INFLUENCE OF THE TRANSITION FROM ACUTE TO CHRONIC HYPOXIA ON PYRUVATE DEHYDROGENASE KINASE 1 AND LACTATE ACCUMULATION IN C2C12 CELLS

**INFLUENCE OF THE TRANSITION FROM ACUTE TO CHRONIC HYPOXIA ON PYRUVATE DEHYDROGENASE KINASE 1 AND LACTATE ACCUMULATION IN C2C12 CELLS**

By

TAYLOR MITCHELL BROOKS, B.SC

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TITLE: Influence of the transition from acute to chronic hypoxia on Pyruvate Dehydrogenase Kinase 1 and lactate accumulation in C2C12 cells

AUTHOR: Taylor M. Brooks

B.Sc. (Mount Allison University, Canada)

SUPERVISOR: Dr. Grant B. McClelland

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**Abstract**

For decades researchers have observed a reduction in exercise-induced blood lactate accumulation transitioning from acute to chronic hypoxia (the ‘lactate paradox’). Hypoxia inducible factor (HIF1-α) has been hypothesized to direct these metabolic changes during hypoxia through the induction of pyruvate dehydrogenase kinase 1 (PDK1). Activation of PDK1 inhibits aerobic glucose metabolism through pyruvate dehydrogenase, and promotes lactate production. Previous work in our lab revealed HIF1-α and PDK1 protein expression correlates to changes in lactate production with the transition from acute to chronic hypoxia in CD-1 mice, revealing a putative mechanism explaining the paradoxical reduction in lactate. We exposed differentiated C2C12 cells to 1% O2 for 4h, 24h, 96h and compared them to time-matched controls in 21% O2. In addition, we used 1 mM of the HIF-agonist DMOG at 20.95% O2 and 25 µM of the HIF antagonist PX-478 in 1% O2. We found that C2C12 myotubes decreased the rate of lactate accumulation and release with long-term hypoxia, similar to observations *in vivo*. This also corresponds to changes in LDH enzyme activity and PDK1 protein expression. However, DMOG-induced PDK1 expression does not match changes in lactate accumulation. Our findings confirm the existence of the lactate paradox at the cellular level and suggest a role for HIF signaling in the decline of lactate with chronic hypoxia.

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**Thesis Organization**

This thesis is organized in a “sandwich” format and consists of three main chapters. Chapter one provides a general introduction and outlines the objectives of my thesis research. Chapter two is a manuscript that is prepared for submission to a peer-reviewed scientific journal. Chapter three discusses these findings and their implications in cellular hypoxia acclimation.

**Chapter 1: General Introduction**

**Chapter 2: Changes in PDK1 expression and lactate accumulation with acute and chronic hypoxia in C2C12 cells**

Authors: Taylor M. Brooks, Amber E. Schlater, Grant B. McClelland

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**List of Abbreviations**

AMPK Adenosine monophosphate activated kinase

AMP Adenosine monophosphate

ADP Adenosine diphosphate

ATP Adenosine triphosphate

C2C12 Immortalized mouse myoblast cell line

CoA Coenzyme A

CS Citrate synthase

Cyt c Cytochrome c

DMEM Dulbecco’s Modified Eagle’s medium

DMOG Dimethyloxalyl Glycine

ETC Electron transport chain

FBS Fetal bovine serum

HIF Hypoxia-inducible factor

HRE Hypoxia response element

LDH Lactate dehydrogenase

MCT Monocarboxylate transporter

NAD Nicotinamide adenine dinucleotide

NADH Reduced nicotinamide adenine dinucleotide

OXPHOS Oxidative phosphorylation

Pi inorganic phosphate

PDH Pyruvate dehydrogenase

PDK Pyruvate dehydrogenase kinase

PO2 Partial pressure of oxygen

PX-478 S-2-amino-3-[4’-N,N,-bis(chloroethyl)amino]phenyl propionic acid N-oxide dihydrochloride

TCA Tricarboxylic acid

qPCR Quantitative polymerase chain reaction

VO2 Maximum volume of oxygen

Vmax Maximal enzyme velocity

**Chapter One: General Introduction**

*Hypoxia as a stressor*

Atmospheric hypoxia refers to a reduced partial pressure of oxygen (PO2) relative to the total composition of all other contributing gases. At sea level, O2 accounts for 20.95% of total atmospheric gases. However, even at sea level, environmental hypoxia occurs naturally in subterranean burrows. Also, atmospheric pressure declines with elevations in altitude and low oxygen in both situations is physiologically challenging by potentially limiting the aerobic capacity of animals.

O2-dependence for oxidative phosphorylation of metazoan species has resulted in the proliferation of elaborate systems to ventilate and circulate O2 to the hundreds of millions of cells in mammals. Hypoxia-tolerant mammals have evolved a variety of adaptations to increase O2 supply when its availability is reduced. This is achieved by making adjustments to the ventilatory, cardiovascular and hematological systems. So too, may changes occur at the level of the cell in an attempt to balance aerobic ATP supply with ATP demand.

A sudden drop in PO2 promotes the hypoxic ventilatory response (HVR), which signals for an increase in ventilation to increase alveolar PO2 (Lahiri 1996; Teppema and Dahan 2010), while an increased heart rate helps mediate O2 delivery to tissues (West et al. 1983). However, depending on the level of hypoxia this compensation may never be complete. A resultant mismatch of O2-supply to O2-demand at the cellular level impairs an aerobic animal’s ability to produce ATP by oxidative phosphorylation. This may result in an accumulation of metabolites within the tricarboxylic acid (TCA) cycle, whereby increases in pyruvate concentration promote its conversion to lactate via LDH by mass action. ATP generation thus becomes more reliant on anaerobic pathways such as glycolysis. This is especially true in tissues capable of sustaining high rates of ATP turnover, such as skeletal muscle, and results in the accumulation of lactate in the tissue, which then spills over into the blood. The maintenance of ATP turnover rate with a switch to anaerobic glycolysis is referred to as the “Pasteur Effect” and is typical of an acute hypoxia response.

This process however cannot continue indefinitely as anaerobic glycolysis only yields ~2 moles ATP per mole of glucose, and rapidly depletes muscle glycogen reserves. While this response to acute hypoxia ensures short-term survival, the long-term survival of an animal depends on its ability to make adequate physiological adjustments to compensate for reduced O2 availability. Whereas metabolic depression is not a viable option for active animals, restoring aerobic means of ATP production is necessary to sustain metabolic demands during long-term hypoxic stress.

*Hypoxia Acclimatization*

In mammals, blood lactate levels are known to increase upon sudden hypoxic exposure due to the limitations in O2-availability to cells and insufficient time for any plastic changes in physiology to enhance O2-supply. Lactate is produced as a result of a mismatch between glycolytic flux and pyruvate oxidation (Reeves et al. 1992; Brooks, 2016). It is also known as a bi-product of anaerobic glycolysis in working skeletal muscle (Hochachka 1988). For decades researchers have observed that blood lactate accumulation increases in humans exercising in acute hypoxic conditions as a manifestation of the Pasteur effect. As early as the 1930s, researchers also observed that after a period of acclimatization to hypoxia at high altitude, these blood lactate levels declined to those observed in normoxia at the same work rate ( Dill et al. 1931; Edwards, 1936). Later, this phenomenon was referred to in the literature as the ‘lactate paradox’ (Appendix Fig. 1; Hochachka 1988; West et al. 1983; Reeves et al. 1992). The paradox is that one might expect blood lactate concentrations to remain elevated at the same reduced atmospheric pressure of O2, but the reduction in lactate accumulation in acclimatized lowlanders is reminiscent to those exercising at the same incremental work rates at sea level. This metabolic arrangement observed is paradoxical because cellular oxygenation may not be improved (Reeves et al. 1992) despite adjustments to respiratory and cardiovascular systems (Calbet et al. 2017). A reduction in blood lactate at the same incremental work rate would suggest that aerobic metabolism is being restored with chronic hypoxia.

*HIF1-α & Oxygen Homeostasis*

While cardio-respiratory changes help buffer variation in ambient O2 concentrations and tissue specific O2-demand, at the level of the cell animals respond to acute hypoxic episodes through the highly conserved transcriptional complex, hypoxia inducible factor (HIF; Semenza and Wang 1992). HIF is regulated at the post-transcriptional level by cellular O2 level and its function depends on the heterodimerization of its two primary subunits, HIF1-α and HIF1-β. While HIF1- α is constitutively synthesized in the cytosol, in the presence of O2 and 2-oxoglutarate, prolyl hydroxylases (PHD) hydroxylate HIF1-α, tagging it for proteosomal degradation by the von Hippel-Lindau tumor suppressor. However, during hypoxic stress PHD hydroxylation rates are reduced as O2 is lackingas a substrate. This permits the stabilization of HIF1-α and its subsequent dimerization. Here, HIF associates with transcriptional coactivators p300 and CBP to bind to Hypoxia Response Elements (HREs) of target genes and thereby activating transcription (Appelhoffl et al. 2004). This union regulates more than 100 genes (Ke and Costa 2006), but pyruvate dehydrogenase kinase isoenzyme 1 (PDK1) has garnered much attention due to its role in regulating aerobic glucose metabolism (Appendix Fig. 2). The activation of HIF stimulates PDK1 expression, which leads to the phosphorylation and deactivation of the pyruvate dehydrogenase complex (PDH) (Papandreou et al. 2006; Mulukutla et al. 2014; Favier et al. 2015). This results in the down regulation of pyruvate oxidation and its mitochondrial respiration, thereby reducing the O2 dependence and directing pyruvate to lactate for continued ATP synthesis (Papandreou et al. 2006).

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Acclimatization to chronic hypoxia in humans promotes compensation for reduced O2 availability by increases in ventilation and hematocrit. While these changes contribute to improved arterial O2 saturation, findings by Reeves and colleagues (1992) also show a reduced blood flow to the exercising leg muscle in humans. This serves to offset improved muscle O2 delivery by the increase in O2 content in the blood. Moreover, myoglobin appears to be never full desaturated, even at VO2max. Thus, the resulting fall in lactate as described in the lactate paradox appears to be not fully dependent on muscle oxygenation. Therefore, it is difficult to distinguish whether oxygen is not necessarily limiting at the myofibrillar level, or whether lactate declines even when muscle PO2 does not improve with acclimatization.Le Moine and colleagues (2011) examined the transition from acute to chronic hypoxia in CD-1 mice *in vivo*. They demonstrated that in exercising CD-1 mice, blood lactate accumulation declined after exposure to hypobaric hypoxia simulating 4,300m from 24h (acute) to 1week (chronic) and that the lactate paradox exists for laboratory mice. They also showed that changes in protein expression of HIF1-α and PDK1 corresponded to the reduction in blood lactate accumulation with the transition from acute to chronic hypoxia. Moreover, enzyme activity of PDH in the active form (PDHa) was inversely related to both HIF1-α and PDK1 protein expression. The authors suggested that a reduction in HIF1-α protein expression plays a role in reducing lactate accumulation by restoring the capacity for pyruvate oxidation after chronic hypoxic acclimation compared to those exercised in normoxia. These data suggest that modification in the regulation of HIF expression may occur after chronic hypoxia acclimation compared to HIF protein stabilization during acute hypoxia. In fact, Ginouvès and colleagues (2008) showed that chronic hypoxic exposure of mice *in vivo* as well as a variety of cell lines, results in increased PHD expression with subsequent reduction of HIF1-α expression. In contrast, acute hypoxia has the opposite effect where PHD is low due to the lack of O2 available as a substrate, allowing for HIF1- α stabilization. However, it is not yet clear whether a decline in lactate production is due to a change in regulation of HIF1-α expression or due to changes in the ability of muscle fibres to extract O2 after hypoxia acclimation (Le Moine et al. 2011). During chronic hypoxia, one could predict cumulative changes in O2 extraction and delivery due to compensatory changes in the cardio-pulmonary system (Hochachka 1994; Hochachka et al. 1998) and increased capillarization. Currently data on hypoxia acclimation is equivocal when it comes to changes in skeletal muscle capacity for O2 transport. Work by Lundby and colleagues (2004) showed a reduction in O2 conductance and diffusion capacity in the working muscle of hypoxia-acclimatized humans, which they interpret to be a delay in aerobically generated ATP at the beginning of exercise (van Hall et al. 2009). Another report distinguishing capillary number from capillary density, showed a loss of muscle fiber cross-sectional area resulted in the same number of capillaries supplying less tissue, but with reduced mitochondrial volume (Hopperler et al. 2002). Furthermore, neither low-altitude nor high-altitude native deer mice (Peromyscus maniculatus) appear to respond to hypoxia acclimation through increased capillarity, or mitochondrial volume (Lui et al. 2015; Mahalingam et al. 2017). In contrast, Theije and colleagues (2011) showed that both glycolytic and oxidative muscles of hypoxia-acclimated C57BL/6J mice showed enhanced capillarization per fiber. However, the two fiber-types achieved this differently; glycolytic fiber cross sectional area and mass were reduced, while oxidative muscles showed increased capillary contact to each individual fiber. This phenotype appears to be a feature of both endurance training (Mason et al. 2007) and hypoxia acclimation (Theije et al. 2015). Together these studies suggest that the acclimated state in chronic hypoxia is still faced with O2 delivery limitations, and that this points to the importance of hypoxia sensing and plasticity of the muscle cell.

*Muscle Phenotype & ATP Supply-Demand Coupling*

Muscle fibres are specialized for different tasks. Fast-twitch glycolytic fibers achieve a high force production for only short duration work, relying extensively on anaerobic ATP production and rapid cross bridge cycling and releasing large quantities of high-energy phosphates in the process. This arrangement of ATP supply and ATP demand is considered loosely coupled, where rapid accumulation of ADP and Pi elevates the phosphate potential and thus stimulating ATP-generating pathways (Hochachka 1988). This makes fast-twitch muscle better suited for rapid and intermittent work rates where rapid upregulation of ATP supply is necessary for fast shortening velocities. Interestingly, these changes in high-energy phosphates stimulate glycolysis, to a greater degree than aerobic metabolism, and drives increased lactate production even under O2-saturating conditions. Such high work rates, however, are unsustainable, especially under hypoxic stress as they rapidly deplete limited phosphocreatine and subsequent muscle glycogen stores. In addition to increasing the phosphate potential in these fibers, ATP hydrolysis also causes an accumulation of protons intracellularly, requiring sufficient means to buffer these changes in pH (Hochachka 1994). Slow-twitch oxidative fibres on the other hand are important for low force but long duration work that relies principally on aerobic ATP production. Thus, they have high volume densities of mitochondria, less intensive demands of rapid force production limit rates of ATP catabolism and the accumulation of phosphate intermediates. Therefore, glycolytic production of pyruvate is more closely matched to the activation of PDH, resulting in less lactate produced and less perturbations of cellular pH. The product is a tighter coupling of ATP demand-supply, which more reliably maintains energy homeostasis.

This hypothesis posits that restoring aerobic metabolism in the muscle of acclimatized lowlanders, requires a phenotypic change from loosely coupled, fast-twitch-like fibers to a more tightly coupled, oxidative-like fibers, (Hochachka 1988). Such a change would promote a transition from metabolic flux with a disproportionate stimulation of glycolysis lending to increased lactate accumulation to one where oxidative phosphorylation more closely matches glycolytic flux (Hochachka 1988). Lui and colleagues (2015) demonstrated in wild mice that muscle fiber-type is different as an evolved characteristic between highland and lowland populations, but that it is not changed after hypoxia acclimation. However, Nguyen and colleauges (2016), showed that while intermittent hypoxia caused a shift in muscle fiber type, sustained hypoxia resulted in a fatigue resistant type IIa fiber composition in the geniohyoid muscle of rats. The latter data support a more oxidative phenotype under chronic hypoxia when compared to rats in cycles of intermittent hypoxia, which showed elevations of fatigue-prone type IIb fiber transcript abundance. Moreover, type IIa transcript abundance corresponds with an elevated PDK1 to HIF1-α protein expression ratio. This phenotype of type IIa fibers fits well with the concept of improved ATP demand-supply coupling. Given that HIF1-α expression is more pronounced in glycolytic fibers, this led the authors to hypothesize that PDK1 is crucial for maintaining ATP levels (Papandreou et al. 2006).

*Cellular Oxygenation*

At each transport level of the O2 cascade PO2 declines, from the alveoli to the level of the myocyte (Richardson et al. 1995). As denoted by the term ‘hypoxia’, hypo- is the Greek prefix meaning ‘under’, while oxia refers to the condition of oxygenation. Therein, hypoxia means under oxygenated. A reduction in inspired ambient PO2 should, in theory, make the succeeding components of oxygen delivery hypoxic as well (Richardson et al. 1995). As O2 in the blood circulates through the capillaries to the tissues, its partial pressure drops due in part by the tissue type and metabolic rate of the cell.

Past studies have measured resting intracellular PO2 *in vivo* using microcatheters in humans (Jung et al. 1999), optical method in rats (Johnson et al. 2005) surface electrodes in dogs (Hutter et al. 1999), and magnetic resonance spectroscopy (Richardson et al. 1995; Greenbaum et al. 1997; Richardson et al. 1998, 2006). These studies point to a normoxic resting intramuscular PO2 of approximately 34 mmHg. Few studies have examined intracellular PO2 during hypoxia, which are further constrained to logistical problems and replication. Thus far, Johnson and colleagues (2005) have reported a value of ~10 mmHg after 1 min of 7% O2 in rat cremaster muscle, while Richardson and colleagues (2006)reported a mean value of 23 mmHg in humans respiring 10% O2. Furthermore, measurements performed in the resting tibialis anterior of rabbits place normoxic intracellular PO2 in the range of 4.7 and 31.3 mmHg (Greenbaum et al. 1997), though this range of PO2 is perhaps too ambiguous. Muscle oxygenation however, is not expected to be homogenous given the variability in fiber-types. A spatial PO2 gradient exists within the myocytes depending on microcirculation and mitochondrial proximity (Hopperler et al. 2002). These studies show that environmental hypoxia results in a reduction in muscle fiber PO2, and that this is likely the primary stimuli for HIF-mediated compensation through the stabilization of HIF1-α.

It is interesting to note that increased O2-demand during only moderately intense exercise in normoxia, can drop the intracellular PO2 to ~3-5 mmHg (Richardson et al. 1995). This is significantly less than that at rest even during hypoxia and provides different contextual insight. During exercise, intracellular PO2 declines as a function of elevated metabolic rate and subsequent O2 consumption, thereby widening ΔPO2 and facilitating the diffusive capacity of O2. Ambient hypoxic stress on the other hand, does not reduce resting intracellular PO2 as effectively though, limiting diffusion to a more confined ΔPO2.

*Objectives*

The primary objective of this thesis was to track changes in muscle lactate production while controlling for cellular oxygenation *in vitro* to assess changes in metabolic pathways. The secondary objective was to identify the functional role that the cellular hypoxic response plays in the reduction of lactate production from acute to chronic hypoxia observed as the lactate paradox. I tested the hypothesis that reductions in lactate from acute to chronic hypoxia are due to a change in regulation of the HIF pathway. I looked at the rates of lactate appearance within the C2C12 myotube and the extracellular media in an attempt replicate the lactate paradox at the level of the myotube. I also looked at enzymatic activities of LDH and CS as markers for the capacity of cells to engage in either anaerobic or aerobic metabolism. I predicted that an aerobic phenotype would re-emerge in long-term hypoxia, and that this may be indicative of a more tightly coupled ATP demand-supply arrangement.

**Chapter Two: Changes in PDK1 expression and lactate accumulation with acute and chronic hypoxia in C2C12 cells**

*Abstract*

For decades researchers have observed a reduction in exercise-induced blood lactate accumulation transitioning from acute to chronic hypoxia (the ‘lactate paradox’). However, the underlying mechanisms that explain these changes in lactate production are still unclear. Hypoxia inducible factor (HIF1-α) is stabilized in low O2 and helps mediate responses to acute hypoxic stress, in part by enhancing glycolytic capacity. It also stimulates pyruvate dehydrogenase kinase 1 (PDK1), which inhibits PDH, and promotes lactate production. Previous work in our lab revealed HIF1-α and PDK1 protein expression correlates with lactate production in acute and chronic hypoxia in mice, revealing a putative mechanism explaining the paradoxical reduction in lactate. To further define the mechanisms involved we turned to cultured C2C12 myocytes. We exposed differentiated C2C12 cells to 1% O2 for 4h, 24h, and 96h and compared them to time-matched control cells in 21% O2. In addition, we used 1 mM of the HIF-agonist at 21% O2 and 25 µM of the HIF-antagonist, PX-478 to shed light on this mechanism. We found that the lactate paradox is observable at the level of the cell, where the rate of lactate accumulation and release increases in acute hypoxia but then subsides with long-term hypoxia acclimation. This also corresponds to similar changes in LDH enzyme activity, and PDK1 protein expression in hypoxia. However, DMOG induced PDK1 but did not alter lactate accumulation, while dynamic lactate changes occurred despite PX-478 repression of PDK1. Our findings confirm the existence of the lactate paradox at the cellular level and reveal that PDK1 is an important player in explaining lactate production in hypoxia over time, but in the absence of hypoxia, PDK1 stimulation does not always lead to lactate production.

*Introduction*

Cellular hypoxia occurs when there is a deficit of O2 supply in relation to O2 demand. Cellular hypoxia is a common feature in ischemic tissue and diffusion-limited tumors. It can also occur through increased metabolic rate, such as with exercise or with exposure to ambient hypoxia at high altitude and in underground burrows, where limited O2-availability transcends to levels of O2-extraction to circulation and diffusion. These challenge cellular metabolism by limiting aerobic capacity. Anaerobic glycolysis thence becomes the preferred method of ATP production and produces lactate as bi-product.

Though hypoxia-tolerant mammals may increase O2 supply through ventilatory, cardiovascular and hematological adjustments, so too do changes occur at the level of the cell in an attempt to balance ATP supply with demand. However, depending on the level of hypoxia this compensation may never be complete. Since anaerobic glycolysis yields less than 6% of the ATP generated from oxidative phosphorylation, restoration of aerobic ATP production is essential to ensure the viability of the animal. This results in an accumulation of metabolites within the tricarboxylic acid (TCA) cycle, inhibiting aerobic pyruvate metabolism. This is especially true in tissues capable of sustaining high-rates of ATP turnover, such as skeletal muscle. The resultant accumulation of lactate in the tissue then spills over into the blood. This is termed the “Pasteur Effect” and is typical of acute hypoxia.

With a reduced yield in ATP synthesis during anaerobic glycolysis, muscle cells are especially susceptible to hypoxic stress as even momentary exertion can rapidly deplete energy reserves. While glycolysis helps buffer sudden and immediate hypoxic episodes, anaerobic means of regenerating ATP are inefficient and through the reduction of pyruvate, produce lactate in excess. While this response to acute hypoxia ensures short-term survival, the long-term survival of an animal depends on its ability to make adequate physiological adjustments to compensate for reduced O2 availability. Whereas metabolic depression is not a viable option for active animals, restoring aerobic means of ATP production is necessary to sustain metabolic demands during long-term hypoxic stress. Phenotypic plasticity to chronic hypoxia results in observably improved arterial O2 saturation (SaO2), but this response coincides with reduced overall blood flow, resulting in no net improvement of muscle oxygenation (Reeves et al. 1992). More current findings suggest that improvements in muscle PO2 may not occur with acclimatization (Hopperler et al. 2002; Lundby et al. 2004; Haseler et al. 2007; Lui et al. 2015). Thus the resulting fall in lactate as described in the lactate paradox appears to be independent of muscle oxygenation.

This level of hypoxia at the level of the cell is expected to disrupt mitochondrial respiration (Papandreou et al. 2006). Hypoxia-inducible factor (HIF) responds to cellular hypoxia in an acute rescue response. One of the things that happens is that the negative regulator of HIF1-α, prolyl hydroxylase (PHD) 2 is inhibited when O2 is limiting as a substrate. This permits the stabilization of HIF1-α, which then dimerizes to its HIF1-β counterpart. This union is translocated into the nucleus, promoting the transcription of Hypoxia Response Elements (HREs). HREs mediate hypoxia primarily through induction of glycolytic enzymes, as well as well as pro-angiogenic factors and red blood cell formation (as reviewed by Favier et al. 2015).

Previously, the transition from acute to chronic hypoxia was examined in CD-1 mice *in vivo*, demonstrating that the lactate paradox could be replicated in mice (Le Moine et al. 2011). They showed that changes protein expression of HIF1-α and PDK1 corresponded to the reduction in blood lactate accumulation with the transition from acute to chronic hypoxia. Moreover, enzyme activity of PDH in the active form was inversely related to both HIF1-α and PDK1 protein expression. This reduction in HIF1-α protein expression is suspected to play a role in reducing lactate accumulation by restoring the capacity for pyruvate oxidation after chronic hypoxic acclimation compared to those exercised in normoxia. This is consistent with findings from Ginouvès and colleagues (2008) who showed that chronic hypoxic exposure of mice *in vivo* as well as a variety of cell lines, resulted in increased PHD expression with a subsequent destabilization of HIF1-α in hypoxia. However, it is not yet clear whether lactate production declines due to a change in regulation of HIF1-α expression or due to enhanced O2 extraction of the muscle after hypoxia acclimation (Le Moine et al. 2011).

Given HIF1-α levels decline as a function of chronic hypoxia acclimationcompared to HIF protein stabilization during acute hypoxia when intramuscular PO2 may not be improved, these data suggest that a modification in the regulation of HIF expression might be influencing blood lactate levels. In this study, we used cultured C2C12 myotubes exposed to normoxic (21% O2) and hypoxic (1% O2) conditions for 4h, 24h and 96h. We also used pharmacological intervention of HIF expression using the agonist, Dimethyloxalyl Glycine (DMOG) in normoxia and the HIF-antagonist, PX-478 in hypoxia to see how perturbations in hypoxia signaling affect cellular metabolism. We tracked lactate accumulation within the cellular fraction and extracellular media at each time point, and tested for the capacity for either anaerobic or aerobic metabolism by measuring enzyme activities of LDH and CS, respectively. We then measured total protein expression of PDK1 to gain insight into how it influences the transition between anaerobic and aerobic states. Lastly, we looked at the transcriptional abundance of the endogenous negative HIF-regulator, PHD2 to gain insight into how hypoxia acclimation changes the metabolic phenotype over time. We hypothesized that changes in cellular lactate accumulation will be dictated by changes in PDK1.

*Materials & Methods*

**Cell Culture**

C2C12 cells (ATCC) were plated from frozen stock onto a 75mm dish (For Western blotting and enzyme assays) and 6 x 35mm dish (for qPCR) and incubated in 20% fetal bovine serum (FBS) high glucose Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 1% sodium pyruvate and a 1% mixture of streptomycin and penicillin at 37**°**C at 5% humidity. Media was changed daily and cells were split to unique passage numbers at approximately 40% confluency. Differentiation was induced at approximately 80-90% confluency by switching to a 5% equine serum high glucose DMEM solution supplemented with 0.02 µg mL-1 insulin, 0.02 µg mL-1 transferrin, and a 1% mixture of streptomycin and penicillin for 5 days. Experiments were carried out from days 0 after differentiation onwards. A positive control group treated with 100 µM CoCl2 for 4h was used to pharmacologically to promote HIF-1α expression and activity (Ciafrè et al. 2007; Rovetta et al. 2013). We used 20.95% ambient O2 as a normoxic controls ( Di Carlo et al. 2004; Pisani and Dechesne 2005; Ono et al. 2006; Li et al. 2013; Slot et al. 2014), and 1% O2 (PO2 = 7.6 mmHg) to induce hypoxia at the level of the cell as previously described (Basic et al. 2014; Nguyen, Kim, and Pae 2016; Ginouvès et al. 2008; Li et al. 2013)

Hypoxic (Hx) treatments were 4h 1% O2 (4h Hx), 24h at 1% O2 (24h Hx), and 96h at 1% O2 (96h Hx). These groups were time-matched against their respective normoxic (Nx) controls at 20.95% O2.

HIF expression was pharmacologically induced at each time-point through the dosage of 1 mM of Dimethyloxalyl Glycine (DMOG; as previously shown by Dehne et al. 2007) in differentiation media at 20.95% O2 and designated 4h Nx + DMOG; 24h Nx + DMOG; 96h Nx + DMOG. 25 µM of the HIF-antagonist, S-2-amino-3-[4V-N,N,-bis(2-chloroethyl)amino]phenyl propionic acid N-oxide dihydrochloride (PX-478; as previously shown by Koh et al. (2008) and Jacoby et al. (2010)) was also used during hypoxic exposure at 1% O2; 4h Hx + PX-478; 24h Hx + PX-478; 96h Hx + PX-478.

Each treatment group consisted of at least 4 samples from unique passages. At each time point cell culture plates from each treatment were removed from the incubator and immediately placed on ice. Media was collected in 1.5 mL micro-centrifuge tubes and the remaining media was aspirated off before the addition of 150 µL of ice cold Buffer A [10 mM Hepes pH 7.6. 10 mM KCl, 1 mM EDTA pH 8.0, EGTA pH 8.0, 1 mM DTT plus protease inhibitors (complete: Roche scientific)] onto each plate. Cultured cells were scraped off with a rubber policeman and collected into micro-centrifuge tubes before centrifugation at 13,000 rpm for 30s at 4**°**C, after which the supernatant was collected in a separate micro-centrifuge tube for cellular fractions containing everything but the nucleus. The remaining pellet was resuspended in 50 uL of Buffer C [20 mM pH 7.6. 0.4 NaCl, 1 mM EDTA pH 8.0, EGTA pH 8.0, 1 mM DTT, 5% glycerol and protease inhibitors (Complete; Roche scientific)] (Brown et al. 2008). The pellets were then stored frozen at -80**°**C until further analysis. The cellular lysate was then thawed on ice and centrifuged at 13,000 rpm for 5 min and the supernatant containing the nuclear fraction was collected into a new micro-centrifuge tube. Protein yield for both fragments was determined using a Bradford assay with bovine serum albumin standard curve (Bio-Rad Labrotories).

Lactate assays were performed in triplicate using both the cellular fraction and extracellular media by the hydrazine sink method to trap pyruvate and at pH 9.2 to favour lactate conversion to pyruvate. Ten µL of cellular fraction sample or cell culture media was combined with 10 µL of 12% perchloric acid to remove residual proteins, and this mixture was diluted in 50 µL of 2x GHE buffer (0.4M Hydrazine H2O, 1 M glycine, 2 mM EDTA, 1M KOH) plus 20 µL of nanopure dH2O. An endpoint reading was measured at absorbance of 340 nm at 37**°**C, after which the addition of 10 µL of 1 U/µL LDH was added and read every 10s during a kinetic run to ensure reaction completion where a final endpoint measurement was taken after 10 min.

**Enzyme Assays**

Lactate Dehydrogenase

Ten µL of the cellular fraction was added to of 180 µL of 40 mM Tris-HCl (pH 7.4) and 0.28 mM NADH. Samples were added with a final dilution between 50-100x and assays were initiated with the addition of 1mM sodium pyruvate and performed in triplicates plus a substrate only control to normalize background activity.

Citrate Synthase

Cellular fractions containing mitochondria were sonicated and freeze-thawed twice to lyse mitochondrial membranes. Ten µL of the cellular fraction was added to 180 µL of 40 mM Tris, 0.05% Triton X-100, 0.1 mM DTNB and 0.22 mM acetyl-CoA. Samples were added with a final dilution between 10-50x and assays were initiated with the addition of 50 mM oxaloacetate and performed in triplicates plus a substrate only control to normalize background activity.

**Western Blotting**

To determine protein expression 35 µg of total protein homogenized in buffer containing (in mM) 10 HEPES pH 7.6. 10 KCl, 1 EDTA pH 8.0, 1 EGTA pH 8.0, were used for Western blotting. To the homogenate, 2x Laemmli buffer (Bio-Rad) containing β-mercaptoethanol (1:20) was added and heated for 5 min at 95**°**C. PDK1 analysis was performed by loading 35 µg of protein into 30 µL wells against 5 µL of precision plus protein all blue prestained protein standard on a 4-15% gradient acrylamide gel (Bio-Rad) and run at 120v until the dye ran out of the gel. Gels were transferred onto a PVDF membrane using the Trans-Blot Turbo transfer system (Bio-Rad) at 25V and 2.5A for 7 min. The membrane was then washed with 1:1000 dilution of PDK1 primary antibody (Abcam: ab110025) overnight at 4**°**C followed by 1 x 15 min wash plus 3 x 5 min wash in TBS-T. Membranes were then incubated in 1:10000 secondary antibody goat anti-mouse IgG-HRP followed by 1 x 15 min wash plus 3 x 5 min wash in TBS-T. Antibody binding was imaged using Bio-Rad ChemiDoc Hi Resolution signal accumulation application. Images were collected just prior to signal saturation. The membranes were then rinsed with diH2O 1 x 15 min and 1 x 1 min before staining with coommassie blue (Bio-Rad) for 2 min. The membranes were then destained using 50% methanol and 1% acetic acid 3 x 3 min and then washed with 10% methanol for 3 min before being air dried to completion. Dried membranes were then imaged using Bio-Rad ChemiDoc colorimetric function. Band signal intensity was divided by its respective total lane protein intensity. The final relative expression was calculated by further dividing the sample band:lane signal intensity ratio by the positive control band:lane signal intensity ratio of a positive control lane on the same gel loaded with protein derived from C2C12 myotubes treated with 100 µM CoCl2 for the final relative expression.

**Real-time PCR**

Total RNA was isolated from cells in 6-well plates using Trizol reagent using the method recommended by the manufacturer (Life Technologies). Cell media was aspirated before the addition of 250 µL of Trizol to each well. Samples were then homogenized by drawing the Trizol containing cells through a 23-gauge needle and syringe and then transferred to RNase-free micro-centrifuge tubes. 100 µL of chloroform was added to each tube and vortexed for 15s and incubated at room temperature for 3 minutes. Tubes were then spun at 12,000 x g for 15 min at 4**°**C. The top aqueous phase was then transferred to a new tube and 1 µL of RNase free glycogen and 200 µL of isopropanol were added. Tubes were vortexed prior to freezing each sample at -80**°**C. Samples were thawed and spun at 12,000g for 1h at 4**°**C. The supernatant was removed, and the pellet was then rinsed with 500 µL of ice cold 75% ethanol prior to vortexing. Samples were then spun at 7,500 x g for 5 min at 4**°**C. The pellet was then rinse, vortexed and spun three consecutive times. The supernatant was removed using a vacuum pump attached to a sterile needle to remove residual ethanol. Samples were allowed to air dry for 5 min. 100 µL of PCR water (Sigma-Aldrich) was added before dissolving the pellet of each sample at 60**°**C for 10 min. Total RNA concentrations were then determined using ND-1000 spectrophotometer (Thermo-Fisher). An Invitrogen cDNAsynthesis kit was used to synthesize cDNA by starting with 8 µg of total RNA and adding 2 µL of primary master mix containing 1 µL of 10x reaction buffer, 1 µL of DNase I plus 1 µL of 25 mM EDTA per sample prior to heating at 70**°**C for 10 min. Samples were immediately placed on ice and then pulse spun. Addition of 2 µL of secondary master mix containing 1 µL of 0.5 µg/µL random hexamers plus 1 µL of 10 mM dNTP mix per sample, after which they were heated at 65**°**C for 5 min. Samples were immediately placed on ice for 10 min. The addition of 7.3 µL tertiary master mix was performed by combining 4 µL of 5x first strand buffer, 2 µL of 0.1 M DTT, 1 µL of RNase-Out plus 0.3 Super Script II (minus no reverse transcriptase control) to each sample. Samples were then heat cycled at 42**°**C for 5 min and 70**°**C for 10 min.

Primers were designed (Table 1) using Primer3 software (Rozen and Skaletsky 2000) and synthesized by Mobix Lab at McMaster University. Standard curves for each gene of interest was assessed using Real-time PCR of a common cDNA sample composed of samples from each treatment group and diluting to 5x, 10x, 20x, 40x, 80x, 160x and 640x. A master mix for each gene of interest was prepared by adding 5.5 µL of PCR H2O (Sigma-Aldrich), 1 µL of both forward and reverse primers from 100 µM stock plus 12.5 µL of SYBR-green (Bio-Rad). Reactions using 5 µL of the diluted common sample plus 20 µL of master mix were performed in triplicates and thermal cycled on the Bio-Rad CFX Connect Real-Time System in 96-well format elevated at a temperature of 95**°**C for 3 min before 40x between 95**°**C and 55**°**C for 10s each. After dilutions were confirmed, individual treatment samples were tested in triplicates in the same manner using the following primer sets: PHD2, HIF1-α. Calculation of mRNA expression was performed by log transformation of the difference between the sample averages and the y-intercept divided by the slope of the standard curve. Values were normalized against the respective treatments groups using the house-keeping gene, β-Actin to achieve relative expression (arbitrary units).

**Table 1**. Primer sequences used for mRNA quantification through real-time PCR in C2C12 cells. Primers were designed from cDNA sequences from the *Mus musculus* genome using Primer-BLAST.

|  |  |  |
| --- | --- | --- |
| **Gene** | **Forward Primer 5’ 🡪 3’** | **Reverse Primer 5’ 🡪 3’** |
| Actin | GTCGTACCACTGGCATTGG | AGGGCAACATAGCACAGCTT |
| PHD2 | GCCCAGTTTGCTGACATTGAAC | CCCTCACACCTTTCTCACCTG TTAG |
| HIF1-α | CAAGATCTGGCGAAGCAA | GGTGAGCCTCATAACAGAAGCTTT |

**Statistical analyses**

All data were analysed on Prism (version 5.0; Graph Pad. 2007). Treatment groups were measured using One-way ANOVA with Bonferonni’s selected pair post-hoc analysis; p<0.05. Selected pair analyses were measured as an effect of treatment where treatments were measured against time-matched controls, and as a measure of time within treatment groups. Data are reported as means ± s.e.m and a significance level of p < 0.05 was considered significant. A significant effect of treatment is denoted using an asterisk (\*) and a significant effect of time is denoted between columns using an octothorpe (#). In the analyses of lactate presence, the absolute values (µmol mg protein-1) were normalized by the time available to produce the lactate, which yields the rate of lactate accumulation (intracellular) or release (extracellular; µmol mg protein-1 hr-1). Enzyme assays producing activity level were also measured as a rate (µmol mg protein-1 min-1). Relative expression for real-time PCR was log10 transformed and expressed as arbitrary units (A.U.). Relative expression for western blots were log10 transformed for analysis (A.U.) using a Two-Way ANOVA. Different letters indicate a significant difference between treatments within a time-group.

**Results**

To determine if C2C12 cells in culture show a change in glycolytic flux we measured the rates of lactate accumulation within the intracellular (IC) contents and lactate released into the extracellular (EC) culture media. We found that after 4h at 1% O2 the rate of intracellular lactate accumulation was 5.5-fold greater compared to time-matched normoxic controls (Table 2A, Fig. 1A).

With what appears as changes in lactate production and PDK1 protein expression being driven by HIF1-α, we hypothesized that a change in metabolic regulation will occur due to hypoxic acclimation at the level of the cell where O2-delivery can be controlled. This would suggest that a change in metabolic regulation has occurred somewhere along the HIF signalling pathway. On the other hand, if metabolic regulation remains constant at the level of the cell, it would point to enhancements in O2 extraction or delivery has been improved in the hypoxic whole animal model. Our objective was to replicate the phenotype described in the lactate paradox and to compare our results more closely to those data from hypoxia acclimated CD-1 mice (Le Moine et al. 2011). We then used a pharmacological approach to either promote the HIF response during normoxia or inhibit the HIF response during hypoxia to identify transitionary features of hypoxic acclimation.

*Long-term hypoxia reduces intracellular and extracellular lactate accumulation*

Since one of the ways that acute hypoxia influences muscle metabolism is by increasing glycolytic flux rates, we examined the rate of lactate accumulation within C2C12 cells (Table 2, Fig. 1A) and in the extracellular media (Table 2, Fig. 1B). We found 4h Hx (0.138 ± 0.008 µmol mg protein-1hr-1) caused a 5.5-fold increase in IC lactate accumulation compared to time-matched normoxic controls (0.025 ± 0.012 µmol mg protein-1hr-1; p>0.05). 4h Hx also showed significant elevations against 24h Hx (0.018 ± 0.003 µmol mg protein-1hr-1; p>0.05), and 96h hypoxic (0.024 ± 0.002 µmol mg protein-1hr-1; p>0.05). This was accompanied by a 29% increase in extracellular lactate accumulation in 4h Hx cultures (0.069 ± 0.002 µmol mg protein-1hr-1) compared to their normoxic controls (0.047 ± 0.003 µmol mg protein-1hr-1; p>0.05), and a significant elevation against 24h Hx (0.025 ± 0.002 µmol mg protein-1hr-1; p>0.05) and 96h Hx (0.012 ± 0.001 µmol mg protein-1hr-1; p>0.05) groups. When expressed as a ratio of intracellular to extracellular lactate accumulation (Table 3, Fig. 2), the 4h Hx (2.004 ± 0.154) maintains a significant elevation over its time-matched normoxic control (0.58 ± 0.323; p>0.05), as well as significant elevations over both 24h hypoxic (0.734 ± 0.054; p>0.05) and 96h Hx (1.115 ± 0.031; p>0.05) groups. To shed light on the potential role of HIF signalling on elevated lactate accumulation during acute hypoxia, we performed pharmacological experiments to alter the hypoxic response, aimed at manipulating HIF1-α. Using the same time-course trials, we dosed the cell culture media with known HIF-mediators to either promote HIF function using 1 mM DMOG in normoxia, or to inhibit HIF function using 25 µM of PX-478 in hypoxia, to see how hypoxia signalling impacted lactate accumulation. We found a significant increase in intracellular lactate accumulation at 4h Nx + DMOG (0.163 ± 0.031 µmol mg protein-1hr-1) compared to 24h Nx + DMOG (0.016 ± 0.002 µmol mg protein-1hr-1; p>0.05) and 96h Nx + DMOG (0.043 ± 0.004 µmol mg protein-1hr-1; p>0.05). The same trend was observed in the extracellular media, where 4h Nx + DMOG (0.093 ± 0.002 µmol mg protein-1hr-1) were significantly greater than both 24h Nx + DMOG (0.013 ± 0.001 µmol mg protein-1 hr-1; p>0.05) and 96h Nx + DMOG (0.034 ± 0.003 µmol mg protein-1hr-1; p>0.05). In addition, intracellular lactate accumulation in 4h Hx + PX-478 (0.070 ± 0.01 µmol mg protein-1 hr-1) showed significant elevations compared to 24h Hx + PX-478 (0.026 ± 0.002 µmol mg protein-1 hr-1; p>0.05) and 96h PX-478 (0.01 ± 0.002 µmol mg protein-1 hr-1; p>0.05), and extracellular lactate accumulation was significantly higher in 4h Hx + PX-478 (0.124 ± 0.008 µmol mg protein-1 hr-1)compared to 24h Hx + PX-478 (0.044 ± 0.004 µmol mg protein-1 hr-1; p>0.05) and 96h PX-478 (0.014 ± 0.003 µmol mg protein-1 hr-1; p>0.05). No change was observed between intracellular to extracellular lactate accumulation ratios.

*LDH:CS enzyme flux remains constant throughout hypoxia*

To help explain the biochemical underpinnings of these results, we looked at the enzymatic activities of both LDH as a marker of pyruvate to lactate capacity flux (Table 4, Fig. 3A) and CS as a marker of mitochondrial volume density (Table 5, Fig. 3B). LDH activity was augmented in each hypoxic treatment group in comparison to the respective time-match controls. 4h Hx LDH activity (4231.61 ± 561.42 µmol mg protein-1 min-1) was 3.4-fold greater in comparison to the time-matched 4h Nx control (1245.38 ± 0.205 µmol mg protein-1 min-1; p>0.05). This was mirrored by a 3.5-fold increase from 24h Nx (994.58 ± 82.26 µmol mg protein-1 min-1) to 24h Hx (3497.93 ± 299.92 µmol mg protein-1 min-1; p>0.05)as well as a 2.5-fold increase from 96h Nx (4189.82 ± 455.97 µmol mg protein-1 min-1) to 96h Hx (1668.34 ± 153.49 µmol mg protein-1 min-1; p>0.05). No effect of time was observed in hypoxia.

No significant differences were observed between any time points when cultures were treated with 1 mM DMOG. However, when cultures were treated under hypoxia with 25 mM PX-478, the 4h group (4661.18 ± 597.15 µmol mg protein-1 min-1) displayed a 2.97-fold increase compared to the 24h group (1848.58 ± 415.60 µmol mg protein-1 min-1; p>0.05). No significant difference was observed between the 4h Hx + PX-478 and the 96h Hx + PX-478.

A 3.5-fold increase in CS activity was observed from 4h Nx (65.15± 8.60 µmol mg protein-1 min-1) to 4h Hx (228.02± 18.95 µmol mg protein-1 min-1; p>0.05), and a 2.14-fold change was observed from 96h Nx (96.09 ± 2.06 µmol mg protein-1 min-1) to 96h Hx (204.90 ± 17.23 µmol mg protein-1 min-1; p>0.05). No significant differences emerged between 24h Nx and 24h Hx, nor were any significant changes observed between hypoxic time-points. Moreover, pharmacological treatments across time points using either 1 µM of DMOG or 25 mM PX-478 revealed no effect of time.

It is noteworthy however, that the LDH:CS ratio (Table 6, Fig. 4) is maintained between normoxia and hypoxia, as well as across all hypoxic time-points. This is also true for cultures treated with 1 mM DMOG and 25 mM PX-478.

*PDK1 protein expression*

PDK1 is a key regulatory kinase capable of directing the fate of pyruvate to acetyl Co-A and entering the TCA cycle or being directed to lactate. Western blot assays (Table 7, Fig. 5) revealed a significant 3.3-fold increase in relative expression from 4h Nx (0.403 ± 0.14 A.U.) to 4h Hx (1.35 ± 0.126 A.U.; p>0.001).

Treatment of cultures with 1 mM of DMOG significantly increased PDK1 expression across all time-points (4h, 2.883 ± 0.805 A.U. p>0.001; 24h, 2.39 ± 0.0.231 A.U. p>0.01; 96h, 4.343 ± 1.044 A.U. p>0.001) when compared to normoxic groups and within timed-treatment. In addition, a 2.2-fold increase was observed from 24h Nx + DMOG to 96h Nx + DMOG (p>0.001). Treatment with PX-478 in hypoxic cultures blunted the hypoxic effect as no significant changes emerged between this group and time-matched normoxic controls.

*Gene regulation of hypoxic response elements is unchanged throughout hypoxia*

To see how the regulation of the hypoxic response changed throughout hypoxia acclimation *in vitro*, we examined transcript levels of the negative HIF-regulator PHD2 (Table 8, Fig. 6A). A significant upregulation of PHD2 transcript abundance was observed at 96h Hx (8.056 ± 1.45 A.U.) compared to 96h Nx (3.529 ± 0.668 A.U.; p>0.05). Significant differences were also observed when compared to 4h Hx (2.737 ± 0.689 A.U.), 24h Hx (4.012 ± 0.375A.U.; p>0.05) and 96h Nx control (3.529 ± 0.668 A.U. ; p>0.05). HIF1-α transcript abundance was also different at 96h Hx (2.398 ± 0.316 A.U.), showing a significant reduction when compared to 96h Nx control (5.391± 1.386 A.U.; Table 8, Fig. 6B; p>0.05).

**Table 2.** Rates of lactate appearance within C2C12 myotubes and in the extracellular media (µmol mgprotein-1 hr-1).

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Intracellular** | | | | **Extracellular** | | | |
|  | **4h** | **24h** | **96h** | **4h** | **24h** | **96h** |
| Nx | 0.025 ± 0.012 | 0.018 ± 0.001 | 0.007 ± 0.002 | 0.047 ± 0.003 | 0.025 ± 0.001 | 0.012 ± 0.001 |
| Hx | 0.138 ± 0.008\*,# | 0.029 ± 0.002 | 0.024 ± 0.002 | 0.069 ± 0.002\*,# | 0.028 ± 0.002 | 0.022 ± 0.001 |
| Nx + DMOG | 0.163 ± 0.031\*,# | 0.016 ± 0.002 | 0.043 ± 0.004 | 0.093 ± 0.02\*,# | 0.013 ± 0.001\*,# | 0.034 ± 0.003 |
| Hx + PX-478 | 0.07 ± 0.01\*,# | 0.026 ± 0.002 | 0.01 ± 0.002 | 0.124 ± 0.009\*,# | 0.044 ± 0.004 | 0.014 ± 0.003 |

Values are expressed in µmol mg protein-1 hr-1 ± s.e.m. \* indicates a significant change from control and # indicates a difference of time (ANOVA; Bonferroni’s selected pairs post-hoc; p<0.05).

**Table 3.** Ratio of intracellular to extracellular lactate appearance (no units).

|  |  |  |  |
| --- | --- | --- | --- |
|  | **4h** | **24h** | **96h** |
| Nx | 0.58 ± 0.323 | 0.734 ± 0.054 | 0.572 ± 0.12 |
| Hx | 2.00 ± 0.154\*,# | 0.903 ± 0.131 | 1.115 ± 0.031 |
| Nx + DMOG | 1.779 ± 0.172\* | 1.248 ± 0.105 | 1.301 ± 0.212 |
| Hx + PX-478 | 0.583 ± 0.121 | 0.592 ± 0.049 | 0.857 ± 0.184 |

Values are expressed as mean ± s.e.m. \* indicates a significant change from control and # indicates a difference of time (ANOVA; Bonferroni’s selected pairs post-hoc; p<0.05).

**Table 4.** Rates of LDH enzyme activity (µmol mgprotein-1 min-1).

|  |  |  |  |
| --- | --- | --- | --- |
|  | **4h** | **24h** | **96h** |
| Nx | 1245.38 ± 248.70 | 994.58 ± 82.46 | 1668.38 ± 62.66 |
| Hx | 4231.61 ± 561.42\* | 3497.93 ± 299.92\* | 4189.82 ± 455.97\* |
| Nx + DMOG | 2245.74 ± 214.81 | 2634.45 ± 91.94 | 3170.05 ± 588.26 |
| Hx + PX-478 | 4661.18 ± 597.15\* | 1848.58 ± 496.59 | 4278.42 ± 1086.83 |

Values are expressed in µmol mg protein-1 hr-1 ± s.e.m. \* indicates a significant change from control and # indicates a difference of time (ANOVA; Bonferroni’s selected pairs post-hoc; p<0.05).

**Table 5.** Rates of CS enzyme activity (µmol mgprotein-1 min-1).

|  |  |  |  |
| --- | --- | --- | --- |
|  | **4h** | **24h** | **96h** |
| Nx | 65.15 ± 8.61 | 226.01 ± 11.71 | 96.09 ± 2.06 |
| Hx | 228.02 ± 18.95\* | 190.97 ± 11.59 | 204.9 ± 17.23\* |
| Nx + DMOG | 155.36 ± 20.52\* | 142.64 ± 13.41\* | 118.41 ± 39.75 |
| Hx + PX-478 | 180.18 ± 24.22\* | 147.93 ± 11.86\* | 122.78 ± 32.75 |

Values are expressed in µmol mg protein-1 hr-1 ± s.e.m. \* indicates a significant change from control and # indicates a difference of time (ANOVA; Bonferroni’s selected pairs post-hoc; p<0.05).

**Table 6.** Ratio of LDH to CS enzyme activity (no units).

|  |  |  |  |
| --- | --- | --- | --- |
|  | **4h** | **24h** | **96h** |
| Nx | 22.87 ± 7.62 | 4.54 ± 0.57 | 17.36 ± 0.52 |
| Hx | 20.18 ± 4.62 | 18.47 ± 1.60 | 21.52 ± 3.31 |
| Nx + DMOG | 14.77 ± 0.9 | 19.06 ± 1.45 | 39.45 ± 18.92 |
| Hx + PX-478 | 26.5 ± 2.32 | 9.55 ± 3.63 | 35.11 ± 4.37 |

Values are expressed as mean ± SEM. \* indicates a significant change from control and # indicates a difference of time (ANOVA; Bonferonni’s selected pairs post-hoc; p<0.05).

**Table 7.** Relative PDK1 protein expression (relative units)

|  |  |  |  |
| --- | --- | --- | --- |
|  | **4h** | **24h** | **96h** |
| Nx | 0.402 ± 0.137a | 0.808 ± 0.203e | 0.814 ± 0.220g |
| Hx | 1.35 ± 0.126b | 0.717 ± 0.294e | 1.04 ± 0.119g |
| Nx + DMOG | 2.883 ± 0.805b,c | 2.391 ± 0.228f | 4.343 ± 2.087h |
| Hx + PX-478 | 0.795 ± 0.177a,b | 0.583 ± 0.086e | 0.910 ± 0.058g |

Different letters indicate a significant difference between treatments within time-groups. (Two-way ANOVA; p<0.05).

**Table 8.** Relative mRNA gene expression of PHD2 and HIF1-α (no units).

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **PHD2** | | | | **HIF1-α** | | |
|  | **4h** | **24h** | **96h** | **4h** | **24h** | **96h** |
| Nx | 3.401 ± 0.562 | 3.73 ± 0.150 | 3.529 ± 0.668 | 4.667 ± 0.508 | 3.582 ± 0.416 | 5.392 ± 1.386 |
| Hx | 2.737 ± 0.690\*,# | 2.605 ± 0.399 | 8.056 ± 1.450 | 2.195 ± 0.553\*,# | 2.605 ± 0.399 | 2.398 ± 0.316 |

Values are expressed mean ± SEM. \* indicates a significant change from control and # indicates a difference of time (ANOVA; Bonferroni’s selected pairs post-hoc; p<0.05).

**Figure 1. Lactate concentration expressed as an hourly rate of appearance** in A) cellular fractions tested at 4h in normoxia (n=6), hypoxia (n=6), normoxia + 1 mM DMOG (n=4), hypoxia + 25 µM PX-478, at 24h normoxia (n=6), hypoxia (n=7), normoxia + 1 mM DMOG (n=6), hypoxia + 25 µM PX-478 (n=7) and at 96h, normoxia (n=6), hypoxia (n=6), normoxia + 1 mM DMOG (n=4), hypoxia + 25 µM PX-478 (n=5); B) extracellular media, tested at 4h in normoxia (n=6), hypoxia (n=6), normoxia + 1 mM DMOG (n=4), hypoxia + 25 µM PX-478, at 24h normoxia (n=6), hypoxia (n=7), normoxia + 1 mM DMOG (n=6), hypoxia + 25 µM PX-478 (n=7) and at 96h, normoxia (n=6), hypoxia (n=6), normoxia + 1 mM DMOG (n=4), hypoxia + 25 µM PX-478 (n=5). Data was normalized per mg of protein in all cells used for measurement. White bars indicate normoxia, black bars indicate hypoxia, light gray bars indicate normoxia + 1 mM DMOG, and dark gray bars indicate hypoxia + 25 µM PX-478. Values are expressed as means ± SEM. \* indicates a significant difference from time-matched control. # indicates significant effect of treatment over time (One-way ANOVA; p<0.05).

**A)**



**B)**



**Figure 2. Ratio of intracellular to extracellular lactate appearance rate in C2C12 myotubes** tested at 4h in normoxia (n=6), hypoxia (n=6), normoxia + 1 mM DMOG (n=4), hypoxia + 25 µM PX-478, at 24h normoxia (n=6), hypoxia (n=7), normoxia + 1 mM DMOG (n=6), hypoxia + 25 µM PX-478 (n=7) and at 96h, normoxia (n=6), hypoxia (n=6), normoxia + 1 mM DMOG (n=4), hypoxia + 25 µM PX-478 (n=5).. White bars indicate normoxia, black bars indicate hypoxia, light gray bars indicate normoxia + 1 mM DMOG, and dark gray bars indicate hypoxia + 25 µM PX-478. Values are expressed as means ± SEM. \* indicates a significant difference from time-matched control. # indicates significant effect of treatment over time (One-way ANOVA; Bonferroni’s selected pairs post-hoc; p<0.05).



**Figure 3. Enzyme activity in C2C12 myotubes of A) LDH and B) CS**, tested at 4h in normoxia (n=6), hypoxia (n=6), normoxia + 1 mM DMOG (n=4), hypoxia + 25 µM PX-478, at 24h normoxia (n=6), hypoxia (n=7), normoxia + 1 mM DMOG (n=6), hypoxia + 25 µM PX-478 (n=7) and at 96h, normoxia (n=6), hypoxia (n=6), normoxia + 1 mM DMOG (n=4), hypoxia + 25 µM PX-478 (n=3); B) LDH, tested at 4h in normoxia (n=6), hypoxia (n=6), normoxia + 1 mM DMOG (n=4), hypoxia + 25 µM PX-478, at 24h normoxia (n=6), hypoxia (n=7), normoxia + 1 mM DMOG (n=6), hypoxia + 25 µM PX-478 (n=7) and at 96h, normoxia (n=6), hypoxia (n=6), normoxia + 1 mM DMOG (n=4), hypoxia + 25 µM PX-478 (n=5). Data was normalized per mg of protein in all cells used for measurement. White bars indicate normoxia, black bars indicate hypoxia, light gray bars indicate normoxia + 1 mM DMOG, and dark gray bars indicate hypoxia + 25 µM PX-478. Values are expressed as means ± SEM. \* indicates a significant difference from time-matched control. # indicates significant effect of treatment over time (One-way ANOVA; p<0.05 Bonferroni’s selected pairs post-hoc; p<0.05).

**A**



**B)**

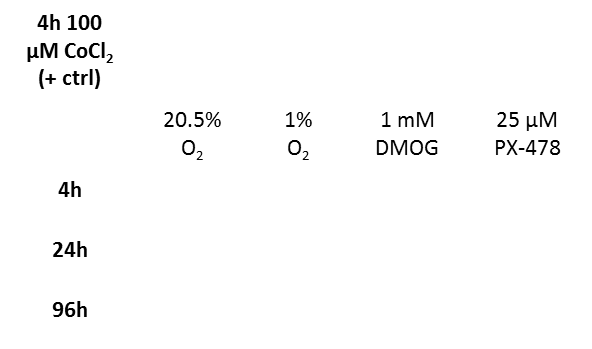


**Figure 4. Ratio of LDH to CS enzyme activity in C2C12 myotubes** tested at 4h in normoxia (n=6), hypoxia (n=6), normoxia + 1 mM DMOG (n=4), hypoxia + 25 µM PX-478, at 24h normoxia (n=6), hypoxia (n=7), normoxia + 1 mM DMOG (n=6), hypoxia + 25 µM PX-478 (n=6) and at 96h, normoxia (n=6), hypoxia (n=7), normoxia + 1 mM DMOG (n=4), hypoxia + 25 µM PX-478 (n=3).. White bars indicate normoxia, black bars indicate hypoxia, light gray bars indicate normoxia + 1 mM DMOG, and dark gray bars indicate hypoxia + 25 µM PX-478. Values are expressed as means ± SEM. \* indicates a significant difference from time-matched control. # indicates significant effect of treatment over time (One-way ANOVA; Bonferroni’s selected pairs post-hoc; p<0.05).



**Figure 5. Western blot protein expression in C2C12 myotubes** A) as representative blots and B) relative expression, determined at 4h in normoxia (n=6), hypoxia (n=6), normoxia + 1 mM DMOG (n=4), hypoxia + 25 µM PX-478, at 24h normoxia (n=6), hypoxia (n=5), normoxia + 1 mM DMOG (n=5), hypoxia + 25 µM PX-478 (n=6) and at 96h, normoxia (n=6), hypoxia (n=6), normoxia + 1 mM DMOG (n=4), hypoxia + 25 µM PX-478 (n=4). PDK1 protein expression levels expressed against total lane protein and normalized against PDK1 protein expression of the positive control PDK1 expression against total lane protein in HIF-stimulated C2C12 treated with 100 µM of CoCl2 for 4h. White bars indicate normoxia, black bars indicate hypoxia, light gray bars indicate normoxia + 1 mM DMOG, and dark gray bars indicate hypoxia + 25 µM PX-478. Values are expressed as mean ± SEM and were log10 transformed for analysis. Different letters indicate a significant difference between treatments within time-groups. (Two-way ANOVA; p<0.05).

**A)**



**B)**



**Figure 6. Real-time PCR mRNA expression of PHD2, and HIF1-α in C2C12 myotubes** acclimated to normoxia (20.95% O2) or hypoxia (1% O2) at 4h, 24h or 96h. n=4.mRNA levels expressed relative to β-Actin mRNA and normalized to control values for A) PHD2, and B) HIF1-α. White bars indicate normoxia, black bars indicate hypoxia. Values are expressed as mean ± SEM. \* indicates a significant difference from time-matched control. # indicates significant effect of treatment over time (One-way ANOVA; Bonferroni’s selected pairs post-hoc; p<0.05).





**Discussion**

We report an increase in the rate of lactate accumulation and release by C2C12 myotubes after 4h of hypoxia, which declines at 24h and 96h of hypoxia. This is consistent with our current understanding that muscle cells are major contributors to hypoxia-mediated lactate accumulation in the blood *in vivo* and with the lactate paradox phenomenon. These findings correlate with both maximal LDH enzyme activity and with PDK1 protein expression. Induction of HIF activity using DMOG increased PDK1 expression and lactate accumulation in 4h normoxic cells. However, despite elevated PDK1 levels at 24h and 96h, lactate levels were no different than in normoxic controls. Inhibition of HIF activity using PX-478 in 4h Hx cells eliminated the hypoxic induction of PDK1 expression and IC/EC lactate levels no different compared to time-matched control. PDK1 expression and lactate accumulation remained at controls levels in 24h and 96h Hx cells treated with PX-478. These data suggest a role for HIF signalling in the decline in lactate with chronic hypoxia, but that PDK1 alone cannot explain the reductions in lactate accumulation over time.

Lactate is the metabolic by-product of anaerobic glycolysis and functions by reducing pyruvate and regenerating NAD+ in the process. The LDH isoform that predominates in muscle (LDHa) has a high affinity for pyruvate and NADH, favoring lactate formation. Product inhibition may otherwise impair this process if not for efflux of lactate out of the cell. The transport of lactate is facilitated by a the family of monocarboxylate transporters (MCTs), and the MCT4 isoform is the predominant variant present in skeletal muscle (McClelland and Brooks 2002). In the whole animal, this allows lactate to be expelled into the blood where it can be taken up in the liver for gluconeogenesis. These processes extend the utility of anaerobic glycolysis, but can deplete muscle glycogen stores and cause lactic acidosis in the blood (Hochachka and Mommsen 1983).

Hypoxia stimulates LDH enzyme activity (Fig. 3A), but only relates to lactate accumulation at 4h Hx. CS enzyme activity (Fig. 3B) also appears to be stimulated by hypoxia and, remained relatively constant across all time groups. CS enzyme activity is a broad marker of aerobic metabolism, but it does not directly measure the rate of aerobic metabolism. This does however suggest that the enzymatic capacity of CS is unaffected by hypoxia or by our pharmacological treatments. Taken together, hypoxia acclimation in C2C12 cells may even shift cellular metabolism towards a more oxidative phenotype, where if PDH is in its active form, can adequately oxidize pyruvate to acetyl CoA for entry into the Krebs cycle. This would be consistent with the idea of improved ATP supply-demand coupling, whereby a shift to more aerobic means of ATP generation would reduce swings of high energy phosphates that stimulate glycolytic flux (Hochachka, 1988). This should be more achievable in sedentary myotubes, as ATP demand is not expected to rise through contraction-induced stimulation where aerobic demands are minimal. However, continual elevations in LDH activity during hypoxia might suggest a priming mechanism to supply ATP if demand suddenly and unexpectedly rises in hypoxia. Such might be the case in working muscle *in vivo*.

Given PDK1s ability to phosphorylate PDH (Brown et al. 1992), we hypothesized that changes observed in metabolic activity would correlate with PDK1 protein expression. We observed a significant upregulation in PDK1 protein expression at 4h Hx and 4h Nx + DMOG compared to time-matched controls. Interestingly, PDK1 returns to normoxic levels at 24h and 96h of hypoxia, but DMOG treatment preserves PDK1 expression. At 4h Hx, lactate increases correspond with increases in PDK1 protein expression. This supports our hypothesis that PDK1 expression would be immediately stimulated upon hypoxic exposure, favouring lactate formation. The same is true in HIF-stabilized DMOG cell in normoxia at 4h. However, DMOG-induced PDK1 expression can not explain changes in the rate of lactate accumulation at 24h and 96h, meaning that the induction of PDK1 alone is likely not what is driving changes in lactate accumulation. This may mean that despite PDK1’s potential to severely impair aerobic respiration that DMOG does not stimulate glycolytic flux in the way hypoxia does, or that other compensatory mechanisms to maintain active PDH are at play. One such mechanism may be through the cellular energy sensory, AMP-activated protein kinase (AMPK) which is stimulated by elevated levels of ADP, AMP and Pi compared to ATP (Hardie et al. 2006), which may override PDK1-dampening of PDH. Moreover, it is possible that HIF2-α may play a role in the transition from acute to chronic hypoxia acclimation, but it may not be as important as HIF1-α is in the skeletal muscle.

A significant rise in the rate of lactate accumulation was observed in PX-478 cells in hypoxia at 4h, but it wasn’t as pronounced as with hypoxia alone and the ratio of IC:EC lactate accumulation remains the lowest at all time points in this treatment group. This corresponds to no change in LDH activity compared to 4h Nx. This suggests that HIF-1α antagonism resulting in a repression of PDK1 is perhaps blunting the drive for anaerobic metabolism despite sudden exposure to hypoxia, and preventing lactate from accumulating in excess. This is consistent with findings from Seagroves and colleagues (2001) who report overall reduced lactate production in mouse embryonic fibroblasts in hypoxia with knock down of HIF1-α. It may be that high glucose culture media is likely protective from the sudden onset of hypoxia without HIF1-α mediating a change in metabolism. Total knockout of HIF1-α should be cautioned in as this is reported to cause extensive muscle damage after exercise in mice (Mason et al. 2007) where presumably damaged blood vessels disrupt O2 diffusion to repairing muscle beds. HIF-antagonism using PX-478 however, may prove to be a useful in combatting lactic acidosis through HIF-antagonism in mountaineers at rest, and perhaps in the tumours of cancer patients. Supposing however that the cell still remains hypoxic, one might anticipate an accumulation of free radicals within the mitochondria under chronic exposure of PX-478.

To gain an understanding of how HIF-signalling might be regulated throughout hypoxia, we performed real-time PCR for PHD2 (Fig.6a) and HIF1-α (Fig. 6b). As HIF1-α protein is constitutively made and degraded in the cytosol (Favier et al. 2015), we did not expect to see any significant changes in HIF1-α mRNA expression. An effect of treatment was observed at 96h, where HIF1-α protein expression may negatively feedback to reduce transcription, but no effect of time was observed in either normoxic or hypoxic treated samples. One way that HIF1-α is degraded is through ubiquitination by PHD2 (Ginouvès et al. 2008). We did see a significant effect of treatment for PHD2 at 96h of hypoxia when compared to its time-matched control, as well as an effect of time when compared to 4h and 96h Hx. It is interesting to note however that PHD2 mRNA expression is only significantly upregulated at the 96h Hx mark. This might indicate that there exists a latency to promote PHD2 transcription that subsequently degrades HIF1-α. This would support our hypothesis that HIF activity affecting lactate accumulation is alternatively regulated in chronic hypoxia.

Our results show that the manifestation of the lactate paradox occurs at the cellular level and that muscle cells are large contributors of this phenomenon. We provide evidence that HIF signalling influences lactate accumulation, but that these changes are not exclusively coordinated by PDK1. Future studies would benefit from investigating how AMPK activity responds to hypoxia-induced declines in cellular energy, and to see if this is capable of overriding the PDK1 inhibition of aerobic metabolism.

**Chapter Three: General Discussion**

Researchers have long been interested in the response to environmental hypoxia and how phenotypic changes help compensate for a cascade of reduced O2 tension from the level of the pulmonary arteries, down to the mitochondria. This is especially true given the popularity of climbing, interest in evolutionary adaptations of animals living in alpine regions and diseases that impair tissue O2 delivery.

The reduction in exercise-induced blood lactate accumulation from acute to chronic hypoxia, known as the lactate paradox, has been observed for over 80 years. Past studies looking at this phenomenon in human test subjects have struggled to find a consensus on the underlying mechanisms that blunt lactate formation. However, experimental reproducibility in mountaineering expeditions have been identified as a flaw in our comprehension of this physiological problem. These objections include, normalized fitness of test subjects through acclimatization (MacRae et al. 1992), failure to maintain volitional exhaustion at altitude between tests (Wagner and Lundby 2007), consistent fraction of inspired O2 across studies (Wagner and Lundby 2007), as well as an overall reduced muscle mass emitting proportional reductions in lactate (West et al. 1983). Our cell culture approach stands contrary to these objections as our myotubes were unstimulated in an environment where O2 diffusion was controlled for and that lactate accumulation was measured as a function of protein mass.

One hypothesis links the correlation between reduced blood lactate concentrations with reduced sympathetic activity after chronic hypoxia, and thus reduced rates of glycolysis (Brooks et al. 1992). Though the two are correlated, blockade of sympathetic activity cannot fully prevent a progressive decline in blood lactate in exercising humans exercised at altitude (Mazzeo et al. 1994). It also does not account for molecular changes occurring in the whole animal, such as the blunted levels HIF1-α and PDK1 after hypoxia acclimation in work performed on mice (Le Moine et al. 2011). These results are supportive of changes in hypoxia sensing within the muscle. In a cell culture approach, our findings reveal a dynamic transition in lactate accumulation from acute to chronic hypoxia in a model that is independent of hormonal or neural stimulation. It would be interesting to further challenge this hypothesis by adrenergically stimulating cultured myotubes throughout hypoxic exposure and measuring rates of lactate accumulation. If doing so increased lactate accumulation in chronic hypoxia as is observed in acute 4h hypoxia, then it would challenge the causal relationship appointed by this hypothesis.

Though lactate production occurs even under aerobic metabolism (Brooks, 2016), the rate at which it is produced depends heavily on glycolytic flux as well. It also depends on LDH activity and the availability of NADH as an oxidizing agent of pyruvate under anaerobic conditions. LDH is a two-way enzyme, but the muscle isoform (LDHa) has a low Km for pyruvate and NADH, favouring the reduction of pyruvate and producing reducing equivalent in the form of NAD+. This allows continued output of anaerobic glycolysis, while continued ATP usage and lactate efflux from the cell promote a more unidirectional flux of glycolysis, helping to maintain cellular energy homeostasis. The transport of lactate out of the cell is made possible by the transmembrane monocarboxylate transporter (MCT) protein isoform 4. MCT4 is the predominating isoform in glycolytic muscles favouring lactate efflux from the cell (McClelland and Brooks 2002). Ullah and colleagues (2006) found that hypoxia stimulated an increase in mRNA, promoter and protein expression of MCT4 in both HeLa and COS cells. While the function of MCT4 was not directly measured, the lactate present in the extracellular media is a good indication of whether the myotubes cope with the build up of this metabolic by-product. Lactate sequestration influences cellular redox state, either through its oxidation to pyruvate, or through ROS formation (George A. Brooks 2009). The former is not likely the case as LDHa has a high affinity for pyruvate, and changes in the rates of lactate accumulation follow the same trend as with changes in the rates lactate release in all treatment groups.

Lactate, in the presence of high glucose can promote ROS formation, causing oxidative damage and mitophagy (Papandreou et al. 2006; Favier et al. 2015). To deal with this, HIF1-α promotes a cytochrome c oxidase (COX) complex isoform switch from COX4-1 to COX4-2 which helps reduce ROS production through improved electron transfer (Fukuda et al. 2007). We show that HIF signalling is likely reduced, owing to the observed PDK1 downregulation, and that this may be a function of altered HIF regulation through the upregulation of PHD2 transcription. This may suggest that a reduction in lactate production plays a more crucial role in curtailing ROS production than the isoform shift of COX4-1 to COX4-2 driven by HIF activity.

There appear to be clear differential changes occurring throughout hypoxic exposure. A change in phenotype is most notable at 4h of hypoxia before an apparent return to levels similar to normoxic controls. Clear phenotypic changes are observed across various hypoxic time points, likely owing to changes in HIF regulation driving metabolic changes. PDK1 is specifically induced by HIF and plays a significant role in the determination of metabolic fate by influencing mitochondrial function (Papandreou et al. 2006). We predicted that PDK1 would be elevated in acute hypoxia and diminish thereafter as observed in CD-1 mice (Le Moine et al. 2011). Hypoxia at 4h significantly stimulated PDK1 expression, though to a much lesser magnitude than that observed in all DMOG-treated time-points. However, mitochondria of human colon carcinoma cells have been reported to be highly sensitive to changes in PDK1 levels where even slight increases reduced oxygen consumption (Papandreou et al. 2006). We did not measure oxygen consumption, but the gravity of lactate accumulation at 4h hypoxia compared to 24h and 96h hypoxia provides a broad indication of changing oxygen utilization.

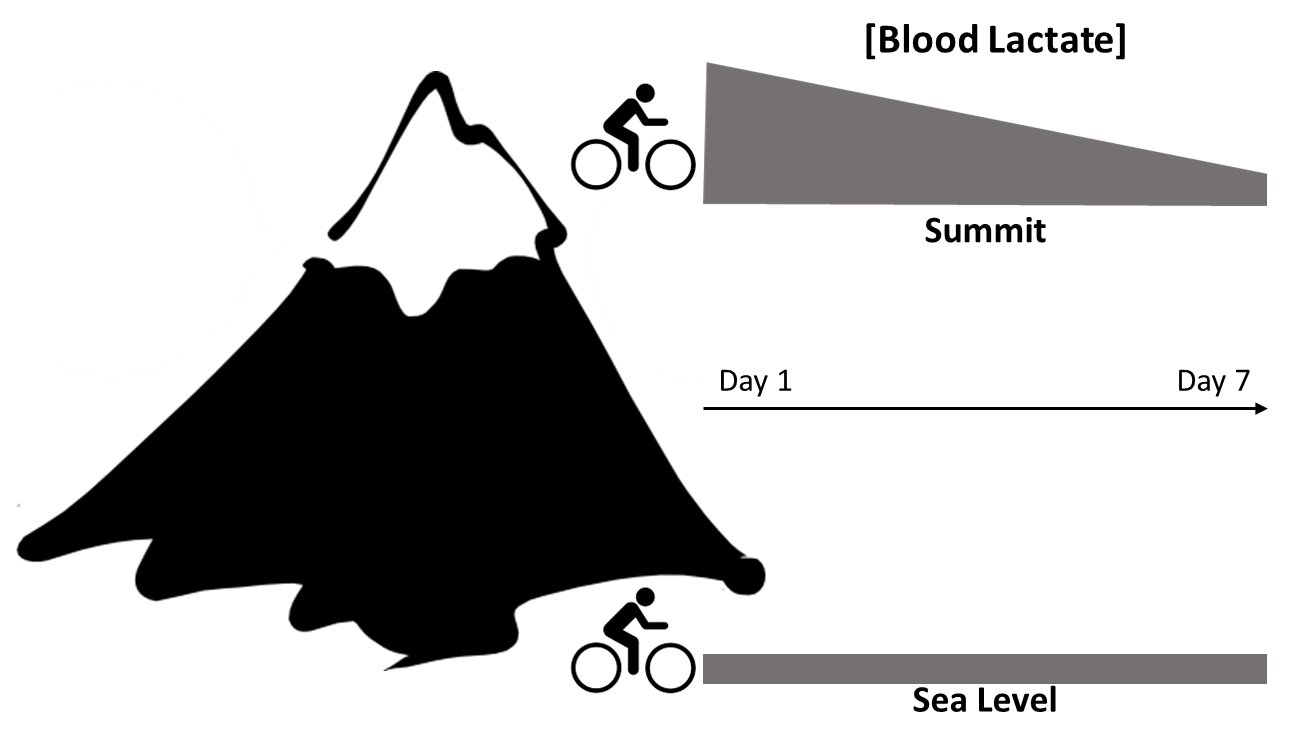
Knockout of HIF1-α is reported to reduce intracellular lactate levels in mouse embryonic fibroblasts cells grown in hypoxia (Seagroves et al. 2001). HIF-antagonism using PX-478 under hypoxia was the only group to show no changes in intracellular to extracellular lactate ratio across all time points, which lends support for reductions in total lactate being a function of dampened HIF signaling. DMOG-induced HIF activity at equal time points showed tonic upregulation of PDK1 expression but this treatment was not successful in driving lactate production at all time points. This suggests that PDK1 does not act alone in driving lactate production and that other energy-state sensors are at play between anaerobic and aerobic metabolic flux.

To gain more insight into how the hypoxic phenotype emerges, we pursued real-time PCR mRNA expression of HIF1-α and its negative regulator, PHD2. An effect of treatment was observed at 96h, which may suggest that translation of HIF1-α negatively feeds back to reduce its transcription. We did not however, observe an effect of time in HIF1-α mRNA, likely due in part by its regulation through post-translational ubiquitination (Favier et al. 2015). This supports the notion that constitutive HIF1-α mRNA presence in the cytosol is critically important for responding to sudden and unexpected drops in O2 availability, where hydroxylation of HIF1-α continuously prevents its transcriptional activity under O2-saturating conditions. We demonstrated and increased PHD2 transcriptional activity at 96h Hx compared to its time-matched control and to 4h Hx. However, another possibility of altering HIF activity is through action on the von Hippel Lindau complex. Transcript abundance of this unit of the proteasome pathway has been reported to increase in short-term hypoxia (Panepistēmio tēs Krētēs. et al. 2014). Moreover, Arthur and colleagues (2000) report that under hypoxia the rate of protein synthesis in C2C12 cells is unaffected when hypoxia was defined as 10 µM O2. They suggest that because oxygen consumption appears to be reduced, that mRNA/DNA synthesis may be down-regulated in favour of more critical cellular functions. If this is to be true, it is interesting that increased PHD2 transcription did not occur at 24 Hx, especially since PDK1 and lactate accumulation had already subsided by then. This might suggest a role that alternative HIF regulators such as factor inhibiting HIF (FIH) play in hypoxia acclimation.

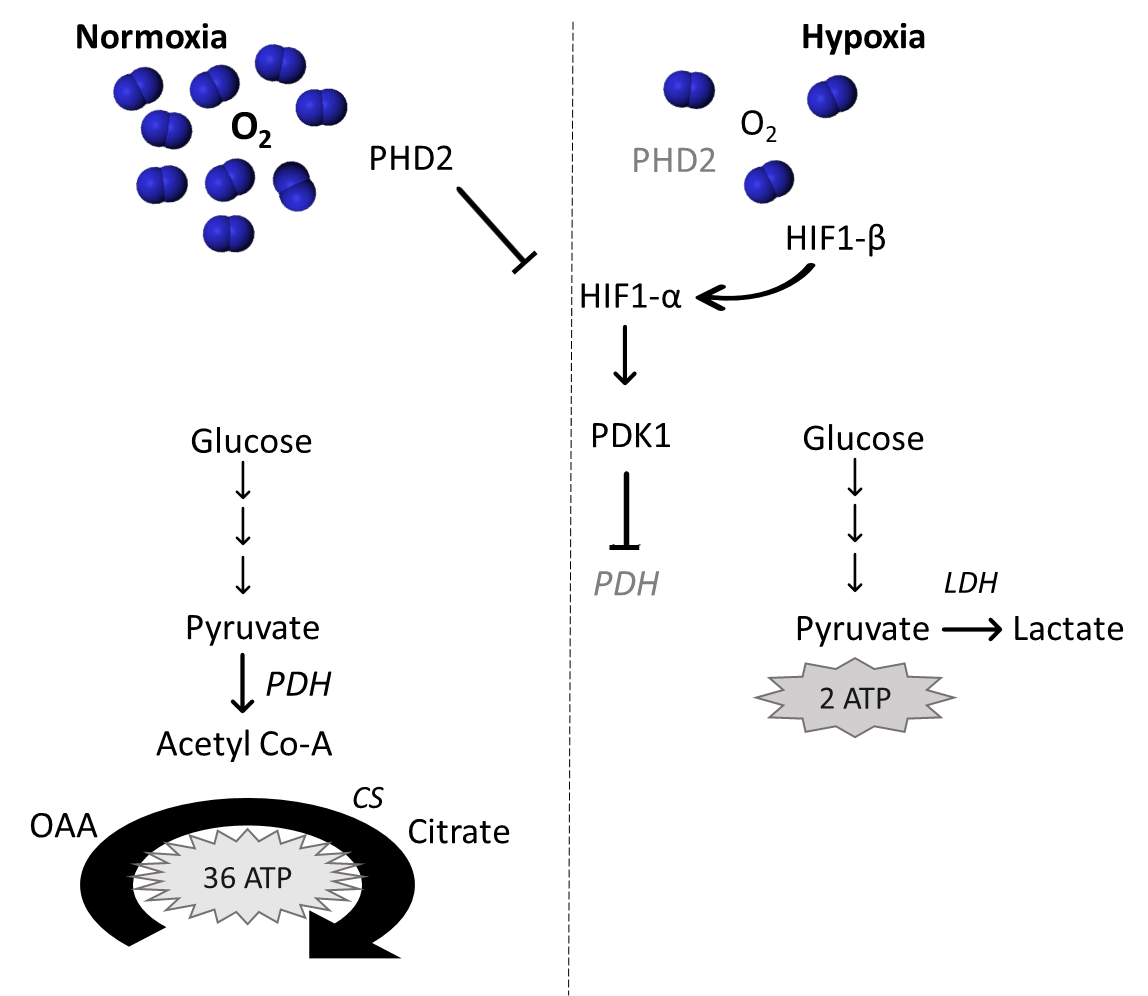
Although the cells used were not stimulated to contract, increased lactate production indicates that aerobic respiration was reduced in acute hypoxia and that high-energy phosphates had likely accumulated; a feature of a loosely coupled ATP demand-supply arrangement. However, the fact that LDH activity remains elevated throughout hypoxic time-points might suggest a back-up mechanism to supply ATP if demand suddenly and unexpectedly rises in hypoxia. Such might be the case in working muscle *in vivo*. Moreover, the enzymatic activity of CS was not affected by hypoxia, but the restoration of aerobic metabolism would likely have occurred with a blunted PDK1 expression exhibited at 24h and 96h hypoxia. The subsequent reduction in lactate accumulation is suggestive that ATP demand-supply is more tightly coupled through oxidative phosphorylation. This is consistent with findings from Nguyen and colleagues (2016) who report that sustained hypoxia preserves oxidative fibers better than in intermittent hypoxia. However, PX-478 successfully inhibited hypoxia-induced PDK1 expression in conjunction with no change in the IC:EC lactate accumulation rate. This might indicate that HIF-antagonism improves ATP demand-supply coupling in hypoxia by allowing oxidative phosphorylation to proceed unimpeded by PDK1 inhibition.

Our findings are consistent with whole animal findings of blunted lactate accumulation in response to chronic hypoxia, which is commonly referred to as the lactate paradox. We also provide evidence that this is a function of altered HIF signalling, as evidenced by expression changes in the HIF-responsive kinase, PDK1. Lastly, we show that increased PHD2 transcriptional activity is likely linked to reduced HIF signalling but that its action only correlates in the chronic 96h state of hypoxia. Future studies should investigate how this translates to changing protein activity by assessing PHD2 and HIF1-α as well as looking at the simultaneous function of VHL in regulating HIF activity throughout hypoxia acclimation. Additionally, further investigations should examine the link between PDK1 function and cellular energy state from ATP levels to see if compensatory mechanisms overriding PDK1 activity can prevent lactate accumulation.

**Appendix Figure 1. Conceptual diagram of the lactate paradox**. Exercising humans at equal relative work rates yield a much greater increase in blood lactate concentration upon sudden exposure to hypobaric hypoxia than that occurring at sea level. Paradoxically, acclimatization to hypobaric hypoxia consistently yields blood lactate levels that are reminiscent to those achieved with equal work rates at sea level. The study was originally performed by West et al. (1983).

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**Appendix Figure 2. Conceptual diagram of cellular hypoxia sensing.** Under normoxic conditions, with saturating molecular O2 availability (in blue) prolyl hydoxylases ubiquinate HIF1-α, preventing it from dimerizing to its HIF1-β counterpart. It is then degraded in the cystosol by the Von Hippel-Lindau proteosome (not shown). Pyruvate oxidation remains undisturbed permitting oxidative phosphorylation of substrates with high ATP yield. Under hypoxic conditions, with a deficit of molecular O2 availability, HIF1-α escapes hydroxylation, permitting its stabilization and dimerization to its HIF1-β counterpart. The active complex gets translocated to the nucleus where it participates in the transcriptional activity of HREs (not shown). One of these HREs, PDK1, phosphorylates PDH, preventing pyruvate oxidation and instead favouring pyruvate reduction to lactate with low ATP yield.

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