THESIS

TEMPORAL EXAMINATION OF MYOGLOBIN AND MYOSIN HEAVY CHAIN EXPRESSION PATTERNS *IN VITRO*

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ABSTRACT

TEMPORAL EXAMINATION OF MYOGLOBIN AND MYOSIN HEAVY CHAIN EXPRESSION PATTERNS *IN VITRO*

Myoglobin is a hemoprotein expressed in vertebrate muscle that is typically known to follow an established oxidative muscle fiber type, found in aerobic muscles. However, more recent evidence has demonstrated changes in myoglobin expression without a change in fiber type, indicating myoglobin expression could be regulated by different pathways and may not always be dependent on a prior expressed fiber type. Myoglobin structure is characterized by a globin backbone that supports a nonprotein heme prosthetic group containing iron, which is responsible for the reversible binding of several ligands such as oxygen, nitric oxide, and carbon monoxide. Naturally, it has been implicated in oxygen transport and storage, nitric oxide and reactive oxygen species scavenging, and cellular lipid transport. Mixed lipid supplementation alone and coupled with hypoxia elevates skeletal muscle myoglobin levels, but it is unknown how these culture treatments affect myoglobin expression relative to the fiber type. Given the uncertainty of when myoglobin is expressed independently from oxidative fiber types, we aimed to determine when differentiating C_2C_{12} cells begin to express myoglobin compared to when they express oxidative isoforms of myosin heavy chain when subjected to factors known to increase myoglobin expression; hypoxia, lipid and/or caffeine treatments. We found that under control and hypoxic conditions, regardless of lipid supplementation, myoglobin expression occurred before oxidative fiber expression. Conversely, cells receiving caffeine stimulation expressed myoglobin following oxidative fiber type expression. Cells exposed to hypoxia and lipid supplementation displayed elevated functional myoglobin expression compared to caffeine stimulated cells, suggesting that this combination of treatments may be more effective at increasing myoglobin than stimulation alone. Overall, the work presented here has determined conditions under which expression of myoglobin precedes oxidative fiber

type expression and within these treatments, conditions that also increase functional myoglobin concentration. These findings can act as a step in the process to assist in revealing more about how myoglobin can be expressed in skeletal muscle. Examination of alternate routes of myoglobin expression that are not reliant on prior expression of a particular fiber type could yield potential therapeutic benefits of expressing myoglobin in tissues to combat ischemic diseases seen in humans.

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CHAPTER 1: INTRODUCTION: THE RELEVANCE, FUNCTION, AND EXPRESSION OF MYOGLOBIN

Myoglobin is an intensively studied hemoprotein found and utilized primarily in vertebrate muscular tissue. The characteristic globin backbone of myoglobin supports a nonprotein heme prosthetic group containing iron, responsible for the reversible binding of a number of ligands such as oxygen, nitric oxide, and carbon monoxide (Garry and Mammen 2007). As anticipated, it has been implicated in oxygen transport and storage, nitric oxide (NO) and reactive oxygen species (ROS) scavenging, and demonstrated the ability to transport lipids (Flögel et al. 2004; Flögel et al. 2001; Kanatous and Mammen 2010; Millikan 1939; Shih et al. 2014; Wittenberg and Wittenberg 2003). Myoglobin is now known to be expressed within all vertebrate species except amphibians and is found in many tissues other than muscle including brain, liver, and cancer cells. Myoglobin and its myriad of functional properties appear to have a strong correlation with the physiology and metabolic requirements of the tissues in which it has been found (Helbo et al. 2013; Kanatous and Mammen 2010). Regarding binding oxygen, myoglobin provides oxygen to working muscles when blood oxygen delivery is insufficient (Gros et al. 2010; Wittenberg and Wittenberg 1989). In fact, myoglobin has a strong affinity for oxygen and is only able to release oxygen for aerobic metabolism when the partial pressure of oxygen is very low, as in hypoxic conditions like ischemia (Nichols and Weber 1989; Schenkman et al. 1997).

Myoglobin in human health

Ischemia is a shortage of blood supply to working tissues in the body, in so decreasing the delivery of vital components such as oxygen, metabolites, and removal of waste (Mallick et al. 2004). This restriction can cause damage to many tissues and organs within minutes however, enhanced oxygen stores bound to myoglobin could be a way to resolve the hypoxic conditions that result from ischemia. Elevated myoglobin levels have been shown to ameliorate the detrimental effects of ischemia in diving vertebrates, and in this capacity, could be implicated in human health application (Kanatous and Mammen 2010). In

humans, ischemia can lead to heart attack, stroke, and is seen in patients that develop peripheral artery disease secondarily due to age, smoking, diabetes, and other common medical conditions.

Elevated myoglobin in tissues alleviates hypoxic stress by providing oxygen to working tissues (Helbo et al. 2013). For example, increased levels of myoglobin serve a protective role in the heart in hypertrophied cardiomyocytes and when blood supply to the heart is interrupted during normal systole when the coronary arteries are compressed by the beating heart (Helbo et al. 2013; Peters et al. 2016). Additionally, tissue hypoxia typically invokes a response characterized by a strong stimulation of angiogenesis primarily driven by the vascular endothelial growth factor (VEGF), but also by NO signaling (Hendgen-Cotta et al 2010). Myoglobin either scavenges NO under normoxic conditions or assists in production of NO in hypoxic conditions, making it a prime candidate to be present in tissue that may experience ischemia for support (Kuleva and Krasovskaya 2016). Further, during hypoxia and following when reperfusion occurs, myoglobin can scavenge the ROS that are produced in tissues before they have a detrimental effect on tissues (Helbo et al. 2013).

An increasing amount of studies are finding that myoglobin can be expressed in non-muscle tissues; even in highly aggressive carcinomas found in humans like breast, colon, lung, ovary, and head and neck cancers (Braganza et al. 2019; Helbo et la. 2013). The presence of myoglobin in these cancer types can positively or negatively affect prognostic outcomes, depending on the role that myoglobin plays in the tumorous tissue (Bicker et al. 2020). The presence of myoglobin could reduce the hypoxia in carcinomas which typically promote tumor progression and resistance to therapy through the hypoxia-inducible factor axis (Ju et al. 2017). Furthermore, in some types of cancer, myoglobin expression causes cell cycle arrest and inhibition of proliferation by way of mitochondrial hyperfusion (Braganza et al. 2019). Although the mechanisms underlying myoglobin expression in these instances has yet to be characterized, the presence of myoglobin could provide a way to combat ischemia in non-cancerous cells and even tumor growth in cancerous cells as a therapeutic intervention (Hendgen-Cotta et al. 2010).

Myoglobin expression regulation and patterns

In skeletal muscle, more myoglobin is expressed in type I slow-oxidative and type IIA fast-oxidative fiber types, while there is little myoglobin in type IIX fast-glycolytic fibers and none in IIB fast-glycolytic fibers. Muscle fibers are given type designations depending on which myosin heavy chain (MHC) isoform they contain (Chin et al. 1998; Kanatous and Mammen 2010; Ordway and Garry 2004; Schiaffino and Reggiani 1996). The pathway that leads to the expression of myoglobin within these fiber types is the calcineurin-NFAT (Nuclear Factor of Activated T cell) pathway, which is a mediator of calcium signaling in many cell systems. The phosphatase calcineurin dephosphorylates and thus activates NFAT proteins, which allows NFAT to translocate from the cytoplasm to the nucleus where it can control target gene expression by activation or deactivation depending on its binding partners (Im and Rao 2004; Pan et al. 2013). C_2C_{12} cells (immortalized mouse skeletal muscle cells) respond to the addition of activated calcineurin by increasing the activity of myoglobin and troponin I slow promoters, both known to be found in slow-twitch oxidative fibers containing MHC I. This group also found that inhibited calcineurin in mice causes a slow to fast change in fiber type (Chin et al. 1998; Dolmetsch et al. 1997; Staron and Pette 1986; Timmerman et al. 1996). These correlations suggest that myoglobin expression is dependent on the fiber type of the tissue, because slow-twitch fibers are able to receive the appropriate calcium signaling (a sustained high concentration) to activate calcineurin and thus effect the expression of myoglobin (Chin et al. 1998).

In oxidative fibers, activation of the myoglobin gene occurs at a positive control element within the 2kb myoglobin promoter region. There are two NFAT response elements located in the proximal portion of the promoter region, that bind calcineurin-dephosphorylated NFAT that has entered the nucleus. The CCAC-box motif located downstream of the NFAT response elements binds several transcription factors, but only binding of Sp1 is required for the expression of myoglobin. At a site downstream of the CCAC-box, there lies an A/T element which binds dephosphorylated myocyte enhancing factor-2 (MEF2) (Chin et al. 1998; Grayson et al. 1998; Kanatous and Mammen 2010). Increases in intracellular calcium activate the phosphatase calcineurin, which not only dephosphorylates NFAT, but also MEF2. These activated

factors then translocate to the nucleus where they are able to bind to the myoglobin regulatory motifs in the promoter to aid in transcription at the myoglobin gene (Chin et al. 1998; Kanatous and Mammen 2010; Kanatous et al. 2009). Additionally, the peroxisome-proliferator-activated receptor-g co-activator-1 (PGC-1γ) has been shown to play a role in the expression of myoglobin. PGC-1 γ is involved in regulation of mitochondrial biogenesis and promoting cellular adaptations to exercise such as expression of slow fiber types, and in fact plays a role in myoglobin expression through a calcineurin-MEF2 dependent pathway (Kanatous and Mammen 2010; Karlsson et al. 2019; Lin et al. 2002).

Besides expression of myoglobin being correlated to a specific fiber type, changes in the levels of expression of the protein has been shown to be attributed to other factors such as hypoxia, NO generation, and *in vitro* lipid supplementation (e.g. adding a mixed lipid composition to a lipid free media) (De Miranda et al. 2012; Kanatous et al. 2009; Rayner et al. 2009; Schlater et al. 2014). For instance, hypoxia acting with another stimulus like muscular contraction was found to be necessary to release calcium from the sarcoplasmic reticulum via ryanodine receptors, to signal properly to initiate the expression of myoglobin. Hypoxia acting alone, however, has been shown to induce a decrease in myoglobin protein levels by causing a release of calcium solely from the endoplasmic reticulum, which causes NFAT to remain phosphorylated and thus unable to enter the nucleus and initiate transcription of myoglobin (Kanatous et al. 2009; Stiber et al. 2005). Surprisingly, in this hypoxia-related protein the myoglobin promoter region does not contain a hypoxia inducible factor-1 (HIF-1) binding motif and is not responsive to HIF-1 (Kanatous et al. 2009, Helbo et al. 2013). HIF-1 is a central regulator of oxygen detection and adaptation in a variety of cell types, so it is interesting that this protein is not related to myoglobin in a response to hypoxia (Choudhry and Harris 2018; Kanatous et al. 2009; Helbo et al. 2013).

Changes in myoglobin expression have also been correlated with other stimuli. For instance, pathological NO concentrations in vascular smooth muscle cells yield an increase in myoglobin concentration (Rayner et al. 2009). Similarly, lipid supplementation to mammalian muscle cells *in vitro* and rats *in vivo* correlates with an increase in myoglobin concentration in the skeletal tissue (De Miranda et al. 2012; Schlater et al. 2014). These studies indicate the levels of myoglobin expressed under these

varying conditions and among different species can be altered, but a more specific timeline of the expression of myoglobin and fiber type indicators is warranted to fully understand the regulatory mechanisms that govern when and how myoglobin is expressed.

As far as differences between species, myoglobin expression is commonly enhanced in the skeletal muscles of hypoxia-tolerant birds and mammals such as in divers, burrowers, or some that are active at high altitudes with low ambient oxygen (Avivi et al. 2010; Helbo et al 2013; Ponganis 2011). In other vertebrates like fish and reptiles, elevated levels of myoglobin appear to be correlated to a high activity level (Helbo et al. 2013). The myoglobin expression patterns across all these species and tissues and their utilities of myoglobin are complex and diverse, but the role of myoglobin in these various locations may help in further investigations of underlying pathways to expression.

Roles of myoglobin in tissues

Myoglobin is similar in structure to other heme proteins that display a globin fold backbone surrounding a heme prosthetic group. The globin backbone of myoglobin forms four cavities that facilitate reversible binding of a number of ligands by concentrating ligands within the cavities (Garry and Mammen 2007). This structural evidence for ligand binding led early investigators to discover the most notable role of myoglobin, binding oxygen (Garry and Mammen 2007; Hill et al. 1936; Millikan 1939). Since then, researchers have found that myoglobin is involved in intracellular oxygen storage and oxygen transport from the sarcolemma to the mitochondria in muscle for aerobic metabolism (Gros et al. 2010; Postnikova and Shekhovtsova 2018; Wittenberg and Wittenberg 1989).

Due to its strong affinity for oxygen, myoglobin can only release oxygen when the oxygen tension in cells becomes very low, lending to its role in assisting vertebrates in combating or avoiding tissue hypoxia (Helbo et al. 2013; Nichols and Weber 1989; Schenkman et al. 1997). Endurance exercise training in terrestrial mammals involving prolonged aerobic metabolism that utilizes oxygen delivered by hemoglobin and bound to myoglobin often correlates with an increase in myoglobin concentration within the working muscle (Beyer and Fattore 1984; Duteil et al. 2004; Harms and Hickson 1983; Pattengale and

Holloszy 1967). Further, hypertrophied striated muscle working at maximal oxygen uptake increases myoglobin concentration in an effort to increase intracellular oxygen supply to hypoxic cell cores (Peters et al. 2016; Peters et al. 2017; Van Wessel et al. 2010). In fact, myoglobin not only delivers oxygen through the cell to mitochondria, but also interacts directly with mitochondria and is able to co-localize with and regulate mitochondrial complex IV (COX IV), suggesting that myoglobin is a central modulator of mitochondrial respiration (Postnikova et al. 2009; Yamada et al. 2013; Yamada et al. 2016). Additionally, in some animal models low oxygen at high altitudes is correlated with increased myoglobin concentration and diving birds and mammals rely on similar increased oxygen to extend aerobic dive performance (Gimenez et al. 1977; Gros et al. 2010; Kooyman and Ponganis 1998; Reynafarje and Morrison 1962; Weber 2007).

Besides a role in oxygen storage and transport, myoglobin has been implicated in the binding of other ligands. For instance, depending on the oxygenation of tissues myoglobin and NO display different interactions with one another. Under normal oxygenation in many vertebrates, myoglobin binds and converts NO to inert nitrate to prevent this gaseous signaling molecule from binding to cytochrome-c-oxidase and inhibiting mitochondrial respiration (Bicker et al. 2020; Flögel et al. 2001; Gödecke et al. 2003; Helbo et al. 2013; Kuleva and Krasovskaya 2016; Moncada and Erusalimsky 2002). Furthermore, pathological NO concentrations in vascular smooth muscle cells yield an increase in myoglobin concentration, presumably to scavenge the excess of NO and prevent it from binding reactive oxygen species to create peroxynitrite. Peroxynitrite is an unstable structural isomer of nitrate that is cytotoxic and causes oxidative stress, protein nitration, and other cellular stress (Lind et al. 2017; Rayner et al. 2009). Alternatively, under hypoxic conditions when NO synthases become less efficient, deoxymyoglobin acts as a nitrite reductase that converts endogenous nitrite to NO. NO produced during hypoxia can reversibly bind to cytochrome-c-oxidase to inhibit mitochondrial respiration, and in this instance be beneficial to the cell. Mitochondrial inhibition during hypoxic conditions limits oxygen consumption by the cell and has been shown to act as a protective measure to limit the production of ROS (Helbo et al. 2013; Hendgen-Cotta et al. 2008; Kuleva and Krasovskaya 2016; Shiva et al. 2007).

ROS can be important signaling molecules in many instances particularly for exercise-induced skeletal muscle adaptation. However, when ROS production from places in the cell like mitochondria, NADPH (nicotinamide adenine dinucleotide phosphate) oxidase sites, and xanthine oxidase sites increases, it can become damaging to the cell (Horn et al. 2017; Powers et al. 2011). ROS increase in muscle during exercise by way of aerobic metabolism, during hypoxia, and additionally during the reoxygenation (reperfusion) phase following hypoxic events (Chouchani et al 2016; Flögel et al. 2004; Helbo et al. 2013). However, the accumulation of ROS can cause many harmful effects, in particular, the impairment of calcium handling and contractile dysfunction in skeletal muscle (Mannino et al. 2019; Powers et al. 2011; Redpath et al 2013). Myoglobin is able to scavenge excess ROS and ultimately yields ferric (met) Mb through reacting with ROS (Yusa and Shikama 1987).

Within the cell fatty acid binding proteins are typically the main chaperone of fatty acids, which are insoluble in the aqueous interior of the cell (Dowling et al. 2020; van der Vusse et al. 2000). Myoglobin competes with fatty acid binding protein when concentrations of unsaturated fatty acids rise above a particular concentration, and therefore enhances fatty acid solubility in the sarcoplasm to channel fatty acids to β-oxidation (Jue et al. 2016; Sriram et al. 2008). Fatty acids bind to myoglobin in the vicinity of the heme pocket and were thought to impact the oxygen affinity of myoglobin however, recent studies have found that fatty acid binding does not have an impact on oxygen affinity (Jue et al. 2016). Fatty acids in fact interact differently with physiological states of myoglobin, and deoxymyoglobin is unable to bind fatty acids. This suggests a mechanism where oxygen and fatty acids can be loaded onto myoglobin at the highly oxygenated sarcolemma, and then dissociate from myoglobin when myoglobin becomes deoxygenated at the mitochondria (Shih et al. 2014).

Tissue without myoglobin

Many of the functional roles of myoglobin were discovered by way of experiments investigating animals without myoglobin. For example, some notothenioid fishes are natural myoglobin “knockouts” and do not express hemoglobin and in some cases, do not express myoglobin either. These fish are able to meet their

oxygen needs primarily because the water column around Antarctica where these fish exist is well mixed and all depths are near to complete saturation of oxygen and is already primed to be oxygen rich due to the cold temperature of the water. Being ectotherms, these fish have cold blood and are able to pass oxygen through the blood stream to tissues and through intracellular spaces in solution. Additionally, these myoglobin-lacking fish have increased mitochondrial densities with more prolific inner mitochondrial membranes which likely serve as an important pathway for oxygen, and they also have less enzymatically active cytochrome-c-oxidase. These fish also produce higher levels of NO that are not apparently detrimental to the fish and might instead act to enhance angiogenesis, mitochondrial biogenesis, and muscle hypertrophy that may assist in the ability of their heart to pump a greater volume of blood (Grove et al 2004; Sidell and O'Brein 2006).

Before the discovery of these myoglobin-less fish, researchers developed a gene disruption strategy to produce transgenic mice that carry two copies of a mutant allele that causes a complete lack of myoglobin expression. Mice that are heterozygous for the null allele had increased embryonic lethality due to several developmental heart defects. The mice homozygous for the null allele that were able to survive gestation without myoglobin proved to have healthy hearts that have no alteration in contractile parameters even after stimulation of a maximal heart rate, and exhibit normal contractile capacity in skeletal muscle across a range of work conditions and oxygen availability (Garry et al. 1998). These transgenic mice also display a number of skeletal muscle adaptations like increased expression of hypoxia-inducible transcription factors and VEGF, and the skeletal muscle fiber type in soleus muscle switches from type I to II (Grange et al. 2001). These mice appear to develop adaptations that rescue their lack of myoglobin including increased hemoglobin, increased vascular density and coronary blood flow, and adaptations unrelated to oxygen availability, all of which are adaptations that allow these animals to persist into adulthood (Garry et al. 1998; Garry et al. 2000; Gödecke et al 1999; Meeson et al. 2001).

Myoglobin knockout mice show a number of adaptations to circumvent the lack of myoglobin and its oxygen storage and transport properties, and other studies have found that these knockout mice possess additional compensatory mechanisms to cope with the absence of myoglobin (Garry and Mammen 2007).

Knockout mice exposed to chronic hypoxia have a significant decrease in left ventricular function in the heart that is rescued by re-exposure to normoxic conditions. Left ventricular function can also be rescued by treatment with an inhibitor of NO, suggesting a role for myoglobin as a NO scavenger in the heart (Mammen et al. 2003). These results were corroborated by another group that was able to directly measure the oxidation of NO by myoglobin occurring in wild type mouse hearts, but not occurring in knockout mice (Flögel et al. 2001). Further, others have developed myoglobin deficient mice using CRISPR-Cas9 mediated gene targeting and found similar results of a decrease in oxidation of NO and elevated expression of proteins involved in NO metabolism (Park et al. 2019). Knockout mice also develop decreased left ventricular function and an accumulation of ROS when their hearts are subjected to oxidative stress by way of infusion of hyperoxide or induced ischemia-reperfusion injury, implicating myoglobin in ROS scavenging (Flögel et al. 2004). Lastly, more recent studies found diminished fatty acid oxidation and enhanced triglyceride accumulation in myoglobin deficient mice, suggesting a role for myoglobin in fatty acid transport (Hendgen-Cotta et al. 2017).

Myoglobin and fiber type disconnect

While myoglobin has been found to perform many functions in skeletal muscle and commonly associated with a particular fiber type, there is recent evidence to suggest alternate pathways by which myoglobin can be expressed. Recent findings have indicated that myoglobin expression is not solely linked to fiber type or calcium signaling in regard to calcineurin activation and that other uncharacterized pathways exist (Kanatous et al. 2009; Oh et al. 2005; Schlater et al. 2014). Specifically, hypoxic conditions that decrease myoglobin expression do not produce a concomitant change in fiber type of the tissue, indicating an alternate pathway for myoglobin not dependent on the fiber type of the muscle. Further, by blocking known NFAT binding sites, myoglobin expression ceases in the myotubes, indicating that the expression of myoglobin under hypoxia may still be reliant on an NFAT pathway (Kanatous et al. 2009).

Further in support of an alternate pathway to myoglobin expression, inhibiting the phosphatase activity of calcineurin in mice causes MHC I and troponin I slow expression to cease, while myoglobin

expression is altogether unaltered. This suggests that the actions of calcineurin have a direct effect on maintaining fiber type of muscular tissue but may not always affect the production of myoglobin (Oh et al. 2005). Additionally, increases in myoglobin protein in skeletal muscle do not correlate to an increase in calcineurin expression, indicating that myoglobin can be stimulated in the absence of calcineurin expression (Schlater et al. 2014). Given the apparent disconnect between fiber type and myoglobin expression, finding specific cell culture conditions under which myoglobin expression is independent from oxidative fiber type expression could serve to better investigate regulatory mechanisms of alternate pathways to myoglobin expression.

Additional considerations for research

In investigating relative myoglobin and fiber type expression in skeletal muscle, there are a couple of additional tools to manipulate *in vitro* experiments, and previous work to be considered. Caffeine is known to be used in cell culture to study *in vitro* exercise-induced skeletal muscle adaptations, acting as a calcium ionophore mimicking exercise activation, exercise signals, or to trigger calcium fluctuations as would be caused by exercise (Carter and Solomon 2019). Physiological levels of caffeine induce regulators of oxidative metabolism and mitochondrial biogenesis and are also implicated in activation of the slow oxidative fiber program (Gan et al. 2013; Schnuck et al. 2018). Caffeine reacts directly with ryanodine receptors causing releases of calcium from the sarcoplasmic reticulum. Calcium released in this way is responsible for the signaling of many biological functions, including differentiation and contraction within striated muscle (Ebashi and Endo 1968; Ogawa et al. 2020). Further, caffeine not only induces regulators of oxidative metabolism but also enhances lipid metabolism and reduces lipid content in skeletal muscle (Schnuck et al. 2018). Collectively, these suggest that caffeine promotes a slow oxidative fiber type and can increase lipid oxidation however, the extent of myoglobin expression and associated fiber types with this stimulus have not been examined.

Cyclosporine is a cyclic peptide commonly used to inhibit the calcineurin signaling pathway by inhibiting calcineurin directly, and could be used to further study the regulation of myoglobin expression

by removing the action of calcineurin to examine if myoglobin can still be produced (Matsuda and Koyasu 2000). However, we know that the calcineurin signaling pathway is responsible for the regulation of many cellular processes, including skeletal muscle cell differentiation (Friday et al. 2000; Pan et al. 2013). Calcineurin has been found to be a key transcriptional activator of myogenin and is crucial in the initiation of differentiation of myoblasts, while other calcium-dependent pathways are also necessary to collectively initiate myoblast differentiation (Friday et al. 2000). Calcineurin has also been shown to be responsible for the development and maintenance of slow fibers in skeletal muscle (Chin et al. 1998). In addition to inhibiting calcineurin and the development of skeletal muscle cells, cyclosporine may have other cellular targets. Cyclosporine inhibits JNK and p38 signaling pathways, which are involved in cellular stress responses including inflammation, apoptosis, cell differentiation, and cell cycle regulation (Huang et al. 2009; Matsuda and Koyasu 2000). Cyclosporine appears to have several other cellular targets aside from calcineurin, so in order to implement this peptide to study myoglobin expression it would be helpful to know when exactly myoglobin is expressed in cells under particular experimental conditions in an effort to inhibit calcineurin activity related to myoglobin expression.

Investigation of when myoblasts begin to express myoglobin in comparison to when they express different isoforms of MHC could reveal more about how myoglobin is eventually expressed in skeletal muscle and if it is dependent on prior expression of a particular fiber type. Analysis of rat and mouse myoblasts grown *in vitro* has shown that myoglobin mRNA or protein is not expressed until after myoblast fusion and differentiation (Devlin et al. 1989; Graber and Woodworth 1986; Ordway and Garry 2004; Van der Giessen et al. 2003; Weller et al. 1986). More specifically, L6 cells (derived from rat skeletal muscle) begin to express myoglobin protein beginning on day three after the initiation of differentiation and expression increased thereafter when samples were collected every two-three days (Graber and Woodworth 1986). Additionally, myoglobin was expressed on day three and MHC on day two after differentiation initiation. The antibody used to determine the presence of MHCs in this instance was MF-20 (DSHB) which recognizes the rod-like tail region of the MHC protein found in all isoforms of

MHC. Therefore, it is unclear which specific isoform of MHC was being expressed, and if it was an oxidative fiber type (Bader et al. 1982; Van der Giessen et al. 2003).

There are currently eleven known mammalian MHC isoforms in skeletal muscle that are expressed differentially throughout the differentiation of muscle (Oh et al. 2005; Reiser 2019). Regarding the most notable isoforms, MHC I is expressed embryonically in developing skeletal muscle and in adult tissue while the fast twitch MHC isoforms have embryonic forms, embryonic MHC (MHCemb) and neonatal MHC (MHCneo), that are replaced by adult-specific isoforms after birth (Oh et al. 2005). Similar developmental patterns of MHC isoform expression have been recorded in skeletal muscle cells *in vitro* (Weydert et al. 1987). The fiber types developed in C_2C_{12} cells have been inconsistently reported, and the development of specific MHC isoforms appears to be affected by the cell culture environment (Allen et al. 2001; LaFramboise et al. 2003; Zebedin et al. 2004).

Given the uncertainty regarding the expression of myoglobin relative to MHCs, this project aimed to integrate investigations of expression of myoglobin in concordance with fiber type indicators throughout the progression of differentiation to create a timeline of expression using C_2C_{12} skeletal muscle cells. Based on the timing of expression of these proteins, we can learn more about how myoglobin expression is regulated or can be induced. We hypothesize that factors that correlate with increased myoglobin expression, will initiate myoglobin expression prior to the expression of MHCs. More specifically, we hypothesize that lipid supplementation alone and coupled with hypoxia will initiate myoglobin expression prior to an oxidative fiber type, and caffeine treatments will result in myoglobin expression following an oxidative fiber type. The results of this study will provide more information about how myoglobin can be eventually expressed in skeletal muscle and when it is preceded by the expression of an oxidative fiber type.

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CHAPTER 2: IN THE ABSENCE OF CONTRACTILE STIMULATION MYOGLOBIN EXPRESSION PRECEDES OXIDATIVE FIBER TYPE EXPRESSION[1]

Summary

Myoglobin is a hemoprotein expressed in vertebrate muscle that is typically known to follow an established oxidative muscle fiber type found in aerobic muscles. Recent evidence has shown changes in myoglobin expression without a change in fiber type, indicating that myoglobin expression may not always be fiber type dependent and might be regulated by other stimulatory pathways. Myoglobin increases in response to mixed lipid supplements and is known to protect muscle from reactive oxygen species produced from oxidative metabolism. Caffeine promotes a slow oxidative fiber type and can increase lipid oxidation. Currently, it is unknown how caffeine or lipid affect myoglobin expression relative to the fiber type of cultured tissue. To investigate, we have cultured and differentiated C_2C_{12} myoblasts in the presence and absence of lipid, and/or caffeine to simulate contraction. Cells were then harvested each day after differentiation initiation. Western blots were conducted to determine the expression of myoglobin and various oxidative fiber myosin heavy chains. We have found that control conditions and the addition of lipid result in myoglobin expression before that of oxidative fiber expression, while caffeine treatments result in oxidative fiber type expression before myoglobin. Cells exposed to lipid supplementation showed a significant increase in functional myoglobin expression, while those that were subjected to caffeine treatments showed less functional myoglobin expression. Examination of alternate routes of myoglobin expression, involving lipid supplementation, that precede oxidative fiber types could yield potential therapeutic benefits to combat ischemic diseases seen in humans.

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Introduction

Myoglobin is an extensively characterized cytoplasmic hemoprotein found and utilized primarily in vertebrate muscular tissue. The globin backbone of this protein supports a heme prosthetic group containing iron, which is responsible for the reversible binding of a number of ligands such as oxygen, nitric oxide (NO), and carbon monoxide (Garry and Mammen 2007). Most notably, myoglobin functions in oxygen storage and provides oxygen to working muscles when blood oxygen delivery is insufficient. Due to its ability to bind many ligands, myoglobin has been implicated in NO and reactive oxygen species (ROS) scavenging and has been shown to have a role in cellular lipid transport (Flögel et al. 2001; Flögel et al. 2004; Kanatous and Mammen 2010; Shih et al. 2014). Myoglobin releases oxygen for aerobic metabolism when the partial pressure of oxygen is very low, as in hypoxic conditions, due to its strong affinity for oxygen (Schenkman et al. 1997). High concentrations of myoglobin ameliorate the detrimental effects of ischemia in diving vertebrates, and in this capacity, could be implicated in human health application (Kanatous and Mammen 2010). Ischemia is a shortage of blood supply to working tissues in the body, resulting in a decrease in the delivery of vital components such as oxygen, metabolites, and removal of waste (Mallick et al. 2004). The hypoxic conditions associated with ischemia can cause damage and even cell death to many tissues and organs within minutes however, enhanced oxygen stores bound to myoglobin can keep tissues from becoming hypoxic, particularly as a therapeutic intervention in human ischemic illnesses.

Myoglobin expression has been correlated with specific muscle fiber types; such that there is more myoglobin found in slow oxidative fiber types (type I) and fast oxidative (type IIA) containing type I and type IIA fibers, respectively. Compared to oxidative fiber types, fast glycolytic fiber types express very little myoglobin in IIX fiber types and none in IIB fiber types. Fiber types are identified as type I, IIA, IIX, and IIB designations depending on which myosin heavy chain (MHC) isoform they express (Kanatous and Mammen 2010; Ordway and Garry 2004; Schiaffino and Reggiani 1996).

The pathway that leads to the expression of myoglobin within these fiber types is the calcineurin-NFAT (Nuclear Factor of Activated T cell) pathway, which is a mediator of calcium signaling in many cell systems. Nerve-induced fluctuations in calcium activate calcium/calmodulin-dependent protein kinase (CaMK, type IV) and the phosphatase calcineurin. CaMK and calcineurin facilitate the transactivating function of myocyte enhancer factor-2 (MEF2), while calcineurin activates NFAT proteins by dephosphorylation. This action allows NFAT to translocate from the cytoplasm to the nucleus where it can control target gene expression of the slow fiber program by collaborating with MEF2 and additional factors (Meissner et al. 2011; Pan et al. 2013; Wu et al. 2000). Further, the slow fiber program can be stimulated by growth factors working through the mitogen-activated protein kinase/extracellular signal-related kinase (MAPK/ERK) pathway (Baldwin et al 2013, Fig 2-1). One group found that the addition of

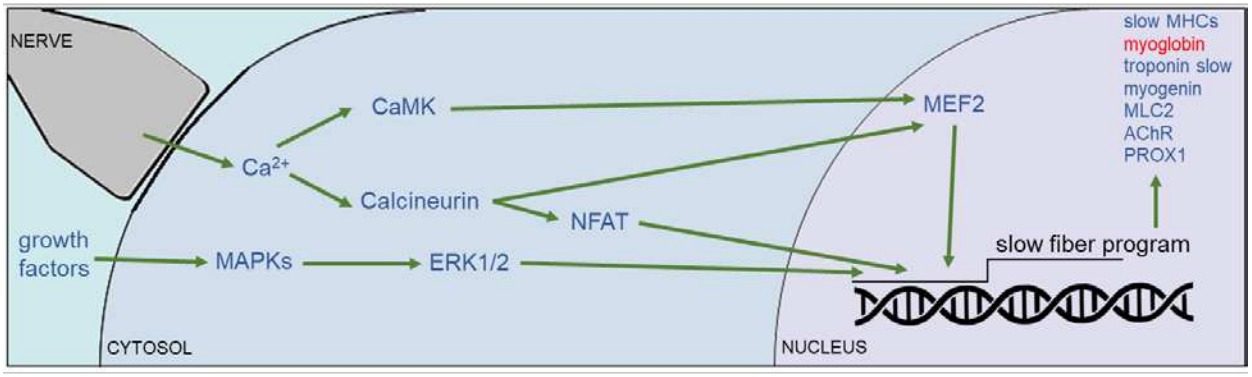

Fig. 2-1 Traditional myosin heavy chain (MHC) and myoglobin expression pathway *in vivo*. Myoglobin expression is thought to follow the induction of the slow fiber program. This can be achieved through growth factors working through the mitogen-activated protein kinase/extracellular signal-related kinase (MAPK/ERK) pathway (seen *in vitro*), and additionally through nerve stimulated Ca2+ signaling, eventually translocating nuclear factor of activated T cell (NFAT) and myocyte enhancer factor-2 (MEF2) to the nucleus to initiate the slow fiber program. This induction of the slow fiber program is thought to lead to the expression of myoglobin, and other slow fiber-related proteins (Myosin regulatory light chain 2: MLC2; acetylcholine receptor: AChR; Prospero homeobox protein 1: PROX1)

activated calcineurin, C_2C_{12} cells (immortalized mouse skeletal muscle cells) showed an increase in the activity of myoglobin and troponin I slow promoters, known to be found in slow-twitch oxidative fibers expressing MHC I. They also found that when they inhibited calcineurin activity in mice, there was a slow to fast change in fiber type (Chin et al. 1998). These correlations suggested that myoglobin expression is dependent on the fiber type of the tissue, because slow-twitch type I fibers were able to

receive the appropriate calcium signaling (a sustained high concentration) to activate calcineurin and affect the expression of myoglobin (Chin et al. 1998; Dolmetsch et al. 1997).

More recent evidence has shown that myoglobin expression is not solely dependent on calcium signaling in regard to calcineurin activation. This suggests that other uncharacterized pathways exist (Oh et al. 2005; Schlater et al. 2014). Specifically, inhibiting the phosphatase activity of calcineurin in mice inhibits MHC I and troponin I slow expression, while myoglobin expression is unaltered. This suggests that calcineurin has a direct effect on maintaining a fiber type of muscular tissue but does not always affect myoglobin production (Oh et al. 2005). Additionally, increases in myoglobin protein in skeletal muscle did not correlate with calcineurin expression, indicating that myoglobin can be stimulated in the absence of calcineurin expression (Schlater et al. 2014). Finding specific conditions under which myoglobin expression is independent from oxidative fiber type expression could serve to investigate regulatory mechanisms of this alternate pathway for myoglobin expression.

Besides expression levels of myoglobin being correlated to fiber type, changes in expression of the protein has been attributed to other factors such as NO generation, *in vitro* lipid supplementation (e.g. adding a mixed lipid composition to a lipid free media), hypoxia, and muscle contraction; suggesting possible alternate regulatory pathways for myoglobin expression (De Miranda et al. 2012; Kanatous et al. 2009; Rayner et al. 2009; Schlater et al. 2014). Pathological NO concentrations in vascular smooth muscle cells increase myoglobin concentration, while lipid supplementation to mammalian muscle correlates with increased myoglobin concentration (De Miranda et al. 2012; Rayner et al. 2009; Schlater et al. 2014). Hypoxia acting with another stimulus like muscular contraction is necessary to release calcium from the sarcoplasmic reticulum via ryanodine receptors and start the calcineurin-NFAT cascade to initiate the expression of myoglobin. Hypoxia acting without another stimulus induces a decrease in myoglobin expression by causing a release of calcium instead from the endoplasmic reticulum, causing NFAT to remain phosphorylated and unable to enter the nucleus and initiate transcription of myoglobin (Kanatous et al. 2009; Stiber et al. 2005). These studies identify conditions that alter myoglobin expression, but a targeted approach investigating increases in myoglobin expression and corresponding

fiber type indicators can help to fully understand the regulatory mechanisms that control when and how myoglobin is expressed in muscle.

Physiological levels of caffeine induce regulators of oxidative metabolism and mitochondrial biogenesis and are also implicated in activation of the slow oxidative fiber program (Gan et al. 2013; Schnuck et al. 2018). Caffeine reacts directly with ryanodine receptors causing releases of calcium from the sarcoplasmic reticulum that is responsible for the signaling of many biological functions, including differentiation and contraction within striated muscle (Ebashi and Endo 1968; Ogawa et al. 2020). Further, caffeine not only induces regulators of oxidative metabolism but also enhances lipid metabolism and reduces lipid content in skeletal muscle (Schnuck et al. 2018). Collectively, these suggest that caffeine promotes a slow oxidative fiber type and can increase lipid oxidation however, the extent of myoglobin expression and associated fiber types with these treatments have not been examined.

Given the uncertainty in the timing of fiber type and myoglobin expression, this study aimed to investigate when differentiating C_2C_{12} cells begin to express myoglobin in comparison to when they express different oxidative isoforms of MHC when subjected to lipid and/or caffeine treatments. We hypothesize that lipid supplementation will initiate myoglobin expression prior to an oxidative fiber type, and caffeine treatments will result in myoglobin expression following an oxidative fiber type. This study will provide more information as a stepping-stone to better understand how myoglobin can be expressed in skeletal muscle and when it is preceded by the expression of an oxidative fiber type. Western blots showed that under normal oxygen conditions with and without lipid supplementation, myoglobin expression preceded MHC I and IIA expression. Cells that received caffeine treatments with and without lipid supplementation began expressing MHC I and IIA before expressing myoglobin. Myoglobin assays showed that compared to other treatments, lipid supplemented cells had a significant increase in myoglobin. Caffeine treated cells that received lipid supplementation showed a slight increase in functional myoglobin expression compared to cells that only received caffeine treatment.

Methods

Cell culture

C_2C_{12} myoblasts were grown under standard conditions (37°C at 21% O_2 and 5% CO_2) to allow for proliferation as described previously by Schlater et al. (2014). Proliferation was achieved using a standard growth media consisting of high glucose Dulbecco's modified eagle's media (DMEM), 20% fetal bovine serum, 1% sodium pyruvate, 1% penicillin/streptomycin antibiotic. When the cells reached 90% confluency, a standard (control) differentiation media containing high glucose DMEM, 5% equine serum, 10µg/ml insulin, and 10µg/ml transferrin was applied to elicit differentiation of the myoblasts into myotubes (Schlater et al. 2014). This differentiation media for lipid groups additionally contained 2.5% of a lipid mixture (Sigma Aldrich) and caffeine treatment plates received control media with added 0.01M caffeine (Mallinckrodt Baker Inc.) for 20min every day, followed by a media change. Cells were harvested on sequential days after the initiation of differentiation, except for caffeine supplemented groups which were only harvested through day six, for subsequent analyses. There were three replicates of each sample group per harvested day, while the entire experiment was replicated three times to ensure valid results. Cells were harvested and western blot control muscles were homogenized (donated from samples collected by Colorado State University Laboratory Animal Resource Center under IACUC approval) with a lysis buffer consisting of 79% PBS, 20% Glycerol, 1% Tween, 0.001 M dithiothreitol, and a protease inhibitor tablet for later protein concentration determinations and western blot analysis as described previously (Schlater et al. 2014). Protein concentrations of harvested cells were analyzed using a Coomassie Plus Protein Assay (Thermo Scientific).

Western blots

To determine changes in protein expression western blots were performed as previously described (Kanatous et al. 2009). Each sample was run with two (caffeine groups harvested day four-six) to three replicates (all other sample groups). Controls were either mouse soleus or tibialis anterior homogenates (donated from samples collected by Colorado State University Laboratory Animal Resource Center under

IACUC approval). Samples were mixed in a 1:1 ratio with SDS and 0.01% bromophenol blue. Samples were then boiled for 3 minutes, then placed on ice for 2 minutes, and spun though glass wool spin columns for 30 seconds at 16,100 rcf. Following that step, 20µg of protein was loaded into precast wells (4-20% Mini-Protean TGX gels, Bio-Rad) and samples were run in a standard running buffer (1X tris-glycine SDS) at 150V for around 40 minutes or until the dye front reached the bottom of the gel. Resulting gels were transferred onto nitrocellulose membranes and were probed with primary antibodies. Rabbit anti-myoglobin (1:1,000; Pierce), Mouse IgG anti-MHCI (0.5ug/ml, BA-D5, Developmental Studies Hybridoma Bank (DSHB)), and Mouse IgG anti-MHCIIA (0.5ug/ml, 2F7, DSHB) were the primary antibodies utilized. To detect the primary antibodies, a horseradish peroxidase-conjugated Goat anti-Rabbit IgG and Goat anti-mouse IgG/IgM secondary antibodies (Invitrogen) and after were developed using SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific). The resulting protein bands were visualized using a BioRad ChemiDoc XRS+ imaging system and band intensity was quantified using Image Studio Lite version 5.2 software. The recorded band intensities for all experimental groups were arranged per sample type and day and plotted in Fig 2-2a and 2-3a.

Myoglobin protein concentration

Myoglobin concentrations of the samples was determined using a method modified from Kanatous et al. (2002) and Reynafarje (1963). Controls were either rat soleus or tibialis anterior homogenates (donated from samples collected by Colorado State University Laboratory Animal Resource Center under IACUC approval). Samples were added to a 0.04M potassium phosphate buffer and the mixes were centrifuged for 50 minutes at 28,000 x G. The supernatant was collected from the samples after centrifugation and bubbled for 3 minutes with carbon monoxide gas. To ensure complete myoglobin reduction, 0.01g of sodium dithionite was added to each sample after the initial 3 minutes of bubbling and samples were bubbled in carbon monoxide for 2 minutes thereafter (Schlater et al. 2014). After the bubbling procedure, samples were read at 538 and 568 nanometers (in a Beckman-Coulter DU-800 Spectrophotometer). Myoglobin protein concentration procedures were performed in triplicate. The resulting readings were

used in calculations to determine myoglobin concentrations and normalized to total protein concentration expressed in mg/mg because wet muscle mass measurements of cell lysates were not collected, as described by Reynafarje (1963) and De Miranda (2012). This assay acquires a myoglobin concentration of a sample based on myoglobin's functional capability to bind gasses, enabling our later reports of 'functional myoglobin expression.'

Statistical analysis

One-way ANOVA with a Tukey's post hoc test were used for statistical analysis of the data using RStudio version 3.6.1. All data presented are as means ± standard error, and significance between treatment groups on the same day was reported for values of $P \leq 0.05$. Figures were rendered with Biovinci version 1.1.5, r20181005.

Results

Lipid treatments

We found that cells differentiating under control conditions expressed myoglobin protein on day two after differentiation initiation (2.78 ± 0.82 relative units, $n = 9$) before the expression of MHC I protein on day three (0.23 ± 0.11 relative units, $n = 12$) or MHC IIA. In addition, MHC IIA did not appear on day two or day three after differentiation initiation (Fig. 2-2). Cells grown in under control conditions and receiving mixed lipid supplements also expressed myoglobin protein on day two (0.92 ± 0.31 relative units, $n = 9$), and showed a non-significant trend towards less myoglobin present than the day two control group not supplemented with mixed lipids. The lipid supplemented cells did not express MHC I until day three (0.62 ± 0.30 relative units, $n = 9$), showing a non-significant trend of increased expression with lipid supplementation compared to day three cells without lipid supplementation. Under control conditions with additional mixed lipid supplementation, MHC IIA did not appear on day two or day three after differentiation initiation (Fig. 2-2).

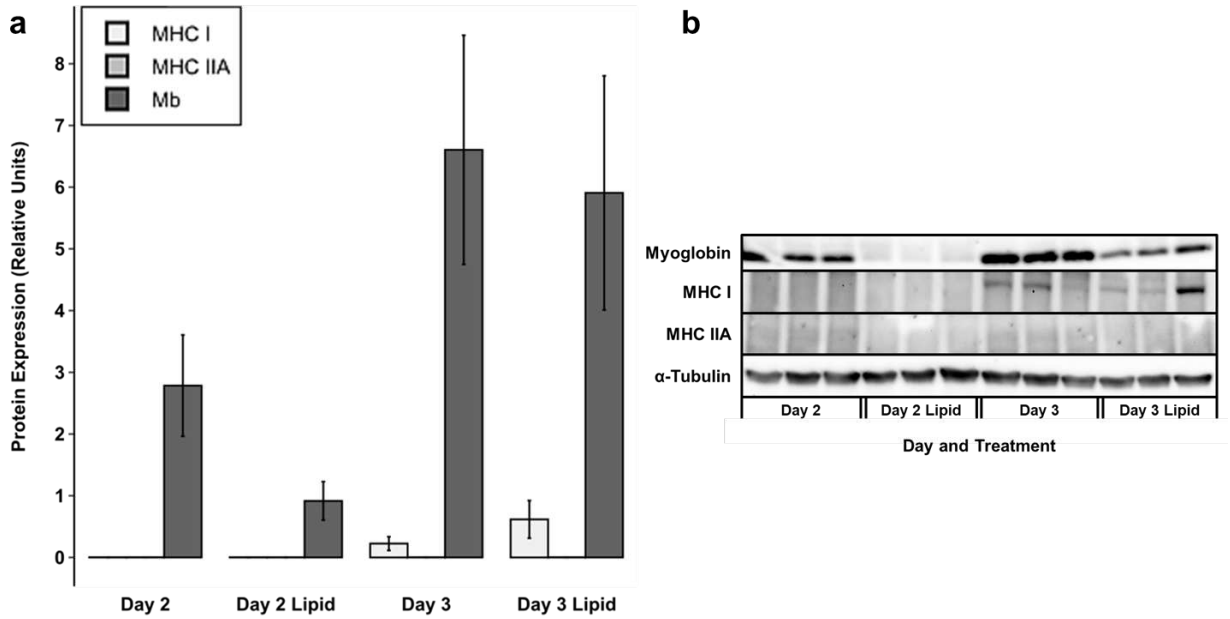

Fig. 2-2 Under control conditions, myoglobin is expressed before oxidative fibers in control and lipid supplemented cells. Western blot analysis to show myoglobin (Mb), myosin heavy chain (MHC) I, and IIA protein expression in C_2C_{12} cells under control conditions and with lipid supplementation. Total myoglobin and MHC protein expression was normalized to α-tubulin and quantified; day 2 $n = 9$, day 2 lipid $n = 9$, day 3 $n = 12$, and day 3 lipid $n = 12$ (**a**). Representative western blot from those used for quantifications; $n = 3$ per sample group (**b**)

Myoglobin assays showed functional myoglobin present on day two in cells differentiated under control conditions and control conditions with lipid supplementation (0.04 ± 0.005 and 0.05 ± 0.007, mg/mg protein, respectively, $n = 27$). Cells showed a significant increase in functional myoglobin expression in lipid supplemented cells compared to cells without supplementation (Day 3, $P = 0.005$, $n = 27$; Day 4, $P = 0.0003$, $n = 33$; Day 5, $P = 0.0002$, $n = 32$; Day 6, $P = 0.0008$, $n = 33$) (Fig. 2-3 and Table 2-1).

Lipid and caffeine treatments

We found that differentiating cells subjected to caffeine treatments expressed MHC I and IIA protein on day two after the initiation of differentiation (0.64 ± 0.21 and 0.189 ± 0.05 relative units, respectively, $n = 6$), before the expression of myoglobin protein on day three (0.04 ± 0.01 relative units, $n = 6$) (Fig. 2-4). Differentiating cells receiving lipid supplements and subjected to caffeine treatments also expressed MHC

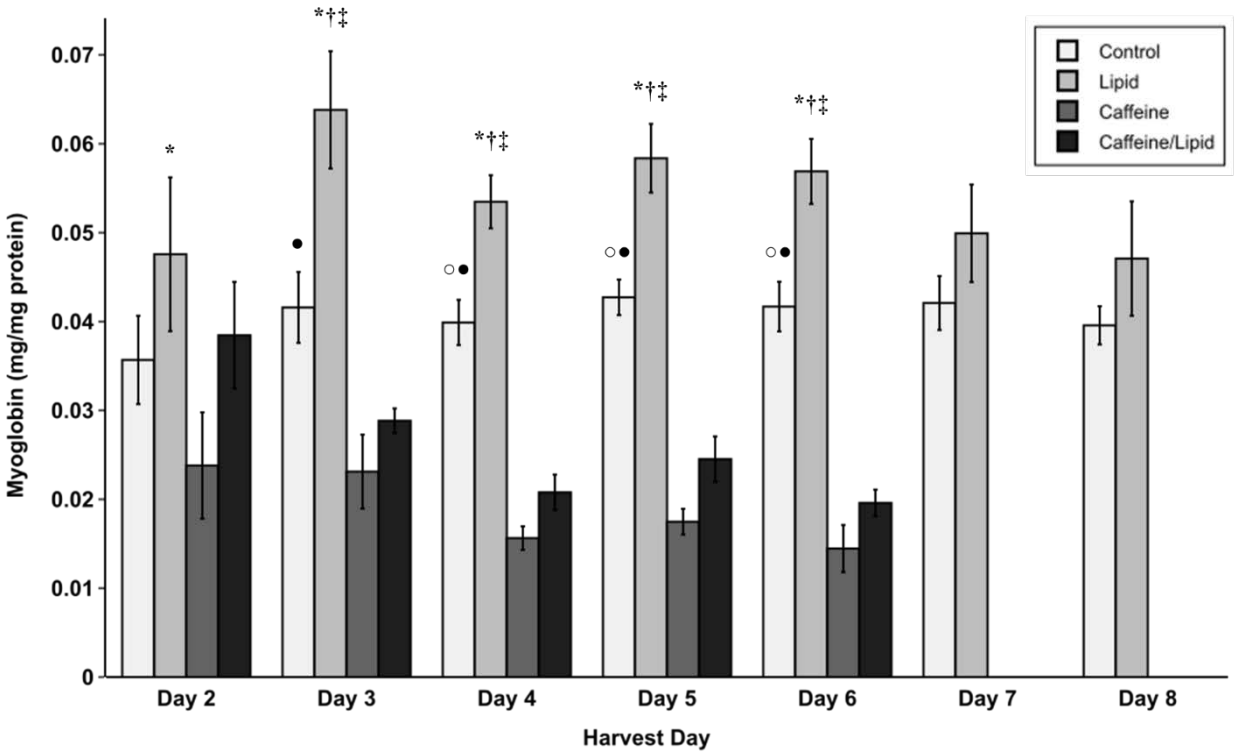

Fig. 2-3 Myoglobin concentration in C_2C_{12} cells. In control conditions with added lipid supplementation, cells expressed more myoglobin than other treatment groups on the same day. Cells receiving lipid and caffeine supplementation expressed slightly more myoglobin than same day cells only supplemented with caffeine. (*significantly different than same day caffeine, $P \leq 0.05$) († significantly different than same day lipid/caffeine $P \leq 0.0001$) (‡ significantly different than same day control $P \leq 0.005$) (● significantly different than same day caffeine $P \leq 0.05$) (○ significantly different that same day lipid/caffeine $P \leq 0.0001$)

I and IIA protein on day two after differentiation initiation (0.83 ± 0.3 and 0.13 ± 0.02 relative units, respectively, $n = 6$) before very faint expression of myoglobin protein on day three (0.01 ± 0.004 relative units, $n = 6$), followed by slightly more expression on day four (0.5 ± 0.15 relative units, $n = 6$) (Fig. 2-4).

Myoglobin assays detected functional myoglobin present on day two in differentiating cells receiving caffeine treatments and cells receiving lipid supplementation and caffeine treatments (0.02 ± 0.006 and 0.04 ± 0.006 mg/mg protein, respectively, $n = 25$). Cells showed a non-significant trend of increased functional myoglobin lipid and caffeine supplemented groups on each day following initiation of differentiation. On each day after differentiation initiation, cells differentiated in control conditions also receiving mixed lipid supplementation showed a significant increase in functional myoglobin when compared to same day cells receiving caffeine treatments (Day 2, $P = 0.05$, $n = 27$; Day 3, $P < 0.0001$, n

Table 1 Summary of myoglobin concentrations of C_2C_{12} skeletal muscle cell treatment groups

Harvest Day	Treatment Groups	Myoglobin Concentration (mg/mg protein)	*n*
Day 2	Control	0.04 ± 0.005	27
	Lipid	0.05 ± 0.009*	27
	Caffeine	0.02 ± 0.006	27
	Caffeine/Lipid	0.04 ± 0.006	25
Day 3	Control	0.04 ± 0.004●	27
	Lipid	0.06 ± 0.007*†‡	32
	Caffeine	0.02 ± 0.004	23
	Caffeine/Lipid	0.03 ± 0.001	24
Day 4	Control	0.04 ± 0.003●○	36
	Lipid	0.05 ± 0.003*†‡	33
	Caffeine	0.02 ± 0.001	27
	Caffeine/Lipid	0.02 ± 0.002	27
Day 5	Control	0.04 ± 0.002●○	35
	Lipid	0.06 ± 0.004*†‡	32
	Caffeine	0.02 ± 0.001	24
	Caffeine/Lipid	0.02 ± 0.003	24
Day 6	Control	0.04 ± 0.003●○	35
	Lipid	0.06 ± 0.004*†‡	33
	Caffeine	0.01 ± 0.003	27
	Caffeine/Lipid	0.02 ± 0.002	26
Day 7	Control	0.04 ± 0.003	24
	Lipid	0.05 ± 0.005	21
Day 8	Control	0.04 ± 0.002	27
	Lipid	0.05 ± 0.006	17

* significantly different than same day Caffeine P = 0.05
† significantly different than same day Caffeine/Lipid P < 0.0001
‡ significantly different than same day Control P = 0.005
● significantly different than same day Caffeine P = 0.05
○ significantly different that same day Caffeine/Lipid P = 0.0001

= 23; Day 4, $P < 0.0001$, $n = 27$; Day 5, $P < 0.0001$, $n = 24$; Day 6, $P < 0.0001$, $n = 27$). On several days after differentiation initiation, cells differentiated in control conditions also receiving mixed lipid supplementation showed a significant increase in functional myoglobin when compared to same day cells receiving lipid and caffeine supplements (Day 3, $P < 0.0001$, $n = 24$; Day 4, $P < 0.0001$, $n = 27$; Day 5, $P < 0.0001$, $n = 24$; Day 6, $P < 0.0001$, $n = 26$). On several days following the initiation of differentiation, cells differentiated under control conditions showed a significant increase in functional myoglobin when compared to same day cells receiving caffeine treatments (Day 3, $P = 0.05$, $n = 23$; Day 4, $P < 0.0001$, $n = 27$; Day 5, $P < 0.0001$, $n = 24$; Day 6, $P < 0.0001$, $n = 27$). Lastly, on several days after differentiation initiation, cells differentiated in control conditions showed a significant increase in functional myoglobin

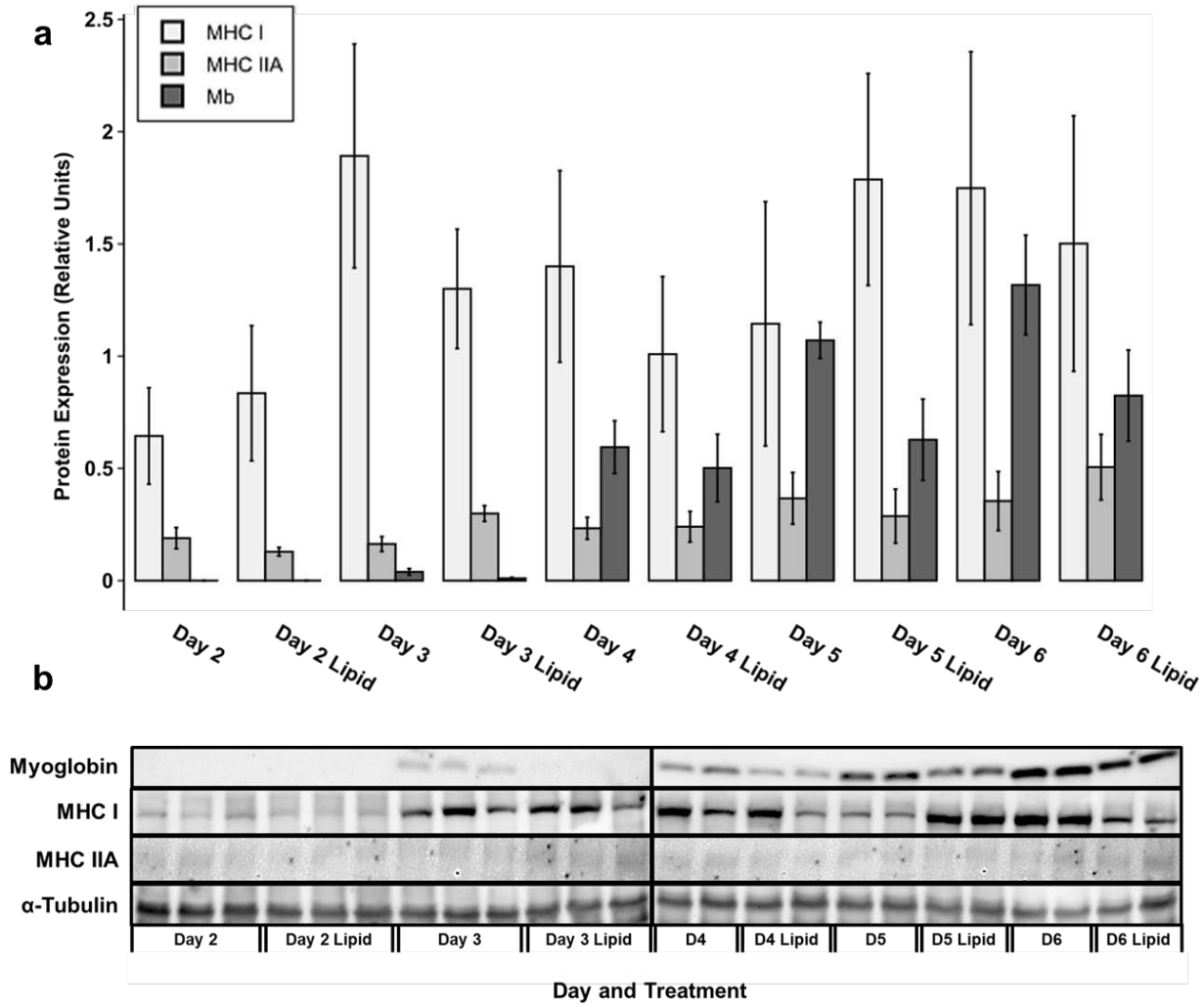

Fig. 2-4 In differentiating cells exposed to caffeine stimulation, oxidative fibers are expressed before myoglobin. Western blot analysis to show myoglobin (Mb), myosin heavy chain (MHC) I, and IIA protein expression in caffeine supplemented C_2C_{12} cells with and without lipid supplementation, determined by western blot analysis. Total myoglobin and MHC protein expression was normalized to α-tubulin and quantified; $n = 6$ for all experimental groups (**a**). Representative western blot from those used for quantifications; harvest days 2-3 $n = 3$ and harvest days 4-6 $n = 2$ (**b**)

when compared to same day cells receiving lipid and caffeine supplements (Day 4, $P < 0.0001$, $n = 27$; Day 5, $P = 0.0001$, $n = 24$; Day 6, $P < 0.0001$, $n = 26$) (Fig. 2-3 and Table 2-1).

Discussion

The results of this study provide researchers with a timeline of expression of myoglobin and oxidative MHCs with corresponding cell culture conditions. In providing this timeline, we have found that: (1)

under control conditions, myoglobin expression precedes that of oxidative fibers in control and lipid supplemented cells; (2) with the addition of caffeine, expression of oxidative fibers precedes that of myoglobin in cells without or with lipid supplementation; and (3) functional myoglobin is reduced in cells receiving caffeine treatments without and with lipid supplements compared to cells under standard (control) differentiation conditions without and with lipid supplements (Fig. 2-5). Our results support our hypothesis that lipid supplements will initiate myoglobin expression prior to an oxidative fiber type and caffeine treatments will result in myoglobin expression following an oxidative fiber type, but we also found that our control conditions resulted in myoglobin expression before an oxidative fiber type was expressed. These results indicate that without stimulation there are alternate pathways to myoglobin

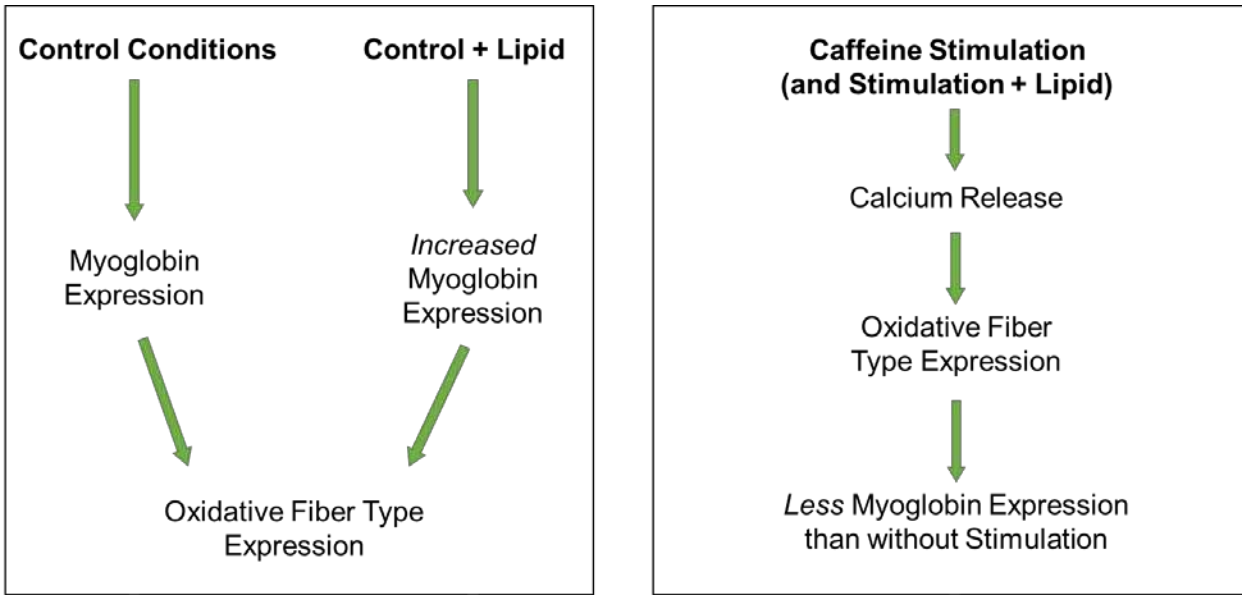

Fig. 2-5 In control conditions, myoglobin is expressed before oxidative fiber types, and while the same results were seen with lipid-supplemented cells, functional myoglobin assays indicated increased levels of functional myoglobin. In caffeine-stimulated cells, caffeine-initiated calcium signaling induces the development of oxidative fiber types, which are followed by myoglobin expression, regardless of lipid supplementation. Myoglobin assays of caffeine groups showed less functional myoglobin than control groups

expression that do not require a prior oxidative fiber type expression in unstimulated muscle cells with and without lipid supplementation, but with stimulation as in our caffeine treatment groups, cells express myoglobin likely based on prior expression of the oxidative fiber type.

Our principal finding was that under standard culture conditions in C_2C_{12} cells, myoglobin expression preceded the expression of MHC I and IIA, and that we observed the same expression patterns in cells receiving lipid supplementation during differentiation. Enhanced myoglobin expression in I or IIA fiber types has led to the assumption that myoglobin expression is always preceded by an oxidative fiber type (Chin et al. 1998; Kanatous and Mammen 2010; Ordway and Garry 2004; Schiaffino and Reggiani 1996). This idea is based on the correlations identified between activated calcineurin in a slow fiber type and elevated myoglobin expression, but our findings suggest that myoglobin expression can precede a specific oxidative fiber type (Chin et al. 1998; Dolmetsch et al. 1997; Kanatous and Mammen 2010; Kanatous et al. 2009). Our principal findings corroborate other studies that found myoglobin expression regulation independent from fiber type and calcineurin signaling, and further investigates the timing of myoglobin and MHC expression (Kanatous et al. 2009; Oh et al. 2005; Schlater et al. 2014).

Myoglobin mRNA or protein is not expressed until after myoblast fusion and differentiation in rodent myoblasts grown *in vitro*, but reports vary as to specifically when after differentiation initiation myoglobin is produced and do not include corresponding fiber type information (Devlin et al. 1989; Graber and Woodworth 1986; Ordway and Garry 2004; Van der Giessen et al. 2003; Weller et al. 1986). For instance, investigators found that myoglobin expression in C_2C_{12} cells under standard culture conditions was preceded by MHC, disagreeing with our results. The antibody used to determine the presence of MHCs was MF-20 (DSHB) which recognizes the rod-like tail region of the MHC protein found in all isoforms of MHC. In this instance it is unclear which specific isoform of MHC was being expressed (Bader et al. 1982; Van der Giessen et al. 2003). Primary myotubes express several embryonic-type MHCs before their fate is set to a slow or fast fiber type (Weiss and Leinwand 1996). Perhaps another MHC isoform is being expressed in our cells before myoglobin expression besides an oxidative fiber type.

While our *in vitro* controls in this experiment were our cells differentiating under control conditions with standard differentiation media, our caffeine treated cells may be a more *in vivo* representation of myoglobin and fiber type expression where contractile stimulation of muscle cells would occur. Caffeine

causes releases of calcium from distinct pools in skeletal muscle. These changes in intracellular calcium are responsible for the signaling of many biological functions, including differentiation initiation and contraction within striated muscle cells (Ebashi and Endo 1968; Friday et al. 2000; Ogawa et al. 2020). However, many have implicated caffeine in several detrimental effects like blockage of insulin signaling, decreases in muscle fiber size, decreasing protein synthesis, increasing protein degradation, and promoting autophagy (Enyart et al. 2020; Moore et al. 2017; Hughes et al. 2017). We did not find that caffeine impaired our cell differentiation, likely due to our physiologically relevant caffeine concentration. Physiological levels of caffeine do not affect anabolic signaling or myoblast proliferation or differentiation and are able to elicit calcium releases in cells (Kordosky-Herrera and Grow 2009; Moore et al. 2017). Notably, calcineurin is a protein activated by a sustained high concentration of calcium and is responsible for the development and maintenance of slow fibers in skeletal muscle (Chin et al. 1998; Delling et al. 2000). Additionally, caffeine stimulates lipid metabolism and also contraction-stimulated glucose transport through AMPK (Schnuck et al. 2018; Tsuda et al. 2015). We did not find differences in fiber type expression between our caffeine groups with or without lipid supplementation. Although we did not measure calcineurin expression or metabolic indices, we suspect that the enhanced differentiation and development of oxidative fibers followed by the expression of myoglobin in our caffeine groups is likely due to the caffeine-induced calcium fluctuations occurring within the cells.

Our functional myoglobin assay results showed decreased levels of myoglobin expression in caffeine-treated cells, and that lipid supplements elevated myoglobin expression in lipid groups but could not reverse the dampened myoglobin expression in caffeine groups. Our functional myoglobin under standard culture conditions at day seven was 0.04 mg/mg protein (Fig. 2-4 and Table 2-1), which closely mirrors day seven functional myoglobin values found by others under replicate culture conditions (De Miranda et al. 2012; Schlater et al. 2014). Our functional myoglobin levels for cells differentiated under control conditions that additionally received lipid supplementation were less than those found in other experiments however, these other researchers supplemented their cells with 5% lipid as compared to our 2.5% lipid supplementation. These researchers also found a significant increase in functional myoglobin

in 5% lipid supplemented cells, whereas we did not see significant changes from standard culture conditions with our 2.5% lipid supplemented group on day seven, this could be due to a potential graded response to lipid (Schlater et al. 2014).

An increase in mixed lipid availability to C_2C_{12} cells increases functional myoglobin, but decreased markers of aerobic metabolism, such that myoglobin increases in this instance were due to reasons aside from enhancing muscular oxygen stores (Schlater et al. 2014). Mitochondrial ROS increase with an increase in β-oxidation of fatty acids, which may stimulate myoglobin expression due to its reactive oxygen scavenging ability (Flögel et al. 2004; Helbo et al. 2013; Mannino et al. 2019; St-Pierre et. al 2002). Other reasons for enhanced myoglobin expression with lipid supplementation could be due to fatty acid binding. Myoglobin competes with fatty acid binding protein when concentrations of unsaturated fatty acids rise above a concentration threshold, and therefore enhances fatty acid solubility in the sarcoplasm to channel fatty acids to β-oxidation (Jue et al. 2016; Sriram et al. 2008). Additionally, the hearts of myoglobin knockout mice show diminished fatty acid oxidation and augmented triglyceride accumulation and glucose utilization by mitochondria (Flögel et al. 2005; Hendgen-Cotta et al. 2017). Myoglobin also induces mitochondrial fusion and decreases cell proliferation in specific types of cancer, which often uptake lipids for growth, indicating another possible role where myoglobin may be elevated to regulate lipid-enhanced cell proliferation and differentiation (Braganza et al. 2019; Snaebjornsson et al. 2020). While we did not measure ROS and β-oxidation or other indices of metabolism, we suspect that lipid-induced increases in myoglobin expression could be due to reasons aside from enhancing muscular oxygen stores.

Our caffeine treated cells showed an interesting decreased amount of functional myoglobin compared to our control cells. Physiological levels of caffeine have been shown to induce mitochondrial biogenesis through mechanisms dependent on activation of PPARβ/δ, oxidative metabolism, and activate the slow oxidative fiber program (Gan et al. 2013; Schnuck et al. 2018). Slow oxidative fibers are known to receive a sustained high concentration of calcium signaling to activate calcineurin and thus effect the expression of myoglobin (Chin et al. 1998). However, we found an upregulation of oxidative fiber type

indicators, but our functional myoglobin assays did not reflect a corresponding myoglobin enhancement relative to our control group. Further, caffeine not only induces regulators of oxidative metabolism but also enhances lipid metabolism in skeletal muscle (Schnuck et al. 2018). Although we did not measure lipid interaction with myoglobin, we think that caffeine causes lipid metabolism in a way that negates the need for myoglobin as a lipid transporter, causing a dampened expression following an established oxidative fiber type.

In this study, the C_2C_{12} cells that were differentiated in control conditions without and with lipid supplementation showed myoglobin expression before the expression of MHC I and IIA, indicating an underlying pathway to myoglobin expression before the expression of an oxidative fiber type. Cells receiving daily doses of caffeine treatment (to stimulate contraction) showed myoglobin expression following the expression of MHC I and IIA, indicating that with appropriate calcium signaling myoglobin expression follows an established oxidative fiber type pathway to expression. Functional myoglobin assays showed that cells receiving lipid supplementation had more enhanced myoglobin expression without caffeine treatments, suggesting that lipids are in some way more effective at increasing functional myoglobin expression than caffeine within cultured muscle tissue. In our lipid groups, there may be many roles for myoglobin to fill including ROS scavenging and lipid transport, whereas caffeine groups may not need enhanced myoglobin for lipid transport. This has yet to be investigated. Further research utilizing similar culture conditions is warranted to investigate the molecular drivers of pathways to myoglobin expression that are not reliant on a prior expression of an oxidative fiber type.

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CHAPTER 3: MYOGLOBIN EXPRESSION UNDER HYPOXIA PRECEDES OXIDATIVE FIBER TYPE EXPRESSION[2]

Summary

Myoglobin is a hemoprotein expressed in vertebrate muscle and is typically known to follow an established oxidative muscle fiber type found in aerobic muscles. More recent evidence has displayed changes in myoglobin expression without a change in fiber type, indicating that myoglobin expression could be regulated by different pathways and may not always be dependent on a prior expressed fiber type. Mixed lipid supplements, and hypoxia coupled with lipid supplements have been shown to elevate myoglobin levels in skeletal muscle, but it is unknown how these treatments affect myoglobin expression relative to the fiber type of the cultured tissue. To investigate, we have cultured and differentiated C_2C_{12} myoblasts in the presence and absence of lipid, and/or in hypoxic conditions and cells were harvested each day after the initiation of differentiation. Western blots were conducted to determine the expression of myoglobin and various oxidative fiber myosin heavy chains. Under control conditions or hypoxia regardless of lipid supplementation, myoglobin expression occurred before that of oxidative fiber expression. Cells exposed to hypoxia and lipid supplementation showed a significant increase in functional myoglobin expression, while cells exposed to solely lipid supplementation showed a less significant increase. Collectively, we see conditions under which there are pathways to myoglobin expression that precede oxidative fiber type expression. Examination of alternate routes of myoglobin expression involving hypoxic exposure that precede oxidative fiber types could yield potential therapeutic benefits to combat ischemic diseases seen in humans.

[2] To be Submitted to the Journal of Muscle Research and Cell Motility (In Preparation)

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Introduction

Myoglobin is an extensively characterized cytoplasmic hemoprotein primarily found in vertebrate muscular tissue. The structure of myoglobin consists of a globin backbone that supports an iron-containing heme prosthetic group, responsible for the reversible binding of several ligands like oxygen, nitric oxide (NO), and carbon monoxide (Garry and Mammen 2007) Myoglobin is most well known as a protein involved in oxygen storage and provides oxygen to working muscles when oxygen delivery from the blood is insufficient. Due to its ability to reversibly bind ligands, myoglobin has also been implicated in nitric oxide and reactive oxygen species (ROS) scavenging and has a role in cellular lipid transport (Flögel et al. 2001; Flögel et al. 2004; Kanatous and Mammen 2010; Shih et al. 2014). Myoglobin releases oxygen for aerobic metabolism only when the partial pressure of oxygen is very low, due to its high affinity for oxygen (Schenkman et al. 1997). High concentrations of myoglobin ameliorate the detrimental effects of ischemia in diving vertebrates, and in this capacity, could be implicated in human health application (Kanatous and Mammen 2010). Ischemia is a shortage of blood supply to working tissues in the body, where the delivery of vital components such as oxygen, metabolites, and removal of waste is decreased (Mallick et al. 2004). The hypoxic conditions associated with ischemia can cause damage even cell death to many tissues and organs within minutes however, enhanced oxygen stores bound to myoglobin could be a way to resolve this issue and keep tissues from becoming hypoxic as seen in diving mammals, particularly as a therapeutic intervention in human ischemic illnesses.

Myoglobin expression has been correlated with specific muscle fiber types; such that there is more myoglobin found in slow oxidative fiber types (type I) and fast oxidative (type IIA) containing type I and type IIA fibers, respectively. Compared to oxidative fiber types, fast glycolytic fiber types express very little myoglobin in IIX fiber types and none in IIB fiber types. Fiber types are identified as type I, IIA, IIX, and IIB designations depending on which myosin heavy chain (MHC) isoform they express (Kanatous and Mammen 2010; Ordway and Garry 2004; Schiaffino and Reggiani 1996).

Myoglobin is typically expressed in these fiber types through the calcineurin-NFAT (Nuclear Factor of Activated T cell) pathway, which mediates calcium signaling in many biological systems. This system

is characterized by the activation of NFAT proteins via dephosphorylation by the phosphatase calcineurin, which controls target gene expression by allowing NFAT to translocate from the cytoplasm to the nucleus where it can collaborate with other factors (Pan et al. 2013). One group found in C_2C_{12} cells (immortalized mouse skeletal muscle cells) that the addition of activated calcineurin correlated with an increase the activity of myoglobin and troponin I slow promoters, known to be found in slow-twitch oxidative fibers expressing MHC I. They also found that when calcineurin activity was inhibited in mice, the fiber type switched from slow to fast (Chin et al. 1998). These correlations suggested that myoglobin expression is dependent on and followed the fiber type expression of the tissue, because slow-twitch

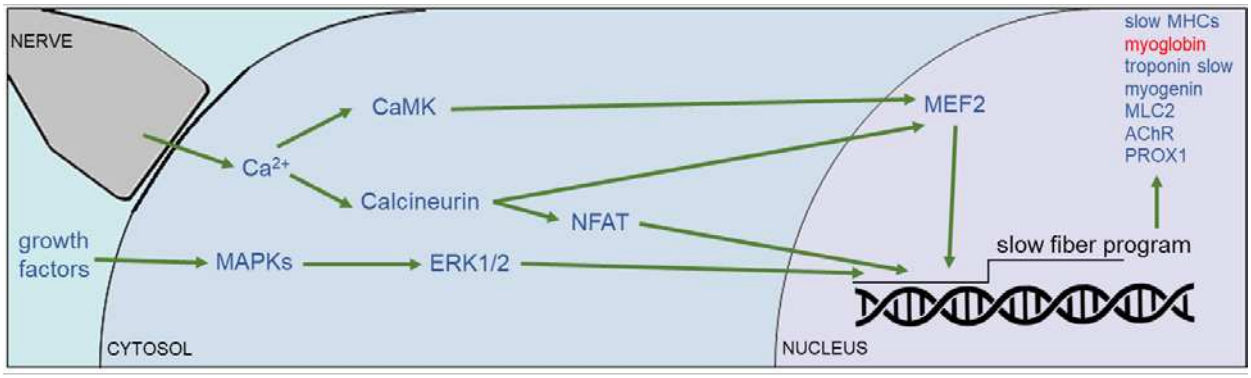

Fig. 3-1 Traditional *in vivo* myosin heavy chain (MHC) and myoglobin expression pathway. Myoglobin expression is thought to follow the induction of the slow fiber program which can be achieved through growth factors working through the mitogen-activated protein kinase/extracellular signal-related kinase (MAPK/ERK) pathway (seen *in vitro*), and additionally through nerve stimulated Ca2+ signaling, eventually translocating nuclear factor of activated T cell (NFAT) and myocyte enhancer factor-2 (MEF2) to the nucleus to initiate the slow fiber program. This induction of the slow fiber program is thought to lead to the expression of myoglobin, and other slow fiber-related proteins (Myosin regulatory light chain 2: MLC2; acetylcholine receptor: AChR; Prospero homeobox protein 1: PROX1) (Chapter 2)

fibers were able to receive the appropriate calcium signaling (a sustained high concentration) to activate calcineurin and effect the expression of myoglobin (Chin et al. 1998; Dolmetsch et al. 1997).

Alternatively, more recent evidence has shown that myoglobin expression is not solely dependent on calcium signaling by calcineurin activation and that other uncharacterized pathways exist (Oh et al. 2005; Schlater et al. 2014). One group found that myoglobin expression does not change when the phosphatase activity of calcineurin is inhibited in mice, but this causes MHC I and troponin I slow expression to cease. This suggests calcineurin has a direct effect on maintaining fiber type of muscular tissue but does not

always affect myoglobin expression (Oh et al. 2005). Further, increases in myoglobin protein in skeletal muscle does not correlate to an increase in the expression of calcineurin, indicating that myoglobin can be stimulated without calcineurin expression (Schlater et al. 2014). Finding specific conditions under which myoglobin expression is independent from oxidative fiber type expression could serve to elucidate novel regulatory pathways for myoglobin expression.

Besides expression levels of myoglobin being correlated to fiber type, other factors such as NO generation, *in vitro* lipid supplementation (e.g. adding a mixed lipid composition to a lipid free media), hypoxia, and muscle contraction have resulted increased myoglobin; suggesting possible alternate regulatory pathways for myoglobin expression (De Miranda et al. 2012; Kanatous et al. 2009; Rayner et al. 2009; Schlater et al. 2014). Pathological NO concentrations in vascular smooth muscle cells yield an increase in myoglobin concentration, while lipid supplementation to mammalian muscle was correlated to an increase in myoglobin concentration (De Miranda et al. 2012; Rayner et al. 2009; Schlater et al. 2014). To properly initiate myoglobin expression, hypoxia acting with another stimulus like muscular contraction is necessary to release calcium from the sarcoplasmic reticulum via ryanodine receptors (Kanatous et al. 2009). These studies identify conditions that alter myoglobin expression, but a targeted approach investigating increases in expression of myoglobin and corresponding fiber type indicators can help to fully understand the regulatory mechanisms that control when and how myoglobin is expressed in muscle.

Muscle cells show several responses to hypoxic exposure. In one study, hypoxia released calcium solely from the endoplasmic reticulum, causing NFAT to remain phosphorylated and unable to enter the nucleus and initiate transcription of myoglobin (Kanatous et al. 2009; Stiber et al. 2005). They also found that a decrease in myoglobin expression does not correspond to a change in fiber type, suggesting that myoglobin and fiber type can be regulated independently (Kanatous et al. 2009). Other groups have found that hypoxia coupled with a secondary stimulus like lipid is sufficient to enhance myoglobin expression. Additionally, applying a ROS scavenger to lipid supplemented cells results in a decrease in myoglobin expression, while the same ROS scavenger treatment to lipid supplemented cells in hypoxia results a

much milder decrease in myoglobin expression. This suggests that myoglobin plays a larger role in ROS scavenging in lipid supplemented cells as compared to those in hypoxia (Schlater et al. 2014). In hypoxia, deoxygenated myoglobin acts as a nitrate reductase to produce NO from nitrate which reversibly binds to cytochrome-c-oxidase to inhibit mitochondrial respiration. Mitochondrial inhibition limits oxygen consumption and limits the production of ROS (Shiva et al. 2007; Hendgen-Cotta et al. 2008). Collectively, these findings suggest that lipid treatment under hypoxia can increase myoglobin expression however, changes in myoglobin expression under hypoxia are not always tied to the expression of a fiber type.

Given the uncertainty in the timing of fiber type and myoglobin expression, this study aimed to investigate when differentiating C_2C_{12} cells begin to express myoglobin in comparison to when they express different oxidative isoforms of MHC when subjected to lipid treatments and/or hypoxia. We hypothesize that lipid and/or hypoxia treatments will result in myoglobin expression before an oxidative fiber type is expressed. This study will provide more information as a stepping-stone to understand how myoglobin can be expressed in skeletal muscle and when it is preceded by the expression of an oxidative fiber type. Western blots showed that under normal oxygen and low oxygen conditions with and without lipid supplementation, myoglobin expression preceded MHC I and IIA expression. Myoglobin assays showed significantly higher functional myoglobin expression in lipid supplemented cells compared to control groups, and even more elevated myoglobin expression in cells differentiated in hypoxia and receiving lipid supplements compared to all other treatment groups.

Methods

Cell culture

C_2C_{12} myoblasts were grown in a standard, normoxic (37°C at 21% O_2 and 5% CO_2) environment to allow for proliferation as described previously (Schlater et al. 2014). The standard proliferation media used consisted of high glucose Dulbecco's modified eagle's media (DMEM), 20% fetal bovine serum, 1% sodium pyruvate, 1% penicillin/streptomycin antibiotic. When the cells reached 90% confluency, a

standard differentiation media containing high glucose DMEM, 5% equine serum, 10µg/ml insulin, and 10µg/ml transferrin was applied to elicit differentiation of the myoblasts into myotubes (Schlater et al. 2014). Treatment groups were placed in either control (normoxia, 37°C at 21% O_2 and 5% CO_2) or hypoxic (37°C at 1% O_2, 5% CO_2, and 94% nitrogen) conditions upon the addition of differentiation media at 90% confluency. Hypoxic cells were differentiated in a hypoxic chamber (Coy Laboratories) and were kept in hypoxic conditions until they were harvested. The differentiation media of the lipid groups also contained 2.5% of a lipid mixture (Sigma Aldrich). Control cells were harvested on days 2, 3, 4, 5, 6, 7, and 8 and hypoxic cells on days 3, 4, 5, and 6 after the initiation of differentiation for subsequent analyses. Hypoxic cells were not harvested on day two because preliminary data collected indicated that our proteins of interest were not expressed on this early day (Fig. 3-2a). There were three replicates of each sample group per harvested day, while the entire experiment was replicated three times to ensure valid results. Cells were harvested and western blot control muscles were homogenized (donated from samples collected by Colorado State University Laboratory Animal Resource Center under IACUC approval) with a lysis buffer consisting of 79% PBS, 20% Glycerol, 1% Tween, 0.001 M dithiothreitol, and a protease inhibitor tablet for later protein concentration determinations and western blot analysis as described previously (Schlater et al. 2014). Protein concentrations of harvested cells were analyzed using a Coomassie Plus Protein Assay (Thermo Scientific).

Western blots

To determine changes in protein expression western blots were performed as previously described (Kanatous et al. 2009). Each sample was run with three replicates. Controls were either mouse soleus or tibialis anterior homogenates (donated from samples collected by Colorado State University Laboratory Animal Resource Center under IACUC approval). Samples were mixed in a 1:1 ratio with SDS and 0.01% bromophenol blue. Samples were then boiled for 3 minutes, then placed on ice for 2 minutes, and spun though glass wool spin columns for 30 seconds at 16,100 rcf. Following that step, 20µg of protein was loaded into precast wells (4-20% Mini-Protean TGX gels, Bio-Rad) and samples were run in a

standard running buffer (1X tris-glycine SDS) at 150V for around 40 minutes or until the dye front reached the bottom of the gel. Resulting gels were transferred onto nitrocellulose membranes and were probed with primary antibodies. Rabbit anti-myoglobin (1:1,000; Pierce), Mouse IgG anti-MHCI (0.5ug/ml, BA-D5, Developmental Studies Hybridoma Bank (DSHB)), and Mouse IgG anti-MHCIIA (0.5ug/ml, 2F7, DSHB) were the primary antibodies utilized. To detect the primary antibodies, a horseradish peroxidase-conjugated Goat anti-Rabbit IgG and Goat anti-mouse IgG/IgM secondary antibodies (Invitrogen) and after were developed using SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific). The resulting protein bands were visualized using a BioRad ChemiDoc XRS+ imaging system and band intensity was quantified using Image Studio Lite version 5.2 software. The recorded band intensities for all experimental groups were arranged per sample type and day and plotted in Fig 3-2a.

Myoglobin protein concentration

Myoglobin concentrations of the samples was determined using a method modified from Kanatous et al. (2002) and Reynafarje (1963). Controls were either rat soleus or tibialis anterior homogenates (donated from samples collected by Colorado State University Laboratory Animal Resource Center under IACUC approval). Samples were added to a 0.04M potassium phosphate buffer and the mixes were centrifuged for 50 minutes at 28,000 x G. The supernatant was collected from the samples after centrifugation and bubbled for 3 minutes with carbon monoxide gas. To ensure complete myoglobin reduction, 0.01g of sodium dithionite was added to each sample after the initial 3 minutes of bubbling and samples were bubbled in carbon monoxide for 2 minutes thereafter (Schlater et al. 2014). After the bubbling procedure, samples were read at 538 and 568 nanometers (in a Beckman-Coulter DU-800 Spectrophotometer). Myoglobin protein concentration procedures were performed in triplicate. The resulting readings were used in calculations to determine myoglobin concentrations and normalized to total protein concentration expressed in mg/mg because wet muscle mass measurements of cell lysates were not collected, as described by Reynafarje (1963) and De Miranda (2012). This assay acquires a myoglobin concentration

of a sample based on myoglobin's functional capability to bind gasses, enabling our later reports of 'functional myoglobin expression.'

Statistical analysis

One-way ANOVA with a Tukey's post hoc test were used for statistical analysis of the data using RStudio version 3.6.1. All data presented are as means ± standard error, and significance between treatment groups on the same day was reported for values of $P \leq 0.05$. Figures were rendered with Biovinci version 1.1.5, r20181005.

Results

Western Blots

We found that cells differentiating under control conditions expressed myoglobin protein on day two after differentiation initiation (2.78 ± 0.82 relative units, $n = 9$) before the expression of MHC I protein on day three (0.23 ± 0.11 relative units, $n = 12$). Under control conditions MHC IIA did not appear on day two or day three after differentiation initiation (Fig. 3-2a and 3-2b). Cells grown in control conditions receiving mixed lipid supplements also expressed myoglobin protein on day two (0.92 ± 0.31 relative units, $n = 9$), although showed a non-significant trend towards a less myoglobin present than the day two control group not supplemented with mixed lipids. The lipid supplemented cells did not express MHC I until day three (0.62 ± 0.30 relative units, $n = 9$), showing a non-significant trend of increased expression with lipid supplementation compared to day three cells without lipid supplementation. Under control conditions with mixed lipid supplementation, MHC IIA did not appear on day two or day three after differentiation initiation (Fig. 3-2a and 3-2b).

We found that cells differentiating under hypoxic conditions expressed myoglobin on day three after the initiation of differentiation (0.16 ± 0.06 relative units, $n = 8$), before the significant expression of MHC I

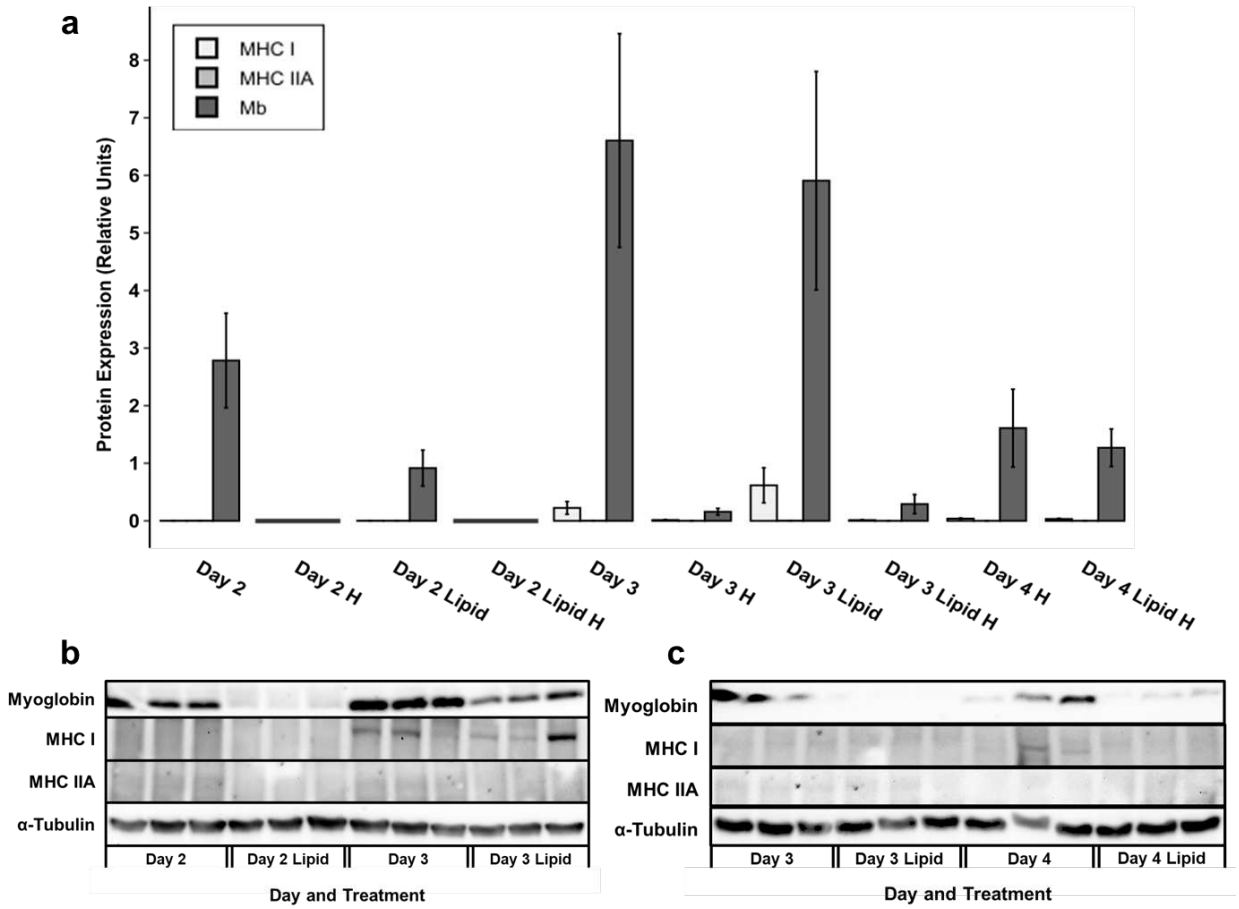

Fig. 3-2 Under control conditions and hypoxia, myoglobin is expressed before oxidative fibers in without and with lipid supplementation. Myoglobin (Mb), myosin heavy chain (MHC) I, and IIA protein expression in C_2C_{12} cells under control or hypoxic (H) conditions and with lipid supplementation (Lipid), determined by western blot analysis. Total myoglobin and myosin heavy chain protein expression was normalized to α-tubulin and quantified; day 2 $n = 9$, day 2 H $n = 3$, day 2 lipid $n = 9$, day 2 lipid H $n = 3$, day 3 $n = 12$, day 3 H $n = 8$, day 3 lipid $n = 12$, day 3 lipid H $n = 8$, day 4 H $n = 9$, day 4 lipid H $n = 9$ (**a**). Representative western blot from control (**b**) and hypoxic (**c**) cells from those used for quantifications

on day four (0.04 ± 0.01 relative units, $n = 8$). Cells differentiating under hypoxic conditions receiving lipid supplements also expressed myoglobin protein on day three after differentiation initiation (0.29 ± 0.17 relative units, $n = 8$) before the significant expression of MHC I on day four (0.03 ± 0.01 relative units, $n = 9$). Under hypoxic conditions without and with lipid supplementation, MHC IIA did not appear on day two, three, or four after differentiation initiation (Fig. 3-2a and 3-2c). These results are similar to our control groups (without and with lipid) but delayed by one day throughout the progression of differentiation (Fig. 3-2a through 3-2c).

Myoglobin Assays

With these assays, we found functional myoglobin present on day two in cells differentiated under control conditions and cells under standard control conditions with lipid supplementation (0.036 ± 0.005 and 0.05 ± 0.009, mg/mg protein, respectively, $n = 27$). Functional myoglobin was identified in hypoxic cells harvested on day three for groups without and with lipid supplementation (0.05 ± 0.004 and 0.09 ± 0.006 mg/mg protein, respectively, $n = 23$) (Fig. 3-3 and Table 3-1).

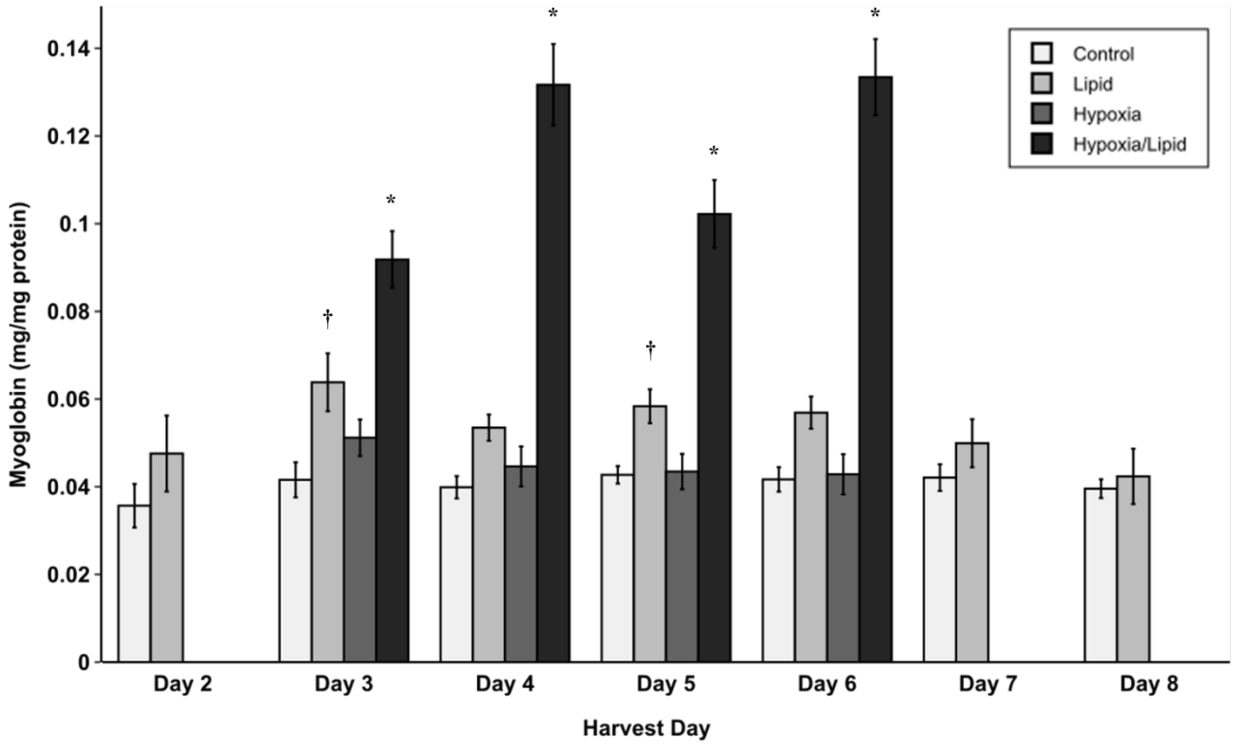

Fig. 3-3 Myoglobin concentration in C_2C_{12} cells. Under hypoxia with added lipid, cells contained more myoglobin than any other treatment groups on the same day. Under control conditions with added lipid, cells contained slightly more myoglobin than same day cells only subjected to control conditions. (*significantly different than all other same day treatment groups: Hypoxia $P < 0.0001$, Lipid $P \leq 0.003$, Control $P < 0.0001$) (†significantly different than same day Control group $P \leq 0.04$)

Cells showed a non-significant trend of increased functional myoglobin in lipid supplemented cells compared to cells without lipid supplementation on each day following differentiation initiation, except for day three and five, when functional myoglobin expression in lipid supplemented cells was significantly higher than cell without supplementation (Day 3, $P = 0.02$, $n = 27$; Day 5, $P = 0.04$, $n = 32$) (Fig. 3-3 and Table 3-1).

Table 3-1 Summary of myoglobin concentrations of C_2C_{12} skeletal muscle cell treatment groups

Harvest Day	Treatment Groups	Myoglobin Concentration (mg/mg protein)	*n*
Day 2	Control	0.04 ± 0.005	27
	Lipid	0.05 ± 0.009	27
Day 3	Control	0.04 ± 0.004	27
	Lipid	0.06 ± 0.007†	32
	Hypoxia	0.05 ± 0.004	23
	Hypoxia/Lipid	0.09 ± 0.006*	24
Day 4	Control	0.04 ± 0.003	36
	Lipid	0.05 ± 0.003	33
	Hypoxia	0.04 ± 0.005	27
	Hypoxia/Lipid	0.13 ± 0.009*	27
Day 5	Control	0.04 ± 0.002	35
	Lipid	0.06 ± 0.004†	32
	Hypoxia	0.04 ± 0.004	24
	Hypoxia/Lipid	0.10 ± 0.008*	24
Day 6	Control	0.04 ± 0.003	35
	Lipid	0.06 ± 0.004	33
	Hypoxia	0.04 ± 0.005	27
	Hypoxia/Lipid	0.13 ± 0.009*	26
Day 7	Control	0.04 ± 0.003	24
	Lipid	0.05 ± 0.005	21
Day 8	Control	0.04 ± 0.002	27
	Lipid	0.04 ± 0.006	17

* significantly different than all other same day treatment groups (Hypoxia $P < 0.0001$, Lipid $P \leq 0.003$, Control $P < 0.0001$)
† significantly different than same day Control group $P \leq 0.03$

Myoglobin assays of all hypoxic cells compared to control cells showed a significant increase in functional myoglobin expression in cells differentiated under hypoxic conditions also receiving mixed lipid supplements when compared to; hypoxic cells without lipid supplementation (Day 3, $P < 0.0001$, $n = 23$; Day 4, $P < 0.0001$, $n = 27$; Day 5, $P < 0.0001$, $n = 24$; Day 6, $P < 0.0001$, $n = 26$), control cells with additional lipid supplementation (Day 3, $P = 0.003$, $n = 24$; Day 4, $P < 0.0001$, $n = 27$; Day 5, $P < 0.0001$, $n = 24$; Day 6, $P < 0.0001$, $n = 26$), and control cells without additional lipid supplementation (Day 3, $P < 0.0001$, $n = 24$; Day 4, $P < 0.0001$, $n = 27$; Day 5, $P < 0.0001$, $n = 24$; Day 6, $P < 0.0001$, $n = 26$) (Fig. 3-2 and Table 3-1).

Discussion

The results of this study provide researchers with a timeline of expression of myoglobin and oxidative MHCs cultured under hypoxic conditions. In doing so, we have found that: (1) under hypoxia, myoglobin

expression precedes that of oxidative fibers in cells without and with lipid supplementation; (2) under hypoxia, expression of myoglobin and oxidative fiber types is delayed by one day compared to control groups; and (3) lipid supplementation coupled with hypoxia correlated with significantly enhanced functional myoglobin expression when compared to all other experimental groups (Fig. 3-4). These findings support our hypothesis that lipid and/or hypoxia treatments will result in myoglobin expression before the expression of an oxidative fiber type. Results involving control groups will not be extensively discussed here as they have already been reported (Chapter 2) and serve as a control here in this study.

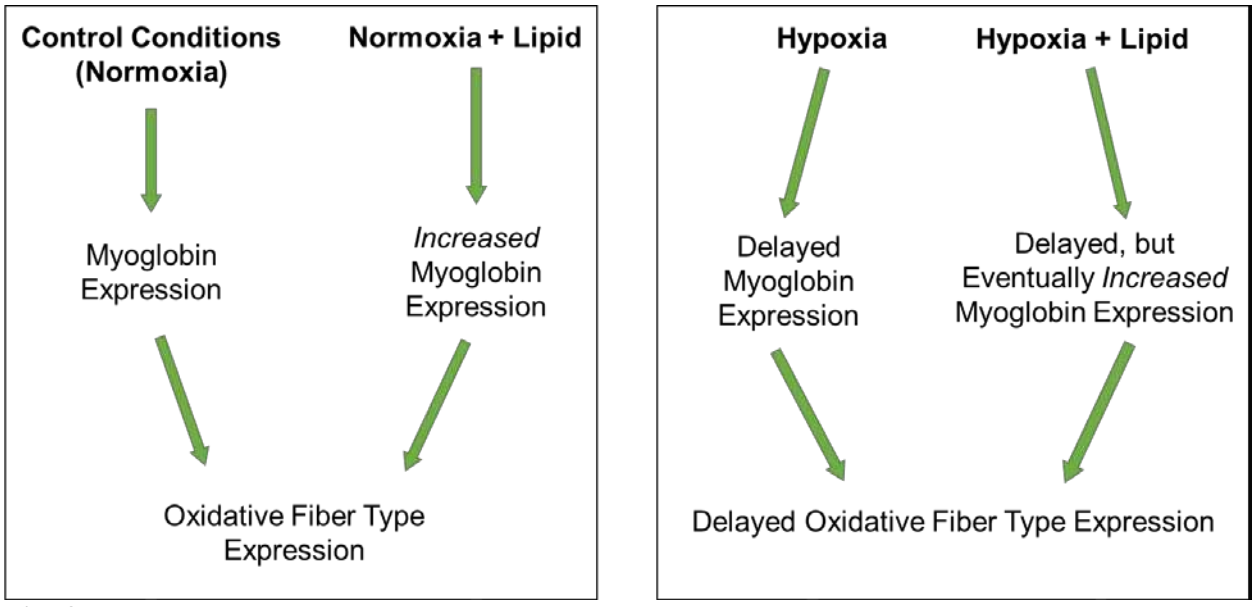

Fig. 4 In control conditions, myoglobin is expressed before oxidative fiber types, and while the same results were seen with lipid-supplemented cells, functional myoglobin assays indicated increased levels of functional myoglobin. In hypoxic cells similar results were recorded regardless of lipid supplementation, although the hypoxic conditions caused a delay in the expression of myoglobin and oxidative fiber type indicators. Myoglobin assays of hypoxic + lipid groups showed more functional myoglobin than all other experimental groups

Our results indicate that under hypoxia there are alternate pathways to myoglobin expression that do not require expression of an oxidative fiber type, and that hypoxia with a secondary stimulus of lipid supplementation produces a significant ($P \leq 0.003$) increase in functional myoglobin expression in C_2C_{12} cells.

Our principal finding was that under control and hypoxic culture conditions in C_2C_{12} cells, myoglobin expression preceded the expression of MHC I and IIA isoforms, and that we observed the same

expression patterns in cells receiving lipid supplementation during differentiation (Chapter 2). Enhanced expression of myoglobin has been attributed to slow-twitch oxidative fibers that have specific calcium signaling to activate calcineurin and trigger myoglobin expression, but our findings indicate that under hypoxia (and control conditions), myoglobin is expressed before the expression of an oxidative fiber type (Chin et al. 1998; Dolmetsch et al. 1997; Kanatous and Mammen 2010; Kanatous et al. 2009). Another group found that cancer cells exposed to hypoxia transcribed myoglobin from an alternate upstream promoter region, further corroborating the idea of an alternate pathway to myoglobin expression (Bicker et al. 2020). Our main findings corroborate other studies displaying myoglobin expression independent from calcineurin signaling and fiber type expression, but additionally investigate the timing of myoglobin and MHC expression (Kanatous et al. 2009; Oh et al. 2005; Schlater et al. 2014).

In rodent myoblasts grown *in vitro* under standard culture conditions, myoglobin mRNA or protein is not expressed until after myoblast fusion and differentiation. Reports vary as to specifically when after differentiation initiation myoglobin is expressed and do not include corresponding fiber type information, or investigations of the effect of hypoxia (Devlin et al. 1989; Graber and Woodworth 1986; Ordway and Garry 2004; Van der Giessen et al. 2003; Weller et al. 1986). Investigators found that myoglobin expression in C_2C_{12} cells under standard culture conditions was preceded by MHC, disagreeing with our results. The antibody used to determine the presence of MHCs was MF-20 (DSHB) which is an antibody that recognizes the rod-like tail region of the MHC protein and is found in all isoforms of MHC. It is unclear which specific isoform of MHC was being expressed in this experiment (Bader et al. 1982; Van der Giessen et al. 2003). Primary myotubes express several embryonic-type MHCs before their fate is set to a particular slow or fast fiber type (Weiss and Leinwand 1996). There could be another isoform of MHC besides MHC I or IIA being expressed in our cells before myoglobin expression.

Myoglobin is known for its role in oxygen storage and transport to mitochondria in muscular tissue, and this could be why we found myoglobin expression before an oxidative fiber type within our hypoxic groups (Helbo et al 2013; Gros et al. 2010). However, myoglobin has other roles within tissue, so myoglobin expressed before an oxidative fiber type in our hypoxic cells could be serving another purpose

besides oxygen storage and transport. During hypoxia, deoxygenated myoglobin helps to produce NO from nitrate by acting as a nitrite reductase and the NO produced can reversibly bind to cytochrome-c-oxidase to inhibit mitochondrial respiration. This mitochondrial inhibition limits oxygen consumption and has been shown to act as a protective measure to limit the production of ROS during hypoxia (Hendgen-Cotta et al. 2008; Shiva et al. 2007). Additionally, myoglobin plays a role in antioxidant defense through its ability to scavenge ROS produced from places like mitochondria, NADPH oxidase sites, and xanthine oxidase sites. Skeletal muscle hypoxia enhances ROS production and accumulation of ROS can cause many harmful effects, in particular, the impairment of calcium handling and contractile dysfunction in skeletal muscle (Clanton and Klawitter 2001; Flögel et al. 2004; Mannino et al. 2019; Powers et al. 2011; Redpath et al 2013). Although we did not measure NO, ROS, or mitochondrial respiration in our experiment, we suspect that delayed myoglobin is appearing before oxidative fibers due to the necessity of myoglobin in another role besides oxygen storage and transport.

The hypoxia imposed on the cells in our experiment caused a delay in the expression of myoglobin and MHCs compared to our control groups, suggesting that hypoxia affects differentiation of C_2C_{12} myoblasts. In C_2C_{12} cells, the rate of mRNA translation decreases with hypoxic treatment and several studies have noted a negative effect on protein expression and myoblast differentiation (Di Carlo et al. 2004; Hardie and Sakamoto 2006; Li et al. 2007; Peters et al. 2017; Yun et al. 2005). Hypoxic conditions causing an increase in the AMP:ATP ratio are sufficient to activate AMP-activated protein kinase (AMPK) in muscle tissue. There are many downstream targets of AMPK, but its inhibition of mammalian target of rapamycin (mTOR) is of particular interest regarding our delay in protein expression under hypoxic conditions. More specifically, AMPK inhibits mTOR which is responsible for mediating translation initiation and thus protein synthesis which could be why we saw a delay in the expression of MHCs and myoglobin within our hypoxic experimental groups (Bolster et al. 2002; Hardie and Sakamoto 2006; Marsin et al. 2000). MyoD is a key transcription factor that regulates gene expression during muscle differentiation and is inhibited by hypoxia. One study found that MyoD inhibition correlated with a decrease in MHC expression, and MHC expression is rescued when cells are returned to normoxic

conditions (Di Carlo et al. 2004; Weintraub 1993). We did not observe the same differentiation inhibition or decrease in MHCs, likely because the hypoxia in the previous experiment was carried out to day three after differentiation, whereas our hypoxic treatment occurred until day six (Di Carlo et al. 2004). Additionally, another group found similar decreased differentiation and MHC expression, but hypoxic treatments past day three resulted in later myogenesis. This suggests that cells are able to adapt to the chronic hypoxia (Yun et al. 2005). The results of these studies described here could explain why we saw a delay, but eventual expression of our proteins of interest in this study. While we did not measure the expression of all MHCs or MyoD, we think that the expression of myoglobin and MHCs in our study was delayed likely due to our cells adapting to chronic hypoxia.

Our functional myoglobin assay results showed significantly increased levels of myoglobin expression in hypoxic, lipid-treated cells compared to all other groups while cells harvested on day three and five in control conditions receiving lipid supplements showed significant enhanced myoglobin expression as compared to the control. Our functional myoglobin under standard culture conditions at day seven was 0.04 mg/mg protein (Fig. 3-2 and Table 3-1), which closely mirrors day seven functional myoglobin values found by others under replicate culture conditions (De Miranda et al. 2012; Schlater et al. 2014). Our functional myoglobin levels for cells differentiated under control conditions that received lipid supplementation were less than those found in other experiments however, these other researchers supplemented their cells with 5% lipid as compared to our 2.5% lipid supplementation. This group also found a significant increase in functional myoglobin in 5% lipid supplemented cells, whereas we did not observe significant changes from standard culture conditions with our 2.5% lipid supplemented group on day seven, likely due to a potential graded response to lipids (Schlater et al. 2014). Values for functional myoglobin in our hypoxic treated cells were comparable to values found by others for cells without and with lipid treatment, despite their use of 5% lipid coupled with hypoxia as compared to our 2.5% lipid (De Miranda et al. 2012; Schlater et al. 2014).

Lipid supplementation to C_2C_{12} cells increases expression of functional myoglobin, but decreased indices of aerobic metabolism, suggesting that myoglobin increases in this instance were due to reasons

besides enhancing muscular oxygen stores. Further, lipid supplemented cells that receive a ROS scavenger treatment decrease functional myoglobin, indicating that elevated myoglobin in lipid supplemented cells is likely due to the presence of ROS generated perhaps from β-oxidation of fatty acids (Flögel et al. 2004; Helbo et al. 2013; Schlater et al. 2014; St-Pierre et. al 2002). Myoglobin competes with fatty acid binding protein when concentrations of unsaturated fatty acids rise above a particular concentration, and therefore enhances fatty acid solubility in the sarcoplasm to channel fatty acids to β-oxidation (Jue et al. 2016; Sriram et al. 2008). Additionally, hearts in myoglobin knockout mice show diminished fatty acid oxidation and augmented triglyceride accumulation and glucose metabolism (Flogel et al. 2005; Hendgen-Cotta et al. 2017). Myoglobin also induces mitochondrial fusion and decreases cell proliferation in specific types of cancer, which commonly uptake lipids for growth, suggesting another possible instance where myoglobin in may be involved in alternate pathways regulating cell proliferation and differentiation (Braganza et al. 2019; Snaebjornsson et al. 2020). Although we did not measure ROS and β-oxidation or lipid transport, we think that lipid-induced increases in functional myoglobin could be due to reasons besides enhancing muscular oxygen stores.

Hypoxia acting alone on muscle tissue does not produce increases in myoglobin, but often seems to require another stimulus such as exercise or in some cases lipid, which is likely why we see our highest myoglobin values in our hypoxic/lipid group (De Miranda et al. 2012; Helbo et al. 2013; Kanatous et al. 2009; Schlater et al. 2014). Further, AMPK activated by hypoxia induces metabolic changes such as increased uptake and oxidation of glucose and fatty acids (Hardie and Sakamoto 2006; Marsin et al. 2000). So perhaps elevated myoglobin in this instance is due to the necessity for lipid transport or even in response to ROS generation from AMPK-upregulated glucose and fatty acid metabolism. However, the group noted earlier that applied a ROS scavenger to lipid supplemented cells applied the same scavenger to their hypoxic lipid supplemented cells and did not see a drastic decrease in myoglobin expression compared to their hypoxic lipid supplemented cells. This suggests that the increases in myoglobin in the hypoxic lipid supplemented cells was unlikely due to the presence of ROS (Schlater et al. 2014). Additionally, more oxygen is required for complete oxidation of fatty acids compared to

glucose oxidation (Flögel et al. 2005). It could be that the enhanced functional myoglobin expression in our hypoxic cells with lipid supplementation is due to a need for elevated oxygen supply for the combination of low oxygen conditions and higher requirement for oxygen for metabolism of the lipids present in the media.

In the present study, the C_2C_{12} cells differentiated in control and hypoxic conditions without and with lipid supplementation showed myoglobin expression before the expression of MHC I and IIA, indicating an underlying pathway to myoglobin expression that does not require the prior expression of an oxidative fiber type. Cells receiving lipid supplementation had more enhanced functional myoglobin expression with lipid supplementation, and even more enhanced myoglobin expression was observed when lipid supplemented cells were also differentiated under hypoxic conditions. This suggests that hypoxia and lipids are in are in some way more effective at increasing functional myoglobin expression within cultured muscle tissue, perhaps with myoglobin acting in another role besides or in addition to oxygen storage and transport. Lastly, research utilizing similar culture conditions is suggested to properly investigate the molecular drivers of pathways to myoglobin expression that precedes the expression of an oxidative fiber type.

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CHAPTER 4: SUMMARIZING DISCUSSION AND FUTURE DIRECTIONS

Dissertation aim and major findings

Myoglobin is an intensively studied protein expressed in vertebrate muscle that typically follows an established oxidative muscle fiber type, which contains myosin heavy chain (MHC) I or IIA isoforms (Chin et al. 1998; Kanatous and Mammen 2010). In spite of this, recent evidence has found changes in myoglobin expression without a change in fiber type, suggesting myoglobin expression may not be dependent on a prior expressed fiber type and their respective signaling pathways (Oh et al. 2005; Schlater et al. 2014). Mixed lipid supplementation, and hypoxia coupled with lipid supplementation or muscular contraction (stimulated by caffeine) elevate myoglobin levels in skeletal muscle, and in some cases these elevations in myoglobin did not correspond to a change in fiber type, suggesting possible alternate regulatory pathways for myoglobin expression (De Miranda et al. 2012; Kanatous et al. 2009; Rayner et al. 2009; Schlater et al. 2014).

Given the uncertainty of when myoglobin is expressed independently from oxidative fiber types, the overarching goal of this study aimed to find when differentiating C_2C_{12} cells begin to express myoglobin in comparison to when they express different oxidative isoforms of MHC. We also aimed to observe the timing of expression of these proteins when the cells were subjected to factors known to increase myoglobin expression such as hypoxia, lipid and/or caffeine treatments. While other studies correlate myoglobin expression with a slow oxidative fiber type, our study is the first to create a timeline of myoglobin expression with added stimuli to establish potential timepoints for therapeutic intervention without altering fiber type.

As we found in Chapter 2 and 3, under control conditions and hypoxia regardless of lipid supplementation, myoglobin expression occurred before oxidative fiber expression, while cells receiving caffeine stimulation resulted in myoglobin expression following the expression of an oxidative fiber type. When conducting a myoglobin assay to determine functional myoglobin content we found that cells exposed to hypoxia and lipid supplementation displayed more enhanced functional myoglobin expression

than those receiving caffeine stimulation, suggesting that the combination of lipid and hypoxia may be more effective at increasing myoglobin than stimulation alone.

Overall, the work presented in this dissertation has: (1) determined conditions under which expression of myoglobin precedes oxidative fiber type expression; (2) shown that with stimulation, myoglobin expression follows the prior expression of an oxidative fiber type; and (3) identified cell culture treatments that not only increase myoglobin concentration, but also produce myoglobin before an oxidative fiber type is expressed.

Myoglobin expression relative to oxidative fiber types is differentially affected by contractile stimulation versus lack of stimulation

In Chapter 2, we found differential expression of myoglobin relative to oxidative fiber type expression with and without caffeine stimulation. Cells without stimulation showed myoglobin protein present on day two with and without lipid supplementation, while MHC I was not expressed until day three and MHC IIA was not expressed at all. Caffeine stimulation however, resulted in cells producing MHC I and IIA on day two before expression of myoglobin on day three.

Our findings indicating differential expression of myoglobin and MHC corroborate other studies that found myoglobin expression regulation independent from fiber type and calcineurin signaling (Kanatous et al. 2009; Oh et al. 2005; Schlater et al. 2014). Myoglobin performs many roles within tissues, and if myoglobin is expressed before an oxidative fiber type it may be serving another purpose besides acting in oxygen storage and transport to support oxidative metabolism however, we did not measure other ligands indicative of the roles of myoglobin in the cell like nitric oxide (NO), reactive oxygen species (ROS), or lipid in our experiment (Flögel et al. 2001; Flögel et al. 2004; Kanatous and Mammen 2010; Shih et al. 2014).

We reasoned that our results from caffeine stimulation were likely due to the ability of caffeine to elicit calcium release in cells, which resulted in the activation of calcineurin to initiate the expression and development of the slow oxidative fibers (Chin et al. 1998; Kordosky-Herrera and Grow 2009; Moore et

al. 2017). Additionally, caffeine is known to stimulate lipid metabolism and also contraction-stimulated glucose transport, further suggesting that our stimulated cells may have had enhanced differentiation through an expedited uptake of nutrients (Schnuck et al. 2018; Tsuda et al. 2015).

Myoglobin expression under hypoxia precedes oxidative fiber type expression

In Chapter 3, we again found expression of myoglobin before oxidative fiber type expression, but this time in cells under hypoxic conditions with and without lipid supplementation. Cells exposed to hypoxia displayed a delay in protein expression, with myoglobin present on day three, while MHC I did not appear until day four and MHC IIA did not appear at all.

Myoglobin expression before oxidative MHCs under hypoxia additionally corroborate other studies that discovered a disconnect in myoglobin and a slow fiber type and thus calcineurin signaling (Kanatous et al. 2009; Oh et al. 2005; Schlater et al. 2014). Myoglobin being expressed here could be serving a role in oxygen support in the cells differentiating under low oxygen conditions however, myoglobin has also displayed other roles for support in hypoxic tissues such as enhanced fuel support if the cells are staying active (Helbo et al 2013; Gros et al. 2010). Myoglobin can also support hypoxic tissues by scavenging ROS and assisting in the production of NO which can serve to inhibit mitochondrial respiration and decrease oxygen consumption by the cell (Flögel et al. 2004.; Hendgen-Cotta et al. 2008; Shiva et al. 2007).

The hypoxic conditions imposed upon our cells resulted in a delay in the expression of myoglobin and MHCs compared to control groups, suggesting that hypoxia may have been affecting differentiation. Hypoxia has been shown to decrease the rate of mRNA translation, differentiation, and expression of MHCs however, others found that as hypoxic treatment progresses cells are eventually able to differentiate and express MHCs. Collectively, cells appear to adapt to chronic hypoxia and ultimately commit to myogenesis (Di Carlo et al. 2004; Peters et al. 2017; Yun et al. 2005).

Hypoxia combined with lipid is more effective for enhanced myoglobin expression than stimulation

In Chapter 2, our functional myoglobin assays showed that cells receiving lipid supplementation had more enhanced myoglobin expression without caffeine stimulation, and that lipid supplements elevated myoglobin expression in (control) lipid groups but could not reverse the dampened myoglobin concentration in stimulated groups. In Chapter 3 we found that cells receiving lipid in hypoxic conditions were able to produce the most myoglobin compared to all other experimental groups, including those from Chapter 2. This displays that hypoxia and lipids are in some way more effective at increasing functional myoglobin expression than stimulation within cultured muscle tissue.

Previous work suggested that lipid-induced myoglobin increases were due to reasons aside from enhancing muscular oxygen stores due to decreased indices of aerobic metabolism (Schlater et al. 2014). Instead, myoglobin may be acting either to transport fatty acids to mitochondria or as a ROS scavenger if the cells are producing more ROS due to increased β-oxidation of fatty acids (Flögel et al. 2004; Jue et al. 2016; Mannino et al. 2019). Our stimulated groups still showed myoglobin expression likely in concordance with the fiber type-calcineurin cascade of expression (Chin et al. 1998), but myoglobin concentration was less in these groups compared to unstimulated groups. Caffeine stimulation enhances lipid metabolism in skeletal muscle, so perhaps caffeine enhances lipid metabolism in a way that negates the need for myoglobin, which could be the reason why we did not see a significant increase in myoglobin in our stimulated cells with lipid supplementation (Schnuck et al. 2018).

Hypoxia acting alone on muscle tissue does not increase myoglobin, but often seems to require another stimulus such as exercise (contractions) or in some cases lipid, which is likely why we see our highest myoglobin values in our lipid group under hypoxic conditions (De Miranda et al. 2012; Helbo et al. 2013; Kanatous et al. 2009; Schlater et al. 2014). When a ROS scavenger was applied to hypoxic lipid supplemented cells, myoglobin levels did not sharply decrease compared to hypoxic lipid supplemented cells without a ROS scavenger. This suggests that heightened myoglobin levels in hypoxic lipid supplemented cells is unlikely due to the presence of ROS (Schlater et al. 2014). Alternatively, more oxygen is required for complete oxidation of fatty acids compared to glucose oxidation, so elevated

myoglobin could be due to a need for elevated oxygen supply for the combination of low oxygen conditions and higher requirement for oxygen for metabolism of lipids present in the media (Flögel et al. 2005).

Outlook and future directions

How is myoglobin expressed outside of an oxidative fiber type? Our experiments resulted in the identification of several culture conditions by which myoglobin is expressed prior to the expression of oxidative fiber types, which will be used to further investigate the molecular drivers of pathways for myoglobin in these instances. While this project included data regarding protein expression, a transcriptome generated by RNA-Seq will be a useful comparison to further investigate myoglobin and myosin heavy chain gene expression and other upregulated genes that may shed some light on pathways for myoglobin protein production when it precedes fiber type expression (Wang et al. 2009).

Primarily, we aim to investigate if myoglobin and myosin heavy chain transcripts are increasing with similar trends to our protein data collected in this study. In this way we can find if our increases in protein are correlated to an increase in transcripts at specific harvest days. Our experimental manipulations from this study such as lipid, caffeine, or hypoxia will also lend some insight into what could be causing potential upregulation of transcripts. RNA-Seq will also be useful to see if other genes besides myoglobin and myosin heavy chains are differentially expressed as the cells progress through differentiation. Upregulation of lipid biosynthetic pathways was observed in mammalian muscle with a high lipid content as compared to muscle with low lipid content, so we would expect an upregulation of these same pathways within our lipid groups (Cardoso et al. 2017). Cardiomyocytes subjected to caffeine treatments displayed an upregulation of structural genes such as myosin heavy chains and troponin, and given our observations of increased protein expression of myosin heavy chains in our caffeine groups, we would expect similar results of increased expression of structural genes (Fang et al. 2014). In muscle subjected to intermittent hypoxia training, upregulated genes were those involved with the hypoxic response (hypoxia inducible factor-1, HIF-1), oxidative metabolism (peroxisome proliferator-activated receptor

gamma coactivator 1-alpha, PGC-1α; cytochrome c oxidase, COX; and citrate synthase, CS), glucose transport (glucose transporter type 4, GLUT-4), oxidative stress defense (mitochondrial superoxide dismutase, MnSOD), and pH regulation (carbonic anhydrase, CA) (Zoll et al. 2006). We would expect increased transcripts similar to these pathways in our hypoxic groups.

Other researchers found myoglobin expression was preceded by MHC in C_2C_{12} cells under standard culture conditions preceded by MHC, disagreeing with our results. The antibody used to determine the presence of MHCs in this previous experiment was MF-20 (DSHB) which recognizes the rod-like tail region of the MHC protein which is found in all isoforms of MHC. In this instance it is unclear which specific isoform of MHC was being expressed (Bader et al. 1982; Van der Giessen et al. 2003). With this in mind, we have begun to investigate the expression of other MHCs in our set of experiments and found that in all culture conditions unspecified isoforms of MHC are expressed in cells before myoglobin expression (Fig. 4-1a-c). In our caffeine stimulation experiments, both MHC I and IIA are expressed in addition to other unspecified MHC isoforms prior to myoglobin expression (Fig. 4-1b). In our control and hypoxic conditions MHC I or IIA is not expressed before myoglobin, but some other MHC isoform is expressed in these cells on the same day or maybe before myoglobin (Fig. 4-1a and 4-1c). Further analysis will be done to investigate which isoforms are being expressed and identified by the MF-20 antibody. Primary myotubes express several embryonic-type MHCs before their fate is set to a slow or fast fiber type, so perhaps one of these are the isoform(s) that we are observing in our experiments (Weiss and Leinwand 1996). Expression of an embryonic-type MHC then giving rise to myoglobin could explain why we can see adult fiber type switching without a concomitant change in the expression of myoglobin (Kanatous et al. 2009; Oh et al. 2005).

What is the role of this myoglobin expression before an oxidative fiber type? Investigating the role that myoglobin is playing in these experiments will uncover more information regarding why we see myoglobin appearing in tissue before an oxidative fiber type is expressed. Investigations will start with evaluating indicators of known roles of myoglobin including ROS and NO scavenging, and lipid transport

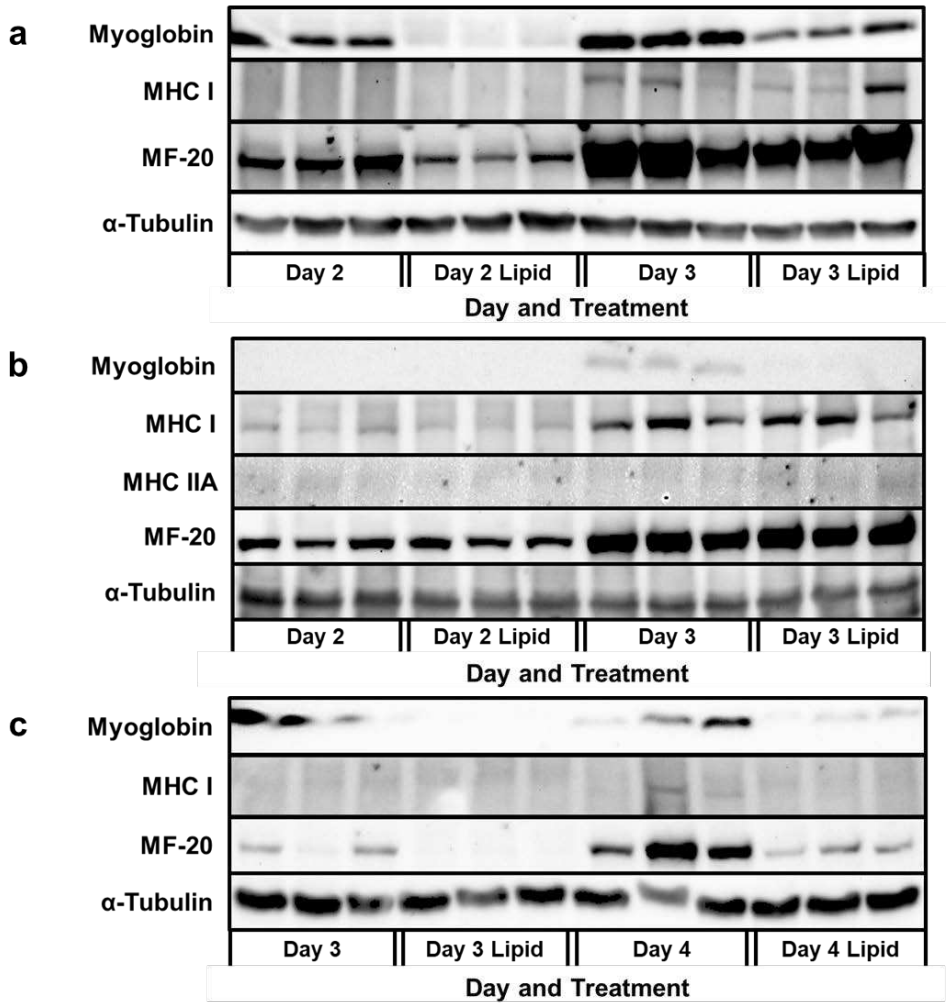

Fig. 4-1 In all culture conditions, unspecified isoforms of MHC are expressed in cells before myoglobin expression, MHC I and IIA are expressed in addition to other unspecified isoforms in caffeine stimulation experiments prior to myoglobin expression. Representative western blot analysis from control experiments with MHC IIA removed for simplicity (**a**), caffeine stimulation experiments (**b**), and hypoxia experiments with MHC IIA removed for simplicity (**c**). Myosin heavy chain (MHC), MF-20 (all MHC isoforms)

(Flögel et al. 2001; Flögel et al. 2004; Kanatous and Mammen 2010; Shih et al. 2014). On sequential days, these indicators will be measured and compared to myoglobin expression on that same day. Firstly, endogenous O^{-}_{2} will be quantified and compared across days to determine changes in ROS, as previously described (Drossos et al. 1995). Additionally, NO will be labeled *in situ* and compared across days to compare changes in NO in the cells in specific locations, as previously described (Pitts and Mykles 2015).

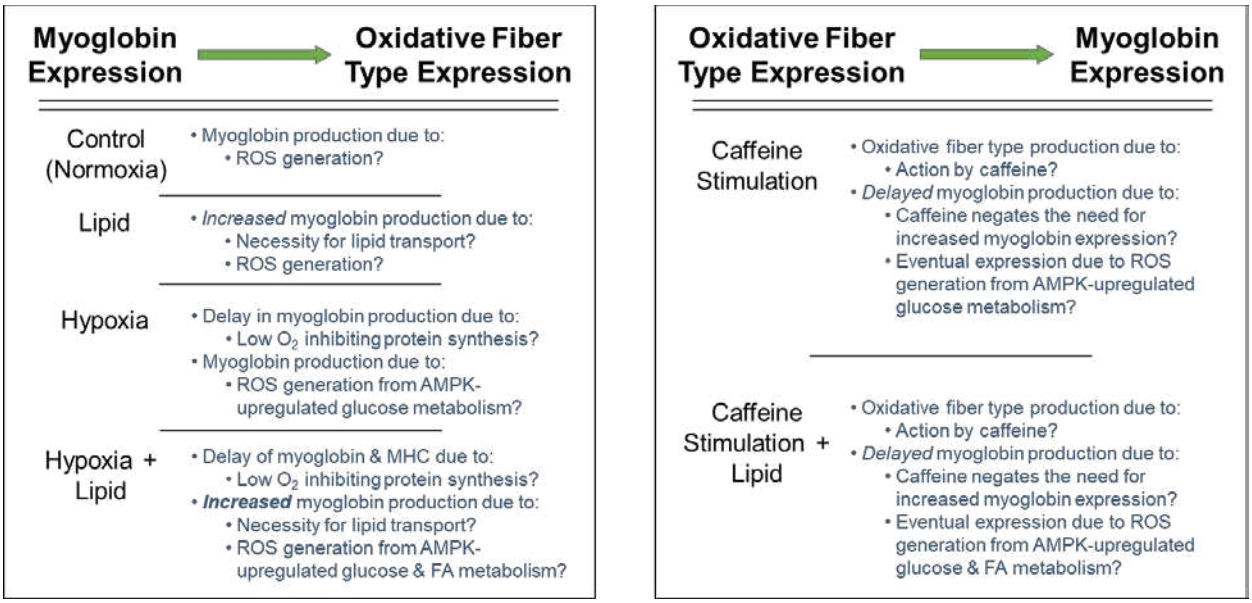

Fig. 4-2 Cell culture conditions in which myoglobin expression preceded oxidative fiber type expression include control conditions, lipid supplemented cells, hypoxic conditions, and lipid supplemented cells in hypoxic conditions. Cell culture conditions in which myoglobin expression followed the expression of oxidative fiber types include caffeine-stimulated cells and lipid-supplemented/caffeine-stimulated cells. Proposed questions to further investigate mechanisms by which myoglobin and oxidative fibers are expressed under each culture condition appear in blue

Lastly, we will determine if myoglobin is interacting with fatty acids and compare across days, as previously described (Jue et al. 2016; Sriram et al. 2008) (Fig. 4-2).

This research is of importance because if we can elucidate how myoglobin is expressed independently from the type of muscle, expressing myoglobin in this way could have more relevant therapeutic applications for ischemia seen in humans that can lead to heart attack, stroke, and is seen in patients that develop peripheral artery disease secondarily due to age, smoking, diabetes, and other common medical conditions.

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