THESIS

GLUCOCORTICOID RECEPTOR SIGNALING IS REQUIRED FOR ACCLIMATION OF SKELETAL MUSCLE TO HYPOBARIC HYPOXIA

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ABSTRACT

GLUCOCORTICOID RECEPTOR SIGNALING IS REQUIRED FOR ACCLIMATION OF SKELETAL MUSCLE TO HYPOBARIC HYPOXIA

Hypobaric hypoxia (HH) encountered at high altitudes acutely impairs aerobic exercise capacity, which partially recovers following 1-2 weeks of acclimation to chronic HH. Persistent elevations in serum glucocorticoids occur during HH exposure, but their role in these acute and chronic physiological responses is unclear. We tested the hypothesis that glucocorticoid signaling is essential for the acclimation of aerobic exercise capacity to chronic HH, in part by mediating adaptive changes in skeletal muscle metabolism. Male F344 rats were administered the glucocorticoid receptor antagonist RU486 (RU; 60 mg/kg/d in chow) or no drug for 5 days prior to 15 days of continued normoxia (Fort Collins, CO; elevation 5,003 feet) or HH (simulated 17,200 feet in a hypobaric chamber) with or without continuous RU treatment (N=4-8/group). Graded treadmill exercise tests (GXT) were conducted on a motorized treadmill in normoxia, during acute HH exposure, and in HH after 15 days of HH acclimation. As expected, acute HH reduced GXT performance compared to normoxia in all rats, which improved following 15 days of acclimation to HH. RU pretreatment did not impact hypoxic GXT performance, but continuous treatment abolished improvements in GXT performance following chronic HH. RU attenuated HH-induced increases in hematocrit and muscle fatty acid oxidation efficiency assessed by high-resolution respirometry ex vivo, suggesting that glucocorticoid signaling may improve muscle oxygen utilization in response to chronic HH. RU also prevented HH-induced decreases in pyruvate dehydrogenase expression and increases in Krüppel-like factor 15, proteolysis and branched-chain amino acid aminotransferase in glycolytic muscle, implicating glucocorticoid signaling in a rewiring of glucose and protein catabolism to rid the cell of excess nitrogen in HH. In conclusion, these results demonstrate that glucocorticoid receptor signaling is essential for the acclimation of aerobic exercise capacity to HH, perhaps by mediating improvements in the bioenergetic efficiency of skeletal muscle metabolism.

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CHAPTER I

SKELETAL MUSCLE RESPONSES TO HYPOXEMIA: AN INTEGRATIVE PERSPECTIVE

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I. Introduction

Skeletal muscle is a primary contributor to organismal oxygen consumption throughout a typical human lifespan, being a major site of aerobic energy metabolism during locomotion and resting conditions (Wang et al., 2010, Heymsfield et al., 2021). Consequently, a myriad of physiological, neurohumoral, and biochemical processes have evolved to maintain oxygen supply to skeletal muscle under conditions of increased energy demand or reduced oxygen availability. Failure of these process is often assoc[1]iated with changes in skeletal muscle metabolism and function, such as in cardiopulmonary disease or chronic exposure to high-altitude hypoxia. While cellular responses to low oxygen conditions are well characterized *in vitro*, exactly how changes in systemic oxygen levels impact skeletal muscle is poorly understood. The purpose of this review is to provide an integrative perspective to understanding how skeletal muscle responds to reductions in oxygen supply *in vivo*, with a particular focus on energy metabolism and mitochondrial function.

II. Physiological basis of oxygen uptake and metabolism

II.A. Oxidative metabolism and cellular respiration

Mitochondrial oxidative phosphorylation (OXPHOS) accounts for approximately 90% of oxygen consumption by humans, which produces the vast majority of cellular adenosine

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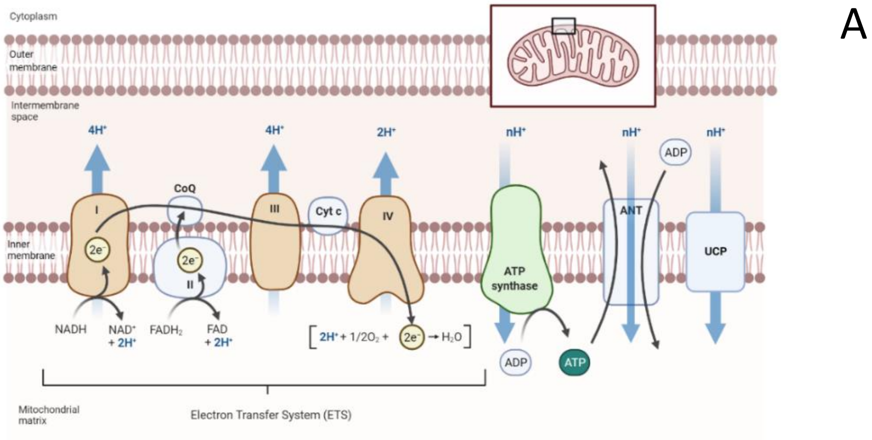
triphosphate (ATP) used to meet human energy demands (Heymsfield et al., 2021). In this process, electrons extracted during the catabolism of high energy substrates, such as glucose and fatty acids, are shuttled by reduced electron carriers through a series of Electron Transfer System (ETS) protein complexes in the mitochondrial inner membrane. Oxygen serves as the final electron acceptor of the ETS when it is reduced to H_2O by cytochrome *c* oxidase (Complex IV), thus enabling the continuous flow of electrons from oxidized substrates through the ETS in the process known as mitochondrial respiration. As electrons are passed between redox centers of ETS complexes I, III and IV, hydrogen ions are translocated to the inner membrane space to form an electrochemical gradient known as the proton motive force (*pmf*). Phosphorylation of ADP is catalyzed by the F_1 subunit of ATP synthase (Complex V) and is powered by the flow of protons driven by the *pmf* to pass through the enzyme complex into the matrix. Therefore, the oxidation of metabolic substrates (OX) is "coupled" to the mitochondrial ATP synthesis (PHOS) by a *pmf* that is facilitated by mitochondrial oxygen consumption (Figure 1).

The efficiency of OXPHOS coupling in imperfect, and varies across cell types, organisms, and physiological conditions (Rolfe et al., 1994, Porter and Brand, 1993, Cheng et al., 2017). The system is considered "coupled" when O_2 consumption results in ATP generation, and "uncoupled" when O_2 is consumed without powering ATP synthesis (Mitchell, 1961). Uncoupling results from an imperfect generation of the *pmf* by the ETC or imperfect harnessing of the *pmf* by the ATP synthase. A major source of uncoupling in mammalian mitochondria is thought to be the "leak" of protons from the inner membrane space into the matrix after being translocated by ETS machinery. Indeed, it is estimated that futile cycling of mitochondrial protons contributes to 20-25% of whole-body resting energy consumption (Rolfe and Brand, 1996). Mechanisms of proton leak include direct movement across the membrane phospholipid bilayer, the activity of uncoupling proteins that enable translocation protons across the inner membrane, or adenine nucleotide translocase (ANT) inducible transport (Brand et al., 2005, Jastroch et al., 2010). Accounting for between 1-10% of total mitochondrial protein (Brand et al., 2005), ANT plays a crucial role during cellular respiration by mediating the intermembrane exchange of ADP and ATP. However, ANT can also exert a protonophoric effect by facilitating transbilayer passage of deprotonated long-chain fatty acids, which are readily re-protonated within the inner membrane space, then able to pass back through the inner membrane (Skulachev, 1999). AMP and alkenals are also known to activate ANT-mediated proton leak (Cadenas et al., 2000, Echtay et al., 2003).

The imperfect coupling of mitochondrial respiration and ATP production in mammals is thought to be important for facilitating endothermy and limiting production of reactive oxygen species, but also increases the O_2 demand of mitochondrial ATP synthesis. Consequently, adaptations in OXPHOS coupling efficiency may confer physiological benefits to organisms native to low oxygen environments (Horscroft et al., 2017). However, due to O_2's central role in maintaining eukaryotic energy homeostasis under nearly all conditions, many local and systemic mechanisms have also evolved to defend adequate cellular O_2 bioavailability in the face of changing metabolic and environmental demands.



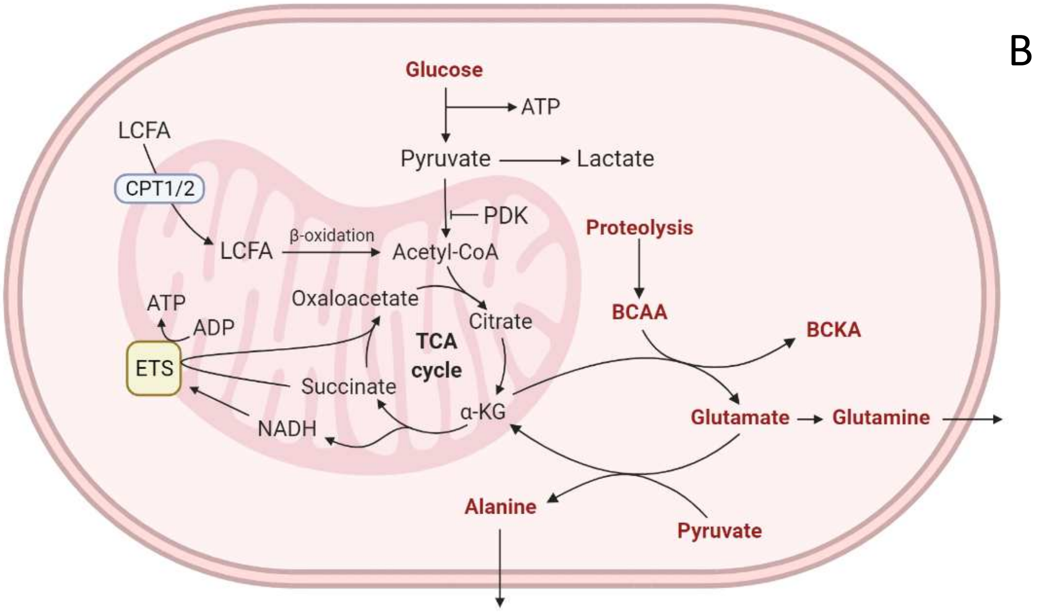


Figure 1. Basic schematics of A) Electron Transfer System, which facilitates Oxidative Phosphorylation (OXPHOS). Electron carrier molecule NADH, generated throughout the oxidation of pyruvate and fatty acids, is oxidized by NADH Dehydrogenase (Complex I, CI). Succinate, a key constituent of the TCA cycle, is oxidized by Succinate Dehydrogenase (SDH, Complex II, CII) through the intermediary electron carrier $FADH_2$. Electrons from CI and CII are forwarded to Coenzyme Q (CoQ), then on to Q-cytochrome *c* oxidoreductase (Complex III, CIII) and Cytochrome c (cyt *c*) before finally being passed to cytochrome *c* oxidase (Complex IV, CIV). CIV catalyzes the final transfer of electrons from cyt *c* to molecular oxygen (1/2 O_2). Complexes I, III and IV facilitate the translocation of H^+ ions from the mitochondrial matrix to the inner membrane space, thus generating the electrochemical gradient (or proton motive force, *pmf*). ATP-Synthase (Complex V, CV) utilizes the *pmf* to phosphorylate ADP to ATP. Oxidative enzymes that directly contribute to *pmf* generation ("OX" component) are shown in orange, while ATP-Synthase ("PHOS" component) is shown in green. Enzymes that contribute to proton leak, such as Adenine Nucleotide Translocase (ANT) and Uncoupling Protein (UCP) are colored grey. B) Key metabolic pathways contributing to energy production. Long-chain fatty acids (LCFA) are translocated to the mitochondrial matrix via Carnitine palmitoyltransferase 1 and 2 (CPT1/2), where they undergo β-oxidation producing Acetyl-CoA. Pyruvate produced in the cytosol can be converted to Acetyl-CoA via pyruvate dehydrogenase, which is inhibited by pyruvate dehydrogenase kinase (PDK), within the mitochondria. Muscle proteolysis results in increased abundance of free branch chain amino acids (BCAA), which can be converted to glutamine and alanine for export from the cell. Created in BioRender.

II.B. The physiological oxygen transport cascade

II.B.1 Pulmonary O_2 uptake

The partial pressure of oxygen (PO_2) in dry air at sea level is 159 mmHg, representing around 21% of the inspired gas (FiO_2) and corresponding atmospheric pressure (P_B; 760 mmHg).

This creates a relatively steep PO_2 gradient between the atmosphere and tissue mitochondria that drives oxygen transport through a series of biological compartments regulated by a variety of intrinsic and extrinsic mechanisms. Upon inspiration, PO_2 is decreased first by water vapor in the upper airway (PH_2O), then by CO_2 diffusing from the pulmonary capillaries surrounding the alveoli. The resulting alveolar oxygen partial pressure (P_AO_2) is generally around 100 mmHg. In healthy individuals, these relatively high P_AO_2s create a steep alveolar-capillary PO_2 diffusion gradient that favors rapid equilibration across these compartments during typical pulmonary capillary transit times (~1 s). Therefore, the efficiency of pulmonary gas exchange is typically limited by how well pulmonary ventilation is matched with alveolar capillary perfusion ($\dot{V}/\dot{Q}$ matching).

Upon diffusing into the arterial blood supply, most oxygen molecules (>98%) bind to hemoglobin within red blood cells, driven by the high alveolar-capillary PO_2 gradient maintained in part by convection of blood flow across the capillary beds. Even during period of high cardiac output, when capillary transit times can be reduced by as much as 50% (Warren et al., 1991), arterial oxyhemoglobin saturation (S_aO_2) is well-defended under physiological conditions (Gale et al., 1985, Warren et al., 1991, Zavorsky et al., 2002). However, pathologies that increase pulmonary diffusion distance (e.g., pulmonary edema), reduce functional alveolar surface area (e.g., emphysema), impair pulmonary ventilation (e.g., asthma), or otherwise decrease the alveolar-capillary PO_2 gradient (e.g., low ambient PO_2) can significantly impair pulmonary gas exchange, leading to significant decreases in P_aO_2 and S_aO2, particularly while engaging in physical activity (Soguel Schenkel et al., 1996, Papiris et al., 2002, Casanova et al., 2008, Fisher et al., 2019).

II.B.2 Hb-mediated O_2 transport

In addition to changes in P_aO_2, the binding of Hb to O_2 is influenced by physicochemical and allosteric mechanisms that regulate pulmonary O_2 uptake and release/delivery to peripheral tissues. The unique heterotetrameric architecture of Hb enables dynamic transitions between two distinct energetic states: a tensed (T) state where Hb-O_2 affinity is lowest when no O_2 molecules are bound, and a relaxed (R) state where Hb-O_2 affinity is greatest when all O_2 binding sites are occupied (Mihailescu and Russu, 2001). As a result, variations in P_aO_2 in O_2-rich environments, such as within the pulmonary circulation (~ 90-100 mmHg), results in comparatively minor changes in S_aO_2 as blood enters the systemic circulation. As the systemic arterial supply branches into smaller vessels downstream of the heart, P_aO_2 begins to decline as O_2 diffuses from capillary blood into lower PO_2 regions of active tissue beds. In skeletal muscle under resting conditions, interstitial PO_2 is typically 8-13 mmHg less than capillary blood (Hirai et al., 2018, Colburn et al., 2020), driven by a intramyocyte PO_2 ranging between 20-30 mmHg at rest (Richardson et al., 2001, Richardson et al., 2006) to ~3 mmHg during maximal exercise breathing hypoxic air (12% F_iO_2) (Richardson et al., 1999b). This steep PO_2 gradient between the capillary blood and myocyte favors release of O_2 from Hb, which is aided by progressively lower Hb-O_2 affinity as O_2 molecules leave Hb binding sites, resulting in a greater off-loading of O_2 (lower S_aO_2) for a given mmHg decrease in P_aO_2 in active tissue beds.

II.B.3 Skeletal muscle O_2 uptake

Upon dissociation from Hb within tissue microvasculature, O_2 diffuses across the RBC membrane and into the plasma (Michenkova et al., 2021). Limited capillary surface area and spatial hematocrit capacity, along with lack of O_2 transport molecules between RBCs and the subsarcolemmal space, intrinsically limits O_2 efflux into myocytes (Poole et al., 2022). However,

O_2 consumption by myocyte mitochondria provides a strong driving force for O_2 uptake into skeletal muscle. Once O_2 enters the myocyte, it binds myoglobin (Mb) in the cytosol, which aids in myocellular O_2 uptake and maintenance of an intracellular PO_2 gradient between the cytosol and mitochondria. Muscle mitochondrial OXPHOS capacity only becomes limited at a PO_2 of ~1 mmHg (Richmond et al., 1997), which is below the lowest recorded values of myocellular PO_2 obtained during maximal exercise in low oxygen conditions (Richardson et al., 1999b, Hirai et al., 2018). Therefore, O_2 consumption by mitochondria maintains a steep PO_2 gradient that favors sufficient O_2 uptake from arterial blood to meet the metabolic demands of skeletal muscle under nearly all physiological conditions (Hirai et al., 2018, Richardson et al., 2006). Consequently, rates of muscle O_2 uptake are ultimately limited by O_2 delivery, not mitochondrial OXPHOS capacity (Richardson et al., 1999a).

II.B.4 Augmentation of O_2 uptake during exercise

The rate of oxygen consumption ($\dot{V}O_2$) by a tissue or organism can be described by the Fick Principle (Fick, 1870) which states that organismal (or tissue) O_2 uptake is determined by the product of O_2 delivery (cardiac output; $\dot{Q}$) and tissue O_2 extraction (difference between arterial and venous O_2 content; A-VO_{2diff}):

$$\dot{V}O_2 = \dot{Q} * A\text{-}\dot{V}O_{2diff}$$

A variety of factors, including systemic energy demand, cardiopulmonary function and environmental conditions, can impact $\dot{V}O_2$ by altering the uptake, transport and delivery of O_2 to body tissues. Under physiological conditions, the increased metabolic demands of physical exercise trigger classic adjustments in nearly every component of the O_2 cascade to maintain O_2 diffusion gradients needed to increase the supply of oxygen to working skeletal muscle. These include increases in pulmonary ventilation ($\dot{V}_E$) and $\dot{Q}$ driven by sympathetic nervous system stimulation of the medullary cardiorespiratory control centers, augmented by afferents from arterial and central chemoreceptors in response to lower P_aO_2 or hypercapnia (higher P_aCO_2), respectively (Guyenet, 2014, Iturriaga et al., 2016). This serves to maintain high PO_2 gradients from the lungs to working tissues and increase the supply of O_2 needed to facilitate greater muscle OXPHOS rates, resulting in 10-20-fold increases in systemic VO_2 during high-intensity exercise.

At the tissue level, elevated temperature, hypercapnia and hydrogen ion levels (lower pH) encountered during exercise also reduce Hb-O_2 binding affinity, which favors greater O_2 unloading from arterial blood perfusing active skeletal muscle (Bohr, 1904). Intracellularly, Mb serves as an important facilitator of augmented diffusivity during periods of high muscle O_2 demand. Because the P_{O2} at which myoglobin is half saturated (P_{50}) is between ~2-5 mmHg (Schenkman et al., 1997, Honig et al., 1992), near-full saturation is maintained under normoxic resting conditions. During maximal exercise however, intramyocyte PO_2 declines to around ~2-3 mmHg (Richardson et al., 2001, Richardson et al., 1995) causing rapid Mb desaturation which contributes powerfully to increased rest-exercise O_2 delivery. This phenomenon is even more pronounced during exercise under low oxygen conditions (e.g., high altitude), where small (1 mmHg) reductions in intracellular PO_2 relative to normoxic exercise leads to even greater Mb desaturation, particularly during submaximal exercise (Richardson et al., 2001). Such reduced intramuscular PO_2 also causes a greater microvascular-myocyte ΔPO_2, driving more rapid O_2 diffusion. Within capillaries, blood flow is increased via upstream arteriolar dilation that defends microvascular PO_2 in the face of increased muscle O_2 uptake and demand (Richardson et al., 1995, Laughlin et al., 2012). Enhanced blood supply to these arterioles is achieved through increased cardiac output and redistribution of flow toward active skeletal muscle through an integration of tissue-specific vasodilatory and vasoconstrictor signals reviewed in detail elsewhere (Thomas and Segal, 2004).

III. Hypoxia: Definitions and Biological Manifestations

III.A. Normoxia, Hypoxia and Hypoxemia

Hypoxia is a state in which the local bioavailability of oxygen (PO_2) is insufficient to meet homeostatic demands, leading to altered physiologic function compared to baseline conditions, followed by activation of compensatory mechanisms to improve O_2 supply and/or reduce demand. "Normoxia" (or euoxia) can be defined as the PO_2 at which a cell or system routinely functions without homeostatic perturbation. Low alveolar PO_2 can result from either a reduced fraction of inspired oxygen (FiO_2) or lower ambient barometric pressure (P_B). The most common biological manifestation of ambient hypoxia *in vivo* is hypoxemia, or low arterial partial pressure of oxygen (P_aO_2), which can diminish the PO_2 gradient that drives O_2 diffusion to tissues downstream. The clinical threshold for hypoxemia is contested and context dependent, but is generally considered lower than 90-95% S_aO_2 (American Thoracic and American College of Chest, 2003). Importantly, hypoxemia resulting from decreases in FiO_2 or P_B do not necessarily result in tissue hypoxia, given the several physiological adjustments discussed above that serve to preserve O_2 uptake and delivery to body tissues. Therefore, caution should be taken when comparing results of studies on cells or tissue deprived of O_2 *in vitro* to tissue responses of humans or animals exposed to ambient hypoxia or hypoxemia *in vivo*.

III.B. Causes of hypoxemia

III.B.1 Pathological causes of hypoxemia

Conditions that impair components of the physiological oxygen transport cascade often result in hypoxemia. Chronic obstructive pulmonary diseases (COPD), characterized by limitations in pulmonary ventilation and gas exchange, reduce the transport of oxygen from ambient air to the pulmonary circulation favoring $\dot{V}/\dot{Q}$ mismatch and lower SaO_2 (Wagner et al., 1977, Rabe et al., 2007). Hypoxemia resulting from acute lower upper respiratory infection has also been well-documented (Lozano, 2001, Orimadegun et al., 2013). Circulatory impairments resulting from decompensated heart failure (Puri et al., 1995, Tamisier et al., 2019) or pulmonary embolism (Huet et al., 1985) may also cause hypoxemia, particularly in patients with previously diagnosed sleep-disordered breathing (Resta et al., 1999, Tamisier et al., 2019). Finally, impairments in circulating O_2 carrying capacity associated with various forms of anemia also reduce the availably of oxygen to peripheral tissues, but are associated with normal PaO_2 that fail to evoke classic physiological response to hypoxemia (e.g., chemoreceptor activation) (Chinawa et al., 2013, Orimadegun et al., 2013).

III.B.2 High-altitude induced hypoxemia

In healthy individuals, ascent to high altitude (HA) is the most common and biologically relevant cause of hypoxemia (Burtscher et al., 2018). Though F_iO_2 is unchanged, P_B is inversely and curvilinearly related to altitude gain, leading to a decline in ambient PO_2 known as hypobaric hypoxia (HH; Figure 2). This leads to hypoxemia due to a lower alveolar-capillary PO_2 gradient, which is exacerbated by exercise when pulmonary capillary transit time is elevated and P_ACO_2 increases. Exercise-induced hypoxemia has also been reported in normoxia conditions in highly trained athletes, though this is generally mild (S_aO_2 between 88-93%) and only seen during near maximal exercise intensity (Dempsey and Wagner, 1999, Durand and Raberin, 2021). Importantly, ascent to HA is often accompanied by increased physical activity (e.g., mountaineering or trekking), which significantly increases organismal O_2 demands and introduces other stressors such as cold exposure, sleep disturbance and caloric deficits that likely influence acute and chronic physiological responses. Therefore, it is important to interpret results of studies utilizing this experimental design with caution, as it can be difficult or impossible to resolve the independent effects of hypoxemia from acute and chronic impacts of other stressors in these contexts. In the following sections, the physiological responses to hypoxemia are reviewed based on the results of well-controlled studies of HH, with some discussion of how potentially confounding variables inherent to some HA field studies may have impacted current thinking in this field.

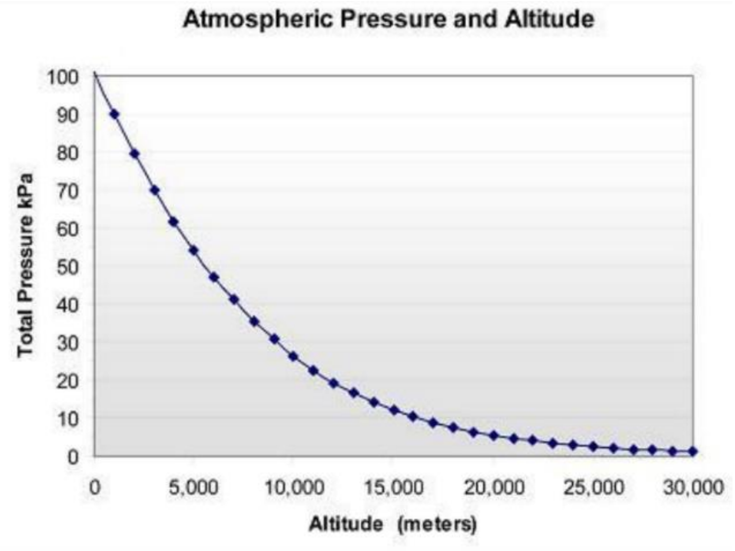


Figure 2. Atmospheric pressure (P_B) declines exponentially with ascending altitude. 1 kPa = ~7.5 mmHg. Citation: (Paul and Ferl, 2007).

IV. Physiological Responses to Hypoxemia

IV.A. Acute Hypoxemia

Acute ascent to HA decreases maximal aerobic exercise capacity ($\dot{V}O_{2max}$) by ~1% for every 100 m above 1500 m in elevation (Bartsch and Saltin, 2008) which results in a greater percent of $\dot{V}O_{2max}$ required to perform a given absolute submaximal workload at high altitude. This triggers a variety of adjustments that improve O_2 uptake, transport and delivery to tissues in response to acute (less than 24h) and chronic (greater than 24h) HH. Immediately upon hypoxemia-induced stimulation, peripheral chemoreceptors in the carotid arteries and aorta signal cardiorespiratory control center in the medulla oblongata to increase pulmonary ventilation and cardiac output. Augmented ventilation increases P_AO_2 and increases expiration of CO_2 from the lungs, causing blood pH to become more alkaline by favoring a leftward shift in the carbonic anhydrase reaction shown below:

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$$

This hypocapnic respiratory alkalosis is a classic physiological signature of acute HA exposure in humans (Winslow et al., 1984, Singh et al., 2003, Grocott et al., 2009, Zouboules et al., 2018), which favors greater Hb-O_2 binding affinity at lower alveolar PO_2s encountered at extreme altitudes (<30 mmHg at 8400 m; (Grocott et al., 2009), but may also limit cerebral oxygenation and contribute to the symptoms of acute mountain sickness (Harvey et al., 1988, Stepanek et al., 2020). Parallel to these effects, O_2 sensing by arterial chemoreceptors activates tracts of the sympathetic nervous system and hypothalamus-pituitary-adrenal (HPA) axis, which increases sympathetic outflow (Xie et al., 2001) and result in greater circulating levels of catecholamines (Dhar et al., 2014, Richalet et al., 2010) and cortisol. These elicit diverse effects on cardiovascular function and metabolism, including an increase in cardiac output that helps to match increases in pulmonary ventilation. Alveolar hypoxia induces pulmonary vasoconstriction, which shunts blood flow to higher P_AO_2 regions to further improve $\dot{V}/\dot{Q}$ matching (Marquis et al., 2021, Sylvester et al., 2012, Penaloza and Arias-Stella, 2007), but can also favor development of pulmonary edema if alveolar hypoxia is severe (e.g., at high altitudes) (Maggiorini et al., 2001).

In the peripheral vasculature, hypoxemia induces arteriolar vasodilation that increases blood flow to active tissue beds such as skeletal muscle, which maintains normal muscle oxygen consumption rates even during hypoxic exercise (Wilkins et al., 2006). Local vasodilation is balanced somewhat by sympathetic (α_1-adrenergic receptor-mediated) vasoconstriction, favoring a re-distribution of blood flow away from regions of lower energy demand (e.g., abdominal viscera) towards those of greater demand (e.g., exercising skeletal muscle). This further supports maintenance of oxygen delivery to skeletal muscle despite hypoxemia, maintaining myocellular PO_2 levels >20 mmHg despite >50% decreases in P_aO_2 in subjects breathing hypoxic air (12% F_IO_2) (Richardson et al., 2006). Consequently, resting muscle O_2 extraction is maintained in HH up to at least 5260 m (Calbet et al., 2003), indicating that adjustments in tissue O_2 supply are sufficient to meet the basal O_2 demands of skeletal muscle even with severe hypoxemia under physiological conditions. However, during maximal exercise in HH, reductions in skeletal muscle vascular conductance may limit O_2 uptake due to a suboptimal balance of vasoconstrictor and vasodilatory signals regulating blood flow to active tissues (Lundby et al., 2006b).

IV.B. Physiological adjustments to chronic HH: acclimation/acclimatization

Physiological responses to chronic hypoxemia have been investigated in humans for decades, primarily in the context of prolonged exposures to hypobaric hypoxia (HH). These are often opportunistic studies of mountaineers or lowlanders during expeditions to high-altitude locations, which should be interpreted carefully due to the presence of other stressors related to prolonged exercise (e.g., trekking or climbing) and environmental exposure (e.g., cold, dehydration, sleep disturbances) that might also influence adaptive physiological responses. However, well-controlled studies using hypobaric chambers and carefully designed field studies have helped to establish some consensus around the process of HH adaptation, often called acclimatization (to a natural environment) or acclimation (to laboratory environment).

IV.B.1. Central and cardiorespiratory adaptations

While $\dot{V}O_2$max remains lower than normoxic levels after chronic exposure to HH, adjustments in several components of O_2 transport cascade facilitate improvements in resting, submaximal and maximal O_2 uptake and transport in HH (Fulco et al., 1998, Chicco et al., 2018, Latshang et al., 2013). Sustained hyperventilation leads to higher P_AO_2 (West, 2006), which partially recovers P_aO_2 following acclimation. This stated, P_aO_2 typically remains lower than in normoxia, contributing to persistent carotid chemoreceptors signaling augmented by greater chemoreceptor O_2-sensitivity and responsiveness of the medullary cardiorespiratory control center (Powell, 2007). This results in sustained elevations of sympathetic outflow with higher pulmonary ventilation and heart rates during rest and submaximal exercise (West, 1982, Naeije, 2010). Despite persistent hyperventilation, acutely alkaline blood pH becomes more acidic during HH acclimation, primarily due to increased renal secretion of bicarbonate (Weiskopf and Severinghaus, 1972, Zubieta-Calleja et al., 2011, Tashi et al., 2014). Lower arterial blood pH improves Hb-O_2 offloading to working tissues, which combined with hemoconcentration from hematopoiesis (Leon-Velarde et al., 2000), significantly improves the O_2 transport capacity. Resting cardiac output returns to normoxic levels during HH acclimation despite sustained reductions in stroke volume due to persistent elevations in resting heart rate (Naeije, 2010). Continued hypoxemic vasodilation in skeletal muscle vasculature further improves O_2 supply to working muscle (Joyner and Casey, 2014, Casey and Joyner, 2011, Dinenno, 2016), but is evidently insufficient to balance sympathetic vasoconstriction signals to meet O_2 demands during maximal exercise (Lundby et al., 2006b). Moreover, maximal cardiac output declines during HH acclimatization due to lower maximal heart rate and perhaps increases in pulmonary vascular resistance secondary to persistent hypoxic pulmonary vasoconstriction (West, 1982). This leads to right ventricular overload and diastolic bulging of the interventricular septum that may impair left ventricular filling (Tanaka et al., 1980, Naeije, 2010, Burkett et al., 2016) and reduce cardiac output.

While HH acclimation is associated with sustained increases in sympathetic tone evidenced by high circulating catecholamines (Bogaard et al., 2002, Calbet, 2003) and elevated sympathetic nerve activity (Hansen and Sander, 2003), effects on HPA axis activity are less clear. Most studies report an initial increase in circulating glucocorticoids consistent with robust HPA axis activation during short-term HH (Rattner et al., 1980, Marinelli et al., 1994, Humpeler et al., 1980, Richalet et al., 2010), which gradually declines to normoxic concentrations after acclimation in the absence of extreme physiologic distress (Benso et al., 2007, von Wolff et al., 2018). Therefore, HPA axis activation may contribute to the early stages of HH acclimation, but delineating its contribution to more sustained responses requires further investigation.

IV.B.2. Skeletal muscle adaptations

Skeletal muscle is a highly plastic organ system capable of undergoing morphologic and metabolic remodeling in response to changes in neural stimulation, disease status or environmental conditions. Muscle fiber types are often categorized by their expression of distinct myosin ATPase isoforms that generally corresponds to features such as contractile velocity and metabolic phenotype. There are two broad categories of muscle fiber types: Type I oxidative fibers tend to be smaller with slower contraction speed, but are more fatigue resistant due to greater mitochondrial density, capillary supply, and expression of oxidative enzymes. Type II glycolytic fibers tend to be larger and contract with greater force and velocity, but fatigue more quickly due to lower mitochondrial density and capillarization and greater reliance upon anaerobic energy production. Fiber type distribution in and between skeletal muscle is driven largely by motor unit size and functional requirements. For example, musculature involved in tonic/postural or low-intensity activities (e.g. erector spinae, soleus) tends to express high amount of Type I fibers, while muscles involved in explosive movements (triceps, gastrocnemius) tend to contain more Type II fibers (Johnson et al., 1973, Schiaffino and Reggiani, 2011).

It has long been understood that prolonged HA trekking and mountaineering reduces fat-free mass and muscle cross sectional area (CSA) (Boyer and Blume, 1984, Hoppeler et al., 1990). However, sustained, intense physical activity associated with such endeavors induces muscle catabolism even in normoxia (Kumar et al., 2009, Pasiakos and Carbone, 2014). Further, negative energy balance associated with prolonged exercise and HH-induced anorexia impact protein turnover (Sartori et al., 2021) and factors such as circadian rhythm disruption, training status, and nutrition may also influence muscle nitrogen balance. While comparatively few HH studies have been performed under controlled laboratory conditions in the absence of these confounding stressors, available data suggests that extreme HH alone is also sufficient to reduce muscle CSA (Green et al., 1989, MacDougall et al., 1991). The impact of HH acclimation on muscle fiber type-specific changes is less clear. While some studies in rodents have shown a preferential loss of oxidative fibers with maintenance of glycolytic fibers (Chaillou, 2018), human *vastus lateralis* biopsies taken following multiple weeks of HH revealed no evidence for a preferential fiber type degradation following HA acclimation (Green et al., 1989, MacDougall et al., 1991, Juel et al., 2003). However, an overall decrease in skeletal muscle CSA may aid in maximizing muscle oxygen delivery in the face of diminished microvascular PO_2, as capillarization is maintained or slightly increased relative to CSA in HH (Levett et al., 2012, Green et al., 1989, MacDougall et al., 1991).

Changes in skeletal muscle mitochondrial density and oxidative enzyme abundance following chronic HH, however, differ among reports. Human field studies conducted at extreme altitudes have largely shown reduced density with acclimation (Hoppeler et al., 1990, Levett et al., 2012, Horscroft et al., 2017). At more moderate altitudes, mitochondrial density seems largely unaffected (Jacobs et al., 2012). This stated, changes in metabolic protein abundance, enzymatic activity, and substrate serum concentration suggests that chronic HH may dramatically rewire mitochondrial bioenergetics. For example, some reports have demonstrated reduced fatty acid uptake (Roberts et al., 1996), attenuated enzymatic activity of Carnitine palmitoyltransferase 1 (CPT1) and hydroxyacyl-CoA dehydrogenase (Levett et al., 2012, Dutta et al., 2009), and lower protein abundance of CPT1β (Horscroft et al., 2017), citrate synthase (Levett et al., 2012) and various ETS components (Vigano et al., 2008, Levett et al., 2012), following acclimation. In contrast, several other reports in humans (Braun et al., 2000, Chicco et al., 2018, Gelfi et al., 2004) and animals (Cheviron et al., 2012, Ou and Leiter, 2004) native or acclimated to high altitudes have concluded that FA oxidation may be favored or improved in the absence of exhaustive exercise. The discrepancies may reflect differences in the species being studied, the extent and duration of altitude exposure, and/or the metabolic demands of subjects at the time of sampling. In addition, static measures of selected metabolic "marker" enzymes might not accurately reflect the integrated function of metabolic pathways that control skeletal muscle substrate utilization. Over the past 5-10 years, the development of high-resolution respirometry has enabled a detailed study of mitochondrial substrate oxidation capacity and efficiency in permeabilized skeletal muscle fibers from humans (summarized in Table 1). This new approach, recently combined with modern "omics" technologies, have provided new insights to the complexity of metabolic adjustments that occur in skeletal muscle HH acclimation.

Table 1: Summary of studies performing high-resolution respirometry analysis of human skeletal muscle mitochondria after HA acclimation

Author	Altitude (m)	Duration	Subject age; Conditions	Major findings	Citation
Jacobs, 2012	3,454	28d	26 ± 4 yr (n=8); transported by train from 432m to 3454m	↓ total ADP-dependent FA oxidation, ↑ FA coupling control	(Jacobs et al., 2012)
Jacobs, 2013	4,559	9-11d	26 ± 1 years (n=10); flown from 42m to 3,647 m, then trekked from 3,647m to 4,559m	trending ↓ total ADP-dependent FA oxidation, ↔ FA coupling control	(Jacobs et al., 2013)
Jacobs, 2016	3,454	28d	26.9 ± 3.7 (n=9) years, transported by train from 432m to 3,454m	trending ↑ total ADP-dependent FA oxidation, ↔ FA coupling control, ↑ intermyofibrillar mito volume density, ↔ subsarcolemmal mito volume density	(Jacobs et al., 2016)
Horscroft, 2017	5,300	54-59d	28.0 ± 1.6 y (n=10), active lowlander; gradual ascent from 2,800m to Everest Base Camp (5,300m)	↔ total ADP-dependent FA oxidation, ↑ FA coupling control	(Horscroft et al., 2017)
Chicco, 2018	5,260	16d	21.0 ± 2y (n=14); flown from sea level to 5,260m	↑ total ADP-dependent FA oxidation; ↑ FA coupling control, ↑ resting ATP/ADP ratio	(Chicco et al., 2018)

While a full discussion of data from studies summarized in Table 1 is beyond the scope of this review, there are a few findings that merit some discussion. There is a lack of consensus regarding changes in ADP-dependent fatty acid oxidation following high altitude acclimation: studies to date have shown that respiratory rate can improve (Jacobs et al., 2016, Chicco et al., 2018) (Jacobs et al., 2012, Jacobs et al., 2013) or remain the same (Horscroft et al., 2017). This stated, all of these studies reported either improved measures of "mitochondrial efficiency" through augmented OXPHOS coupling (ADP-control of respiration) or maintain coupling control in the face of extreme physiologic challenge (HH). When considered alongside reduced CSA and maintenance of capillary and mitochondrial density in HH, it is feasible that altitude-mediated morphologic and metabolic changes within the myocytes may function to maximize the efficiency of oxygen utilization by skeletal muscle after acclimation.

V. Mechanisms governing responses to chronic hypoxemia

V.A. Central adjustments

V.A.1. Sympathetic outflow and catecholamines

As noted above, increased carotid body chemoreceptor signaling induced by prolonged hypoxemia plays a key role in orchestrating whole-body acclimatization responses through activation of the sympathetic nervous system. Within arterial chemoreceptors, enhanced O_2 sensitivity and increased frequency of action potential bursts is mediated by glomus cell hypertrophy and increased luminal surface area exposure (Kusakabe et al., 1993). Intracellularly, changes in the abundance and activity of transmembrane ion channels alters membrane potential and contributes to increase excitability at a given PO_2 (Stea et al., 1992). Greater afferent output has several important consequences. In addition to augmenting ventilation, carotid body signaling causes sympathetically mediated vasoconstriction in splanchnic viscera, skeletal muscle, kidneys, pulmonary circulations, leading to increased arterial pressure (Morrison, 2001) and contributing to pulmonary hypertension. Sympathetic simulation of cardiac pacemaker cells maintains elevated HR, while adrenal gland stimulation causes the continued release of catecholamines (Johnson et al., 1983, Marshall, 1994, Cao and Morrison, 2001).

β-adrenergic receptor (β-AR) signaling has been identified as a potential mediator of cellular adaptation to hypoxemia, particularly within the cardiovascular system (Simpson et al., 2021). Use of pharmacologic β-AR inhibitors (“beta-blockers”) have been shown to suppress

HH-induced hypertension, though this was accompanied by reduced SaO_2 (Bilo et al., 2011). β-AR blockade may also inhibit renal hypoxia inducible factor-1α (HIF-1α) activation and subsequent upregulation of erythropoietin in mice after acute ambient hypoxia (Cheong et al., 2016); however, no difference in EPO concentration or red cell volume have been observed among humans after 3 weeks of HA exposure (Grover et al., 1998). In the heart, mammals native to extreme altitudes may exhibit increased capacity for β-AR cardiac stimulation, in addition to improved ejection fraction (Wearing et al., 2022). The intracellular mechanisms underpinning tissue-specific effects of catecholamines, however, remain unclear.

V.A.2. Hypothalamic-pituitary-adrenal (HPA) Axis

Parallel to activation of the sympathetic nervous system, *tractus solitarii* neurons in the paraventricular nucleus of the hypothalamus release corticotropin-releasing hormone (CRH) into portal circulation in response to hypoxemia (Ruyle et al., 2018). After triggering release of adrenocorticotropic hormone (ACTH) from the anterior pituitary into the systemic circulation, ACTH travels to the Zona Fasciculata of the adrenal cortex to stimulate glucocorticoid (GC) production. GCs are synthesized from precursor cholesterol, obtained predominantly from circulating LDL (Gwynne and Strauss, 1982) and are converted from inactive corticosterone to biologically active cortisol via 11β-HSD1 (Du et al., 2013, Svendsen et al., 2009). Contrary to acute exposure, long-term HH often results in normalization of plasma GC concentrations, even among high-altitude trekkers and mountaineers (Rattner et al., 1980, Basu et al., 1997, Benso et al., 2007, von Wolff et al., 2018). However, during caloric restriction, intense exercise, environmental stress, or extreme altitude, GC concentrations may remain elevated (Marinelli et al., 1994, Barnholt et al., 2006, Rattner et al., 1980, von Wolff et al., 2018). Because GCs are released in response to perceived homeostatic threat, this suggests that an "adaptive threshold" exists at which biological compensatory mechanisms are incomplete or insufficient (Rattner et al., 1980).

Finally, it is worth noting that chronic hypoxemia may impact other hormonal axes as well. Although data is heterogenous, it can generally be concluded that hypoxemia activates the thyroid axis in humans, evidenced primarily by elevated circulating T4 levels (Basu et al., 1995, Nepal et al., 2013, von Wolff et al., 2018). T3 abundance is largely unchanged after chronic HH (von Wolff et al., 2018, Nepal et al., 2013) though this may be due to its relatively short half-life *in vivo*. Interestingly, thyroid hormone has been seen to upregulate HIF-1α through both nuclear receptor and nongenomic mechanisms (Ma et al., 2004, Moeller et al., 2005, Lin et al., 2009) and plays a role in hypoxemia-induced erythropoiesis (Ma et al., 2004) and erythroblasts 2,3-bisphosphoglycerate synthesis (Gonzalez-Cinca et al., 2004). Therefore, the neurohormonal regulation of hypoxemic response likely involves the integration of multiple metabolic, transcriptional and cell signaling responses along the entire O_2 transport cascade.

V.B. Hypoxia inducible factor-1alpha (HIF-1α)

V.B.1. Regulation of HIF-1α transcriptional activity

The transcriptional regulator hypoxia inducible factor-1 (HIF-1) plays a central role in orchestrating cellular responses to low oxygen conditions (Semenza, 2012). During normoxic physiological conditions, HIF-1α is hydroxylated by prolyl hydroxylase domain-containing enzymes (PHDs), which utilize alpha-ketoglutarate and O_2 as substrate. The E3 ubiquitin ligase von Hipple Lindau protein (VHL) complex then recognizes this hydroxylation and targets HIF-1α for proteolytic degradation (Maxwell et al., 1999). Similarly, O_2-dependent hydroxylation of HIF-1α asparaginyl residues is mediated by factor-inhibiting HIF-1α (FIH1), resulting in disruption of HIF-1α cofactor recruitment and inactivation (Lando et al., 2002, Webb et al., 2009). During tissue O_2 deprivation, hydroxylase activity becomes limited, allowing for HIF-1α stabilization (Jaakkola et al., 2001). However, there are many O_2-independent mechanisms for regulating HIF-1α stability. For example, increased reactive oxygen species (ROS) generation from electron transport chain enzymes has been shown to inhibit PHDs (Klimova and Chandel, 2008). Competitive inhibition of PHDs and HIF-1α stabilization can also occur from succinate accumulation, even in the presence of abundant O_2 (Lee et al., 2005, Selak et al., 2005), while administering cell-permeating α-ketoglutarate derivatives to cells *in vitro* can rescue PHD activity in both high succinate (MacKenzie et al., 2007) and hypoxic environments (Tennant et al., 2009). Consistent with these findings, inhibition of the tricarboxylic acid cycle flux at isocitrate dehydrogenase also stabilizes HIF-1α and increases expression of its target genes in the absence of hypoxia (Zhao et al., 2009). HIF-1α expression can also be transcriptionally regulated through NF-κB (Rius et al., 2008), STAT3 (Papadakis et al., 2010) and sp1 (Vlaminck et al., 2007), suggesting links to inflammatory signaling. After translocation to the nucleus, HIF-1α heterodimerizes with HIF-1β at hypoxia response elements (HREs), where they are involved in regulating many of the cellular programs implicated in the systemic adaptive response to cellular hypoxia. Famously among these is the erythropoietin (*EPO*) gene, whose corresponding protein stimulates red blood cell production in the bone marrow (Semenza and Wang, 1992). Transferrin (Rolfs et al., 1997) and vascular endothelial growth factor (Forsythe et al., 1996) upregulation are also canonical HIF-1α targets.

In a number of cell lines, HIF-1α-mediated reprogramming generally serves to increase anaerobic ATP production and shunt substrates towards one carbon metabolism and pentose phosphate pathway for DNA repair and redox homeostasis maintenance during tissue hypoxia

(Zhao et al., 2009). For example, pyruvate dehydrogenase (PDH) kinase 1 (*PDK1)* is upregulated by HIF-1α, which favors inhibition of the PDH enzyme complex and reduced conversion of glycolytic end-products into acetyl-CoA within mitochondria (Papandreou et al., 2006). Similarly, mitochondrial oxidative capacity is attenuated, perhaps to better regulate redox balance and reduce ROS generation (Semenza et al., 2001, Li et al., 2019). This stated, specific transcriptional programs activated by HIF-1α are highly dependent on cell type and hypoxic stimulus, and may differ between *in vitro* and *in vivo* contexts.

V.B.2. Role of HIF-1α in adaptive response to HH

While HIF-1α is often discussed in the context of HH, evidence linking HIF-1α stabilization to adaptive responses in HH is surprisingly limited. In *Hif1a*$^{+/-}$ mice exposed to normobaric hypoxia (10% O_2) for multiple weeks, significantly delayed development of polycythemia, right ventricular hypertrophy, pulmonary hypertension, and pulmonary vascular remodeling and significantly greater weight loss was observed (Yu et al., 1999). Consistent with its proposed role as an "oxygen sensor", some studies have suggested that HIF-1α stabilization may be transient in the context of HH. Increased protein abundance of HIF-1α has been reported in muscle tissue taken from rodents exposed to both acute (Stroka et al., 2001) and chronic (Chaudhary et al., 2012) hypobaric hypoxia. It should be noted however, that to date, HIF-1α has not been shown in humans after HH exposure (Vigano et al., 2008, Chicco et al., 2018) and significantly augmented protein expression in rodents has only been seen >7,600 m in simulated altitude.

This stated, the same study reported detectable HIF-1α protein in mouse skeletal muscle under even resting normoxic conditions (Stroka et al., 2001), with future investigations revealing greater HIF-1α mRNA and protein in Type II skeletal muscle fibers relative to Type I (Pisani and Dechesne, 2005). Perhaps in agreeance with these findings, HIF-1α over-expression in rat skeletal muscle has been shown to induce slow-to-fast fiber type shifts (Lunde et al., 2011). Significant detectable stabilization at baseline, however, does not necessarily translate to meaningful activation during acclimation to HH. Indeed, given the abundance of protective physiologic mechanisms which defend skeletal muscle O_2 supply in the face of HH-induced hypoxemia, it is unlikely that resting myocytes are sufficiently hypoxic to induced HIF-1α stabilization under sedentary physiological conditions. However, sufficient myocellular hypoxia may occur transiently during metabolic challenges such as acute physical exercise, which has been shown to increase both HIF-1α protein (Ameln et al., 2005, Nava et al., 2022) and mRNA levels (Lundby et al., 2006a).

Given attenuated $\dot{V}O_2$max and increased submaximal $\dot{V}O_2$ in HH, it is possible that the intense, prolonged physical activity at high altitude (such as mountaineering or trekking expeditions) at least transiently exceeds the body's capacity to maintain stable intramyocyte PO_2, resulting in more sustained HIF-1α stabilization. Circadian disturbances, common to HA trekking, have also been recently shown *in vitro* to increase HIF-1α activation in skeletal myotubes (Peek et al., 2017). Collectively, this may help to explain differences in skeletal muscle responses between studies performed in simulated hypobaric chambers and those involving alpine mountaineering. Indeed, in most controlled studies of HH, there has been no significant loss of muscle mitochondrial density, change in fiber type distribution or clear downregulation of oxidative metabolism (describe above), which are all canonical features of sustained HIF-1α signaling. Furthermore, the only study to date which has measured HIF-1α protein after multiple weeks of HH exposure demonstrated no significant stabilization (Chicco et al., 2018). Taken together, the current body of HH literature suggests that HIF-1α stabilization in skeletal muscle is largely transient, related to either increased O_2 demand within myocytes during exercise or perhaps early-phase acclimation to hypoxemia. This stated, other factors beyond intramyocyte PO_2 may have significant ability to modulate O_2 activity in response to sustained hypoxemia.

V.C. Glucocorticoids

V.C.1. Glucocorticoid Receptor (GR) signaling

Hypoxemia triggers activation of the HPA axis, leading to the release of glucocorticoids such as cortisol (in humans) and corticosterone (in rodents) into the circulation that bind GC receptors (GRs) in target cells. GRs are expressed almost ubiquitously and contain motifs common to other members of the steroid receptor superfamily: a highly conserved zinc-finger DNA Binding Domain (DBD), a C-terminal ligand binding domain (LBD), and a divergent amino terminal domain (NTD) (Heitzer et al., 2007). *In vivo*, GRs are embedded within a multiprotein complex containing the ATP-dependent heat shock protein 90 (HSP90) along with HSP70, HSP56, immunophilins, FKBP51/2 and p23 (Cheung and Smith, 2000). These extra-receptor components are required to facilitate ligand binding and efficient translocation to the nucleus via intact microtubule networks (Picard et al., 1990, Galigniana et al., 1998). Subsequently, distinct GR nuclear localization signals (Picard and Yamamoto, 1987, Cadepond et al., 1992) interact with importins (Echeverria et al., 2009, Freedman and Yamamoto, 2004) to facilitate passage across through the nuclear pore complex (Madan and DeFranco, 1993). There, the GR can either homodimerize on DNA palindromes known as glucocorticoid response elements (GREs) or bind with other transcription factors to induce transactivation or transgression of cellular programs (Beato et al., 1996a, Beato et al., 1996b). For example, direct tethering to NF-κB and activating factor-1 (AP1) are canonical non-genomic targets for GR activation, suppressing inflammatory pathways and regulating cell fate, respectively (Garces de Los Fayos Alonso et al., 2018).

V.C.2. Potential role of GR signaling in the adaptive responses to hypoxemia

GR signaling is responsible for many systemic adaptations to stress that may be beneficial in the context of hypoxemia. For example, GR signaling mediates adaptive erythropoiesis (Bauer et al., 1999, Wang et al., 2021) through EPO and c-Kit activation (Wessely et al., 1997, von Lindern et al., 1999), inducing proliferation of committed erythroid progenitors (Chute et al., 2010). Hemopoietic stem cell homing (Guo et al., 2017) and migration from bone marrow (Pierce et al., 2017) is also reportedly mediated by GR signaling *in vitro*. In the heart, GR contributes to normal systolic function and restrains maladaptive cardiac growth in response to physiological stress (Richardson et al., 2017, Oakley et al., 2013), while chronically elevated GCs contribute to the development of cardiac hypertrophy and cardiac fibrosis (Muiesan et al., 2003, Frustaci et al., 2016). Mitochondrial function may also be directly modulated by GR signaling in a concentration-dependent manner. Acute GC administration to mitochondria at low doses *in vitro* increase substrate oxidation, membrane potential, and calcium tolerance, while the opposite effects are seen following higher, more chronic doses (Du et al., 2009).

Skeletal muscle proteolysis is nearly always associated with chronic HH exposure, and has been suggested to be beneficial in this context by modulating metabolism and systemic energy homeostasis (Murray, 2014). GR activation is a primary stimulus for skeletal muscle atrophy by dampening anabolic programs and augmenting proteolysis, particularly during chronic GC exposure (Falduto et al., 1990, Lofberg et al., 2002, Sandri et al., 2006). Degradation is most pronounced in Type II (glycolytic) muscle fibers, which have a higher relative abundance of GR protein (Sandri et al., 2006, Shimizu et al., 2011). Following ligand binding, GRs transactivate ubiquitin-protease system genes REDD1 and FoxO1/3, which in turn regulate the expression of the protein ligases Murf1 and Atrogin-1 to trigger proteolysis (Zhao et al., 2007, Clarke et al., 2007, Shimizu et al., 2011, Schakman et al., 2013). GR signaling also exhibits anti-anabolic action by inhibiting protein synthesis machinery stimulated by insulin, free amino acids, and other growth factors (Shah et al., 2000, Short et al., 2009). The key anabolic regulator mechanistic target of rapamycin (mTOR) is also suppressed indirectly through GR-mediated akt/PKB inhibition (Morgan et al., 2009) and KLF15 activation (Wang et al., 2006, Shimizu et al., 2011), further promoting the catabolic effects of GCs on target tissues.

Despite this well-established role in promoting skeletal muscle atrophy, short term GC administration can elicit ergogenic effects which improve whole-body exercise performance (Duclos, 2010, Casuso et al., 2014, Tacey et al., 2017). This may be due in part to systemic mobilization of energy resources during states of metabolic challenge. GR signaling reduces muscle glucose uptake while inducing glycogenolysis (Coderre et al., 1991) and hepatic gluconeogenesis (van Raalte et al., 2009), collectively functioning to defend blood glucose concentrations. GR signaling also increases lipase expression (LIPE, ATGL, MGLL) within adipocytes, resulting in greater release of free fatty acids and glycerol into circulation (Beaupere et al., 2021). This stated, reports detailing GR-mediated ergogenic adaptations in skeletal muscle are few. Krüppel-like factor 15 (KLF15) is a direct target of GR signaling (Kuo et al., 2012, Sasse et al., 2013) and has been shown to favor lipid uptake, transport and oxidation in myocytes (Haldar et al., 2012, Fan et al., 2021). Further, KLF15 has been shown to play a crucial role in endurance exercise capacity in response to elevated GCs (Morrison-Nozik et al., 2015).

Therefore, future research is needed to elucidate the potential impacts of GR-mediated mechanisms on skeletal muscle metabolism and function.

V.C.3. Emerging link between GR signaling and HIF-1α stabilization?

Accumulating evidence suggests that complex cross-talk between GR signaling and HIF-1α may coordinate cellular responses to hypoxemia. For example, HREs have been discovered on GR promoter-reporter constructs of DNA, with some evidence suggesting HIF-1α may play a role in facilitating and amplifying GR transcription during tissue hypoxia (Leonard et al., 2005, Zhang et al., 2015). Other studies have shown that GC are capable of stabilizing HIF-1α through either pVHL degradation (Vettori et al., 2017) or through binding to the HIF-1α transactivational domain (Kodama et al., 2003). Therefore, it is plausible that GCs may trigger HIF-1α activation in skeletal muscle in response to hypoxemia.

VI. Summary and conclusions

In summary, several physiological mechanisms defend skeletal muscle PO_2 in response to hypoxemia, including hematopoiesis, hyperventilation, tachycardia, altered oxyhemoglobin binding dynamics, and adjustments in pulmonary and peripheral blood flow. Adaptations within skeletal muscle also occur to reduce energy demands and improve O_2 uptake and utilization, including increased proteolysis with maintained or enhanced capillary:CSA ratio and improved efficiency of fatty acid oxidation. Though HIF-1α appears to be essential for initiating many of the systemic adaptive responses to chronic hypoxemia, its role in skeletal muscle appears to be largely limited to transient periods of heightened energy demand (e.g., intense exercise) where tissue O_2 demand exceeds O_2 supply. However, glucocorticoids are known to play an important role in maintaining basal energy homeostasis during many forms of physiologic stress, including regulation of muscle proteolysis and fuel mobilization during hypoxemia. Therefore, future research should aim to better understand the role that glucocorticoid signaling plays in the adaptive response of skeletal muscle to chronic HH. This is the basis for my original thesis research described in the following chapter.

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CHAPTER 2

GLUCOCORTICOID RECEPTOR SIGNALING IS REQUIRED FOR ACCLIMATION OF SKELETAL MUSCLE TO HYPOBARIC HYPOXIA

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I. Introduction

Hypobaric hypoxia (HH) encountered at high altitudes represents the most common physiological cause of sustained hypoxemia in mammals. Given the essential role of oxygen in mitochondrial energy metabolism, the resulting decrease in arterial blood oxyhemoglobin saturation (S_aO_2) significantly impairs aerobic exercise capacity at high altitudes (Calbet, 2003, Chaudhary et al., 2012, Wehrlin and Hallen, 2006). Therefore, a major aim of physiologic adaptation to hypoxemia is the maintenance of adequate oxygen delivery to working tissues such as the heart and skeletal muscle. Acutely, this response includes increased ventilation and elevated heart rate, as well as hemoconcentration and functional redistribution of blood flow towards active skeletal muscle (Wilkins et al., 2006, Naeije, 2010, Dill et al., 1969). Prolonged HH exposure results in augmented hematopoiesis (Haas[2]e, 2010, Faura et al., 1969), increased bicarbonate secretion (Zouboules et al., 2018), and greater chemoreceptor O_2-sensitivity (Powell, 2007). Though the function of the sympathetic nervous system in mediating acclimation is well-understood (Hainsworth et al., 2007), the potential role of the HPA axis is less clear. Most *in vivo* studies in humans (Humpeler et al., 1980, Marinelli et al., 1994, Richalet et al., 2010) and

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rats (Rattner et al., 1980) have shown that glucocorticoids (GCs) are released in response to acute hypoxemia and decline over time in the absence of extreme physical distress (Rattner et al., 1980, von Wolff et al., 2018, Benso et al., 2007). Perhaps most significantly, glucocorticoid receptor (GR) stimulation is known to mediate adaptive erythropoiesis during acclimation to high altitude (Bauer et al., 1999, Wang et al., 2021).

In skeletal muscle, chronic HH induces changes in both morphology and function. The current body of literature suggests that chronic HH reduces skeletal muscle cross-sectional area in humans without loss of capillary density (Green et al., 1989, MacDougall et al., 1991, Juel et al., 2003, Levett et al., 2012), while effects on measures of mitochondrial density and metabolism have been less consistent (Levett et al., 2012, Hoppeler et al., 1990, Jacobs et al., 2012, Chicco et al., 2018, Horscroft et al., 2017). It should be noted that most human studies to date have utilized subjects involved in high altitude mountaineering and trekking expeditions. Extreme variation in participant training status, daily exercise volume, duration of exposure and specific altitude reached may help to explain differences in findings. Though many characterizations of skeletal muscle following HH acclimation have been made, surprisingly little is known about the mechanisms responsible. Some have speculated that HIF-1α signaling may play a role in driving mitochondrial loss and downregulating oxidative metabolism in skeletal muscle (Murray, 2009), though *in vivo* evidence collected thus far has only been in the context on extreme, acute HH exposure rather than long-term acclimation (Stroka et al., 2001). In contrast, the impact of GR signaling is well known to enhanced proteolysis and inhibit anabolic action in skeletal muscle (Falduto et al., 1990, Lofberg et al., 2002, Sandri et al., 2006), which may lead to adaptive metabolic rewiring (Murray and Montgomery, 2014, Chicco et al., 2018) and improved exercise performance (Morrison-Nozik et al., 2015).

The present study seeks to elucidate the role of glucocorticoid signaling in the physiologic response to hypobaric hypoxia. Specifically, we aim to A) determine if glucocorticoid receptor blockade impacts exercise performance in acute HH, B) determine if GR signaling is necessary for improvement in exercise performance realized following HH acclimation and C) evaluate fiber type-specific effects of GR signaling on skeletal muscle which may help to explain differences in exercise performance related to HH acclimation.

II. Methods

II.A. Animal model and experimental design

Male Fisher 344 rats (aged 2-3 months) were housed in a temperature-controlled environment on traditional 12 hour light-dark cycle (5pm-5am) with *ad libitum* access to food and water. Only male rats were used in this study because the GR antagonist RU-486 also antagonizes progesterone receptors at therapeutic concentrations (Meyer et al., 1990, Baulieu, 1991), which could impact muscle metabolism and responses to hypoxemia in females (Rosa-Caldwell and Greene, 2019). Rats were randomly assigned to one of four experimental groups: 1) Normoxia Control (C), remaining in normoxic conditions without treatment for the duration of the study; 2) C + RU486 (RU), remaining in normoxia, receiving the GR antagonist RU486 (also known as Mifepristone, Sigma M8046) administered in chow at 60 mg/kg/d (based on an average food intake of ~20 g/d) beginning 5 days prior to the first hypoxia exercise test to ensure full ablation of GR signaling *in vivo* (Kroon et al., 2017); 3) Hypobaric Hypoxia Control (HC), exposed to 15 days of continuous HH following the first hypoxic exercise test without RU treatment; and 4) HC + RU486 (HRU). A schematic summary of the experimental design is presented below in Figure 1.

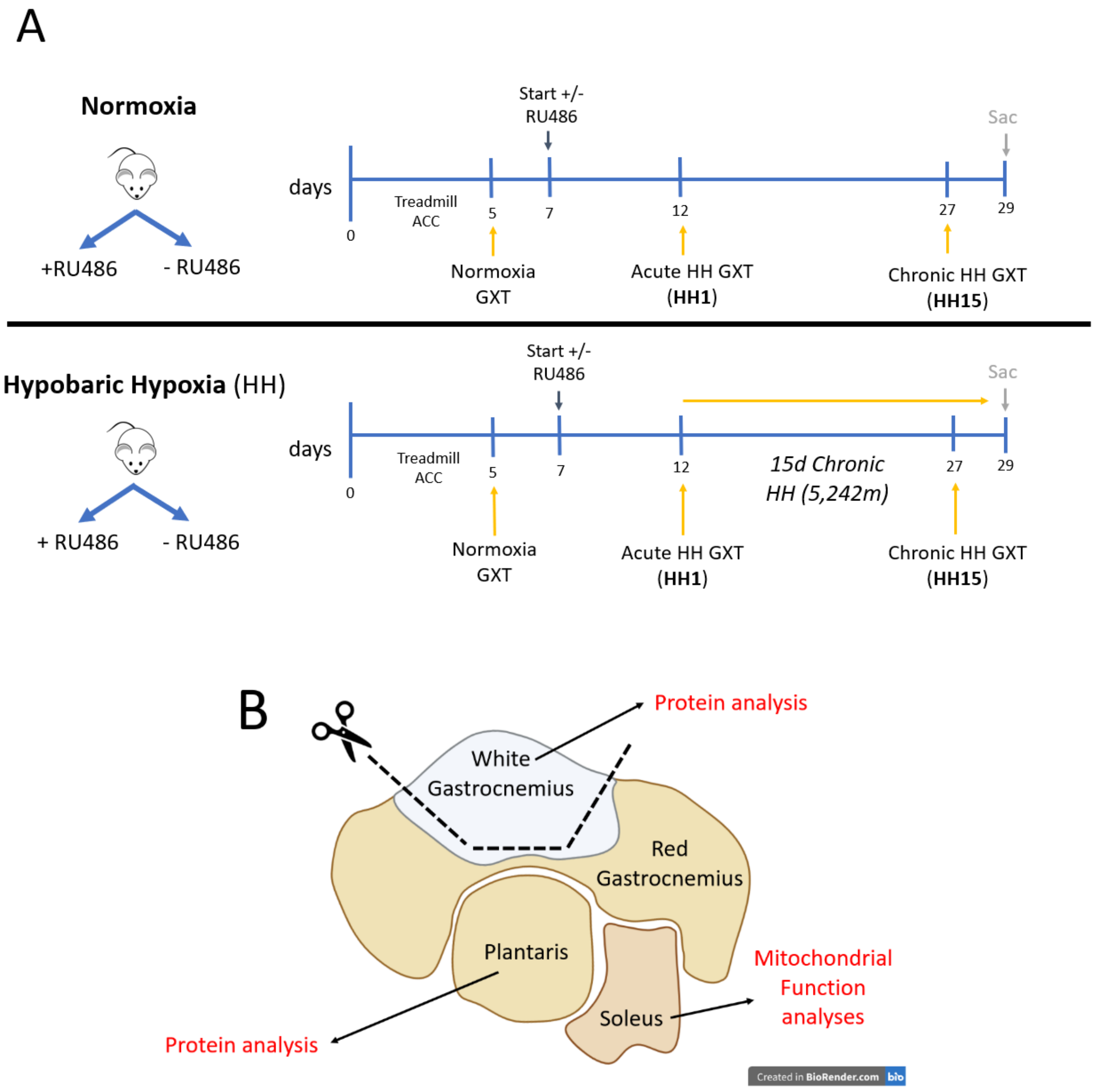


Figure 3. A) Schematic summary of experimental design. Rats were first divided up into four groups: Normoxia +/- RU486 and HH +/- RU486, then given five days of treadmill acclimation (Treadmill ACC) prior two normoxia graded exercise test (Normoxia GXT). After a two-day wash period, RU486 groups began drug treatment (60mg/kg/d in chow). Five days later all rats were given graded exercise tests in acute hypobaric hypoxia (HH1). Rats in HH groups remained at simulated altitude for an additional 15 days. All rats then performed a second graded exercise test (HH2). A final two-day wash period was allowed before sacrifice and tissue collection. B) Simplified schematic of rat hindlimb depicting muscles excised for analysis. Fill color represents the relative fiber type composition of each muscle, with soleus containing the most Type I oxidative (*red fill*), plantaris and red gastrocnemius containing a mixed amount of each fiber type (*orange fill*) and white gastrocnemius containing a high percent of Type I glycolytic fibers (*white fill*).

II.B. Induction of hypobaric hypoxia

A Hypo-Hyperbaric Chamber housed at Colorado State University (Fort Collins, CO; 1,525m above sea level; barometric pressure 636 mmHg at 20 degrees Celsius) was used to simulate an altitude of 5,242 m (17,200 ft; barometric pressure 413 mmHg at 20 degrees Celsius) above sea level for all acute and chronic HH exposures in this study. Rate of chamber depressurization to target simulated altitude was around ~1,000 ft/min. An ante-chamber capable of equalizing to either external or chamber pressure was used to enable technicians to enter and exit without disrupting the average pressure within the main compartment. Temperature was maintained at 20-22°C on the same 12-hour light/dark cycle as the normoxic group.

II.C. Graded Exercise Test (GXT)

To assess aerobic exercise capacity in normoxia and hypoxia, rats were trained to perform a graded exercise test (GXT) on a 5-lane motorized treadmill (Harvard Apparatus) based on a previously published protocol (Gonzalez et al., 1994). The treadmill was equipped with a 0.2V shock grid at the posterior end, and set at a 10% fixed incline for the duration of the GXT. Prior to the first test, animals were acclimated to the treadmill by allowing them to briefly explore the lane and walk at varying speeds encountered during the GXT. All acclimation sessions were limited to 10 minutes total and did not include long-duration bouts of intense exercise, so as to prevent an effect of pre-training on Baseline (BL) GXT measurement. As summarized in Figure 1A, a total of three GXTs were administered per animal: BL (two days treadmill post-acclimation), GXT1 (performed in acute HH, 7 days after BL GXT), and GXT2 (performed in HH after 15 days of HH or normoxia). Animals were given a 90 second warm-up period at a speed of 7cm/s, followed by a 90 second break, before beginning the graded protocol (shown in Table 2). The test was terminated upon individual animal fatigue, which we defined as 10 touches on the shock grid (set to 0.2V) within a 10 second period.

Table 2: Graded aerobic exercise test protocol

Stage	GXT time (min)	Time spent in Stage (min)	Speed (cm/s)	Speed (m/min)
Warm Up	0	1.5	0	0
Warm Up	0	1.5	7	4.2
Warm Up	0	1.5	0	0
Stage 1	1 – 1.5	1.5	15	9
Stage 2	1.5 – 3.0	1.5	18	10.8
Stage 3	3.0 – 4.5	1.5	21	12.6
Stage 4	4.5 – 6.5	2.0	28	16.8
Stage 5	6.5 – 8.5	2.0	35	21
Stage 6	8.5 – 10.30	2.0	42	25.2
Stage 7	10.30 – 14.30	4.0	50	30
Stage 8	∞	∞	60	36

Protocol adapted from Gonzalez et al. 1994. Shock grid set to 0.2V. Exercise fatigue defined as 10 consecutive touches on shock pad in 10 seconds.

II.D. Tissue collection and sample preparation

Rats were sacrificed in their respective experimental conditions (normoxia or HH) following confirmation of deep anesthesia with sodium pentobarbital (100 mg/kg i.p.; Vortech; 0298-9373) by midline thoracotomy and removal of the heart 48 hours after their final GXT to minimize the impact of acute exercise stress on endpoint outcomes. Blood was immediately extracted from the beating heart at the pulmonary trunk and placed on ice prior to centrifugation at 10,000 x g for 10 min at 4°C for hematocrit measurement and serum collection. Skeletal muscles were carefully dissected (Figure 1B), weighed and prepared for various experimental procedures as follows: The left soleus muscle was immediately placed in ice-cold BIOPS buffer containing 10 mM Ca-EGTA (0.1 µM free calcium), 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5mM DTT, 6.56 mM $MgCl_2$, 5.77 mM ATP, and 15 mM phosphocreatine) for preparation of permeabilized fibers. The right soleus, right plantaris and right gastrocnemius muscles were fixed in 10% formalin for future histological evaluation. The left plantaris, left white gastrocnemius and left red gastrocnemius section were snap-frozen in liquid nitrogen and stored at -80°C for biochemical analyses. Heart, spleen, kidney, and liver were also weighed and sectioned for storage at -80°C or fixed in 10% formalin.

II.E. Serum Corticosterone Assay

Circulating levels of corticosterone were measured in serum collected at sacrifice using a Corticosterone ELISA kit (Enzo; CAT# ADI-900-097), according to the manufacturer's instructions.

II.F. Western Blotting

Frozen tissue sections (25-50 mg) were thawed on ice and homogenized in Thermo Scientific M-PER™ Mammalian Protein Extraction Reagent lysis buffer (1:10 w/v; Cat# 78501) with Thermo Scientific Halt™ Protease and Phosphatase Inhibitor Single-Use Cocktail (100X; Cat# 78442) using glass-on-glass mortar and pestle on ice. Homogenates were then sonicated (Branson 250 Digital Sonifier Ultrasonic Cell Disruptor, Branson Ultrasonics Corporation) for five 0.5 second pulses on medium setting on ice, then centrifuged at 10,000 x g for 10 min at 4°C before transferring supernatants to fresh tubes for storage at -80°C. The total protein concentration of the supernatant was determined using a Bicinchoninic acid (BCA) assay (Thermo Scientific #23225), and 30ug of muscle proteins were electrophoresed in 4-12% Bis-Tris polyacrylamide gels (Invitrogen, CAT# NW04122BOX) at 150V for 1 hour, then transferred to polyvinylidene difluoride (PVDF) membranes for immunoblotting with antibodies specific to rat glucocorticoid receptor (GR; Invitrogen; PA1-511A), branch chain amino acid transferase 1 (BCAT1; Abcam; ab-232700), pyruvate dehydrogenase 1a (PDHa1; Abcam; ab-

92696), hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit alpha (HADHA; Abcam; ab-203114), muscle RING-finger protein-1 (MuRF1; Santa Cruz; sc-398608), and Krüppel-like factor 15 (KLF1-15; Santa Cruz; sc-271675), or an antibody cocktail recognizing subunits from Complexes I-V of the mitochondrial respiratory chain (Abcam; MS604).

Membranes were blocked with 5% non-fat milk for 1 hour, then incubated with primary antibodies incubated overnight at 4° Celsius, washing 3 x 4 minutes with Tris-buffered Saline + Tween (TBST; 20 mm Tris-base, 150 mm NaCl, pH 7.4) between blocking and antibody applications. Membranes were incubated with HRP-conjugated secondary antibody for 1 hour before adding chemiluminescence (Fisher SuperSignal™ West Dura Extended Duration Substrate, Cat# 34075) for protein band visualization. Densitometric analysis (Image-J, NIH) of detected bands was standardized to total protein content using Amido Black peptide staining (Sigma; A8181) as loading control. This stated, as it became apparent that white gastrocnemius tissue exposed to chronic hypoxia demonstrated a consistently low content of higher-molecular weight proteins, an region of interest of lower-weight stained proteins (between 30-55 kDa) was chosen as a more consistent loading control between groups.

II.G. Mitochondrial Respirometry

Skeletal muscle mitochondrial metabolism was evaluated in permeabilized soleus fiber bundles prepared as previously described (Pesta and Gnaiger, 2012) using two Oxygraph-2k high-resolution respirometers (Oroboros Instruments, Innsbruck, Austria). Briefly, excised soleus fibers were gently teased in ice-cold BIOPS, then permeabilized by incubation in BIOPS containing 50 μg saponin rocking on ice for 20 min, followed by two 15 min washes in ice-cold MiR05 buffer containing 110 mM sucrose, 20 mM HEPES, 10 mM KH_2PO_4, 20 mM taurine, 20 mM lactobionic acid, 3 mM $MgCl_2$ $6H_2O$, 0.6 mM EGTA, 1 mg/ml bovine serum albumin. Oxygen flux was monitored in real-time by resolving changes in the negative time derivative of the chamber oxygen concentration signal normalized to fiber wet weight following standardized instrumental and chemical background calibrations performed daily (instrumental) or before every experiment (chemical background). Respirometry experiments were performed in MiR05 buffer maintained at 37°C in a hyperoxygenated environment (275–400 μM O_2) to avoid potential limitations in oxygen diffusion on respiratory capacity of permeabilized fiber bundles (Li Puma et al., 2020). Several multi-substrate protocols were performed to elucidate effects on specific substrate oxidation capacities and efficiencies discussed further in the Results.

II.H. Statistical Analyses

Two-tailed t-tests were used to compare normoxia and HH groups, as well as make comparisons between PL and WG. The effect of RU486 treatment in both normoxia and HH was assessed using a 2 (environment) X 2 (GR status) analysis of variance with Tukey correction for multiple comparisons. A standard significance threshold of $p < 0.05$ was used in all physiology and respirometry analyses. A Grubbs' test, or ESD method (extreme studentized deviate), was used to identify and exclude statistical outliers.

III. Results

III.A. Impaired exercise capacity in acute HH is independent of GR signaling

To evaluate the effect of acute HH on aerobic exercise performance, GXTs were administered first in normoxia, then again in acute HH simulating 5,240 m above sea level. Consistent with established literature (Deb et al., 2018), aerobic capacity was significantly

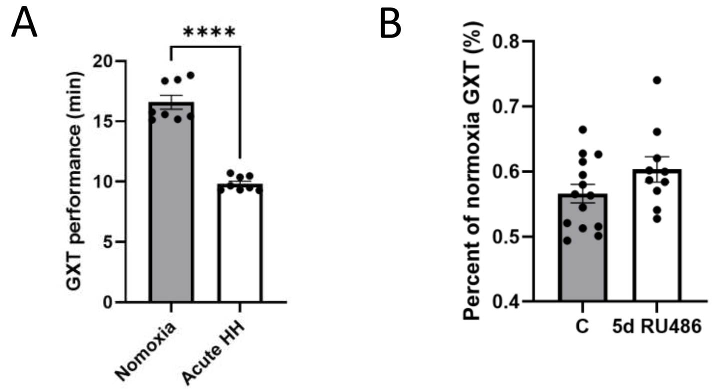


Figure 4. Effect of acute HH (17,000 ft) on Graded Exercise Time Trial (GXT). (A) Paired 2-tailed t-test, n=8,8. (B) Effect of 5 day pre-treatment of RU486 on acute HH GXT challenge. Unpaired 2-tailed t-test, n=14,10. ****p<0.0001

impaired in acute HH (Figure 2A). Five days of pretreatment with RU486 had no significant effect on HH exercise performance compared to non-treated animals (Figure 2B).

III.B. Improvements in HH exercise performance following HH acclimation are GR-dependent

Following the acute HH GXT, rats were maintained in either normoxic or HH conditions for 15 days before performing a second HH GXT. Each individual performance was compared to the initail HH GXT to derive a percent change (Figure 3A) and is also presented as absolute

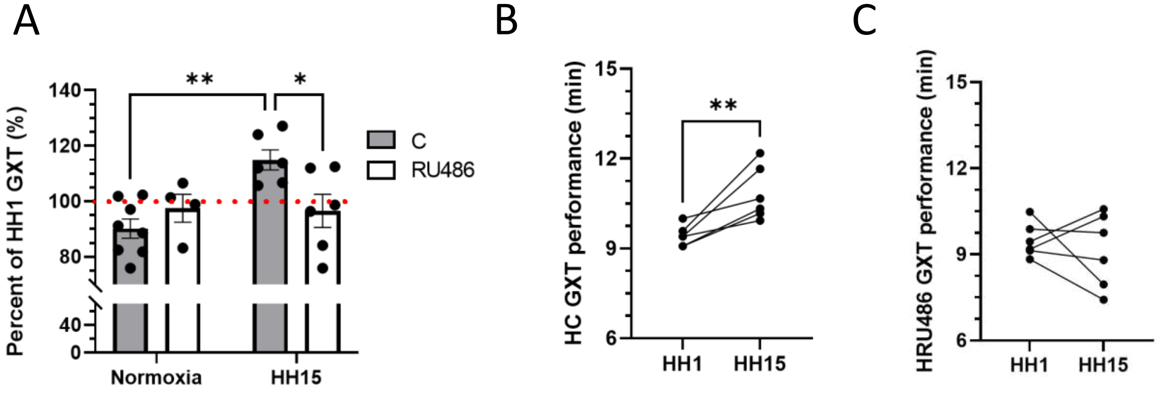


Figure 5. Effect of chronic HH exposure on post-acclimation Hypoxic Graded Exercise Time Trial (HH15). (A) Percent improvement in GXT performance after HH acclimation (15d) relative to acute HH GXT (HH1). 2-way ANOVA with Tukey post-hoc correction. (B) Paired t-test of HC rat exercise performance during acute (HH1) and post-acclimation (HH15) GXT. (C) Paired t-test of HRU486 rat exercise performance during acute (HH1) and post-acclimation (HH15) GXT. Significance *p<0.05, **p<0.01.

GXT time (Figures 3B, C). Chronic HH exposure significantly improved HH GXT performance compared with those that remained in normoxia, but this improvement was abolished by chronic administration of RU486 in HH. This suggests that GR signaling is required for adapative improvement in HH exercise performance after HH acclimation.

III.C. GR blockade attenuates hematopoiesis during HH acclimation

Hematopoiesis is a well-established adaptive response to chronic HH (Hannon et al., 1969, Sawka et al., 2000, Siebenmann et al., 2015) and has been shown to be partially dependent on GR signaling (Paulson et al., 2020). As expected, hematocrit was significantly greater among rats exposure to chronic HH in the present study (Figure 4). RU486 significantly attenuated hematocrit levels under both normoxic and HH conditions, but the effect in HH was not sufficient to return concentrations to normoxic levels.

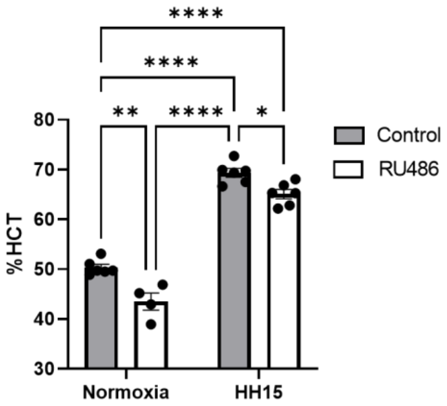
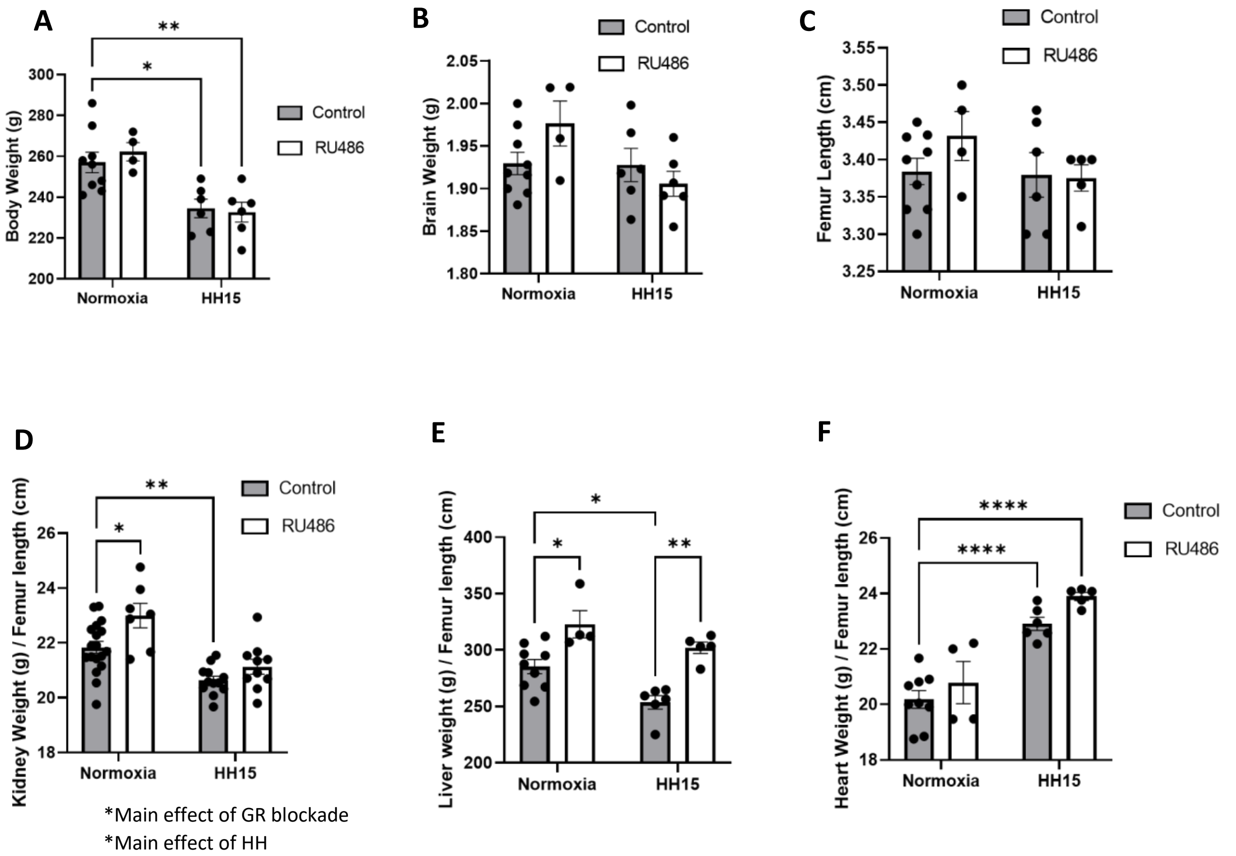


Figure 6. Percent hematocrit (%HCT). 2-way ANOVA with Tukey correction for multiple comparisons. *p<0.05, **p<0.01, ****p<0.0001.

III.D. Impact of HH acclimation and GR blockade on body and organ weights

Rats exposed to fifteen days of HH weighed significantly less than those in which remained in normoxia (Figure 5A). However, no significant difference in brain weight or femur length were observed (Figure 5B, C). All other organ weights were standardized to femur length, which served as a marker of fat free mass. Standardized kidney and liver weight were higher among RU486 treated rats in normoxia, but significantly lower among non-treated HH Control rats (Figure 5D, E). Consistent with other reports (Genovese et al., 1985, Nehra et al., 2016, Paddenberg et al., 2007), heart mass was significantly higher following chronic HH exposure (Figure 5F).



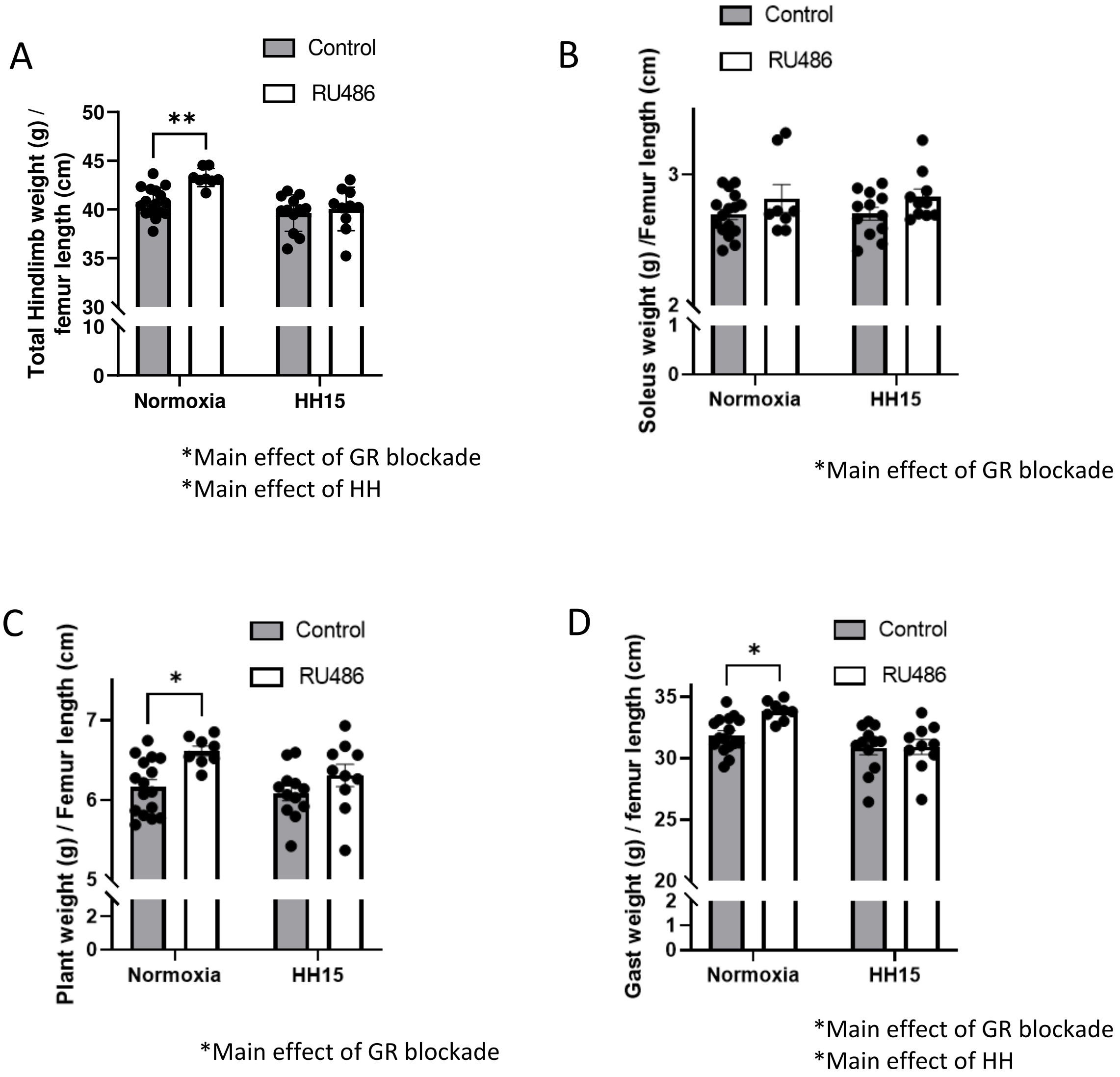
*Main effect of GR blockade
*Main effect of HH

Figure 7. Tissue weight among rats exposed to normoxia and HH with and without RU486 administration: (A) total body weight; (B) Brain weight; (C) femur length; (D) Kidney, (E) liver and (F) heart weights were standardized to individual femur length. All comparisons made using 2-way ANOVA with Tukey correction for multiple comparisons. (n= 9,4,6,6). *p<0.05, **p<0.01, ****p<0.0001.

III.E. Impact of HH acclimation and GR signaling on skeletal muscle morphology

After sacrifice, soleus, plantaris and gastrocnemius muscles where carefully excised and weighed (Figure 6). “Total hindlimb” (Figure 5A) represents the sum of all three muscles and was significantly increased in normoxia with RU486 treatment. Slightly lower total hindlimb mass, driven by marginally less gastrocnemius muscle, was also observed in HH (Figure 5 B-D).

Of note, soleus muscle, which is mostly composed of Type I skeletal fibers (Novak et al., 2010), was not significantly different between groups.



*Main effect of GR blockade
*Main effect of HH

*Main effect of GR blockade

*Main effect of GR blockade

*Main effect of GR blockade
*Main effect of HH

Figure 8. Muscle weights standardized to femur length. (A) Total hindlimb weight, (B) Soleus muscle, (C) Plantaris, and (D) gastrocnemius. Each data point represents an individual muscle collected from each rat at the time of sacrifice. All comparisons: 2-way ANOVA with Tukey post-hoc correction. *$p<0.05$

Further, HH-mediated improvements in running performance are not correlated to either total hindlimb or average gastrocnemius weight (Figure 7A, B). Collectively, these data suggest that GR-mediated improvements in GXT performance after 15 days of HH exposure are not attributable to gross differences in muscle mass. However, increased sensitivity to HH and GR blockade among muscles with greater density of Type II fibers suggests a potential role of GR in intracellular remodeling. This lead us to evaluate the concentration of GR in both red (predominantly Type I) or white (mostly Type II) muscles, as well as several mitochondrial enzymes key in substrate metabolic processes.

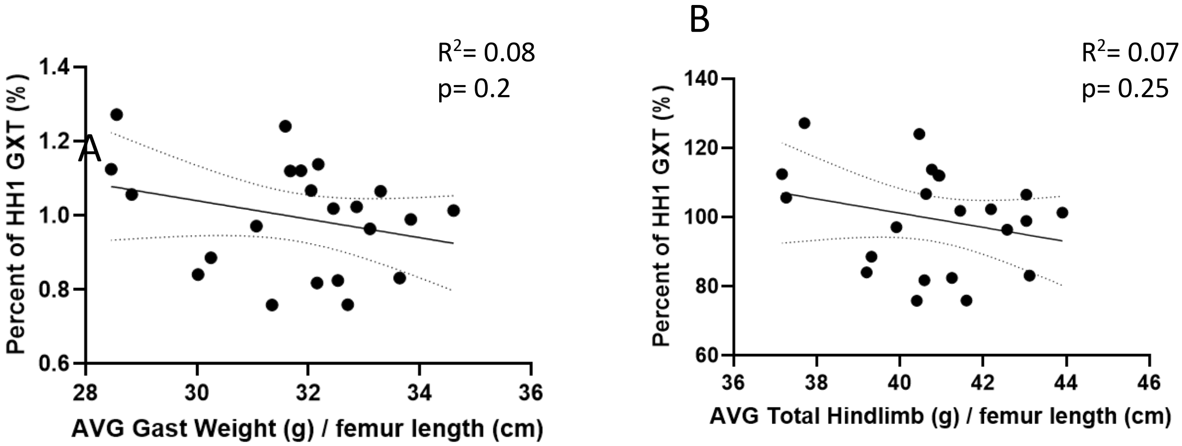


Figure 9. Correlation plot between %GXT performance after 15d of normoxia/HH exposure and (A) average gastrocnemius weight standardized to femur length or (B) total hindlimb weight standardized to femur length.

III.F. GR expression is >3-fold greater in white gastrocnemius than red plantaris muscles

GR protein content in the plantaris (PL) and white gastrocnemius (WG) was evaluated in untreated normoxia and HH groups (Figure 8). Under both barometric conditions, higher concentrations GR were observed in WG compared with PL muscles.

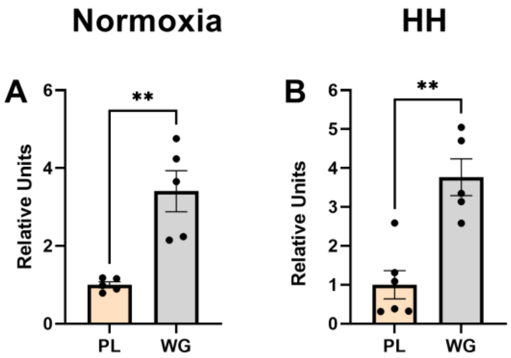


Figure 10. Glucocorticoid receptor (GR) protein content in plantaris (PL) and white gastrocnemius (WG) measured by Western Blot (immunofluorescence) in (A) normoxia control and (B) hypobaric hypoxia (HH) untreated control rats. Data standardized to plantaris group in both normoxia and HH. 2-tailed t-test. **p<0.01.

III.H. Muscle proteolysis following HH acclimation is GR-dependent and fiber-type specific

To determine if HH and GR-signaling influenced muscle proteolysis, we measured the relative expression of proteins involved in GR-dependent protein catabolism and nitrogen handling across groups. HH increased levels of the proteolytic E3 ubiquitin ligase Muscle RING-finger protein-1 (MuRF1) in white gastrocnemius, which was abolished by GR-blockade with RU486 (Figure 9A). MuRF1 expression is regulated by the GR-regulated transcription factor Krüppel-like factor (KLF15) (Shimizu et al., 2011), which was similarly increased in the WG with HH acclimation in a GR-dependent manner (Figure 9B). Muscle protein breakdown results in higher intracellular concentrations a free amino acids, particularly branched chain amino acids (BCAA) (Neinast et al., 2019, Suryawan et al., 1998). Because excessive ammonia can disrupt myocyte function, excess BCAAs are exported from the cell in the form of alanine and glutamine, which can used as a hepatic gluconeogenic substrate or catabolized in the kidney, respectively (Mann et al., 2021). Branch chain amino acid transferase (BCAT1) plays a key role in this process by catalyzing the transfer of amine groups from BCAAs onto α-KG molecules, the expression of which is regulated by KLF15 (Biswas et al., 2019). Accordingly, BCAT1 protein levels in the WG were also elevated in HH in a GR-dependent fashion (Figure 9C). Interestingly, no significant differences in any of these proteins were observed in the plantaris muscle across the four experimental groups (Figure 9D-F). Taken together, these results demonstrate that activation of the skeletal muscle ubiquitin protease system in HH is GR-dependent and fiber type-specific, perhaps being governed by tissue GR expression rather than exposure to hypoxemia, *per se*.

III.I. HH acclimation results in GR-dependent loss of mitochondrial enzymes in the white gastrocnemius, but not red plantaris muscle

Previous studies have suggested that high altitude acclimation changes expression patterns of enzymes involved in substation oxidative in myocytes (Levett et al., 2012, Chicco et al., 2018). To determine if such changes occurred the present study, the protein expression of several mitochondrial enzymes was evaluated by immunoblotting. As expected, combined expression of single subunits from each of the five mitochondrial electron transfer system (ETS) complexes was significantly lower in white gastrocnemius than plantaris muscle under both

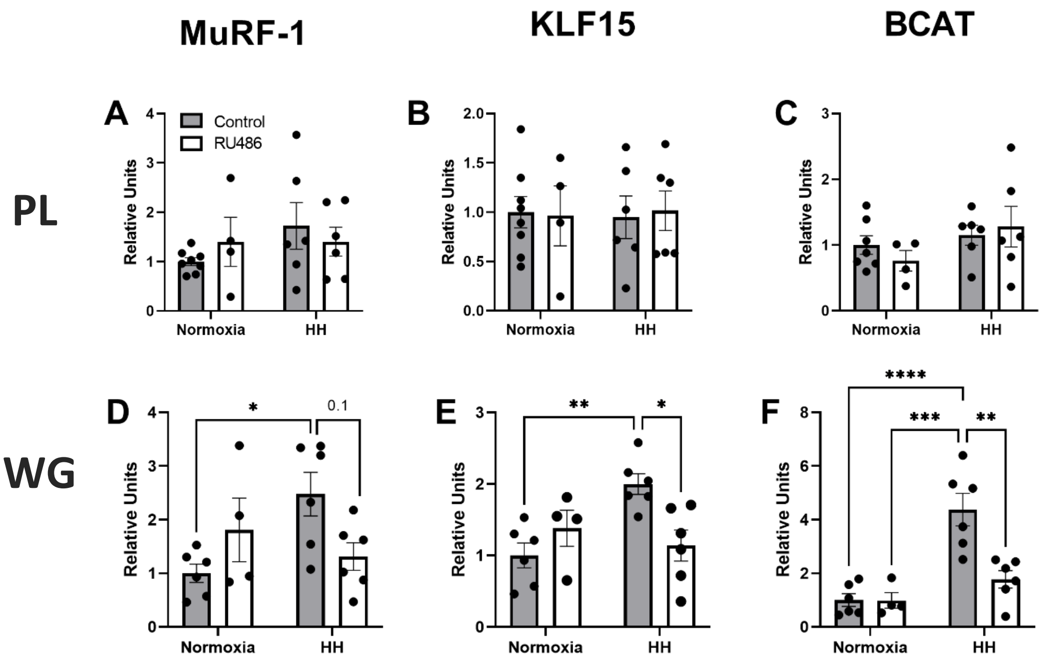


Figure 11. Muscle protein expression of MuRF-1, KLF15 and BCAT in plantaris (PL, panels A-C) and white gastrocnemius (WG, panels D-F). All comparisons standardized to normoxia control group. 2-way ANOVA with Tukey post-hoc correction. *p<0.05, **p<0.01, ***p<0.001, ****p<0.00001.

normoxia (Figure 10A) and HH conditions (Figure 10B), consistent with a much lower mitochondrial density in predominantly glycolytic/Type I versus oxidative/Type II muscles, respectively. After acclimation to HH, significant GR-dependent reductions in ETS protein were observed in the WG (Figure 10C), but not PL (Figure 10D).

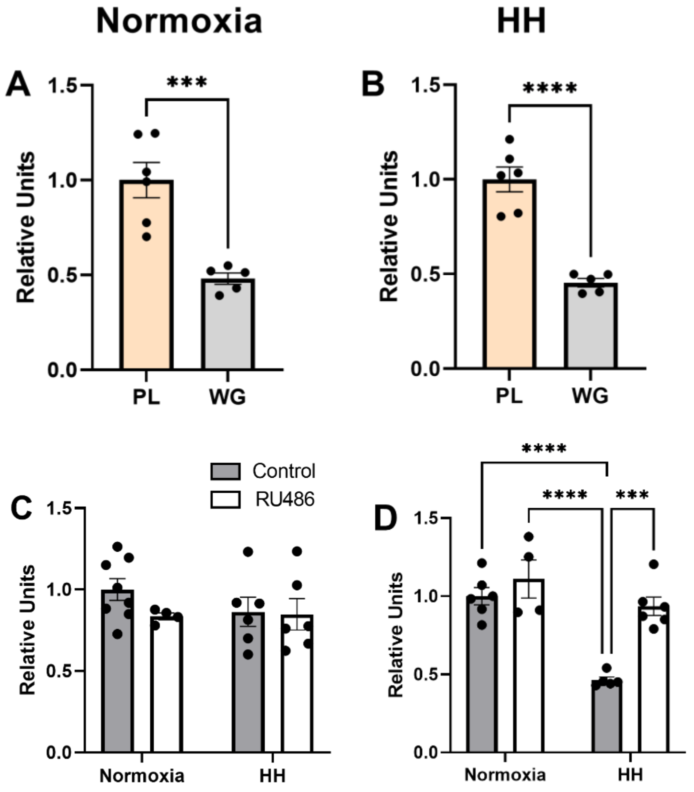


Figure 12. Relative ETS protein concentration in both normoxia and HH. (A, B) comparison of PL and WG muscle under both barometric conditions. Two-tailed t-test. ***p<0.001, ****p<0.0001. (C, D) Differences between normoxia and HH +/- RU486 in both WG and PL fibers. Comparisons in panels A and B are standardized to PL. Comparisons in panels C and D are standardized to normoxia control group. 2-way ANOVA with Tukey post-hoc correction. ***p<0.001, ****p<0.0001.

Similarly, expression of Hydroxyacyl-CoA Dehydrogenase (HADHA, or Trifunctional enzyme subunit alpha), which catalyzes two steps of the long-chain fatty acid β-oxidation cycle, and Pyruvate Dehydrogenase (PDH), which converts pyruvate to Acetyl-CoA in the

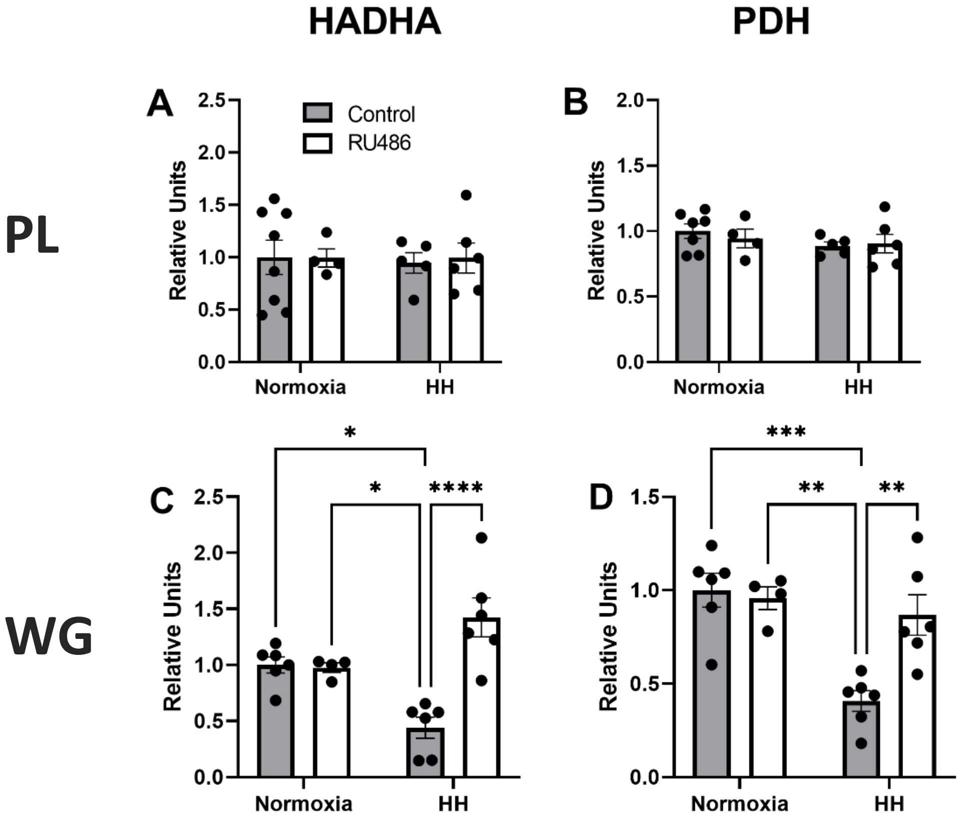


Figure 13. Relative protein concentration of Hydroxyacyl-CoA Dehydrogenase (HADHA) and Pyruvate Dehydrogenase (PDH). (A, B) comparison of PL and WG muscle under both barometric conditions (C, D) Differences between normoxia and HH ± RU486 in both WG and PL fibers. All comparisons standardized to normoxia control group. 2-way ANOVA with Tukey post-hoc correction. Significance *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

mitochondria, exhibited GR-dependent reductions after chronic HH in WG, but not in PL (Figure 11).

III.J. HH acclimation elicits GR-dependent improvements in long-chain fatty acid oxidation efficiency

Given reductions in ETS, carbohydrate and lipid enzyme concentrations, we next sought to understand how changes in these metabolic protein concentrations may impact functional parameters of mitochondrial bioenergetics. To investigate, high-resolution respirometry was performed on permeabilized muscle fibers. Soleus muscle was chosen for analysis due its essentially uniform distribution of mitochondria-dense Type I fibers, which make up between

87-100% of the muscle in rats (Soukup et al., 2002, Novak et al., 2010, Mizunoya et al., 2014, Sawano et al., 2016). To detect potential differences in substrate-specific oxygen consumption, we used four different protocols outlined in Table 1. No significant differences were observed across experimental groups using any substrates except the long-chain (16-carbon) fatty acid, palmitoyl-carnitine (PAL). During this experiment, “Leak” respiration resulting dissipation of the inner membrane proton gradient fueled by PAL oxidation was significantly lower in HH Control (2.5 ± 0.5 pmol O_2/mg/s) compared to Normoxia Control (4.7 ± 1.9 pmol O_2/mg/s) ($P < 0.05$), but not when compared to Normoxia RU486 (3.7 ± 0.4 pmol O_2/mg/s) or HH RU486 (4.3 ± 0.6 pmol O_2/mg/s).

ADP-dependent respiration with the same substrates also tended to be lower in HH Controls (20.7 ± 3.3 pmol O_2/mg/s) than Normoxia Control (21.2 ± 6.5 pmol O_2/mg/s) for, Normoxia RU486 (18.1 ± 1.8 pmol O_2/mg/s), and HH RU486 (15.3 ± 2.8 pmol O_2/mg/s), but differences were not statistically significant. However, ADP control of PAL oxidation, an estimate of OXPHOS coupling efficiency calculated as [1-(leak respiration/ADP-dependent respiration)] (Pesta and Gnaiger, 2012), was significantly higher in HH compared to normoxia, and was abolished by RU486 treatment (Figure 12A). The same trends were not observed when the medium chain (8 carbon) octanoylcarnitine (Figure 12B) or pyruvate (Figure 12C) were used as substrates, indicating a selective improvement the efficiency of long-chain fatty acid oxidation in response to long-term HH that is also mediated by GR signaling.

Table 3: Mitochondrial respirometry protocols

Protocol	Outcome
M | P | ADP | G | S | *c*	No change across groups
M | PAL | ADP | P | G | S | *c*	GR-mediated improvement in PAL CCR
M | OCT | ADP | P | G | S | *c*	No change across groups
M | G | ADP | P | S | *c*	No change across groups
M | P | G | S | ADP | *c*	No change across groups

M = 1 mM malate, P = 5 mM pyruvate, ADP = 3 mM adenosine diphosphate, G= 10 mM glutamate, S = 20 mM succinate, PAL = 0.4 mM palmitoyl-carnitine, OCT = 0.5 mM octanoyl-carnitine, and *c* = 10 µM cytochrome *c*. CCR = Coupling Control Ratio, represented by the equation: [1-(MPAL/ATP), where "MPAL is rate of oxygen consumption (*J*O2) after the addition of malate + palmitoyl-carnitine and "ATP" is the subsequent *J*O2 after the addition of ADP.

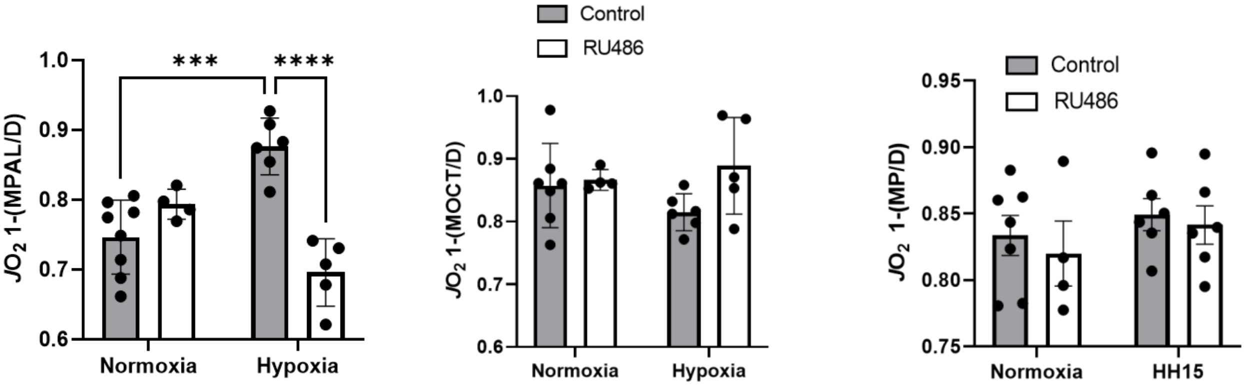


Figure 14. ADP control of PAL oxidation calculated as [1 - (leak respiration/ADP-dependent respiration)] for A) palmitoyl-carnitine and B) octanoyl-carnitine. 2-Way ANOVA with Tukey post-hoc correction. ***p<0.001, ****p<0.0001.

III.K. GR signaling may mediate skeletal muscle HIF-1α stabilization during HH acclimation

Preliminary data on HIF-1α protein expression in WG and PL muscles (shown below) demonstrate a GR-mediated increase after HH acclimation in WG, but not PL. Future experiments will seek to confirm tissue-specific HIF-1α stabilization through additional immunoblotting. Additionally, to test the hypothesis that HIF-1α stabilization is driven predominantly by low intramyocyte concentration of α-KG in WG due to GR-mediated proteolysis, direct quantification of metabolites will be conducted using GC-MS metabolomics.

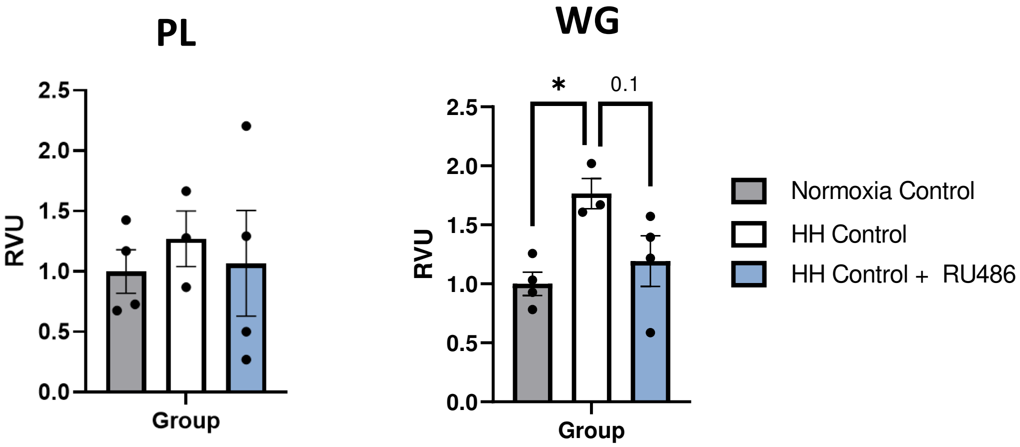


Figure 15. Preliminary protein quantification of HIF-1α in plantaris (PL, A) and white gastrocnemius (WG, B) muscle. 1-way ANOVA, Tukey correction. *P<0.05

V. Discussion

Physiological adaptation to HH is a biologically essential and evolutionarily conserved process that serves to attenuate hypoxemia and prevent tissue hypoxia following chronic exposure. Though many components of the acclimation response are well understood, there is currently a lack of consensus regarding skeletal muscle responses to chronic HH. Most studies to date using humans have involved high altitude mountaineering or trekking expeditions involving wide variability in the extent and duration of HH exposure, and may be confounded by variations subject training status, concomitant metabolic demands of strenuous physical exercise volume/intensity, and significant environmental stressors (e.g., disrupted sleep and food intake, extreme cold, etc.). Furthermore, even well-controlled studies of skeletal muscle metabolism using methods such as high-resolution respirometry have thus far ignored potential fiber type-specific effects. Indeed, the *vastus lateralis* muscle, which has a highly heterogenous fiber type composition, has been used exclusively in human high-altitude acclimation studies. Additionally, though changes in various hormone concentrations during and following ascent to high altitude have been studied (von Wolff et al., 2018, Benso et al., 2007, Calbet, 2003, Bogaard et al., 2002), impact of their signaling on skeletal muscle response and exercise performance within this context has not been investigated. Collectively, these factors may explain the wide variation in metabolic function seen in biopsied skeletal muscle after prolonged exposure to HH.

The present study is the first to demonstrate that GR signaling is necessary for improved exercise performance after 15 days of HH acclimation, and is responsible for marked changes in skeletal muscle metabolism in this context. Although there are many GR-mediated processes that could improve aerobic exercise capacity in HH, including increased hematocrit (Bauer et al., 1999, Wang et al., 2021) and increased nutrient availability (Coderre et al., 1991, van Raalte et al., 2009, Beaupere et al., 2021), changes in muscle morphology and metabolic function might also play an important role. We found that muscle mitochondrial respiratory capacity is unchanged in skeletal muscle containing nearly 100% Type I fibers (soleus) following HH acclimation, while the efficiency of long-chain fatty acid oxidation is improved. This finding is consistent with data from humans acclimated to 5260 m for 16 days (Chicco et al., 2018) and in general agreement other studies in humans (Jacobs et al., 2012, Horscroft et al., 2017) and rats (Ou and Leiter, 2004) demonstrating improvements in muscle fatty acid utilization following acclimation to altitudes >4300 m. In contrast, we found a marked reduction in muscle mitochondrial enzymes that mediate both fatty acid and carbohydrate oxidation in the Type II fiber-rich WG, which are consistent with several previous studies of high-altitude climbers and trekkers (Horscroft et al., 2017, Levett et al., 2012, Vigano et al., 2008). Our study is the first to demonstrate that these seemingly conflicting responses of muscle to HH may be explained simply by fiber type-specific differences in GR signaling (Figure 7) (Sandri et al., 2006, Shimizu et al., 2011). Moreover, activation of muscle proteolysis, which has been widely associated with HH acclimation in humans and animal models (Przygodda et al., 2017, Schakman et al., 2013, de Theije et al., 2018), is similarly GR-dependent and largely restricted to the WG. This degradation of Type II muscle protein may be adaptive in HH (Murray and Montgomery, 2014), and along with the loss of mitochondrial enzymes in these fibers, may serve to reduce oxygen consumption by muscles rich in Type II fibers while shunting oxygen supply to Type I-rich muscle where oxidative capacity is preserved. Given that Type I fibers support the bulk of low-to-moderate intensity aerobic exercise, these impacts of GR signaling may serve to maximize the efficiency of skeletal muscle oxygen utilization in response to chronic hypoxemia.

While the present studies demonstrate the importance of GR signaling in skeletal muscle responses to HH, the mechanisms responsible for these effects remain incompletely understood. Hypoxia inducible factor 1 alpha (HIF-1α) is the canonical regulator of the cellular response to hypoxia (Semenza, 2012), and has often been implicated in skeletal muscle responses to hypoxemia associated with HH such as reduced mitochondrial oxidative capacity and increased glycolytic activity (Murray, 2009). Interestingly, we observed higher HIF-1α protein following HH acclimation in WG, but not PL, which was dependent on GR signaling. This finding is consistent with a previous report in rodents, which noted greater abundance of HIF-1α in muscle containing mostly Type II fibers relative those containing mostly Type I (Pisani and Dechesne, 2005). Increased HIF-1α activation in white skeletal muscle may at least partially explain attenuated abundance of oxidative enzymes in GR-rich white muscle (Dutta et al., 2009). However, studies which have measured intramuscular PO_2 at rest in acute HH have noted only modest reductions compared to normoxic conditions (Richardson et al., 2006, Johnson et al., 2005). Indeed, it is likely that the well-established cardiopulmonary, hematological adjustments to HH are sufficient to defend intramyocyte PO_2 under resting conditions. The GR-dependence and fiber-type specific expression of HIF-1α following HH acclimation in the present study further implicates mechanisms of HIF-1α stabilization others than tissue hypoxia. Exactly how

GR signaling may regulated HIF-1α levels is unclear from our studies, but emerging evidence has provided some clues to this potential interaction. Studies using cell lines have suggested an emerging link between GR signaling and HIF-1α stabilization through either degradation of von Hippel-Lindau protein (pVHL), a E3-ligase which recognizes hydroxylated HIF-1α protein for proteolytic destruction (Vettori et al., 2017), or through direct GR binding to HIF-1α activation domains (Kodama et al., 2003).

HIF-1α is hydroxylated via prolyl hydroxylase domain-containing enzymes (PHDs) activity, which require molecular oxygen and alpha-ketoglutarate (α-KG) as substrate (Maxwell et al., 1999). Though HIF-1α is canonically stabilized during tissue hypoxia, low cellular concentrations of α-KG may also play an important role. Reduced intramyocyte concentrations of α-KG may rewire citric acid cycle flux to favor HIF-1α stabilization through reduced PHD activity in the absence of hypoxia (Tennant et al., 2009). Our lab has previously that increased HH-induced proteolysis can contribute to α-KG cataplerosis through increased aminotransferase and purine nucleotide cycle activity (Chicco et al., 2018). Though α-KG was not measured in the current study, increased GR-mediated protein expression of MuRF-1, BCAT, and KLF-15 in WG suggest similarly increased myofibril proteolysis, export of amine nitrogen via transaminase activity and reduced intramyocyte α-KG (Figure 14). Therefore, it is plausible that GR-mediated shifts in muscle protein metabolism are sufficient to alter HIF-1α protein stabilization independent of changes in muscle PO_2.

In conclusion, improvements in exercise performance and changes in skeletal muscle function realized after prolonged exposure to HH are mediated by GR signaling. Specific skeletal muscle changes depend predominantly on fiber type composition due to differential expression of the GR. Our results suggest that the acclimation response acts to defend muscle oxygen utilization by maintaining respiratory capacity and fatty acid oxidation efficiency in Type I muscle fibers, and perhaps reducing OXPHOS capacity of Type II skeletal fibers. These GR-mediated changes, alongside other systemic adaptations, may meaningfully contribute to observed improvements in exercise capacity following HH acclimation.

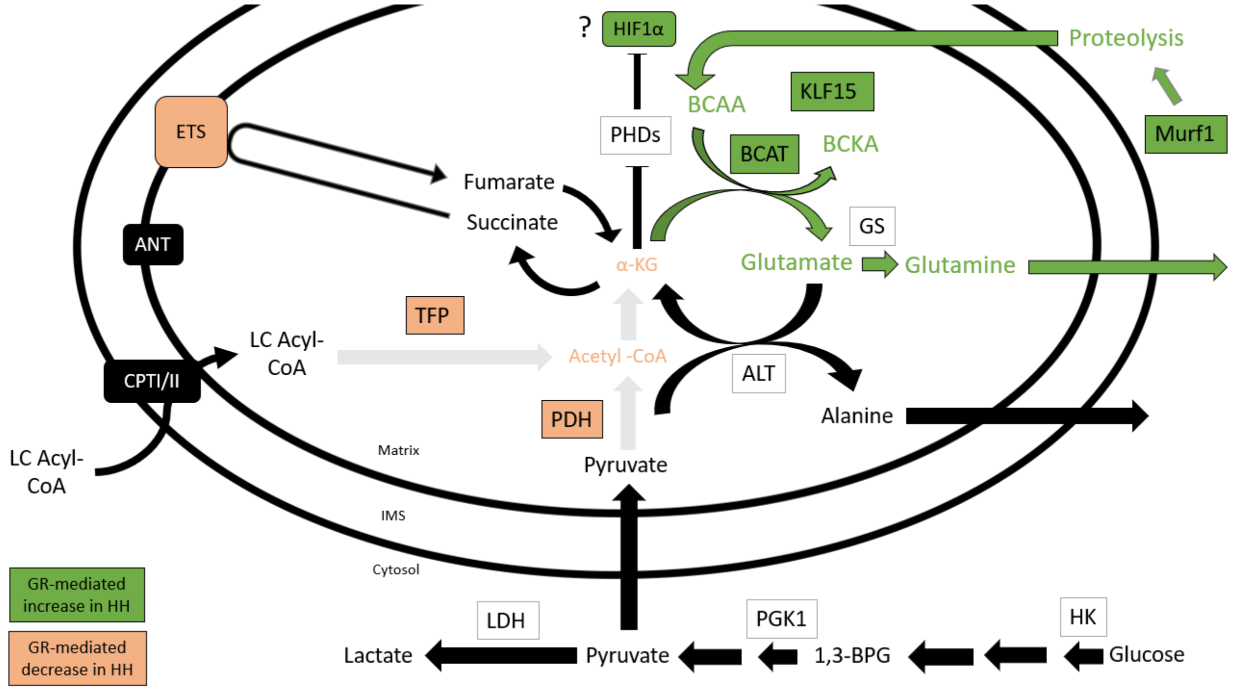


Figure 16. Simple schematic of α-KG regulation in WG mitochondria after chronic HH. Enzymes or enzyme complexes which exhibited GR-mediated reduction in HH are represented by *orange fill*, while those that exhibited GR-mediated increases are shown in *green fill*. Metabolites are shown in free text and colored based on possible intramyocyte concentration (*orange* represents lower concentration, *green* represents higher). Arrows denote possible means of HIF1α upregulation via reduced α-KG concentrations.

VI. Limitations and Future Directions

Although protein expression of key mitochondrial enzymes was significantly lower in WG muscle following chronic HH, mitochondrial respiratory analysis was not performed on these tissues. Future respirometry experiments using WG may confirm differences in mitochondrial function in response to HH acclimation between WG and soleus mitochondrial populations. Furthermore, measures of mitochondrial density, vascularization, and GR protein distribution (captured using immunohistochemical techniques) among gastrocnemius muscles following HH may provide additional insight into differential changes in oxidative capacity between red and white segments.

Anorexia is a well-established consequence of chronic HH which can exacerbate muscle loss (Rose et al., 1988, Hamad and Travis, 2006, Westerterp et al., 2000). Even under normoxic conditions, reduced caloric intake over prolonged periods can lead to reduced skeletal muscle growth and augmented catabolism, though specific implication for exercise performance are nuanced (el Haj et al., 1986, Biolo et al., 2007, Friedlander et al., 2005). In the present study, rats in both HH groups consumed less chow than those in normoxia, regardless of RU486 treatment status. Future studies should seek to elucidate the role of HH-induced anorexia on skeletal muscle, proteolysis, remodeling, and exercise performance following acclimation.

Male rats were used in the present study to control for potential effects of progesterone receptor antagonism via RU486 (Meyer et al., 1990), including gluconeogenic downregulation and reduced glycogenolysis (Campbell and Febbraio, 2002, Masuyama and Hiramatsu, 2011). This stated, muscle phenotype and physiology differ between sexes (Rosa-Caldwell and Greene, 2019). Notably, a greater percent of muscle is composed of Type II skeletal muscle fibers in males compared with females (Haizlip et al., 2015). Additionally, myocytes exhibit greater ubiquitin-protease activity relative to autophagy programs in males, while in females the autophagy system seems to be dominant (Ogawa et al., 2017, Ogawa et al., 2015). Given that data from the present study suggests GR-mediated upregulation of ubiquitin-protease activity in Type II fibers, it is possible that HH-induced GR signaling during acclimation impacts muscle function and whole-body exercise performance somewhat differentially between sexes.

Data collected on circulating corticosteroid concentrations post-acclimation were challenging to interpret (Figure 15). Corticosteroid concentrations were significantly lower in HH control rats compared with those in normoxia (28.4 ng/mL ± 21.8 vs 87.27 ng/mL ± 53.8, respectively). Although we did not collect blood samples throughout HH exposure, elevated GC plasma is a canonical response to acute hypoxemia (Rattner et al., 1980, von Wolff et al., 2018). Although statistically significant attenuation of plasma GC content following chronic HH has not been in previous reports, studies in both humans (Basu et al., 1997, Benso et al., 2007, von Wolff et al., 2018) and rats (Rattner et al., 1980) have described normalization of high GC concentrations in the absence of extreme calorie restriction or physical distress. This most likely occurs through gradual negative feedback inhibition of hypothalamic CRH release over time (Yuen et al., 2017). In our study, rats lived in a temperature-controlled environment with *ad libitum* food access and essentially no physical activity throughout the acclimation period. In addition, evidence of many canonical adaptations to HH were indeed observed in HC animals, such as high percent hematocrit (Figure 4) and greater cardiac mass (Figure 5F). Significantly lower GC concentrations seen after 15 days of HH exposure may therefore suggest hormonal feedback-inhibition and successful acclimation to HH-induced hypoxemia.

Interestingly, twenty-two total days of RU486 administration in both normoxia and hypoxia resulted in attenuated concentration of plasma GCs (Figure 13A). In humans, treatment with RU486 for 1-2 weeks has been shown to interfere with negative feedback inhibition of CRH and ACTH, leading to cortisol overproduction (Klijn et al., 1989, Bertagna et al., 1994, Yuen et al., 2017). Contrastingly, 4-15 days of RU486 treatment has not been shown to significantly alter circulating GCs in rats (Albertson et al., 1994, Eshkevari et al., 2015, Ding et al., 2019) or mice (Dalm et al., 2019). Furthermore, there is evidence of RU486-induced enzymatic inhibition of adrenal glucocorticoid synthesis (Albertson et al., 1994). To our knowledge, this is the first study to date which measured plasma GC concentration in rats after over 3 weeks of RU486 administration. Though dramatically reduced GC concentrations with RU486 treatment may indeed result from greatly attenuated HPA axis activity, mechanisms remain unclear. Collectively, these results highlight the need for follow-up studies which include serial blood draws throughout chronic HH exposure, in addition to skeletal muscle analysis following acute (24 hours) exposure, to better understand the relationship between changing GC blood content and GR mediated skeletal muscle adaptation over time.

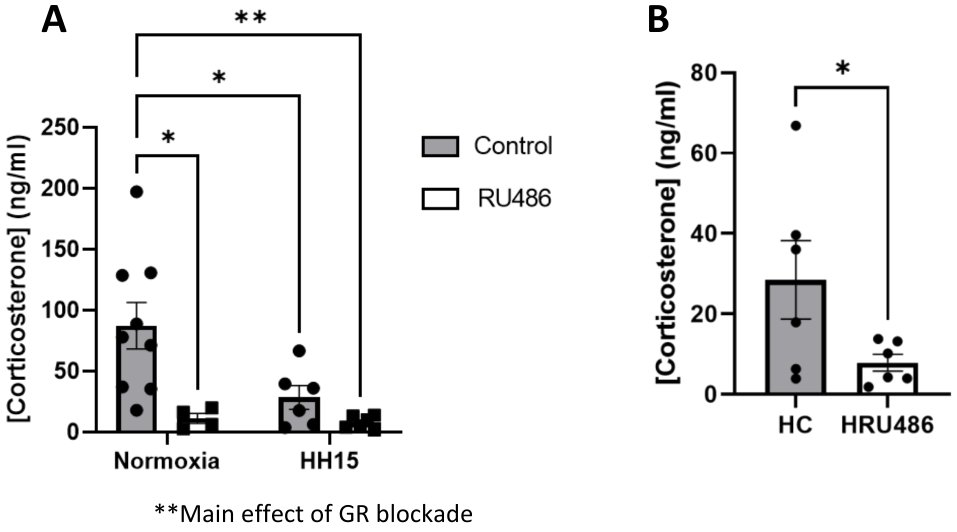


Figure 17. Plasma corticosterone concentration and GR protein relative abundance in normoxia and HH between white gastrocnemius (WG) and plantaris (PL) tissues. (A) 2-way ANOVA of plasma corticosterone concentrations after 15 days of either normoxia or hypobaric hypoxia (HH15) +/- RU486 treatment, with Turkey correction. (B) One-tailed t-test comparing GC concentration +/- RU486 in HH. *P<0.05, **P<0.01.

Finally, although the present study demonstrates that GR signaling is necessary to improve exercise performance following HH acclimation, future studies should investigate whether it is also sufficient to induce these effects. The GR agonist dexamethasone (DEX) has long been used to induce GR signaling cascades in both *in vitro* and *in vivo*. Replicating the present study design with DEX treatment groups instead of RU486 may more completely elucidate the role of GR signaling in the adaptive response to HH and yield translational insights for high-altitude exercise performance and clinical practice.

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