate to the increased expression of fatty-acid transporters as well, with the low glucose/lipid displaying a considerable increase in expression of transporters as compared to the other two groups. Finally, we expected this low glucose/lipid treatment to demonstrate significant oxygen consumption, an index of lipid usage that could be driving higher myoglobin.

The purpose of the following studies elucidates the link between supplemental lipids in media and the expression of the protein myoglobin. Overall, these specific effects have not been investigated, particularly within skeletal muscle. The relationship between lipids and myoglobin expression is one that is crucial to understanding alternate pathways of the protein's expression, removed from the calcineurin/NFAT pathway prevalent throughout the literature. The additional metabolic consequences through altering the glucose and lipid concentrations present throughout the media can further our understanding of the relationships of the fatty acid-glucose cycle and the metabolic consequences associated with increasing or decreasing glucose levels; a metabolic substrate that C2C12 cells preferentially run on. Through this work, we can further understand the requirements and pathways driving myoglobin expression, and the conditions that encourage or detract from its expression and subsequent physiological parameters in terrestrial mammals.

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CHAPTER 2: Where do they come from, where do they go? Uncovering lipid uptake within media, high-glucose and low

For submission to the Journal of Experimental Biology, June 2021

Summary

Lipids are utilized within multiple roles in mammalian skeletal muscle: from their usage as metabolic substrates, storage in lipid droplets for energy, sequestering into cell phospholipid membranes, or even as ligands to achieve gene regulation. The correlation between an increase in myoglobin expression resulting from the addition of lipids into the media leaves a gap in evidence of whether the lipids are directly affecting myoglobin expression through intracellular methods within skeletal muscle cells or stimulating cells extracellularly. Lipids themselves must be mobilized in a particular fashion for usage within the skeletal muscle, and even further for their usage within the cell itself. As a result, investigating the direct uptake and usage, or lack thereof, will provide an intrinsic link to connect the pieces of the protein puzzle. We hypothesized the increase in myoglobin within lipid-supplemented C2C12 mouse skeletal muscle cells would be a consequence of lipid uptake and the gathered lipids intracellularly stimulating FABPpm transporters to influence the continued uptake of lipids. Furthermore, we expected a varied uptake in lipid amounts dependent on glucose concentrations, as we hypothesized that limiting the amount of glucose would result in cells having to conditionally adapt to a higher lipid concentration, resulting in a higher lipid uptake in low glucose/lipid treatments and a subsequent increase in myoglobin expression. Our results demonstrated a significant difference within the high glucose and low glucose lipid-supplemented groups across days 1 and 7, but not between groups, indicating similar lipid uptake. In addition, cellular lipid concentration was

significantly different between the control and high glucose group. Furthermore, no significant change in FABPpm transporters was observed among the three treatments. Overall, we determined the capacity for C2C12s in multiple metabolic conditions to take up lipids. However, this uptake was found to be independent of treatment, suggesting that metabolic availability played a role in a cell's ability to express or downregulate myoglobin. The lack of a significant change in transporters was another finding that lipids were not intracellularly stimulating expected pathways, and the uptake was measured at different concentrations within the cells. This internal concentration of lipids and similar lipid uptake profiles, even though the cells were provided different metabolites, suggests an influence of both glucose and lipid on uptake and usage; and uptake and usage does not necessarily rely on availability. Overall, we found the ability of C2C12 cells to take up lipid, as they were taking them up similarly, but using lipids differently.

Introduction

Lipid introduction into mammalian skeletal muscle cells is a process facilitated by different pathways, dependent on metabolic demands and conditions ranging from fasting, rest, or exercise intensity (Stanley 2011, Purdom et al. 2018). The cellular machinery is also responsible for facilitating the transport of lipids (in the blood bound to albumin as free fatty acids, or FFA) across the cell membrane. Due to the hydrophobic structure characteristic of lipids, a transporter or chaperone is necessary for the introduction of lipids within the intermembranous area of the cell. This movement into the membrane is typically facilitated by fatty acid transport proteins (FATP) or fatty acid binding proteins (FABP), of which two transporters, plasma membrane fatty-acid binding protein (FABPpm) or Cluster of

Differentiation 36/Fatty Acid Translocase (CD36/FAT), are the primary chaperones within mammalian skeletal muscle (Bastie et al. 2004, Luiken et al. 2002). Once inside the cell, the fate of the lipids is dependent on multiple aforementioned factors.

Our lab has established an alternate pathway linking lipids with the expression of myoglobin. In addition to the primarily studied pathway involving the activation of calcineurin (directed from skeletal muscle activation), then dephosphorylating NFAT and MEF2 and activating myoglobin expression (Kanatous et al. 2008), a lipid link providing a myoglobin increase independent of skeletal muscle activation has been uncovered. Lipid supplementation in media has provided an increase in myoglobin expression in both marine and terrestrial mammal cells (DeMiranda et al 2012, Schlater et al. 2014). However, the exploration regarding the introduction of lipids and the response of increased myoglobin expression requires analysis. These specific mechanisms remain uninvestigated, and this work aims to further uncover the direct effect lipids hold over this protein's expression. To understand these effects, we must identify exactly what the fate of the lipids was within the culture conditions. Specifically, the investigation of the supplemental lipid destination at the level of uptake has yet to be completed. We were unsure whether uptake at the membrane level was occurring, or if lipids outside the membrane were somehow externally influencing cellular processes.

C2C12 cells are an immortal mouse skeletal muscle cell line that preferentially feed off glucose (Abdelmoez et al. 2020, Wong et al. 2020). Experimentally, glucose uptake has been demonstrated in C2C12s through simulated muscle contraction by electrical stimulation or the addition of caffeine (Rosser et al. 2009, Kolawole et al. 2011). Glucose uptake can also be triggered through the addition of insulin, which also promotes the growth of cells (Demetriadis et al. 2011, Nisr and Affourti, 2014,Wong et al. 2020). The activation of metabolic pathways by

insulin includes influencing GLUT4 receptors, the primary chaperone facilitating glucose entry into the muscle cells (Nedachi and Kanzaki, 2006, Stahl et al. 2002). Insulin also works to stimulate the uptake of fatty acids, mainly through inducing the translocation of fatty acid transporters (Luiken 2002, Nickerson et al. 2009). High lipid concentrations exterior to the cell have also experimentally correlated with increased fatty acid uptake (Alsabeeh et al. 2017, Glatz et al. 2010).

Lipid uptake has thus been experimentally shown to be achieved through multiple factors and investigating the specific amounts of uptake can elucidate the effects of a variable metabolic substrate environment. In an environment restricted of glucose, the system switches to lipolysis and fatty acid uptake/usage to satisfy metabolic demands (Schulz 2013), or increase in lipid resulting in reduction of glycolysis and increase in fatty acid oxidation (Morales et al. 2017). With our lipid studies, we have never investigated the effects of glucose restriction on lipid uptake. Restriction of glucose was achieved through low glucose DMEM, to encourage the uptake of lipids, which we hypothesized would facilitate increased myoglobin expression in the low glucose/lipid treatment group.

Based on these results, we expected to see an increased uptake in the low glucose/lipid treatment relative to the high glucose/lipid treatment. Lipid levels were maintained at 2.5% concentration to provide a basal measurement of lipid influences. We also examined the internal cellular lipid concentration after seven days of differentiation. This data was compared with the level of uptake to generate an understanding of the metabolic influences of lipids: was uptake happening, and if it was, did uptake particularly correlate with increased concentration of lipids within the cells?

Overall, we hypothesized that the low glucose and high glucose treatments with supplemented lipid would take up more lipids than the control condition, with the low glucose/lipid treatment taking up the largest amount out of the three groups. We also expected to see this uptake reflected in the internal cellular lipid concentration, with the highest amount of internal cellular lipids within the low glucose/lipid treatment group. Our results indicated the opposite, with the high glucose/lipid treatment group featuring an uptake rate that was not significantly different then the uptake rate of the low glucose/lipid group. The internal concentrations themselves was variable, with the control condition sequestering the most lipids within the cell, and the high glucose featuring the lowest internal concentration of all three groups.

This demonstrates an interesting balance between glucose and lipids affecting lipid uptake within skeletal muscle, as internal lipid concentrations could reflect eventual metabolism or membranous/cytotoxic elements with lipid uptake. Particularly with supplemented lipid, the lack of glucose restricted cells rapidly taking up lipids to account for an energetic change relative to the other two treatment groups gives way to new questions involving energetic availability and budgeting of glucose limited cells. Within marine mammals that rely on a lipid-based diet and conditions that facilitate myoglobin expression, the regulators of a protein that delivers a supply of oxygen during hypoxic diving bouts has been an active source of intrigue, and the discovery of similar cues within terrestrial mammals that live different lifestyles than marine mammals invokes investigation into the ubiquitous triggers that stimulate myoglobin expression in mammalian systems.

Materials and Methods

Cell culture

C2C12 cells are an immortal cell line isolated from mouse skeletal muscle. They were grown in P100 plates with standard high glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma Aldrich), 20% fetal bovine serum (FBS), 1% penicillin/streptomycin as an antibiotic, and 1% sodium pyruvate. Cells were grown in normoxic (21% O2) conditions and incubated at 5% CO2 and 37 degrees Celsius. They were passed at ~50% confluence and at an appropriate number plated onto 0.1% gel-coated P150 plates and differentiated once they reached ~90-95% confluence. Experimental groups consisted of nine P150 plates for a total of 27 plates (cell amt).

Differentiation media consisted of three treatments. The standard differentiation media was composed of high glucose DMEM, 5% equine serum, 10 ug ml-1 transferrin and 10ug ml-1 of insulin. The low glucose differentiation media with supplemented lipid consisted of low glucose (1 mg/mL) DMEM, 5% equine serum, 10 ug ml-1 transferrin 10ug ml-1 of insulin, and 2.5% lipid. High glucose differentiation media was composed of high glucose DMEM, 5% equine serum, 10 ug ml-1 transferrin and 10ug ml-1 of insulin, and 2.5% lipid cocktail. The lipid cocktail consisted of 2 μg/ml arachidonic and 10 μg/ml each linoleic, linolenic, myristic, oleic, palmitic and stearic acid, 0.22 mg/ml cholesterol from New Zealand sheep′s wool, 2.2 mg/ml Tween-80, 70 μg/ml tocopherol acetate and 100 mg/ml Pluronic F-68 solubilized in cell culture water (Sigma-Aldrich, SLCC5594). Cells were differentiated into myotubes for 7 days, with new media replenished every 24 hours. Harvesting took place within a laminar flow cell culture unit (Labconco Corporation Purifier Class II Biosafety Cabinet).

Harvesting took place with a lysis homogenization buffer composed of 79% phosphate-buffered saline, 20% glycerol, 1% Tween-20, 1mmol dithiothreitol and a protease inhibitor tablet. Plates were scraped with a rubber policeman and plates harvested into a microcentrifuge tube and then frozen at -80 degrees Celsius. Cells were subsequently thawed and centrifuged at 10,000 g, 4 degrees Celsius for 10 minutes, with the supernatant collected for protein and crude fat extraction.

Crude Fat Extraction

Crude Fat Analysis was performed based on a revised protocol from Folch et al. (Folch, 1957). 1mL of cell supernatant was weighed to amount to ~1-2g of weight. 20mL of 2:1 Choloroform:Methanol mixture was added to supernatant sample, then homogenized for 30 seconds. Sample was shaken for 20 minutes at ~100 RPM, then filtered through Whatman Filter Paper. 4mL of 0.9% NaCL was added and samples refrigerated overnight. After separating for 24 hours, scintillation vials were weighed, and lipid layer collected into vial from separated sample. Samples were placed in the NEvap until dry, dried in a hood for 2 hours, and then in a dry matter oven at 100 degrees C overnight. After desiccation, vials were then weighed, with the final weight subtracted from the initial vial weight. Percentages were calculated by dividing the final crude fat weight by the original sample weight, samples sizes of n=16

Media was collected at Days 1 and 7 of differentiation by pulling media off P150 plates (1-13 mL) and frozen at -20 degrees Celsius. Day 1 media was collected after the first 24h of differentiation and day 7 media collected after 24h on the 7^{th} day of differentiation. Media crude

fat analysis was performed similarly to cell crude fat extraction using crude fat extraction methods detailed previously.

Western Blots

Harvested protein was measured from Western blot methods previously utilized in our lab (Kanatous et al. 2009). Samples were normalized to the ubiquitous mammalian cytoskeletal protein, vinculin (124 kDa), (MH24 was deposited to the DSHB by Waterston, R.H. (DSHB Hybridoma Product MH24)). Fluorescence was detected utilizing a primary antibody for Asparatate aminotransferase, mitochondrial (47.5 kDa) (AFFN-GOT2-6B2 was deposited to the DSHB by EU Program Affinomics (DSHB Hybridoma Product AFFN-GOT2-6B2)), a genetic alias for FABPpm. Samples were controlled to rat tibialis anterior or soleus muscle homogenates (donated from samples donated by Colorado State University Laboratory Animal Resource Center, IACUC approved). Sample preparation involved a 1:1 ratio of SDS and 0.01% bromophenol blue, boiling for three minutes, icing for 2 minutes, and spinning through prepared glass wool columns at 16,000 rpm for 30 seconds. SDS-samples were then loaded into pre-made acrylamide gels (Bio-Rad) at 20 ug per well. Samples were then run on ice at 200V for 45 minutes in 1x tris-glycine SDS running buffer. Following transfer to nitrocellulose membranes, samples were probed with primary antibodies (detailed above). To allow for fluorescence, horseradish-conjugated secondary antibodies of goat anti mouse IgG (Invitrogen) were then combined with 1mL total SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific) and imaged with a BioRad ChemiDoc XRS+. Fluorescence was then quantified through the Image Studio Lite program.

Statistical Analysis

Statistics were assessed utilizing either one or two-way analysis of variance (ANOVA) and pairwise t-tests. Significance was set at a P value < 0.05, and all data presented as means ±standard error. Samples were run in triplicate and statistical tests were performed in R Studio, and all figures generated within R Studio.

Results

Lipid concentration within cells

Crude fat extraction methods to examine lipids within the cellular concentrations demonstrated a significant difference between crude fat percentage in the control condition (0.408 ±0.0714, n=16) as compared to the high glucose/lipid condition (0.2501 ±0.0347, n=16, $p<0.05$). Low glucose/lipid conditions exhibited a trend that was lower in concentration than the control and higher than the high glucose/lipid treatments, but was not significantly different (0.3449 ±0.042, n=16). Overall, there was a trend of decreased fat content within the cells across control>low glucose> high glucose (Fig. 2-1).

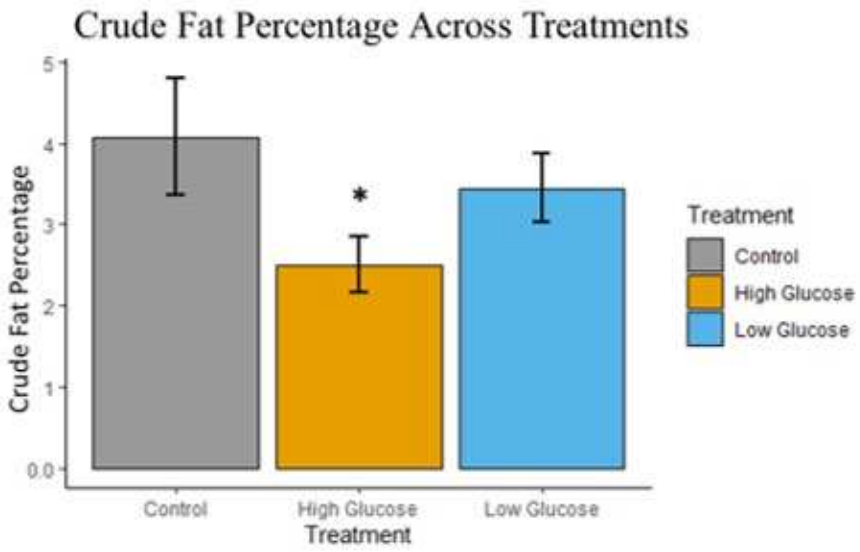

Figure 2-1: Internal cellular crude fat percentage across treatments. A * denotes significance from other groups, with significance determined at p≤ 0.05. High glucose/2.5% lipid displayed a significant difference in crude fat percentage (p=0.0306)

Lipid Change Within Media

Crude fat levels within the media across treatment groups demonstrated a significantly (p=0.0306) elevated crude fat percentage within lipid-supplemented treatments at Day 1 (High Glucose= 0.3688±0.049, n=15; Low Glucose= 0.4043±0.0373, n=14) as compared to the control condition (0.1807±0.043, n=15), indicating success of the crude fat extraction in detecting supplemental lipid. A pairwise comparison between crude fat percentage for low glucose/lipid and high glucose/lipid treatments also did not show a significant difference (p=0.8577). From Day 1 to Day 7, lipid within the media decreased across all treatments (see Fig. 2-2). Both the low glucose/lipid and high glucose/lipid treatment demonstrated a change that was not statistically significant from Day 1 to Day 7 (low p=0.1686, high p= 0.3370, n=9). The control condition featured a decrease in lipid concentration between Day 1 and Day 7 that was also not significantly different (p=0.0864) from both lipid supplemented treatments in final crude fat

percentage (0.0774±0.0244, n=9), (Fig. 2-2). Overall, while there is a trend for a decrease in fat across all three treatments, it was not a statistically significant one. However, when treatments were compared by Day, the control conditions featured a significant difference in overall crude fat percentage than the lipid supplemented groups ($p<0.0001$), (Table 2-1).

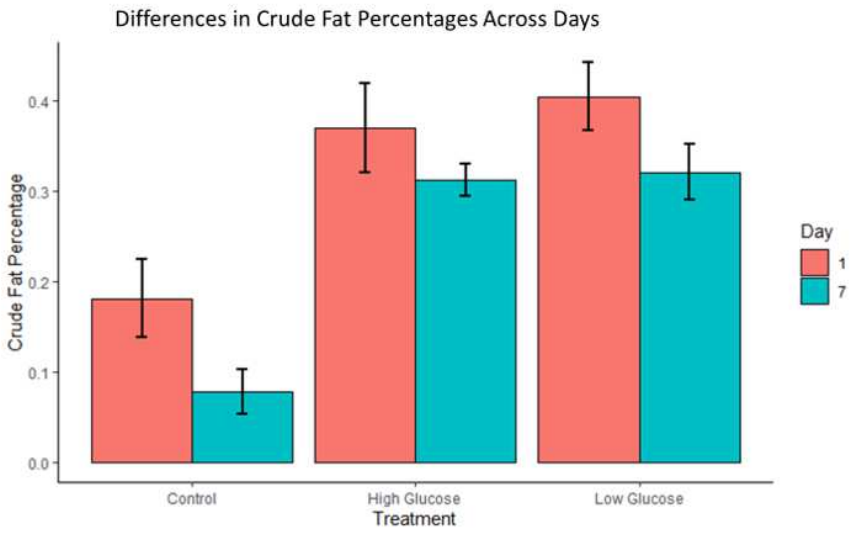

Figure 2-2: Crude fat percentage within cell culture media across Day 1 (24 h on the first day of differentiation) and Day 7 (24 h on the last day of differentiation). A downward trend across treatments was observed, but no significant difference detected across groups.

When running pairwise comparisons,, comparing just days gives a significant difference from Day 1 to Day 7 ($p=0.0209$) (Fig. 2-3).

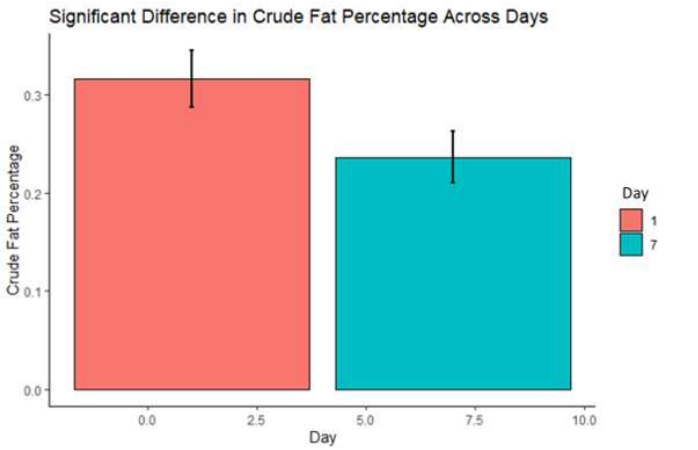

Figure 2-3: Significant Differences in Crude Fat Percentage Across Days. Pairwise comparison of days 1 and 7 independent of treatment displayed a significant difference (p=0.021) in crude fat percentage across days. A * denotes significance, established at p<0.05.

Table 2-1

a: Pairwise comparisons between Treatments across Days 1 and 7, showing no significant difference in fat uptake

b: Control conditions demonstrate significant differences in crude fat percentage across Days 1-7 from both lipid supplemented groups. (* denotes significance of p <0.0001)

c: Averages of just days independent of treatment show a significant difference in crude fat percentage (* denotes significance of p<0.05)

Pairwise Comparison	Groups	P-value
a) Treatments: Days 1-7	Control 1-7	0.0884
	High Glucose 1-7	0.3370
	Low Glucose 1-7	0.1686
b) Treatments: Treatments	Control-High Glucose	<0.0001**
	Control-Low Glucose	<0.0001**
	High Glucose- Low Glucose	0.8577
c) Days, No Treatments	1-7	0.0209 *

Western Blots

Western blots to analyze the protein expression of FABPpm yielded no significant difference (p=0.273) between the high glucose (n=6) and low glucose (n=4) treatments, or the control (n=6)from the low or high lipid supplemented treatments (p=0.5894 and 0.5213, respectively). A two-way ANOVA comparing all three groups indicated no significant difference (p=0.5301) in relative protein expression of FABPpm. Overall, there was a trend towards decreased protein expression in low glucose/treatment groups as compared to the control condition, and a relative increase in the high glucose/lipid treatment as compared to both the control and low glucose/lipid group (Fig. 2-4 and 2-5).

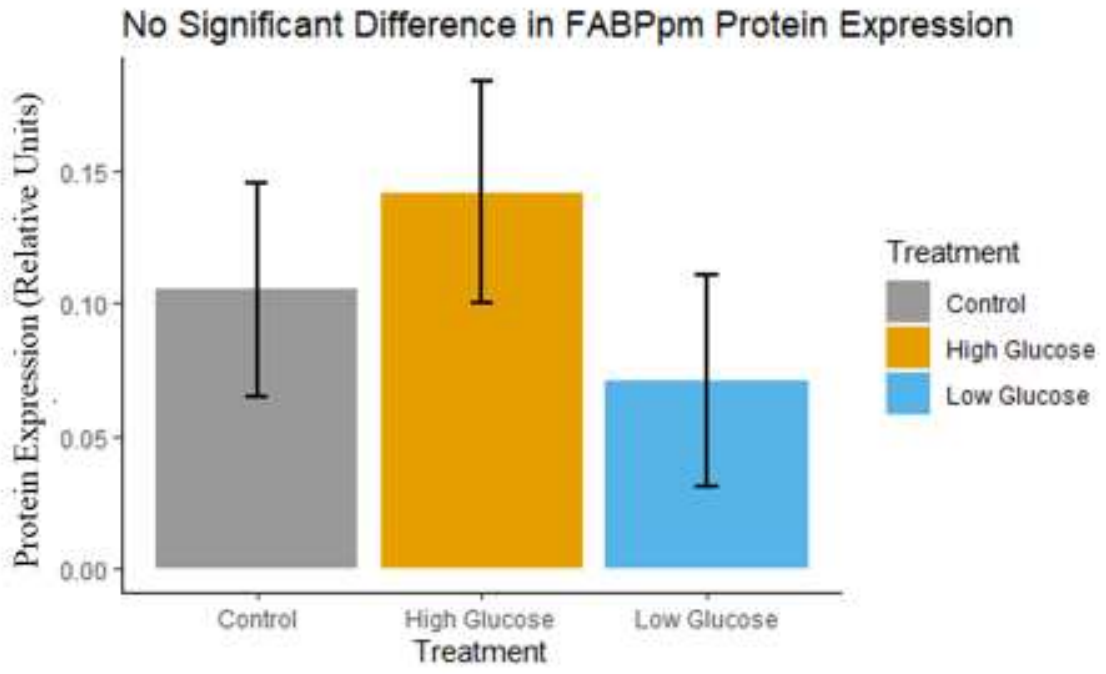

Figure 2-4: Plasma Membrane Fatty Acid Binding Protein (FABPpm) expression across treatment groups. Protein expression is notated in relative units. Significance was determined at $p \leq 0.05$. No significant difference in expression was observed across three treatments.

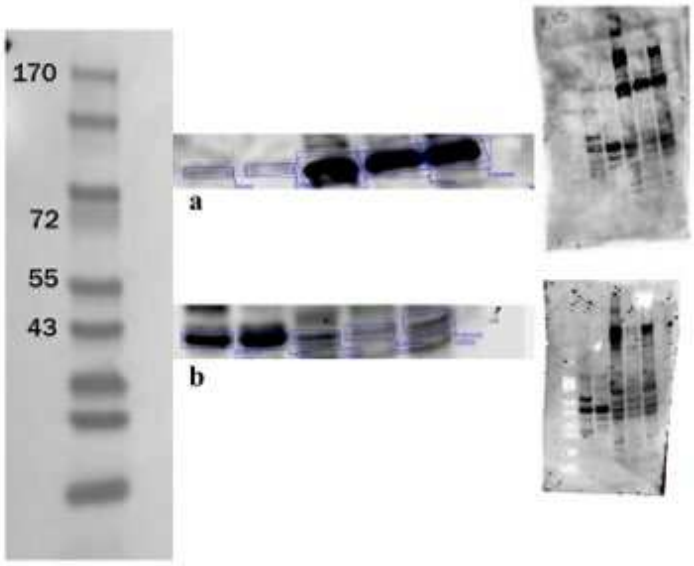

Figure 2-5: Western blots for FABPpm (43 kDa) normalized to vinculin (124 kDA) and visualized next to whole western images to detail fluorescence

Discussion

This work allows for further understanding of the specific habits of lipids under differential glucose conditions in mouse skeletal muscle cells, demonstrating the uptake differences and reflected transporter regulation under these conditions. The main findings *include 1) similar fatty acid uptake profiles among lipid supplemented treatments, regardless of high or low glucose level 2) variable internal fatty acid profiles among C2C12 cells under lipid supplementation and 3) no significant upregulation of FABPpm transporters during lipid supplementation.* While we saw a trend towards lipid uptake in all three groups, contrary to our hypotheses, the low glucose/lipid treatment did not display a higher amount of lipid uptake relative to the other two groups. In addition, the lack of significant upregulations in FABPpm transporter expression in either lipid supplemented treated compared with the control condition was also a departure from our initial hypothesis. These findings indicate that lipid uptake and

cellular transporter response is a complex response that requires conditions beyond lipid availability, and metabolic conditioning at the cellular levels is subject to additional factors that drive cellular processes to adapt to a lipid-supplemented environment.

Comparing Days 1 to 7, while there were no significant differences in lipid uptake across the three groups, the control condition featured a slightly increased uptake profile compared with the lipid-supplemented groups. This was also contrary to our hypotheses, as we expected lipid-supplemented groups to take up more lipid as an available substrate, particularly the low glucose group. However, when ignoring treatments, there is a significant difference between Days 1 and 7, showing that lipid uptake is happening, and whatever uptake is happening is independent of treatment (see Table 2-1). As C2C12 cells preferentially run off glucose, we were unsure if they would even have the capacity to bring in fat, and while they are, it seems that different metabolic profiles and varying amounts of glucose does not entice cells to bring in more lipid. Particularly as we see variable responses in lipid profiles within the cells and different responses of myoglobin (see Chapter 3), we would have expected treatment to have a much larger effect on lipid uptake. Particularly as both lipid-supplemented groups took up lipid at nearly the same rate, yet expressed differential internal concentrations (and no effects on FABPpm transporters), this suggests that there are more intricacies to cellular metabolism than just the introduction of lipids, and perhaps other stimuli are needed for particular responses.

These results suggest that the detraction from expectations that lipid presence alone (in conjunction with insulin, which typically induces substrate uptake) is not adequate to promote a significant intracellular response to environmental metabolic conditions. This is contrary to other research that indicates consumption of a high fat diet drives expression of FABPpm transporters (Roepstorff et al. 2004). However, this research has mainly been a function of whole-body

systems, with a larger temporal scale over several months rather than days. These results are most likely a function of a cellular environment, and we can continue to tease apart the specific conditions that provoke the results at a larger level, such as hormonal and whole-body exercise responses.

The low glucose and lipid condition were a new exploration investigating if we could metabolically drive lipid uptake by limiting glucose availability, which based on these results appears to be an incorrect assumption. The similar uptake levels seen in the high glucose/lipid treatment indicate that regardless of glucose levels in skeletal muscle, lipid uptake remains relatively constant without the introduction of additional physiological parameters. Moreover, the appearance and overall differentiation of the low glucose/lipid cells demonstrated an adaptive struggle, as the cells had a large turnover period and visible loss of density during the differentiation period (Figure 2-6).

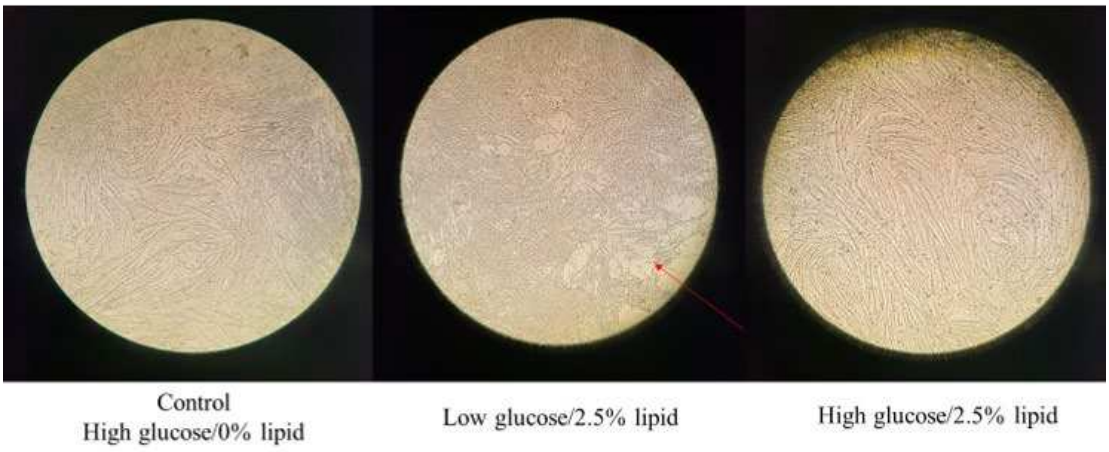

Figure 2-6: Microscopic photographs of fully differentiated myotubes. Red arrows denote cell death areas. Low glucose/2.5% myotubes exhibited pockets of dead cells and significant cell waste.

Following differentiation, there were marked microscopic differences between cell treatments. While the control and high glucose/lipid treatments featured marked cell growth and activity in response to their respective media, the low glucose/lipid treatment initially featured an intense cell turnover period between 1-3 days of differentiation. There was increased cell waste relative to the other conditions, and visual pockets where cells were missing was apparent. However, after this initial 'slough' stage, low glucose/lipid cells were able to maintain the remaining distribution in the final days of differentiation. The control and high glucose condition were extraordinarily receptive to their respective differentiation media, reflected in the high protein concentrations and maintenance of activity and confluence throughout seven days of differentiation.

At an observable level, the pH change across media was also readily noticeable, as DMEM changes from a reddish to a pink or orange in response to less or more acidity, respectively, owing to cellular activity and metabolism producing byproducts affecting the pH. The high glucose/lipid condition demonstrated the highest activity, reflected in the light orange color of the pH after 24h of differentiation, whereas the control remained the standard reddish hue and the low glucose/lipid condition exhibited a pink color.

It has been demonstrated that it is difficult for C2C12 cells to adapt to lipid concentrations beyond 5% (DeMiranda et al. 2012), but to our knowledge this is the first visualization of the effects of low glucose/lipid on C2C12 skeletal muscle cells. It is very likely that the low transporter expression and confluency struggles are a consequence of this difficulty, as the cells may not have had the energetic budget to dedicate to production of new machinery or utilization of oxygen to metabolize a lipid-heavy media. However, as seen in the high glucose/lipid treatment, which were healthy cells based on their growth and activity rate, even

healthy cells did not move lipids to a large degree with their supplementation, indicating the lipid uptake results we saw were not a function of health and more one of the metabolic needs of the cells independent of treatment.

The ability of the cells to conditionally adapt to the lipid-supplemented conditions were generally minimal. While we expected an increased response in FABPpm transporter expression, with the exception of the low glucose treatment (addressed above), there was no significant difference in expression between the control and high glucose/lipid condition. Even so, the high glucose/lipid condition contained a lower internal lipid concentration than the other treatments and based on those results we would have expected a larger transporter response. The cells were differentiated for seven days, with research establishing that four days of differentiation is adequate for the expression of FABPpm transporters (Newsom et al. 2015). There was an upward trend within high glucose/lipid cells, but not significant enough to vary from the control condition.

Within skeletal muscle, fatty acid uptake is also regulated by CD36. Translocation of this transporter regulates fatty acid uptake and has also been experimentally shown to be upregulated with fatty acid presence (Nickerson et al. 2009). However, as FABPpm and CD36 can work in tandem, and expression or ablation of one reflects the other(Glatz et al. 2001), it is very likely that the responses we see in a lack of upregulation of FABPpm could also extend to CD36 as well. Future directions will explore the expression of CD36 to examine this relationship at the cellular level. Based on these results and low differences in uptake with lipid supplementation, it is likely that there will not be a significant difference. Overall, transporter response is most likely more of a function than simply supplementing with lipids and investigating other links to

other proteins that correlate with lipid supplementation is key to understanding why lipids function to regulate expression of some proteins and not others.

Through past studies in our lab, we have been investigating the links between lipid between lipid supplementation and expression, and this work adds information previously assumed. It is necessary to emphasize that while the low and the high glucose treatments with supplemented lipid took up fat across Day 1 and Day 7, both essentially took up the same percentage of crude fat, regardless of glucose concentration. This lack of significant uptake of lipids, even with variable glucose concentrations, suggests that even with different metabolic environments, the intracellular response (if one at all) is relatively similar on the basis of lipid uptake, and instead other factors most likely regulate the responses we have observed.

An additional point to be made focuses on the similar minimal lipid uptake, yet variable internal cellular lipid concentrations for both lipid groups. The treatments which contained 2.5% lipid concentration and high or low glucose provided for different observed differences in intracellular lipid concentration. The metabolic effect of variable glucose most likely had a significant effect on the storage or consumption of lipids. Especially as insulin is a stimulant for glucose uptake, so to does it function to also bring in fatty acids, and what minimal uptake there was most likely functioned because of factors that provide for both glucose and lipid uptake.

Finally, while FABPpm and lipid uptake profiles were not significantly different in the groups, the results that were substantially different were the internal lipid concentrations of the cells. Particularly in the high glucose/lipid condition, which exhibited the lowest level of three treatments, investigating the fate of the lipids is key to understanding effects of lipids when they do get into the cell. As the extraction would have picked up any lipids put into droplets or the phospholipid membrane, the likely fate of the lipids was most likely metabolic purposes. It is

worth noting that the control condition, which exhibited the highest internal lipid concentration, also featured the most significant lipid uptake between days 1 and 7. This suggests that the control condition would feature a higher lipid concentration, and the 'low' internal lipid concentration in high glucose is just a factor of minimal lipid uptake rather than a stark deviation from the control condition. Establishing the metabolic profile of these cells will help further understand and provide concrete evidence of the metabolic habits of C2C12 skeletal muscle cells under differential substrate conditions.

Overall, this work has demonstrated that lipid supplementation is not adequate to promote whole body effects of transporter expression, and insulin itself, while experimentally shown to induce lipid uptake, may not be enough to promote a significant lipid uptake that reflects these experimental effects. Instead, other factors in conjunction with lipids must be required to exhibit the physiological effects that in the past have been associated with simple lipid supplementation and teasing apart these effects is key to understanding this diverse substrate. Especially as palmitic acid provokes insulin resistance and restrict facets of fatty acid metabolism (Cotta et al. 2017), exploring avenues devoid of this fatty acid, or even unsaturated ones that experimentally demonstrated their promoting effects on fatty acid metabolism (Nakamura et al. 2001) is a necessary next step to ensure results investigating lipids and their effects are not compounded. Lipids are not a solitary monolith with numerous carbon chains and types of fatty acids, and exploration of different types of fats is an exciting avenue to understand the specificity of lipids within mammalian systems.

Future Directions

Future directions of this work will involve building a metabolic profile for the treatment groups by collecting oxygen consumption data and respiratory quotient to establish if the differential lipid concentration within cells is a result of beta-oxidation and lipid metabolism. In addition, studying the expression of CD36 will be a two-pronged approach to determine if there is an increased expression of the protein in response to supplemental lipid, or if, like previous studies, additional data can determine if CD36 and FABPpm indeed work in tandem, and expression of one indicates the other. The expression of carnitine palmitoyl transferase (CPT), that facilitates the movement of fatty acids into the mitochondria for beta oxidation can also give an indication of whether or not the lipids taken up are a) destined for beta oxidation and b) if this uptake correlates with transporters beyond the plasma membrane. Finally, the effects of specific types of fats or variable combinations could give further insight into effects of fats on uptake, expression, or metabolism, as past research indicates some fatty acids have complicating or promotional qualities on cellular processes (Nisr and Affourti, 2014, Kazantzis and Stahl, 2013).

Funding

Funding was made possible due to the National Science Foundation

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Chapter 3: What's In the β-Ox: Does metabolizing lipids achieve myoglobin expression?

For submission to the Journal of Experimental Biology, June 2021

Summary

Myoglobin, an oxygen-binding protein that facilitates oxygen delivery during ischemic or low oxygen periods is a protein whose expression patterns are pervasive. Typically, in marine mammals the expression of this protein can be up to ten-fold the amount seen in terrestrial mammals. Particularly in marine mammals, a lipid-based diet has long been thought to somehow contribute to their aerobic performance, aided by the expression of myoglobin, which exhibits a close relationship with lipid supplementation. Experiments have shown that cues that regulate its expression in marine mammals seem to possess the same effect in terrestrial mammals as well, and that lipids alone exhibit an influence on myoglobin expression independent of the most well-studied pathways involving skeletal muscle activating calcineurin. However, the threads tying together this complex web of diet and molecular responses has hardly been explored, and the specific effects lipids hold over directing myoglobin expression remain to be answered. The present study explores the role of lipids and glucose in terrestrial animal skeletal muscle, exploring the effects of lipid uptake on myoglobin expression. The use of respirometry to establish oxygen consumption and any metabolism of lipids was also performed. These experiments served to establish if the direct metabolism of lipids can account for myoglobin increases in terrestrial mammal cells, or if the lipids influence other aspects of cellular response. It is possible that the complete metabolism of lipids in the beta-oxidation process stimulates myoglobin, either through the production of reactive oxygen species (ROS) or a still unexplored

mechanism. The exploration of its expression patterns holds important information regarding the role of lipids as an intracellular influencer and the ability to determine whole animal physiological implications from cellular mechanisms. Based on previous work, we hypothesized the high glucose/lipid supplemented treatment that experimentally demonstrated an increased myoglobin concentration would exhibit the highest oxygen consumption and subsequent ROS production. Initial data demonstrates that the high glucose/lipid treatment is the most active with the largest oxygen consumption of the three treatments and the highest myoglobin expression. These results indicate that it is not simply lipid uptake or direct ligand influence, but instead cellular activity facilitated by metabolic aspects of diet that is responsible for myoglobin upregulation.

Introduction

Lipids provide multiple roles within the mammalian physiological pathway, either through storage as lipid droplets (Welte and Gould, 2017), ligands that can promote upregulation of proteins (Hardie 2007, Ravnskjaer et al. 2010, Wolfrum et al. 2001, Varga et al. 2011), or their oft studied role as a metabolic substrate providing cellular energy (Alsabeeh et al. 2017, Askew 1984, Cooney et al. 2014, Horowitz et al. 2000, Kanatous et al. 2002). Lipids must be mobilized from adipocytes or storage droplets for cellular energy, which requires their binding to a carrier protein, typically albumin (Glatz 2001, Stahl and Kazantizis 2011). This complex travels through the bloodstream to transport proteins on the cell membrane, both allowing for the hydrophobic lipid to move to its destination effectively and safely without producing cytotoxicity once inside (Schaffer 2003).

Depending on metabolic needs of the cell, the lipid (mobilized as a free fatty acid) is destined for different fates. In a cellular environment where they must be metabolized, lipids are the primary fuel so as to reserve glucose stores for glucose-exclusive organs such as the brain. This can change depending on energetic needs and exercise intensity (Stanley 2011, Purdom et al. 2018). The movement of lipids into the beta oxidation process produces two NADH molecules and acetyl CoA that can be shunted into the tricarboxylic acid cycle, eventually producing adenosine triphosphate (ATP) in the electron transport chain (ETC), providing energy to power muscular work and other processes.

Within the beta oxidation process, as in other metabolic pathways, oxygen is the primary electron acceptor to produce ATP and CO2 (Talley and Mohiuddin, 2021), and we can derive which specific metabolic substrates being metabolized for energy. In the beta oxidation process and the electron transport chain, CO2 as well as reactive oxygen species (ROS) like hydrogen peroxide (H2O2) are produced, as the movement of electrons generates conditions that produce different types of ROS. ROS are not uncommon to cellular aerobic processes and can be found in large supply throughout the beta oxidation process (Cortassa et al. 2017, Schlater et al. 2014), as well as periods of oxidative stress, conditions ripe within working skeletal muscle (Serra et al. 2018). Experimentally, just as lipids correlate with increased myoglobin production, so to does myoglobin expression accompany conditions prevalent with ROS (Schlater et al. 2014).

We have discovered myoglobin's function not only as an oxygen-binding protein, but also as a ROS scavenger (Schlater et al. 2014). Introduction of an antioxidant that reduces ROS numbers also decreases the amount of myoglobin present, suggesting that myoglobin may not be regulated directly by lipids intracellularly, but instead as a function of their complete metabolism. Completion through the beta oxidation process generating ROS could subsequently

activate myoglobin as a dual approach through increased activity and oxidative stress brought on by a large accumulation of ROS.

The role of oxygen in processing lipids is well documented, and in relation to glucose requires a higher overall oxygen cost to process (Leverve et al. 2007). In addition, different fatty acids require different amounts of oxygen for their complete metabolism (Savage and Goldstone, 1965). Biochemically, a higher oxygen consumption can be indicative of increased reliance on lipids as fuel. We have hypothesized that cells with the highest myoglobin expression will also feature the highest oxygen consumption, indicating the utilization of lipids as fuel and potential increased ROS production (Fig. 3-1).

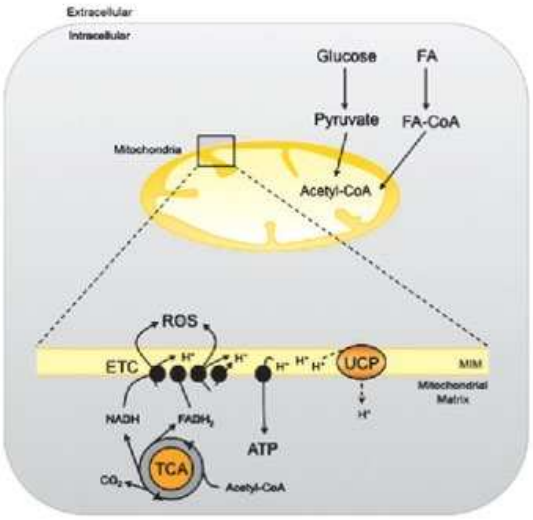

Fig. 3-1: The interplay between glucose and fatty acids and their production of acetyl coA. Upon shuttling of acetyl-CoA int the mitochondria, the breakdown of acetyl coA in the TCA cycle and entry of produced units into the ETC generates usable ATP for energy as well as ROS *(Serra et al. Mitochondrial fatty acid oxidation in obesity, 2013).*

We hypothesized that all lipid-supplemented groups would have an increase in myoglobin relative to the control condition. Experimentally we have demonstrated that high glucose media with 2.5% lipid supplementation significantly increased myoglobin production

relative to the standard high glucose/no lipid condition C2C12 cells are normally grown in. The low glucose/lipid treatment was a significant source of intrigue, as we hypothesized that restriction of glucose would force the uptake of lipids and demonstrate a much higher increase in myoglobin as compared to the other two groups. Finally, we expected this low glucose/lipid treatment to demonstrate significant oxygen consumption, an index of lipid usage that could be driving higher myoglobin. Our results instead indicate the opposite, as the high glucose/lipid condition featured the highest myoglobin concentration, with myoglobin in the low glucose/lipid condition seemingly downregulated (as compared to the control). However, within the oxygen consumption measurements we saw a significant amount of oxygen consumption in the high glucose/lipid cells relative to the control that featured no lipids.

Understanding the role of lipid in myoglobin regulation is critical to understanding the role of metabolic substrates on overall physiological capabilities. We have shown that myoglobin is privy to similar cues in animals that live radically different lifestyles, and more likely than not these lifestyles can contribute to the expression of the protein rather than an innate natural expression of large quantities of myoglobin. In short: diet as a part of these lifestyles cannot be discounted on overall aerobic performance, function, and survival in mammalian systems, and understanding the specific effects and consequences of diet can resolve numerous questions around lipid's role as an intracellular influencer or metabolic maestro.

Materials and Methods

Cell culture

C2C12 cells are an immortal cell line isolated from mouse skeletal muscle. They were grown on P100 plates with standard high glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma Aldrich), 20% fetal bovine serum (FBS), 1% penicillin/streptomycin as an antibiotic, and 1% sodium pyruvate. Cells were grown in normoxic (21% O2) conditions and incubated at 5% CO2 and 37 degrees Celsius. They were passed at ~50% confluence and at an appropriate number plated onto 0.1% gel-coated P150 plates or 12-well plates with 13mL or 1mL of differentiation media, respectively. Cells were then differentiated once they reached ~90-95% confluence and allowed to differentiate for seven days. One 12-well plate generated three samples per treatment.

Differentiation media consisted of three treatments. The standard differentiation media was composed of high glucose (4.4 mg/mL DMEM, 5% equine serum, 10 ug ml-1 transferrin and 10ug ml-1 of insulin. The low glucose differentiation media with supplemented lipid consisted of low glucose (1 mg/mL) DMEM, 5% equine serum, 10 ug ml-1 transferrin 10ug ml-1 of insulin, and 2.5% lipid. High glucose differentiation media was composed of high glucose (4.5 mg/mL) DMEM, 5% equine serum, 10 ug ml-1 transferrin and 10ug ml-1 of insulin, and 2.5% lipid cocktail. The lipid cocktail consisted of 2 μg/ml arachidonic and 10 μg/ml each linoleic, linolenic, myristic, oleic, palmitic and stearic acid, 0.22 mg/ml cholesterol from New Zealand sheep′s wool, 2.2 mg/ml Tween-80, 70 μg/ml tocopherol acetate and 100 mg/ml Pluronic F-68 solubilized in cell culture water (Sigma-Aldrich, SLCC5594). As results in our lab previously indicated, a lack of glucose with supplemental lipid would have resulted in no cell growth and a waste of resources, so this condition was not used. Cells were differentiated into myotubes for 7

days, with new media replenished every 24 hours. Harvesting took place within a laminar flow cell culture unit (Labconco Corporation Purifier Class II Biosafety Cabinet).

Harvesting took place with a lysis homogenization buffer composed of 79% phosphate-buffered saline, 20% glycerol, 1% Tween-20, 1mmol dithiothreitol and a protease inhibitor tablet . Plates were scraped with a rubber cell scraper and plates harvested into a microcentrifuge tube and then frozen at -80 degrees Celsius. Cells were subsequently thawed and centrifuged at 10,000 g, 4 degrees Celsius for 10 minutes, with the supernatant collected for protein analysis. Protein concentration was measured in a DU800 spectrophotometer analyzing absorbance with Pierce Coomassie Plus Protein Assay Reagent.

Myoglobin Expression

Myoglobin measurements were obtained through previous lab protocols (Kanatous et al. 1999, Kanatous et al. 2002), modified from Reynafarje, 1963. 0.04 mmol-1 potassium phosphate buffer was added to sample and centrifuged at 28,000 g for 50 minutes at 4 degrees C. The resulting supernatant was bubbled with 99.9% carbon monoxide for 3 minutes. Absorbance was then read at 538 nm and 568 nm. After normalizing to original protein concentration, functional sample myoglobin is reported in mg myoglobin per mg protein and standardized to rat tibialis anterior muscle.

Respirometry

Respirometry analysis was performed in a quad mass 497 spectrometer (Pfeiffer PrismaPlus QMS200; PTM28612), utilizing methods from Andersson et al. 2019. Cells were harvested from plates ~5 minutes before respirometry analysis. Media was removed from plates, 0.5 mL of PBS added and cells were scraped with a rubber policeman and collected into a microcentrifuge tube. 12-well samples were collected into one tube/well, while P150 plates were collected into three tubes to be run in triplicate. Samples were resuspended in fresh media and maintained at 37 degrees Celsius in a Styrofoam box during transport. Cell samples were analyzed in cuvette and oxygen allowed to normalize before reading consumption rates for ~300-400s. Samples were recollected, respun, and analyzed for protein in a DU800 spectrophotometer analysing absorbance at 595 nm with Pierce Coomassie Plus Protein Assay Reagent.

Statistical Analysis

All samples were run in triplicate. Statistics were assessed utilizing one-way analysis of variance (ANOVA) and pairwise t-tests. Significance was set at a $P < 0.05$. Samples were run in triplicate and all statistical tests were performed in R Studio.

Results

Myoglobin concentration

Myoglobin concentrations indicated an apparent increasing trend ($p=0.07$) in the high glucose/supplemented lipid condition, as we have seen in previous work (DeMiranda et al. 2012,

Schlater et al. 2014), although there was statistically no significance between high glucose/lipid and control (n=16) conditions (p=0.1616). There was a significant difference in myoglobin concentration between high glucose/lipid (1.343±0.349, n=18) and low glucose/lipid (0.5642±0.1239, n=19) conditions (p=0.024) (Fig 3-2).

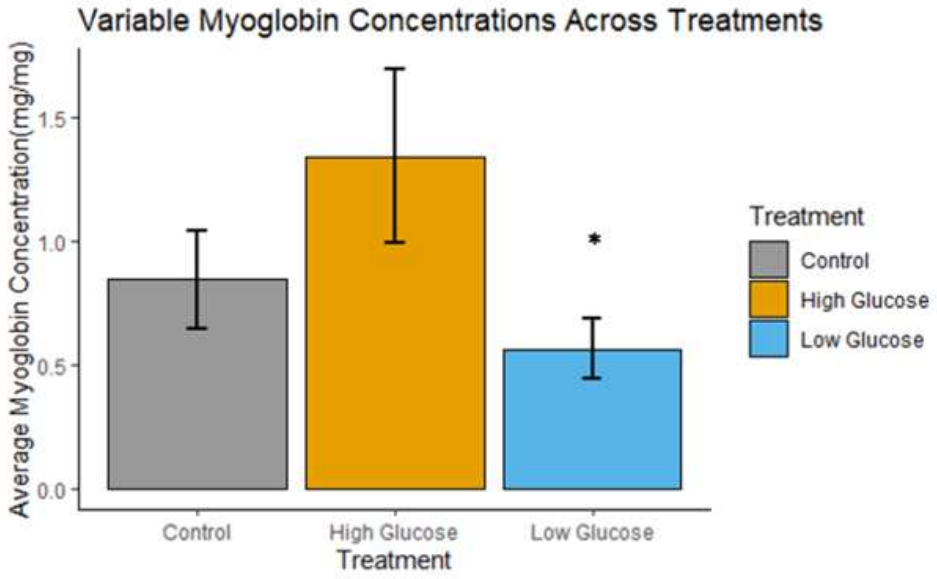

Figure 3-2: Average myoglobin concentrations across treatment groups, presented in mg myoglobin/mg protein. There was an upward trend between control and high glucose/lipid conditions, with a significant decrease in low glucose/lipid from the other two conditions. A * denotes significance, with significance determined at $p \leq 0.05$.

Oxygen consumption across treatment groups

Oxygen consumption demonstrated an upward trend between the control and high glucose condition, but was not a statistically significant difference (p=0.076) between control (0.3256±0.1689, n=3) and high glucose/lipid (4.4126±2.026, n=3). Low glucose/lipid displayed an upwards trend from the control condition (1.734±0.0756, n=2) (Fig. 3-3).

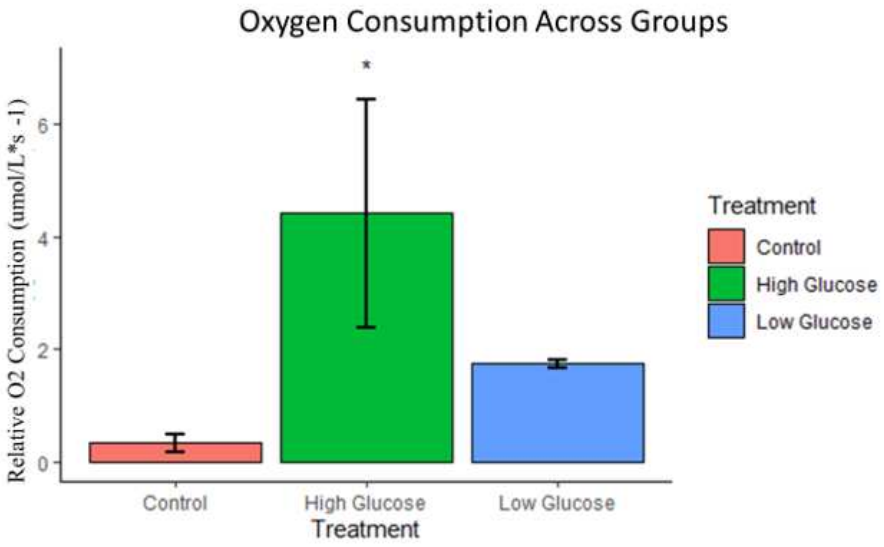

Figure 3-3: Oxygen consumption across treatments. There is an upwards trend of the two lipid-supplemented groups from the control condition. Significance determined at $p<0.05$.

Discussion

Our results are an addition to our previous work clarifying the link between lipids and myoglobin expression. Regarding previous work, we have found that both high and low glucose supplemented cells feature a significant rate of lipid uptake across days, but not across treatments. We aimed to extend that knowledge to this work, observing if similar lipid uptake but differential glucose concentrations resulted in variable intracellular response, particularly as it related to myoglobin expression. Our lab has previously reported a link between supplemental lipid/high glucose treatment and myoglobin expression in both marine (DeMiranda et al. 2012) and terrestrial mammal cells (Schlater et al. 2014). Additionally, we wanted to observe the effects of limiting glucose and supplementing with 2.5% lipid. We expected a more expressed myoglobin in the low glucose/lipid condition over the other two groups. Instead, we saw the opposite, where, although high glucose/lipid expressed more myoglobin than the control

condition, the low glucose/lipid presented a significantly lower amount of myoglobin than both the control and high glucose conditions. To our knowledge, this is the first study documenting the decrease of myoglobin in low glucose/lipid supplemented treatment. As it was contrary to our hypothesis, where we were expecting a significant increase of myoglobin in low glucose conditions, as we believed restricting glucose availability would force the uptake and subsequent oxidation of lipids for cellular metabolic requirements.

The low glucose and lipid condition was a new exploration investigating if we could metabolically drive lipid uptake through limiting glucose availability, which based on these results appears incorrect. Moreover, the appearance and overall differentiation of the low glucose/lipid cells demonstrated an adaptive struggle, as the cells had a large turnover period and visible loss of density during the differentiation period (Figure 3-4).

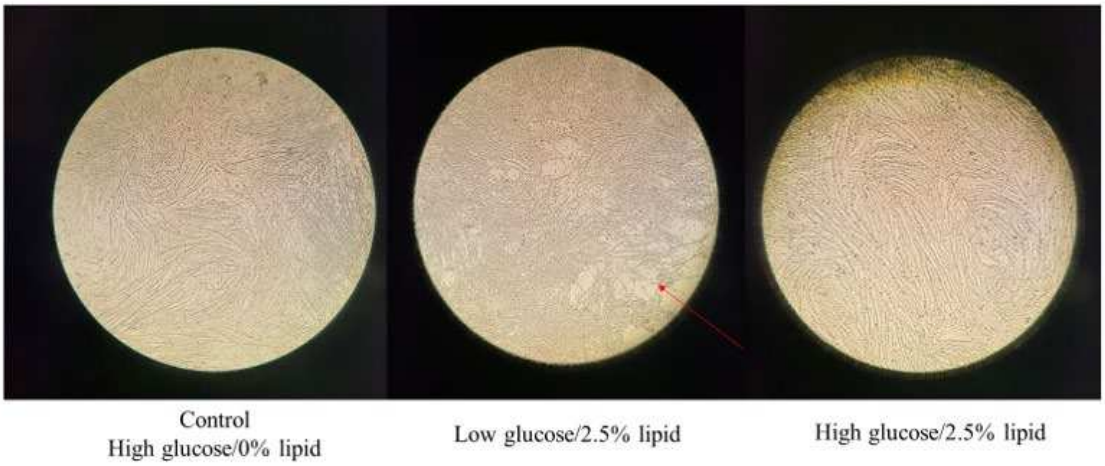

Figure 3-4: Microscopic photographs of fully differentiated myotubes. Red arrows denote cell death areas. Low glucose/2.5% myotubes exhibited pockets of dead cells and significant cell waste.

Following differentiation, there were marked microscopic differences between cell treatments. While the control and high glucose/lipid treatments featured marked cell growth and activity in response to their respective media, the low glucose/lipid treatment initially featured an

intense cell turnover period between 1-3 days of differentiation. There was increased cell waste relative to the other conditions, and visual pockets where cells were missing was apparent. However, after this initial 'slough' stage, low glucose/lipid cells were able to regroup and maintain distribution in the remaining days of differentiation (seven days total). The control and high glucose condition were extraordinarily receptive to their respective differentiation media, reflected in the high protein concentrations and maintenance of activity and confluence throughout seven days of differentiation.

On an observational note, the pH change across media was also readily noticeable, as DMEM changes from a reddish to a pink or orange in response to less or more acidity, respectively, owing to cellular activity and metabolism producing byproducts affecting the pH. The high glucose/lipid condition demonstrated the highest activity, reflected in the light orange color of the pH after 24h of differentiation, whereas the control remained the standard reddish hue and the low glucose/lipid condition exhibited a pink color.

Our lab has demonstrated the struggle of cells to adapt to lipid concentrations beyond 5% (DeMiranda et al. 2012), but to our knowledge this is the first visualization of the effects of low glucose/lipid on C2C12 skeletal muscle cells. It is very likely that the lower myoglobin expression and confluency struggles are a consequence of this difficulty, as the cells may not have had the energetic budget to dedicate to production of new proteins, essentially existing in a survival mode. The reduction of myoglobin provides an interesting avenue, as investigating the patterns of expression in myoglobin also entails understanding the triggers that detract from it as well. Even with lower cell density, the normalization to protein concentration indicates that although the cell protein was low in low glucose/lipid conditions, normalizing to this low

number still reveals a decrease, or shutting down, of an important oxygen-binding protein in conditions where it was expected to thrive.

Overall, in establishing a link between lipids and myoglobin, it seems at least in C2C12 cells a specific metabolic environment is required, as high glucose/lipid cells proliferated modestly and low glucose/lipid cells struggled, indicating that lipids alone are not enough to sustain myoglobin expression in every condition. However, we still add further conclusion that supplemental lipid in high glucose media promotes myoglobin expression in terrestrial mammal cells, a pathway independent of skeletal muscle activity and one previously proposed by Schlater et al. (2014).

Previous work in our lab has proposed that the expression of myoglobin in lipid supplemented conditions can be explained by the metabolizing of lipids, a process that consumes considerably more oxygen over glycolysis as lipids are oxidized for fuel. Research shows that lipid oxidation is accompanied by increased ROS production (as metabolizing lipids results in higher electron transfer over glycolysis) (Cortassa et al. 2017). Myoglobin's role as a ROS scavenger has previously been investigated (Schlater et al. 2014), and it has been proposed that lipid's link to myoglobin expression could lie in ROS production, creating oxidative stress conditions that facilitate myoglobin's role both as a ROS scavenger and a relief protein to better handle conditions of oxidative stress. Our data on oxygen consumption indicate both lipid supplemented groups display a higher oxygen consumption than the control condition that featured no additional lipids.

In particular, the high glucose/lipid condition that displayed the highest myoglobin concentration also exhibited the highest oxygen consumption, and based on past work, the lowest internal lipid concentration (Chapter 2). While high oxygen consumption is not a definitive

measure of lipid oxidation, it is still an indicator of lipid oxidation, especially relative to the control condition that featured the same high glucose concentration as the high glucose/lipid supplemented group, suggesting the metabolizing of a substrate other than glucose. Though this result is not *de novo*, this higher oxygen consumption is still reflective of a higher metabolic rate, providing substrate like lipid accounts for this increased consumption. This result was not de novo However, the low glucose/lipid supplemented group featured higher oxygen consumption than the control condition, yet still featured a lower myoglobin concentration than the other two groups. This suggests perhaps lipid oxidation is required along with an additional stimulus, or more likely, the poor health of the low glucose/lipid condition left little availability to develop metabolic machinery and thus an inability to facilitate increased myoglobin expression.

Past research displays evidence that lipid accumulation can occur in the absence of myoglobin (Cotta et al. 2017), which based on our previous work with lipid percentages, could explain why we see lipid accumulation in low glucose/lipid cells that is higher than the high glucose condition but with a decrease in myoglobin concentration. It is worth noting the observation that beta oxidation is not impaired by myoglobin ablation (Cotta et al. 2017), so as myoglobin may be tied to beta oxidation, the protein's absence does not denote a subsequent decrease in beta oxidation. Overall, it is not so much lipid oxidation, but the development of ROS that accompanies it that is most likely driving myoglobin expression, and future work will investigate ROS production amongst the three treatments as well as the respiratory quotient to determine the specific metabolic profile of each of the three conditions.

Future Directions

Future directions of this work will generate a robust metabolic profile, specifically generating ROS production and respiratory quotient. Overall, we aim to follow the role of lipids

and subsequent expression of myoglobin. Our previous work has demonstrated the viability of cells to take up lipids, and oxygen consumption indicates the potential for increased lipid oxidation as a result of availability (additionally in a predominantly glucose-preferential system). In addition, the role of lipids and their oxidation and the expression of myoglobin can be investigated by blocking key lipid metabolism pathways in future cell culture research. Finally, this work allows us to directly investigate the metabolism of lipids in a system that experimentally prefers glucose. Investigating the role of lipids in a marine mammal system that expresses myoglobin in large concentrations relative to terrestrial mammals, and are already well-primed to take up and oxidize lipids can give further information into the efficiency of marine mammal metabolism and the promoters of myoglobin expression that facilitates impressive physiological performance.

Funding

Funding was made possible through the National Science Foundation.

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Chapter 4: Conclusions and Future Directions

The aims of this thesis work were to explore the specific links between lipid supplementation and myoglobin in terrestrial mammal cells. In past work in our lab, this link has been established and a new paradigm put forward of an alternate pathway to myoglobin expression (Schlater et al. 2014). This work challenged the previous notion of exclusively skeletal muscle activity activating the calcineurin pathway that led to myoglobin expression (Kanatous et al. 2008). In addition, an alternate pathway was now proposed, completely independent of skeletal muscle activity, to which lipids alone somehow promoted myoglobin expression. This work explored this space in between lipids and myoglobin expression, teasing out the intracellular communication of lipids, and investigating their specific role as a primary facilitator or passive generator of myoglobin expression. Investigating if lipids were taken up was the first task, as this research also focused on visualizing the specific path of lipids throughout different treatment conditions. Identifying specific intracellular effects of lipids was also key, as well as determining if cells were metabolizing lipids and thus potentially driving myoglobin expression.

The main findings of this work confirm **that 1) lipid uptake in cells supplemented with lipid is observable, however 2) altering glucose availability had no effect on amount of lipid uptake. In addition, we further confirm 3) increased myoglobin expression in response to supplemental lipid in high glucose media, but did not see the same effect of increased myoglobin expression in low glucose and supplemental lipid treated cells**. As far as intracellular effects go, we observed no response in FABPpm expression in response to increased

lipid supplementation or lipid uptake but did confirm increased cellular activity and oxygen consumption in lipid supplemented cells.

The relationship between marine mammals and myoglobin expression is an interesting one, with marine mammals expressing significantly more myoglobin when compared to terrestrial mammals. However, although the quantities may differ, whether as a result of genetics, lifestyle, or both, we have observed the same conditions (with the exception of solely hypoxia in terrestrial mammals) promoting myoglobin expression in both marine and terrestrial mammals. This provokes certain questions to be answered as to the specific pathways in which myoglobin is expressed. Myoglobin as an oxygen-binding protein is appealing to study for multiple reasons: its appearance in aerobically-adapted mammals provides physiological answers to performance, and particularly in marine mammals, the facilitation of performance in the wild to operate in an extreme environment during breath hold dives (Costa 2004, Kanatous et al. 1999, Wright 2006).

Even so, the same conditions facilitating myoglobin expression being consistent even in terrestrial mammals, that often do not experience the same hypoxic conditions characteristic of a marine mammal lifestyle, is a fascinating avenue to explore the basis of mammalian physiology in response to diet. In essence, are you what you eat? We were specifically curious if we could force a system of C2C12 cells that is observably reliant on glucose to conditionally adapt to a new metabolic environment. In Chapter 2 and 3, the uptake of lipids and their potential oxidation as seen in the uptake across days in both lipid supplemented treatment, and their oxidative fate as suggested by diminished internal cellular lipid concentrations paired with increased oxygen consumption indicates that a system traditionally seen as glucose-dependent indeed has the capacity to take up and even metabolize lipids. However, as all three conditions essentially took

up lipids in the same manner, regardless of other substrate availability leaves the question open as to if they can conditionally adapt to high lipid conditions.

The link between lipid and myoglobin is a little less obscured, however the dual approach of lipid and glucose and its effect on myoglobin in terrestrial mammals may present an interesting avenue of study for the future. It is important to emphasize this work has elucidated the effects of lipids on myoglobin expression where there were previously assumptions. Previous work established some sort of lipid-myoglobin link, but the intricacies of what was happening in the cells was unclear. Was the presence of the lipids indirectly influencing the cells? Was oxidation of lipids driving ROS production? Did the cells even possess the capacity to take up lipids?

In particular, our results in Chapter 3 indicated that lipid uptake and potential oxidation does not necessitate myoglobin expression, and the effects of cellular health and energetic availability opens avenues for the specific energetics and environmental conditions conducive to myoglobin expression. The interplay between two metabolic substrates also provides interesting metabolic questions, and as a previously glucose-preferential system displays capabilities of lipid uptake, can we also provide certain energetic conditions in which we can drive lipid metabolism? Previous work has utilized caffeine to imitate skeletal muscle contraction and investigating the effect of exercise and its intensity on cellular activity and resulting protein expression is the next logical research step.

The effect on transporters was a surprise, as we expected increased lipid uptake to drive transporter expression. The results of Chapter 2 indicated no significant change in FABPpm expression, the first line of transporters that lipids would encounter, and is influenced by dietary lipid intake (Roepstorff 2004, Frohnert and Bernlohr 2000). We expected the low glucose/lipid

condition to take up more lipid due to concentration and effectively express more transporters than the other two conditions. While we observed a significant lipid uptake, it did not defer substantially from the lipid uptake seen in the other two conditions, and no difference in FABPpm transporters was observed. There are numerous fatty acid transporters in skeletal muscle and observing lipid supplementation and its effects on the others can further close the loop on whether or not cells conditionally adapt to the surrounding media environment.

Based on previous research that indicates FABPpm and the next likely transporter, CD36, express in tandem, we would expect little change in transporter usage. However, our data on oxygen consumption detailed in Chapter 3 indicates that potential oxidation of fatty acids is taking place, particularly in the lipid supplemented groups, and documenting the expression of the carnitine palmitoyl transferase-1 protein (CPT-1) that facilitates entry of fatty acids into the mitochondria could further demonstrate intracellular effects of lipids on expression of proteins other than myoglobin. As these transporters, particularly the translocation of CD36, are reliant (but not wholly dependent) on skeletal muscle activity or exercise, the facilitation of similar conditions will also give insight into the specific conditions that express or fail to affect myoglobin expression as a result of lipid and its intracellular effects.

A main takeaway from this study is now the capacity to investigate lipid fate inside cell culture and applying these methods and observations to marine mammal cells. Particularly as marine mammals are heavily reliant on lipid metabolism relative to other mammals (DeMiranda et al. 2012), performing comparative studies with the results of these studies allow for a further profile of aerobic adaptation of marine mammals to terrestrial mammals that increased myoglobin expression can provide. In addition, we can observe any potential efficiency and change from the terrestrial condition concerning lipid uptake and usage. We have observed both

in terrestrial mammals and resulting myoglobin expression, and the marine mammal system that seems well-primed to move and use lipids could provide key data on the requirements of altering lipid uptake or usage. Further, investigating the specific transporters and their usage in marine mammal cells would be a novel exploration into how marine mammals are primed for such high-lipid conditions. In particular, our hypothesis that the C2C12 cells in the low glucose/lipid condition would take up more lipid and express more myoglobin was proven incorrect, and it would be prudent to investigate if a similar response is observed in a system that may have better adaptations to a high lipid, low glucose metabolic environment. The addition of knockouts or blocking fatty acid oxidation could also elucidate the necessity for lipid oxidation or just the presence of ROS influencing myoglobin expression.

The uptake and movement of lipids in a cellular environment is a subject of great interest, particularly since lipids are capable of different roles inside the cell rather than just substrate. Future work should involve investigating the patterns of specific fatty acid effects on the expression of myoglobin or transporters. In particular regards to marine mammals, this data can be extrapolated to the prey specific fatty acid concentrations and their effect on myoglobin expression and thus aerobic performance. Particularly in an environment where survival is dependent on performance, the conditions that promote or detract from the expression of an important protein can give further insight into ecological roles of diet.

Our work focuses on understanding the workings at the cellular level and its applications to the whole system, and this work is a furthering of these research interests. This work adds to the influence of diet on performance, and the mammalian system that presents similar physiological influences regardless of life history. Nutritional physiology is an exciting avenue, especially as lipids serve a vital role in aerobic capabilities under particular exercise intensities.

We further the linkage between myoglobin and lipids and propose additional factors that must work in tandem with lipids to achieve myoglobin expression, at least in C2C12 cells. The results of this thesis further enhance the principles that diet can affect performance, and it is necessary to understand the intricate networks beyond just cellular workings in order to understand these effects from the cell, to the whole animal, and continue to answer the adage of if you indeed are what you eat.

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