THE EFFECTS OF ACUTE AND CHRONIC HYPOXIA ON MUSCLE METABOLISM IN MICE

By

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ABSTRACT

At high altitude mammals face a multitude of challenges, the most prominent being hypoxic conditions. Using CD-1 mice I examined the phenotypic plasticity of skeletal muscle in response to chronic hypoxia and acutely to exercise. I tested the hypotheses that 1. acclimation to chronic hypoxia will result in an adjustment in the proportion of cytochrome c oxidase (COX) subunits suggesting an increase in the rate of electrons transferred to O_2, 2. the degree of PDH phosphorylation will be similar between resting controls and acclimated mice indicating comparable rates of aerobic metabolism, and 3. relative post-exercise changes in muscle metabolites and the degree of PDH phosphorylation will be similar between control and acclimated mice reflective of a common mechanism regulating fuel use. Based on cell culture experiments, mammals are predicted to improve the efficiency of ATP production under hypoxia by increasing the levels of the 4-2 isoform and decreasing the levels of the 4-1 isoform of COX. Acclimated mice exhibited a significant elevation in the mRNA levels of COX4-2 after 24hrs and a noticeable decrease in COX4-1 mRNA after 2hrs of acclimation. There was, however, no significant change at the protein level for the two subunits. I also examined the phosphorylation state of pyruvate dehydrogenase (PDH) in resting mice acclimated to chronic hypoxia as an indicator of overall PDH activity. The degree of PDH phosphorylation relative to total PDH content increased at site 1, Ser^{293}, in acclimated mice compared to controls, suggesting a reduced basal activity of PDH. A decrease in activity may indicate a decreased reliance on carbohydrate derived acetyl-CoA for energy production. These results indicate that the skeletal muscle of mice is plastic under

hypoxia. I also wanted to see how acclimation affected muscle response to acute submaximal exercise. In many mammals, a conserved pattern of fuel use exists and common mechanisms dictating fuel selection have been proposed. To explore the possibility of a common controlling mechanism I examined the phosphorylation state of PDH as well as levels of key muscle metabolites in the gastrocnemius of acclimated and control pre and post-exercise mice. There were no significant changes in PDH phosphorylation due to exercise at varying intensities which could help to explain fuel use patterns. Further, changes in muscle metabolites in post-exercise mice suggest that acclimated mice were relying more heavily on anaerobic metabolism than controls.

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THESIS ORGANIZATION AND FORMAT

This thesis is organized in a sandwich format, as recommended and approved by members of my supervisory committee, consisting of three chapters. Chapter one is a general introduction outlining relevant background information and the objectives of my study. Chapter two consists of a manuscript that is in preparation for submission to a peer-reviewed scientific journal. To conclude, chapter three summarizes the major findings of this thesis, placing them in the context of current knowledge and suggests future steps to further this research.

Chapter 1:		**General introduction**
Chapter 2:		**Skeletal muscle phenotypic plasticity during chronic hypoxia and its effects on whole animal muscle metabolism during acute exercise in small mammals.**
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Chapter 3:		**General summary and conclusions**

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CHAPTER 1: GENERAL INTRODUCTION

Energy production is vital to the survival of all living organisms. A variety of mechanisms exist across different species to accomplish this task. Under normal physiological conditions most mammals mainly employ a process referred to as oxidative phosphorylation to produce energy in the form of adenosine triphosphate (ATP) (Mitchell, 1961; figure 1). Oxidative phosphorylation can occur in various ways depending on the substrate or fuel being utilized. However, the unifying goal is to produce acetyl-CoA for the tricarboxylic acid cycle (TCA) and further generate reducing equivalents (NADH and $FADH_2$) to donate electrons to pass along the electron transport chain (ETC; Mitchell, 1961; figure 1). The ETC consists of four main complexes (complex I – IV) and a single F1F0-ATP synthase (Mitchell, 1961). The movement of NADH, $FADH_2$, and thus electrons, through the complexes of the ETC drives the pumping of protons across the inner mitochondrial membrane, creating a proton gradient (Mitchell, 1961). The movement of electrons through the ETC terminates with the transfer of electrons to oxygen (O_2) via cytochrome c oxidase (COX) at complex IV, producing water (H_2O; Mitchell, 1961). The proton gradient that has been established is then used to power the production of ATP via ATP synthase (Mitchell, 1961). To generate reducing equivalents such as NADH and $FADH_2$, substrates must be catabolized. In mammals, the main fuel sources used for the oxidative production of ATP are carbohydrates, lipids, and to a lesser extent, proteins.

Lipids, or fats, are a group of highly reduced and condensed hydrophobic molecules. Some of the most prevalent groups of lipids in mammals include

phospholipids, steroids, and triacylglycerols (Frayn, 1996). Triacylglycerols are the major storage form of metabolic fuels and are composed of a glycerol back bone linked through an ester linkage to three fatty acids (FAs), which are broken down to produce metabolic intermediates (non-esterified fatty acids or NEFA and glycerol) and ATP (Frayn, 1996; Nelson and Cox, 2008). The NEFAs used most readily during metabolism are long-chain, unbranched carboxylic acids with an even amount of carbon atoms (Frayn, 1996). For lipids to be oxidized they must be mobilized from storage cites (such as adipocytes) and transported throughout the body by circulation (reviewed in McClelland, 2004). Lipid stores are liberated from triacylglycerides into NEFAs which are, due to their hydrophobic nature, subsequently bound to the transport protein albumin to be sent throughout the body via the blood stream (reviewed in McClelland, 2004). Once the NEFAs reach their destination, they are transported into the cell through membrane transporters such as the fatty acid binding protein plasma membrane (FABPpm) as well as the fatty acid transporter (CD36/FAT; reviewed in McClelland, 2004; figure 1). Once inside the cell NEFAs are bound by fatty acid binding proteins (FABP) allowing them to travel throughout the cell (reviewed in McClelland, 2004). NEFAs are converted to fatty acyl-CoA (FA-CoA) by acyl-CoA synthase (ACS) to ensure concentration gradients are maintained between the blood and cytosol (reviewed in McClelland, 2004). The now formed FA-CoA is bound by acyl-CoA binding protein (ACBP) for transport to the mitochondria (reviewed in McClelland, 2004). To be transported into the mitochondria, the FA-CoA molecule must be converted to fatty acyl-carnitine (FA-Carn) by carnitine palmitoyltransferase (CPT) I located on the inner side of the outer mitochondrial membrane (reviewed in McClelland, 2004). Transport of FA-

Carn into the mitochondrial matrix is accomplished by carnitine acyl-transferase (CAT) where it is converted back to FA-CoA by CPTII (reviewed in McClelland, 2004). FA-CoA is then able to undergo β-oxidation to produce acetyl-CoA (reviewed in McClelland, 2004; figure 1). One molecule of acetyl-CoA is produced for every two carbons to pass through the β-oxidation cycle (Nelson and Cox, 2008). Acetyl-CoA is then fed through the TCA and oxidative phosphorylation continues (Nelson and Cox, 2008; figure 1). Lipids, compared to other major fuel sources, produce the greatest amount of ATP, 104 molecules, per mole of substrate oxidized (Brand, 2005; Nelson and Cox, 2008). However, the oxidation of lipids also consumes a great deal of O_2, producing only 4.32 molecules of ATP per mole O_2 consumed (Brand, 2005; Nelson and Cox, 2008).

In their simplest form carbohydrates (CHOs) are referred to as monosaccharides, with glucose being the most prevalent in metabolism. CHOs are a more resourceful energy source compared to others due to their ability to contribute to both aerobic and anaerobic metabolism (Spriet and Watt, 2003). However, CHO stores within the body are not large, lasting for only about 2hrs of vigorous exercise in humans (Spriet and Watt, 2003). Therefore, any fuel use strategy which avoids this depletion is favorable (Spriet and Watt, 2003). Glucose can enter the blood stream through many different pathways including the breakdown of liver glycogen stores, and from gluconeogenesis (Frayn, 1998; figure 1). Glucose is able to travel through the blood stream without the aid of transport proteins due to its hydrophilic nature. After reaching the tissue or target cell, such as the myocyte, glucose molecules are transported into the cell via facilitated diffusion through glucose transporters, the most important in muscle during exercise being Glucose Transporter 4 (GLUT4; Rose and Richter, 2005; figure 1). Once inside

the cell, glucose molecules are phosphorylated to form glucose-6-phosphate (G-6-P) and subjected to glycolysis where they are broken down to form 2 pyruvate molecules, liberating 2 ATP as well as 2 NADH molecules (figure 1). The 2 pyruvate molecules are then converted to acetyl-CoA by pyruvate dehydrogenase (PDH) which is then sent through the TCA and ETC (figure 1). The oxidation of one mole of glucose produces only 31 molecules of ATP, a much lower yield than that obtained from a mole of lipids (Brand, 2005). However, the oxidation of one mole of CHOs consumes ≈ 15% less O_2, producing 4.99 molecules of ATP per mole of O_2 consumed (Brand, 1994).

Proteins are long chains of amino acids linked by peptide bonds which are folded into 3-dimentional secondary, tertiary, or quantinary structures. Proteins mainly play a structural role within the body, with the majority being found in skeletal muscles (Frayn, 1998). Unlike CHOs and lipids, the body does not store protein for later use (Frayn, 1998). Due to this lack of storage and a significant structural role, the denaturation of proteins for energy production is avoided if possible in the postabsorptive state (Frayn, 1998). However, after periods of high protein intake, proteins are more readily available in the blood stream and may significantly contribute to energy production (Forslund *et al*., 1999).

The choice of fuel/mixture of fuels used by an organism varies depending on the situation faced. Under normal physiological conditions mammals tend to replenish their fuel stores, ending the day in the state in which they began (Frayn, 1998). Some circumstances, however, may require an individual to mobilize stores more rapidly, to a greater extent, or in a different way (Frayn, 1998). Such situations occur when an individual is subjected to vigorous exercise or limited O_2 tensions, referred to as hypoxia

(Frayn, 1998; Spriet and Watt, 2003; McClelland *et al*., 1998; Romijn *et al*., 1993).

Fuel Use in Hypoxic Conditions

Hypoxia is defined as a reduction in O_2 availability in one state or condition compared to another (Prabhakar and Semenza, 2012). When exposed to short bouts of hypoxia, organisms must limit their O_2 use (reviewed in Ramirez *et al*., 2007). Some, such as diving mammals, will reduce their metabolism and enter a hypometabolic state in order to reduce the amount to O_2 consumed (Hochachka, 2000; reviewed in Ramirez *et al*., 2007). Others will resort to anaerobic metabolism because of the reduced rate of oxidative phosphorylation caused by reduced O_2 tensions in the mitochondria (Kim *et al*., 2006). Pyruvate is converted to lactate by lactate dehydrogenase (LDH) producing lactate instead of being sent through the TCA (figure 1), acting to decrease the number of reducing equivalents sent through the ETC and thus the reliance on oxidative phosphorylation (Kim *et al*., 2006). Although this is a suitable short-term strategy it would quickly exhaust limited CHO stores, therefore, a more viable long-term strategy is needed (Hochachka, 1985). To survive long-term hypoxic conditions organisms are predicted to adopt the following strategies, 1) to rely on aerobic rather than anaerobic metabolism due to the greater yield of ATP per mole of fuel used and 2) to increase the efficiency of ATP production to reduce the amount of O_2 used per ATP formed (Hochachka, 1985). To accomplish a greater efficiency of ATP production mammals are predicted to alter the mix of fuels used by relying more heavily on CHOs and to increase the efficiency of O_2 use by the mitochondria (Hochachka *et al*., 1996; Hochachka *et al*., 2002; McClelland *et al*., 1998; Schippers *et al*., 2012).

Phenotypic Plasticity in Skeletal Muscle in Response to Chronic Hypoxia

Humans adapted to hypoxia appear to be more efficient at ATP production than sea level natives (reviewed in Cerretelli *et al*., 2009). P/O ratios (the number of ATP molecules produced per pair of electrons passed through the ETC) measured in isolated rat liver mitochondria supplied with excess α-ketoglutarate under hypoxic conditions were found to be 15% higher than those observed under normoxic conditions (Solien *et al*., 2005). This increase indicates the energy transfer from glycolysis and the TCA to the ETC has become more efficiently coupled (Solien *et al*., 2005; Cerretelli *et al*., 2009). Furthermore, humans which are native to high altitude have been shown to possess a lower VO_2 (oxygen consumption) than humans native to sea level at the same relative workload (reviewed in Cerretelli *et al*., 2009; Cerretelli and Gelfi, 2010). This lowered VO_2 suggests that there is a more efficient use of ATP per O_2 consumed (reviewed in Cerretelli *et al*., 2009; Cerretelli and Gelfi, 2010). This increased efficiency of O_2 use in individuals experiencing prolonged periods of hypoxia cannot be explained by changes in running mechanics or contributions from anaerobic metabolism (Cerretelli and Gelfi, 2010). However, changes of a metabolic nature in muscle have been observed during acclimatization to hypoxia in lowlanders, which could help to explain the increased efficiency of energy production in high altitude natives (Cerretelli and Gelfi, 2010; Fukuda *et al*., 2007; Le Moine *et al*, 2011).

The hypoxia inducible factor 1 (HIF-1) is the main transcription factor responsible for inducing many of the metabolic changes observed in response to hypoxia (Prabhakar and Semenza, 2012). HIF-1 is a heterodimeric protein comprised of HIF-1α and HIF-1β, also known as the aryl hydrocarbon nuclear translocator (ARNT), subunits (Semenza, 2000).

The amino terminal portion of each individual subunit contains helix-loop-helix (HLH) as well as PER-ARNT-SIM (PAS) homology domains (Semenza, 2000). The basic HLH domain is the site of DNA interaction and binding (Semenza, 2000). Activity of HIF-1 is mediated by the expression and activity of the O_2 sensitive HIF-1α subunit (Semenza, 2000). The regulation of HIF-1α activity can occur at many levels including mRNA expression, protein expression, nuclear localization, and transactivation (Semenza, 2000). However, regulation of HIF-1α expression at the protein level is the most widely studied and accepted form of HIF-1 regulation (Semenza, 2000). Within HIF-1α is an oxygen-dependent degradation domain (ODD) which contains proline residues 402 and 564 (Lee, 2004). During normal O_2 tensions prolyl hydroxylases (PHD) hydroxylate these proline residues (Lee, 2004). The hydroxylated HIF-1α is then recognized by the von Hippel-Lindau tumor suppressor protein (pVHL) which ubiquinates HIF-1α, targeting it for destruction via the ubiquitin mediated proteosomal degradation pathway (Lee, 2004). The degradation of HIF-1α prevents the buildup of HIF-1 and thus lowers the amount of HIF-1 bound DNA (Lee, 2004). However, when cells are exposed to hypoxic conditions proline residues 402 and 564 are not hydroxylated due to the lack of available O_2 atoms, and HIF-1 protein accumulates within the cell (Lee, 2004). When levels of HIF-1 rise it is able to enter the cell's nucleus (Lee, 2004). Once inside the nucleus, HIF-1 binds to hypoxic response elements (HRE) located in the promoter regions of hypoxia-inducible genes, including important ETC proteins, inducing expression or repression (Nikinmaa and Rees, 2005; Semenza, 2000; Lee 2004).

It has previously been shown that acclimation to chronic hypoxia results in little to no change in overall oxidative capacity in skeletal muscle in mammals (Desplanches *et*

al., 1996; Hochachka, 1992; Kayser *et al*., 1991; Kennedy *et al*., 2001; Le Moine *et al*., 2011; Perhonen *et al*., 1996). However, little information about the response of CHO and lipid metabolism to chronic hypoxia in skeletal muscle exists (Schippers, 2011). Recently, the rates of glucose and lipid oxidation in response to chronic hypoxia in CD-1 mice were investigated using maximal activities (Vmax) of key enzymes in each pathway as indicators of overall pathway flux (Schippers, 2011). It was found that there were no observable changes in the activities of hexokinase (HK), phosphofructokinase (PFK), and pyruvate kinase (PK) suggesting that there were no changes in the flux through glycolysis (Schippers, 2011). Further, there were no changes in the activities of citrate synthase (CS) or isocitrate dehydrogenase (IDH) indicating no changes in the flux through the TCA (Schippers, 2011). Also, there was no change in the rate of β-oxidation predicted though β-hydroxyacyl-CoA dehydrogenase (HOAD) activity (Schippers, 2011). These results suggest that there are no changes in the rates of CHO or lipid oxidation in response to chronic hypoxia (Schippers, 2011). Furthermore, there were no changes in the activity of COX, suggesting that there was no overall change in oxidative capacity in response to chronic hypoxia (Schippers, 2011). Although these results suggest that there is no observable change in the capacity for fuel oxidation rates in response to chronic hypoxia, others have observed alterations to the flux through metabolic pathways in response to hypoxia on a smaller time scale (24hrs; Le Moine *et al*., 2011). Further, there is recent evidence for a change in the composition of COX in cells exposed to hypoxia for 24hrs which may alter the oxidative capacity of cells when exposed to chronic hypoxia (Fukuda *et al*., 2007).

It is believed that modifications of the complexes in the ETC, especially complex

IV, contribute to this adjustment (Cerretelli and Gelfi, 2010). As previously mentioned, when mammals are acutely exposed to hypoxia, there are not sufficient amounts of O_2 present to accept electrons in the ETC and organisms are forced to switch to anaerobic metabolism (Kim *et al*., 2006). However, when subjected to chronic hypoxia this strategy is not a viable option and organisms are forced to make other long-term cellular changes, such as an alteration in the composition of the subunits of COX (Cerretelli and Gelfi, 2010; Fukuda *et al*., 2007; figure 2).

COX exists as a dimer with each monomer being composed of 13 subunits (Capaldi, 1990; Tsukihara *et al*., 1996). Only 3 subunits, subunits 1, 2, and 3, are encoded by mitochondrial DNA with the remaining 10 encoded by nuclear DNA (Capaldi, 1990; Tsukihara *et al*., 1996). Subunits 1 and 2 are responsible for coordinating the transfer of electrons through the interior of the enzyme (Capaldi, 1990). To facilitate the movement of electrons, subunits 1 and 2 contain heme groups, a and a_3, as well as 2 redox-active copper containing metal centers, CuB and CuA (Capaldi, 1990). The crystal structure of bovine COX shows that there is an interaction between subunit IV (COX4) and subunits I (COX1) and II (COX2; Tsukihara *et al*., 1996). Therefore, it is believed that COX4 plays an integral role in regulating the catalytic rate of the enzyme (Huttemann *et al*., 2001; Li *et al*., 2006). COX4 has been observed to exist as 2 different isoforms, COX4-1 and COX4-2. These isoforms have been found to react differently to varying O_2 tensions (Fukuda *et al*., 2007; figure 2). Under normoxic conditions, mammalian COX is predominantly composed of the COX4-1 isoform (Fukuda *et al*., 2007). Under hypoxic conditions, however, COX4-1 is degraded by the mitochondrial protease LON whose transcription is induced by HIF-1 (Cerretelli and Gelfi, 2009;

Fukuda *et al*., 2007; figure 2). As COX4-1 is degraded, HIF-1 induces the transcription of the COX4-2 isoform causing it to become the primary isoform present (Cerretelli and Gelfi, 2009; figure 2). When COX4-2 is in abundance, the catalytic properties of COX are predicted to become better suited for low O_2 respiration (Fukuda *et al*., 2007). When COX4-2 is in the majority, an increase in the rate of internal electron transfer between heme groups has been observed (Poyton *et al*., 1988; Waterland *et al*., 1991). An increased turnover rate of O_2 to H_2O has also been observed in the COX4-2 dominant enzyme (Poyton *et al*., 1988; Waterland *et al*., 1991). These modifications of COX are believed to aid in limiting the early transfer of electrons to O_2 at complex I and III and reduce the production of reactive oxygen species (ROS; Cerretelli and Gelfi, 2010). Furthermore, it is believed that the increase in efficiency of the ETC allows organisms to rely more heavily on oxidative phosphorylation for energy production instead of anaerobic metabolism under hypoxic conditions, thus reducing the amount of lactate produced (Cerretelli and Gelfi, 2010). However, a switch to lactate production is not solely dictated by reduced O_2 tensions and can be initiated by changes in the activity of key enzymes such as PDH (Le Moine *et al*., 2011; Wieland, 1983). PDH acts as a major branch point between the conversion of pyruvate to acetyl-CoA or to lactate, and thus the extent of aerobic and anaerobic metabolism, respectively (Wieland, 1983). Furthermore, the activity of PDH is regulated by HIF-1, which when present leads to a decrease in activity (Le Moine *et al*., 2011; Papandreou *et al*., 2006; figure 2).

PDH is one of the largest multienzyme complexes found in eukaryotic cells (Wieland, 1983). PDH is composed of multiple copies of three catalytic components (E_1, E_2, and E_3; Wieland, 1983). The complex's core is composed of a lipoate

acetyltransferase (E_2) to which all of the other enzymes are attached through noncovalent bonding (Wieland, 1983). E_3 consists of two identical subunits housing a molecule of FAD^+ (Wieland, 1983). E_1 is made up of nonidentical polypeptide chains, α and β, which form $\alpha_2\beta_2$ tetramers (Wieland, 1983). PDH is located within the mitochondrial inner membrane-matrix space and is involved in the linking of glycolysis to the TCA (Wieland, 1983). PDH is responsible for catalyzing the oxidative decarboxylation of pyruvate, producing acetyl-CoA, NADH, H^+, and CO_2 (Wieland, 1983). When active, PDH sends acetyl-CoA through the TCA to generate reducing equivalents (NADH, $FADH_2$) which are subsequently passed through the ETC to generate ATP, completing oxidative phosphorylation (Mitchell, 1961; Wieland, 1983; figure 3). However, when the activity of active PDH cannot keep pace with the rate of pyruvate production, as in the case when PDH is inactivated, pyruvate is not reduced to acetyl-CoA and is instead shuttled off to LDH which catalyzes the oxidation of pyruvate to lactate, producing ATP at a much lower yield (Wieland, 1983; figure 3). Thus, PDH acts as a master switch, regulating the degree of glucose and fatty acid oxidation (Rardin *et al*., 2009). Furthermore, in tissues where FA synthesis is active (e.g. liver), PDH is also producing precursors for cystolic acetyl-CoA through the formation of mitochondrial acetyl-CoA via citrate formation and efflux (Sugden and Holness, 2006). Due to substrate competition between glucose and FAs, PDH's role in facilitating the production of malonyl-CoA, a lipogenic intermediate, suggests that high PDH activity has the potential to limit mitochondrial FA uptake and subsequently oxidation through the inhibition of CPTI (Sugden and Holness, 2006). Also, since the conversion of acetyl-CoA to glucose is non-existent in mammals, inactivation of PDH is important to conserve glucose and facilitate FA oxidation when

glucose levels are low (Sugden and Holness, 2006).

The activity of PDH is modulated through reversible phosphorylation at one of three phosphorylation sites (site 1: Ser^{293}, site 2: Ser^{300}, and site 3: Ser^{232}) located on serine residues of the PDHE1α subunit (Patel and Korotchkina, 2006; Wieland, 1983). Phosphorylation is catalyzed by one of four pyruvate dehydrogenase kinases (PDK1-4) which have a tissue specific distribution, and dephosphorylation is catalyzed by pyruvate dehydrogenase phosphatase (PDP) of which two isoforms exist in mammalian cells, PDP1 and 2 (Rardin *et al*., 2009). All four isoforms of PDK are believed to be capable of inactivating PDH through phosphorylation of sites designated site one, Ser^{293}, and site two , Ser^{300}, although with varying affinities (Korotchkina and Patel, 2001). However, Ser^{232} is specifically phosphorylated by the PDK1 isoform which has been observed to be induced by hypoxic stress via HIF-1 (Le Moine *et al*., 2011; Papandreou *et al*., 2006; figure 2). Furthermore, PDK1 is the only isoform observed to be able to phosphorylate all three sites (Rardin *et al*., 2009). PDH activity is also under acute regulation by various metabolic end products such as acetyl-CoA, NADH, and ATP which activate PDK expression and thus decreases PDH activity; and pyruvate which inhibits PDK expression and thus increases PDH activity (Rardin *et al*., 2009; Spriet and Heigenhauser, 2002). PDK activity has also been shown to be affected by FA concentrations, becoming more active with an increase in FA concentrations (Sugden and Holness, 2006). The increase in PDK activity in the presence of increased levels of FAs is believed to increase FA utilization (Sugden and Holness, 2006). It also been shown that PDK can be regulated both transcriptionally and post-translationally (Sugden and Holness, 2003).

Muscle Phenotype May Affect Whole Animal Metabolic Response to Acute Exercise

Fuel use in the muscle may vary due to many factors including diet, exercise, and changes in environment such as a move to hypoxic conditions (Hill *et al*., 1991; Schippers *et al*., 2012; Weber and Haman, 2004). One way in which fuel use is modified is through changes in the activity of key metabolic enzymes (Saurez *et al*., 2005). Enzyme activity can be altered through changes in the amount of enzyme present (hierarchal regulation) or by changing how existing enzymes are regulated (metabolic regulation; Suarez *et al*., 2005). Also, changes in the composition of existing enzymes, such as changes in protein isoforms with different kinetic properties, have been shown to alter the flux through metabolic pathways (Fukuda *et al*., 2007). A recent study examining the concentration of various enzymes involved in CHO and lipid oxidation in CD-1 mice found no significant changes with acclimation to chronic hypoxia or exercise (Schippers, 2011). Therefore, it is predicted that changes in fuel use may be due, in part, to changes in enzymes regulation.

During acute hypoxia PDH becomes inactivated and pyruvate is converted to lactate by LDH (Papandreau *et al*., 2006; figure 3). A recent study conducted by Le Moine *et al*. (2011) examined the levels of HIF-1α, PDK1, and the degree of PDH phosphorylation in response to hypoxia and exercise. When an individual is exposed to hypoxia, PDK1 activity is believed to be up-regulated by HIF-1 (Cerretelli *et al*., 2009; Le Moine *et al*., 2011; Mason *et al*., 2007; figure 2). It was found that protein levels of HIF-1α, PDK1, and PDH phosphorylated at the Ser^{232} site all showed the same pattern of first increasing under acute hypoxia (24hr) and then decreasing to levels closer to those

observed under normoxia during chronic hypoxia (1 week; Le Moine *et al*., 2011). Total PDH activity mirrored the changes observed in the degree of phosphorylation, first decreasing at 24hrs and then returning to normoxic control levels after 1 week (Le Moine *et al*., 2011). This pattern of protein expression and PDH activity suggests that when exercising, individuals exposed to acute hypoxia rely more on anaerobic metabolism rather than aerobic metabolism and *vise versa* during chronic hypoxia due to the dephosphorylation of PDH (Le Moine *et al*., 2011). This hypothesis is also supported by changes in muscle lactate levels during exercise (Le Moine *et al*., 2011).

When an individual exercises there is an increased demand for ATP, especially in skeletal muscle. At moderate exercise intensities the body is usually able to meet this demand through increased rates of oxidative phosphorylation. In exercising mammals the acetyl-CoA required for increased ETC flux can be derived from glucose or FA oxidation (Frayn, 1996). As exercise intensity increases, however, O_2 delivery cannot meet energy demand and the body is forced to rely almost solely on CHO oxidation and anaerobic metabolism (Frayn, 1996). As discussed above, the switch between aerobic and anaerobic metabolism is largely dictated by the degree of phosphorylation and thus activity of PDH (Rardin *et al*., 2009; Wieland, 1983). A decrease in total phosphorylation has been observed at each of PDH's phosphorylation sites in response to one – legged extensor exercise at moderate intensities (Kiilerich *et al*., 2011). To my knowledge, this is the only study examining the degree of phosphorylation at each of PDHs phosphorylation sites in response to exercise. Furthermore, an increase in PDH activity has also been observed in response to exercise suggesting a greater rate of pyruvate oxidation through the aerobic pathway (Putman *et al*., 1995). The degree of

PDH activation in the vastus lateralis muscle of humans cycling over varying exercise intensities (35%, 65%, and 90% VO_2max) has also been investigated (Howlett *et al*., 1998). The activation of PDH was observed to progressively increase with increasing exercise intensity (Howlett *et al*., 1998). This increase in PDH activation was predicted to lead to an increased aerobic production of ATP through oxidative phosphorylation to meet the increasing energy demand accompanying greater exercise intensities (Howlett *et al*., 1998). There has, however, been an observed tissue specific effect of exercise on PDH activation (Denyer *et al*., 1991). Denyer *et al*. observed the activity of PDH in the heart, red quadriceps, diaphragm, and liver of rats run on a treadmill for 30min (Denyer *et al*., 1991). A variation in PDH activity was observed between the different muscle types examined (Denyer *et al*., 1991). The difference in activation was suggested to be a product of the level of glucose required by the specific muscle groups during exercise (Denyer *et al*., 1991).

As previously discussed, mammals generally rely mainly on CHOs and lipids for energy production within the muscle during exercise (Venables *et al*., 2005). The proportion of each, however, can vary due to many factors such as diet composition, muscle glycogen content, exercise intensity, duration, and on previous exercise training (Brooks, 1998; McClelland, 2004; Venables *et al*., 2005). Generally, exercise intensity is based on the amount of O_2 being consumed compared to an individual's maximal O_2 consumption during exercise (VO_2max), expressed as %VO_2max. While at rest or moderate exercise intensities (≈ 60% VO_2max) mammals consume primarily lipids with, a lesser contribution from CHOs (Brooks, 1998; Roberts *et al*., 1996; figure 3). In dogs, a highly aerobic animal, and goats, a more sedentary animal, it was observed that

maximal rates of fat oxidation occurred while exercising at ≈ 40% of their maximal O_2 consumption (Roberts *et al*., 1996). As exercise intensity increases, whole animal VO_2 increases and the relative rates of lipid oxidation decrease while the relative rate of CHO oxidation increases, becoming the primary fuel source at high exercise intensities (≈85-90% VO_2max; Brooks, 1998; Roberts *et al*., 1996; figure 3). Again, in dogs and goats it was observed that CHO oxidation reaches a maximum at exercise intensities eliciting maximal O_2 consumption (Roberts *et al*., 1996; figure 3). This fuel use pattern during exercise has been observed to be conserved among many mammals (Roberts *et al*., 1996). However, individuals that are acclimated to and exercised under hypoxic conditions were originally predicted to place a higher dependence on CHOs for energy production during exercise due to the greater amount of ATP produced during oxidation per molecule of O_2 consumed (McClelland *et al*., 1998). This prediction was tested and shown to not be the case with acclimation (McClelland *et al*., 1998). Hypoxia acclimated rats which were exercised at the same relative VO_2max in fact showed the same pattern of fuel use as rats exercised under normoxic conditions, utilizing lipids at low intensities and CHOs at high intensities (McClelland *et al*., 1998; figure 3). These results suggest that mammals act to conserve their small but valuable CHO reserves rather than reduce O_2 consumption during exercise (Weber and Haman, 2004). However, a recent study conducted by Schippers and colleagues (2012) provided compelling evidence for increased CHO oxidation in high-altitude adapted Andean mice. It was found that high-altitude native species of Andean mice (genus *Phyllotis*) used a greater proportion of CHOs relative to total fuel use both at rest and when exercised at 75% of their VO_2max under hypoxic conditions (12% O_2; Schippers *et al*., 2012). However, this is the only example of a

deviation from the conserved fuel use pattern shared by other mammals. Due to the highly conserved nature of fuel use present in most mammals, a common controlling mechanism has been suggested to be present (Spriet and Watt, 2003).

The flux through the various metabolic pathways in mammals is largely controlled through feedback inhibition from products of the same or competing pathway (Balaban, 1990; Jeukendrup, 2002). When ATP levels are high there is a decreased need for its production so the processes responsible for the initiation of oxidative phosphorylation, glycolysis and β-oxidation, are scaled down (Chance *et al*., 1985). Further, the rates of glycolysis and β-oxidation have been predicted to be reduced when lipid or glucose oxidation, respectively, is the dominant source of acetyl-CoA production (Jeukendrup, 2002). One of the most prominent forms of regulation for glycolysis is through feedback inhibition of three key enzymes, HK, PFK, and PK. High ATP/ADP ratios act to inhibit HK, PFK, and PK thus decreasing the overall flux through glycolysis (Chance *et al*., 1985). Also, phosphocreatine (PCr), which is involved in the production of ATP during burst exercise, has also been shown to inhibit PFK and PK at high concentrations, reducing glycolytic flux (Krzanowski and Matschinsky, 1969; Wu and Yeung, 1978). When glycolysis is slowed at downstream points such as PFK or PK the end products of upstream reactions build-up inhibiting their respective enzymes (Nelson and Cox, 2008). For example, as the rate of glycolysis is reduced there is a buildup of G-6-P inhibiting HK, thus preventing the further conversion of glucose to G-6-P (Jeukendrup, 2002). Therefore, the levels of these metabolites, as well as others, can provide key insights into what metabolic pathway is in use and which ones are inhibited (Green *et al.*, 1992). Further, the patterns of muscle fiber recruitment and rates of CHO

and lipid transport have also been hypothesized to show insight into the specific metabolic pathways being utilized and thus the type of fuel being oxidized (Jeukendrup, 2002; Roberts *et al*., 1996; Romijn *et al*., 1993).

Muscle Fiber Recruitment

It is believed that when an individual transitions from low intensity exercise to high intensity exercise there is a shift in muscle fiber recruitment from slow oxidative to fast glycolytic fibers (Brooks, 1998). This change in fiber recruitment is predicted to lead to a greater reliance on CHOs for energy production with increasing exercise intensities (Roberts *et al*., 1996). A progressive shift has been observed with increasing exercise intensity from slow twitch oxidative muscle fibers located deep within the muscle to more peripheral fast twitch glycolytic fibers (Armstrong and Laughlin, 1985). Furthermore, Vollestad and Blom saw in humans a shift from type I fibers at 43% VO_2max, to type I and IIA fibers at 61% VO_2max, and finally to type I, IIA, IIAB, and IIB fibers (Vollestad and Blom, 1985). Therefore, since type I muscle fibers are generally considered oxidative, and type II glycolytic there seems to be some evidence for a progressive shift in use to more glycolytic muscle types as exercise intensity increases (Armstrong and Laughlin, 1985; Vollestad and Blom, 1985).

Limitations to CHO and FA Transport

As exercise intensity increases there is a decrease in the appearance of free FAs in the blood stream, suggesting that there is a reduced ability to break down lipids (lipolysis) at higher exercise intensities (Romijn *et al*., 1993). However, Romijn and company observed an increase in overall lypolitic rate in individuals cycling at high intensities. This observation leads to the prediction that there is a reduction in the ability to transport

FAs throughout the blood at high exercise intensities rather than a limit on lipolysis (Romijn *et al*., 1993). Since lipids are hydrophobic, they need to be bound to carrier proteins (albumin) during transport in the blood stream (Romijn *et al*., 1993). Albumin molecules have limited binding capacities for FAs, causing a restriction point for FA transport (Romijn *et al*., 1993). Furthermore, some evidence exists for a reduction in blood flow to the skin and adipose tissue during exercise, further limiting the body's ability to provide muscles with FAs for oxidation (Romijn *et al*., 1993; Bulow and Madsen, 1981). Therefore, it is conceivable that a reduction in adipose blood flow coupled with the limited binding capacity of albumin could limit the amount of FAs available for oxidation at the muscle, forcing the body to switch to a more readily available fuel source, CHOs (Romijn *et al*., 1993; Spriet and Watt, 2003; McClelland *et al*., 1998). Odland and company (1998) observed a 4-fold increase in leg FA uptake and a 23% decrease in glycogenolysis by increasing FA availability through the infusion of triacylglycerides (TAG). The exact cause of this increase could not be pinpointed, however, it was concluded the regulation occurred at the level of glycogen phosphorylase and/or PDH (Odland *et al*., 1998).

Although some evidence exists for a common mechanism dictating fuel use during exercise across mammalian species, a definitive mechanism remains to be discovered (Jeukendrup, 2002; Romijn *et al*., 1993; Vollestad and Blom, 1985). As discussed above, acclimation to hypoxia does not alter the conserved pattern of fuel use as originally predicted, however, it does decrease VO_2max (Ferretti *et al*., 1990). This aspect of hypoxia makes it possible to make comparisons between animals with different VO_2maxs within a single species, thus controlling for any interspecific differences in

muscle metabolism. Furthermore, muscle phenotypic changes in response to acute and chronic hypoxia are not fully understood, nor are the changes in metabolic regulation that might result because of them (McClelland *et al*., 1998; Schippers, 2011). Therefore, hypoxia is a useful model for the investigation into a common mechanism controlling fuel selection in mammals (McClelland *et al*., 1998)

OBJECTIVES

The aim of the current study was to investigate the phenotypic changes resulting from acclimation to acute and chronic hypoxia and whether these changes affect the whole animal metabolic responses to acute exercise. To carry out this investigation my objectives were:

1.) To assess the temporal patterns of gene and protein expression which occur in response to hypobaric hypoxia acclimation.

2.) To assess acute responses to exercise that may help explain conserved exercise fuel use patterns between control and acclimated mice

Since the response of muscle metabolism to acute and chronic hypoxia is not well understood, and the mechanism of fuel selection remains to be elucidated, I set out to test the following hypotheses.

1.) Acclimation to chronic hypoxia will result in an adjustment in the proportion of cytochrome c oxidase subunits as a way to increase the efficiency of oxidative phosphorylation.

2.) The degree of PDH phosphorylation will be similar between resting control and acclimated mice indicating comparable rates of aerobic metabolism.

3.) Relative post-exercise changes in muscle metabolites and the degree of PDH phosphorylation will be similar between control and acclimated mice reflective of a common mechanism regulating fuel use.

Figure 1. The various pathways involved in mammalian muscle metabolism during exercise. Energy production in the form of adenosine triphosphate (ATP) begins with the oxidation of two main fuel types during exercise, (a) carbohydrates (CHO) and (b) lipids, to produce acetyl-CoA. (a) Glucose-6-phosphate (G-6-P) is created within the myocyte either from circulating glucose molecules or from the breakdown of intramuscular glycogen stores. G-6-P is then fed into glycolysis, which ends with the production of ATP, nicotinamide adenine dinucleotide (NADH), and pyruvate. The fate of pyruvate is under the control of pyruvate dehydrogenase (PDH) which, when active, converts pyruvate to acetyl-CoA and when inactive, to lactate through the lactate dehydrogenase (LDH) reaction. Acetyl-CoA may also be produced through the oxidation of lipids (b). Non-esterified fatty acids (NEFAs) are transported into the cell and subsequently the mitochondria. Once inside the mitochondria, the fatty acids (FAs) go through a series of reactions referred to as β-oxidation to produce the end product of acetyl-CoA. Once acetyl-CoA is formed, it is sent through the tricarboxylic acid cycle (TCA) where it is further oxidized to produce NADH and flavin adenine dinucleotide ($FADH_2$), marking the beginning and oxidative phosphorylation (c). After their formation, NADH and $FADH_2$ donate electrons to the complexes of the electron transport chain (ETC) which drives the transport of protons from the mitochondrial matrix to the intermembrane space, creating an electrochemical gradient which is then used to power the production of ATP. ATP can also be formed in the cytosol of the myocyte through the phosphagen energy system (d) which involves the breakdown of phosphocreatine (PCr) to creatine (Cr) to produce ATP. The phosphagen system also acts to shuttle ATP from site of production (e.g. mitochondria) to the site of use (e.g. myosin).

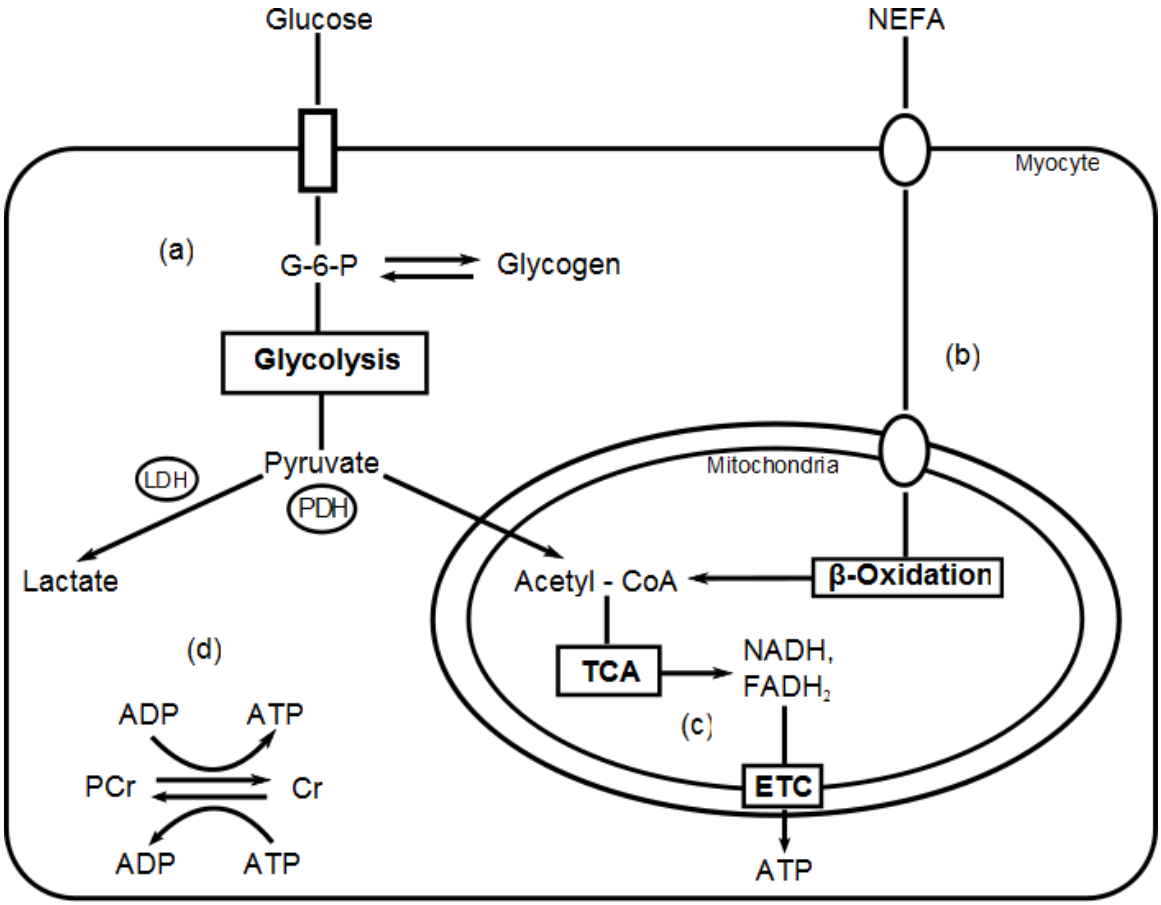

Glucose
NEFA
Myocyte
(a)
G-6-P
Glycogen
Glycolysis
(b)
Pyruvate
LDH
PDH
Mitochondria
Lactate
Acetyl - CoA
β-Oxidation
(d)
TCA
NADH,
FADH2
(c)
ADP
ATP
PCr
Cr
ADP
ATP
ETC
ATP

Figure 2. The effects of the hypoxia inducible factor 1 (HIF-1) on pyruvate dehydrogenase (PDH) phosphorylation and cytochrome c oxidase (COX) subunit composition. Under hypoxic conditions the transcription factor hypoxia inducible factor 1 (HIF-1) is stabilized. HIF-1 leads to an increase in pyruvate dehydrogenase (PDH) phosphorylation (a) through an increase in pyruvate dehydrogenase kinase 1 (PDK1) protein levels and thus activity. The increase in PDH phosphorylation leads to a decrease in the amount of active PDH present. Further, HIF-1 causes a change in the composition of the subunits of cytochrome c oxidase (COX), COX4-1 and COX4-2 (b). HIF-1 induces the expression of mitochondrial LON protease (LON) which subsequently degrades the COX4-1 -isoform. As COX4-1 is degraded, HIF-1 causes a rise in the levels of COX4-2.

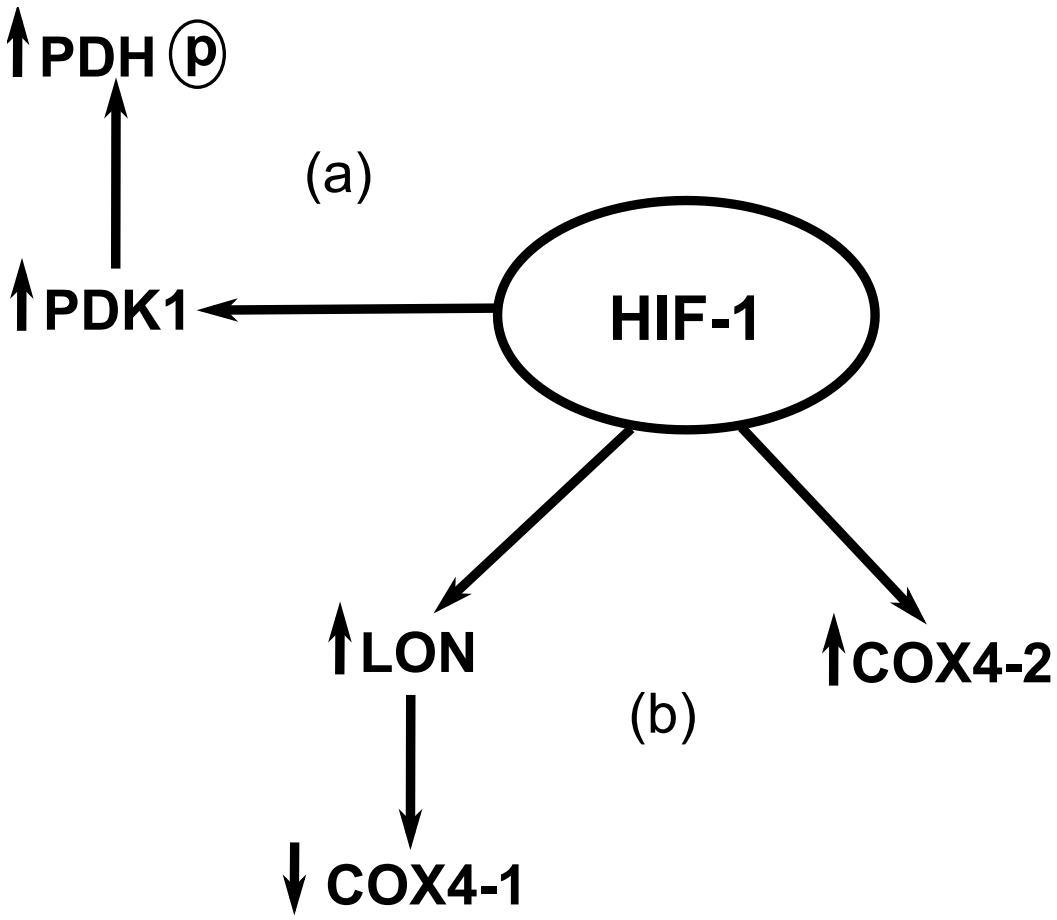
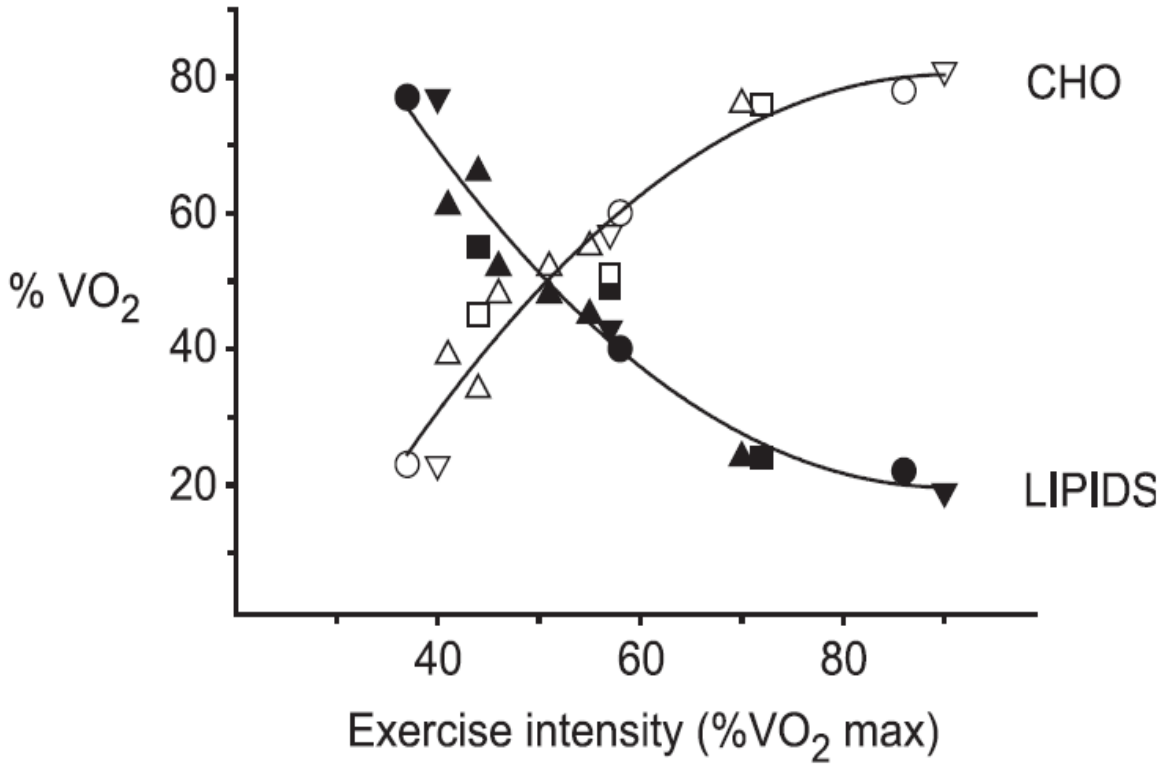

PDH ⓟ
(a)
PDK1
HIF-1
LON
COX4-2
(b)
COX4-1

Figure 3. Fuel section pattern during exercise in the mammalian model. Relative contribution of carbohydrate (CHO) oxidation (open symbols) and lipid oxidation (closed symbols) to total oxygen consumption (VO_2) over varying exercise intensities expressed as a % of maximal oxygen consumption (%VO_2max). (○ and ●) represent dogs, (▽and ▼) represent goats, (□ and ■) represent humans, and (△ and ▲) represent rats. Trained and untrained rats acclimated to hypoxia were also included. Adapted from Weber and Haman, 2004.


80
60
40
20
% VO2
CHO
LIPIDS
40
60
80
Exercise intensity (%VO2 max)

CHAPTER 2: SKELETAL MUSCLE PHENOTYPIC PLASTICITY DURING CHRONIC HYPOXIA AND ITS EFFECTS ON WHOLE ANIMAL MUSCLE METABOLISM DURING ACUTE EXERCISE IN SMALL MAMMALS

Abstract

Mammals exposed to hypoxic stress face many challenges, especially in terms of energy production. To conserve O_2 for the most essential bodily processes mammals may employ strategies such as entering a hypometabolic state or relying on anaerobic metabolism rather that aerobic. However, the latter strategy is not a viable option for long stays in hypoxic conditions and long lasting cellular changes are needed. Under chronic hypoxia, mammals have been predicted to alter their metabolic machinery in an attempt to increase the efficiency of ATP production to reduce the amount of O_2 used per ATP formed by the mitochondria. One way mammals are predicted to increase the efficiency of ATP production is through a change in the proportion of the isoforms of cytochrome c oxidase (COX). In cell culture experiments it has been observed that there is a decrease in the COX4-1 isoform and an increase in the COX4-2 isoform under hypoxia, leading to an increase in the turnover rate and reaction efficiency of COX. In the present study, I observed a significant increase in the mRNA levels of COX4-2 with 24hrs of hypoxia. However, this change was not mirrored by corresponding changes at the protein level. Further, I examined the phosphorylation state of pyruvate dehydrogenase (PDH) as an indicator of the amount of PDH in the active form. Under chronic hypoxia resting mice exhibited a significant rise in PDH phosphorylation. This

increase may represent a decrease in overall PDH activity and a decreased reliance on carbohydrate derived acetyl-CoA.

I also explored the effects plastic changes in muscle during chronic hypoxia had on muscle metabolism during acute exercise. In hypoxic post-exercise mice, a significant increase in muscle lactate levels was observed compared to rest. This rise was not present in control mice, suggesting that acclimated mice were relying more heavily on anaerobic metabolism. However, there were no significant changes in PDH phosphorylation in post-exercise mice which could help to explain elevated muscle lactate levels.

Introduction

Mammals residing at high altitudes face a multitude of challenges, including low temperatures, increased UV exposure, and of course hypoxic conditions. Hypoxia is defined as a local reduction in oxygen (O_2) availability, either at a cellular, tissue, or environmental level (Prabhakar and Semenza, 2012). One of the most striking challenges of hypoxic stress is in terms of energy production, especially during times of intense physical activity (Hochachka *et al*., 1996; Hochachka *et al*., 2002; McClelland *et al*., 1998). Normally, when carrying out activities which do not require maximal O_2 consumption, mammals mainly produce ATP through the process of oxidative phosphorylation. Oxidative phosphorylation involves the production of acetyl-CoA through the oxidation of either proteins, carbohydrates (CHO; glycolysis) or lipids (β-oxidation) which is further oxidized through the tricarboxylic acid cycle (TCA) producing reducing equivalents (NADH and $FADH_2$; Mitchell, 1961). The electrons from NADH and $FADH_2$ are subsequently passed through the complexes of the electron

transport chain (ETC) which terminates with the transfer of electrons to O_2 producing H_2O at complex IV also called cytochrome c oxidase (COX; Mitchell, 1961). Under hypoxic stress, however, O_2 tensions are too low to dedicate such a large portion of the total O_2 available to energy production in this manner (reviewed in Ramirez *et al*., 2007). Therefore, mammals which routinely experience hypoxic stress have developed strategies to either reduce their total ATP consumption or to produce it through a different route (Hochachka, 2000; Kim *et al*., 2006; reviewed in Ramirez *et al*., 2007). Diving mammals, such as certain whales, have been observed to reduce their energy consumption by entering a state of hypometabolism (Hochachka, 2000; reviewed in Ramirez *et al*., 2007). A state of hypometabolism is a reduction in metabolism allowing ATP to be dedicated to only essential processes (Hochachka, 2000; reviewed in Ramirez *et al*., 2007). Others will resort to anaerobic metabolism, thus bypassing the O_2 consuming step of the ETC (Kim *et al*., 2006). Anaerobic metabolism, however, ends with the production of lactic acid, which when at high levels can be detrimental to the cell and slow the rate of glycolysis (Prampero and Ferretti, 1999). Further, the rate of glycolysis must increase greatly, due to the reduced yield of only 2 ATP, to match ATP demand leading to the rapid depletion of limited CHO stores. Therefore, this strategy is not a viable option when mammals are subjected to long periods of hypoxic stress (Hochachka, 1985).

When animals are exposed to chronic hypoxia they are predicted to accomplish two things, 1) to rely on aerobic rather than anaerobic metabolism due to the greater yield of ATP per mole of fuel used and 2) to increase the efficiency of ATP production to reduce the amount of O_2 used per ATP formed (Hochachka, 1985). To accomplish these

goals, mammals have been predicted to alter the mix of fuels used by relying more heavily on carbohydrates (CHOs) as well as to increase the efficiency of O_2 use by the mitochondria through changes in muscle phenotype (Hochachka *et al*., 1996; Hochachka *et al*., 2002; McClelland *et al*., 1998; Schippers *et al*., 2012).

Plastic Changes in Muscle Phenotype in Response to Chronic Hypoxia

Previous investigators exploring changes in muscle phenotype in response to chronic hypoxia have found few to no changes (Abdelmalki *et al*., 1996; Schippers, 2011). Schippers recently examined changes in enzyme activity, *in vitro*, within the gastrocnemius of CD-1 mice, and thus content, of key enzymes involved in glycolysis, the TCA, ETC, and β-oxidation, finding no change in enzyme activity after 4 weeks of acclimation (Schippers, 2011). However, studies examining the effects of acclimation for shorter periods of time (1 week) have observed changes in enzyme regulation and activity (Le Moine *et al*., 2011). Le Moine and colleagues observed changes in the phosphorylation state and activity of pyruvate dehydrogenase (PDH) with the transition from acute to chronic hypoxia in the muscle of mice (Le Moine *et al*., 2011). Therefore it is predicted that plastic changes may occur prior to 4 weeks of acclimation which are abolished with long term acclimation. Further, changes in other key enzymes controlling the flux through oxidative phosphorylation, such as PDH, may be occurring. As previously discussed, an initial response of some mammals to hypoxic stress is to revert to anaerobic metabolism (Kim *et al*., 2006; Papandreau *et al*., 2006). This switch has been shown to be largely dependent on the regulation of PDH (Papandreau *et al*., 2006). PDH is a large multisubunit protein located in the mitochondrial inner membrane-matrix space (Wieland, 1983). It is responsible for linking glycolysis with the TCA through the

conversion of pyruvate to acetyl-CoA (Wieland, 1983). However, when PDH is inactivated through phosphorylation and pyruvate production exceeds acetyl-CoA production by PDH, pyruvate is converted to lactate by lactate dehydrogenase (LDH), completing anaerobic metabolism (Rardin *et al*., 2009). Thus, PDH acts as a master switch regulating the degree of aerobic and anaerobic carbohydrate metabolism (Rardin *et al*., 2009).

The activity of PDH is controlled through reversible phosphorylation at one of its three phosphorylation sites (site 1: Ser^{293}, site 2: Ser^{300}, and site 3: Ser^{232}) which are found on the serine residues of the PDHElα subunit (Patel and Korotchkina, 2006; Wieland, 1983). Pyruvate dehydrogenase phosphatases (PDPs) are responsible for dephosphorylating PDH leading to its activation and pyruvate dehydrogenase kinases (PDKs) are responsible for phosphorylating PDH leading to its inactivation (Rardin *et al*., 2009). A total of 4 isoforms of PDK exist (1 – 4) which have a tissue specific distribution (Rardin *et al*., 2009). Each isoform is capable of depressing PDH activity, with varying affinities, through phosphorylation at site 1, Ser^{293}, and site 2, Ser^{300} (Korotchkina and Patel, 2001). PDK1, however, is capable of phosphorylating all three phosphorylation sites and is the only isoform capable of phosphorylating PDH at site 3, Ser^{232} (Le Moine *et al*., 2011; Papandreou *et al*., 2006). Further, PDK1 expression has been observed to be up-regulated by the hypoxia inducible factor 1 (HIF-1) in response to hypoxic stress (Le Moine *et al*., 2011; Papandreou *et al*., 2006). The activity of PDH may also be modulated through the buildup of various metabolic end products (Rardin *et al*., 2009; Spriet and Heigenhauser, 2002). PDK expression is elevated when the levels of acetyl-CoA, NADH, and/or ATP become elevated increasing the acetyl-CoA/CoA,

$NADH/NAD^+$, and ATP/ADP ratios, respectively (Rardin *et al*., 2009; Spriet and Heigenhauser, 2002). Further, PDK expression is depressed when there are high levels of pyruvate within the cell, thus leading to an increase in PDH activity (Rardin *et al*., 2009; Spriet and Heigenhauser, 2002).

Efficiency of ATP Production

Humans native to high altitudes have been observed to consume lower amounts of O_2 (VO_2) than sea level natives while biking at a given work load under hypoxic conditions (reviewed in Cerretelli *et al*., 2009). This lowered VO_2 during exercise suggests that theses natives have a higher mechanical work output or metabolic input under hypoxia (Cerretelli and Gelfi, 2010). Furthermore, an increased ratio of ATP molecules produced per O_2 molecule consumed (P/O) has been observed in isolated mitochondria from rat livers under hypoxic conditions (Solien *et al*., 2005). This increased P/O ratio suggests that the energy transfer between glycolysis, the TCA, and the ETC has become more tightly coupled, allowing the mitochondrion to produce more ATP per O_2 consumed (Solien *et al*., 2005). Together, these results suggest that organisms may be able to increase the efficiency of ATP production, producing more ATP per molecule of O_2, while exposed to chronic hypoxia (Cerretelli and Gelfi, 2010). It has previously been predicted that changes in exercise mechanics or contributions from anaerobic metabolism may be causing this apparent increase in efficiency (Cerretelli and Gelfi, 2010). However, it has been shown that these situations are not the case (Cerretelli and Gelfi, 2010). Recently, changes of a metabolic nature leading to changes in muscle phenotype have been proposed (Cerretelli and Gelfi, 2010; Fukuda *et al*., 2007; Le Moine *et al*, 2011). One of the predicted changes in muscle phenotype is in the subunit

composition of COX, specifically in the proportion of the COX4-1 and COX4-2 isoforms (Cerretelli and Gelfi, 2010; Fukuda *et al*., 2007).

COX is the final complex in the ETC responsible for facilitating the transfer of electrons to O_2 forming H_2O (Mitchell, 1961). COX is a dimer with each monomer being composed of 13 different subunits (Tsukihara *et al*., 1996). Of the 13 subunits, 3 are encoded by mitochondrial DNA (subunits 1, 2, and 3) with the remaining 10, including COX4, encoded by nuclear DNA (Capaldi, 1990). Subunits 1 and 2 contain the heme groups a and a_3 as well as 2 redox-active copper containing metal centers CuB and CuA which are responsible for the transfer of electrons through the protein (Capaldi, 1990). COX4 is believed to play an integral role in regulating the catalytic rate of the enzyme (Huttemann *et al*., 2001; Li *et al*., 2006). Further, two different isoforms of the COX4 subunit have been identified in mammalian cells, COX4-1 and COX4-2, which have been observed to respond differently to varying O_2 tensions (Fukuda *et al*., 2007). Under normal O_2 tensions, COX4-1 is observed to be the primary isoform present (Fukuda *et al*., 2007). Under hypoxic conditions, however, expression of the COX4-2 isoform has been shown to be up-regulated causing it to become the dominant isoform expressed (Fukuda *et al*., 2007). This change in isoforms is facilitated by the transcription factor HIF-1 (Fukuda *et al*., 2007). Under hypoxia, HIF-1 induces the expression of LON protease, which is responsible for the degradation of the COX4-1 isoform (Fukuda *et al*., 2007). HIF also directly induces the expression of the COX4-2 isoform (Fukuda *et al*., 2007). When the COX4-2 isoform is in majority an increase in the rate of internal electron transfer between the heme groups has been observed (Poyton *et al*., 1988; Waterland *et al*., 1991). Furthermore, it has been found that when COX4-2 is the primary

isoform present, the COX enzyme shows an increased turnover rate of O_2 to H_2O (Poyton *et al*., 1988; Waterland *et al*., 1991). These results suggest that this switch in COX isoforms under hypoxic conditions causes COX to become more efficient, allowing electron transfer through the ETC to continue (Poyton *et al*., 1988; Waterland *et al*., 1991).

Phenotypic Plasticity and the Response of Skeletal Muscle to Acute Exercise

During exercise, the body's demand for ATP increases. Unless O_2 tensions become limiting within the muscle, this demand is met through an increased production of acetyl-CoA from either CHOs or lipids and an increase in the flux though the TCA and ETC (Frayn, 1996). The degree of PDH phosphorylation, and thus PDH activity, dictates the extent of aerobic and anaerobic CHO metabolism (Rardin *et al*., 2009; Wieland, 1983). In humans performing a one-legged extensor exercise at 60% VO_2max a decrease in the phosphorylation state at all three of the phosphorylation sites on PDH was observed (Kiilerich *et al*., 2011). Furthermore, the activity of PDH has been observed to increase in response to cycling in humans (Putman *et al*., 1995). The activity of PDH has been observed to increase even further in response to increasing exercise intensities (Howlett *et al*., 1998). This increase in PDH activity suggests that there is an increase in acetyl-CoA derived from CHOs leading to an increase in the amount of ATP produced through aerobic metabolism while exercising (Howlett *et al*., 1998). However, changes in muscle phenotype in response to hypoxic stress have been observed to alter the response of PDH to exercise affecting whole animal muscle metabolism (Le Moine, *et al*., 2011).

Recently, Le Moine and colleagues investigated whether acclimation to hypoxia lead to changes in muscle phenotype, specifically at the level of PDH activation, and

what affects, if any, those changes had on energy metabolism in mice during acute exercise (Le Moine *et al*., 2011). It was found that in mice acclimated to acute hypoxia (24hrs) there was a significant rise in the protein levels of HIF-1α, and PDK1 which led to an increase in the degree of PDH phosphorylation at site 3, Ser^{232}, compared to normoxic controls when run on a treadmill at a constant speed of 26m/min (Le Moine *et al*., 2011). Interestingly, after 1 week of hypoxic acclimation the levels of HIF-1α, PDK1, and PDH phosphorylation returned levels not different from control mice (Le Moine, *et al*., 2011). Furthermore, overall PDH activity during exercise was found to decrease in mice acclimated to acute hypoxia and then return to levels not different from controls in mice acclimated to chronic hypoxia (Le Moine *et al*., 2011). These results suggest that there was an alteration in muscle phenotype in response to acute hypoxia, an increase in HIF-1 protein and PDH phosphorylation, causing the body to rely more heavily on anaerobic metabolism during exercise, which was then reversed during a chronic stay in hypoxia (Le Moine, *et al*., 2011). Furthermore, muscle and blood lactate levels were observed to rise during exercise under acute hypoxia and then decrease to control levels during chronic hypoxia, supporting this prediction (Le Moine *et al*., 2011).

Fuel Selection During Exercise

Mammals rely mainly on the oxidation of CHOs and lipids for energy production with minimal contribution from protein oxidation (<5%) during exercise (Carraro *et al*., 1994; Rennie *et al*., 1981). The proportion of CHOs or lipids oxidized can vary due to many factors such as diet and exercise intensity (Edwards *et al*., 1934; Brooks, 1998; McClelland, 2004; Venables *et al*., 2005). When exercising at low intensities (≈ 60% VO_2max) mammals have been observed to rely primarily on the oxidation of lipids to

power energy production (Brooks, 1998; Roberts *et al*., 1996). However, with increasing exercise intensity there is an increase in VO_2 and a decrease in the relative levels of lipid oxidation coupled with a progressive increase in CHO oxidation (Brooks, 1998; Roberts *et al*., 1996). This progressive decline of relative lipid oxidation and increase in CHO oxidation continues as exercise intensity increases, with CHOs becoming the primary fuel choice at high exercise intensities (85 – 90% VO_2max; Brooks, 1998; Roberts *et al*., 1996). This pattern of fuel use has been observed to be conserved among many mammals under normal physiological conditions (Roberts *et al*., 1996; Weber and Haman, 2004; McClelland, 2004). Under hypoxia, however, mammals acclimated to chronic hypoxia were originally predicted to rely more heavily on CHO oxidation during exercise due to the greater amount of ATP produced during oxidation per molecule of O_2 consumed (McClelland *et al*., 1998). When the confounding effect of exercise intensity was taken into account this prediction has since been tested and proven to be false (McClelland *et al*., 1998). Hypoxia and normoxia acclimated rats were run at the same relative VO_2max and observed to follow the same fuel use pattern, relying mainly on lipids at low exercise intensities and CHOs at high intensities (McClelland *et al*., 1998). Due to the highly conserved nature of this fuel selection pattern, a common controlling mechanism has been suggested to be present (Brooks, 1998; Jeukendrup, 2002; McClelland, 2004; Randle *et al*., 1963; Romijn *et al*., 1993). Changes in the recruitment pattern of muscle fibers, limitations to lipid transport, and feedback inhibition loops between CHO and lipid oxidation have all been proposed as possible mechanisms (Brooks, 1998; Jeukendrup, 2002; Randle *et al*., 1963; McClelland, 2004; Romijn *et al*., 1993; Weber, 2011). However, evidence for the aforementioned mechanisms remains

scarce and a common mechanism remains to be discovered.

Therefore, in the current study I set out to determine if acclimation to chronic hypoxia resulted in changes in muscle phenotype and whether these changes affected muscle metabolism during exercise in mice. In resting mice, I examined changes in the composition of COX, specifically changes in the levels of the 4-1 and 4-2 isoforms in the gastrocnemius muscle. I also determined the phosphorylation state of PDH in the gastrocnemius of resting mice and whether changes in the phenotype of these two enzymes resulted in an alteration in muscle metabolism during acute exercise. Further, I investigated the possibility of a common controlling mechanism for fuel selection during exercise through the comparison of the post-exercise levels of various metabolites in hypoxic and control mice.

Therefore, the objectives of my study were:

1.) To assess the temporal patterns of gene and protein expression which occur in muscle in response to hypobaric hypoxia acclimation.

2.) To assess acute responses to exercise that may help explain conserved exercise fuel use patterns between control and acclimated mice

Since the response of muscle metabolism to acute and chronic hypoxia is not well understood, and the mechanism of fuel selection remains to be elucidated I set out to test the following hypotheses.

1.) Acclimation to chronic hypoxia will result in an adjustment in the proportion of cytochrome c oxidase subunits as a way to increase the efficiency of oxidative phosphorylation.

2.) The degree of PDH phosphorylation will be similar between resting controls and acclimated mice indicating comparable rates of aerobic metabolism.

3.) Relative post-exercise changes in muscle metabolites and the degree of PDH phosphorylation will be similar between control and acclimated mice reflective of a common mechanism regulating fuel use.

Materials and Methods

Animals

All experimental measures were approved by the Animal Research and Ethics Board at McMaster University according to the Canadian Council of Animal Care guidelines. Female and male CD-1 mice were used to investigate changes in the isoforms of cytochrome c oxidase (COX) and the whole animal response to acute exercise, respectively, in response to chronic hypoxia acclimation. Mice were obtained from a commercial supplier (Charles River, Wilmington, MA) at 7 – 8 weeks of age and housed at 25°C with a 12:12hr light cycle and given food (Harlan's Teklad Animal Diet) and water *ad libitum* unless otherwise mentioned. Half of these mice were acclimated to simulated altitude using hypobaric chambers as described below.

Acclimation to Hypobaric Hypoxia

24 female CD-1 mice were acclimated at the equivalent of ≈ 6500m (≈ 300mmHg) for 2hrs (n=6), 24hrs (n=6), 3 days (n=6), and 1 week (n=6) to asses changes in the isoforms of COX in response to chronic hypoxia acclimation. 21 male CD-1 mice were acclimated at the equivalent of ≈ 4300m (≈ 450 mmHg) for 4 weeks to assess the effects of chronic hypoxia acclimation on the whole animal response to acute exercise. 13 of these mice

were run on a treadmill to obtain post-exercise samples as described below.

Treadmill Running

As described previously (Templeman *et al*., 2012), prior to exercise all mice were fasted for 4 – 6 hours allowing them to become post-absorptive. Individuals from each treatment group were subjected to 30min of exercise on a small plexiglass-enclosed animal treadmill (Columbus Instruments) modified to have a working volume of ≈ 800ml (Schippers *et al*., 2012; Templeman *et al*., 2012). Normoxic mice were run under standard O_2 tensions and pressure (≈21% and 750mmHg) at a speed of 14m/min (predicted 75% VO_2max; Schippers, pers comm.; n=8) and 20m/min (predicted 85% VO_2max; Schippers, pers comm.; n=5) at a treadmill incline of 10°. Acclimated individuals were run under hypoxic conditions (≈12% O_2) at a speed of 8m/min (predicted 75% VO_2max; Schippers, pers comm.; n=8) and 12 m/min (predicted 85% VO_2max; Schippers, pers comm.; n=5) at a treadmill incline of 10°.

Sample Preparation

After 2hrs, 24hrs, 3 days, and 1 week of hypoxia acclimation mice were anesthetized at rest using isoflourane, and sacrificed via cervical dislocation. The left gastrocnemius muscle was excised and freeze clamped with liquid N_2-cooled aluminum tongs. Normoxic control mice were sampled at the same time as the 1 week hypoxia group, using the same protocol. Mice acclimated for 4 weeks were divided into their respective rest and exercise groups. Resting mice were quickly removed from their respective hypobaric chambers and immediately sacrificed with exercised mice being sacrificed immediately after exercise via cervical dislocation. The right gastrocnemius was excised from both resting and post-exercise mice, taking on average 55s ± 3.1 (mean ± SEM) and

freeze clamped with liquid N_2-cooled tongs.

All samples were kept at -80°C prior to analysis.

mRNA Quantification

Real-time PCR was implemented to determine the relative levels of cytochrome c oxidase subunit 4 isoform 1 (COX4-1), cytochrome c oxidase subunit 4 isoform 2 (COX4-2), LON protease (LON), and vascular endothelial growth factor (VEGF) mRNA within the left gastrocnemius, using 18S ribosomal protein as the housekeeping gene. 18S was used as the housekeeping gene due to its stable nature throughout hypoxic exposure. In the right gastrocnemius of 4 week individuals, relative levels of pyruvate dehydrogenase kinase 1 (PDK1), pyruvate dehydrogenase kinase 2 (PDK2), and pyruvate dehydrogenase kinase 4 (PDK4) mRNA were determined using 18S ribosomal protein as the housekeeping gene. Primer sequences were either designed using Primer 3 and tested with RT-PCR or previously published (Fukuda *et al*., 2007; Leick *et al*., 2009; Templeman *et al*., 2012; table 1).

Total RNA was isolated from 30-40mg of powdered tissue through homogenization with TRIzol using a derivative of the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). cDNA was synthesized from 1 µg of total RNA treated with DNase I. Reverse transcription was accomplished by SuperScript II RNase H^- reverse transcriptase using a mixture of random primers and Oligo dT primers in the presence of a dNTP mixture (Invitrogen, Burlington, ON).

To perform real-time PCR, the resulting cDNA product was diluted 5x using RNase and DNase-free water (Invitrogen). For each real-time reaction 5 µL of diluted cDNA was combined with 12.5 µL SYBR green mix (Bio-Rad Laboratories,

Mississauga, ON), 1 µL of each the forward and reverse primers at a concentration of 5 µM, and 5.5 µL of RNase and DNase-free water. A no template control containing RNase and DNase-free water in place of cDNA was run for each primer set to check for the presence of contaminants. Each reaction was run in duplicate using a Stratagene MX3000P system (Stratagene, La Jolla, CA, USA) using ROX as the reference dye. Amplification for all primer sets was accomplished using a 3-step method. The thermal profile was as follows: a 3 min initial denaturation at 95°C, 40 cycles of 95°C for 15s, 60°C for 45s, and 72°C for 30s. After amplification a dissociation curve was performed in order to insure reaction specificity. Relative amounts of mRNA were calculated based on the amplification efficiency using the comparative Ct method (mRNA quantity = $1/(E+1)^{Ct}$; Cikos *et al*., 2007).

Western Blot Analysis

For measurements of COX isoforms, powdered left gastrocnemius samples were briefly homogenized on ice in cold homogenization buffer at pH 7.4 containing in mM: 30 Hepes, 40 NaCl, 210 sucrose, 2 EGTA, and a protease inhibitor cocktail (Complete MINI, Roche Diagnostics, Laval, Quebec) using a cooled glass-on-glass homogenizer. After homogenization, samples were centrifuged at 600 x g for 10 min to remove structural proteins. Equal amounts of soluble protein (50-60 µg) were loaded into pre-cast 15% polyacrylamide gels (Bio-Rad Laboratories) and run at 150 volt for ≈ 80min. Protein was transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories), and blocked with 10% skim milk in PBST (in mM: 1.5 NaH_2PO_4-● H_2O, 8.1 Na_2HPO_4, 145.5 NaCl, 0.05% Tween 20, pH 7.4) overnight at 4°C. Following blocking, membranes were washed with PBST (4 x 5min), and then incubated in primary

antibody, diluted 1:6000 for COX4-1 (ab14744; Abcam Inc. Cambridge, MA) and 1:2000 for COX4-2 (ab70112; Abcam Inc) for 1hr at room temperature. After incubation, membranes were washed with PBST (1 x 15min, 3 x 5 min) then incubated in secondary antibody for 1hr, HRP-conjugated Rabbit Anti-Mouse (NEF822; Perkin Elmer, MA, USA) diluted 1:150000 for COX 4-1, and HRP-conjugated Donkey Anti-Rabbit (sc-2313; Santa Cruz Biotechnology Inc., CA, USA) diluted 1:80000 for COX4-2. Following the second incubation, membranes were washed once again in PBST (1 x 15min, 3 x 5min). Chemiluminescence was detected using autoradiographic films (Kodak, XAR) and analyzed using a ChemiDoc Gel Imaging system (Bio-Rad Laboratories). Levels of β – Actin protein were also determined for each blot and used to control for variations in protein loading between lanes. Protein levels were determined based on band volumes calculated from pixel density which were found using the ChemiDoc Gel Imaging software (Bio-Rad Laboratories). Total COX activity (Vmax) determined in the right gastrocnemius of the same mouse was used as a common denominator for the levels of COX4-1 and COX4-2 protein as a way to control for any differences in the amount of total COX present within the muscle. A common sample was run on each gel to allow for standardization between blots.

To determine the total PDH protein present and to quantify the phosphorylation state of PDH, samples were prepared as before with slight modification. Powdered right gastrocnemius were homogenized in cold homogenization buffer containing in mM: 50 Hepes pH 7.5, 150 NaCl, 10% glycerol, 1% Triton X-100, 1.5 $MgCl_2$, 1 EGTA, 10 NaP_2O_7, 100 NaF, and a protease inhibitor cocktail (Complete MINI, Roche Diagnostics). Equal amounts of soluble protein (30-40µg) were separated on 10% polyacrylamide gels.

Following transfer, PDVF membranes were treated as before with antibody dilutions as follows, PDHE1α (sc-133898; Santa Cruz Biotechnology Inc.) 1:200, PDHSer^{293} (AP1062; EMD Chemicals, Inc. San Diego, CA, USA) 1:2000, PDHSer^{300} (AP1064; EMD Chemicals) 1:1000, and PDHSer^{232} (AP1063; EMD Chemicals) 1:2000. HRP-conjugated Donkey Anti-Rabbit (sc-2313; Santa Cruz Biotechnology Inc.) secondary antibody was diluted 1:5000, 1:10000, 1:70000, and 1:70000, respectively. The degree of phosphorylation at each of the phosphorylation sites of PDH (Ser^{293}, Ser^{300}, Ser^{232}) were standardized to total PDH protein content. Normoxic rest and 4 week acclimated rest samples were also analyzed for COX4-1 protein levels as described above. A common sample was run on each gel to allow for standardization between blots.

Muscle Metabolites

The levels of adenosine triphosphate (ATP), creatine (Cr), creatine phosphate (PCr), pyruvate, glucose-6-phosphate (G-6-P), lactate, and glycogen were determined in the right gastrocnemius in both rest and post-exercise control and 4 week hypoxia acclimated mice. Metabolites were extracted and quantified following standard methods (Bergmeyer, 1974) adapted to a 96-well format for the Spectramax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA). Briefly, muscle samples were powdered using a liquid N_2-cooled mortar and pestle and stored at -80°C prior to analysis. Powdered tissue was subjected to perchloric acid extraction through homogenization in 500 µl of 6% perchloric acid using a motorized homogenizer (PowerGen 125, Fisher Scientific, Whitby, ON). 100 µl of the homogenate was removed and stored at -80°C to determine glycogen content. The remaining homogenate slurry was centrifuged at 10 000 x g for 10 min at 4°C. The supernatant was removed and subsequently neutralized with

K_2CO_3 (3M). Samples were then centrifuged again at 10 000 x g for 10 min at 4°C. The resulting supernatant was used for metabolite quantification.

Final assay conditions in mM were as follow: for ATP and PCr, 20 Tris (pH 8.0), 5 $MgCl_2$, 5 glucose, 2 NAD^+, 1 U/ml glucose-6-phosphate dehydrogenase (G6PDH from *L. mesenteriodes*, Roche Diagnostics), and for PCr only, 2 ADP and 1.5 U/ml Hexokinase (HK from yeast, Roche Diagnostics). Reactions were started with 1U of HK and 2.5 U of creatine phosphokinase (CPK from rabbit muscle, Sigma, Oakville, ON) for ATP and PCr, respectively. Cr levels were determined in 500 triethanolamine (TEA; pH 7.5), 5 ATP, 5 PEP, 0.15 NADH, 5 $MgCl_2$, 1 U/ml lactate dehydrogenase (LDH from rabbit muscle, Roche Diagnostics), 1 U/ml pyruvate kinase (PK from rabbit muscle, Sigma) with the reaction initiated with 2.5 U CPK. For pyruvate: the reaction buffer consisted of 500 TEA and 5 EDTA (pH 7.6), with reaction conditions of 0.15 NADH; the reaction was started with 5 U LDH (from rabbit muscle, Roche Diagnostics). G-6-P levels were assayed in 20 imidazole (pH 7.4), 1 ATP, 0.5 NADP, 5 $MgCl_2$, with the reaction initiated with 1 U G6PDH (Roche diagnostics). For lactate: 2 NAD^+ in Glycine/Hydrazine buffer (pH 9.2, Sigma), with the reaction started with the addition of 8 U LDH (from rabbit muscle, Roche Diagnostics).

For glycogen content, samples were mixed with 50 µl of 1 M K_2HCO_3 and 100 µl of 400 mM acetate buffer (pH 4.8). Half of the mixture was set aside to measure free glucose content. The remaining 125 µl was mixed with 7 µl of amyloglucosidase enzyme (4 U/µl; Roche Diagnostics). All samples were incubated at 40°C for 2hrs. After incubation samples were neutralized with 1M K_2CO_3 and assayed for glucose content. Assay conditions in mM for glucose were: 20 imidazole, 1 ATP, 0.5 NADP, 5 $MgCl_2$,

and 1 U G6PDH (Roche Diagnostics) suspended in TRA buffer (300 TEA-HCl, 4.05 $MgSO_4$, pH 7.5; Roche Diagnostics). The reaction was started with the addition of 1 U HK suspended in TRA buffer (Roche Diagnostics).

Maximal Activity of Cytochrome c Oxidase (Vmax)

Right gastrocnemius muscles from control and acclimated mice (2hrs, 24hrs, 3 days, and 1 week) were powdered using a liquid N_2-cooled mortar and pestle. Approximately 20 mg was homogenized using a glass-on-glass homogenizer on ice for 1 min in 20 volumes of homogenization buffer containing in mM: 100 potassium phosphate (pH 7.2), 5 EDTA, and 0.1% Triton X-100. To determine maximal enzyme activities assays were done in triplicate with background rates determined for each assay at 37°C using a Spectromax Plus 384, 96-well microplate reader (Molecular Devices). Assay conditions in mM for COX were as follows: 100 KH_2PO_4 (pH 7.0), and 0.1 reduced cytochrome c as a substrate.

Statistical Analysis

All statistical analysis was carried out using either the program Sigma Stat 3.5 (Systat Software Inc., San Jose, CA) or JMP 10 (Statistical Analysis Software, Cary, NC). One-way ANOVAs and one-tailed student t-tests were used to test the significance of changes observed in the levels of COX4-1 and COX4-2 mRNA and protein as well as LON and VEGF mRNA. ANCOVAs were implemented including weight as a co-factor when analyzing changes in PDH phosphorylation and muscle metabolite levels in response to exercise. Student t-tests were used when comparing PDH phosphorylation and metabolite levels between acclimation treatments.

Table 1. Primer sequences used for mRNA quantification through real-time PCR in mouse left and right gastrocnemius.

Gene	Forward Primer 5' → 3'	Reverse Primer 5' → 3'
COX4-1[1]	AGTGTTGTGAAGAGTGAAGAC	GCGGTACAACTGAACTTTCTC
COX4-2[1]	GTTGACTGCTACGCCCAGCGC	CCGGTACAAGGCCACCTTCTC
LON	TGCTGGAGGAGGACCACTAC	CATGCCACCAACACTGAAAC
VEGF[2]	ACCCTGGCTTTACTGCTGTACCT	TCATGGGACTTCTGCTCTCCTT
PDK1	CAATGATGTCATTCCCACCA	TTCCAATGTGCTTTCGATGA
PDK2	TCCATCCGAATGCTAATCAA	GTGGCATTGACTTCCTGGAT
PDK4	GCCTTGGGAGAAATGTGTGT	CACTGGCTTTTTGAGTGCAA
18S[3]	TGTGGTGTTGAGGAAAGCAG	TCCCATCCTTCACATCCTTC

[1]Fukuda *et al*., 2007

[2]Leick *et al*., 2009

[3]Templemen *et al*., 2012

Results

Whole animal weights of all individuals used in this study were recorded prior to sampling and found to be significantly different from one another ($p<0.05$; table 2). Normoxic resting controls had an average weight of 35.37 ± 1.42g and hypoxic resting controls had an average weight of 32.88 ± 1.01g (table 2). Normoxic mice exercised at 75% VO_2max had an average body weight of 36.17 ± 1.6g which significantly differed from hypoxic mice exercised at 85% VO_2max (29.78 ± 0.43g; $p<0.05$; table 2). Individuals exercised at 85%VO_2max under normoxic conditions were significantly heavier (38.7 ± 1.73g; $p<0.05$) than their hypoxic counter parts (table 2). Finally, acclimated mice exercised at 75%VO_2max weighed significantly less (32.3 ± 1.02g) than normoxic mice exercised at 85% VO_2max ($p<0.05$; table 2).

Physiological Plasticity of Resting Muscle to Acclimation

Cytochrome c Oxidase Activity (Vmax)

To gain a better understanding of the total amount of COX present within the gastrocnemius muscle as well as changes in total aerobic capacity, I measured the apparent Vmax of COX after acute and chronic hypoxia acclimation (figure 4). Total COX activity increased significantly after 2hrs of acclimation ($p<0.05$) but returned to levels not significantly different from controls by 24hrs ($p>0.05$) and remained unchanged after 1 week of chronic hypoxia (figure 4).

COX4-1 and 4-2 mRNA Expression and Protein Content

Changes in the 4-1 and 4-2 isoforms of COX were assessed with respect to mRNA expression and total protein content (figure 5). A decrease in COX4-1 mRNA levels was

observed after 2hrs of hypoxic exposure which approached significance ($p=0.053$; figure 5 (A)). Levels of COX4-1 mRNA returned to levels not significantly different from controls after 24hrs of acclimation ($p>0.05$; figure 5 (A)). No significant changes in the protein levels of COX4-1 were observed after 1 week of acclimation ($p>0.05$; figure 5 (A)). Further, a longer acclimation at a lower simulated altitude (≈4300m) did not significantly alter the levels of COX4-1 protein ($p>0.05$). Changes in COX4-1 mRNA with acclimation were not accompanied by significant increases in LON protease, at least at the mRNA level ($p>0.05$; figure 6 (A)). There was a trend for COX4-2 mRNA to increase at 2hrs which reached a level significantly higher than controls (≈ 0.5 fold) at 24hrs of acclimation ($p<0.05$; figure 5 (B)). However, although there was a trend for COX4-2 protein levels to decrease at 2hrs and increase again by 3 days of acclimation, it never reached levels significantly different from unacclimated controls ($p>0.05$; figure 5 (B)). In mice acclimated for 2hr, 24hr, 3 days, and 1 week total COX4-1 and COX4-2 protein levels were normalized to total COX activity from the opposite gastrocnemius, used as an indicator of the total amount of COX protein present, of the same mouse (figure 5).

VEGF mRNA Expression

To determine if the gastrocnemius was responsive to the hypoxic exposures, I used a hypoxia responsive gene, VEGF, as a positive control. There was a significant rise in total VEGF mRNA content in the gastrocnemius of mice acclimated to hypobaric hypoxia (figure 6 (B)). After 2hrs of hypoxia acclimation a ≈ 8 fold increase in VEGF mRNA compared to controls was observed ($p<0.05$; figure 6 (B)). Total VEGF mRNA levels decreased after 24hrs of hypoxia acclimation to values not different than those

observed in controls ($p>0.05$; figure 6 (B)).

PDK1, 2, and 4 mRNA Expression

To help assess regulation of muscle metabolic regulation during exercise after chronic hypoxia I measured the mRNA levels of 3 kinases important in regulating the activity of PDH. Levels of PDK1, PDK2, and PDK4 mRNA were determined in the gastrocnemius of control and 4 week acclimated CD-1 mice at rest (figure 7). There were no significant changes in relative mRNA expression for PDK1, PDK2, or PDK4 with 4 weeks of chronic hypoxia acclimation ($p>0.05$; figure 7). There was a trend for PDK4 mRNA to increase in response to 4 weeks of acclimation, however, levels never reached a level statistically different than controls due to high individual variability ($p>0.05$; figure 7).

Total PDH (PDHE1α) and the Degree of PDH Phosphorylation

Total PDH protein content was assessed by Western blot and although the total amount of PDH protein appeared to have a low abundance in hypoxia at 85% VO_2max, there was no significant effect of either acclimation or exercise ($p>0.05$; figure 8 and figure 11). To get a better understanding of changes in the activity of PDH, I measured the degree of phosphorylation, a good indicator of the amount of PDH in the active/inactive form, at each of its phosphorylation sites. After 4 weeks of acclimation there was a significant increase in the degree of PDH phosphorylation in unexercised mice at Ser^{293} (site1; $p<0.05$; figure 9 and figure 11). Although Ser^{232} showed a trend of increasing phosphorylation with acclimation it was determined to be non-significant ($p>0.05$; figure 9 and figure 11).

Muscle Metabolites – Rest

Compared to normoxic controls, acclimation to hypoxia for 4 weeks did not significantly alter the resting levels of ATP (3.49 ± 0.19 vs. 3.64 ± 0.27 µmol/g), PCr (12.40 ± 1.10 vs. 10.53 ± 1.15 µmol/g), Cr (15.82 ± 0.59 vs. 16.49 ± 0.32 µmol/g), G-6-P (1.57 ± 0.28 vs. 1.08 ± 0.22 µmol/g), pyruvate (0.46 ± 0.043 vs. 0.46 ± 0.05 µmol/g), glycogen (10.34 ± 1.80 vs. 8.03 ± 0.61 µmol/g), or lactate (2.60 ± 0.12 vs. 2.60 ± 0.12 µmol/g; $p>0.05$; table 3).

Effect of Phenotypic Plasticity on the Response of Muscle to Exercise

Phosphorylation of Ser^{293}, Ser^{300}, Ser^{232} in Response to Exercise

Exercise at 2 different intensities (75% and 85% VO_2max) did not significantly affect the phosphorylation state at any site regardless of acclimation state ($p>0.05$; figure 10). However, there were trends for phosphorylation to increase at Ser^{293} (site 1) in controls but a decrease in phosphorylation in hypoxia with both exercise intensities ($p>0.05$; figure 10). At Ser^{300} (site 2) and Ser^{232} (site 3) there were trends for a decrease in phosphorylation with exercise in both conditions except at the higher exercise intensity where site 2 showed an increasing trend ($p>0.05$; figure 10).

Muscle Metabolites – Post-Exercise

To better assess the fuel pathways used and mechanisms employed to select said pathways, muscle metabolites were measure and compared in pre and post-exercise mice acclimated to either normoxia or hypoxia.

ATP, Cr, and PCr

Under normoxia and hypoxia, ATP levels were not significantly altered in mice exercised

at either intensity ($p>0.05$; table 3; figure 12). In control mice, however, a trend for decreasing ATP levels was observed in mice exercised at 85% VO_2max compared to rest ($p>0.05$; table 3; figure 12). Cr levels did not significantly change in response to exercise at either intensity, regardless of acclimation state ($p>0.05$; table 3; figure 12). There was, however, a trend for Cr levels to increase at both exercise intensities under hypoxia ($p>0.05$; table 3; figure 12). Furthermore, a trend for PCr levels to decrease was observed in response to exercise in normoxic mice and to increase in hypoxic mice but did not reach levels significantly different than resting controls ($p>0.05$; table 3; figure 12).

G-6-P and Pyruvate

Regardless of acclimation state, there were no significant effects of exercise at either intensity on the levels of G-6-P or pyruvate when compared to rest ($p>0.05$; table 3; figure 12). However, a trend for G-6-P to decrease and to increase was present under normoxia and hypoxia, respectively ($p>0.05$; table 3; figure 12). The levels of G-6-P in normoxia acclimated mice exercised at 85% VO_2max (0.59 ± 0.13 µmol/g) were found to be significantly different from those in hypoxic mice exercised at 85% VO_2max (1.46 ± 0.22 µmol/g; $p<0.05$; table 3; figure 12). Pyruvate levels showed a trend to decrease in response to exercise under normoxia and to increase under hypoxia, but never reached levels significantly different from their respective resting controls ($p>0.05$; table 3; figure 12).

Lactate and Glycogen

A trend for lactate levels to increase in response to exercise at both intensities was present under normoxia and hypoxia ($p>0.05$; table 3; figure 13). However, lactate only reached

levels significantly higher than resting controls in hypoxic mice exercised at 85% VO_2max (6.99 ± 1.40 μmol/g; $p<0.05$; table 3; figure 13). Although there was a trend for glycogen levels to decrease in mice exercised at both intensities regardless of acclimation state they did not reach a point significantly different than resting controls ($p>0.05$; table 3; figure 13).

Lactate/Pyruvate

To better assess the extent of anaerobic metabolism I measured the ratio of lactate/pyruvate. The ratio of lactate/pyruvate showed a trend to increase in response to exercise, under both hypoxia and normoxia but did not reach levels significantly different than controls ($p>0.05$; figure 14).

PCr/ATP

To gain a better understanding of the flux through the glycolytic pathway, I measured the ratio of PCr/ATP. Under normoxia, the ratio of PCr/ATP decreased significantly ($p=0.05$) in mice exercised at 75% VO_2max but remained unchanged in mice exercised at 85% VO_2max ($p>0.05$; figure 15). Under hypoxia, there were no observable effects of exercise at either intensity on the PCr/ATP ratio ($p>0.05$; figure 15).

Table 2. Whole animal weight (g) of male CD-1 mice acclimated to hypoxia or normoxia and exercised at 75% or 85% VO_2max.

	Normoxia			Hypoxia		
	Rest	**75% VO_2max**	**85% VO_2max**	**Rest**	**75% VO_2max**	**85% VO_2max**
Weight (g)	35.37 ± 1.42 a,b,c	36.17 ± 1.6 a,b	38.7 ± 1.73 a	32.88 ± 1.01 a,b,c	32.3 ± 1.02 b,c	29.78 ± 0.43 c

Values are expressed as mean ± SEM. Means not connected by the same letter are significantly different from one another (ANOVA; $p<0.05$).

Table 3. Levels of metabolites (µmol/g wet weight) in the right gastrocnemius of male CD-1 mice acclimated to hypoxia or normoxia and exercised at 75% or 85% VO_2max.

	Normoxia			Hypoxia		
	Rest	**75% VO_2max**	**85% VO_2max**	**Rest**	**75% VO_2max**	**85% VO_2max**
ATP	3.49 ± 0.19	3.64 ± 0.27	2.96 ± 0.3	3.70 ± 0.31	3.76 ± 0.31	3.48 ± 0.23
PCr	12.40 ± 1.10	8.05 ± 1.42	10.39 ± 0.42	10.53 ± 1.15	13.03 ± 1.58	12.54 ± 1.02
Cr	15.82 ± 0.59	17.22 ± 0.46	15.07 ± 1.38	16.49 ± 0.32	19.98 ± 1.88	17.89 ± 0.67
Pyruvate	0.46 ± 0.043	0.44 ± 0.03	0.37 ± 0.08	0.46 ± 0.05	0.54 ± 0.13	0.45 ± 0.11
G-6-P	1.57 ± 0.28	1.24 ± 0.36	0.59 ± 0.13#	1.08 ± 0.22	1.06 ± 0.15	1.46 ± 0.22#
Lactate	2.60 ± 0.12	3.74 ± 0.97	4.74 ± 0.80	2.62 ± 0.49	3.45 ± 1.0	6.99 ± 1.40*
Glycogen	10.34 ± 1.80	6.94 ± 1.39	3.04 ± 0.68	8.03 ± 0.61	6.82 ± 0.68	5.24± 1.59

Values are expressed in µmol/g wet weight ± SEM. * indicates a significant change from rest (ANCOVA; $p<0.05$) and # indicates a difference between treatments (t-test; $p<0.05$).

Figure 4. The maximal enzyme activity (Vmax) for cytochrome c oxidase (COX) in the left gastrocnemius of mice acclimated to hypobaric hypoxia for 0hrs (normoxia), 2hrs (2Hr), 24hrs (24Hr), 3 days (3 Day), and 1 week (1 week). COX activity (Vmax) in the left gastrocnemius of mice acclimated to hypobaric hypoxia for 0hrs (Normoxia), 2hrs (2Hr), 24hrs (24Hr), 3 days (3 Day), and 1 week (1 week). White bars represent normoxic controls, light grey bars represent 2hrs of hypoxia acclimation, black bars represent 24hrs of hypoxia acclimation, dark grey bars represent 3 days of hypoxia acclimation, and cross-hatched grey bars represent 1 week of hypoxia acclimation. * indicates a significant deviation from rest (ANOVA; $p<0.05$). Values are expressed as mean ± SEM.

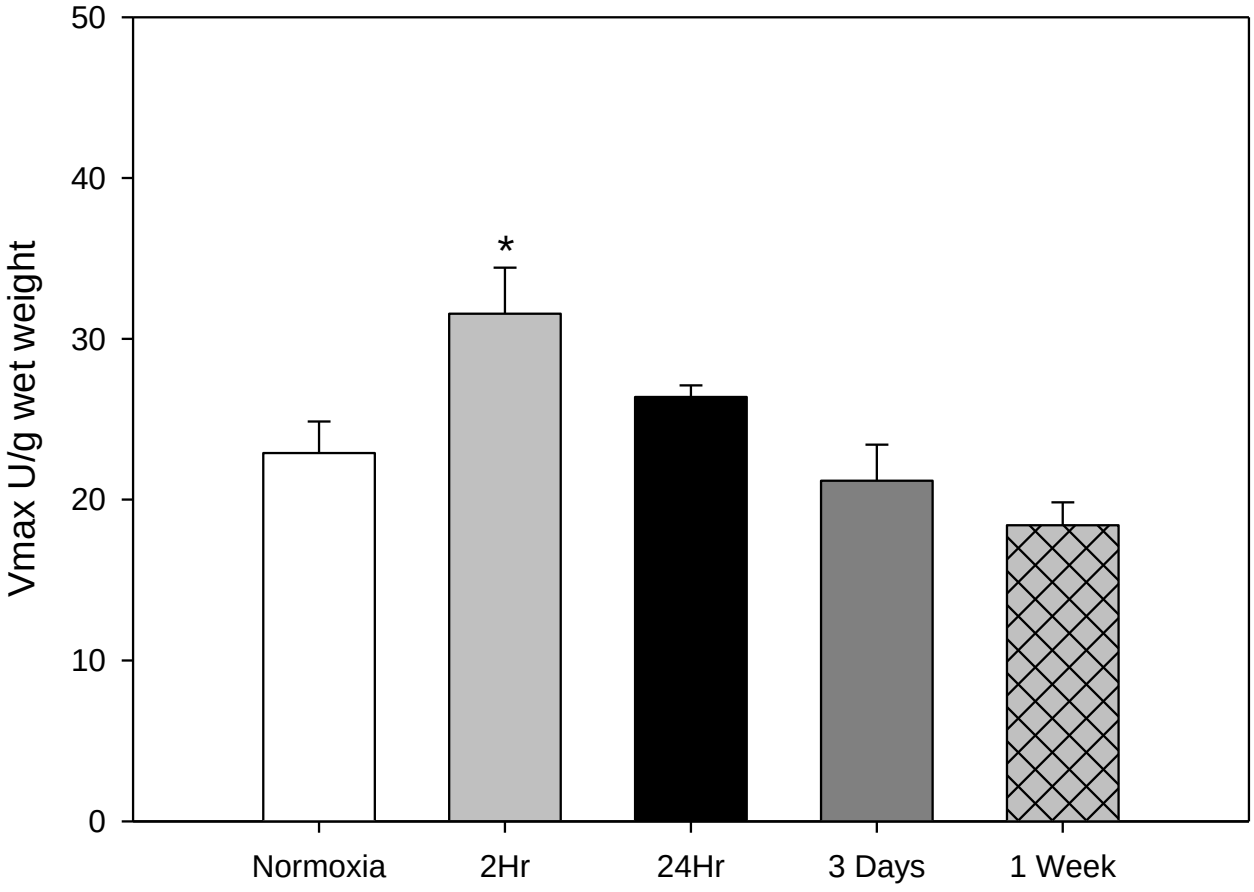
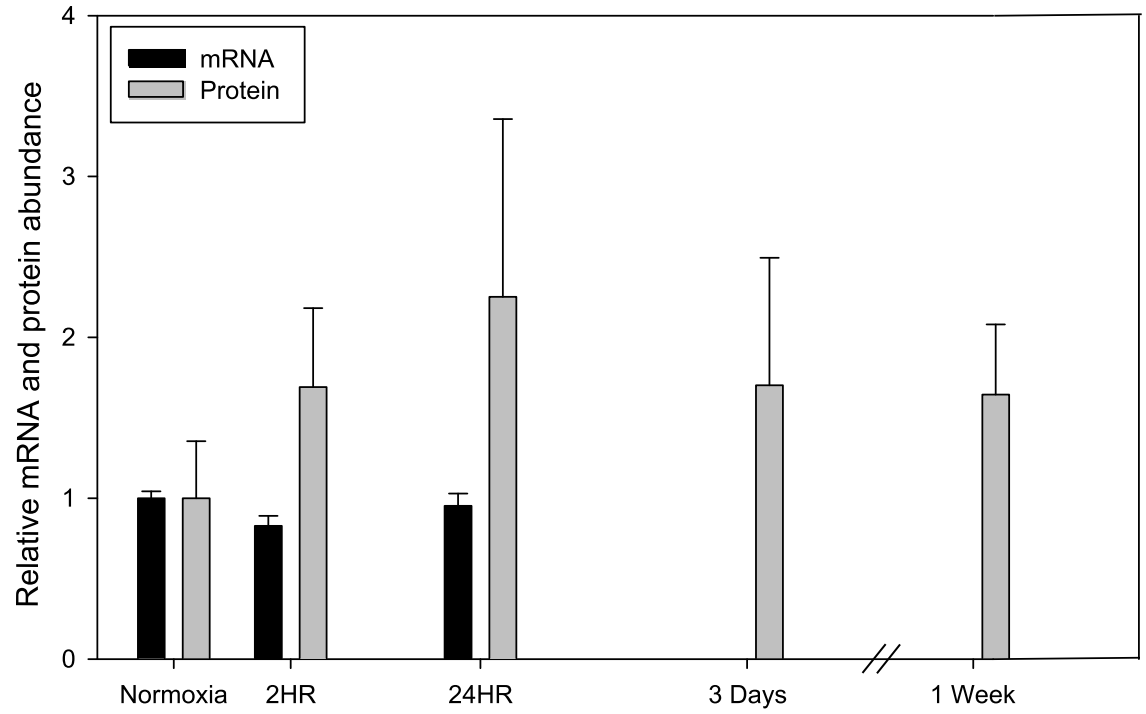
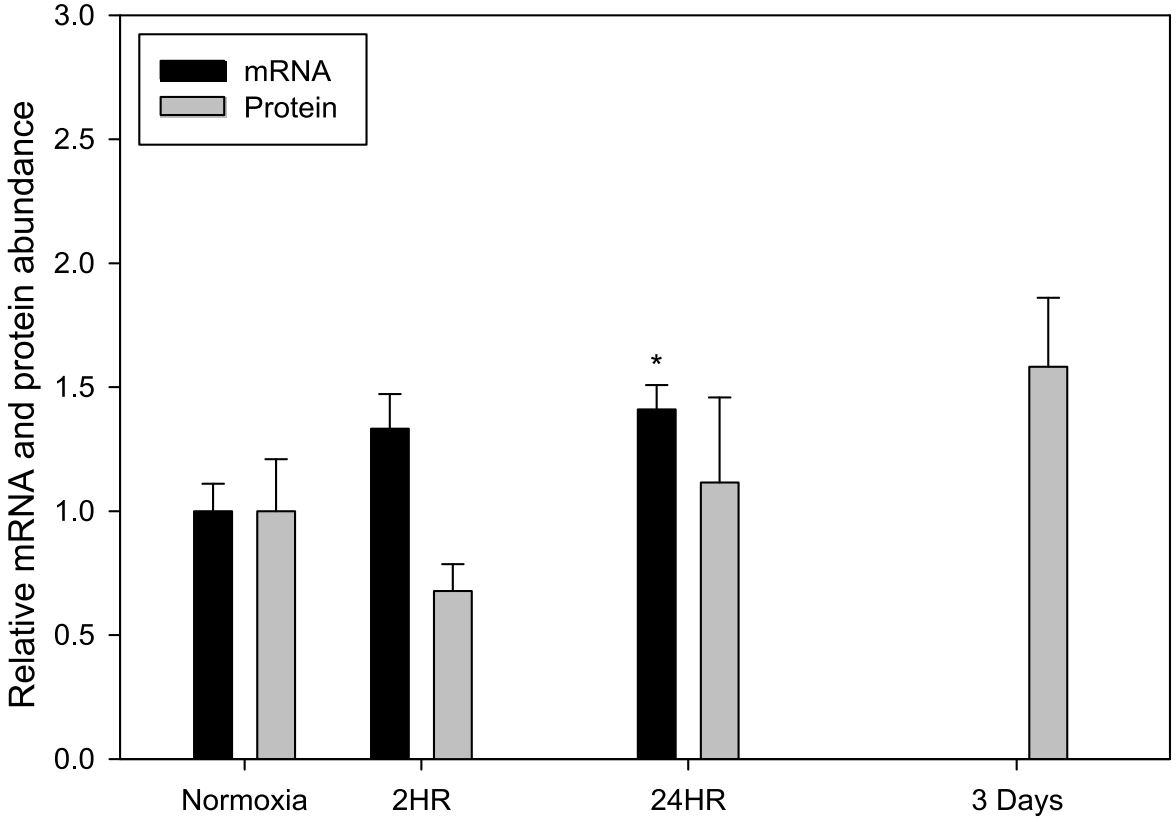

50
40
30
20
10
0
Vmax U/g wet weight
*
Normoxia
2Hr
24Hr
3 Days
1 Week

Figure 5. Real-time PCR expression data and protein content for COX4-1 and COX4-2 in the left gastrocnemius of female CD-1 mice acclimated to 0hr (n=6), 2hrs (n=6), 24hrs (n=6), 3 days (n=6), 1 week (n=6). mRNA expression and protein levels for (A) cytochrome c oxidase subunit 4 isoform 1 (COX4-1; mol. weight ≈ 16 kDa) after 2hrs, 24hrs, 3 days, and 1 week of acclimation, and (B) cytochrome c oxidase subunit 4 isoform 2 **(**COX4- 2; mol. weight ≈ 20 kDa) after 2hrs, 24hrs, and 3 days of acclimation. (C) Representative Western blot images for COX4-1 and COX 4-2. Black bars represent mRNA expression and grey bars represent protein abundance. Protein levels are normalized to total COX activity (Vmax) and mRNA expression is normalized to 18S ribosomal mRNA. Data is standardized to normoxic controls and presented as means ± SEM. * indicates a significant change from control (ANOVA; $p<0.05$).

(A)


mRNA
Protein
Relative mRNA and protein abundance
0
1
2
3
4
Normoxia
2HR
24HR
3 Days
1 Week

(B)


mRNA
Protein
Relative mRNA and protein abundance
0.0
0.5
1.0
1.5
2.0
2.5
3.0
*
Normoxia
2HR
24HR
3 Days

(C)

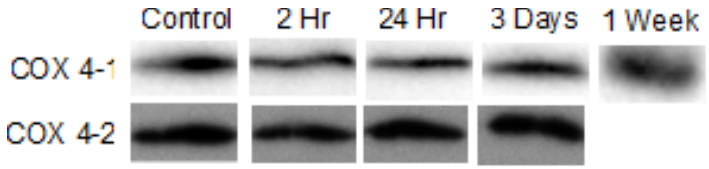
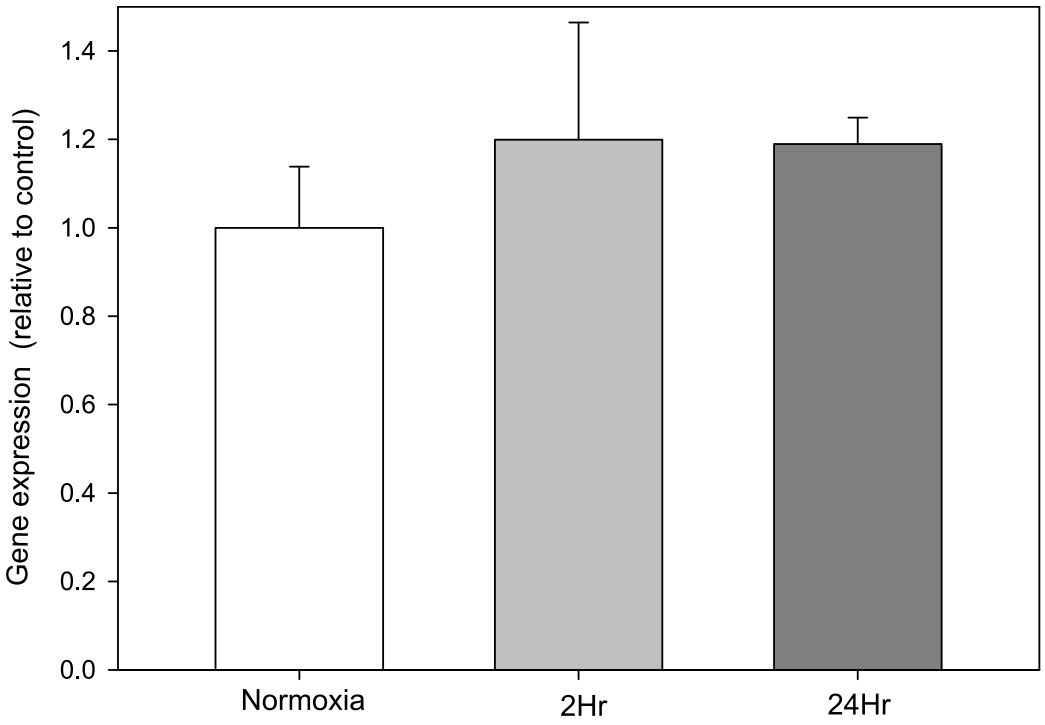


Figure 6. Real-time PCR gene expression for LON protease, and VEGF in the left gastrocnemius of female CD-1 mice acclimated to 0hr (n=6), 2hrs (n=6), and 24hrs (n=6) of hypobaric hypoxia (≈ 6500 m.a.s). mRNA levels expressed against 18S ribosomal mRNA and normalized to control values for (A) LON protease (LON), and (B) vascular endothelial growth factor (VEGF). White bars indicate hypoxic rest, light grey bars indicate 2hrs of hypoxic acclimation, and dark grey bars indicate 24hrs of hypoxic acclimation. Value are expressed as means ± SEM. * indicates a significant difference from control (ANOVA; $p<0.05$).

(A) LON


Gene expression (relative to control)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Normoxia
2Hr
24Hr

(B) VEGF

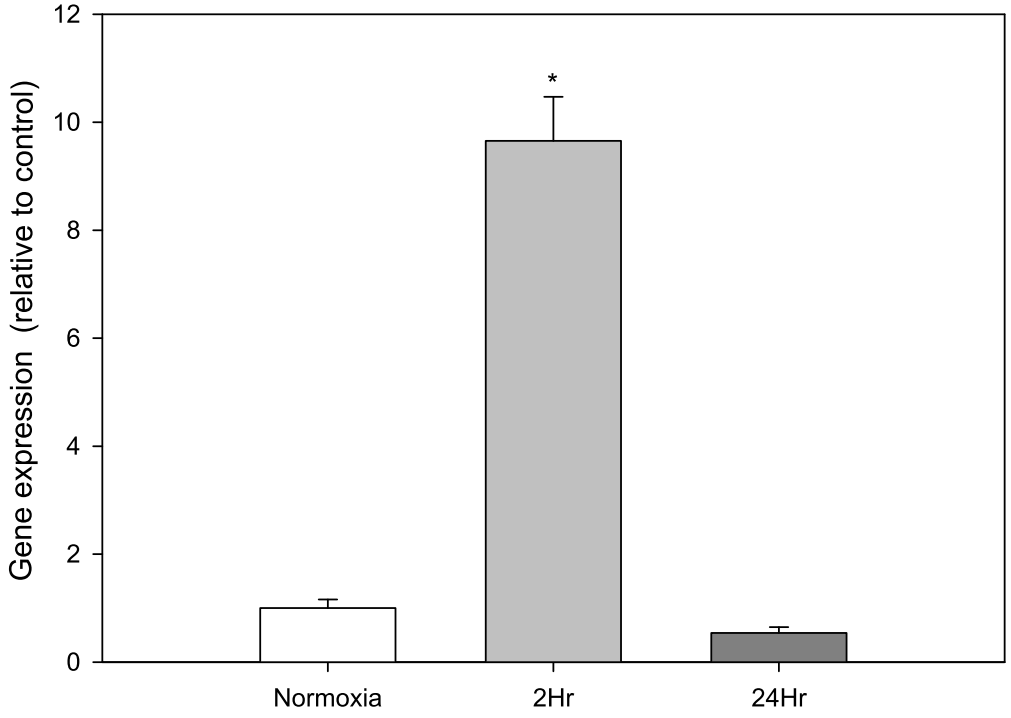

Gene expression (relative to control)
12
10
8
6
4
2
0
*
Normoxia
2Hr
24Hr

Figure 7. Real-time PCR gene expression data for pyruvate dehydrogenase kinase 1, 2, and 4 (PDK1, 2, and 4) in the right gastrocnemius of male CD-1 mice acclimated to normoxia (n=5) or hypobaric hypoxia (n=4; 4300 m.a.s.) for 4 weeks. mRNA levels expressed against 18S ribosomal mRNA normalized to control values for pyruvate dehydrogenase kinase 1, 2, and 4 (PDK1, 2, and 4) in the right gastrocnemius of male CD-1 mice acclimated to normoxia (n=5) or hypobaric hypoxia (n=4; 4300 m.a.s.). Black bars represent normoxic controls and grey bars represent hypoxia acclimated mice. Values are expressed as means ± SEM.

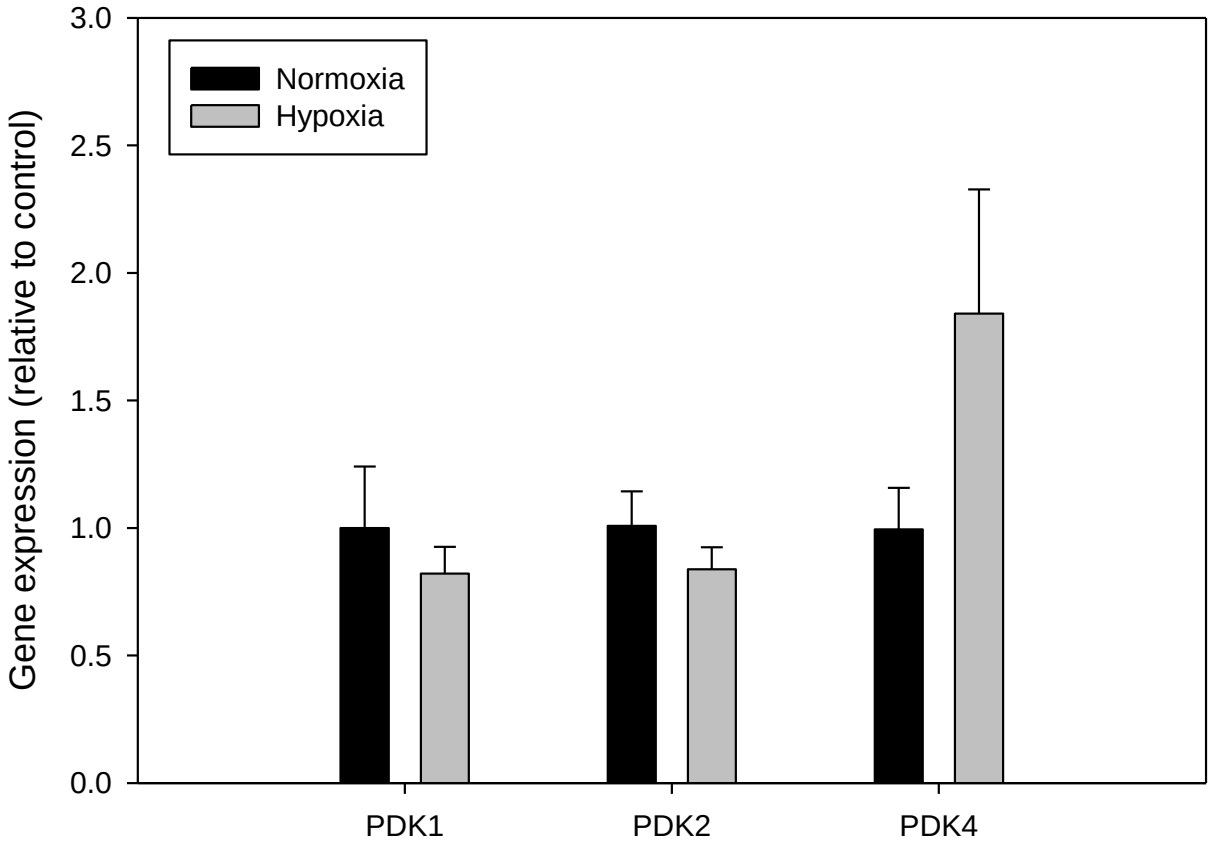

Normoxia
Hypoxia
Gene expression (relative to control)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
PDK1
PDK2
PDK4

Figure 8. Total pyruvate dehydrogenase (PDHE1α) protein levels in the right gastrocnemius of resting and post-exercise male CD-1 mice acclimated to normoxia or hypobaric hypoxia (4300 m.a.s.) for 4 weeks. Total protein content for total pyruvate dehydrogenase (PDHE1α) in the right gastrocnemius of post-exercise mice acclimated to normoxia or hypobaric hypoxia exercised at 75% (n=7-8) and 85% (n=5) VO_2max. White bars represent resting controls, black bars represent 75% VO_2max, and grey bars represent 85% VO_2max. Values are standardized to normoxic resting controls and presented as means ± SEM.

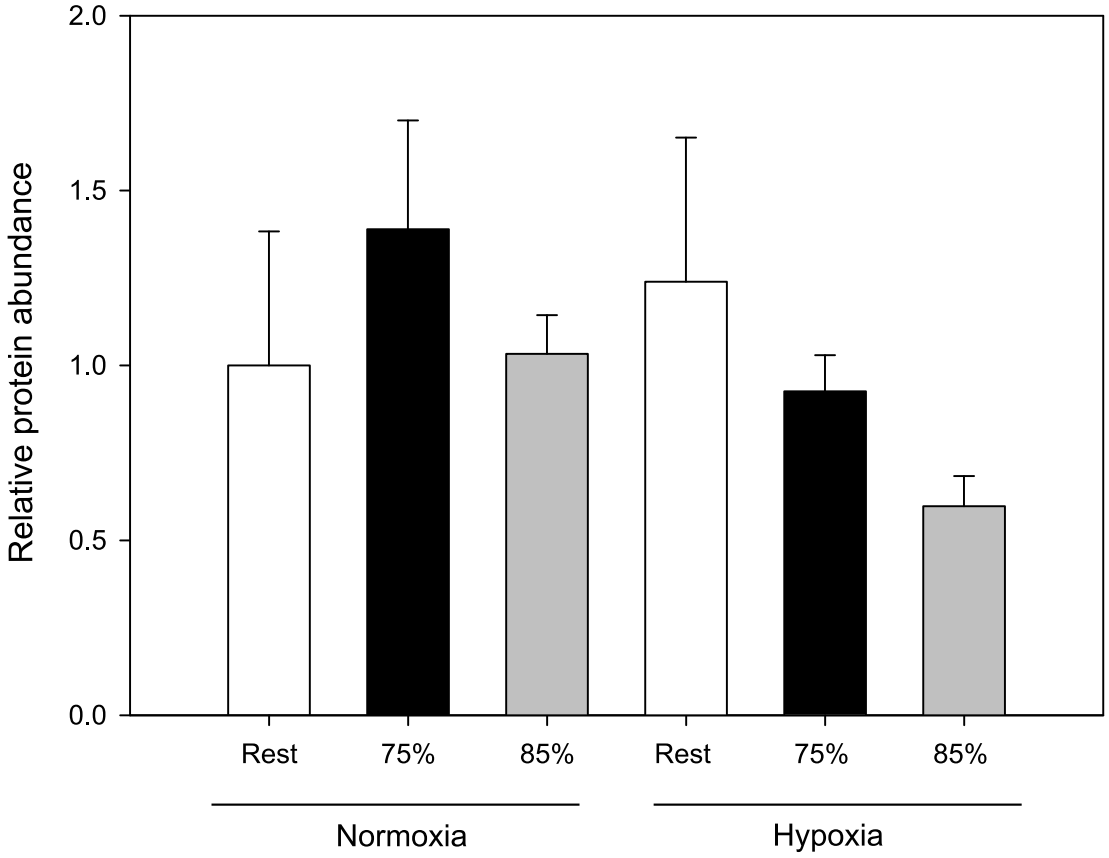

2.0
1.5
1.0
0.5
0.0
Relative protein abundance
Rest
75%
85%
Rest
75%
85%
Normoxia
Hypoxia

Figure 9. The degree of phosphorylation measured through total protein content for the three phosphorylation sites of pyruvate dehydrogenase (PDH; Ser^{293}, Ser^{300}, and Ser^{232}) expressed against total PDH content and standardized to controls (normoxic rest). Degree of phosphorylation measured through total protein content for Ser^{293} (mol. weight ≈ 43 kDa) Ser^{300} (mol. weight ≈ 43 kDa), and Ser^{232} (mol. weight ≈ 43 kDa) in the right gastrocnemius of resting mice acclimated to normoxia (n=7-8) and hypobaric hypoxia (≈ 4300 m.a.s.; n=6-7). Black bars represent normoxic mice, and grey bars represent hypoxic mice. Values are normalized to total PDH content (PDHE1α; mol. weight ≈ 43 kDa) and standardized to controls. Data is presented as means ± SEM. * represents a significant difference between treatments (t-test; $p<0.05$).

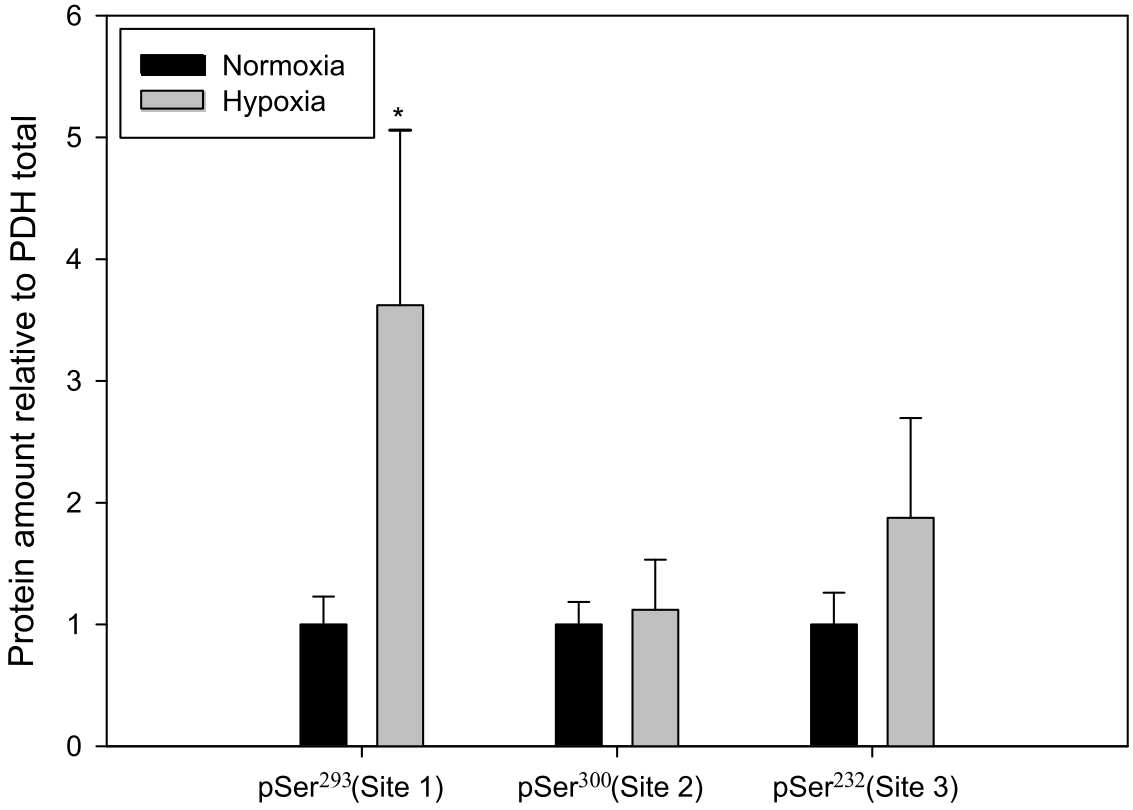

Normoxia
Hypoxia
*
Protein amount relative to PDH total
0
1
2
3
4
5
6
pSer293(Site 1)
pSer300(Site 2)
pSer232(Site 3)

Figure 10. The degree of phosphorylation measured through total protein content for the three phosphorylation sites of PDH (Ser^{293}, Ser^{300}, and Ser^{232}) expressed against total PDH content and standardized to controls (normoxic rest). The degree of phosphorylation measured through total protein content at (A) site 1, Ser^{293} (mol. weight ≈ 43 kDa), (B) site 2, Ser^{300} (mol. weight ≈ 43 kDa), and (C) site 3, Ser^{232} (mol. weight ≈ 43 kDa) in the right gastrocnemius of post-exercise mice acclimated to normoxia or hypobaric hypoxia exercised at 75% (n=7-8) and 85% (n=5) VO_2max. Black bars represent 75% VO_2max and grey bars represent 85% VO_2max. Values normalized to total PDH content (mol. weight ≈ 43 kDa) and expressed as the fold change from resting controls (0 mark). Data is presented as means ± SEM.

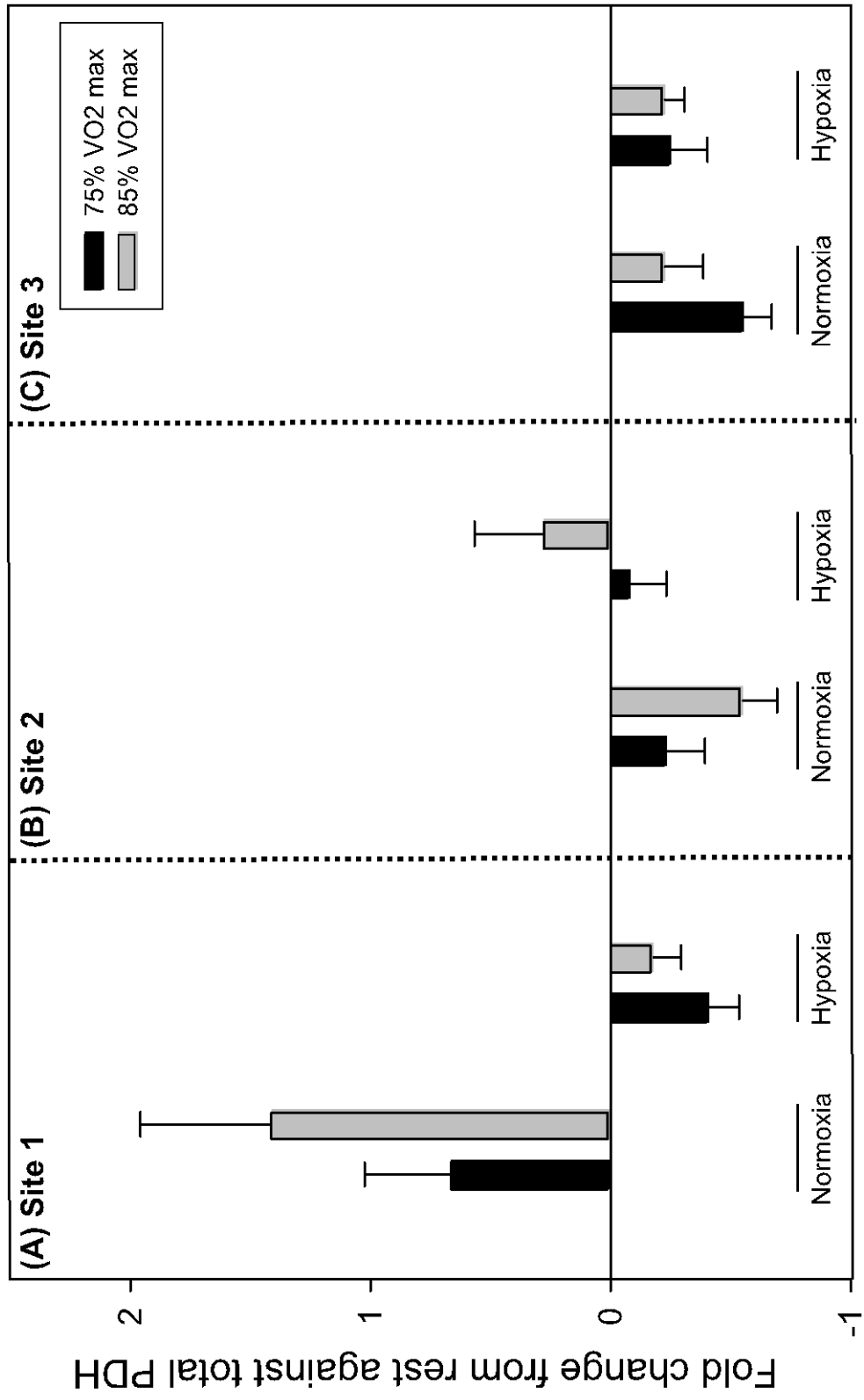

(A) Site 1
(B) Site 2
(C) Site 3
75% VO2 max
85% VO2 max
Normoxia
Hypoxia
Normoxia
Hypoxia
Normoxia
Hypoxia
Fold change from rest against total PDH
2
1
0
-1

Figure 11. Representative Western blots for the three phosphorylation sites of PDH (Ser^{293}, Ser^{300}, and Ser^{232}) and total pyruvate dehydrogenase (PDHE1α). Western blot images of total protein content for total pyruvate dehydrogenase (PDHE1α) and its three phosphorylation sites (Ser^{293}, Ser^{300}, Ser^{232}) in the right gastrocnemius of post-exercise mice acclimated to normoxia or hypobaric hypoxia exercised at 75% (n=7-8) and 85% (n=5) VO_2max.

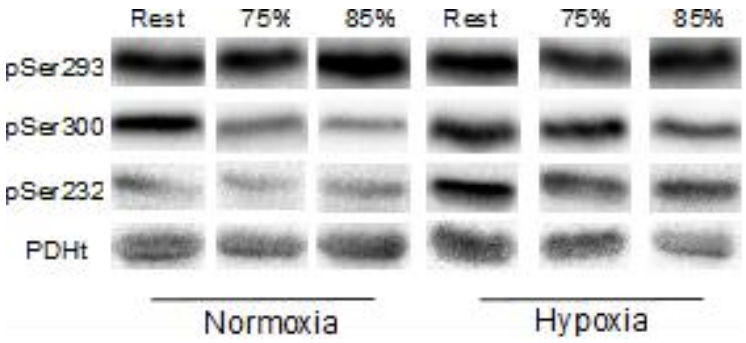

Rest
75%
85%
Rest
75%
85%
pSer293
pSer300
pSer232
PDHt
Normoxia
Hypoxia

Figure 12. Total ATP, PCr, Cr, pyruvate, and G-6-P content in the right gastrocnemius of resting and post-exercise male CD-1 mice acclimated to normoxia or hypobaric hypoxia (4300 m.a.s.) for 4 weeks. Total ATP, PCr, Cr, pyruvate, and G-6-P in the right gastrocnemius of resting mice acclimated to normoxia (n=5) and hypobaric hypoxia (n=5) and post-exercise mice acclimated to normoxia (n=5-6) or hypobaric hypoxia (n=5) exercised at 75% and 85% VO_2max. Black bars represent 75% VO_2max and grey bars represent 85% VO_2max. Values are presented as mean fold difference from resting controls (0 mark) ± SEM. # indicates a significant difference between treatments (t-test; $p<0.05$).

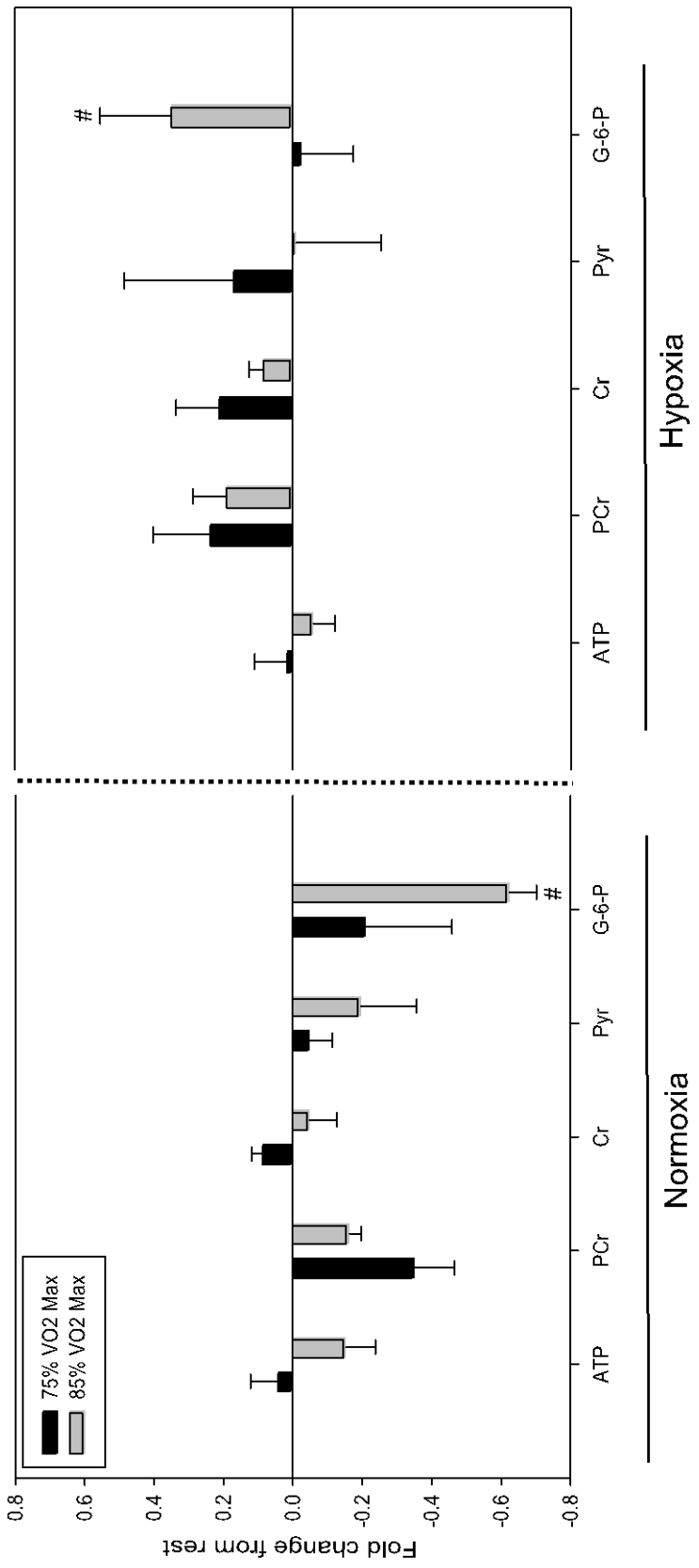

Fold change from rest
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
75% VO2 Max
85% VO2 Max
ATP
PCr
Cr
Pyr
G-6-P
#
Normoxia
Hypoxia

Figure 13. Total lactate and glycogen content in the right gastrocnemius of resting and post-exercise male CD-1 mice acclimated to normoxia or hypobaric hypoxia (4300 m.a.s.) for 4 weeks. Total glycogen and lactate content in the right gastrocnemius of resting mice acclimated to normoxia (n=5) and hypobaric hypoxia (n=5) and post-exercise mice acclimated to normoxia (n=5-6) or hypobaric hypoxia (n=5) exercised at 75% and 85% VO_2max. Black bars represent 75% VO_2max and grey bars represent 85% VO_2max. Values are presented as mean fold difference from resting controls ± SEM. * indicates a significant deviation from rest (ANCOVA; $p<0.05$).

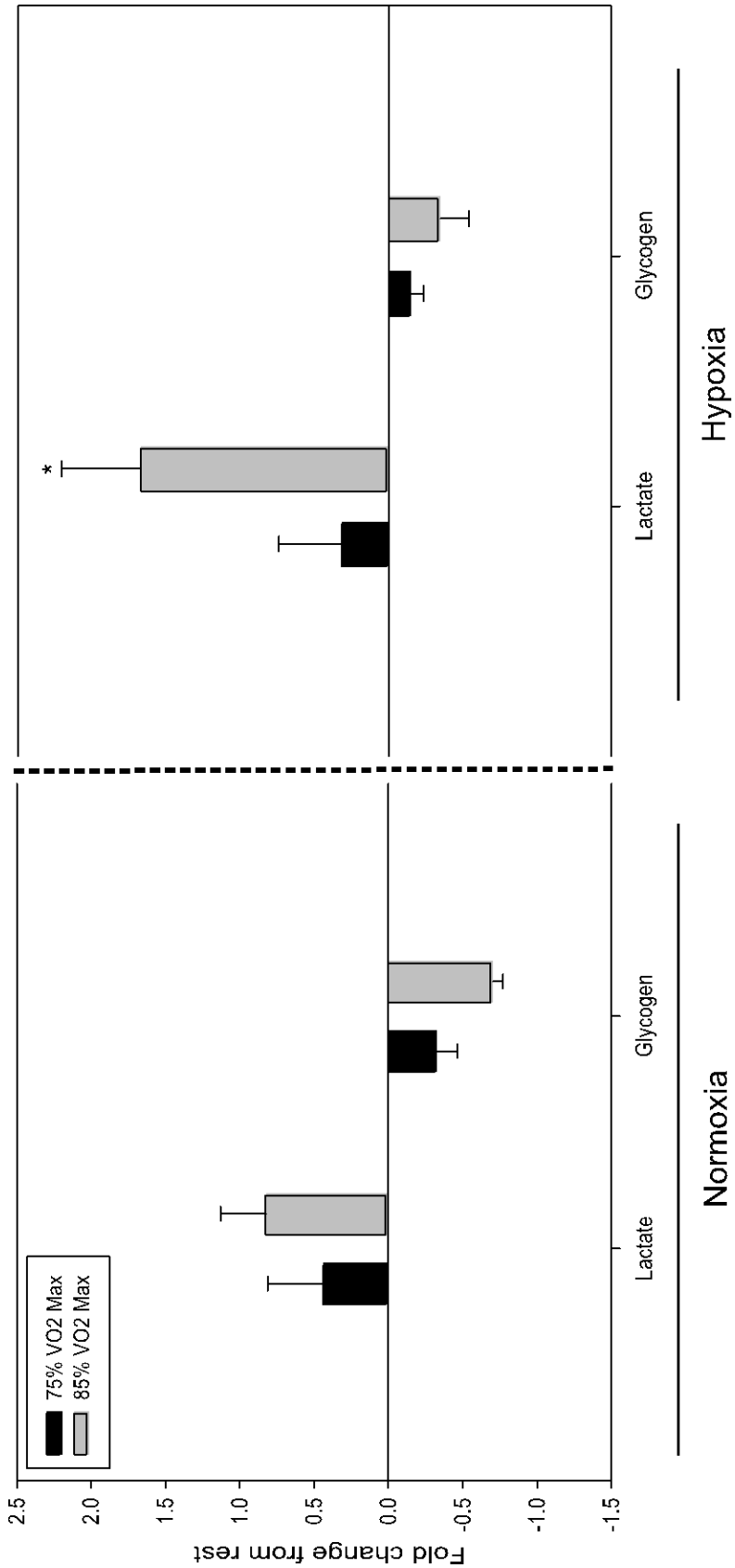

Fold change from rest
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
75% VO2 Max
85% VO2 Max
*
Lactate
Glycogen
Lactate
Glycogen
Normoxia
Hypoxia

Figure 14. The ratio of total lactate and pyruvate content in the right gastrocnemius of resting and post-exercise male CD-1 mice acclimated to normoxia or hypobaric hypoxia (4300 m.a.s.) for 4 weeks. The ratio of total lactate and pyruvate in the right gastrocnemius of resting mice acclimated to normoxia (n=5) and hypobaric hypoxia (n=5) and post-exercise mice acclimated to normoxia (n=5-6) or hypobaric hypoxia (n=5) exercised at 75% and 85% VO_2max. White bars represent resting controls, black bars represent 75% VO_2max, and grey bars represent 85% VO_2max. Values are presented as means ± SEM.

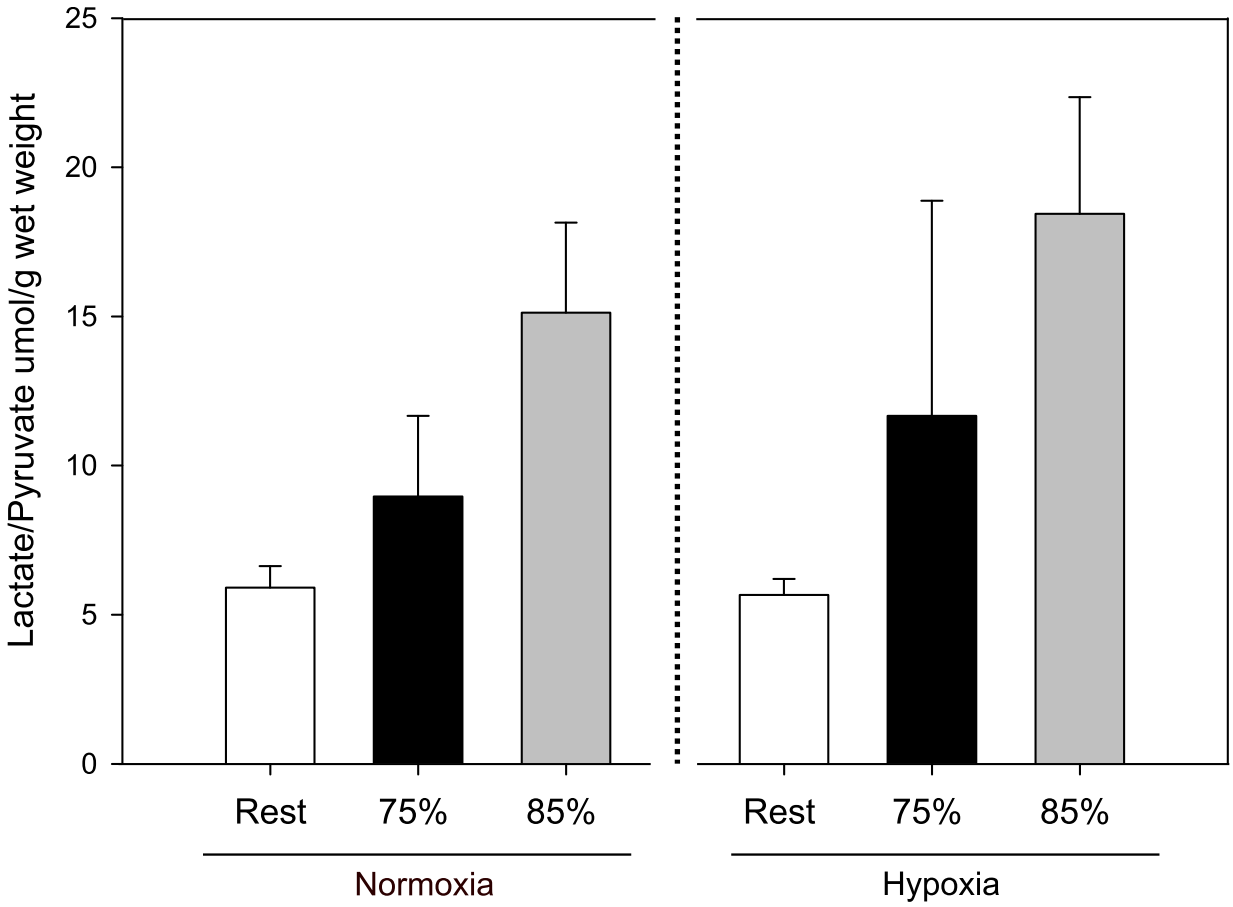
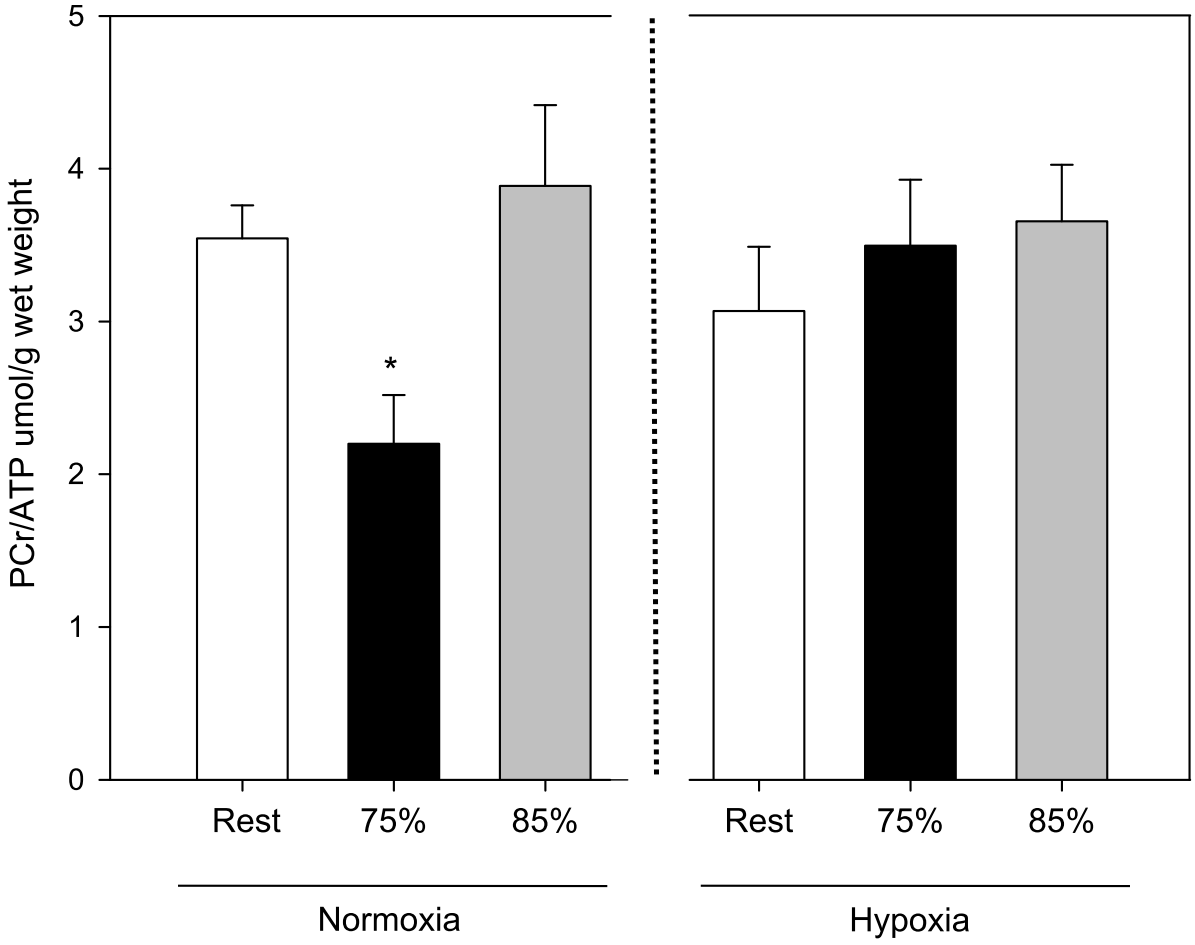

Lactate/Pyruvate umol/g wet weight
25
20
15
10
5
0
Rest
75%
85%
Normoxia
Rest
75%
85%
Hypoxia

Figure 15. The ratio of total PCr and ATP content in the right gastrocnemius of resting and post-exercise male CD-1 mice acclimated to normoxia or hypobaric hypoxia (4300 m.a.s.) for 4 weeks. The ratio of total PCr and ATP in the right gastrocnemius of resting mice acclimated to normoxia (n=5) and hypobaric hypoxia (n=5) and post-exercise mice acclimated to normoxia (n=5-6) or hypobaric hypoxia (n=5) exercised at 75% and 85% of their VO_2max. White bars represent resting controls, black bars represent 75% VO_2max, and grey bars represent 85% VO_2max. Values are presented as means ± SEM. * indicates a significant difference from resting control (ANCOVA; $p<0.05$).


PCr/ATP umol/g wet weight
5
4
3
2
1
0
*
Rest
75%
85%
Rest
75%
85%
Normoxia
Hypoxia

Discussion

In the present study, I explored the plastic response of muscle phenotype to hypoxia to determine if acclimation results in a change in the machinery used for energy production. Specifically, I investigated the abundance of subunit IV isoforms, 4-1 and 4-2, of COX and the phosphorylation state of PDH at each of its phosphorylation sites. I hypothesized because cell culture studies have observed an increase in the 4-2 isoform and a decrease in the 4-1 isoform of COX in hypoxic conditions (Fukuda *et al*., 2007), that there would be a change in the abundance of COX isoforms after hypoxia acclimation in mice, acting as a strategy to increase the efficiency of ATP production. In my investigation I have provided some support for this hypothesis in the gastrocnemius of mice under chronic hypoxia. However, the changes observed were much more subtle than those previously observed in cell culture (Fukuda *et al*., 2007). Further, I hypothesized that the degree of PDH phosphorylation would be similar in resting control and acclimated mice, suggesting a similar fuel use strategy. However, in my study I provided evidence that the degree of PDH phosphorylation was significantly different in the gastrocnemius of resting control and acclimated mice.

I also set out to determine if phenotypic changes in skeletal muscle during acclimation resulted in any changes in muscle metabolism and fuel selection during acute exercise. I accomplished this through examining the phosphorylation state of PDH as an indicator of the capacity for aerobic glucose use as well as the post-exercise changes in various metabolites as possible indicators of the fuel pathways being activated in the muscle. I hypothesized that relative post-exercise changes in metabolites would be similar, suggesting a similar mechanism of fuel selection between control and acclimated

mice. However, the post-exercise levels of metabolites differed between acclimation groups, suggesting either a difference in muscle recruitment or in the regulation of fuel use at the muscle fiber level. Furthermore, I hypothesized that PDH would be phosphorylated to similar extents in both control and acclimated post-exercise mice. In my investigation, no significant changes in PDH phosphorylation with exercise in either acclimation group were observed.

Mitochondrial Oxidative Capacity and COX Isoform Expression

The results obtained from real-time PCR analysis show that there was an increase in amount of COX4-2 mRNA in response to hypoxia acclimation, relative to normoxic controls (figure 5). The increase in VEGF mRNA observed over the same time period confirms cellular hypoxia and a HIF mediated response was elicited (figure 6). These changes in mRNA levels suggest that there may be an increase in the abundance of the COX4-2 subunit under hypoxic conditions. There was, however, no significant change in levels of COX4-2 or COX4-1 protein present after acclimation to hypoxia (figure 5). The small changes in mRNA expression observed may not have translated to measureable differences in protein levels through Western blot analysis. Interestingly, there was a rise in total COX enzymatic activity (Vmax) at 2hrs of acclimation (figure 4). The activity of COX is known to be affected by various factors such as enzyme concentration, the binding of allosteric effectors such as ATP, and enzyme subunit composition (Fukuda *et al*., 2007; Ludwig *et al*., 2001; Suarez *et al*., 2005). An ATP binding site is found on the matrix side of the COX4 subunit and as the ATP/ADP ratio increases, the binding of ATP to this site results in a decrease in COX activity (reviewed in Ludwig *et al*., 2001). However, the majority of support for this mechanism has been found in heart tissue with

little evidence in other tissues (reviewed in Ludwig *et al*., 2001; Frank and Kadenbach, 1996). Furthermore, there is evidence for a phosphorylation site on the COX4 subunit, but whether the phosphorylation/dephosphorylation of this site affects activity remains to be fully understood (reviewed in Ludwig *et al*., 2001). It has been suggested that the cAMP-dependent phosphorylation and Ca^{2+} activated dephosphorylation of this site makes the allosteric control by ATP possible (reviewed in Ludwig *et al*., 2001). Also, as previously discussed, it has been shown that the 4-2 isoform of COX increases during hypoxic stress (Fukuda *et al*., 2007). Since previous studies have found that the turnover rate of O_2 to H_2O increases when the 4-2 isoform is in the majority, the observed increase in COX activity may be due to an increase in the COX 4-2 isoform (Poyton *et al*., 1988; Waterland *et al*., 1991). However, activity was not maintained at an elevated level throughout the acclimation period, returning to levels comparable to controls at 1 week (figure 4). Furthermore, the changes in mRNA expression observed in this study were notably smaller than those observed in cell culture experiments (Fukuda *et al*., 2007). This rapid and subtle response may have been due to an up regulation of other HIF-1 regulated genes, such as erythropoietin (EPO) and VEGF, increasing O_2 delivery to the muscle and thus reducing cellular hypoxia (Ramirez *et al*., 2007; Semenza, 2007; Shweiki *et al*., 1992). EPO is a glycoprotein hormone/growth factor which controls the production of erythrocytes (Jelkmann, 1992). During acute hypoxia, rises in HIF-1 protein have been shown to cause a rise in EPO mRNA transcripts and thus protein content (Forsythe *et al*., 1996; Schuster *et al*., 1989). This rise in EPO leads to a greater rate of erythropoiesis and subsequently a higher O_2 carrying capacity of the blood (Forsythe *et al*., 1996; Schuster *et al*., 1989). VEGF is a growth factor responsible for

stimulating angiogensis/arteriogensis within the cell, leading to an increase in capillary formation and hence blood flow (Breen *et al*., 2008; Ramirez *et al*., 2007; Shweiki *et al*., 1992). The increase in VEGF mRNA observed (figure 5) may represent an increase in VEGF protein levels and an increase in the rate of vessel growth as seen with hypoxia exposure in birds (Mathieu-Costello *et al*., 1998; Semenza, 2007; Shweiki *et al*., 1992). This increase in vessel growth may have increased blood perfusion to the hypoxic tissue (Mathieu-Costello *et al*., 1998; Semenza, 2007; Shweiki *et al*., 1992). Increased blood flow and red blood cell formation could have increased the supply of O_2 and nutrients to the hypoxic area, thus decreasing the hypoxic stress created by the environment (Semenza, 2007; Shweiki *et al*., 1992). In addition to these molecular changes, behavioral changes have also been observed to be present in mammals subjected to acute hypoxia (Gordon and Fogelson, 1991). When subjected to acute hypoxia mammals have been noted to increase their breathing rate, allowing the lungs to take up more O_2 from the environment (Gordon and Fogelson, 1991). Furthermore, mammals, such as mice and rats, have been observed to decrease their set point for body temperature, allowing them to maintain a lower than normal core body temperature and thus decrease their overall metabolism (Gordon and Fogelson, 1991). Thus, some of the aforementioned molecular and behavioral changes may have been implemented, allowing the skeletal muscle to remain normoxic even while being subjected to hypobaric hypoxia, thus decreasing the need to adjust metabolic machinery.

Mitochondrial Capacity for Pyruvate Oxidation (PDH Phosphorylation)

To asses muscle capacity for the phosphorylation of PDH I measured the mRNA levels of PDK1, 2, and 4. Changes in the levels of PDK mRNA and protein have

previously been shown to be indicative of changes in total PDK activity (Wu *et al*., 1998). Therefore, PDK mRNA levels can serve as accurate predictors of PDK activity. Acclimation to hypobaric hypoxia for 4 weeks resulted in no significant changes in the mRNA levels of PDK1, 2, or 4 (figure 7). The increasing trend in PDK4 mRNA expression may indicate a higher level of PDK4 activity and potentially a greater level of PDH phosphorylation, leading to decrease in PDH activity (Rardin *et al*., 2009). Upon further examination, the degree of PDH phosphorylation at site 1, Ser^{293} was found to be significantly elevated in resting mice acclimated to chronic hypoxia (figure 9). This increase in phosphorylation suggests that the activity of any of the PDK isoforms may have increased (Korotchkina and Patel, 2001; Papandreou *et al*., 2006). The increase in phosphorylation observed at Ser^{293} was not mirrored at all phosphorylation sites (figure 9). However, it has been previously documented that the activity of PDH can be affected through changes in the phosphorylation state of only a single phosphorylation site (Le Moine *et al*., 2011). The observed increase in the degree of phosphorylation suggests that total PDH activity has decreased and the flux of acetyl-CoA derived from CHOs has been reduced (Denyer *et al*., 1991; Rardin *et al*., 2009; Wieland, 1983). This reduction in CHO derived acetyl-CoA may be acting as a strategy to conserve small CHO reserves for other activities such as exercise.

Muscle plasticity in response to acute and chronic hypoxia has previously been investigated in terms of the contribution of aerobic and anaerobic metabolism to energy production during exercise (Le Moine *et al*., 2011). Recently, Le Moine and company investigated the phosphorylation state of PDH in mice acclimated to either acute or chronic hypoxia and exercised at the same speed (26 m/min; Le Moine *et al*., 2011). It

was found that the phosphorylation state of PDH is increased with exercise under acute hypoxia, leading to an increase in lactate production (Le Moine *et al*., 2011). The phosphorylation state of PDH was subsequently observed to decrease to levels comparable to controls after 1 week of acclimation, leading to a reduction in lactate production (Le Moine *et al*., 2011). This change in phosphorylation state was predicted to contribute to the lactate paradox, which describes a situation of reduced blood lactate accumulation during exercise with acclimation to chronic hypoxia as opposed to acute hypoxia (Le Moine *et al*., 2011). However, the degree of PDH phosphorylation or lactate production was not determined past 1 week of acclimation (Le Moine *et al*., 2011). During longer periods of hypoxic stress (5 – 9 weeks) reversals in certain aspects of acclimation, such as the observed reduction in lactate production during exercise, have been noted (Hall *et al*., 2001; Lundby *et al*., 2000). Some studies investigating the lactate paradox for longer periods of time (5 – 9 weeks of hypoxia acclimation) suggest that it is a transient effect of acclimation, disappearing with prolonged bouts of hypoxic stress (Hall *et al*., 2001; Lundby *et al*., 2000). In humans acclimated to hypoxia for 9 weeks, blood lactate levels were observed to be elevated compared to normoxic controls, similar to those observed under acute hypoxia during one – legged knee extensor exercise (Hall *et al*., 2001). These results suggest that the lactate paradox is in fact a transient phenomenon, disappearing with longer stays in hypoxia (Hall *et al*., 2001; Lundby *et al*., 2000). Therefore, one can assume that any alteration in muscle phenotype occurring during the first few weeks of hypoxic acclimation allowing for a decrease in lactate production, such as PDH phosphorylation, may have disappeared after prolonged periods of hypoxic stress. Although there was a significant increase in PDH phosphorylation in

non-exercised, resting hypoxic mice there were no changes in PDH phosphorylation observed in exercised, hypoxic mice that could explain elevated muscle lactate levels (figures 10 and figure 13)

Le Moine *et al*., (2011) also showed that the increased PDH phosphorylation observed in mice exercised under acute hypoxia was dictated by an increase in HIF-1 protein. However, as previously mentioned the mice in this study were all run at the same speed and exercise intensity was not taken into account (Le Moine *et al*., 2011). Therefore, I set out to explore the interaction between exercise intensity and hypoxia on the phosphorylation state of PDH by measuring the degree of phosphorylation at each of PDH's three phosphorylation sites in response to exercise at various intensities. To my knowledge, this is one of the first studies to investigate the phosphorylation state of PDH at each of its three phosphorylation sites in response to hypoxia and exercise at varying intensities. At most phosphorylation sites, there was a trend for the degree of phosphorylation to decrease when compared to resting controls in both hypoxia and normoxia acclimated mice in response to exercise at 75% and 85% VO_2max (figure 10). However, this trend never reached levels significantly different from resting controls due to high individual variability. Previous studies investigating the phosphorylation state and the overall activity of PDH in response to exercise have reported an increase in total PDH activity and thus a decrease in the phosphorylation state (Kiilerich *et al*., 2011; Putman *et al*., 1995). The increase in PDH activity was predicted to allow for a greater flux through the TCA and ETC to meet increasing ATP demands (Kiilerich *et al*., 2011; Wieland, 1983). However, these studies were performed mainly on humans, using the vastus lateralis muscle (Denyer *et al*., 1991; Howlett *et al*., 1998; Kiilerich *et al*., 2011;

Putman *et al*., 1995). Furthermore, the response of PDH to exercise has been noted to be specific to the tissue examined, based on its glucose demand (Denyer *et al*., 1991). In the red muscle of the quadriceps, a decrease in overall PDH activity was observed in rats run on a treadmill for 30min at 15m/min (Denyer *et al*., 1991). This decrease in PDH activity was attributed to a decrease in glucose oxidation and an increase in FA oxidation (Denyer *et al*., 1991). Therefore, it is predicted that the trend for PDH phosphorylation to decrease observed in the present study may represent an increase in overall PDH activity and thus a greater extent of CHO oxidation leading to an increased flux through glycolysis (Kiilerich *et al*., 2011; Rardin *et al*., 2009; Wieland, 1983). Further research is needed to confirm this prediction.

Previous investigators have observed a progressive increase in overall PDH activity with increasing exercise intensity due to the accompanied increase in ATP demand (Howlett *et al*., 1998). However, in the present study, there were no significant differences in the phosphorylation state of PDH between exercise intensities suggesting that there was no change in PDH activity (figure 10). This disparity may be due to the exercise protocol or model organism used. Howlett and colleagues investigated PDH activity in the vastus lateralis muscle of humans cycling at various exercise intensities (Howlett *et al*., 1998). Conversely, in the present study PDH phosphorylation was investigated in the gastrocnemius of mice in response to treadmill running. These differences in exercise protocol and species/muscle tissue used may explain the differences in the results obtained.

Muscle Metabolite Changes with Exercise

Many mammals exhibit similar patterns of fuel selection during exercise, relying mainly

on lipids at low intensities and almost exclusively on CHOs at high intensities (Brooks, 1998; McClelland, 2004; Roberts *et al*., 1996). Due to the conserved nature on this pattern, a common mechanism dictating fuel selection has been proposed but remains to be determined (McClelland, 2004; Spriet and Watt, 2003). Flux through the different fuel use pathways are largely controlled through feedback inhibition from products of the same or competing pathways (Balaban, 1990; Jeukendrup, 2002). Therefore, levels of key metabolites may lend insight into the specific pathways being activated during exercise, as well as the regulation of muscle fuel use (Green *et al.,* 1992). In my investigation, a significant difference in G-6-P levels was observed between controls and hypoxic mice exercised at 85% VO_2max, suggesting a difference in the overall rate of glycolysis (figure 11; Jeukendrup, 2002). The elevated levels of G-6-P present with exercise in hypoxic mice may be a result of a decrease in glycolytic rate. As downstream reactions slow, such as the conversion of fructose-6-phosphate to fructose -1, 6-bisphosphate by PFK and phosphoenolpyruvate to pyruvate by PK, there is a buildup of upstream products like G-6-P (Nelson and Cox, 2008). A build-up of G-6-P would subsequently lead to the inhibition of HK, further slowing the flux through glycolysis in terms of blood glucose (Jeukendrup, 2002). It has been found that high levels of lactate within the cell are capable to decreasing cellular pH, leading to a reduction in the activity of PFK and glyceraldehyde-3-phosphate dehydrogenase and thus glycolytic rate (Prampero and Ferretti, 1999; Rovetto *et al*., 1975). Therefore, the increase in muscle lactate observed in hypoxic mice may have led to a reduction in overall glycolytic rate, leading to a build-up of G-6-P (Jeukendrup, 2002; Prampero and Ferretti, 1999).

Other than G-6-P and lactate there were no other significant differences observed

in the levels of muscle metabolites in response to exercise or between acclimation groups (figure 12 and figure 13). Previous studies have shown that there are often quite subtle changes in muscle metabolites even when large changes in ATP turnover rate between rest and exercised individuals are present (Hochachka and McClelland, 1997). Therefore, the large individual variability observed in the present study may have masked any differences in the levels of muscle metabolites. Also, different trends in the levels of muscle metabolites and PDH phosphorylation in response to exercise were often observed between acclimation groups (figure 10, figure 12, and figure 13). This disparity may be due to the fact that only the gastrocnemius was sampled and compared between mice. The gastrocnemius may have been recruited to different extents in the different experimental groups, resulting in varying metabolic demands.

Conclusions

In conclusion, results obtained from real-time PCR analysis suggest that there may be a change in the abundance of the COX4-1 and COX4-2 subunits of cytochrome c oxidase in skeletal muscle of mice acclimated to hypoxia. However, this change in COX mRNA expression did not translate into an increase at the protein level. Further research is needed to confirm the composition of COX after acclimation to hypoxia and if changes in the subunits leads to an increase in the efficiency of ATP production. The use of isolated mitochondria may lend aid in uncovering the subtlety of this phenomenon.

The higher degree of PDH phosphorylation observed in resting hypoxic mice suggests that mice are relying less on CHO oxidation for ATP production. A decreased reliance on CHOs may indicate a higher utilization of lipid derived acetyl-CoA (Denyer *et al*., 1991). This change in fuel preference may act as a strategy to conserve CHOs for

other activities such as burst exercise. Post-exercise changes in the levels of lactate and G-6-P leads to the prediction that hypoxic mice are relying more heavily on anaerobic metabolism for energy production during exercise. Although there were trends in the phosphorylation state of PDH in response to exercise, they did not reach a level of statistical significance to support this prediction.

The results obtained from the present study suggest that the skeletal muscle of small mammals responds to hypoxia through changes in muscle phenotype, more specifically, changes in the structure of COX. This adjustment may act as a possible strategy to increase the efficiency of ATP production. Furthermore, under hypoxia the activity of PDH in the muscle of resting mice may be altered as a way to adjust the flux through different metabolic pathways. Muscle metabolite levels in post-exercise mice suggest that these plastic changes have an effect on the whole animal response to acute exercise, leading to a higher production of lactate in hypoxic mice. Future studies should examine the levels of post-exercise muscle metabolites involved in lipid metabolism to investigate the possibility of an increased reliance on lipid derived acetyl-CoA at rest in mice acclimated to chronic hypoxia.

CHAPTER 3: GENERAL SUMMARY AND CONCLUSIONS

In the present study, I investigated phenotypic changes within skeletal muscle during the transition from acute to chronic hypoxia and whether these changes had an effect on the whole animal response to acute exercise. To determine if phenotypic plasticity occurs during chronic hypoxia I explored 2 main assumptions of mammalian muscle metabolism under hypoxic conditions, 1) that mammals will rely on aerobic rather than anaerobic metabolism due to the greater yield of ATP per mole of fuel used during aerobic metabolism, and 2) that mammals should act to increase the efficiency of ATP production to reduce the amount of O_2 used per ATP formed, relying on CHOs more than lipids for energy production due to their higher ATP/O_2 ratio (Hochachka, 1985). In my study I believe I have provided evidence that these assumptions, in part, hold true in small mammals.

Previous studies investigating the efficiency of ATP production in the mitochondrion in terms of the P/O ratio suggest that efficiency increases under acute hypoxic conditions, indicated by an elevated P/O ratio (Solien *et al*., 2005). Cell culture experiments suggest that an increase in the 4-2 isoform and a decrease in the 4-1 isoform of COX contribute to this change in efficiency (Fukuda *et al*., 2007; Poyton *et al*., 1988; Waterland *et al*., 1991). In my investigation, I examined this phenomenon using hypoxia at the whole animal level and looked in skeletal muscle to determine if these cellular changes hold true in intact animals. I observed a significant increase in mRNA expression of the COX4-2 isoform after 24hrs of hypobaric hypoxic acclimation with no changes in COX4-1 or LON protease mRNA observed over the course of acclimation (figure 5). Previous investigators have documented increased levels of LON protease

expression under hypoxic stress, leading to a decrease in COX4-1 protein levels in cell culture (Fukuda *et al*., 2007). The inability to detect a change in LON mRNA in the present study may have been due to the large degree of individual variability present. Further, while COX4-2 increased the overall abundance of COX isoforms may have increased as COX4-1 remained static. The observed increase in COX4-2 mRNA suggests that the COX4-2 isoform may become more abundant in skeletal muscle under hypoxic conditions. Increasing levels of COX4-2 have been linked to a higher turnover rate of O_2 to H_2O by COX, suggesting an increase in total efficiency (Poyton *et al*., 1988; Waterland *et al*., 1991). Therefore, the observed increase in COX4-2 mRNA may indicate an increase in the COX4-2 isoform leading to an increased efficiency of electron transport and H_2O formation (Poyton *et al*., 1988; Waterland *et al*., 1991). The observed increase in COX enzyme activity after 2hrs of hypoxic exposure further supports this prediction (figure 4). However, I did not observe a significant change in total protein levels for COX4-1 or COX4-2 over the course of 7 days of acclimation (figure 5). This disparity between transcript and protein abundance may be due to the resolution of Western blotting and the inability to pick up subtle changes in protein expression. I also investigated changes in the phosphorylation state of PDH in muscle in response to chronic hypoxia in resting mice. After 4 weeks of hypoxic acclimation I observed a significant increase in the degree of phosphorylation at one of the three phosphorylation sites found within PDH, Ser^{293}(site 1). This increase in phosphorylation may indicate that there was on overall reduction in PDH activity, suggesting that CHO derived acetyl-CoA is being relied on to a lesser extent for energy production (Patel and Korotchkina, 2006; Wieland, 1983).

When mammals are initially exposed to hypoxic stress they act to reduce their ATP consumption using strategies such as entering a hypometabolic state and turning to anaerobic rather that aerobic metabolism for energy production (Hochachka, 2000; Kim *et al*., 2006; reviewed in Ramirez *et al*., 2007). However, continued anaerobic metabolism may deplete the small but valuable CHO stores as well as produce high levels of lactic acid (Hochachka, 1985; Nelson and Cox, 2008). High levels of lactic acid may act to reduce cellular pH and interfere with the normal operations of various glycolytic enzymes (Prampero and Ferretti, 1999; Rovetto *et al*., 1975). Therefore, mammals must adopt other long-term O_2 saving strategies when exposed to chronic hypoxia (Hochachka, 2000; Kim *et al*., 2006; reviewed in Ramirez *et al*., 2007). Mammals have been observed to reduce the rate of anaerobic CHO oxidation under chronic hypoxia and increase the rate of aerobic CHO oxidation to levels comparable to normoxic controls (Le Moine *et al*., 2011; Papandreou *et al*., 2006). A recent study investigated this phenomenon, examining the protein levels of HIF-1α and PDK1 as well as PDH phosphorylation state in an attempt to uncover an underlying cellular mechanism (Le Moine *et al*., 2011). Mice were acclimated to acute and chronic hypoxia and run at a constant speed of 26m/min to maintain the same ATP turnover rate between acclimation groups (Le Moine *et al*., 2011). In mice acclimated to acute hypoxia (24hrs) Le Moine and colleagues observed an initial increase in HIF-1 and PDK1 protein levels as well as an increase in the phosphorylation state of PDH (Le Moine *et al*., 2011). In mice acclimated to chronic hypoxia, however, levels of HIF-1, PDK1, and PDH phosphorylation state had returned to levels not different from controls (Le Moine *et al*., 2011). Furthermore, the activity of PDH was found to decrease under acute hypoxia and then increase back to levels not

different than controls under chronic hypoxia (Le Moine *et al*., 2011). These changes in activity were found to be mirrored by an increase and subsequent decrease in muscle lactate levels (Le Moine *et al*., 2007). These results suggest that the HIF-1, PDK1, and PDH pathway is integral in controlling the extent of anaerobic and aerobic metabolism during exercise in mice under hypoxia (Le Moine *et al*., 2011). However, as previously mentioned, the mice in this study were run at the same constant speed which did not allow the authors to address the control of fuel use at the same $\%VO_2max$ (Le Moine *et al*., 2011). Previous studies have shown that the mix of fuels used ($\%VO_2$) is not altered with acclimation (McClelland *et al*., 1998; Schippers *et al*., unpublished). Furthermore, this study did not examine long-term periods of hypoxic stress ≥ 4 weeks (Le Moine *et al*., 2011). This pattern of energy metabolism under acute and chronic hypoxia, coined the lactate paradox, has been observed to disappear, in a few studies, with longer exposures to hypoxia (≈ 5 – 6 weeks) with post-exercise lactate levels resembling those first observed under acute hypoxia (Hall *et al*., 2001; Lundby *et al*., 2000). Although what is happening at rest may not reflect what happens during exercise, a greater level of phosphorylation at rest may suggest a higher baseline kinase activity which may also occur in exercise. The increased muscle lactate levels observed in post-exercise mice under hypoxia further suggests this prediction.

In my study, I also investigated the possibility of a common controlling mechanism within mice muscle responsible for dictating fuel selection during exercise. To determine this, I compared changes in the phosphorylation state of PDH as well as changes in various metabolites post-exercise in mice exercised at varying intensities in normoxia and hypoxia. Although there were some similarities in the relative changes in

the phosphorylation state of PDH post-exercise in hypoxic and normoxic mice, these data were not statistically significant and so failed to provide insights into the putative common controlling mechanism. Post-exercise metabolite changes were often different between hypoxic and normoxic mice, suggesting that hypoxic mice were relying on anaerobic metabolism to a greater extent than normoxic mice. Common regulatory mechanisms for a similar mix of fuels may take the form of similar relative changes in muscle metabolites because many steps in metabolic pathways and cellular metabolism respond to these changes (Green *et al.*, 1992). However, the relative changes observed in the present study were not indicative of a common controlling mechanism. There are many ways in which fuel pathways can be regulated including changes in enzyme activity, changes in enzyme content and structure, the rate of substrate supply, and changes in membrane conductance (Fukuda *et al*., 2007; Nelson and Cox, 2008; Saurez *et al*., 2005). Therefore, I believe that it is not the case that a mechanism does not exist, but that a more diverse selection of metabolites and parameters should be examined in order to investigate this prediction more thoroughly. Including all of the byproducts of both lipid and glucose metabolism would give a better indication of the extent of each pathway being used. Further, a greater selection of metabolites would allow for the investigation into the possibility of a glucose-FA or FA-glucose cycle which dictates fuel selection during exercise in skeletal muscle (Randle *et al*., 1963).

Future Directions

My study has provided some support for an alteration in metabolic machinery under hypoxic conditions which may lead to an increase in the efficiency of ATP production in mice. However, changes in mRNA expression were not mirrored at the protein level

using Western blot analysis. As previously mentioned, this disparity may have been to the inability of Western blotting to detect small changes in protein content at the whole muscle level. Due to the subtle nature of the change in mRNA expression observed, I believe it would be beneficial to use a more sensitive tool for protein quantification. Exploring the changes in isoform abundance using proteomic analysis or an enzyme – linked immunosorbent assay (ELISA) may allow for the subtle changes in protein expression to be observed. The use of isolated mitochondria may further enhance any changes in protein expression present. Also, using a denominator other than total COX activity, such as the protein content of another COX subunit, to standardize protein levels may give a better indication of the total COX present within the muscle. Furthermore, examining O_2 use and ATP production by the mitochondrion would provide the opportunity to determine if changes in COX composition directly lead to changes in the efficiency of ATP production.

Further research examining the overall activity of PDH as well as the levels of its various controlling proteins and metabolites is needed to confirm the predictions made in the present study. As discussed above, the activity of PDH can be modulated through changes in the levels of key metabolites including acetyl-CoA and NADH. Therefore, I believe that measuring the levels of acetyl-CoA/CoA and NADH/NAD^+ in skeletal muscle at rest and in response to exercise will provide further insight into possible controlling mechanisms of fuel selection, especially in terms of PDH activity. Also, determining the overall activity of PDH would provide direct evidence for changes in the extent of aerobic and/or anaerobic metabolism being used for ATP production at rest and during exercise.

In my study, I uncovered important details regarding the phenotypic plasticity of skeletal muscle to hypoxia and the acute response to exercise. Unfortunately I did not provide support for the hypothesis that muscle fiber metabolism is regulated by a common mechanism resulting in the conserved pattern observed. My results suggest that, at least in the gastrocnemius, control and acclimated mice may use distinct regulation methods to achieve the same fuel use patterns. Further research is needed to explore this possibility. I suggest that a wider array of metabolites, especially those relating to the Randle cycle, be measured in mice exercised at varying intensities. Furthermore, it has previously been suggested that a change in muscle fiber activation is responsible for the classic pattern of mammalian fuel use during exercise (Armstrong and Laughlin, 1985; Brooks, 1998; Roberts *et al*., 1996; Vollestad and Blom, 1985). Therefore, I suggest that glycogen levels be determined in various muscles in the hind limb of mice exercised at varying exercise intensities to determine the level of recruitment. This investigation would provide the opportunity to explore another predicted controlling mechanism of fuel selection.

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