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IS THE SKELETAL MUSCLE HYPOXIC RESPONSE TO EXERCISE DIFFERENT BETWEEN ENDURANCE TRAINED AND UNTRAINED INDIVIDUALS?

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IS THE SKELETAL MUSCLE HYPOXIC RESPONSE TO EXERCISE DIFFERENT BETWEEN ENDURANCE TRAINED AND UNTRAINED INDIVIDUALS?

BY

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DISSERTATION

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DEDICATION

To my mother, Alma Delia Salazar and father, Roberto C. Nava. My successes are the fruits of your unconditional love and support. I am so proud to be your son.

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ABSTRACT

It was previously hypothesized that endurance exercise training blunts the hypoxia inducible factor 1 (HIF-1) response to acute exercise. We investigated whether training status influences the skeletal muscle HIF-1 response to acute exercise. 7 endurance athletes and 8 untrained controls performed a bout of cycling exercise. Skeletal muscle protein levels of HIF-1α and its inhibitors, prolyl hydroxylase 2 (PHD2), factor inhibiting HIF (FIH) and von Hippel-Lindau (VHL) were measured pre, post and 3h post-exercise. HIF-1 target gene expression was assessed via rtPCR and RNA-sequencing. Baseline protein levels of FIH and PHD2 were greater in the trained group. The exercise-induced increase in HIF-1α was not different between groups. Pyruvate dehydrogenase kinase mRNA was higher in the trained group at baseline but was unaffected by exercise. RNA sequencing revealed no group differences in HIF-1 target genes. These data suggest that the HIF-1 response to exercise is not different between trained and untrained individuals.

TABLE OF CONTENTS

LIST OF FIGURES

Review Manuscript

LIST OF TABLES

Review Article

Research Manuscript

SYMBOLS AND ABBREVIATIONS

% of Max Watts: Power expressed relative to max power achieved during VO_2peak test
ADM: Adrenomedullin
AMPK: AMP-activated protein kinase
ANKRD37: Ankyrin Repeat Domain 37
ANOVA: Analysis of Variance
ATF3: Activating Transcription Factor 3
BCA: bicinchoninic acid
BNIP3: BCL2 Interacting Protein 3
CDKN1A: Cyclin Dependent Kinase Inhibitor 1A
cDNA: complementary deoxyribonucleic acid
DMOG: Dimethyloxalylglycine
EDTA: Ethylenediaminetetraacetic acid
EPO: erythropoietin
ERRa Estrogen-Related Receptor alpha
FIH Factor Inhibiting HIF
FiO_2: Fraction of inspired O_2
FOSL2: FOS Like 2, AP-1 Transcription Factor Subunit
FOXO3: Forkhead Box O3
GADD45B: Growth Arrest and DNA Damage Inducible Beta
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GO: Gene Ontology
GYS1: Glycogen Synthase 1
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHb: deoxyhemo(+myo)globin
HIF-1: hypoxia inducible factor
HIIT: High Intensity Interval Training
HMOX1: Heme Oxygenase 1
HR: Heart Rate
HRE: Hypoxia Response Element
kDa: Kilodaltons
MCH: Myosin Heavy Chain
mPO_2: Oxygen Tension of Muscle Tissue
mRNA: Messenger Ribonucleic Acid
MT-CO2: Mitochondrially Encoded Cytochrome C Oxidase II
NOS3: Nitric Oxide Synthase 3
NR4A1: Nuclear Receptor Subfamily 4 Group A Member 1
NRF1/2: nuclear respiratory factor 1 and 2
O_2: Oxygen
O_2Hb: oxy(+myo)hemoglobin)
PCA: Principal component analysis
PDGFA: Platelet Derived Growth Factor Subunit A
PDK1: Pyruvate Dehydrogenase Kinase 1
PDK2: Pyruvate Dehydrogenase Kinase 2
PDK4: Pyruvate Dehydrogenase Kinase 4
PFKB3: 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 enzyme
PFKL: Phosphofructokinase L
PGC-1a: peroxisome proliferator-activated receptor-γ coactivator a
PHD2: prolyl hydroxylase 2
PBS: Phosphate Buffered Saline
PIM1: Proto-Oncogene, Serine/Threonine Kinase
PKM: Pyruvate Kinase Muscle type

PLUAR: Plasminogen Activator, Urokinase Receptor
PPARa: Peroxisome Proliferator-Activated Receptor alpha (
PTGS2: Prostaglandin-Endoperoxide Synthase 2
PVDF: Polyvinylidene Fluoride
RER: Respiratory Exchange Ratio
RPE: Rating of Perceived Exertion
RPM: Revolutions Per Minute
RT-qPCR : Reverse Transcription Polymerase Chain Reaction
SDH: Succinate Dehydrogenase
SERPINE1: Serpin Family E Member 1
SIRT6: Sirtuin 6
SLC2A3: Glucose Transporter Type 3
$SpO_{2:}$ Peripheral Blood Oxygen Saturation
TBST: Tris-buffered saline containing 0.1% Tween 20
TFAM: Mitochondrial Transcription Factor A
tHb: Total Hemo(+myo)globin
TSI: Tissue Saturation Index
VEGF: Vascular Endothelial Growth Factor
VHL: von Hippel-Lindau tumor supressor protein
VO_2max: Maximal Oxygen Consumption
VO_2peak: Peak Oxygen Uptake
W/kg: Relative power in Watts, expressed relative to body mass in kilograms
ΔTSI: Percent change in tissue saturation index from baseline

CHAPTER I: Introduction

Is the skeletal muscle hypoxic response to exercise different between endurance trained and untrained individuals?

Introduction

Skeletal muscle displays remarkable plasticity in response to exercise training. Endurance exercise imposes metabolic (de Freitas, Gerosa-Neto, Zanchi, Lira, & Rossi, 2017), oxidative (Steinbacher & Eckl, 2015) and hypoxic stress (Ameln et al., 2005) within skeletal muscle cells. These stressors stimulate a multitude of transcriptional and post-translational modifications, altering the protein and gene expression profile of skeletal muscle in the hours and days following exercise (Egan & Zierath, 2013). The skeletal muscle adaptive response to exercise training results from the cumulative effect of these events (Perry et al., 2010). Over time, the muscle's ability to take up and utilize oxygen is improved (Prior, Yang, & Terjung, 2004), while substrate utilization is optimized to meet energetic demands (Kiens, Essen-Gustavsson, Christensen, & Saltin, 1993). Because these adaptations underlie training-induced improvements in exercise performance and metabolic health (Egan & Zierath, 2013), understanding the mechanisms by which the skeletal muscle response to exercise is regulated has broad implications for the field of exercise physiology.

Pioneering work by Richardson and colleagues showed that acute exercise results in a 10-fold decrease in the partial pressure of myoglobin-associated O_2 (mPO_2) (Richardson et al., 2006; Richardson, Noyszewski, Kendrick, Leigh, & Wagner, 1995). This decrease in mPO_2 is a robust hypoxic stimulus for the activation of oxygen-sensing pathways. The Hypoxia-Inducible Factor-1 (HIF-1) pathway is the most well characterized oxygen sensing pathway in mammalian cells. As the master regulator of oxygen homeostasis, HIF-1

regulates over 100 genes of varying function, many of which are involved in oxygen carrying and delivery, cell growth and glucose metabolism (Ke & Costa, 2006). Several studies have shown that acute exercise increases protein (Ameln et al., 2005; Van Thienen, Masschelein, D'Hulst, Thomis, & Hespel, 2017) and gene expression (Drummond et al., 2008; Gustafsson et al., 2002; Slivka et al., 2014; Slot et al., 2014) of the oxygen-sensitive subunit of HIF-1, HIF-1α, suggesting that it is involved in the molecular response to exercise.

The current hypothesis regarding the role of HIF-1 in the adaptation to exercise by skeletal muscle is that endurance exercise training increases the abundance of HIF-1 inhibitors, suppressing its activity in response to acute exercise (M. E. Lindholm et al., 2014). This hypothesis is predicated on the following: 1) chronic HIF-1 inhibition induces an endurance-like skeletal muscle phenotype in mice (Mason et al., 2004); 2) endurance training increases resting levels of skeletal muscle HIF-1α inhibitors in humans (M. E. Lindholm et al., 2014); and 3) endurance training blunts the increase in HIF-1α mRNA in skeletal muscle in humans (Lundby, Gassmann, & Pilegaard, 2006). Despite this evidence, how training status impacts the activation of HIF-1α at the protein level has yet to be determined. Discrepancies between HIF-1α mRNA and protein levels were previously documented (Mounier, Pedersen, & Plomgaard, 2010). Therefore, the hypothesis predicated on exercise-induced changes in HIF-1α mRNA levels is tentative. The primary aim of this study is to determine if the exercise-induced activation of the HIF-1α pathway is influenced by training status.

Problem Statement

It was previously reported that endurance exercise training blunts the exercise-induced increase in HIF-1α mRNA in skeletal muscle (Lundby et al., 2006) leading to the

hypothesis that active HIF-1α suppression is part of the skeletal muscle adaptive response to endurance exercise training (Malene E. Lindholm & Rundqvist, 2016). However, HIF-1α mRNA differs considerably from protein expression, the level at which the transcription factor exerts its regulation on gene expression (Mounier et al., 2010). Therefore, it is currently unknown if exercise training status influences the exercise-induced increase in HIF-1α protein and the expression of its target genes in skeletal muscle.

Purpose of the Study

The purpose of this study is to determine if the HIF-1α response to acute exercise is different between endurance trained and untrained individuals.

Hypotheses

Hypothesis #1: Exercise-induced HIF-1α protein expression will be higher in endurance-trained individuals compared to untrained individuals.

Rationale: Increases in oxygen consumption drive the decrease in mPO_2 (O'Hagan et al., 2009). Because trained individuals have a greater capacity to consume oxygen and tolerate high workloads, the exercise-induced increase in HIF-1α will be higher in trained individuals.

Hypothesis #2: The exercise-induced change in HIF-1α mRNA will be inconsistent with protein expression changes.

Rationale: The HIF-1α mRNA response to exercise was blunted after endurance exercise training (Lundby et al., 2006). Fiber type-specific HIF-1α mRNA expression profiles are opposite of protein profiles (Mounier et al., 2010).

Hypothesis #3: The change in expression of HIF-1-regulated genes will be greater in endurance trained individuals compared to untrained individuals.

Rationale: The expression of HIF-1 target genes was augmented by the induction of the oxidative muscle phenotype by overexpression of PGC-1α in cultured muscle cells (O'Hagan et al., 2009).

Hypothesis #4: The decrease in TSI during exercise will be greater in trained individuals compared to untrained individuals.

Rationale: Muscle tissue oxygenation decreases with increases in exercise intensity (Tabira et al., 2012). Because trained participants will likely exercise at greater absolute intensities, the muscle oxygen demand will be higher and oxygen saturation lower in trained compared to untrained participants.

Hypothesis #5: The % decrease in muscle tissue oxygenation (a proxy for muscle hypoxia) will be positively correlated with the increase in HIF-1α protein expression.

Rationale: Upregulation of HIF-1α protein levels is dependent on the hypoxic dose (magnitude and time) (Nguyen et al., 2013). Contraction-mediated increases in HIF-1 are intensity-dependent (Tang et al., 2004).

Hypothesis #6: Aerobic capacity and fiber type composition will be negatively correlated with the increase HIF-1α protein expression.

A higher capacity to consume oxygen will result in a greater decrease in muscle PO_2. At baseline, type I oxidative fibers display higher HIF-1α protein levels than type II fibers (Mounier et al. 2010).

Scope of the Study

Eight untrained and seven endurance-trained individuals between the ages of 18 and 45 years participated in the study. Endurance-trained participants were included if their VO_2peak was above the 90^{th} percentile for their age and sex according to the norms

published by the American College of Sports Medicine (American College of Sports Medicine). Untrained participants were included if their VO_2peak was below the 50^{th} percentile for their age and sex. In order to determine whether training status influenced the exercise-induced increase in HIF-1α and HIF-1-regulated genes, each participant performed an acute bout of exercise. As part of a larger study, the exercise protocol consisted of 20, 'all out' 10-second sprints. Muscle tissue was sampled from the *vastus lateralis* muscle pre, immediately post, and 3 hours post exercise for the measurement of HIF-1α protein and mRNA as well as HIF-1-regulated gene expression. Muscle tissue saturation of the *vastus lateralis* muscle was measured via near-infrared spectroscopy (NIRS).

Limitations

1. The study of trained and untrained groups during an acute bout of exercise to understand the molecular adaptations to training was done as a substitute for a training study.
2. The exercise intensity was not fixed to a relative or absolute workload. It was self-paced exercise and thus the participants were likely exercising at different intensities.
3. The molecular markers were measured in the *vastus lateralis* and thus the responses observed will be specific to that muscle.
4. Both men and women were included. Any sex-specific differences in the molecular markers could have influenced the results of the between-group analysis.

Significance

Skeletal muscle adaptations underlie some of the improvements in metabolic health and exercise performance that occur following a period of endurance exercise training. There is a need to broaden the understanding of how these adaptations occur. HIF-1α regulates

many genes involved in oxygen and substrate metabolism. Therefore, examining how endurance training status affects the HIF-1α response will provide insight into how the adaptive response of skeletal muscle is regulated at the molecular level.

Definitions

HIF-1: Hypoxia-inducible factor 1

Hypoxia: Lower than normal oxygen levels.

mPO_2: The partial pressure of myoglobin-associated O_2, measured in millimeters of mercury (mmHg).

Muscle Oxygen Saturation: The percentage of myoglobin and hemoglobin fully saturated with oxygen within the skeletal muscle and surrounding capillaries.

mRNA: Messenger mRNA. Made from the transcription of DNA into RNA templates.

Phenotype: The observable characteristics of an organism, tissue or cell.

Transcription: The process by which DNA is transcribed into messenger RNA.

Post-translational modification: Modifications of proteins following their formation by ribosomes.

VO_2peak: The highest oxygen consumption value observed during a maximal graded exercise test.

TSI: Tissue Saturation Index

References

Ameln, H., Gustafsson, T., Sundberg, C. J., Okamoto, K., Jansson, E., Poellinger, L., & Makino, Y. (2005). Physiological activation of hypoxia inducible factor-1 in human skeletal muscle. *The FASEB Journal*, *19*(8), 1009–1011. https://doi.org/10.1096/fj.04-2304fje

American College of Sports Medicine. (2018). *ACSM's guidelines for exercise testing and prescription*. (D. Riebe, J. K. Ehrman, G. Liguori, & M. Magal, Eds.) (10th ed., pp. 93-94). Philadelphia: Wolters Kluwer/Lippincott Williams & Wilkins Health.

de Freitas, M. C., Gerosa-Neto, J., Zanchi, N. E., Lira, F. S., & Rossi, F. E. (2017). Role of metabolic stress for enhancing muscle adaptations: Practical applications. *World Journal of Methodology*, *7*(2), 46. https://doi.org/10.5662/wjm.v7.i2.46

Drummond, M. J., Fujita, S., Takashi, A., Dreyer, H. C., Volpi, E., & Rasmussen, B. B. (2008). Human muscle gene expression following resistance exercise and blood flow restriction. *Medicine and Science in Sports and Exercise*, *40*(4), 691–698. https://doi.org/10.1249/MSS.0b013e318160ff84

Egan, B., & Zierath, J. R. (2013). Exercise Metabolism and the Molecular Regulation of Skeletal Muscle Adaptation. *Cell Metabolism*, *17*(2), 162–184. https://doi.org/10.1016/j.cmet.2012.12.012

Gustafsson, T., Knutsson, A., Puntschart, A., Kaijser, L., Nordqvist, S. A. C., Sundberg, C., & Jansson, E. (2002). Increased expression of vascular endothelial growth factor in human skeletal muscle in response to short-term one-legged exercise training. *Pflugers Archiv European Journal of Physiology*, *444*(6), 752–759. https://doi.org/10.1007/s00424-002-0845-6

Ke, Q., & Costa, M. (2006). Hypoxia-Inducible Factor-1 (HIF-1). *Molecular Pharmacology*, *70*(5), 1469–1480. https://doi.org/10.1124/mol.106.027029

Kiens, B., Essen-Gustavsson, B., Christensen, N. J., & Saltin, B. (1993). Skeletal muscle substrate utilization during submaximal exercise in man: effect of endurance training. *The Journal of Physiology*, *469*(1), 459–478. https://doi.org/10.1113/jphysiol.1993.sp019823

Lindholm, M. E., Fischer, H., Poellinger, L., Johnson, R. S., Gustafsson, T., Sundberg, C. J., & Rundqvist, H. (2014). Negative regulation of HIF in skeletal muscle of elite endurance athletes: a tentative mechanism promoting oxidative metabolism. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *307*(3), R248–R255. https://doi.org/10.1152/ajpregu.00036.2013

Lindholm, Malene E., & Rundqvist, H. (2016). Skeletal muscle hypoxia-inducible factor-1 and exercise. *Experimental Physiology*, *101*(1), 28–32. https://doi.org/10.1113/EP085318

Lundby, C., Gassmann, M., & Pilegaard, H. (2006). Regular endurance training reduces the exercise induced HIF-1α and HIF-2α mRNA expression in human skeletal muscle in normoxic conditions. *European Journal of Applied Physiology*, *96*(4), 363–369. https://doi.org/10.1007/s00421-005-0085-5

Mason, S. D., Howlett, R. A., Kim, M. J., Olfert, I. M., Hogan, M. C., McNulty, W., … Johnson, R. S. (2004). Loss of skeletal muscle HIF-1α results in altered exercise endurance. *PLoS Biology*, *2*(10), e288. https://doi.org/10.1371/journal.pbio.0020288

Mounier, R., Pedersen, B. K., & Plomgaard, P. (2010). Muscle-specific expression of hypoxia-inducible factor in human skeletal muscle. *Experimental Physiology*, *95*(8), 899–907. https://doi.org/10.1113/expphysiol.2010.052928

Nguyen, L. K., Cavadas, M. A. S., Scholz, C. C., Fitzpatrick, S. F., Bruning, U., Cummins, E. P., … Cheong, A. (2013). A dynamic model of the hypoxia-inducible factor 1 (HIF-1) network. *Journal of Cell Science*, *126*(6), 1454–1463. https://doi.org/10.1242/jcs.119974

O'Hagan, K. A., Cocchiglia, S., Zhdanov, A. V., Tambuwala, M. M., Cummins, E. P., Monfared, M., … Allan, B. B. (2009). PGC-1 is coupled to HIF-1 -dependent gene expression by increasing mitochondrial oxygen consumption in skeletal muscle cells. *Proceedings of the National Academy of Sciences*, *106*(7), 2188–2193. https://doi.org/10.1073/pnas.0808801106

Perry, C. G. R., Lally, J., Holloway, G. P., Heigenhauser, G. J. F., Bonen, A., & Spriet, L. L. (2010). Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. *The Journal of Physiology*, *588*(23), 4795–4810. https://doi.org/10.1113/jphysiol.2010.199448

Prior, B. M., Yang, H. T., & Terjung, R. L. (2004). What makes vessels grow with exercise training? *Journal of Applied Physiology*, *97*(3), 1119–1128. https://doi.org/10.1152/japplphysiol.00035.2004

Richardson, R. S., Duteil, S., Wary, C., Wray, D. W., Hoff, J., & Carlier, P. G. (2006). Human skeletal muscle intracellular oxygenation: the impact of ambient oxygen availability. *The Journal of Physiology*, *571*(2), 415–424. https://doi.org/10.1113/jphysiol.2005.102327

Richardson, R. S., Noyszewski, E. A., Kendrick, K. F., Leigh, J. S., & Wagner, P. D. (1995). Myoglobin O2 desaturation during exercise. Evidence of limited O2 transport. *Journal of Clinical Investigation*, *96*(4), 1916–1926. https://doi.org/10.1172/JCI118237

Slivka, D. R., Heesch, M. W. S., Dumke, C. L., Cuddy, J. S., Hailes, W. S., & Ruby, B. C. (2014). Human Skeletal Muscle mRNA Response to a Single Hypoxic Exercise Bout. *Wilderness and Environmental Medicine*, *25*(4), 462–465. https://doi.org/10.1016/j.wem.2014.06.011

Slot, I. G. M., Van Den Borst, B., Hellwig, V. A. C. V., Barreiro, E., Schols, A. M. W. J., & Gosker, H. R. (2014). The muscle oxidative regulatory response to acute exercise is not impaired in less advanced COPD despite a decreased oxidative phenotype. *PLoS ONE*, *9*(2), e90150. https://doi.org/10.1371/journal.pone.0090150

Steinbacher, P., & Eckl, P. (2015). Impact of Oxidative Stress on Exercising Skeletal Muscle. *Biomolecules*, *5*(2), 356–377. https://doi.org/10.3390/biom5020356

Tabira, K., Horie, J., Fujii, H., Aida, T., Ito, K., Fukumoto, T., … Ishihara, H. (2012). The Relationship Between Skeletal Muscle Oxygenation and Systemic Oxygen Uptake During Exercise in Subjects With COPD: A Preliminary Study. *Respiratory Care*,

57(10), 1602–1610. https://doi.org/10.4187/respcare.01602

Tang, K., Breen, E. C., Wagner, H., Brutsaert, T. D., Gassmann, M., & Wagner, P. D. (2004). HIF and VEGF relationships in response to hypoxia and sciatic nerve stimulation in rat gastrocnemius. *Respiratory Physiology and Neurobiology*, *144*(1), 71–80. https://doi.org/10.1016/j.resp.2004.04.009

Van Thienen, R., Masschelein, E., D'Hulst, G., Thomis, M., & Hespel, P. (2017). Twin resemblance in muscle HIF-1α responses to hypoxia and exercise. *Frontiers in Physiology*, *7*(JAN), 1–11. https://doi.org/10.3389/fphys.2016.00676

CHAPTER II: Literature Review

This chapter presents a review manuscript written for submission to the *European Journal of Applied Physiology: Call for "State of the Art" Reviews.* The references cited in this review are provided at the end of the manuscript.

The Role of HIF-1 in Adaptation to Exercise: Lessons from Whole Body and Skeletal Muscle-Specific Models

Roberto Carlos Nava, Michael Deyhle, Flávio de Castro Magalhães, Fabiano Amorim, Christine Mermier

Abstract

The adaptive response to exercise in skeletal muscle is the product of repeated, transient changes in gene and protein expression that follow acute exercise. These molecular responses to exercise are driven in part by the hypoxic stress imposed by exercise onto skeletal muscle. At exercise intensities greater than 50% of VO_2max, the partial pressure of oxygen in skeletal muscle tissue decreases, resulting in cellular hypoxia. This activates the oxygen sensing pathway regulated primarily by hypoxia-inducible factor 1α (HIF-1α), leading to the transcriptional modulation of hundreds of genes, some of which are implicated in the metabolic and morphological changes observed in skeletal muscle following a period of exercise training. Data collected over the last two decades shows that exercise alone or in combination with environmental hypoxia is a potent activator of the HIF-1α pathway. The magnitude of HIF-1α activation by exercise appears to be dependent on the intensity and duration of exercise, both of which contribute to the magnitude of the hypoxic stimulus. While it is clear that acute exercise increases HIF-1α activity, how training influences its basal and exercise-induced expression is less clear. It was hypothesized that the adaptations

induced by endurance exercise training blunt the HIF-1α response to acute exercise. However, this hypothesis is limited by the technical and methodological limitations of the experiments used to support it. Here, we review the effect of acute and chronic exercise on HIF-1α expression in skeletal muscle, with special emphasis on the methodological factors that currently limit our understanding of its role in the adaptation to exercise training.

Introduction

Exercise is a potent stimulus for induction of muscular and systemic physiological adaptations. Acute bouts of exercise result in transient changes in gene and protein expression related to skeletal muscle carbohydrate (O'Gorman et al. 2006) and lipid metabolism (Murakami et al. 1998; Coffey et al. 2006). Exercise also stimulates the production of red blood cells (erythropoiesis) and skeletal muscle capillary growth via upregulation of erythropoietin (EPO) (Schwandt et al. 1991) and vascular endothelial growth factor (VEGF) (Richardson et al. 1999), respectively. With exercise training, the cumulative effect of these changes in gene and protein expression alters the morphological and metabolic profile of skeletal muscle while also facilitating systemic oxygen (O_2) transport, delivery and utilization, ultimately improving exercise capacity (Holloszy and Coyle 1984; Egan and Zierath 2013). Although the physiological adaptations to exercise training such as improvements in maximal oxygen consumption (VO_2max) and exercise economy are well documented, the underlying molecular mechanisms by which these adaptations take place are not fully understood.

Exercise drives cellular and molecular responses in skeletal muscle by temporarily imposing metabolic, oxidative, heat and hypoxic stress. At intensities above 50% of VO_2max, the oxygen tension of muscle tissue (mPO_2) drops from resting values of 30 mm

Hg to approximately 2-3 mmHg (Richardson et al. 1995a). The magnitude of exercise-induced hypoxia is highlighted by the fact that environmental hypoxia alone, achieved by reducing the fraction of inspired O_2 (FiO_2) by more than half (from 21% to 10%- equivalent to 6,000 m), only decreases mPO_2 to approximately 23 mmHg (Richardson et al. 2006).

The cellular and molecular responses to hypoxia are primary driven by the activation of hypoxia inducible factor 1 (HIF-1). As a transcription factor, HIF-1 is often referred to as 'the master regulator of O_2 homeostasis' as it is known to regulate over 100 genes related to O_2 transport and delivery as well as both anaerobic and aerobic metabolism (Gallezot 2012). HIF-1 is a heterodimeric protein complex consisting of HIF-1α and HIF-1β subunits. While HIF-1β is constitutively expressed, HIF-1α is tagged for proteasomal degradation at the same rate as it is synthesized in an O_2-dependent manner by the von Hippel-Lindau tumor suppressor protein (VHL). In fact, the half-life of HIF-1α in isolated lung tissue is less than one minute, shorter than any other known protein (Semenza 2004; Yu et al. 2018). VHL tags HIF-1α for proteasomal degradation by recruiting the E3 ubiquitin-protein ligase complex. VHL-mediated degradation of HIF-1α is dependent on prolyl-hydroxylation by prolyl hydroxylase 2 (PHD2). Additionally, its interaction with hypoxia response elements (HREs) on hypoxia-sensitive genes is inhibited by the asparaginyl-hydroxylase, factor inhibiting HIF (FIH) (Nguyen et al. 2013). Reductions in O_2 tension within a given tissue inhibits hydroxylase activity, thus allowing interaction between HIF-1α and HIF-1β subunits and subsequent transcriptional regulation of target genes (Semenza 2004, 2007a).

In 2019, Greg Semanza, William Kaelin Jr, and Peter Ratcliffe were awarded the Nobel Prize in Physiology and Medicine for their research regarding the role of HIF-1 in cancer cell metabolism (Iliopoulos et al. 1995; Maxwell et al. 1999; Semenza 2000). More

recently, it has become clear that skeletal muscle HIF-1 levels are responsive to acute exercise and exposure to environmental hypoxia. Data collected with a variety of experimental models show that HIF-1 plays several roles in the adaptation to exercise training in normoxic and hypoxic conditions. However, there are mixed findings regarding the effect of exercise training on HIF-1 expression as well as its role in the adaptive response to exercise in skeletal muscle. Furthermore, the reductionist models used to study the function of skeletal muscle HIF-1 limits the conclusions that can be drawn from them, especially in the context of exercise. Here we review findings from human, animal and cell models to highlight the important, yet complex role of HIF-1 in the adaptation to exercise training.

The Effect of Acute Exercise on HIF-1α Expression

Shortly after its discovery in 1992, Semenza and colleagues reported the expression of HIF-1α in human skeletal muscle (Wiener et al. 1996). Subsequent studies confirmed that its expression in skeletal muscle is sensitive to acute endurance (Gustafsson et al. 1999; Ameln et al. 2005a; Slivka et al. 2014; Van Thienen et al. 2017) and resistance exercise (Drummond et al. 2008) (**Table 1**). Each study that reported the effect of exercise on HIF-1α expression also evaluated the effect of an additional hypoxic stress on skeletal muscle HIF-1 signaling. Two methods have been used to augment skeletal muscle hypoxia, blood flow restriction via arterial occlusion (Gustafsson et al. 1999; Ameln et al. 2005a; Drummond et al. 2008) and normobaric hypoxia (Slivka et al. 2014; Van Thienen et al. 2017). Using blood flow restriction to reduce skeletal muscle perfusion by 15-20%, Ameln et al. (2005) found a 97% increase in HIF-1α protein, but not mRNA expression in the *vastus lateralis* 30 minutes after a 45-minute bout of low-intensity unilateral cycling. These results were consistent with

the groups' previous finding that the same exercise and blood flow restriction protocol had no significant effect on HIF-1 mRNA (Gustafsson et al. 1999). In contrast, Drummond et al. (2008) showed that low-intensity knee extension, with or without blood flow restriction significantly increased HIF-1α mRNA. In all cases, however, there was no difference in the HIF-1α response between exercise only and blood flow restricted exercise. Considering that even low intensity exercise (50% of VO_2max) results in a 10-fold decrease in muscle mPO_2, the added hypoxic stress from blood flow occlusion is not sufficient to augment hypoxic signaling further than the exercise alone. This appears to be true of intensities below 50% of VO_2max, as these findings were observed from protocols utilizing workloads between 20 and 26% of VO_2max (Gustafsson et al. 1999; Ameln et al. 2005a) or 1 repetition maximum (Drummond et al. 2008).

Although not a universal finding, environmental hypoxia was shown to further decrease mPO_2 during exercise (Richardson et al. 1995b) and thus would be expected to augment HIF-1α signaling. Van Thienen et al. (2017) showed that normobaric hypoxia alone increased HIF-1α protein and mRNA. This response was further augmented following 20 minutes of cycling at 50.7 ± 2.3% of VO_2max. In contrast, Slivka et al. (2014) reported a robust post exercise increase in HIF-1α mRNA following 1 hour of cycling exercise. However, when the same exercise was performed in normobaric hypoxia equivalent of 3000 m, the response was no different than the normoxic trial (Slivka et al. 2014). Further complicating the matter, there was no increase in HIF-1α following exercise alone, suggesting that environmental hypoxia was a more potent stimulus for muscle hypoxia than exercise. How is it that previous researchers (Ameln et al. 2005b; Drummond et al. 2008) showed an increase HIF-1α expression following exercise alone, despite employing exercise

intensities half of that of used by Van Thienen et al. (2017)? The discrepancy between these studies may have been due to the differences in exercise duration. The exercise protocol used by Ameln et al (2004) (Ameln et al. 2005b) and Slivka et al. (2014) (Slivka et al. 2014) were more than twice as long than the protocol employed by Van Thienen et al. (2017). Given that the degradation of HIF-1α is O_2-dependent, the length of time in which the muscle remains hypoxic would presumably determine the degree of HIF-1α stabilization and downstream effects on target genes. This was also evidenced by the fact that Van Thienen's group showed resting HIF-1α protein levels increased after three hours of hypoxic exposure (~5,300 m). Thus, it appears that the amount of time a muscle cell remains hypoxic, whether it be from exercise, reductions in FiO_2 or a combination of both, may determine the magnitude of HIF-1α protein expression.

The magnitude of the hypoxic stimulus may also play an important role in determining the degree of exercise-induced HIF-1. Molé et al. (1999) showed that muscle PO_2 decreases linearly with increasing exercise intensity, dropping from resting values of approximately 30 mmHg to 6.8 mmHg and 3.1 mmHg during submaximal and maximal-intensity exercise, respectively (Molé et al. 1999). Because intramuscular PO_2 decreases in an intensity-dependent manner, higher relative intensities may also provide a greater hypoxic stimulus for HIF-1α activation. Simulating continuous exercise via sciatic nerve stimulation in rats, Tang et al. (2004) found that HIF-1α protein levels increased following maximal muscle contraction but remained near-baseline during submaximal contractions (Tang et al. 2004). While exercise duration appears to influence the skeletal muscle HIF-1 response, Slot et al. (2014) reported an increase HIF-1α mRNA following a maximal graded exercise test (Slot et al. 2014). This increase was achieved after exercise alone, despite it being less than

10 minutes in duration (the shortest duration of continuous exercise employed in the studies included here). These data suggest the existence of an intensity threshold that must be surpassed in order for exercise to adequately decrease mPO_2 to a level that allows for the stabilization of HIF-1α.

The Effect of Exercise Training on HIF-1α

Hypoxic Training Upregulates HIF-1

The acute increase in HIF-1α following exercise corresponds with increases in mRNA levels of VEGF (Richardson et al. 1999; Ameln et al. 2005a; Kopp et al. 2011), and EPO (Semenza 1999; Ameln et al. 2005a; Kopp et al. 2011), suggesting that HIF-1 stabilization plays an important role in regulating angiogenesis and erythropoiesis in response to exercise training (Lindholm and Rundqvist 2016). In consideration of this, live low, train high (LLTH) and live high, train low (LHTL) training methods have been used to supplement exercise training for athletes (Vogt and Hoppeler 2010). Early work by Vogt et al. (2001) revealed that both high-intensity and low-intensity exercise training in normobaric hypoxia (equivalent to 3,859 m) increased HIF-1α mRNA in the *vastus lateralis* of previously untrained males (Vogt et al. 2001) (**Table 2**). More recently, HIF-1 signaling has been suggested to underlie improvements in sprint performance following repeated sprint training in hypoxia (RSH) (Lindholm et al. 2014a). Faiss et al. (2013) reported superior improvements in repeated sprint performance following RSH compared to the same training in normoxia. The authors speculated that the training-induced increases in HIF-1α and decreases in peroxisome proliferator-activated receptor-γ coactivator α (PGC-1α) and mitochondrial transcription factor A (TFAM) suggested an increased reliance on glycolytic metabolism. Using a slightly different approach in which RSH was used in conjunction with

high altitude residence, Brocherie et al. (2018) showed that RSH augmented resting mRNA levels of HIF-1α and VEGF (Brocherie et al. 2018). In contrast to their previous report however, PGC-1α and TFAM mRNA were also greater following RSH. Therefore, while it is clear that RSH results in the induction of HIF-1α (at least at the transcriptional level), it is unclear what effect RSH used alone or in combination with hypoxic residence, has on signaling related to mitochondrial turnover.

The Current Hypothesis Regarding the Effect of Endurance Training on HIF-1α

Given that exercise in normoxia imposes profound hypoxic stress on skeletal muscle, exercise induced-increases in HIF-1α have been proposed to contribute to the functional adaptations to endurance exercise training (Mason et al. 2004; Freyssenet 2007). This hypothesis, however, is complicated by evidence of reduced HIF-1 signaling following endurance training. Lundby et al. (2006) showed that after four weeks of unilateral cycling training, skeletal muscle HIF-1α mRNA remained unaffected after three hours of cycling exercise at 50% of peak power (Lundby et al. 2006). In contrast, HIF-1α mRNA increased in the untrained leg following the same exercise bout, suggesting that the effect of acute exercise on HIF-1α was blunted after training. Further, Lindholm et al. (2014) reported that compared to moderately active controls, elite endurance athletes had higher levels of HIF-1 regulatory proteins, PHD2, FIH, and sirtuin-6 (SIRT-6) (Lindholm and Rundqvist 2016). The authors speculated that the high levels of HIF-1 inhibitors in elite athletes would result in a downregulation of HIF-1 transcriptional activity in response to exercise. However, this was not confirmed by measurements of HIF-1.

The development and utilization of transgenic mouse models for exercise physiology research has enabled researchers to determine how the suppression or overexpression of

individual proteins affects the acute responses and adaptation to exercise. Regarding the role of HIF-1 in the adaptation to exercise training, two transgenic models have been developed: one lacking HIF-1 expression specifically in skeletal muscle tissue (Mason et al. 2004, 2007a) and another with a whole-body PHD2 deficiency that results in greater than normal accumulation of HIF-1 under normoxic conditions. Mason et al. (2004) first showed that swimming and running time to exhaustion was longer in mice with skeletal muscle-specific deletion of HIF-1 compared to controls (Mason et al. 2004). A subsequent investigation by the same group revealed that the deletion of HIF-1 resulted in a muscle phenotype akin to endurance-trained mice, exhibiting lower respiratory exchange ratio (RER), increased AMP-activated protein kinase (AMPK) phosphorylation and higher hexokinase activity than wild-type controls. In agreement, mitochondrially encoded cytochrome c oxidase II (MT-CO2) gene expression was reduced in mice lacking PHD2 (and thus overexpressing HIF-1) compared to controls. Combined with the aforementioned findings of reduced HIF-1 mRNA in skeletal muscle following exercise training, Lindholm et al. (2014) proposed a tentative hypothesis that HIF-1 suppresses mitochondrial metabolism in skeletal muscle and exercise training alleviates such regulation, allowing for the induction of the oxidative phenotype associated with endurance trained muscle (Lindholm et al. 2014b).

A Critical Review of the Current Hypothesis

The current hypothesis that endurance training-induced inhibition of skeletal muscle HIF-1 signaling allows for improvements in aerobic capacity relies on two lines of evidence: 1) chronic hypoxia and/or HIF-1 activation suppresses mitochondrial metabolism (Kim et al. 2006; Mason et al. 2007b), and 2) endurance exercise training increases the abundance of HIF-1 inhibitors and decreases the HIF-1 response to acute exercise (Lundby et al. 2006;

Lindholm et al. 2014b). A close examination of these points in relation to what is known about the adaptive response to exercise training supports the notion that this hypothesis is indeed *tentative*. In cancer cells, HIF-1 mediates the suppression of mitochondrial respiration in favor of glycolysis (known as the Warburg effect) (Semenza 2007b). This appears to be true in skeletal muscle as chronic overexpression of HIF-1 in mice decreases MT-CO2 (Nunomiya et al. 2017) while its deletion results in oxidative phenotype (Mason et al. 2004, 2007a). However, while these models provide profound insight into the molecular roles of HIF-1 in skeletal muscle, it is difficult to relate the results from such experiments to exercise training in humans. It is imperative to consider how adaptations to exercise training occur in skeletal muscle and elsewhere. Exercise imposes *acute* metabolic and hypoxic stress on skeletal muscle resulting in the *transient* post-translational modification of signaling proteins and transcriptional modification of exercise-sensitive genes (Egan and Zierath 2013). This may be especially true of HIF-1α, which was shown to return to baseline levels within 5 to 10 minutes of the termination of hypoxic stress in both *in vitro* (Berra et al. 2001) and *in vivo* (Yu et al. 2018) models. Ameln et al. (2005) reported that HIF-1α protein expression remained elevated for up to 6 hours following acute exercise, although statistical outcomes were only reported for the comparison between pre and immediately post-exercise (Ameln et al. 2005b). Unpublished observations from our lab show that HIF-1α protein levels increase immediately post exercise and decrease to near-resting values after 3 hours of recovery. By comparison, AMPK phosphorylation also peaks immediately post exercise and returns to baseline levels by 3 hours into recovery (Egan et al. 2010). Regardless of the time course of HIF-1α activity following exercise, it is clear that the transient nature of its activation is unlike the chronic activation tested in cancer cells and transgenic mice. Therefore, the role

HIF-1 plays in suppressing mitochondrial respiration is likely to be less pronounced following acute exercise than that observed following its chronic modulation.

There is also a scarcity of data supporting the notion that exercise training suppresses HIF-1 activity at rest or during exercise. The original investigation by Lundby et al. (2006) compared the HIF-1α mRNA response to acute two-legged exercise between the participants' trained and untrained legs before and following a unilateral cycling training protocol. Because HIF-1 gene expression was only elevated in the untrained leg after exercise, it was concluded that exercise training blunts HIF-1 activation by exercise. One caveat of this study is that workload was the same between legs, meaning the trained leg was exercised at a lower relative intensity than the untrained leg. Furthermore, there are discrepancies between the abundance of protein and mRNA (Liu et al. 2016), particularly in regard to HIF-1α (Mounier et al. 2010). In studies that reported an increase in HIF-1α at the protein level in skeletal muscle, there was no observed increase in HIF-1α mRNA (Ameln et al. 2005b; Van Thienen et al. 2017). Indeed, the activation of HIF-1 is considered to occur primarily at the post transcriptional level (Brahimi-Horn et al. 2005). This does not discount the measurement of mRNA levels as a method for evaluating acute responses to exercise. However, if the aim of an experiment is to determine the hypoxic response to exercise, then the measurement of HIF-1α protein expression is key as its stability is directly related to O_2 availability (Masson and Ratcliffe 2003). In contrast to the current hypothesis, De Smet et al. (2018) showed that the HIF-1 response to hypoxia was preserved following 5 weeks of high intensity interval training (De Smet et al. 2018). This was evidenced by the similar increase in HIF-1α positive nuclei in response to blood flow occlusion performed before and after

training, a unique approach that may more closely reflect the transcriptional activity of HIF-1.

It is also unclear whether HIF-1α stability or HIF-1 transcriptional activity is altered at rest. Lindholm et al. (2014) provided strong evidence that endurance exercise training increases resting levels of HIF-1 inhibitors. These include hydroxylases PHD2 and FIH and well as the histone deacetylase SIRT6. However, neither HIF-1α protein nor mRNA were reported, although protein levels were measured in the *in vitro* arm of the study. Resting levels of skeletal muscle HIF-1α mRNA have been shown to be unchanged following training in normoxia (Vogt et al. 2001; Gustafsson et al. 2002; Lundby et al. 2006; Brocherie et al. 2018; Magalhães et al. 2020) and higher following after training in hypoxia (Vogt et al. 2001). Ignoring the pitfalls of equating mRNA to protein levels and considering that HIF-1α mRNA is in part regulated by HIF-1 in an autoregulatory manner (Koslowski et al. 2011), the null effect of training on HIF-1α transcription may indicate unchanged basal HIF-1 activity. This assumption is undermined by the observation that fiber-specific HIF-1α mRNA profiles are incompatible to those shown at the protein level (Mounier et al. 2010), further emphasizing the need to base our assessments of HIF-1α activity by measuring the protein content.

Finally, the hypothesis that HIF-1 activity is reduced in order to allow for the induction of the oxidative phenotype during endurance training is in contrast to evidence of muscle fiber-specific HIF-1α expression. Mounier et al. (2010) reported greater HIF-1α protein levels in the human *soleus* (composed of primarily type I fibers) and *vastus lateralis* (composed of type I and II fibers) than the triceps muscle (composed of primarily type II fibers) (Mounier et al. 2010). In agreement, whole body PHD2 deficiency in mice increases

type I fiber expression in the *gastrocnemius* (Shin et al. 2016; Nunomiya et al. 2017) and *soleus* (Shin et al. 2016) muscles. The greater abundance of type I fibers in mice overexpressing HIF-1α compared to controls is not accompanied by differences in baseline levels of the oxidative enzyme, succinate dehydrogenase (SDH). However, HIF-1α overexpression resulted in a greater increase in SDH levels following training, suggesting that whole-body stabilization of HIF-1α augments training-induced increases in oxidative enzyme activity (Nunomiya et al. 2017). The available evidence therefore constitutes a paradox: HIF-1α appears to positively regulate a shift towards the oxidative muscle phenotype (Mounier et al. 2010; Shin et al. 2016; Nunomiya et al. 2017) but its absence in skeletal muscle improves aerobic capacity (Mason et al. 2004, 2007b). Further experiments that incorporate several markers of mitochondrial metabolism, including high resolution respirometry, are needed to further understand the role of HIF-1α in regulating muscle cell function. Nevertheless, this paradox highlights yet another problem with the assumption that HIF-1α is suppressed by endurance exercise training.

A Revised Hypothesis

We do not regard the current hypothesis as invalid or without empirical support. The hypothesis provided by Lindholm and colleagues is grounded in solid circumstantial evidence and has piqued our interest. However, in light of the aforementioned issues with the current rationales, we propose a revised hypothesis in which the HIF-1α response to exercise may be higher, not lower, following a period of training (**Figure 1**). Recall that the oxygen demand of exercise drives the profound decrease in mPO_2, creating a hypoxic stimulus for HIF-1α stabilization (Richardson et al. 1995b). It is accepted that endurance exercise training augments the muscle's capacity to utilize oxygen (Egan and Zierath 2013), enableling trained

individuals to reach and maintain higher absolute exercise intensities than in their untrained state. At the same relative intensity, improvements in aerobic capacity may cause a greater drop in mPO_2 during exercise in trained compared to untrained muscle, increasing the hypoxic stimulus and thus HIF-1α activation. Evidence of the concurrent increase in HIF-1α following increases in oxidative capacity was reported by O'Hagan et al. (2009), demonstrating that overexpression of PGC-1α increased protein expression of HIF-1α as well as several HIF-1 target genes including VEGF, transferrin receptor (TfR) and the glycolytic enzyme, phosphofructokinase (PFK) in cultured primary human muscle cells (O'Hagan et al. 2009). PGC-1α is activated during exercise by AMPK and is known to upregulate mitochondrial biogenesis through the coactivation of several transcription factors, like nuclear respiratory factors (NRF1/2) (Wu et al. 1999), estrogen-related receptor alpha (ERRα) (Huss et al. 2002), and peroxisome proliferator-activated receptor alpha (PPARα) (Vega et al. 2000). Relevant to our hypothesis, O'Hagan's group showed that upregulation of HIF-1α occurred as a result of increased O_2 utilization and therefore reduction in O_2 tension within human muscle cells following the induction of an oxidative phenotype. These findings contradict the notion that HIF-1α signaling must be suppressed to allow for improvements in oxidative capacity. Instead, training-induced increases in O_2 utilization may result in the stabilization of HIF-1α, particularly during exercise when the O_2 demand of the muscle far exceeds the capacity of the cardiovascular system to deliver it (Bassett and Howley 2000).

How do we reconcile this revised hypothesis with the fact that endurance training increases the abundance of HIF-1 inhibitors (Lindholm et al. 2014)? Intuitively, an increase in regulatory HIF-1 hydroxylases would suggest a suppression of HIF-1 stabilization at rest and during periods of hypoxic stress. However, these enzymes function in an O_2-dependent

manner (Semenza 2004) and thus their inhibition of HIF-1 during exercise is likely to be limited by cytosolic O_2 availability. Due to their higher capacity to extract and utilize oxygen for oxidative phosphorylation, trained individuals have lower muscle tissue oxygen saturation levels than untrained individuals during intense exercise (Van Thienen and Hespel 2016). Therefore, at least during intense exercise, the inhibition of HIF-1 activity by hydroxylases may be limited to a greater degree in trained compared to untrained individuals. Furthermore, trained participants exhibit higher levels of pyruvate dehydrogenase (PDH) activity compared to healthy controls, despite having a greater abundance of inhibitory pyruvate dehydrogenase kinase proteins (PDK1-4) (Gudiksen et al. (2017). This is particularly relevant to the case of HIF-1 because the current hypothesis emphasizes that the suppression of HIF-1 activity and subsequent downregulation of PDK1 mRNA levels allows for improvements in oxidative capacity associated with exercise training (Lindholm et al. 2014). In contrast, increases in inhibitory enzymes like HIF-1 hydroxylases and PDKs more likely represent an improved flexibilty to respond to hypoxic and metabolic stress rather than active supression of their target proteins.

Of course, our rationale for this new hypothesis is not without flaws. The improvements in exercise tolerance and capacity that occur with training result from structural and metabolic changes that reduce the degree of stress imposed by subsequent bouts of exercise (at the same workload). From this perspective, it is reasonable to assume that the hypoxic response to exercise would be blunted following chronic exercise as training improves matching of O_2 supply to demand (Bassett and Howley 2000). This would be especially likely if one were to compare the HIF-1α response to the same absolute workload before and after a period of training. Indeed, the upregulation of several exercise-inducible

proteins and genes was shown to be blunted using this approach (Granata et al. 2020). However, recreationally active individuals and athletes undertake training regimens to improve their ability to exercise at ever-higher relative workloads, not ameliorate the stress of a fixed workload. Therefore, we speculate that training-induced increases in oxidative metabolism augment the capacity of skeletal muscle to produce and maintain a hypoxic environment that stabilizes HIF-1α, provided a sufficiently high exercise stress. Considering that there is an intensity threshold that must be met for HIF-1α to be stabilized (as detailed above), the superior improvements in exercise capacity observed following high intensity interval training (HIIT) compared to traditional, moderate intensity training may be in part due to the greater activation of HIF-1α. Future studies should be designed to examine the impact of exercise intensity on the acute HIF-1α response before and after training.

Conclusions and Future Directions

In conclusion, the available data regarding the effect of acute and chronic exercise on HIF-1α protein and gene expression limit our current understanding to theories based on reductionist experimental designs. HIF-1α mRNA levels do not reflect resting and exercise-induced changes in HIF-1α protein expression. Nevertheless, HIF-1α activity is clearly augmented by acute exercise. Data from human and animal studies show that the exercise-induced increase in HIF-1α is dependent on the intensity and duration of the exercise.

The current hypothesis regarding the role of HIF-1α in the adaptation to endurance exercise training is that chronic exercise suppresses resting and/or exercise induced levels of HIF-1α via the upregulation of several inhibitors of its stability and transcriptional activity of HIF-1α. This hypothesis is undermined by the following evidence: 1) mRNA levels do not reflect HIF-1α activity, 2) chronic modulation of HIF-1 in animals and cells is an

inappropriate model for understanding responses to exercise training, and 3) the paradox that HIF-1 suppresses oxidative metabolism but is also involved in fiber phenotype transition towards type I fibers. In light of these limitations to the current hypothesis, we provide an alternative hypothesis in which exercise training augments the capacity of the muscle to stimulate HIF-1α via increases in O_2 utilization. Future studies should be done to examine basal and exercise-induced changes in HIF-1α to further clarify the effect of chronic exercise on its activity and its role in the adaptive response to training.

Table 1. Description of studies that investigated the effect of acute exercise on skeletal muscle HIF-1α

Author and year	Participants	Intervention/Groups	Measurement Timepoints	HIF-1 responses
Gustafsson 1999	15 male (control; n= 7, BFR; n=8)	45 min of single leg cycling at 24 ± 3 % of one leg peak load in control and blood flow-restricted conditions	Baseline (1 week prior to intervention) and 30 min post exercise	Increase in HIF-1β but not HIF-1α mRNA following exercise. No difference between conditions
Ameln et al. 2005	9 males	45 min of single leg cycling at 26 ± 4 % of one leg peak load in control and blood flow-restricted conditions	Rest, 30-, 120-, and 360-min post-exercise	Protein, but not mRNA, increased immediately after and up to 360 min following exercise. No difference in increase between control and blood flow restricted exercise.
Drummond 2008	6 males	4 sets (30, 15, 15, 15 reps) of knee extension resistance exercise @ 20% 1RM with and without blood flow restriction	Rest and 3 h post exercise	Post-exercise increase in mRNA, no difference between control and blood flow restricted exercise.
Slivka 2014	11 males	1 hr cycling @ 60% of peak power in normoxia (975m) and normobaric hypoxia (3000m)	Rest and 4 h post-exercise	Increase in HIF-1α mRNA in normoxia and hypoxia. No difference between conditions
Slot 2014	15 healthy controls (9 males, 6 females) 28 COPD patients (16 males, 12 females)	Incremental load cycle ergometry test lasting ~10 min for healthy controls and ~7.4 min for COPD patients	Rest and 4 h post-exercise	Increase in HIF-1α mRNA in both groups, no difference between groups.
Van Thienen et al. 2017	11 pairs of identical twins. (males)	Exercise only (20 min of cycling @ 50.7 ± 2.3% of VO_2max in normoxia) Hypoxia only (FiO_2= 10.7%) Hypoxia + Exercise (20 min of cycling @81.4 ± 3.2% VO_2max in hypoxia)	Rest, 3 h hypoxia, Immediately post-exercise	Significant increase in protein and gene expression from baseline during hypoxia only. Hypoxia + Exercise resulted in further increase above baseline.

h: hours

Table 2. Description of studies that investigated the effect of exercise training on basal and/or exercise-induced hypoxia inducible factor 1α (HIF-1α)

Author and year	Participants	Intervention/Groups	Measurement Timepoints	HIF-1 responses
Vogt et al. 2001	39 untrained males	Nor-high (n=8): High intensity training in normoxia Hyp-high (n=7): High intensity training in hypoxia Nor-low (n=8): low intensity training in normoxia Hyp-low (n=7): low intensity training in hypoxia	Baseline and at least 24 h after last training session	Increase in HIF-1α mRNA following Hyp-low and trend towards ($p < .1$) after Hyp-high. Increase in Hifdel (splice variant of HIF-1α) following Hyp-high only. No change in HIF-1α mRNA following normoxic training in either group.
Gustafsson et al. 2002	8 males	7, 45 min sessions of unilateral cycling exercise at 60-70% of maximal single leg cycling capacity	Baseline and 24 hours following 7th training session	No change in HIF-1β but not HIF-1α mRNA following training.
Zoll et al. 2006	15 male runners	-Training in normoxia (n=6) -Intermittent training in hypoxia (2x/week)	Pre and post 6 weeks of training	Increase in HIF-1α mRNA in hypoxic training group only.
Lundby et al. 2006	7 males	4 weeks of unilateral knee extension exercise training	Pre and immediately post, 2 h, 6 h and 24 h post acute exercise in trained and untrained legs.	Increase in HIF-1α mRNA 6h post exercise in untrained leg only.
Mounier et al. 2010	14 males	Comparison of HIF-1 mRNA and protein expression between the triceps (predominately type II fibers), and (predominately type I fibers) and vastus lateralis (equal proportion of type I and type II fibers) muscles	Baseline only	-Higher HIF-1α protein expression in vastus lateralis and soleus than triceps. -HIF-1α mRNA was lower in the soleus than triceps.
De Smet et al. 2018	10 males living in normobaric hypoxia	5 weeks of unilateral knee extension training. One leg trained in hypoxia, the other in normoxia.	-Before and after training in each leg -Pre and post blood flow occlusion in each leg	No change in HIF-1α protein, mRNA or HIF-1α positive nuclei after training in either leg. Post-occlusion increase in HIF-1α was the same across all tests.
Magalhães et al. 2020	17 males	13 sessions of high intensity interval training	Pre and post training at rest	No increase in HIF-1α after training.

Abbreviations: hyp: hypoxia, norm: normoxia, h: hours

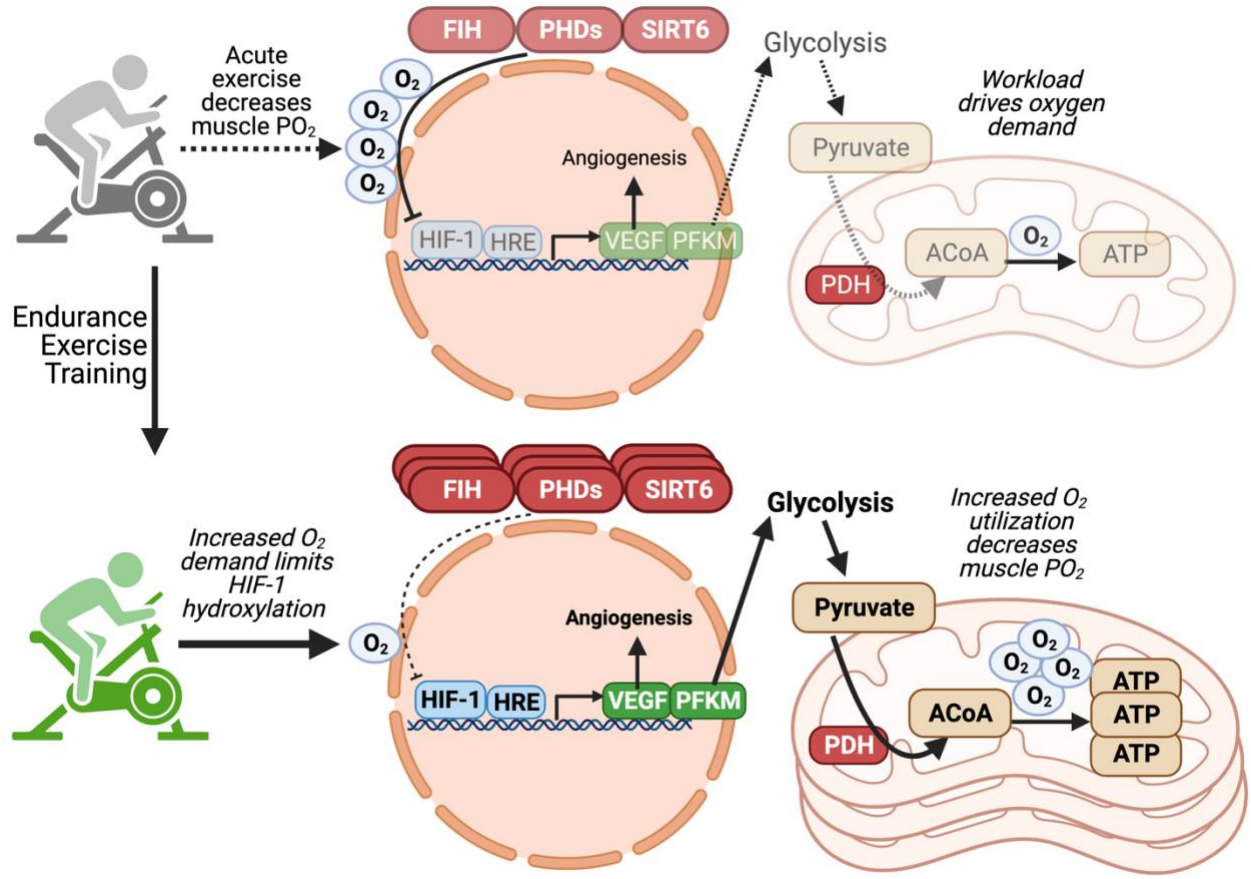

Figure 1. Schematic representation of the revised hypothesis regarding the influence of endurance exercise training on the hypoxia inducible factor 1 (HIF-1) response to acute exercise in skeletal muscle. In the untrained state, HIF-1 is activated by exercise-induced intramuscular hypoxia. HIF-1 increases angiogenic signaling through vascular endothelial growth factor (VEGF) while also augmenting glycolytic capacity through increases in phosphofructokinase (PFKM). Endurance exercise training improves oxygen delivery and utilization through HIF-1-dependent and independent mechanisms. At the same relative intensity or during maximal exercise, the increase in oxygen consumption (compared to the untrained state) further decreases cellular O_2 available for the O_2-dependent inhibition of HIF-1, increasing its activity during acute exercise. FIH: factor inhibiting HIF, PHDs: prolyl dehydrogenases, SIRT6: sirtuin 6, ACoA: acetyl coenzyme A, ATP: adenosine triphosphate, HRE: hypoxia response element.

References

Ameln H, Gustafsson T, Sundberg CJ, et al (2005) Physiological activation of hypoxia inducible factor-1 in human skeletal muscle. FASEB J 19:1009–1011. doi: 10.1096/fj.04-2304fje

Bassett DR, Howley ET (2000) Limiting factors for maximum oxygen uptake and determinants of endurance performance. Med Sci Sports Exerc 32:70–84. doi: 10.1097/00005768-200001000-00012

Berra E, Roux D, Richard DE, Pouysségur J (2001) Hypoxia-inducible factor-1α (HIF-1 escapes O2-driven proteasomal degradation irrespective of its subcellular localization: Nucleus or cytoplasm. EMBO Rep 2:615–620. doi: 10.1093/embo-reports/kve130

Brahimi-Horn C, Mazure N, Pouysségur J (2005) Signalling via the hypoxia-inducible factor-1α requires multiple posttranslational modifications. Cell. Signal. 17:1–9

Brocherie F, Millet GP, D'Hulst G, et al (2018) Repeated maximal-intensity hypoxic exercise superimposed to hypoxic residence boosts skeletal muscle transcriptional responses in elite team-sport athletes. Acta Physiol 222:e12851. doi: 10.1111/apha.12851

Coffey VG, Shield A, Canny BJ, et al (2006) Interaction of contractile activity and training history on mRNA abundance in skeletal muscle from trained athletes. Am J Physiol - Endocrinol Metab 290:E849–E855. doi: 10.1152/ajpendo.00299.2005

De Smet S, D'Hulst G, Poffé C, et al (2018) High-intensity interval training in hypoxia does not affect muscle HIF responses to acute hypoxia in humans. Eur J Appl Physiol 118:847–862. doi: 10.1007/s00421-018-3820-4

Drummond MJ, Fujita S, Takashi A, et al (2008) Human muscle gene expression following resistance exercise and blood flow restriction. Med Sci Sports Exerc 40:691–698. doi: 10.1249/MSS.0b013e318160ff84

Egan B, Carson BP, Garcia-Roves PM, et al (2010) Exercise intensity-dependent regulation of peroxisome proliferator-activated receptor γ coactivator-1α mRNA abundance is associated with differential activation of upstream signalling kinases in human skeletal muscle. J Physiol 588:1779–1790. doi: 10.1113/jphysiol.2010.188011

Egan B, Zierath JR (2013) Exercise Metabolism and the Molecular Regulation of Skeletal Muscle Adaptation. Cell Metab 17:162–184. doi: 10.1016/j.cmet.2012.12.012

Freyssenet D (2007) Energy sensing and regulation of gene expression in skeletal muscle. J. Appl. Physiol. 102:529–540

Gallezot P (2012) Conversion of biomass to selected chemical products. Chem Soc Rev 41:1538–1558. doi: 10.1039/c1cs15147a

Granata C, Oliveira RSF, Little JP, Bishop DJ (2020) Forty high-intensity interval training sessions blunt exercise-induced changes in the nuclear protein content of PGC-1α and p53 in human skeletal muscle. Am J Physiol Metab 318:E224–E236. doi: 10.1152/ajpendo.00233.2019

Gudiksen A, Bertholdt L, Stankiewicz T, et al (2017) Effects of training status on PDH regulation in human skeletal muscle during exercise. Pflügers Arch – Eur J Physiol 469:1615–1630. Doi: 10.1007/s00424-017-2019-6

Gustafsson T, Knutsson A, Puntschart A, et al (2002) Increased expression of vascular endothelial growth factor in human skeletal muscle in response to short-term one-legged exercise training. Pflugers Arch Eur J Physiol 444:752–759. doi: 10.1007/s00424-002-0845-6

Gustafsson T, Puntschart A, Kaijser L, et al (1999) Exercise-induced expression of angiogenesis-related transcription and growth factors in human skeletal muscle. Am J Physiol - Hear Circ Physiol 276:H679–H685. doi: 10.1152/ajpheart.1999.276.2.h679

Holloszy JO, Coyle EF (1984) Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. J. Appl. Physiol. Respir. Environ. Exerc. Physiol. 56:831–838

Huss JM, Kopp RP, Kelly DP (2002) Peroxisome proliferator-activated receptor coactivator-1α (PGC-1α) coactivates the cardiac-enriched nuclear receptors estrogen-related receptor-α and -γ: Identification of novel Leucine-rich interaction motif within PGC-1α. J Biol Chem. doi: 10.1074/jbc.M206324200

Iliopoulos O, Kibel A, Gray S, Kaelin WG (1995) Tumour suppression by the human von Hippel-Lindau gene product. Nat Med 1:822–826. doi: 10.1038/nm0895-822

Kim JW, Tchernyshyov I, Semenza GL, Dang C V. (2006) HIF-1-mediated expression of pyruvate dehydrogenase kinase: A metabolic switch required for cellular adaptation to hypoxia. Cell Metab. doi: 10.1016/j.cmet.2006.02.002

Kopp R, Köblitz L, Egg M, Pelster B (2011) HIF signaling and overall gene expression changes during hypoxia and prolonged exercise differ considerably. Physiol Genomics 43:506–516. doi: 10.1152/physiolgenomics.00250.2010

Koslowski M, Luxemburger U, Türeci Ö, Sahin U (2011) Tumor-associated CpG demethylation augments hypoxia-induced effects by positive autoregulation of HIF-1α. Oncogene 30:876–882. doi: 10.1038/onc.2010.481

Lindholm ME, Fischer H, Poellinger L, et al (2014) Negative regulation of HIF in skeletal muscle of elite endurance athletes: A tentative mechanism promoting oxidative metabolism. Am J Physiol - Regul Integr Comp Physiol 307:R248–R255. doi: 10.1152/ajpregu.00036.2013

Lindholm ME, Rundqvist H (2016) Skeletal muscle hypoxia-inducible factor-1 and exercise. Exp Physiol 101:28–32. doi: 10.1113/EP085318

Liu Y, Beyer A, Aebersold R (2016) On the Dependency of Cellular Protein Levels on mRNA Abundance. Cell 165:535–550

Lundby C, Gassmann M, Pilegaard H (2006) Regular endurance training reduces the exercise induced HIF-1α and HIF-2α mRNA expression in human skeletal muscle in normoxic conditions. Eur J Appl Physiol 96:363–369. doi: 10.1007/s00421-005-0085-5

Magalhães F de C, Aguiar PF, Tossige-Gomes R, et al (2020) High-intensity interval training followed by postexercise cold-water immersion does not alter angiogenic circulating cells, but increases circulating endothelial cells. Appl Physiol Nutr Metab 45:101–111. doi: 10.1139/apnm-2019-0041

Mason SD, Howlett RA, Kim MJ, et al (2004) Loss of skeletal muscle HIF-1α results in altered exercise endurance. PLoS Biol 2:e288. doi: 10.1371/journal.pbio.0020288

Mason SD, Rundqvist H, Papandreou I, et al (2007a) HIF-1α in endurance training: Suppression of oxidative metabolism. Am J Physiol - Regul Integr Comp Physiol 293:R2059–R2069. doi: 10.1152/ajpregu.00335.2007

Mason SD, Rundqvist H, Papandreou I, et al (2007b) HIF-1α in endurance training: Suppression of oxidative metabolism. Am J Physiol - Regul Integr Comp Physiol 293:R2059–R2069. doi: 10.1152/ajpregu.00335.2007

Masson N, Ratcliffe PJ (2003) HIF prolyl and asparaginyl hydroxylases in the biological response to intracellular O2 levels. J. Cell Sci. 116:3041–3049

Maxwell PH, Wiesener MS, Chang G-W, et al (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 399:271–275. doi: 10.1038/20459

Mole PA, Chung Y, Tran TK, et al (1999) Myoglobin Desaturation with Exercise Intensity in Human Gastrocnemius Muscle. Med Sci Sport Exerc 31:S275. doi: 10.1097/00005768-199905001-01341

Mounier R, Pedersen BK, Plomgaard P (2010) Muscle-specific expression of hypoxia-inducible factor in human skeletal muscle. Exp Physiol 95:899–907. doi: 10.1113/expphysiol.2010.052928

Murakami T, Shimomura Y, Yoshimura A, et al (1998) Induction of nuclear respiratory factor-1 expression by an acute bout of exercise in rat muscle. Biochim Biophys Acta - Gen Subj 1381:113–122. doi: 10.1016/S0304-4165(98)00018-X

Nguyen LK, Cavadas MAS, Scholz CC, et al (2013) A dynamic model of the hypoxia-inducible factor 1 (HIF-1) network. J Cell Sci 126:1454–1463. doi: 10.1242/jcs.119974

Nunomiya A, Shin J, Kitajima Y, et al (2017) Activation of the hypoxia-inducible factor pathway induced by prolyl hydroxylase domain 2 deficiency enhances the effect of running training in mice. Acta Physiol 220:99–112. doi: 10.1111/apha.12751

O'Gorman DJ, Karlsson HKR, McQuaid S, et al (2006) Exercise training increases insulin-stimulated glucose disposal and GLUT4 (SLC2A4) protein content in patients with type 2 diabetes. Diabetologia 49:2983–2992. doi: 10.1007/s00125-006-0457-3

O'Hagan KA, Cocchiglia S, Zhdanov A V., et al (2009) PGC-1 is coupled to HIF-1 - dependent gene expression by increasing mitochondrial oxygen consumption in skeletal muscle cells. Proc Natl Acad Sci 106:2188–2193. doi: 10.1073/pnas.0808801106

Richardson RS, Duteil S, Wary C, et al (2006) Human skeletal muscle intracellular oxygenation: The impact of ambient oxygen availability. J Physiol 571:415–424. doi: 10.1113/jphysiol.2005.102327

Richardson RS, Noyszewski EA, Kendrick KF, et al (1995a) Myoglobin O2 desaturation during exercise: Evidence of limited O2 transport. J Clin Invest 96:1916–1926. doi: 10.1172/JCI118237

Richardson RS, Noyszewski EA, Kendrick KF, et al (1995b) Myoglobin O2 desaturation during exercise: Evidence of limited O2 transport. J Clin Invest 96:1916–1926. doi: 10.1172/JCI118237

Richardson RS, Wagner H, Mudaliar SRD, et al (1999) Human VEGF gene expression in skeletal muscle: Effect of acute normoxic and hypoxic exercise. Am J Physiol - Hear Circ Physiol 277:H2247–H2252. doi: 10.1152/ajpheart.1999.277.6.h2247

Schwandt HJ, Heyduck B, Gunga HC, Rocker L (1991) Applied Physiology on the erythropoietin concentration in blood. Eur J Appl Physiol 463–466

Semenza, G. L. (2000). HIF-1 and human disease: one highly involved factor. *Genes & Development*, *14*(16), 1983–1991. https://doi.org/10.1101/gad.14.16.1983

Semenza GL (2004) Hydroxylation of HIF-1: Oxygen sensing at the molecular level. Physiology 19:176–182

Semenza GL (2007a) Life with Oxygen. Science (80-) 318:62–64. doi:10.1126/science.1147949

Semenza GL (2007b) HIF-1 mediates the Warburg effect in clear cell renal carcinoma. J Bioenerg Biomembr 39:231–234. doi: 10.1007/s10863-007-9081-2

Semenza GL (1999) Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Annu. Rev. Cell Dev. Biol. 15:551–578

Shin J, Nunomiya A, Kitajima Y, et al (2016) Prolyl hydroxylase domain 2 deficiency promotes skeletal muscle fiber-type transition via a calcineurin/NFATc1-dependent pathway. Skelet Muscle 6:. doi: 10.1186/s13395-016-0079-5

Slivka DR, Heesch MWS, Dumke CL, et al (2014) Human Skeletal Muscle mRNA Response to a Single Hypoxic Exercise Bout. Wilderness Environ Med 25:462–465. doi:

10.1016/j.wem.2014.06.011

Slot IGM, Van Den Borst B, Hellwig VACV, et al (2014) The muscle oxidative regulatory response to acute exercise is not impaired in less advanced COPD despite a decreased oxidative phenotype. PLoS One 9:e90150. doi: 10.1371/journal.pone.0090150

Tang K, Breen EC, Wagner H, et al (2004) HIF and VEGF relationships in response to hypoxia and sciatic nerve stimulation in rat gastrocnemius. Respir Physiol Neurobiol 144:71–80. doi: 10.1016/j.resp.2004.04.009

Van Thienen R, Hespel P (2016) Enhanced muscular oxygen extraction in athletes exaggerates hypoxemia during exercise in hypoxia. J Appl Physiol 120:351–361. doi: 10.1152/japplphysiol.00210.2015

Van Thienen R, Masschelein E, D'Hulst G, et al (2017) Twin resemblance in muscle HIF-1α responses to hypoxia and exercise. Front Physiol 7:1–11. doi: 10.3389/fphys.2016.00676

Vega RB, Huss JM, Kelly DP (2000) The Coactivator PGC-1 Cooperates with Peroxisome Proliferator-Activated Receptor alpha in Transcriptional Control of Nuclear Genes Encoding Mitochondrial Fatty Acid Oxidation Enzymes. Mol Cell Biol. doi: 10.1128/MCB.20.5.1868-1876.2000

Vogt M, Hoppeler H (2010) Is hypoxia training good for muscles and exercise performance? Prog. Cardiovasc. Dis. 52:525–533

Vogt M, Puntschart A, Geiser J, et al (2001) Molecular adaptations in human skeletal muscle to endurance training under simulated hypoxic conditions. J Appl Physiol 91:173–182. doi: 10.1152/jappl.2001.91.1.173

Wiener CM, Booth G, Semenza GL (1996) In vivo expression of mRNAs encoding hypoxia-inducible factor 1. Biochem Biophys Res Commun 225:485–488. doi: 10.1006/bbrc.1996.1199

Wu Z, Puigserver P, Andersson U, et al (1999) Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell. doi: 10.1016/S0092-8674(00)80611-X

Yu AY, Frid MG, Shimoda LA, et al (2018) Temporal , spatial , and oxygen-regulated expression of hypoxia-inducible factor-1 in the lung. Am J Physiol

CHAPTER III: Research Manuscript

This chapter presents a research manuscript written for submission to the *for the European Journal of Applied Physiology.* The references cited in this review are provided at the end of the manuscript.

The Influence of Training Status on the Exercise Induced HIF-1 Response to Acute Exercise

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Abstract

Background: Hypoxia inducible factor 1 (HIF-1) has been proposed to mediate the skeletal muscle adaptive response to exercise training via its regulation of angiogenesis and glycolysis. However, it was previously hypothesized that endurance exercise training blunts the HIF-1 response to acute exercise. This tentative hypothesis has not been fully explored and the purpose of this study was to determine if training status influences the HIF-1 response to acute exercise in skeletal muscle. **Methods:** 7 endurance athletes and 8 untrained controls performed an acute bout of supramaximal exercise consisting of 20 'all out', 10-second sprints on a cycle ergometer. Muscle tissue samples were collected pre, immediately post, and 3 h post exercise. Protein levels of the oxygen sensitive HIF-1 subunit, HIF-1α and its inhibitors, prolyl hydroxylase 2 (PHD2), factor inhibiting HIF (FIH) and von Hippel-Lindau tumor suppressor protein (VHL) were measured at each timepoint and compared between groups. Group differences in the exercise-induced expression of HIF-1 target genes were assessed via RT-qPCR and next-generation RNA sequencing. **Results:** FIH and PHD2

protein levels were greater in the trained group compared to the untrained group ($P < .05$). The exercise-induced increase in HIF-1α was not different between groups ($P > .05$) and the effect of exercise was similar across trained ($d = 1.64$) and untrained ($d = 1.58$) individuals. Pyruvate dehydrogenase kinase mRNA levels were higher in the trained group ($P < .05$) but was unaffected by acute exercise. RNA sequencing revealed no significant difference between the fold change in HIF-1 target genes, with the exception of forkhead box O3 (FOXO3), which was upregulated in the untrained group and downregulated in the trained group following acute exercise. **Conclusion:** The HIF-1 response to acute, supramaximal exercise is similar between trained and untrained individuals at the protein and gene expression level. These data suggest that given sufficient exercise stress, the molecular responses in skeletal muscle regulated in part by HIF-1 are stimulated in endurance trained athletes, despite increases in regulatory HIF-1 hydroxylases.

Introduction

Skeletal muscle displays remarkable plasticity in response to exercise training. Endurance exercise imposes metabolic (de Freitas et al. 2017), oxidative (Steinbacher and Eckl 2015) and hypoxic stress (Ameln et al. 2005) within skeletal muscle cells. These stressors stimulate a multitude of transcriptional and post-translational modifications, altering the protein and gene expression profile of skeletal muscle in the hours and days following exercise (Egan and Zierath 2013). The skeletal muscle adaptive response to exercise training results from the cumulative effect of these events (Perry et al. 2010). Over time, the muscle's ability to take up and utilize oxygen is improved (Prior et al. 2004) while substrate utilization is optimized to meet energetic demands (Kiens et al. 1993). Because these adaptations underlie training-induced improvements in exercise performance and metabolic health (Egan

and Zierath 2013), understanding the mechanisms by which the skeletal muscle response to exercise is regulated has broad implications for the field of exercise physiology.

During exercise, the increase in oxygen consumption by skeletal muscle cells causes a decrease in the muscle oxygen tension (mPO_2), resulting in intramuscular hypoxia (Richardson et al. 1995). The molecular response to cellular hypoxia is mediated in part by the oxygen-sensitive transcription factor, hypoxia inducible factor 1 (HIF-1) (Semenza 2007a). In normoxic conditions, factor inhibiting HIF (FIH) and prolyl hydroxylase 2 (PHD2) hydroxylate the alpha subunit of HIF-1 (HIF-1α) (Berra 2003; Dayan et al. 2009). Hydroxylation allows the von Hippel-Lindau tumor suppressor protein (VHL) to tag HIF-1α for proteasomal degradation, suppressing its transcriptional activation of target genes during periods of oxygen abundance (Maxwell et al. 1999). During periods of intracellular hypoxia (like exercise), however, hydroxylase activity becomes limited by oxygen availability, allowing HIF-1α to dimerize with the beta subunit and exert its transcriptional regulation of target genes (Nguyen et al. 2013). HIF-1 regulates the transcription of hundreds of genes involved in oxygen transport and substrate metabolism (Benita et al. 2009), implicating it as a prime mediator of the skeletal muscle response to exercise training.

While there is clear evidence that acute exercise increases the mRNA (Lundby et al. 2006; Drummond et al. 2008) and protein (Ameln et al. 2005) abundance of skeletal muscle HIF-1α (the regulatory subunit of the HIF-1 dimer), its role in the adaptive response to exercise training is not well understood. It was previously hypothesized that endurance exercise training suppresses the exercise-induced increase in skeletal muscle HIF-1 activation, blunting its regulation of target genes (Lindholm et al. 2014). This hypothesis is predicated on data showing baseline HIF hydroxylase abundance increases (Lindholm et al.

2014) while the exercise-induced HIF-1α mRNA response to acute exercise decreases following endurance training (Lundby et al. 2006). This hypothesis is intuitive as chronic HIF-1 expression is known to suppress mitochondrial respiration in favor of anaerobic ATP production (Semenza 2007b). Thus, the metabolic changes mediated by HIF-1 oppose the increase in oxidative capacity known to result from endurance training (Egan and Zierath 2013). However, there is no evidence to suggest that mitochondrial respiration is regulated by HIF-1 *during* exercise, nor is it likely that the suppression of oxidative metabolism by HIF-1 needs to be attenuated in order for ATP to be produced aerobically. It is more likely that the activation of HIF-1 during exercise stimulates morphological and metabolic changes in skeletal muscle cells that make them more robust to subsequent hypoxic stress. Indeed, HIF-1 plays a central role in regulating genes that code for angiogenic factors like vascular endothelial growth factor A (VEGFA) (Carbajo-Pescador et al. 2013) and heme oxygenase 1 (HMOX1) (Dunn et al. 2020) as well as glycolytic enzymes, phosphofructokinase L (PFKL) and pyruvate dehydrogenase kinase1 (PDK1) (Kim et al. 2006), all of which are upregulated by endurance exercise training (Olfert et al. 2010; Lindholm et al. 2016; Ropka-Molik et al. 2017; Gudiksen et al. 2017; Toledo-Arruda et al. 2020). An additional limitation to the current hypothesis is that the blunted HIF-1α response to acute exercise was shown only at mRNA level (Lundby et al. 2006). Skeletal muscle HIF-1α mRNA levels are not consistent with HIF-1α protein (Mounier et al. 2010), and thus it is unclear what effect, if any, endurance training has on the HIF-1 protein response to acute exercise.

The purpose of this study was to determine if training status influences the exercise-induced increase in skeletal muscle HIF-1α and the transcription of its target genes. To this end, we measured HIF-1α protein, and gene expression levels of HIF-1 target genes before

and after acute exercise. It was hypothesized that the exercise-induced expression of HIF-1α and its target genes would be higher, not lower in endurance trained individuals compared to untrained participants. The rationale for this was that muscle oxygen saturation at maximal exercise is lower in trained compared to untrained individuals (Van Thienen and Hespel 2016). Therefore, trained individuals may be able to achieve and maintain a higher degree of hypoxic stress during intense exercise than untrained individuals.

Methods

Participants

Participants underwent an informed consent process in accordance with the university institutional review board and the Declaration of Helsinki. Seven endurance trained (5 males, 2 females) and 8 untrained (all males) individuals participated in this study. Endurance trained (hereafter referred to as 'trained') participants were included if they met the following criteria: 1) reported competing in at least one endurance race in the past year (cycling, triathlons, distance running), 2) reported engaging in at least 10 hours of endurance exercise training per week for the past 6 months, 3) had a measured VO_2peak ≥ the 90th percentile for their age and sex according to the American College of Sports Medicine (American College of Sports Medicine 2018). Untrained participants were included if they met the following criteria: 1) reported not engaging in endurance training exercise in the past year, 2) had a measured VO_2peak ≤ the 50th percentile for their age and sex. Baseline demographic and physiological descriptors of the participants are shown in **Table 1**.

Study Design

This was an arm of a larger study that aimed to examine skeletal muscle response to repeated sprint exercise. Baseline testing included measurement of body weight, height, and VO_2peak. Participants performed ten all out sprints for familiarization following the

VO_2peak test. At least 72 hours after the initial visit, participants completed a repeated sprint exercise protocol on a cycle ergometer. Heart rate (HR), peripheral blood oxygen saturation (SpO_2), muscle tissue oxygenation index (TSI) and perceived exertion were measured during the exercise trial. Muscle tissue was sampled from the *vastus lateralis* pre, immediately post and 3h post-exercise for protein and gene expression analysis.

Peak Oxygen Uptake

Participants performed a maximal graded exercise test on a cycle ergometer (Lode Excalibur, Gronigen, Netherlands) for the measurement of VO_2peak. The ramp protocol (20 – 40 W per minute) was determined on an individual basis based on sex, fitness level and body size. The technician designed the test to elicit maximal effort in 8-10 minutes. Oxygen consumption was measured via indirect calorimetry using a metabolic cart (TrueOne 2400, Parvomedics, Salt Lake City, UT, USA). VO_2peak was identified as the highest VO_2 value following data processing using an 11-breath rolling average (Robergs et al. 2010).

Exercise Protocol

The repeated sprint protocol was performed on a Wingate testing ergometer (Model 894E Monark, Vansbro, Sweden). Participants performed a 1-min self-selected warm-up prior to the sprint exercise. A total of 20 (4 sets of 5 sprints), 'all-out' 10-sec sprints were performed. Resistance was adjusted to 7.5% of the participants' bodyweight and set to automatically drop at a cadence (110-140 rpm) determined during familiarization. 30-sec active rest periods (cycling against no external load) were allowed between each sprint and during the 5-min intervals between each set. Wingate testing software (Monark Anaerobic Test Software, Vansbro, Sweden) was used to record second-by-second power output in watts during each sprint. For statistical analyses, power output in watts (W) was averaged

over all 20 sprints and expressed relative to body mass (W/kg) and as a percent of the max watts achieved during the VO_2 peak test (% of Max Watts). HR was measured using a chest strap monitor (Polar Electro, Bethpage, NY, USA). SpO_2 was measured with a finger pulse oximeter (GO2, Phillips, Netherlands). HR and SpO_2 were recorded following each sprint. The mean of these values across the trial was reported as average HR and SpO_2, respectively. Rating of perceived exertion (RPE: 6-20) was recorded after each set of 5 sprints and averaged across the trial.

Measurement of Muscle Tissue Oxygen Saturation:

Muscle oxygen saturation was measured using a portable near-infrared spectroscopy device (MOXY, Hutchinson, MN, USA) secured to the *vastus lateralis* 10 cm above the first muscle biopsy incision. The NIRS device measures oxy(+myo)hemoglobin, (O_2Hb), deoxyhemo(+myo)globin (HHb), and total hemo(+myo)globin (tHb) and records second-by-second % tissue saturation index (TSI; calculated by [O_2Hb/tHb x 100]) using the modified Beer-Lambert law (McManus et al. 2018). TSI was measured for 30 seconds at rest prior to exercise. Mean TSI was determined by identifying the lowest point during each sprint and taking the average of these values across all 20 sprints. The percent change from baseline (ΔTSI) was calculated using the difference between baseline and mean TSI values and reported as the average across all sprints.

Skeletal Muscle Tissue Sampling:

Approximately 20 mg of skeletal muscle was sampled from the *m. vastus lateralis* muscle of each participant's dominant leg using a 14 G micro-biopsy needle at each timepoint (pre, post and 3 h post exercise). Following each biopsy, ~10 mg were washed immediately in ice-cold phosphate buffered saline (PBS), snap frozen in liquid nitrogen and

stored at -80° C for western blot analysis. The remainder was stored in RNAlater (Invitrogen, AM7020) at 4°C overnight then stored at -80°C for gene expression analysis by rtPCR and next-generation RNA sequencing. Due to the short half-life of HIF-1α, great care was taken to collect and freeze the post exercise sample as soon as possible (within approximately 5 minutes after the end of exercise).

Immunoblotting:

Muscle samples were homogenized on ice with a rotor-stable homogenizer (Scilogex, Rocky Hill, CT, US) in a lysis buffer containing 20 mmol L^{-1} HEPES (pH 7.5, Gibco, 15630130), 0.2 mmol L^{-1} EDTA (pH 7.4, Invitrogen, AM9260G), 1.5 mmol L^{-1} $MgCl_2$ (Invitrogen, AM9530G), 100 mmol L^{-1} NaCl (Invitrogen, AM9759), 2 mmol L^{-1} Dithiothreitol (Sigma,10197777001), 0.4 mmol L^{-1} phenylmethylsulfonyl fluoride (Sigma, 78830), 0.5 mmol L^{-1} DMOG (Sigma, D3695) and 1% Triton X-100. (Sigma, T8787), and a protease and phosphatase cocktail (Thermo Scientific, 78446). DMOG was added to the lysis buffer to prevent HIF-1α degradation during sample preparation, a technique adapted from Srinivasan and Dunn (2011) (Srinivasan and Dunn 2011). Lysates were centrifuged at 10,000 g for 10 minutes, 4° C and the supernatant was stored at -80°C. Protein concentration was determined using a BCA protein assay (Thermo Scientific, 23225). Lysates were diluted with 4x Laemmli buffer (Bio-Rad, 1610747) containing 0.5% 2-Mercaptoethanol and boiled at 95°C for 5 minutes. Proteins were loaded on 4-20% polyacrylamide gel containing 0.5% 2,2,2-trichloroethenol (Fisher Scientific, AC139441000), enabling stain-free imaging of total protein on the gel and post-transfer membrane (Ladner et al. 2004). Proteins were then transferred onto a PVDF membrane (Sigma, P2938) and an image of total protein was taken for use as a loading control (Rivero-Gutiérrez et al. 2014). Membranes were blocked for 1

hour at room temperature in Tris-buffered saline containing 0.1% tween 20 (Sigma, 9005-64-5) (TBST) and 5% fat-free milk. Membranes were cut according to the molecular weight of the protein of interest and incubated overnight at 4°C with the following antibodies: HIF-1α (1:500, BD Biosciences, 610958), VHL (Cell Signaling, 48547) and PHD-2 (Cell Signaling, 4835). Membranes were then washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies (diluted 1:1000) for 1 hour at room temperature followed by a second set of washes. Lastly, membranes were incubated in a chemiluminescent substrate containing 2 mmol L^{-1} 4-iodophenylboronic acid (Sigma, 471933), 1.25 mmol L^{-1} luminol (Sigma, 123072), 5.3 mmol L^{-1} hydrogen peroxide (Sigma, 216763) in 100 mmol L^{-1} Tris/HCl pH 8.8 (Invitrogen, 15568025) (Haan and Behrmann 2007). Images were captured on the Chemidoc Imager. ImageLab (Version 6.0, Biorad, Hercules CA) was used to quantify protein expression and total protein on the stain-free membrane. Protein expression was normalized to total protein (McDonough et al. 2015) and expressed relative to pre-exercise. C2C12 (ATCC Cat# CRL-1772, RRID:CVCL_0188) myotubes were exposed to 1% O_2 for 4 hours and used as a positive control for each protein of interest.

Gene Expression Analysis:

We measured mRNA levels of the HIF-1 targets, PDK1, VEGFA and BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) pre and 3 h post exercise. We chose these timepoints based on our previous finding that the expression of these genes remained relatively unchanged immediately post exercise but increased 3 h post exercise. We selected these genes to measure because we found that their transcriptional activation by hypoxia and muscle contraction was dependent on HIF-1 activity in vitro (unpublished data). Amplification efficiency was analyzed for each transcript using 5, 10-

fold dilutions of a single cDNA template, as recommended by Kuang et al. (2018) (Kuang et al. 2018). Assay efficiencies ranged from 94 to 103%. Based on these analyses, cDNA templates were diluted 10-fold. Muscle tissue was lysed in Trizol (Invitrogen, 15596018) using a bead homogenizer (Beadbug 3, Benchmark Scientific, Sayrenville, NJ, USA). RNA was isolated and purified with the Trizol Plus purification kit (Invitrogen, 12183555). RNA concentration and purity were measured on a NanoDrop lite (Thermofisher, Waltham, MA, USA) and the A260/A280 ratio was confirmed to be > 1.8. One RNA sample was excluded from the gene expression analysis because the A260/A280 ratio was below 1.8. One microgram of RNA was reverse transcribed using the Superscript IV VILO cDNA synthesis kit (Invitrogen, 11754050) and diluted in RNAase-free water. Gene expression assays were purchased from Thermofisher. Human assays included: HIF-1α (Hs00153153), PDK1, (Hs01561847), BNIP3 (Hs00969291), and VEGFA (Hs00900055). Gene expression assays were performed using Taqman Advanced Master Mix (Thermofisher, 4444963) in 10μL reactions. Cycle threshold values were measured via rtPCR (Quantstudio 3, Applied Biosystems, Foster City, CA, USA). The cycle threshold (Ct) for the gene of interest was normalized to *18s* as an endogenous control (Hs99999901*)* by calculating the difference (Δ) between the Ct of the gene of interest minus the Ct of 18s. Because the ΔCt value of a gene of interest is negatively related to mRNA abundance (exponentially), ΔCt values expressed negative values (-ΔCt) so that the bar graphs depicting the change in mRNA abundance with exercise reflected an increase in the ΔCt value (Figure 3).

RNA Sequencing

Isolated RNA was delivered to Ademra Health Biopharma (South Plainfield, NJ, USA) for next-generation RNA sequencing. Sample quality was assessed by Agilent RNA

6000 Pico Reagent on Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and quantified by Qubit RNA HS assay (ThermoFisher, Waltham, MA). Ribosomal RNA depletion and library preparation was performed with SMARTer Stranded Total RNA v3 (Takara Bio USA Inc., California, USA) following manufacturer's instructions. Average final library size is approximately 350 bp. Illumina 8-nt unique dual-indices were used for multiplexing. Samples were pooled and sequenced on an Illumina NovaSeq S4 sequencer for 150 bp read length in paired-end mode, with an output of 60 million reads per sample. Only 14 samples were sequenced (7 untrained, 7 trained) because the RNA concentration for one untrained sample was too low for sequencing.

Differential Gene Expression Analysis of RNA-seq data.

Raw FASTQ files was assessed for quality using *FastP* (Chen et al. 2018), which provides quality control data while simultaneously trimming low quality reads and adapter ends. Quality reads were then aligned to the most current version of the human genome (GRCh38.p13, downloaded from https://www.gencodegenes.org/human/) using *STAR* (Dobin and Gingeras 2015). The primary annotation and sequence files were used for alignment and gene annotation. Aligned reads were summarized at the gene level to provide a list of read counts for each gene. Gene counts were filtered down to only genes previously identified as HIF-1 targets (Ke and Costa 2006; Benita et al. 2009). A total of 572 HIF-1 target genes were selected and filtered. A list of these genes is shown in **Supplementary Table 1 (**table is provided as a shared link to save space in this document). Differential gene expression analysis was applied to these gene counts using DESeq2 (Love et al. 2014). First, all samples were analyzed together to determine whether the effect of exercise was different across groups. Specifically, this assessed if the change in gene expression due to exercise was

different between the trained and untrained groups. Following the primary analysis, secondary models were constructed to identify differentially expressed genes between pre and 3 h post exercise in each respective group. Principal component analysis (PCA) was used to determine the degree of variance between groups when analyzed together (both groups, pre and 3 h post exercise) and individual groups analyzed separately (pre and 3 h post exercise). Significantly expressed genes were considered to be those with a Benjamini-Hochberg adjusted P value < .05. Volcano plots were generated using the Enhanced Volcano R package (Blighe et al. 2020).

To determine group differences in muscle fiber type, baseline myosin heavy chain (MCH) isoform gene count data (MYH1, MYH2, MYH4, and MYH7) were analyzed separately from the HIF-1 target gene data set, as described by West et al. 2021 (West et al. 2021). However, DESeq2 was used for the comparison between trained and untrained groups in place of parametric statistical methods. For the graphical representation and linear regression analysis (described below), MYH7 was calculated relative to the total expression of all 4 isoforms (% MYH7).

Functional Enrichment Analysis of Differentially Expressed Genes

The gProfiler web application (Raudvere et al. 2019) was used to link differentially expressed genes to function using the Gene Ontology (GO) biological function (Carbon et al. 2021) and reactome (Jassal et al. 2019) databases. Significantly enriched pathways (g:SCS-adjusted P value < .05) were imported into Cytoscape and visualized using the Enrichment Map application (Merico et al. 2010).

Power Analysis

An *a priori* power analysis was performed in G power (Faul et al. 2007) to estimate the sample size needed to detect a significant difference in HIF-1α protein expression between groups, if there was indeed a difference. The effect size of the difference in HIF-1α expression between trained and untrained participants was estimated from the data reported by Lundby (2006) (Lundby et al. 2006). Using the web application, WebPlotDigitizer *(*https://apps.automeris.io/wpd/*)*, the means and standard deviations were extracted from the bar graph provided in the manuscript that illustrated the fold change in HIF-1α mRNA between trained and untrained legs. The Cohen's d calculated from these values was 3.12 (a very large effect). Using a conservative approach to the power analysis for ANOVA, we used a partial η^2 of .25 (large effect). Assuming 80% power and a significance threshold of .05, the sample size calculated was 6 per group.

Statistical Analyses

Statistical analyses were performed in Rstudio (Version 1.4.1106, (Rstudio 2020)) and Graphpad Prism (version 9, San Diego, CA). Data were assessed for normality using the Shapiro-Wilk method and visual inspection of residual Q-Q plots. Mixed effects linear models were constructed to determine the effect of exercise (pre, post, and 3 h post exercise) and group (trained vs. untrained) on HIF-1α, PHD2, FIH and VHL protein, HIF-1α, PDK1, BNIP3 and VEGFA mRNA, and mean TSI. Data that were not normally distributed were align rank-transformed using the ARTool package in R (Kay and Wobbrock 2015). This method was chosen because it is one of few statistical packages available that accommodate mixed effect designs and is directly compatible with standard mixed effect ANOVA (Feys 2016). Where there was a significant main or interaction effect, pairwise comparisons were performed, and P values were corrected for using the Tukey method. Unpaired t-tests were

used to compare average heart rate, SpO_2, RPE, power and ΔTSI between trained and untrained groups during exercise.

Multiple linear regression analysis was used to determine the main effects of training group, exercising variables (power, delta TSI, and mean SpO2), as well as the interaction between training group and exercising variables on post fold change in HIF-1 protein expression. In addition, a separate multiple linear regression analysis was performed to determine the influence of training group, baseline characteristics (%MYH7 expression and VO2peak, and the interaction between training group and baseline characteristics) on post exercise fold change in HIF-1 protein expression. Where appropriate, variables were centered around the mean to help reduce multicollinearity (i.e., power, mean SpO2, VO2peak, and % MYH7). The assumptions of the multiple linear regression models including multivariate normality, multicollinearity, and homoscedasticity were confirmed by visual inspection of graphs or explicitly tested where appropriate (i.e., Variance Inflation Factor). Diagnostic testing including inspection of boxplots and Cook's distance was used to identify potential outliers or influential data points. In all cases, significance was set to $P < .05$.

Results

Physiological Responses and Performance during Repeated Sprint Exercise

Table 2 shows the statistical comparisons (P values and Cohen's d) between trained and untrained groups for heart rate, SpO_2, RPE, and power during exercise. Heart rate and RPE were not significantly different between groups ($P > .05$). SpO_2 during exercise was lower in the trained group compared to the untrained group ($P < .05$). Mean power relative to body mass was higher in the trained group than the untrained groups ($P < .05$). There was a significant interaction effect between group and exercise on mean TSI ($F_{1,13} = 5.82$, $P = .03$). Compared to baseline, mean TSI was lower during exercise in both untrained ($P = .0001$, d =

3.18) and trained groups (P = .0002, d = 3.30) compared to the respective baseline values (**Figure 1A)**. Despite the interaction effect, there was no significant difference in mean TSI between groups at baseline (P = .35, d = .93) or exercise (P = .45, d = -.83). There was no significant difference in the ΔTSI between groups (P = .12, d = 1.03) (**Figure 1B)**.

Protein Expression

There was a significant main effect of exercise on HIF-1α fold change ($F_{2,26}$ = 2.39, P = .0005). HIF-1α protein was greater immediately post exercise in both untrained (P = .03, d = 1.58) and trained groups (P = .002, d = 1.64) (**Figure 2A)**. There was a main effect of group on PHD2 protein levels ($F_{1,13}$ = 4.89, P = .045). Averaged across time, PHD2 levels were greater in the trained group than the untrained group (P = .002, d = 1.68). PHD2 levels were also greater in the trained group 3 h post exercise (P= .02, d = 1.88) (**Figure 2B)**. There was a main effect of group on FIH protein levels ($F_{1,13}$ = 5.72, P= .032). Averaged across time, FIH protein levels were higher in the trained group compared to the untrained group (P = .032, d = 2.04). FIH protein levels were also greater at 3 h post exercise in the trained group compared to the untrained group (P = .02, d = 1.97) (**Figure 2C)**. There was no main or interaction effects for VHL protein levels (P > .05) (**Figure 2D)**.

PCR Gene Expression

There was a group x exercise interaction effect on HIF-1α mRNA levels ($F_{1,12}$ = 4.84, P = .04). At baseline, HIF-1α mRNA levels were higher in the trained group compared to the untrained group (P = .02, d = 1.63). HIF-1α mRNA was significantly greater at 3 h post exercise compared to baseline in the untrained group only (P = .02, d = 1.5) (**Figure 3A)**. There was a main effect of exercise on VEGFA mRNA levels ($F_{1,12}$ = .8.69, P = .01). Averaged across group, VEGFA mRNA levels were elevated 3 h post exercise (P = .01, d =

.76) (**Figure 3B)**. There was a significant main effect of group on PDK1 mRNA ($F_{1,2} = 5.46$, P = .03). PDK1 levels were higher at baseline in the trained group compared to the untrained group (P = .02, d = 2.1) (**Figure 3C)**. There was no main or interaction effect for BNIP3 mRNA levels (**Figure 3D)**.

Differential Gene Expression Analysis

When groups were analyzed together to determine the influence of training status on the change in HIF-1 target gene expression, PCA showed no specific clustering between groups or timepoint **(Figure 4A**). There was no significant difference in HIF-1 target gene expression between groups at rest. Of the 572 HIF-1 target genes analyzed, only the exercise-induced change in FOXO3 was significantly different between trained and untrained groups (P < .0001) (**Figure 4B)**. The log2 (fold change) in FOXO3 was 0.62 in the untrained group and -.35 in the trained group (**Figure 6A**). When groups were analyzed separately, the untrained group showed some clustering along principal component 2 (**Figure 5A**) while the trained group showed clustering along both principal components 1 and 2 (**Figure 5C**). Volcano plots of differentially expressed genes (Log2(fold change) > .5 and P < .05) for untrained and trained groups are shown in **Figure 5B** and **D**, respectively. Of the 572 HIF-1 target genes analyzed, 45 genes were differentially expressed (26 upregulated, 19 downregulated after exercise) in the trained group only. 17 genes were differentially expressed (14 upregulated, 3 downregulated after exercise) in the untrained group only. 15 genes were differentially expressed in both groups (12 upregulated, 2 downregulated after exercise) (**Figure 6B**). The Log2(fold change) of all differentially expressed genes is shown in **Figure 6A**. The top 10 (largest fold change) commonly expressed genes between trained and untrained groups are shown in **Figure 7** (NR4A1, HMOX1, PIM1, ATF3, CDKN1A,

GADD45B, PDK4, PTGS2, VEGFA, and FOSL2). The top 6 most differentially expressed genes in the trained (GAPDH, GYS1, ANKRD37, PDK2, PKM, PDGFA) and untrained (ADM, NOS3, PFKB3, SERPINE1, PLUAR and SLC2A3) groups are shown in **Figure 8** and **Figure 9**, respectively.

In addition to the analysis of HIF-1 target genes, myosin heavy chain gene expression content was compared between groups at baseline. The relative expression of MYH7 (which codes for the MHC slow isoform and is highly expressed in type I fibers) was greater in the trained group compared to the untrained group ($P < .0001$). Relative MYC1 (which codes for the fast IIx MHC isoform, as is highly expressed in type II fibers) was lower in the trained group compared to the untrained group (**Figure 10**).

Functional Enrichment Analysis of Differentially Expressed HIF-1 Target Genes

The 3 gene sets acquired from differential gene expression analysis (common to both groups, trained only and untrained only) were mapped to their biological function using GO and reactome terms. Gene ontology enrichment analysis revealed 42 enriched pathways common to both groups, 37 pathways enriched only in the trained group, and 5 pathways enriched in the trained group only. These pathways were filtered to reduce the redundancy between pathways for a given data set (common, trained, and untrained only). Further filtering was performed to include only those related to signaling for morphological or metabolic adaptations to exercise (unfiltered enriched terms can be found in the following links: *common pathways, trained only, untrained only)*. Filtering resulted in 8 enriched pathways common to both groups, 5 enriched pathways in the trained group only, and 1 enriched pathway in the untrained group only (**Figure 11**). Pathways common to both groups were involved in the cellular response to exercise stress (*response to nutrient stress, cellular*

response to external stimulus, protein phosphorylation), morphological changes to skeletal muscle tissue and surrounding capillaries (*muscle tissue development, angiogenesis, cell proliferation, apoptosis*) and muscle autocrine/paracrine function (*interleukin signaling).* Pathways significantly enriched in the trained group only were involved primary in the regulation energy turnover via glycolysis (*ATP metabolic process, glycolytic process, generation of precursor metabolites and energy, pyruvate metabolism and gluconeogenesis).* *Negative regulation of blood coagulation* was the only pathway significantly enriched in the untrained group only. In all cases, enriched pathways were significantly up- regulated. A list of genes related to each enriched pathway is shown in **Table 3**.

Prediction of Exercise-Induced HIF-1α Protein Expression

For the prediction of exercise-induced HIF-1α protein expression with exercise variables (power, delta TSI, and mean SpO2), diagnostic testing indicated one potential outlier with a Cook's distance greater than 0.90. This participant was subsequently removed from this analysis. A different participant was removed from the prediction of exercise-induced HIF-1α protein expression with baseline variables (VO_2peak and %MYH7 expression) because one participant was excluded from the RNA-seq analysis. Thus, sample sizes are reported as n=14 for both analyses. The results from the multiple linear regression models are presented in Tables 4 and 5. No variable significantly predicted fold change in HIF-1α protein expression.

Discussion

The purpose of this study was to determine if the skeletal muscle HIF-1 response to exercise was different between endurance trained and untrained individuals. Our main finding was that after supramaximal exercise, activation of the HIF-1 pathway was similar between trained and untrained groups.

The current hypothesis regarding the role of HIF-1 in the adaptation to endurance training is that HIF-1 mediates the early adaptive process in skeletal muscle remodeling by regulating angiogenic signals. Because HIF-1 is known to suppress mitochondrial respiration (in cancer cells), its initial activation by exercise is thought to be suppressed to allow for training-induced improvements in oxidative capacity (Lindholm et al. 2014). This hypothesis is supported by the observation that elite endurance athletes have high levels of HIF-1 hydroxylases, PHD2 and FIH. We are the first to determine if the greater abundance of FIH and PHD2 in endurance athletes influences the exercise-induced increase in HIF-1α at the protein and mRNA level. Consistent with the findings reported by Lindholm et al. (2014), muscle FIH and PHD2 proteins levels were significantly higher in the trained group compared to the untrained group in the current study. HIF-1α mRNA levels were increased by exercise in the untrained group only, which is also consistent with previous observations (Lundby et al. 2006) and the current hypothesis. Because oxygen consumption drives the decrease in mPO_2 and muscle cells with high oxidative capacity display higher HIF-1 activity (27, 51), our alternative hypothesis was that HIF-1α protein expression, and the transcription of its target genes would be higher in the trained group. In contrast to both the previously proposed hypothesis and our own, the fold increase in HIF-1α protein expression immediately post exercise was not significantly different between groups. The effect size between pre and post exercise was also similar across groups (Cohen's d of untrained = 1.58, trained = 1.64). This suggests that training status has a minimal influence on the exercise-induced HIF-1α protein expression, at least when muscle O_2 desaturation during exercise is similar between groups. The hydroxylase activity of FIH and PHD2 depends primarily on the availability of oxygen as a substrate. Therefore, despite the trained group having greater

expression of HIF-1 hydroxylases, HIF-1α stabilization was similar between both groups, probably because the oxygen limitation was also similar. This notion is supported by the observation that the decrease in muscle oxygenation (TSI) was not different between groups. It is worth noting that the trained group showed evidence of moderate exercise-induced hypoxemia (mean SpO_2 = 90% (Dempsey and Wagner 1999)). This did not translate to a greater decrease in muscle oxygenation, as the effect of intense exercise on mean TSI was again similar between trained (d = 3.20) and untrained (d = 3.18) individuals. Taken together, these data show that provided sufficient exercise stress (i.e supramaximal exercise), trained individuals have a similar capacity to activate the HIF-1 protein as untrained individuals.

Utilizing both rtPCR and next-generation RNA sequencing, we provide the most in-depth analysis of the effect of exercise on HIF-1 target gene transcription to date. We first analyzed genes that were validated to be dependent on HIF-1 activity in our lab (PDK1, VEGFA, and BNIP3). Of particular interest was PDK1, because it mediates the HIF-1-dependent shift away from aerobic respiration in favor of anaerobic glycolysis during periods of chronic hypoxia by inactivating (through phosphorylation) pyruvate dehydrogenase (PDH) (Kim et al. 2006). The downregulation of PDK1 is central to the previously proposed hypothesis that endurance training suppresses the HIF-1 response to exercise (Lindholm et al. 2014). In contrast to the report that endurance athletes have lower baseline gene expression of PDK1 compared to active controls (Lindholm et al. 2014), PDK1 mRNA was higher in the trained group compared to the untrained group in the present study. This is consistent with work of Gudiksen et al. (2017), which showed that the protein expression of PDK isoforms 1, 2 and 4 were higher in the skeletal muscle of endurance trained athletes compared to healthy, untrained controls (Gudiksen et al. 2017). In their study, the Gudiksen group showed that the

exercise-induced increase in PHD activity was similar between trained and untrained individuals, despite the greater abundance of PDK proteins at rest. The relationship between the regulatory enzymes (PDKs) and their target (PDH) reported by Gudiksen et al. mirrors the relationship between HIF-1 hydroxylases and HIF-1α in the present study. In both cases, the exercise-induced increase in the regulated protein is not influenced by training status, despite a greater abundance of the baseline regulatory enzymes. Instead of suppressing the activity of the target protein during exercise, the training-associated increases in regulatory enzymes like PDKs and HIF-1 hydroxylases more likely represent an improved flexibility of the regulatory enzymes to respond to metabolic (i.e. PDKs) and hypoxic (i.e. HIF-1 hydroxylases) stress.

Similar to the null effect of training status on HIF-1α protein expression, we found that of the 572 HIF-1 target genes assessed via differential gene expression analysis, only the exercise-induced change in FOXO3 was different between the untrained (upregulated) and trained (downregulated) groups. This finding is consistent with the similar fold increase in HIF-1α between the trained and untrained groups. Taken together, the exercise-induced induction of the HIF-1 pathway, from stabilization of the oxygen-sensitive HIF-1α subunit to regulation of hypoxia-sensitive genes, is not notably different between endurance trained and untrained individuals. To investigate the effect of exercise on HIF-1 target genes in a more sensitive manner, models were constructed to analyze the two groups separately. We identified 45 genes differentially expressed in the trained group only, 17 genes differentially expressed in the trained group only and 15 genes differentially expressed in both groups. Because the fold change in these genes (except for FOXO3) was not significantly different between groups, these data suggest that trained individuals may have a more diverse

molecular response to exercise than untrained individuals. However, we did not observe a group-specific difference in magnitude of increase in these molecular responses.

The differentially expressed genes common to both groups corresponded to biological pathways expected to be regulated by HIF-1, including those involved in angiogenesis, muscle tissue development and the response to nutrient stress. Nuclear receptor subfamily 4 group A member 1 (NR4A1) was one of the most highly upregulated HIF-1 target genes and is involved in 4 out the 8 biological pathways commonly enriched in both groups. A recent meta-analysis of transcriptomic data related to acute exercise and training revealed that the NR4A3 isoform is among the highest differentially expressed genes affected by acute exercise and is likely critical to the positive changes in metabolic health associated with exercise training (Pillon et al. 2020). Skeletal muscle-specific overexpression of NR4A3 in mice induces mitochondrial biogenesis and confers the endurance phenotype in the absence of exercise training (Pearen et al. 2013). Considering that all three NR4A isoforms are regulated by HIF-1 and their function is highly homologous (Beard et al. 2015), HIF-1 may play a diverse role in the adaptation to exercise training, apart from simply regulated genes involved in angiogenesis and glucose metabolism. This is unsurprising given the importance of oxygen homeostasis to health and disease. However, we did not verify that the expression of the HIF-1 target genes like NR4A1 was in fact the result of HIF-1α stabilization by exercise. Verification that the exercise-induced increase in NR4A isoforms is dependent on HIF-1 activity may provide further insight into its role in the skeletal muscle adaptation to exercise.

A secondary aim of this study was to determine if baseline characteristics related to training status (VO_2peak and fiber type composition) predicted the exercise-induced increase

in HIF-1α protein. Because HIF-1α protein expression was shown to be greater in type I fibers compared to type II fibers (Mounier et al. 2010), it was hypothesized that the degree of increase HIF-1α would be related to basal MHCI expression. However, neither the relative expression of MHCI gene expression nor VO_2max significantly predicted the exercise-induced HIF-1α protein response. This suggests that the HIF-1 response to acute exercise is heterogenous among individuals of varied training status and endurance phenotype characteristics. We also hypothesized that physiological variables representing the intensity and hypoxic stress of exercise (mean power, SpO_2 and ΔTSI) might predict the HIF-1 response. No single variable or interaction between variables significantly predicted the fold increase in HIF-1α protein expression. However, prediction of the post exercise HIF-1α protein levels with SpO_2 ($P = .07$) and mean power ($P = .10$) approached statistical significance. It is unlikely that this study was sufficiently powered to draw meaningful conclusions from these analyses. Nevertheless, we believe this information may be useful for those interested in the relationship between exercise intensity and the skeletal muscle HIF-1 response.

In summary, we present multiple lines of evidence from protein, rtPCR and transcriptomic data showing that the skeletal muscle HIF-1 response to supramaximal exercise is retained in endurance athletes. We attribute the null effect of exercise training on the HIF-1 response to the supramaximal nature of the exercise performed by the participants in this study. This suggests that given sufficient exercise stress, the adaptations to endurance exercise training mediated by HIF-1 can still be stimulated in trained athletes. The molecular responses shown here are consistent with those previously attributed to HIF-1 signaling, including the upregulation of genes involved in angiogenesis, glycolytic metabolism, and

cellular responses to metabolic and hypoxic stress. Furthermore, we highlight a large increase in the HIF-1 target gene, NR4A1, which belongs to a family of nuclear receptors that are gaining attention as mediators of the adaptive response to exercise in skeletal muscle. Our understanding of the role of HIF-1 in the skeletal muscle adaptation to exercise will benefit from studies designed to determine the dependence of such genes on HIF-1 activity.

References

Ameln H, Gustafsson T, Sundberg CJ, et al (2005) Physiological activation of hypoxia inducible factor-1 in human skeletal muscle. FASEB J 19:1009–1011. Doi: 10.1096/fj.04-2304fje

American College of Sports Medicine. (2018). *ACSM's guidelines for exercise testing and prescription*. (D. Riebe, J. K. Ehrman, G. Liguori, & M. Magal, Eds.) (10th ed., pp. 93-94). Philadelphia: Wolters Kluwer/Lippincott Williams & Wilkins Health.

Beard JA, Tenga A, Chen T (2015) The interplay of NR4A receptors and the oncogene–tumor suppressor networks in cancer. Cell Signal 27:257–266. Doi: 10.1016/j.cellsig.2014.11.009

Benita Y, Kikuchi H, Smith AD, et al (2009) An integrative genomics approach identifies Hypoxia Inducible Factor-1 (HIF-1)-target genes that form the core response to hypoxia. Nucleic Acids Res 37:4587–4602. Doi: 10.1093/nar/gkp425

Berra E (2003) HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1 in normoxia. EMBO J 22:4082–4090. Doi: 10.1093/emboj/cdg392

Blighe K, Rana S, Lewis M (2020) EnhancedVolcano: Publication-ready volcano plots with enhanced colouring and labeling

Carbajo-Pescador S, Ordoñez R, Benet M, et al (2013) Inhibition of VEGF expression through blockade of Hif1α and STAT3 signalling mediates the anti-angiogenic effect of melatonin in HepG2 liver cancer cells. Br J Cancer 109:83–91. Doi: 10.1038/bjc.2013.285

Carbon S, Douglass E, Good BM, et al (2021) The Gene Ontology resource: Enriching a Gold mine. Nucleic Acids Res 49:D325–D334. Doi: 10.1093/nar/gkaa1113

Chen S, Zhou Y, Chen Y, Gu J (2018) Fastp: An ultra-fast all-in-one FASTQ preprocessor. In: Bioinformatics. Oxford University Press, pp i884–i890

Dayan F, Monticelli M, Pouysségur J, Pécou E (2009) Gene regulation in response to graded hypoxia: The non-redundant roles of the oxygen sensors and FIH in the HIF pathway. J Theor Biol 259:304–316. Doi: 10.1016/j.jtbi.2009.03.009

de Freitas MC, Gerosa-Neto J, Zanchi NE, et al (2017) Role of metabolic stress for enhancing muscle adaptations: Practical applications. World J Methodol 7:46. Doi: 10.5662/wjm.v7.i2.46

Dempsey JA, Wagner PD (1999) Exercise-induced arterial hypoxemia. J Appl Physiol 87:1997–2006. Doi: 10.1152/jappl.1999.87.6.1997

Dobin A, Gingeras TR (2015) Mapping RNA-seq Reads with STAR. Curr Protoc Bioinforma 51:. Doi: 10.1002/0471250953.bi1114s51

Drummond MJ, Fujita S, Takashi A, et al (2008) Human muscle gene expression following resistance exercise and blood flow restriction. Med Sci Sports Exerc 40:691–698. Doi: 10.1249/MSS.0b013e318160ff84

Dunn LL, Kong SMY, Tumanov S, et al (2020) Hmox1 (Heme Oxygenase-1) Protects Against Ischemia-Mediated Injury via Stabilization of HIF-1α (Hypoxia-Inducible Factor-1α). Arterioscler Thromb Vasc Biol. Doi: 10.1161/ATVBAHA.120.315393

Egan B, Zierath JR (2013) Exercise Metabolism and the Molecular Regulation of Skeletal Muscle Adaptation. Cell Metab 17:162–184. Doi: 10.1016/j.cmet.2012.12.012

Faul F, Erdfelder E, Lang AG, Buchner A (2007) G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. In: Behavior Research Methods

Feys J (2016) Nonparametric Tests for the Interaction in Two-way Factorial Designs Using R. R J 8:367. Doi: 10.32614/RJ-2016-027

Gudiksen A, Bertholdt L, Stankiewicz T, et al (2017) Effects of training status on PDH regulation in human skeletal muscle during exercise. Pflügers Arch – Eur J Physiol 469:1615–1630. Doi: 10.1007/s00424-017-2019-6

Haan C, Behrmann I (2007) A cost effective non-commercial ECL-solution for Western blot detections yielding strong signals and low background. J Immunol Methods 318:11–19. Doi: 10.1016/j.jim.2006.07.027

Jassal B, Matthews L, Viteri G, et al (2019) The reactome pathway knowledgebase. Nucleic Acids Res. Doi: 10.1093/nar/gkz1031

Kay M, Wobbrock JO (2015) ARTool: Aligned Rank Transform for Nonparametric Factorial ANOVAs. R Journal.

Ke Q, Costa M (2006) Hypoxia-Inducible Factor-1 (HIF-1). Mol Pharmacol 70:1469–1480. Doi: 10.1124/mol.106.027029

Kiens B, Essen-Gustavsson B, Christensen NJ, Saltin B (1993) Skeletal muscle substrate utilization during submaximal exercise in man: effect of endurance training. J Physiol 469:459–478. Doi: 10.1113/jphysiol.1993.sp019823

Kim JW, Tchernyshyov I, Semenza GL, Dang C V. (2006) HIF-1-mediated expression of pyruvate dehydrogenase kinase: A metabolic switch required for cellular adaptation to hypoxia. Cell Metab. Doi: 10.1016/j.cmet.2006.02.002

Kuang J, Yan X, Genders AJ, et al (2018) An overview of technical considerations when using quantitative real-time PCR analysis of gene expression in human exercise research. PloS One 13:e0196438. Doi: 10.1371/journal.pone.0196438

Ladner CL, Yang J, Turner RJ, Edwards RA (2004) Visible fluorescent detection of proteins

in polyacrylamide gels without staining. Anal Biochem 326:13–20. Doi: 10.1016/j.ab.2003.10.047

Lindholm ME, Fischer H, Poellinger L, et al (2014) Negative regulation of HIF in skeletal muscle of elite endurance athletes: a tentative mechanism promoting oxidative metabolism. Am J Physiol Integr Comp Physiol 307:R248–R255. Doi: 10.1152/ajpregu.00036.2013

Lindholm ME, Giacomello S, Werne Solnestam B, et al (2016) The Impact of Endurance Training on Human Skeletal Muscle Memory, Global Isoform Expression and Novel Transcripts. PloS Genet. Doi: 10.1371/journal.pgen.1006294

Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. Doi: 10.1186/s13059-014-0550-8

Lundby C, Gassmann M, Pilegaard H (2006) Regular endurance training reduces the exercise induced HIF-1α and HIF-2α mRNA expression in human skeletal muscle in normoxic conditions. Eur J Appl Physiol 96:363–369. Doi: 10.1007/s00421-005-0085-5

Maxwell PH, Wiesener MS, Chang G-W, et al (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 399:271–275. Doi: 10.1038/20459

McDonough AA, Veiras LC, Minas JN, Ralph DL (2015) Considerations when quantitating protein abundance by immunoblot. Am J Physiol – Cell Physiol 308:C426–C433. Doi: 10.1152/ajpcell.00400.2014

McManus CJ, Collison J, Cooper CE (2018) Performance comparison of the MOXY and PortaMon near-infrared spectroscopy muscle oximeters at rest and during exercise. J Biomed Opt 23:1. Doi: 10.1117/1.JBO.23.1.015007

Merico D, Isserlin R, Stueker O, et al (2010) Enrichment map: A network-based method for gene-set enrichment visualization and interpretation. PloS One 5:e13984. Doi: 10.1371/journal.pone.0013984

Mounier R, Pedersen BK, Plomgaard P (2010) Muscle-specific expression of hypoxia-inducible factor in human skeletal muscle. Exp Physiol 95:899–907. Doi: 10.1113/expphysiol.2010.052928

Nguyen LK, Cavadas MAS, Scholz CC, et al (2013) A dynamic model of the hypoxia-inducible factor 1 (HIF-1) network. J Cell Sci 126:1454–1463. Doi: 10.1242/jcs.119974

O'Hagan KA, Cocchiglia S, Zhdanov A V., et al (2009) PGC-1 is coupled to HIF-1 -dependent gene expression by increasing mitochondrial oxygen consumption in skeletal muscle cells. Proc Natl Acad Sci 106:2188–2193. Doi: 10.1073/pnas.0808801106

Olfert IM, Howlett RA, Wagner PD, Breen EC (2010) Myocyte vascular endothelial growth factor is required for exercise-induced skeletal muscle angiogenesis. Am J Physiol

Integr Comp Physiol 299:R1059–R1067. Doi: 10.1152/ajpregu.00347.2010

Pearen MA, Goode JM, Fitzsimmons RL, et al (2013) Transgenic muscle-specific Nor-1 expression regulates multiple pathways that effect adiposity, metabolism, and endurance. Mol Endocrinol. Doi: 10.1210/me.2013-1205

Perry CGR, Lally J, Holloway GP, et al (2010) Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. J Physiol 588:4795–4810. Doi: 10.1113/jphysiol.2010.199448

Pillon NJ, Gabriel BM, Dollet L, et al (2020) Transcriptomic profiling of skeletal muscle adaptations to exercise and inactivity. Nat Commun. Doi: 10.1038/s41467-019-13869-w

Prior BM, Yang HT, Terjung RL (2004) What makes vessels grow with exercise training? J Appl Physiol 97:1119–1128. Doi: 10.1152/japplphysiol.00035.2004

Raudvere U, Kolberg L, Kuzmin I, et al (2019) G:Profiler: A web server for functional enrichment analysis and conversions of gene lists (2019 update). Nucleic Acids Res 47:W191–W198. Doi: 10.1093/nar/gkz369

Richardson RS, Noyszewski EA, Kendrick KF, et al (1995) Myoglobin O2 desaturation during exercise. Evidence of limited O2 transport. J Clin Invest 96:1916–1926. Doi: 10.1172/JCI118237

Rivero-Gutiérrez B, Anzola A, Martínez-Augustin O, De Medina FS (2014) Stain-free detection as loading control alternative to Ponceau and housekeeping protein immunodetection in Western blotting. Anal Biochem 467:1–3. Doi: 10.1016/j.ab.2014.08.027

Robergs RA, Dwyer D, Astorino T (2010) Recommendations for improved data processing from expired gas analysis indirect calorimetry. Sport Med 40:95–111. Doi: 10.2165/11319670-000000000-00000

Ropka-Molik K, Stefaniuk-Szmukier M, Z˙ukowski K, et al (2017) Exercise-induced modification of the skeletal muscle transcriptome in Arabian horses. Physiol Genomics 49:318–326. Doi: 10.1152/physiolgenomics.00130.2016

Rstudio T (2020) Rstudio: Integrated Development for R. Rstudio Team, PBC, Boston, MA URL http//www.rstudio.com/

Semenza GL (2007a) Life with Oxygen. Science (80-) 318:62–64. Doi: 10.1126/science.1147949

Semenza GL (2007b) HIF-1 mediates the Warburg effect in clear cell renal carcinoma. J Bioenerg Biomembr 39:231–234. Doi: 10.1007/s10863-007-9081-2

Srinivasan S, Dunn JF (2011) Stabilization of hypoxia-inducible factor-1α in buffer containing cobalt chloride for Western blot analysis. Anal Biochem 416:120–122. Doi:

10.1016/j.ab.2011.04.037

Steinbacher P, Eckl P (2015) Impact of Oxidative Stress on Exercising Skeletal Muscle. Biomolecules 5:356–377. Doi: 10.3390/biom5020356

Van Thienen R, Hespel P (2016) Enhanced muscular oxygen extraction in athletes exaggerates hypoxemia during exercise in hypoxia. J Appl Physiol 120:351–361. doi: 10.1152/japplphysiol.00210.2015

Toledo-Arruda AC, Sousa Neto IV de, Vieira RP, et al (2020) Aerobic exercise training attenuates detrimental effects of cigarette smoke exposure on peripheral muscle through stimulation of the Nrf2 pathway and cytokines: a time-course study in mice. Appl Physiol Nutr Metab 45:978–986. Doi: 10.1139/apnm-2019-0543

West DWD, Doering TM, Thompson JM, et al (2021) Low responders to endurance training exhibit impaired hypertrophy and divergent biological process responses in rat skeletal muscle. Exp Physiol 106:714–725. Doi: 10.1113/EP089301

Table 1. Demographic and physiologic characteristics of endurance trained and untrained participants.

	Untrained (n=8)	Endurance Trained (n=7)	P value	Cohen's d
Age (years)	26 ± 5	30 ± 6	.14	.80
Height (cm)	176 ± 6.4	180.9 ± 6.5	.88	0
Body Mass (kg)	83.8 ± 14.6	73.6 ± 8.3	.11	.91
VO_2Peak (ml/kg/min)	**38.4 ± 5.9**	**54.9 ± 8.3**	**.001**	**2.11**

cm: centimeters kg: kilograms, ml: milliliters, min: minutes. Data are presented as mean ± standard deviation. Bold values indicate statistically significant difference between groups ($P < .05$).

Table 2. Physiological responses and performance during exercise in untrained and trained groups

	Untrained (n = 8)	Trained (n = 7)	P value	Cohen's d
Heart Rate (bpm)	165 ± 10	160± 14	0.53	-0.35
SpO_2 (%)	**94± 1**	**90± 2**	**0.0007**	**-2.22**
RPE	18 ± 2	17± 1	0.31	-0.54
Power (W/kg)	**5.7 ± 1.3**	**8.3± 0.9**	**0.0005**	**2.39**
% of Max Watts	187 ± 32.0	168.5 ± 22.6	0.20	.69

Mean power (W/kg) was calculated as the average power across 20 sprints divided by the subject's body mass. The % of Max Watts was calculated as the average power across 20 sprints divided by the max power achieved during the subject's VO_2peak test. SpO_2: peripheral blood oxygen saturation, bpm: beats per minute, RPE: rating of perceived exertion, W: watts, kg: kilograms. Data are shown as mean ± standard deviation. Bold rows represent a significant difference between groups ($P < .05$).

Table 3. Results from g:Profiler pathway analysis of exercise-induced, hypoxia-sensitive genes common to both trained and untrained groups as well as pathways enriched in each respective group.

	Enrichment Term	Associated Genes
Common	positive regulation of cell population proliferation	NR4A1, HMOX1, PIM1, ATF3, CDKN1A, PTGS2, VEGFA, FOSL2, RARA, SERTAD1
	regulation of apoptotic process	NR4A1, HMOX1, PIM1, ATF3, CDKN1A, GADD45B, PDK4, PTGS2, VEGFA, PHLDA1, RARA
	cellular response to external stimulus	HMOX1, PIM1, ATF3, CDKN1A, PDK4, PTGS2
	response to nutrient stress	HMOX1, PIM1, PTGS2, RARA
	striated muscle tissue development	NR4A1, PIM1, ATF3, VEGFA, RARA
	protein phosphorylation	PIM1, CDKN1A, GADD45B, PDK4, PTGS2, VEGFA, RARA, SERTAD1
	sprouting angiogenesis	NR4A1, HMOX1, PTGS2, VEGFA
	Interleukin-4 and Interleukin-13 signaling	HMOX1, PIM1, CDKN1A, PTGS2, VEGFA
Trained Only	pyruvate metabolic process	GAPDH, PDK2, PKM, TPI1, STAT3, GPI, PGK1
	generation of precursor metabolites and energy	GAPDH, GYS1, PKM, TPI1, PINK1, STAT3, GPI, PGK1, ASPH, CCNB1
	glycolytic process	GAPDH, PKM, TPI1, STAT3, GPI, PGK1
	ATP metabolic process	GAPDH, PKM, TPI1, PINK1, STAT3, GPI, PGK1, CCNB1
	gluconeogenesis	GAPDH, PDK2, TPI1, GPI, PGK1
Untrained Only	negative regulation of blood coagulation	NOS3, SERPINE1, PLAUR

Table 4: Multiple linear regression results for predicting the exercise-induced change in HIF-1α protein expression with baseline physiological characteristics of endurance trained and untrained participants (*N=14).*

Predictor	r^2	Coefficient (β)	Standard Error	t-statistic	P-value
Main effects					
Intercept		0.55	1.40	0.39	0.70
Group (Untrained)		1.63	2.25	0.73	0.49
Centered VO_2peak	0.22	0.10	0.07	1.36	0.21
Centered %MHC7	0.10	0.55	0.04	1.09	0.31
Interaction effects					
Group x VO2peak		0.03	0.23	0.13	0.90
Group x %MHC7		-0.10	0.07	-1.39	0.20
Full Model					
F-statistic: 1.434		P-value: 0.34			
R-squared: 0.63		Adjusted R-squared: 0.19			

Table 5: Multiple linear regression results for predicting the exercise-induced change in HIF-1α protein expression with variables considered to be influence the hypoxic stress of acute exercise (*N=14).*

Predictor	r^2	Coefficient (β)	Standard Error	t-statistic	P-value
Main effects					
Intercept		3.14	2.27	1.38	0.22
Group (Untrained)		1.11	2.99	0.37	0.72
Delta TSI	0.02	0.04	0.04	1.01	0.35
Centered Mean SpO_2	0.27	-0.73	0.33	-2.18	0.07
Centered Power	0.18	0.01	0.01	1.92	0.10
Interaction effects					
Group x Delta TSI		-0.01	0.05	-0.26	0.80
Group X Mean SpO_2		0.16	0.55	0.28	0.79
Group x Power		-0.01	0.01	-1.17	0.29
Full Model					
F-statistic: 1.434		P-value: 0.34			
R-squared: 0.63		Adjusted R-squared: 0.19			

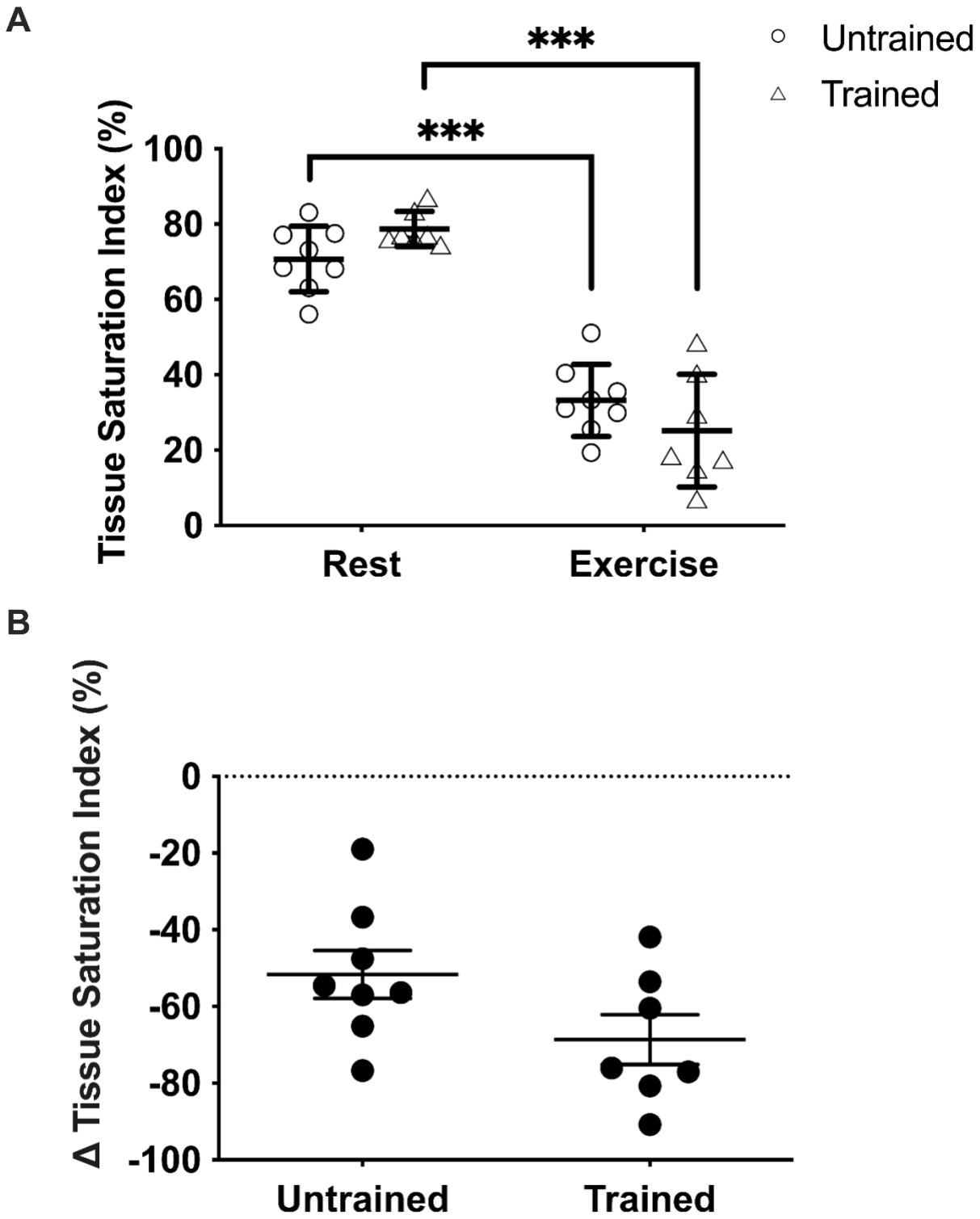

Figure 1. (A) Comparison of muscle tissue saturation index (TSI) at baseline and during exercise between trained and untrained groups. (B) The percent change (Δ) in muscle tissue saturation index between untrained and trained groups. Data are shown as mean ± standard deviation. *** denotes statistically significant difference between groups ($P < .001$)

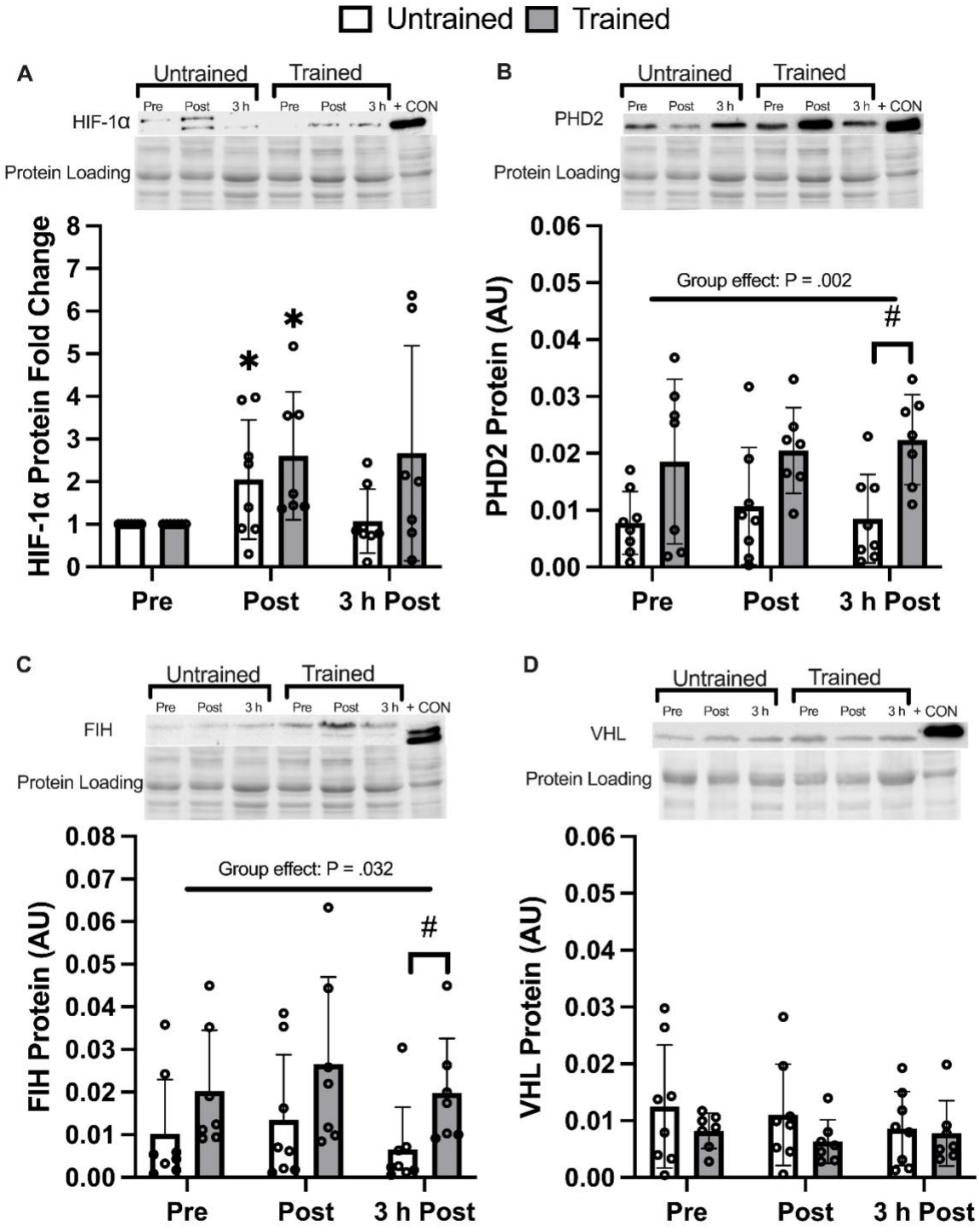

Figure 2. Comparison of exercise-induced protein expression of hypoxia inducible facor 1α (HIF-1α) (A), prolyl hydroxylase 2 (PHD2) (B), factor inhibiting hif (FIH) (C), and von-Hippel-Lindau (VHL) (D) across time (pre, immediately post, and 3 h post exercise. Fold change in HIF-1α was assessed instead of arbitrary units (AU) because basal expression was zero in most samples. Data are expressed as mean ± standard deviation. * denotes significant

difference from baseline (P < .05), # denotes significant difference between trained and untrained groups for that timepoint (P < .05). C2C12 myotubes were exposed to 1% O_2 for 4 h and used as a postivie control.

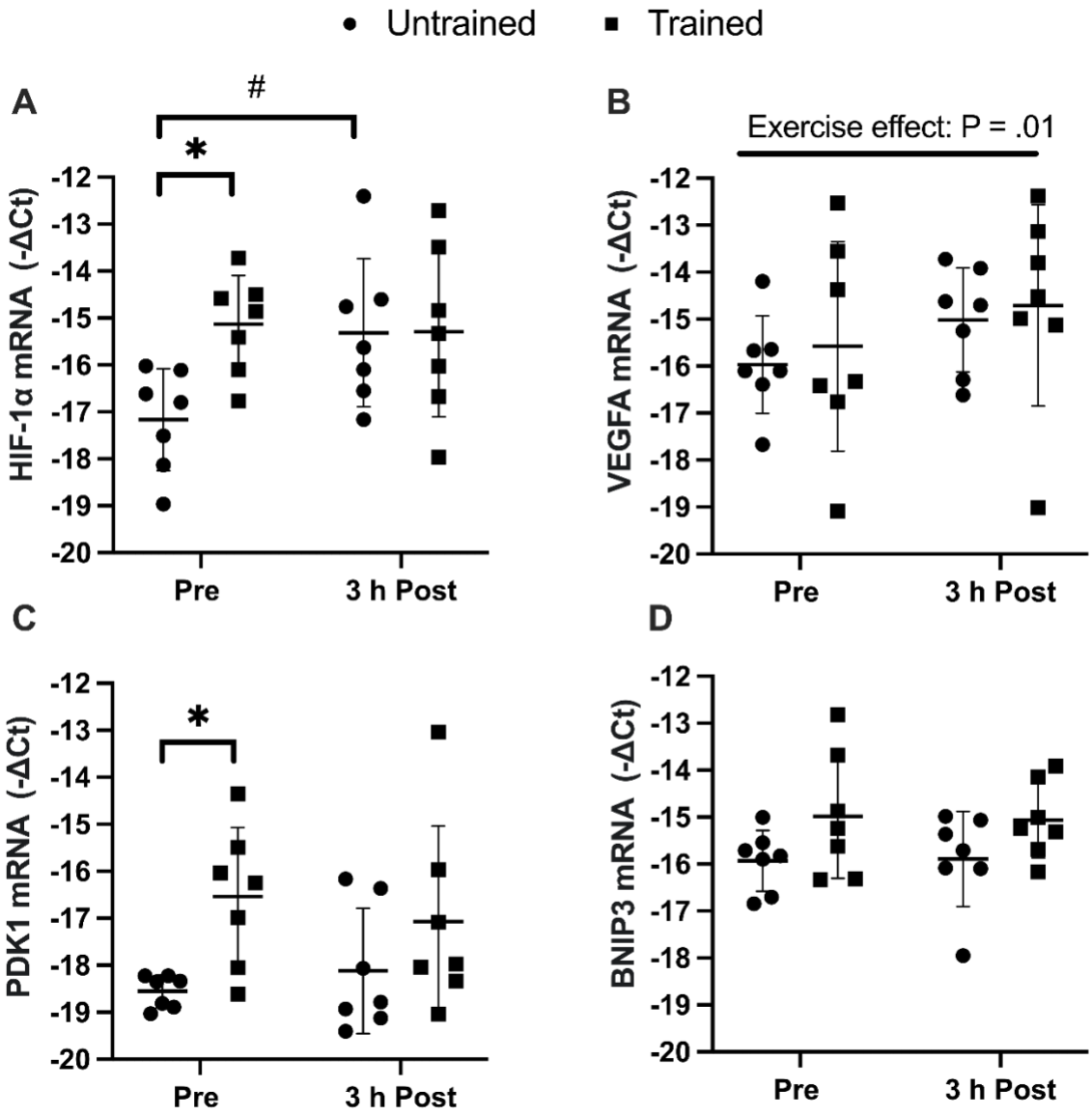

Figure 3. Comparision of baseline and exercise-induced expression of hypoxia inducible facor 1α (HIF-1α) (A), vascular endothelial growth factor A (VEGFA) (B), pyruvate dehydrogenase 1 (PDK1) (C) and BCL2 interacting protein 3 (BNIP3) (D) mRNA levels between trained and untrained groups. Gene expression was measures via RT-qPCR. -ΔCt (cycle threshold) values are depicted to show that a decrease in the ΔCt represents a greater abundance of the mRNA for the gene of interest. Data are shown as mean ± standard deviation (n = 7). * denotes a significant difference between untrained and trained groups at a given timepoint. # depicts a significant difference between pre and 3 hours (3 h) post exercise for an individual group.

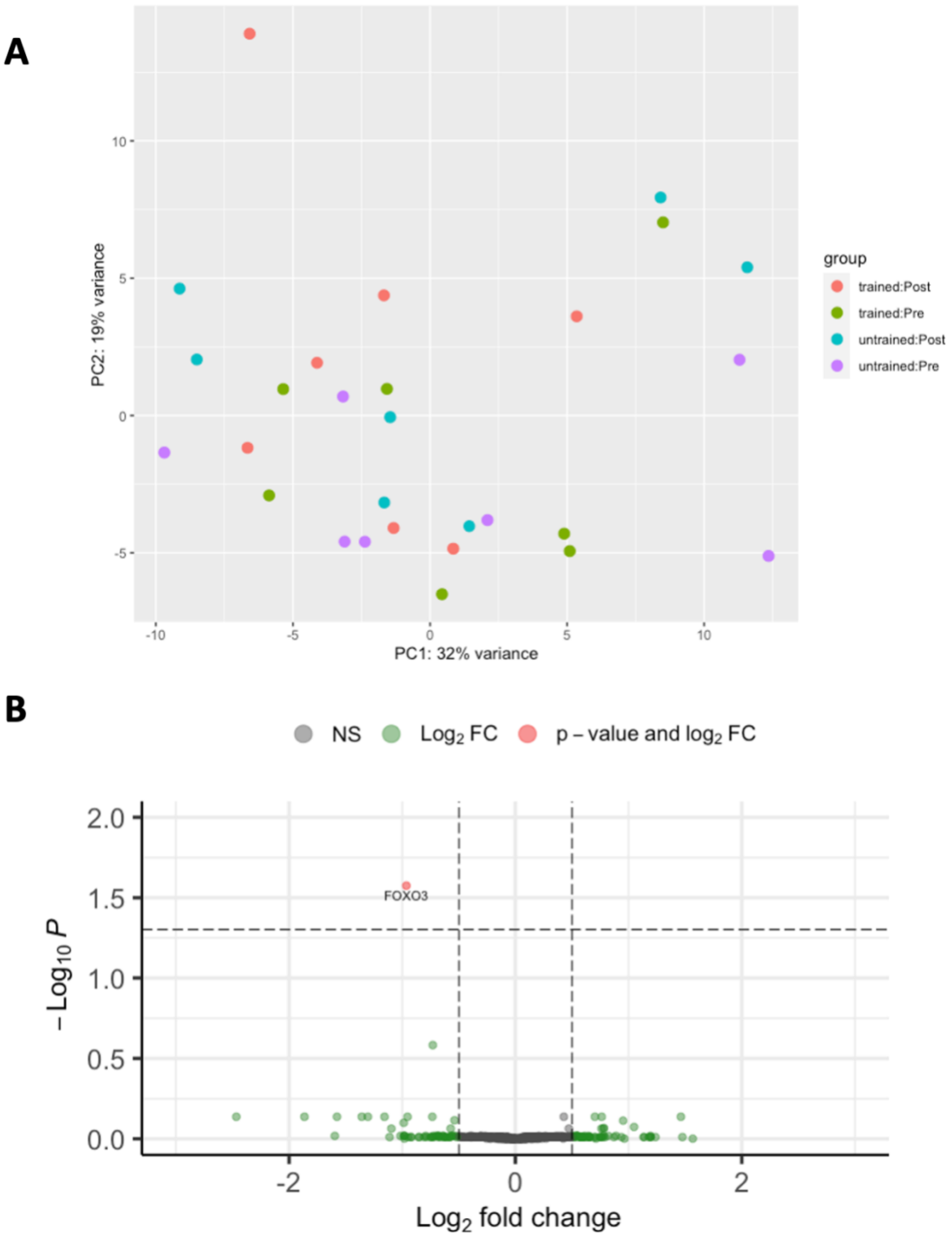

Figure 4. Comparison of the change in skeletal muscle HIF-1 target gene expression between endurance trained (n = 7) and untrained (n = 7) individuals. Principal component analysis of both groups pre and 3 h post exercise shows no specific clustering among groups or timepoints (A). The $\log_2$ fold change in the expression of 572 HIF-1 target genes was not statistically significant between groups ($P < .05$), with the exception of FOXO3 ($P < .0001$) (B). A negative $\log_2$ fold change indicates greater gene expression in the trained group

compared to the untrained group (i.e., FOXO3 increased in the untrained group and decreased in the trained group).

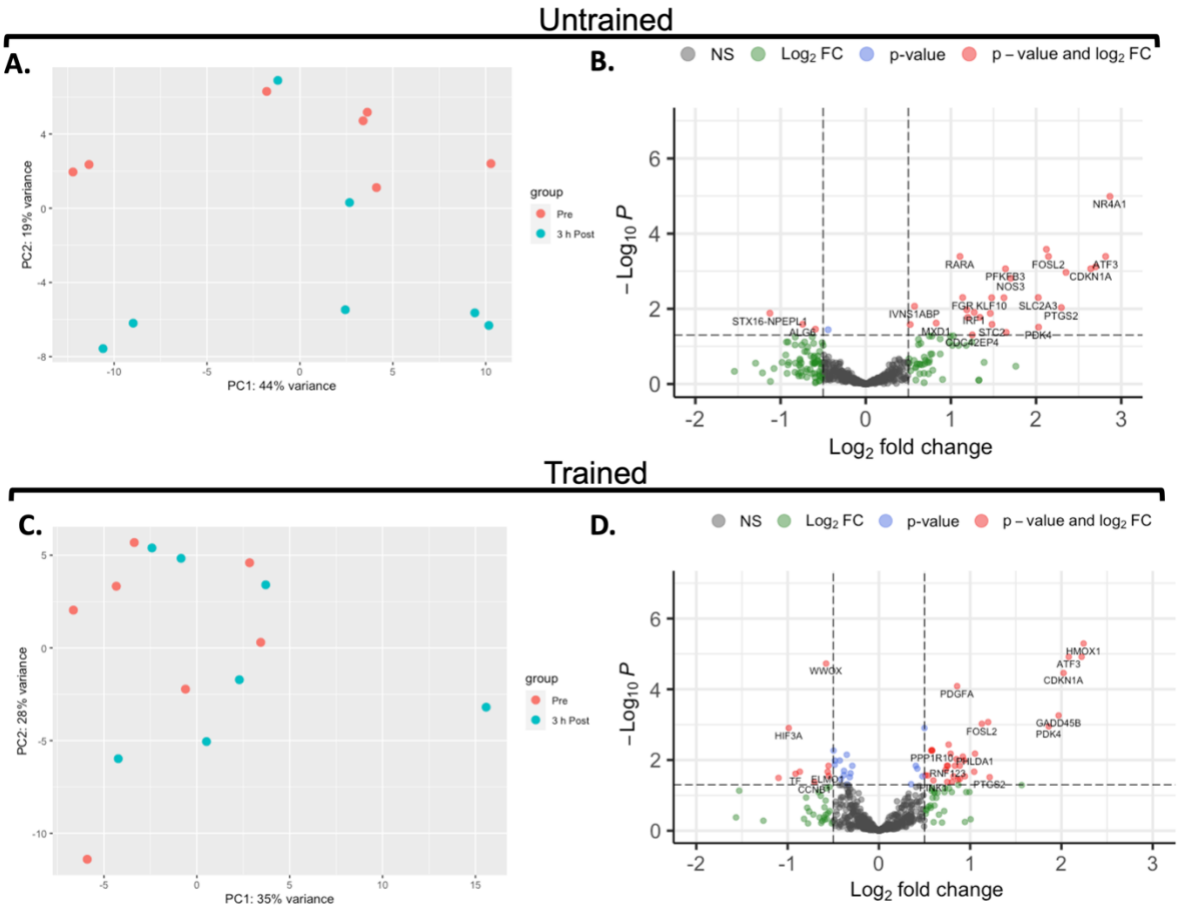

Figure 5. Comparison of exercise-induced skeletal muscle HIF-1 target gene expression between endurance trained (n = 7) and untrained (n = 7) individuals. Principle component analysis was used to indent global differences in gene expression between pre and 3 h post exercise in untrained (A) and trained (C) individuals). Volcano plots show differentially expressed genes from pre to 3 h post exercise in untrained (B) and trained (D) groups.

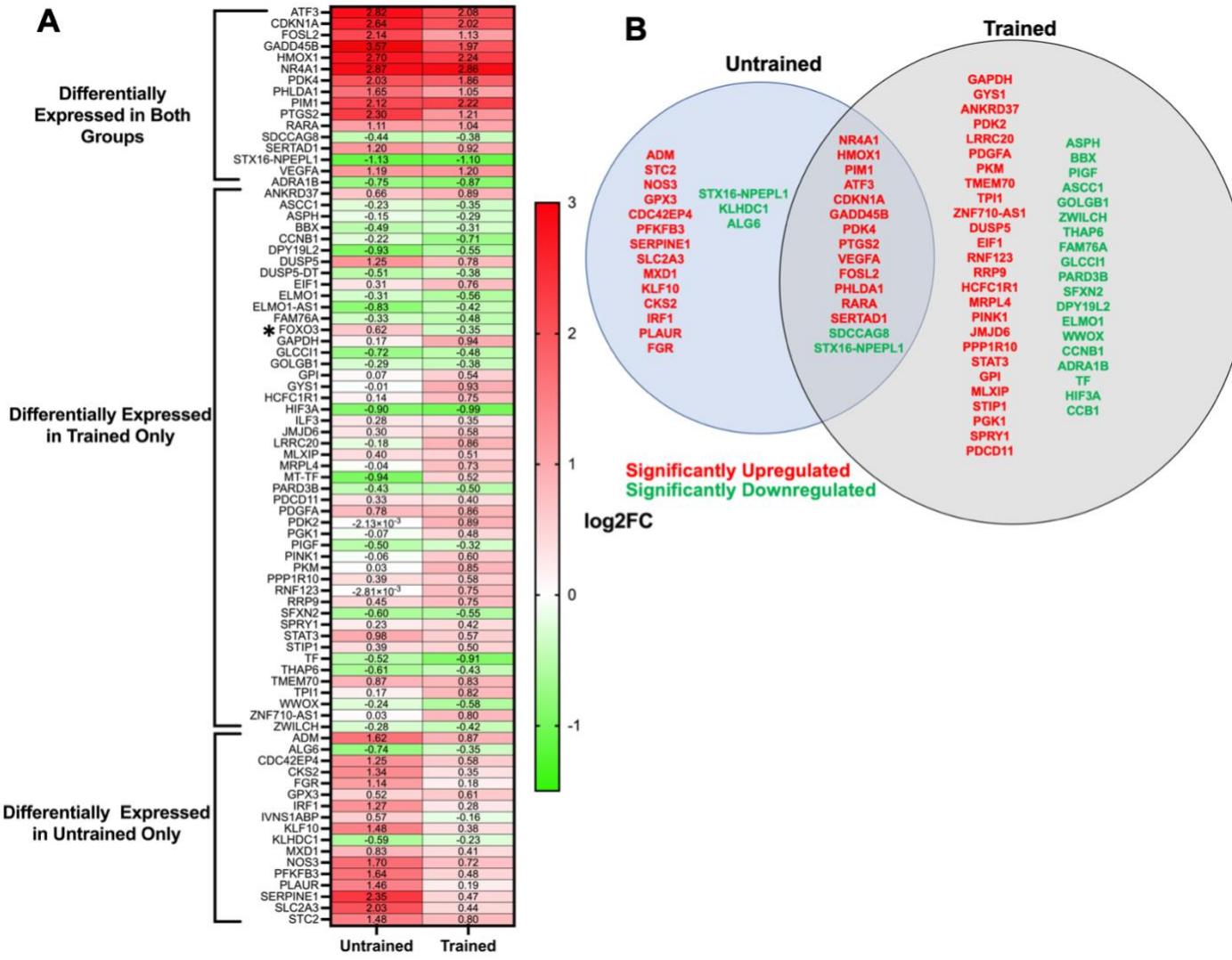

Figure 6. Exercise-induced HIF-1 target gene expression (pre vs 3 h post exercise) in endurance trained (n = 7) and untrained (n = 7) individuals. The heat map (A) shows the log2 (fold change) of differentially expressed genes, sorted by those commonly differentially expressed in both groups and others expressed in the respective groups. Of the 572 HIF-1 target genes analyzed, 26 were upregulated and 19 were downregulated in the trained group only. 14 upregulated and 3 downregulated HIF-1 target genes were identified in the untrained group only. 12 upregulated and 2 downregulated genes were common to both groups (B). * in Figure 6A denotes that the Log2 (fold change) in that gene (FOXO3) was significantly different between groups ($P < .0001$).

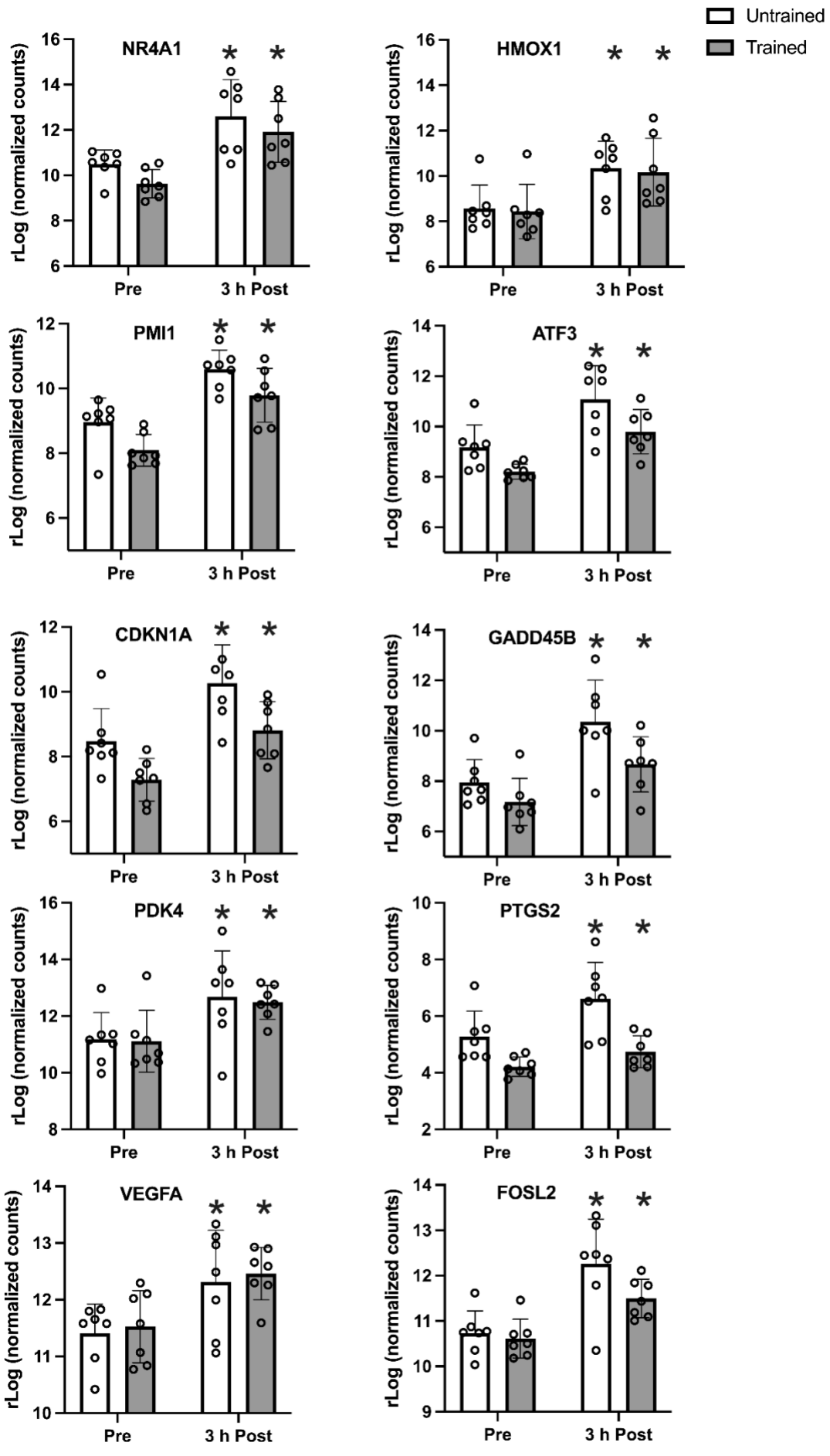
Untrained
Trained
NR4A1
HMOX1
PMI1
ATF3
CDKN1A
GADD45B
PDK4
PTGS2
VEGFA
FOSL2
rLog (normalized counts)
Pre
3 h Post
*

Figure 7. Comparison of normalized gene counts between endurance trained (n = 7) and untrained (n = 7) individuals for the top 10 most differentially expressed HIF-1 target genes common to both groups. Gene counts were generated from next generation RNA-sequencing data. Data are shown as mean ± standard deviation. * indicates a significant difference between pre and 3 h post exercise for a respective group ($P < .05$).

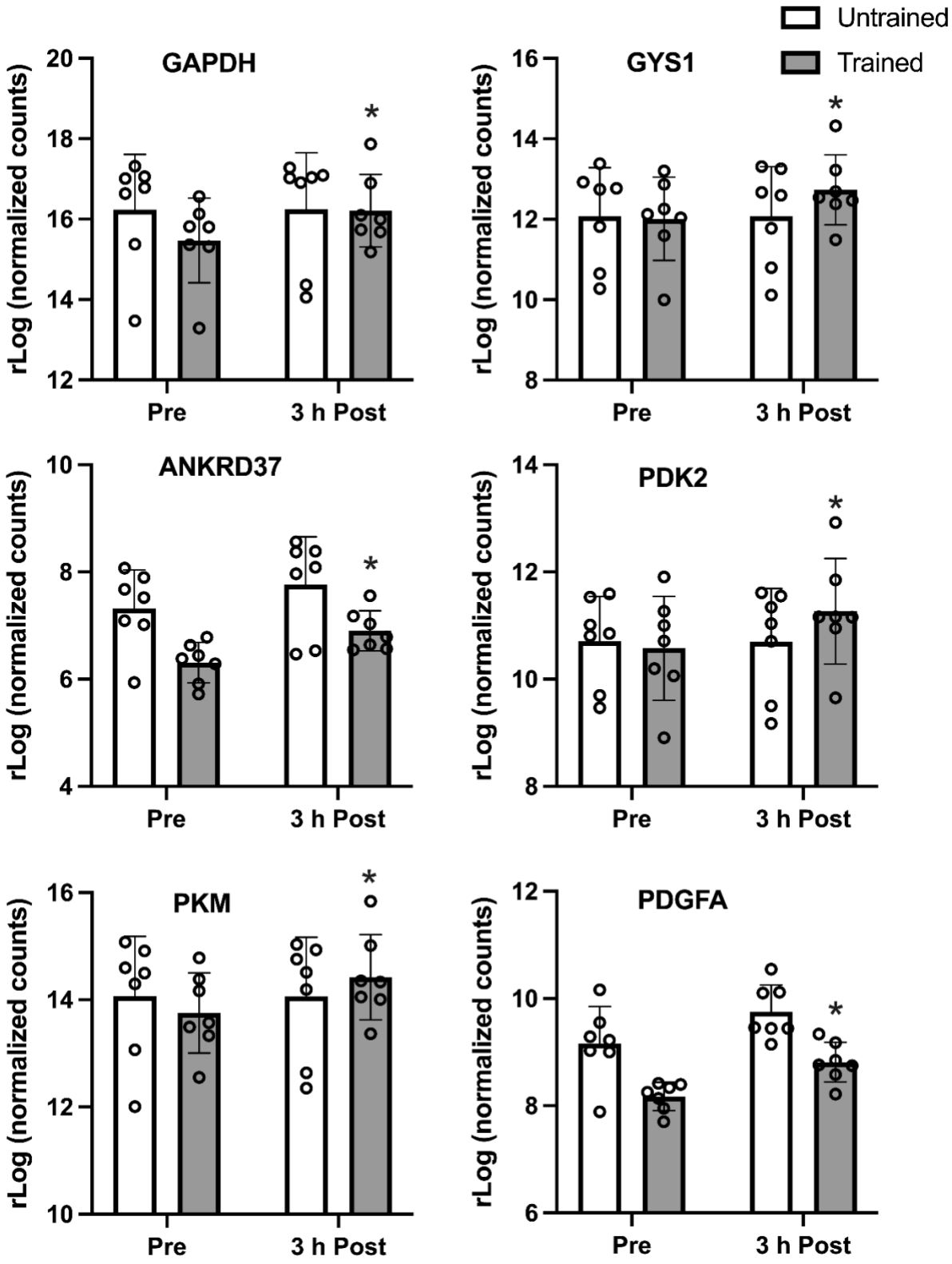

Figure 8. Normalized read counts for the top 6 most exercise-induced HIF-1 target genes in endurance trained (n = 7) individuals. Read counts for untrained individuals (n = 7) are

included for comparision. Gene counts were generated from next generation RNA-sequencing data. Data are shown as mean ± standard deviation. * indicates a significant difference between pre and 3 h post exercise within an individual group ($P < .05$).

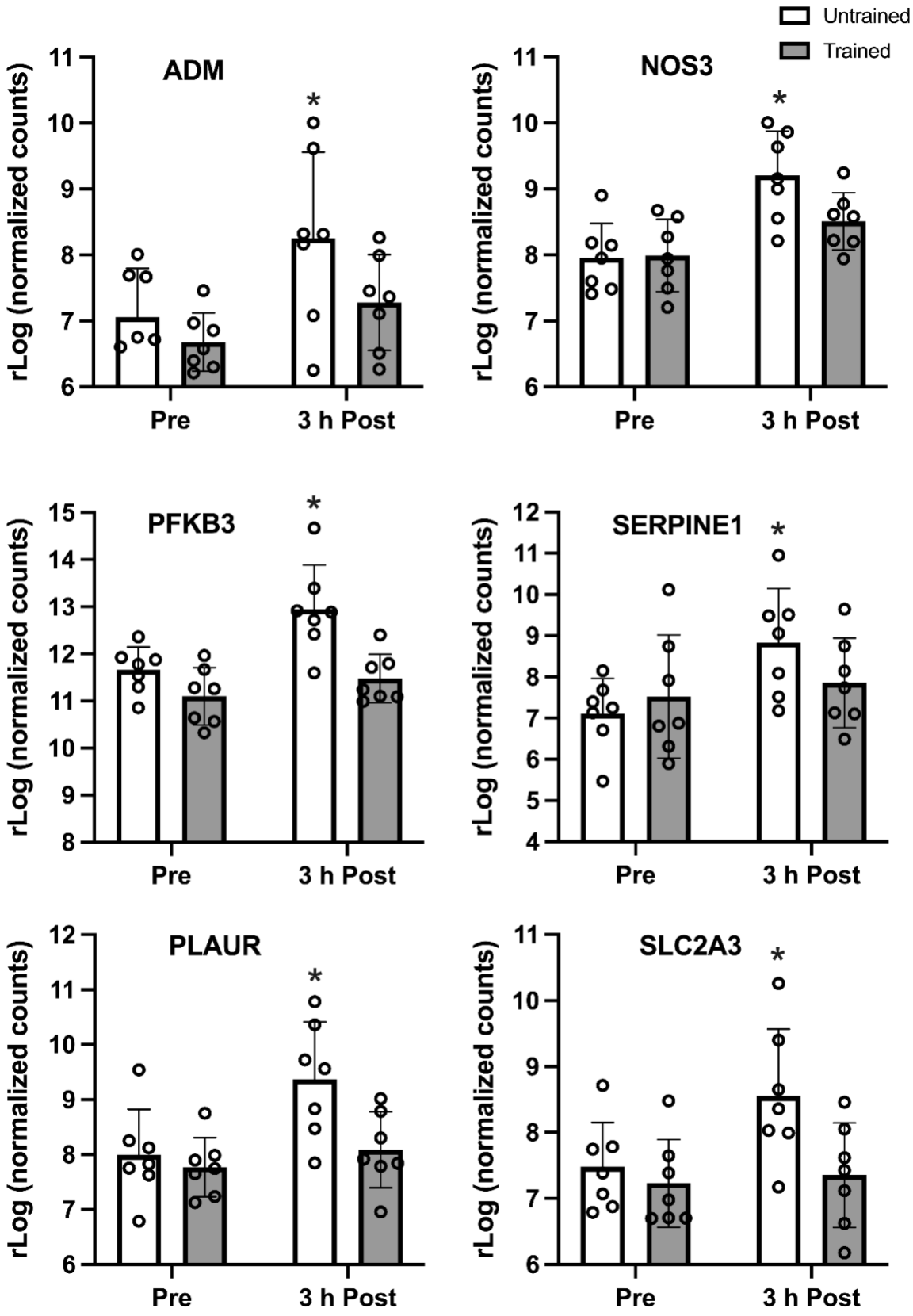
Untrained
Trained
ADM
NOS3
PFKB3
SERPINE1
PLAUR
SLC2A3
rLog (normalized counts)
Pre
3 h Post
*

Figure 9. Normalized read counts for the top 6 most exercise-induced HIF-1 target genes in untrained individuals (n = 7). Read counts for endurance trained individuals (n = 7) are included for comparision. Gene counts were generated from next generation RNA-sequencing data. Data are shown as mean ± standard deviation. * indicates a significant difference between pre and 3 h post exercise for a respective group (P < .05).

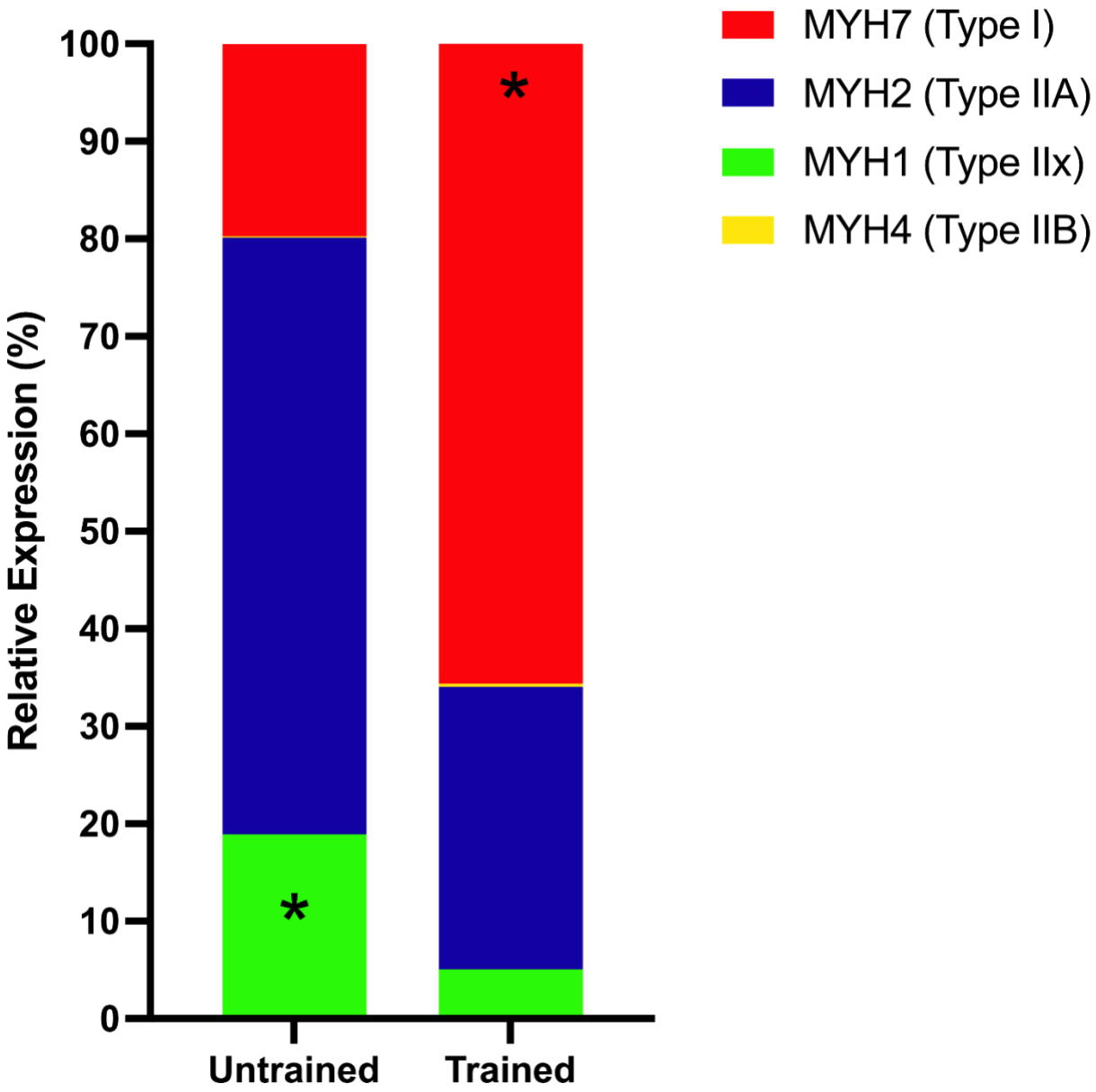

Figure 10. Comparison of the relative expression of myosin heavy chain (MYH) isoforms between endurance trained (n = 7) and untrained (n = 7) groups. The relative expression was calculated as the % of each isoform relative to the total number of MYH counts for an individual. * indicates significantly greater expression of that MYH isoform compared to the opposite group (P < .05).

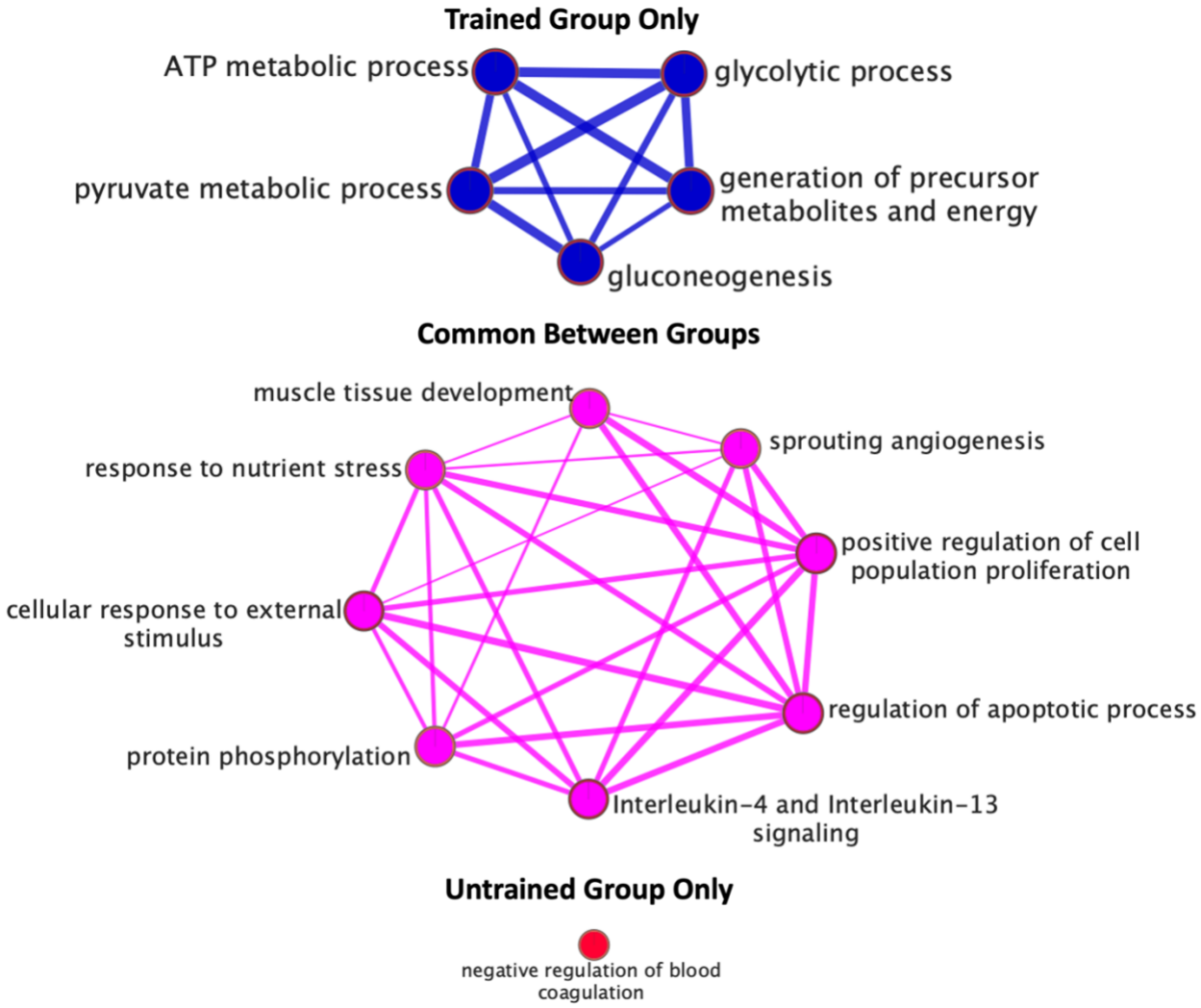

Figure 11. Enrichment networks of hypoxia inducible factor 1 (HIF-1) target genes determined to be differentially expressed between pre and 3 hours post exercise in muscle tissue samples collected from endurance trained (n = 7) and untrained (n = 7) individuals. The network common to both groups represents enriched pathways from genes that were differentially expressed after exercise in both trained and untrained groups. Pathways were determined to be enriched if the g:SCS-adjusted P value was < .05 (Raudvere et al. 2019).

CHAPTER IV: Summary, Conclusions and Recommendations

Summary

We were interested in the mechanisms by which skeletal muscle adapts to endurance exercise training. To this end, we aimed to investigate the role of skeletal muscle HIF-1 in the adaptation to endurance training. First, we reviewed the available studies that determined the effect of acute exercise on HIF-1 protein and gene expression. Highlighted in the review is the importance of exercise duration and intensity in the exercise-induced increase of HIF-1 in skeletal muscle. We then summarized the state of the literature regarding the role of HIF-1 in the adaptation to chronic endurance training. The previously proposed hypothesis was that endurance exercise training results in the inhibition of HIF-1, an adaptation that allows for improvements in oxidative capacity known to occur with training. We found several flaws with this perspective.

To examine this hypothesis, we performed an in-depth investigation to determine the influence of exercise training on the HIF-1 response to acute exercise in both endurance trained and untrained individuals. Because the skeletal muscle adaptations conferred by endurance training result from transient molecular changes following acute exercise, we tested whether the responsiveness of the HIF-1 pathway to a single bout of exercise was different in trained compared to untrained individuals. Using protein, rtPCR and next generation RNA sequencing data, we performed the most comprehensive study on the effect of acute exercise on HIF-1 signaling to date. While we confirmed that endurance athletes have higher levels of HIF-1 inhibitors, the exercise-induced change in HIF-1 protein expression was not different between groups. Furthermore, out of 572 HIF-1 target genes analyzed in pre and post exercise samples, the exercise-induced change was different in only one gene between trained and untrained groups.

Conclusions

Through several lines of evidence at both the gene and protein expression level, it was concluded that the responsiveness of the HIF-1 pathway is retained in the muscle of trained endurance athletes, despite a greater abundance of HIF-1 inhibitors. In addition, the large exercise-induced increase in the HIF-1 target gene, NR4A1, was highlighted because it was recently shown to underlie some beneficial metabolic adaptations to exercise. This finding suggests that the increase in HIF-1 activity following acute exercise upregulates many genes with diverse functions.

Recommendations

A limitation of this study was that the exercise intensity was not fixed to a single relative intensity across groups or individuals. This likely contributed to variability in the molecular responses to exercise, decreasing the power of our analyses. Researchers interested in the topic may choose to compare the HIF-1 response between untrained and trained groups after exercise of a standardized intensity.

BIBLIOGRAPHY

Ameln, H., Gustafsson, T., Sundberg, C. J., Okamoto, K., Jansson, E., Poellinger, L., & Makino, Y. (2005). Physiological activation of hypoxia inducible factor-1 in human skeletal muscle. *The FASEB Journal*, *19*(8), 1009–1011. https://doi.org/10.1096/fj.04-2304fje

American College of Sports Medicine. (2018). *ACSM's guidelines for exercise testing and prescription*. (D. Riebe, J. K. Ehrman, G. Liguori, & M. Magal, Eds.) (10th ed.). Philadelphia: Wolters Kluwer/Lippincott Williams & Wilkins Health.

Bassett, D. R., & Howley, E. T. (2000). Limiting factors for maximum oxygen uptake and determinants of endurance performance. *Medicine and Science in Sports and Exercise*, *32*(1), 70–84. https://doi.org/10.1097/00005768-200001000-00012

Beard, J. A., Tenga, A., & Chen, T. (2015). The interplay of NR4A receptors and the oncogene–tumor suppressor networks in cancer. *Cellular Signalling*, *27*(2), 257–266. https://doi.org/10.1016/j.cellsig.2014.11.009

Benita, Y., Kikuchi, H., Smith, A. D., Zhang, M. Q., Chung, D. C., & Xavier, R. J. (2009). An integrative genomics approach identifies Hypoxia Inducible Factor-1 (HIF-1)-target genes that form the core response to hypoxia. *Nucleic Acids Research*, *37*(14), 4587–4602. https://doi.org/10.1093/nar/gkp425

Berra, E. (2003). HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1 in normoxia. *The EMBO Journal*, *22*(16), 4082–4090. https://doi.org/10.1093/emboj/cdg392

Berra, E., Roux, D., Richard, D. E., & Pouysségur, J. (2001). Hypoxia-inducible factor-1α (HIF-1 escapes O2-driven proteasomal degradation irrespective of its subcellular localization: Nucleus or cytoplasm. *EMBO Reports*, *2*(7), 615–620. https://doi.org/10.1093/embo-reports/kve130

Blighe, K., Rana, S., & Lewis, M. (n.d.). EnhancedVolcano: Publication-ready volcano plots with enhanced colouring and labeling.

Brahimi-Horn, C., Mazure, N., & Pouysségur, J. (2005, January). Signalling via the hypoxia-inducible factor-1α requires multiple posttranslational modifications. *Cellular Signalling*. https://doi.org/10.1016/j.cellsig.2004.04.010

Brocherie, F., Millet, G. P., D'Hulst, G., Van Thienen, R., Deldicque, L., & Girard, O. (2018). Repeated maximal-intensity hypoxic exercise superimposed to hypoxic residence boosts skeletal muscle transcriptional responses in elite team-sport athletes. *Acta Physiologica*, *222*(1), e12851. https://doi.org/10.1111/apha.12851

Carbajo-Pescador, S., Ordoñez, R., Benet, M., Jover, R., García-Palomo, A., Mauriz, J. L., & González-Gallego, J. (2013). Inhibition of VEGF expression through blockade of Hif1α and STAT3 signalling mediates the anti-angiogenic effect of melatonin in HepG2 liver

cancer cells. *British Journal of Cancer*, *109*(1), 83–91. https://doi.org/10.1038/bjc.2013.285

Carbon, S., Douglass, E., Good, B. M., Unni, D. R., Harris, N. L., Mungall, C. J., … Elser, J. (2021). The Gene Ontology resource: Enriching a GOld mine. *Nucleic Acids Research*, *49*(D1), D325–D334. https://doi.org/10.1093/nar/gkaa1113

Chen, S., Zhou, Y., Chen, Y., & Gu, J. (2018). Fastp: An ultra-fast all-in-one FASTQ preprocessor. In *Bioinformatics* (Vol. 34, pp. i884–i890). Oxford University Press. https://doi.org/10.1093/bioinformatics/bty560

Coffey, V. G., Shield, A., Canny, B. J., Carey, K. A., Cameron-Smith, D., & Hawley, J. A. (2006). Interaction of contractile activity and training history on mRNA abundance in skeletal muscle from trained athletes. *American Journal of Physiology - Endocrinology and Metabolism*, *290*(5), E849–E855. https://doi.org/10.1152/ajpendo.00299.2005

Dayan, F., Monticelli, M., Pouysségur, J., & Pécou, E. (2009). Gene regulation in response to graded hypoxia: The non-redundant roles of the oxygen sensors and FIH in the HIF pathway. *Journal of Theoretical Biology*, *259*(2), 304–316. https://doi.org/10.1016/j.jtbi.2009.03.009

de Freitas, M. C., Gerosa-Neto, J., Zanchi, N. E., Lira, F. S., & Rossi, F. E. (2017). Role of metabolic stress for enhancing muscle adaptations: Practical applications. *World Journal of Methodology*, *7*(2), 46. https://doi.org/10.5662/wjm.v7.i2.46

De Smet, S., D'Hulst, G., Poffé, C., Van Thienen, R., Berardi, E., & Hespel, P. (2018). High-intensity interval training in hypoxia does not affect muscle HIF responses to acute hypoxia in humans. *European Journal of Applied Physiology*, *118*(4), 847–862. https://doi.org/10.1007/s00421-018-3820-4

Dempsey, J. A., & Wagner, P. D. (1999). Exercise-induced arterial hypoxemia. *Journal of Applied Physiology*, *87*(6), 1997–2006. https://doi.org/10.1152/jappl.1999.87.6.1997

Dobin, A., & Gingeras, T. R. (2015). Mapping RNA-seq Reads with STAR. *Current Protocols in Bioinformatics*, *51*(1). https://doi.org/10.1002/0471250953.bi1114s51

Drummond, M. J., Fujita, S., Takashi, A., Dreyer, H. C., Volpi, E., & Rasmussen, B. B. (2008). Human muscle gene expression following resistance exercise and blood flow restriction. *Medicine and Science in Sports and Exercise*, *40*(4), 691–698. https://doi.org/10.1249/MSS.0b013e318160ff84

Dunn, L. L., Kong, S. M. Y., Tumanov, S., Chen, W., Cantley, J., Ayer, A., … Stocker, R. (2020). Hmox1 (Heme Oxygenase-1) Protects Against Ischemia-Mediated Injury via Stabilization of HIF-1α (Hypoxia-Inducible Factor-1α). *Arteriosclerosis, Thrombosis, and Vascular Biology*. https://doi.org/10.1161/ATVBAHA.120.315393

Egan, B., Carson, B. P., Garcia-Roves, P. M., Chibalin, A. V., Sarsfield, F. M., Barron, N., … O'Gorman, D. J. (2010). Exercise intensity-dependent regulation of peroxisome

proliferator-activated receptor γ coactivator-1α mRNA abundance is associated with differential activation of upstream signalling kinases in human skeletal muscle. *The Journal of Physiology*, *588*(10), 1779–1790. https://doi.org/10.1113/jphysiol.2010.188011

Egan, B., & Zierath, J. R. (2013). Exercise Metabolism and the Molecular Regulation of Skeletal Muscle Adaptation. *Cell Metabolism*, *17*(2), 162–184. https://doi.org/10.1016/j.cmet.2012.12.012

Faul, F., Erdfelder, E., Lang, A. G., & Buchner, A. (2007). G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. In *Behavior Research Methods*. https://doi.org/10.3758/BF03193146

Feys, J. (2016). Nonparametric Tests for the Interaction in Two-way Factorial Designs Using R. *The R Journal*, *8*(1), 367. https://doi.org/10.32614/RJ-2016-027

Freyssenet, D. (2007, February). Energy sensing and regulation of gene expression in skeletal muscle. *Journal of Applied Physiology*. https://doi.org/10.1152/japplphysiol.01126.2005

Gallezot, P. (2012). Conversion of biomass to selected chemical products. *Chemical Society Reviews*, *41*(4), 1538–1558. https://doi.org/10.1039/c1cs15147a

Granata, C., Oliveira, R. S. F., Little, J. P., & Bishop, D. J. (2020). Forty high-intensity interval training sessions blunt exercise-induced changes in the nuclear protein content of PGC-1α and p53 in human skeletal muscle. *American Journal of Physiology-Endocrinology and Metabolism*, *318*(2), E224–E236. https://doi.org/10.1152/ajpendo.00233.2019

Gudiksen, A., Bertholdt, L., Stankiewicz, T., Tybirk, J., Plomgaard, P., Bangsbo, J., & Pilegaard, H. (2017). Effects of training status on PDH regulation in human skeletal muscle during exercise. *Pflügers Archiv - European Journal of Physiology*, *469*(12), 1615–1630. https://doi.org/10.1007/s00424-017-2019-6

Gustafsson, T., Knutsson, A., Puntschart, A., Kaijser, L., Nordqvist, S. A. C., Sundberg, C., & Jansson, E. (2002). Increased expression of vascular endothelial growth factor in human skeletal muscle in response to short-term one-legged exercise training. *Pflugers Archiv European Journal of Physiology*, *444*(6), 752–759. https://doi.org/10.1007/s00424-002-0845-6

Gustafsson, Thomas, Puntschart, A., Kaijser, L., Jansson, E., & Sundberg, C. J. (1999). Exercise-induced expression of angiogenesis-related transcription and growth factors in human skeletal muscle. *American Journal of Physiology - Heart and Circulatory Physiology*, *276*(2 45-2), H679–H685. https://doi.org/10.1152/ajpheart.1999.276.2.h679

Haan, C., & Behrmann, I. (2007). A cost effective non-commercial ECL-solution for Western blot detections yielding strong signals and low background. *Journal of Immunological Methods*, *318*(1–2), 11–19. https://doi.org/10.1016/j.jim.2006.07.027

Holloszy, J. O., & Coyle, E. F. (1984). Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. *Journal of Applied Physiology Respiratory Environmental and Exercise Physiology*. https://doi.org/10.1152/jappl.1984.56.4.831

Huss, J. M., Kopp, R. P., & Kelly, D. P. (2002). Peroxisome proliferator-activated receptor coactivator-1α (PGC-1α) coactivates the cardiac-enriched nuclear receptors estrogen-related receptor-α and -γ: Identification of novel Leucine-rich interaction motif within PGC-1α. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M206324200

Iliopoulos, O., Kibel, A., Gray, S., & Kaelin, W. G. (1995). Tumour suppression by the human von Hippel-Lindau gene product. *Nature Medicine*, *1*(8), 822–826. https://doi.org/10.1038/nm0895-822

Jassal, B., Matthews, L., Viteri, G., Gong, C., Lorente, P., Fabregat, A., … D'Eustachio, P. (2019). The reactome pathway knowledgebase. *Nucleic Acids Research*. https://doi.org/10.1093/nar/gkz1031

Kay, M., & Wobbrock, J. O. (2015). ARTool: Aligned Rank Transform for Nonparametric Factorial ANOVAs. *The R Journal*.

Ke, Q., & Costa, M. (2006). Hypoxia-Inducible Factor-1 (HIF-1). *Molecular Pharmacology*, *70*(5), 1469–1480. https://doi.org/10.1124/mol.106.027029

Kiens, B., Essen-Gustavsson, B., Christensen, N. J., & Saltin, B. (1993). Skeletal muscle substrate utilization during submaximal exercise in man: effect of endurance training. *The Journal of Physiology*, *469*(1), 459–478. https://doi.org/10.1113/jphysiol.1993.sp019823

Kim, J. W., Tchernyshyov, I., Semenza, G. L., & Dang, C. V. (2006). HIF-1-mediated expression of pyruvate dehydrogenase kinase: A metabolic switch required for cellular adaptation to hypoxia. *Cell Metabolism*. https://doi.org/10.1016/j.cmet.2006.02.002

Kopp, R., Köblitz, L., Egg, M., & Pelster, B. (2011). HIF signaling and overall gene expression changes during hypoxia and prolonged exercise differ considerably. *Physiological Genomics*, *43*(9), 506–516. https://doi.org/10.1152/physiolgenomics.00250.2010

Koslowski, M., Luxemburger, U., Türeci, Ö., & Sahin, U. (2011). Tumor-associated CpG demethylation augments hypoxia-induced effects by positive autoregulation of HIF-1α. *Oncogene*, *30*(7), 876–882. https://doi.org/10.1038/onc.2010.481

Kuang, J., Yan, X., Genders, A. J., Granata, C., & Bishop, D. J. (2018). An overview of technical considerations when using quantitative real-time PCR analysis of gene expression in human exercise research. *PLoS ONE*, *13*(5), e0196438. https://doi.org/10.1371/journal.pone.0196438

Ladner, C. L., Yang, J., Turner, R. J., & Edwards, R. A. (2004). Visible fluorescent detection of proteins in polyacrylamide gels without staining. *Analytical Biochemistry*, *326*(1),

13–20. https://doi.org/10.1016/j.ab.2003.10.047

Lindholm, M. E., Fischer, H., Poellinger, L., Johnson, R. S., Gustafsson, T., Sundberg, C. J., & Rundqvist, H. (2014a). Negative regulation of HIF in skeletal muscle of elite endurance athletes: a tentative mechanism promoting oxidative metabolism. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *307*(3), R248–R255. https://doi.org/10.1152/ajpregu.00036.2013

Lindholm, M. E., Fischer, H., Poellinger, L., Johnson, R. S., Gustafsson, T., Sundberg, C. J., & Rundqvist, H. (2014b). Negative regulation of HIF in skeletal muscle of elite endurance athletes: A tentative mechanism promoting oxidative metabolism. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, *307*(3), R248–R255. https://doi.org/10.1152/ajpregu.00036.2013

Lindholm, Maléne E., Giacomello, S., Werne Solnestam, B., Fischer, H., Huss, M., Kjellqvist, S., & Sundberg, C. J. (2016). The Impact of Endurance Training on Human Skeletal Muscle Memory, Global Isoform Expression and Novel Transcripts. *PLoS Genetics*. https://doi.org/10.1371/journal.pgen.1006294

Lindholm, Malene E., & Rundqvist, H. (2016). Skeletal muscle hypoxia-inducible factor-1 and exercise. *Experimental Physiology*, *101*(1), 28–32. https://doi.org/10.1113/EP085318

Liu, Y., Beyer, A., & Aebersold, R. (2016, April). On the Dependency of Cellular Protein Levels on mRNA Abundance. *Cell*. https://doi.org/10.1016/j.cell.2016.03.014

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*. https://doi.org/10.1186/s13059-014-0550-8

Lundby, C., Gassmann, M., & Pilegaard, H. (2006). Regular endurance training reduces the exercise induced HIF-1α and HIF-2α mRNA expression in human skeletal muscle in normoxic conditions. *European Journal of Applied Physiology*, *96*(4), 363–369. https://doi.org/10.1007/s00421-005-0085-5

Magalhães, F. de C., Aguiar, P. F., Tossige-Gomes, R., Magalhães, S. M., Ottone, V. de O., Fernandes, T., … Amorim, F. T. (2020). High-intensity interval training followed by postexercise cold-water immersion does not alter angiogenic circulating cells, but increases circulating endothelial cells. *Applied Physiology, Nutrition and Metabolism*, *45*(1), 101–111. https://doi.org/10.1139/apnm-2019-0041

Mason, S. D., Howlett, R. A., Kim, M. J., Olfert, I. M., Hogan, M. C., McNulty, W., … Johnson, R. S. (2004). Loss of skeletal muscle HIF-1α results in altered exercise endurance. *PLoS Biology*, *2*(10), e288. https://doi.org/10.1371/journal.pbio.0020288

Mason, S. D., Rundqvist, H., Papandreou, I., Duh, R., McNulty, W. J., Howlett, R. A., … Johnson, R. S. (2007a). HIF-1α in endurance training: Suppression of oxidative metabolism. *American Journal of Physiology - Regulatory Integrative and Comparative*

Physiology, *293*(5), R2059–R2069. https://doi.org/10.1152/ajpregu.00335.2007

Mason, S. D., Rundqvist, H., Papandreou, I., Duh, R., McNulty, W. J., Howlett, R. A., … Johnson, R. S. (2007b). HIF-1α in endurance training: Suppression of oxidative metabolism. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, *293*(5), R2059–R2069. https://doi.org/10.1152/ajpregu.00335.2007

Masson, N., & Ratcliffe, P. J. (2003, August 1). HIF prolyl and asparaginyl hydroxylases in the biological response to intracellular O2 levels. *Journal of Cell Science*. https://doi.org/10.1242/jcs.00655

Maxwell, P. H., Wiesener, M. S., Chang, G.-W., Clifford, S. C., Vaux, E. C., Cockman, M. E., … Ratcliffe, P. J. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature*, *399*(6733), 271–275. https://doi.org/10.1038/20459

McDonough, A. A., Veiras, L. C., Minas, J. N., & Ralph, D. L. (2015). Considerations when quantitating protein abundance by immunoblot. *American Journal of Physiology - Cell Physiology*, *308*(6), C426–C433. https://doi.org/10.1152/ajpcell.00400.2014

McManus, C. J., Collison, J., & Cooper, C. E. (2018). Performance comparison of the MOXY and PortaMon near-infrared spectroscopy muscle oximeters at rest and during exercise. *Journal of Biomedical Optics*, *23*(01), 1. https://doi.org/10.1117/1.JBO.23.1.015007

Merico, D., Isserlin, R., Stueker, O., Emili, A., & Bader, G. D. (2010). Enrichment map: A network-based method for gene-set enrichment visualization and interpretation. *PLoS ONE*, *5*(11), e13984. https://doi.org/10.1371/journal.pone.0013984

Mol??, P. A., Chung, Y., Tran, T. K., Sailasuta, N., Hurd, R., & Jue, T. (1999). Myoglobin Desaturation with Exercise Intensity in Human Gastrocnemius Muscle. *Medicine & Science in Sports & Exercise*, *31*(Supplement), S275. https://doi.org/10.1097/00005768-199905001-01341

Mounier, R., Pedersen, B. K., & Plomgaard, P. (2010). Muscle-specific expression of hypoxia-inducible factor in human skeletal muscle. *Experimental Physiology*, *95*(8), 899–907. https://doi.org/10.1113/expphysiol.2010.052928

Murakami, T., Shimomura, Y., Yoshimura, A., Sokabe, M., & Fujitsuka, N. (1998). Induction of nuclear respiratory factor-1 expression by an acute bout of exercise in rat muscle. *Biochimica et Biophysica Acta - General Subjects*, *1381*(1), 113–122. https://doi.org/10.1016/S0304-4165(98)00018-X

Nguyen, L. K., Cavadas, M. A. S., Scholz, C. C., Fitzpatrick, S. F., Bruning, U., Cummins, E. P., … Cheong, A. (2013). A dynamic model of the hypoxia-inducible factor 1 (HIF-1) network. *Journal of Cell Science*, *126*(6), 1454–1463. https://doi.org/10.1242/jcs.119974

Nunomiya, A., Shin, J., Kitajima, Y., Dan, T., Miyata, T., & Nagatomi, R. (2017). Activation of the hypoxia-inducible factor pathway induced by prolyl hydroxylase domain 2 deficiency enhances the effect of running training in mice. *Acta Physiologica*, *220*(1), 99–112. https://doi.org/10.1111/apha.12751

O'Gorman, D. J., Karlsson, H. K. R., McQuaid, S., Yousif, O., Rahman, Y., Gasparro, D., … Nolan, J. J. (2006). Exercise training increases insulin-stimulated glucose disposal and GLUT4 (SLC2A4) protein content in patients with type 2 diabetes. *Diabetologia*, *49*(12), 2983–2992. https://doi.org/10.1007/s00125-006-0457-3

O'Hagan, K. A., Cocchiglia, S., Zhdanov, A. V., Tambuwala, M. M., Cummins, E. P., Monfared, M., … Allan, B. B. (2009). PGC-1 is coupled to HIF-1 -dependent gene expression by increasing mitochondrial oxygen consumption in skeletal muscle cells. *Proceedings of the National Academy of Sciences*, *106*(7), 2188–2193. https://doi.org/10.1073/pnas.0808801106

Olfert, I. M., Howlett, R. A., Wagner, P. D., & Breen, E. C. (2010). Myocyte vascular endothelial growth factor is required for exercise-induced skeletal muscle angiogenesis. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *299*(4), R1059–R1067. https://doi.org/10.1152/ajpregu.00347.2010

Pearen, M. A., Goode, J. M., Fitzsimmons, R. L., Eriksson, N. A., Thomas, G. P., Cowin, G. J., … Muscat, G. E. O. (2013). Transgenic muscle-specific Nor-1 expression regulates multiple pathways that effect adiposity, metabolism, and endurance. *Molecular Endocrinology*. https://doi.org/10.1210/me.2013-1205

Perry, C. G. R., Lally, J., Holloway, G. P., Heigenhauser, G. J. F., Bonen, A., & Spriet, L. L. (2010). Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. *The Journal of Physiology*, *588*(23), 4795–4810. https://doi.org/10.1113/jphysiol.2010.199448

Pillon, N. J., Gabriel, B. M., Dollet, L., Smith, J. A. B., Sardón Puig, L., Botella, J., … Zierath, J. R. (2020). Transcriptomic profiling of skeletal muscle adaptations to exercise and inactivity. *Nature Communications*. https://doi.org/10.1038/s41467-019-13869-w

Prior, B. M., Yang, H. T., & Terjung, R. L. (2004). What makes vessels grow with exercise training? *Journal of Applied Physiology*, *97*(3), 1119–1128. https://doi.org/10.1152/japplphysiol.00035.2004

Raudvere, U., Kolberg, L., Kuzmin, I., Arak, T., Adler, P., Peterson, H., & Vilo, J. (2019). G:Profiler: A web server for functional enrichment analysis and conversions of gene lists (2019 update). *Nucleic Acids Research*, *47*(W1), W191–W198. https://doi.org/10.1093/nar/gkz369

Richardson, Russell S., Noyszewski, E. A., Kendrick, K. F., Leigh, J. S., & Wagner, P. D. (1995). Myoglobin O2 desaturation during exercise. Evidence of limited O2 transport. *Journal of Clinical Investigation*, *96*(4), 1916–1926. https://doi.org/10.1172/JCI118237

Richardson, R S, Wagner, H., Mudaliar, S. R. D., Henry, R., Noyszewski, E. A., & Wagner, P. D. (1999). Human VEGF gene expression in skeletal muscle: Effect of acute normoxic and hypoxic exercise. *American Journal of Physiology - Heart and Circulatory Physiology*, *277*(6 46-6), H2247–H2252. https://doi.org/10.1152/ajpheart.1999.277.6.h2247

Richardson, Russell S., Duteil, S., Wary, C., Wray, D. W., Hoff, J., & Carlier, P. G. (2006). Human skeletal muscle intracellular oxygenation: the impact of ambient oxygen availability. *The Journal of Physiology*, *571*(2), 415–424. https://doi.org/10.1113/jphysiol.2005.102327

Rivero-Gutiérrez, B., Anzola, A., Martínez-Augustin, O., & De Medina, F. S. (2014). Stain-free detection as loading control alternative to Ponceau and housekeeping protein immunodetection in Western blotting. *Analytical Biochemistry*, *467*(15), 1–3. https://doi.org/10.1016/j.ab.2014.08.027

Robergs, R. A., Dwyer, D., & Astorino, T. (2010). Recommendations for improved data processing from expired gas analysis indirect calorimetry. *Sports Medicine*, *40*(2), 95–111. https://doi.org/10.2165/11319670-000000000-00000

Ropka-Molik, K., Stefaniuk-Szmukier, M., Z˙ukowski, K., Piórkowska, K., & Bugno-Poniewierska, M. (2017). Exercise-induced modification of the skeletal muscle transcriptome in Arabian horses. *Physiological Genomics*, *49*(6), 318–326. https://doi.org/10.1152/physiolgenomics.00130.2016

Rstudio, T. (2020). RStudio: Integrated Development for R. *Rstudio Team, PBC, Boston, MA URL Http://Www.Rstudio.Com/*. https://doi.org/10.1145/3132847.3132886

Schwandt, H. J., Heyduck, B., Gunga, H. C., & Rocker, L. (1991). Applied Physiology on the erythropoietin concentration in blood. *European Journal of Applied Physiology*, (63), 463–466.

Semenza, G. L. (2000). Hif-1 and human disease: One highly involved factor. *Genes and Development*. https://doi.org/10.1101/gad.14.16.1983

Semenza, Gregg L. (2004, August). Hydroxylation of HIF-1: Oxygen sensing at the molecular level. *Physiology*. https://doi.org/10.1152/physiol.00001.2004

Semenza, Gregg L. (2007a). HIF-1 mediates the Warburg effect in clear cell renal carcinoma. *Journal of Bioenergetics and Biomembranes*, *39*(3), 231–234. https://doi.org/10.1007/s10863-007-9081-2

Semenza, Gregg L. (2007b). Life with Oxygen. *Science*, *318*(5847), 62–64. https://doi.org/10.1126/science.1147949

Semenza, Gregg L. (1999). Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. *Annual Review of Cell and Developmental Biology*. https://doi.org/10.1146/annurev.cellbio.15.1.551

Shin, J., Nunomiya, A., Kitajima, Y., Dan, T., Miyata, T., & Nagatomi, R. (2016). Prolyl hydroxylase domain 2 deficiency promotes skeletal muscle fiber-type transition via a calcineurin/NFATc1-dependent pathway. *Skeletal Muscle*, *6*(1). https://doi.org/10.1186/s13395-016-0079-5

Slivka, D. R., Heesch, M. W. S., Dumke, C. L., Cuddy, J. S., Hailes, W. S., & Ruby, B. C. (2014). Human Skeletal Muscle mRNA Response to a Single Hypoxic Exercise Bout. *Wilderness and Environmental Medicine*, *25*(4), 462–465. https://doi.org/10.1016/j.wem.2014.06.011

Slot, I. G. M., Van Den Borst, B., Hellwig, V. A. C. V., Barreiro, E., Schols, A. M. W. J., & Gosker, H. R. (2014). The muscle oxidative regulatory response to acute exercise is not impaired in less advanced COPD despite a decreased oxidative phenotype. *PLoS ONE*, *9*(2), e90150. https://doi.org/10.1371/journal.pone.0090150

Srinivasan, S., & Dunn, J. F. (2011). Stabilization of hypoxia-inducible factor-1α in buffer containing cobalt chloride for Western blot analysis. *Analytical Biochemistry*, *416*(1), 120–122. https://doi.org/10.1016/j.ab.2011.04.037

Steinbacher, P., & Eckl, P. (2015). Impact of Oxidative Stress on Exercising Skeletal Muscle. *Biomolecules*, *5*(2), 356–377. https://doi.org/10.3390/biom5020356

Tabira, K., Horie, J., Fujii, H., Aida, T., Ito, K., Fukumoto, T., … Ishihara, H. (2012). The Relationship Between Skeletal Muscle Oxygenation and Systemic Oxygen Uptake During Exercise in Subjects With COPD: A Preliminary Study. *Respiratory Care*, *57*(10), 1602–1610. https://doi.org/10.4187/respcare.01602

Tang, K., Breen, E. C., Wagner, H., Brutsaert, T. D., Gassmann, M., & Wagner, P. D. (2004). HIF and VEGF relationships in response to hypoxia and sciatic nerve stimulation in rat gastrocnemius. *Respiratory Physiology and Neurobiology*, *144*(1), 71–80. https://doi.org/10.1016/j.resp.2004.04.009

Toledo-Arruda, A. C., Sousa Neto, I. V. de, Vieira, R. P., Guarnier, F. A., Caleman-Neto, A., Suehiro, C. L., … Martins, M. A. (2020). Aerobic exercise training attenuates detrimental effects of cigarette smoke exposure on peripheral muscle through stimulation of the Nrf2 pathway and cytokines: a time-course study in mice. *Applied Physiology, Nutrition, and Metabolism*, *45*(9), 978–986. https://doi.org/10.1139/apnm-2019-0543

Van Thienen, R., Masschelein, E., D'Hulst, G., Thomis, M., & Hespel, P. (2017). Twin resemblance in muscle HIF-1α responses to hypoxia and exercise. *Frontiers in Physiology*, *7*(JAN), 1–11. https://doi.org/10.3389/fphys.2016.00676

Vega, R. B., Huss, J. M., & Kelly, D. P. (2000). The Coactivator PGC-1 Cooperates with Peroxisome Proliferator-Activated Receptor alpha in Transcriptional Control of Nuclear Genes Encoding Mitochondrial Fatty Acid Oxidation Enzymes. *Molecular and Cellular Biology*. https://doi.org/10.1128/MCB.20.5.1868-1876.2000

Vogt, M., Puntschart, A., Geiser, J., Zuleger, C., Billeter, R., & Hoppeler, H. (2001). Molecular adaptations in human skeletal muscle to endurance training under simulated hypoxic conditions. *Journal of Applied Physiology*, *91*(1), 173–182. https://doi.org/10.1152/jappl.2001.91.1.173

Vogt, Michael, & Hoppeler, H. (2010, May). Is hypoxia training good for muscles and exercise performance? *Progress in Cardiovascular Diseases*. Elsevier Inc. https://doi.org/10.1016/j.pcad.2010.02.013

West, D. W. D., Doering, T. M., Thompson, J. M., Budiono, B. P., Lessard, S. J., Koch, L. G., … Coffey, V. G. (2021). Low responders to endurance training exhibit impaired hypertrophy and divergent biological process responses in rat skeletal muscle. *Experimental Physiology*, *106*(3), 714–725. https://doi.org/10.1113/EP089301

Wiener, C. M., Booth, G., & Semenza, G. L. (1996). In vivo expression of mRNAs encoding hypoxia-inducible factor 1. *Biochemical and Biophysical Research Communications*, *225*(2), 485–488. https://doi.org/10.1006/bbrc.1996.1199

Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., … Spiegelman, B. M. (1999). Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell*. https://doi.org/10.1016/S0092-8674(00)80611-X

Yu, A. Y., Frid, M. G., Shimoda, L. A., Wiener, C. M., Stenmark, K., Semenza, G. L., … Se-, G. L. (2018). Temporal , spatial , and oxygen-regulated expression of hypoxia-inducible factor-1 in the lung. *American Journal of Physiology*, (26).

APPENDICES

Appendix A: Informed Consent

A. Informed Consent

Comparison of high intensity interval exercise in normoxia and hypoxia on oxygen sensing and downstream gene regulation
Consent to Participate in Research
Version date: 02/10/21

Purpose of the study: You are being asked to participate in a research study that is being done by Christine Mermier, Ph.D., who is the Principal Investigator, and Roberto Nava from the Department of Health, Exercise & Sport Sciences and their associates. The purpose of this study is to investigate the effect of exercise intensity on the levels of a protein called hypoxia inducible factor-1α (HIF-1α). HIF-1α is important in the regulation of oxygen in the body. We are interested to see of the effect of high intensity interval exercise performed at Albuquerque's altitude or a simulated altitude of approximately 4,700m (~15,000 ft). Though the word "gene" is in the title, will **not** be doing any genetic (DNA) testing related to your health.

You are being asked to participate in this study because you have lived in the Albuquerque area for at least six months, are a healthy (no heart or lung disease or disorders such as diabetes, kidney disease and other diagnosed medical conditions, and no current bone or joint injury) individual between the ages of 18 – 45 years old. We are looking for a group of participants who have **not** engaged in regular aerobic activity (> 150 minutes of moderate to vigorous intensity aerobic activity per week for a minimum of 1 year) and a group of participants who **have** been training regularly doing aerobic exercise for at least one year. You must also not have a bleeding disorder or blood clotting problem or be allergic to Lidocaine. If you have a ruptured ear drum, it will be too uncomfortable for you to perform some of the procedures for the study. Lastly, if you know or think you may be pregnant, you will not be able to participate. Up to 20 people will be recruited for this study at the University of New Mexico.

This form will explain what to expect when joining the research, as well as the possible risks and benefits of participation. If you have any questions at any time during or after reviewing this form, please ask one of the study researchers.

Key information for you to consider:

- We are studying high intensity exercise at high altitude & Albuquerque's altitude
- The study requires 3 bouts of exercise, 6 muscle biopsies and 3 visits of 1-1.5 hours each
- High intensity exercise may cause muscle soreness, fatigue, nausea, or dizziness
- Muscle biopsies may cause you some pain and carries the risk of infection
- Participation in the project will take a total of 3-4 hours over 3 trials across 3 weeks.
- Session 1 will take up to 1 hour and sessions 2 and 3 require up to 1.5 hours of your time for each session

What you will do in the study:

After reading the consent form and discussing the details with the research team, you will be asked to sign this consent form if you decide to participate in the study and before any study activities take place.

You will then complete a Health and Physical Activity Questionnaire to assess your current health status and training history.

You will be required to visit the Exercise Physiology Laboratory once and the altitude chamber located in Carlisle gym twice, for a total of three visits. We will request that you wear exercise attire for the exercise bouts at each visit. You will be performing high intensity exercise, and this study requires you to have allow us to collect 6 muscle samples over the course of the study.

Upon arrival for the first visit, we will measure your resting blood pressure which will involve you sitting upright while a blood pressure cuff is wrapped around your upper arm. A stethoscope will be placed against your arm and the cuff which will be inflated and then deflated while systolic (upper number) and diastolic (lower number) blood pressure is measured. If your resting blood is over 140 (systolic) or 90 (diastolic), you will not be able to participate in the study. Women who are of child bearing age/potential will asked to take a urine pregnancy test. If the test shows that you could be pregnant, you will not be able to participate in the study and you will be referred to your health care provider for follow-up.

You will also have your body composition (body fat) estimated, using the skinfold caliper technique. This will require a slight pinching of the skin at three sites of the body (chest, abdomen and thigh for men and triceps, hip and thigh for women), done two-three times at each site.

VO_2max Testing- Visit 1

You will have refrained from alcohol for 24 hours, exercise for 24 hours, and caffeine for 4 hours.

During the first visit, you will perform a maximal graded (meaning it starts out easy and gets gradually harder) exercise tests on a stationary bike at Albuquerque's altitude. During each of these tests, the resistance you pedal against will gradually increase until you cannot continue exercising, cannot maintain at least 60 revolutions per minute (rpm), or if the researchers determine it is not safe for you to continue. This test assesses the maximum ability of the body to use oxygen to support physical activity (VO_{2max}) which is measured by a gas analyzer by collecting expired air from your lungs. This will require you to wear a mouthpiece that feels like a swimming snorkel and a nose-clamp during the test. Your heart rate and the amount of oxygen in your blood will be measured throughout the test. The heart

rate monitor will require you to wear a strap around your chest and measuring your oxygen will require a small instrument placed at the end of one of your fingers. The exercise tests will take approximately 8 – 12 minutes each.. The time required of you for the first visit will be approximately one hour,

You will be given a food log and asked to track your diet in the 24 hours leading up to your second visit so that you can mimic that diet prior to your third visit.

Exercise Trials- Visits 2 and 3

You will be assigned to perform one of the following high intensity cycling bouts first:

High intensity interval exercise in at Albuquerque's altitude

High intensity interval exercise at high altitude (~15,000 ft)

You will complete both exercise conditions in a randomized order, meaning the order will be determined by flipping a coin. These trials will take up to 1.5 hours each and will be separated by at least 7 days. You will be asked to arrive to the laboratory after refraining from exercising for 72 hours, drinking alcohol for 24 hours and caffeine for 4 hours. Also, we ask that you mimic your diet recorded prior to the 2nd visit on the 3rd visit.

It is important to tell the researchers if you have a nasal congestion, such as from a head cold or allergies if you are scheduled to exercise in the altitude chamber. It will be uncomfortable for you to go into the chamber where the pressure will change. We will reschedule the exercise trial when your congestion clears up.

The high intensity cycling exercise will consist of a 5-minute warm-up followed by 4 sets of 5, 10-second 'all out' sprints with 30-second rest in between sprints and 5-minute rests in between sets. All bouts of exercise will be concluded with a five-minute cool-down while the altitude chamber is going "down".

You will wear a heart rate monitor around your chest during all exercise bouts. During the high-altitude trials, you will also have a small monitor on one finger that measures the amount of oxygen in your blood.

Biopsy: Small skeletal muscle tissue samples will be collected via needle biopsy taken from your upper leg/thigh area before, immediately after and 3 hours after each exercise bout. We require two insertions of the needle in the same location, one right after the other for each biopsy session to obtain adequate muscle tissue for our experiments. You will be allowed to leave following the second biopsy and asked to return 3 hours after the end of the exercise bout for the third biopsy. There will be a total of six biopsies required over the course of the study (~3 weeks). You will be placed on an examination table lying down on your back (supine) so that the muscles of the leg are relaxed. The skin will be cleaned and prepared after which a surgical cover will be placed around the sampling site. A local numbing agent (Lidocaine) will be injected into the tissue under the skin around the site to be sampled. You may experience a slight pinching sensation while the Lidocaine is injected. If you are allergic to the numbing medicine, Lidocaine, you will be disqualified from the study. Once the area has been completely numbed, a small hole will then be made in the skin overlying the muscle using a pilot needle (16-gauge) and a slightly larger (14-gauge) biopsy needle will be inserted into the hole. The biopsy needle will be inserted twice into this hole to obtain two individual samples. There may be some minimal bleeding. If you have a bleeding disorder or blood clotting problem, you will inform the study personnel and not participate in the study. Pressure will be applied to the site immediately after the biopsy. Sterile disposable instruments will be used for the preparation of the site and tissue sampling. In total, approximately 20 mg (size of a 2 sesame seeds) of skeletal muscle tissue will be removed

with each biopsy. You may feel a brief sensation of pressure in the leg and potentially some moderate pain. This pain will quickly go away, and you will likely be able to perform exercise and normal daily activities without a problem. This technique is minimally invasive; however, it is common for participants to feel a sense of tightness and potentially may feel a sensation of a deep bruise or "Charlie Horse" at the biopsy site, although this should get better within 2 days and you may begin exercising immediately. There may be a possibility of a small scar forming. There have been no major complications reported as a result of taking small tissue samples from the skeletal muscle. Following the biopsy, the opening will be cleaned, treated with a sterile dressing, and a wrapped in a bandage to minimize the possibility of a blood related infection. You will also be given written instructions for the proper care of the biopsy site.
Participation in this study will take a total of 4 hours over a period of 3 laboratory visits (~3 weeks).

Your consent is voluntary and may be withdrawn at any time. At the time of withdrawal, your data will be deleted. Your personal data collected for this research will be deleted or anonymized by the end of your participation in the study. Projected future use of your muscle tissue samples includes measuring other proteins related to exercise.

OPTIONAL: The UNM Exercise Physiology Laboratory personnel conducts studies that may include tests and procedures that are involved in this study. Data from this study may be useable in other studies. Your data will be de-identified (your name, date of birth or any other identifiable information will not be associated with any data) and contain only variables such as sex, age, VO_{2max}, body composition, etc. If you are willing to allow the results from this study to be used in other future studies, please initial below. By initialing, you are not required to perform any additional tasks, tests, or paperwork. The researchers will only use your data.
I agree to allow my data to be used in other studies _____
Initials

Risks:
As long as the New Mexico State of Public Health Emergency declared in Executive Order 2020-004 remains in effect, this study will use procedures intended to reduce the risk of transmitting COVID-19. When we schedule your visit and again when you arrive at Johnson Center, we will ask you questions about COVID-19 symptoms. We will also check your temperature using a touchless thermometer. If you have a temperature above 100°F or have any COVID-19 symptoms we will reschedule your visit for another day.
By participating in this study, you are saying that as far as you know that you do not have any current symptoms of COVID-19, including: fever or chills; mild or moderate difficulty breathing; new or worsening cough; sustained loss of smell, taste, or appetite; sore throat; vomiting or diarrhea; aching throughout your body. If you have any of these, you must let us know, and we will delay this study until your symptoms are gone and you feel better. We will test your temperature using a remote thermometer. In addition, if you develop any of these symptoms within two weeks of participating in this study, you should contact us to let

us know. You will be contacted if a research team member you interacted with develops COVID-19.
Any friends or family members who come with you to a visit must wait outside of the building. Use of masks is required; if you do not have a mask or if your mask is not sufficient, one will be provided for you. A social distancing limit of 6' will be followed whenever possible except when necessary for data collection, for example when performing the skinfold measurement, blood draws, muscle temperature readings, and muscle biopsies. We will use additional personal protective equipment like face shields or Plexiglas barriers when we need to remain within 6' of you. Please contact Dr. Mermier immediately (cmermier@unm.edu/ (505) 715-0455) if you test positive for COVID-19 or develop symptoms of COVID-19 within 14 days of your visit to Johnson Center
There are risks associated with maximal exercise testing including the following: muscle soreness, fatigue, nausea, or dizziness during or after completion of exercise. The incidence of risk of fatal and nonfatal events during maximal exercise testing are very low, approximately <0.8 per 10,000 tests or 1 per 10,000 hours of testing. We will minimize these risks by checking your medical history questionnaire for any medical conditions or history that could increase your risk, and by using trained personnel to conduct your testing.
The risks associated with a muscle biopsy include momentary discomfort or moderate pain during the time the needle is inserted. To minimize the occurrence of discomfort and pain, an effective numbing agent (Lidocaine) will be used to numb the area to sampled. You will likely experience a brief and small pinching sensation while the numbing agent (Lidocaine) is injected. A minimal amount of muscle tissue (10 mg) will be extracted from your leg. You may feel a brief sensation of pressure in the leg and potentially some moderate pain during the tissue sampling. This pain will quickly get better, and you will likely be able to perform exercise and normal daily activities without problem. There is a risk that you may feel a sense of dizziness or feeling faint. Your leg may feel tight and you may feel a sensation of a deep bruise or "Charlie Horse" afterwards; however, this tightness in the muscle typically gets better within 2 days and you may begin exercising immediately, and routinely begin exercising at normal capacity within 2 days. There is also a risk of the possible appearance of a scar, bruising or soreness, and infection. To limit the potential risks, only trained technicians using sterilized instruments will perform the biopsy procedure. Additionally, the sampling site will be sterilized prior to the procedure.
Exercising at a higher altitude may make you feel sick to your stomach, have lightheadedness, headache or dizziness. Other risks of exercising at high altitude include feeling tired, shortness of breath and rapid heart rate. During each high-altitude trial we will be monitoring you for any of these problems. Generally, these symptoms do not develop in healthy people until at least 6-hr after ascent, even during heavy exercise. You will only be at higher altitude for approximately one hour, therefore, we do not foresee the development of these symptoms. Additionally, if you do not feel well, we will immediately lower the altitude chamber to Albuquerque's altitude which should relieve any symptoms you are having. Our safety procedures require us to re-pressurize (go down in altitude) and de-pressurize (go up) at no more than 1000 ft/min. Therefore, in the unlikely event that we would need to bring it down (pressurize) faster in the case of an emergency, there is a small chance of eardrum rupture or injury.
There are risks of stress, emotional distress, inconvenience and possible loss of privacy and confidentiality associated with participating in a research study.

Benefits: We will provide you with the results of your body composition assessment and maximal exercise tests. Knowledge of body composition and VO_{2max} are of benefit in that they are indicative of health and aerobic fitness which can be helpful in directing an individual exercise program. Additionally, it is hoped that the information gained from this study will help us understand the underlying mechanisms of exercise adaptation at high altitude.

Confidentiality of your information: Privacy will be maintained by conducting screening and testing in
private rooms in the Johnson Center Exercise Physiology lab and altitude chamber with no access to anyone but the study team. You will be given a random number for confidentiality of your data. Only approved research team members will have access to your information through a password protected computer, with hard copies stored in a locked file cabinet. We will take measures to protect the security of all your personal information, but we cannot guarantee confidentiality of your data. The University of New Mexico Institutional Review Board (IRB) that oversees human subject research may be permitted to access your records. Your name will not be used in any published reports about this study.

What will happen if I am injured or become sick because I took part in this study?
If you are injured or become sick as a result of this study, UNM Health Science Center will provide you with emergency treatment, at your cost. No commitment is made by the University of New Mexico Health Sciences Center (UNMHSC) to provide free medical care or money for injuries to participants in this study. In the event that you have an injury or illness that is caused by your participation in this study, reimbursement for all related costs of care will be sought from your insurer, managed care plan, or other benefits program. If you do not have insurance, you may be responsible for these costs. You will also be responsible for any associated co-payments or deductibles required by your insurance.
It is important for you to tell the investigator immediately if you have been injured or become sick because of taking part in this study. If you have any questions about these issues or believe that you have been treated carelessly in the study, please contact the Office of the Institutional Review Board (OIRB) at the (505) 277-2644 for more information.

Payment: You will be paid up to $100 for your participation ($20 for visit 1 and $40 each for visits 2 & 3). You will be reimbursed by gift card or cash.

Right to withdraw from the study: Your participation in this study is completely voluntary. You have the right to choose not to participate or to withdraw your participation at any point in this study without penalty.

If you have any questions, concerns, or complaints about the research study, please contact the principal investigator: Christine Mermier, Ph.D., Department of Health, Exercise & Sport Sciences, 1 University of New Mexico, Albuquerque, NM, 87131. She may be reached Monday-Friday 8:00 a.m. – 5:00 p.m. at (505) 277-2658, or anytime via email at and cmermier@unm.edu.

If you would like to speak with someone other than the research team or have questions regarding your rights as a research participant, please contact the IRB. The IRB is a group of people from UNM and the community who provide independent oversight of safety and ethical issues related to research involving people:

UNM Office of the IRB, (505) 277-2644, irbmaincampus@unm.edu. Website: http://irb.unm.edu/

CONSENT

You are making a decision whether to participate in this study. Your signature below indicates that you have read this form (or the form was read to you) and that all questions have been answered to your satisfaction. By signing this consent form, you are not waiving any of your legal rights as a research participant. A copy of this consent form will be provided to you.

I agree to participate in this study.

_____ _____

Name of Adult Participant Signature of Adult Participant

Date

Researcher Signature (to be completed at time of informed consent)

I have explained the research to the participant and answered all of his/her questions. I believe that he/she understands the information described in this consent form and freely consents to participate.

_____ _____

Name of Research Team Member Signature of Research Team Member

Date

Appendix B: IRB Protocol

UNM IRB PROTOCOL

TITLE:	Comparison of high intensity interval exercise in normoxia and hypoxia on oxygen sensing and downstream gene regulation
VERSION DATE:	02/10/2021
PRINCIPAL INVESTIGATOR/ RESPONSIBLE FACULTY:	Christine Mermier
STUDENT INVESTIGATOR:	Roberto Nava
FUNDING AGENCY:	Received RAC & OFAC (internal grants)

BACKGROUND/SCIENTIFIC RATIONALE

It is well established that aerobic exercise is potent stimulus for muscular and systemic physiological adaptations. Acute bouts of exercise result in transient changes in gene and protein expression related to skeletal muscle carbohydrate[1] and lipid metabolism[2], and mitochondrial biogenesis[3]. Systemically, acute exercise stimulates the production of red blood cells (erythropoiesis) and skeletal muscle capillary growth via upregulation of erythropoietin (EPO)[4] and vascular endothelial growth factor (VEGF)[5], respectively. With exercise training, the cumulative effect of these changes in gene and protein expression alters the metabolic profile of skeletal muscle and facilitates oxygen (O_2) transport and delivery, resulting in improved exercise capacity [6,7]. Although the overall physiological adaptations to chronic exercise like improvements in maximal oxygen consumption (VO_2max) and exercise economy are well documented, the underlying molecular mechanisms by which these adaptations take place are not fully understood.

The transcription factor hypoxia inducible factor-1 (HIF-1) is often referred to as 'the master regulator of O_2 homeostasis' as it is known to regulate over 100 genes related to O_2 transport and delivery as well as both anaerobic and aerobic metabolism[8]. Under normoxic (normal level of oxygen) conditions, the O_2-sensing subunit of HIF-1, HIF-1α, is quickly degraded by regulatory proteins. However, when O_2 tension drops below a critical threshold, the regulation of HIF-1α is inhibited, leading to a stabilization of the HIF-1 protein complex and subsequent regulation of downstream target genes[9],[10]. EPO and VEGF are among the most physiologically relevant genes regulated by HIF-1. In addition, prolonged or repeated stabilization of HIF-1 has been shown to upregulate proteins related to carbohydrate metabolism, and increase the energy yield per liter of O_2 consumed[11].

While HIF-1α has been mostly studied under normobaric hypoxic (sea-level barometric pressure, reduced ambient O_2 concentration) or hypobaric hypoxic (low pressure, low O_2) conditions, exercise serves as a stressor capable of inducing hypoxia in both normoxic and hypoxic conditions[12,13]. At intensities above 50% of VO_2max, the partial pressure of O_2 in human skeletal muscle drops from resting values of 30mmHg to approximately 2-3mmHg, providing a hypoxic stimulus for the stabilization of HIF-1 and

downstream transcriptional effects[14]. Considering that this exercise-induced decrease in O_2 tension is well below the decrease shown in ambient hypoxia alone[13], exercise-induced HIF-1 may be a central mechanism of adaptation to exercise. These adaptations may also be accelerated by the added effect of ambient hypoxia. Our hypothesis is that some of the improvements in O_2 transport and utilization stimulated by exercise training are mediated the stabilization HIF-1α and that the magnitude of stabilization is dependent on the degree and duration the hypoxic stimulus.

Despite evidence for exercise-induced HIF-1 stabilization in animal models, the effect of exercise on HIF-1 protein expression in human skeletal muscle has been seldom explored. To date, there are only three peer-reviewed manuscripts published regarding the effect of an acute exercise bout on HIF-1 protein expression in human skeletal muscle[12,15,16]. Further, the effect of exercise intensity and duration on HIF-1 protein levels and downstream regulation of hypoxia-inducible genes like VEGF, EPO have never been studied in humans or animals. The degree of exercise-induced HIF-1 is likely dependent on intensity of exercise, as intramuscular hypoxia decreases in an intensity dependent manner[17]. In cell models, the degree to which HIF-1 upregulates target genes under ambient hypoxic conditions is dependent on the length of time in which cells remain hypoxic[15]. Therefore, the duration of an exercise bout may play an important role in the determining the magnitude of exercise-induced adaptations regulated by HIF-1. Lastly, all previous investigations regarding HIF-1 expression have used a hypoxic gas mixture to simulate hypoxia, Fortunately, our lab has access to one of the only hypobaric chambers in the country and thus participants will be tested under hypobaric hypoxic conditions to better mimic high altitude exposure[18].

Therefore, the purpose of this study is to investigate the effect of two different exercise intensities on exercise induced HIF-1 stabilization in both normoxia (Albuquerque altitude of approximately 1600m and hypobaric hypoxia equivalent to 4700m.)

OBJECTIVES/AIMS

To determine the effect of exercise on HIF-1 protein expression and downstream gene expression of hypoxia inducible genes EPO, VEGF and GLUT-4, among others.

The effect of exercise on these outcomes will be evaluated by measuring protein and gene expression before and after high intensity interval exercise.

To determine if there is an added effect of hypobaric hypoxia on exercise-induced HIF-1 protein expression and gene regulation.

This will be evaluated by measuring protein and gene expression before and after high intensity exercise in normoxia 5158 ft (1600m) and hypobaric hypoxia (4700m).

PROJECT DESIGN

Target Population and Inclusion/Exclusion Criteria

Participants will be healthy (as defined below) men and women with diverse ethnic backgrounds between the ages of 18 and 45. All will be able to speak and read English.

Inclusion Criteria:

Answered “no” to all seven questions on the Physical Activity Readiness Questionnaire (PARQ, see appendix).

Living in the Albuquerque area for at least 6 months in an area that deviates no more than ± 600 ft from the altitude of the laboratories on the UNM campus (5158 ft).

Indicates via questionnaire that they, show no signs or symptoms of or are not known to have cardiovascular, renal, metabolic, or pulmonary disease, are not a current smoker or has quit over six months ago, and are not pregnant (confirmed by urine pregnancy testing).
Exclusion Criteria:
Answered "yes" to at least one question on the PARQ.
Has not lived in the Albuquerque area for more than 6 months or lives at an altitude > ± 600 ft compared with the laboratories on UNM campus (5158 ft).
Indicates they are claustrophobic, show signs or symptoms of or are known to have cardiovascular, renal, metabolic, or pulmonary disease as determined by a health questionnaire, are a current smoker.
Are pregnant or could be pregnant.
Have a ruptured eardrum
Participants under 18 years or over 45 years.
Non-English speakers, prisoners, pregnant women, or persons requiring a LAR will not be included
Have a known allergy to lidocaine (very rare-249 adverse reactions in 70 million injections[24])
Have a known bleeding disorder or blood clotting problem.

Participant Enrollment

Based on power analysis using G*Power software the number of participants needed for the study to have a power of 0.80 is 10. Since attrition is expected, the maximum number of participants to be enrolled will be 26, so that at least 15 participants will complete all testing conditions. The effect sizes were determined *a priori* based on the magnitude (effect size) of exercise-induced HIF-1 in human muscle tissue gathered from the findings of Ameln et al (2005)[12], Slot et al. (2014)[16] and Van Thienen et al. (2017)[15]. These researchers reported effect sizes of 1, 0.83, and 1.5, respectively.

Recruitment and Screening Procedures

Recruitment:
Participants will be recruited by word-of-mouth from research team members and posted flyers around UNM campus. Past participants may be asked in they would be interested in participating in the study once to redo all three trials if their samples were inadequate for analysis or data collected was not of sufficient quality. If they agree, they will be re-consented. The original samples will be discarded.

Phone Screening:
Interested participants will email the research team. After interest is known, an in-person or phone appointment will be set between the interested individual and a member of the research team. Potential participants will complete a short screening questionnaire (which will take ~15 minutes). This includes exclusion/inclusion criteria such as a requirement to live in the Albuquerque area for at least 6 months in an area that deviates no more than ± 600 ft from the altitude of the laboratories on the UNM campus (5158 ft), the PARQ, and a health and physical activity questionnaire. If a participant fails the screening process by indicating they have any of the exclusion characteristics, they will be thanked for their interest in the

study and informed that their inability to participate will not impact their status at the University of New Mexico, if applicable. All data collected during a failed screen will be destroyed immediately by shredding the documents. Direct identifiers will not be maintained for failed screens during the study. For those that qualify for the study, there will only be one list with direct identifiers kept in the PI's possession and destroyed once the data analysis is complete. The link to the direct identifiers will be stored in a locked file cabinet in the PI's office.
Potential participants will be emailed and will complete a set of short health and physical activity screening questionnaires as well as a COVID-19 symptom checklist (taking ~15 minutes) and the informed consent. The questionnaires include exclusion/inclusion criteria, health and physical activity questions, and a screening checklist for COVID-19. Participants will need to complete these forms online with a research team member or bring printed copies during their 1st visit. Whether completed online or in person, participants will review the consent documents with a member of the study team during their 1st visit before signing them. Participants will complete the COVID19 checklist before each visit (total of 3). Their skin temperature will be checked upon arrival and they will be asked to reschedule if their temperature is above 100°F or have any other COVID19 symptoms.

Informed Consent Process

The Exercise Physiology Laboratory has confidentiality procedures in place to minimize the risk when potential participants take part in this study. Only an ID number will be kept on the phone screening questionnaire. A separate master list containing names and ID numbers of those enrolled in the study will be kept in a locked cabinet in the PI's office. The phone screening questionnaire would be a useful tool to minimize inconveniencing potential participants who do not meet inclusion criteria by having them speak to a research team member over the phone rather than coming to the laboratory in person.

After phone screening (before face-to-face contact):
Copies of the informed consent will be provided by email or in-person for review to those who pass the screening process.
Visit 1 (first face-to-face contact):
Participants will be verbally informed about the research and given a copy of the consent form. After a period of not less than 24 hours after review of the consent form, in the Exercise Physiology Lab conference room for privacy, they will meet with the research team and/or the PI to discuss the study procedures and risks. After all of their questions are answered, they will be invited to sign the informed consent form and participate in the study. Participants will have opportunities to ask as many questions as they need to the research team members before signing the informed consent form and during their study participation. As stated in the consent, participants will be able to withdraw from the study at any time without consequences.

Data Collection Procedures

The study design will require a total of three visits, one to the Exercise Physiology Laboratory (Johnson gym, B142) and two to the hypobaric chamber (Carlisle gym). The first visit will take up to 60 minutes. Participants will arrive to the laboratory after abstaining from alcohol for 24 hours, caffeine for 4 hours and exercise for 24 hours. Before every visit, a research team member will call the participant and will inquire about COVID symptoms using the COVID-19 symptoms checklist. The participant will be approved to come to the laboratory if there are no signs and symptoms of COVID-19 and/or they have not been exposed to individuals who are COVID-19 positive. For every visit, prior to entering the lab, participants will have their body temperature taken using a no-touch forehead thermometer. During all exercise and experimental sessions, participants and study team members will be required to wear a face mask. Participants will fill out health/exercise history and consent forms and a urine pregnancy test will be given to women of childbearing potential. If the pregnancy test is positive, the participant will be told that she does not qualify for the study in a private room and ensured that the information will be kept confidential. She will then be thanked for her time and referred to her health care provider. Baseline measurements will include; resting blood pressure, 3 site skinfold measurement (chest, abdomen and thigh for men and triceps, hip and thigh for women), determination of dominant leg by having them step up onto a riser. They will then perform a maximal graded exercise test on a cycle ergometer. During the maximal graded exercise test, heart rate will be monitored throughout the exercise test, and the test will be terminated if participants reach any criteria for absolute or relative indications for terminating exercise testing[19]. Expired gases will be collected via Parvo Medics metabolic gas analyzer (TrueOne 2400, Sandy, UT) to determine maximum oxygen consumption (VO_{2max}). This will require that participants wear a mouthpiece and nose clip during exercise. Termination of the maximal graded exercise test will occur upon the participant reaching maximal exertion, which is the point of which the participant is no longer capable of maintaining a pedal cadence of 60 revolutions per minute and typically takes 8-12 minutes. The maximal graded exercise test will be performed under the supervision of individuals well-trained and experienced in exercise testing. Participants will be given a food log and will be asked to track their diets in the 24 hours prior to visit 2. They will be asked to mimic their diet prior to visit 3 to limit the influence of diet on protein and gene expression.

Visits two and three will be separated by at least seven days. These visits will each take up to 90 minutes each. Participants will arrive to the laboratory one hour after consuming a light meal and abstaining from exercise for 72 hours, alcohol for 24 hours and caffeine for 4 hours. Using a randomized, crossover design, participants will perform high-intensity interval cycling in either normoxia (Albuquerque altitude of approximately 1600m) or hypobaric hypoxia (4700m). The high intensity exercise will consist of a 5-minute warm-up followed by 4 sets of 5, 10-second ‘all out’ sprints with 30-seconds rest in between sprints and 5-minute rests in between sets . All bouts of exercise will be concluded with a five-minute cool-down while the chamber is re-pressurizing. Heart rate (Polar H10) and oxygen saturation (Nonin Go2 pulse oximeter) will be monitored continuously by study personnel who will always be with the participant during each trial.

Muscle tissue samples will be collected from the thigh (*vastus lateralis* muscle) pre-, immediately post- and 3 hours post-exercise. Participants will be allowed to leave following the second biopsy and asked to return 3 hours after the end of the exercise trial for the third biopsy. Muscle tissue will be sampled from the dominant leg in both exercise trials (normoxia and hypoxia). Two passes of the needle in the same insertion area will be done to obtain enough sample. The second pass is done immediately after the first sample is extracted from the needle (within 30 seconds). Other researchers have performed multiple passes in the same location with no complications[26]. A total of six muscle biopsies will be performed for each participant over the 3 weeks of the study. Muscle tissue samples will be analyzed for several markers of oxygen and energy sensing including but not limited to; HIF-, VEGF and EPO.

Skeletal Muscle Biopsy Procedures and Storage

The fine needle biopsy (i.e. 14-gauge needle microbiopsy) will involve the extraction of a small piece of muscle tissue from the *m. vastus lateralis* using a sterile hollow needle done by IRB-approved biopsy technicians. The PI will always be in the room to oversee the procedure. This method is minimally invasive which allow the researcher to obtain multiple muscle samples if needed without large incisions that accompany other biopsy methods such as the Bergstrom method. Additionally, the pain level of a fine needle biopsy has been reported to be minimal, with individuals comparing it to a "pushing sensation" and most participants engage in normal physical activity the same day[20]. The area over the *m. vastus lateralis* (outside of the lower thigh muscle) will be carefully cleaned with 70% isopropyl alcohol followed by an antiseptic solution (betadine) and covered with a fenestrated drape. Through the access hole of the drape, approximately 2 – 3 cc of Lidocaine anesthetic will be injected into and under the skin in circular (360˚) fashion. Participants will likely experience a small pinching sensation while the numbing agent is injected. After 2 – 3 minutes to allow for the numbing agent to take effect and verification that the participant did not have superficial sensation at the region of interest, a small puncture to the skin will be made using a 16-gauge pilot needle, perpendicular to the muscle, until the superior fascia of the superficial muscle is pierced. A 14-gauge hollow biopsy needle will be loaded into the biopsy instrument and inserted into the leg via the pilot incision. Skeletal muscle tissue will be obtained by the depression of the trigger button which unloads the spring and activates the needle to extract a small amount of tissue. The needle will be removed from the leg, pressure with sterile gauze will be immediately applied, and the tissue sample will be removed from the biopsy needle using a sterile instrument and placed into a sterile and sealed tube. A second tissue sample will be collected from the same incision. In total, approximately, 20 mg of muscle tissue will be extracted. Participants may feel the sensation of pressure in the thigh and in some instances some mild pain during extraction of muscle tissue. However, the potential discomfort dissipates quickly, and participants are likely capable of performing exercise and normal daily activities unhindered the same day as the sampling. There may be some minimal bleeding while the needle is removed which will be stopped with the application of pressure on the site. Following the biopsy, the incision will be cleaned with 70% isopropyl alcohol, treated with a sterile dressing, and a wrapped in a bandage.

All tissue samples will be immediately frozen in liquid nitrogen and stored in a -80°C freezer in the Exercise Physiology Laboratory for subsequent analysis. Samples will be stored via a

unique study number that will only include participant ID number, collection time point, collection sample type (muscle) and OIRB study number. We may keep these samples for up to five years to complete analyses. Samples will be disposed of in an appropriate biohazard receptacle when analysis is complete. Samples will be analyzed for protein expression and mRNA of HIF-1α and related proteins and genes including, but not limited to, VEGF, EPO and GLUT4.

Biological Sample Analysis

Protein Quantification

Muscle tissue will be homogenized with 300 µl of RIPA commercial lysis buffer. The protein-containing supernatant will be collected and stored in 1.5 ml Eppendorf tubes for protein quantification. Protein quantification will be performed using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). A 4X Laemmli buffer with 5% β-mercaptoethanol will be added to the protein lysate and incubated at 100°C for 10 minutes before gel loading for protein separation.

Western blot protein analysis

Proteins will be separated by electrophoresis on a resolving and stacking sodium dodecyl sulfate polyacrylamide gel. Separated proteins will be transferred to a Trans-blot Turbo transfer PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). Membranes will be blocked for 30 min in 5% dry milk and Tris buffered saline Tween 20 buffer solution, washed in Tris-buffered saline (TBS), and incubated overnight in the corresponding primary antibody at 4°C. All membranes will be washed in TBS with 0.05% Tween 20 (TBS-tween) and incubated with a horseradish peroxidase-conjugated secondary antibody (Invitrogen, Carlsbad, CA) and incubated for 1 hour at room temperature. Santa Cruz Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA) using the ChemiDoc Touch Imaging System (Bio-Rad, Hercules, CA) to develop the membrane. Image Lab software (Bio-Rad, Hercules, CA) will be used to quantify protein expression by determining densitometric values. All proteins will be standardized to endogenous housekeeping protein (β-actin).

Isolation of total RNA from Skeletal Muscle

Total cellular RNA will be isolated from the muscle tissue. A QIA-RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) will be used to disrupt cells, isolate, and purify total RNA content per the manufacturer's protocol.

Reverse transcription for RNA detection

Reverse transcription will be performed to create complementary DNA (cDNA) from single stranded RNA using the Transcription First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). The reactions will be incubated at 65°C for 5 min, placed on ice for 1 min, and then at 25°C for 10 min followed by a 60 min incubation at 50°C, and 85°C for 5 minutes to denature the RNA secondary structure. The cDNA samples will be aliquoted and stored at -20°C. cDNAs will be amplified in a StepOnePlus Lightcycler (Grand Island, NY, USA).

Oxidative Stress in Serum

The degree of oxidative stress induced by exercise and/or hypoxia will be determined by a reactive oxygen species (ROS) colorimetric assay (Cell Biolabs, San Diego, CA)

Anticipated End Date

The total participant duration in this study will be approximately eight hours over six sessions. The two initial baseline testing sessions will last up to 60 minutes while the four exercise trials may last up to 90 minutes each. Given the number of subjects and experimental measures required for this study, a total study duration of one year from approval will be required.

Project Location(s)

The study will take place at the University of New Mexico Exercise Physiology Lab located in Johnson gym as well as the hypobaric chamber located at the north end of Carlisle gym. Samples will be stored and analyzed for protein and gene expression at the Exercise Physiology Lab.

Participant Compensation

Participants will be given paid **up to** $100 for participation **($20 for visit 1 and $40 each for visits 2 and 3)**.

Project Resources

The PI's is a Ph.D.-level professor who has many years of experience in exercise testing and tissue extraction and processing. Other staff include phlebotomists and doctoral students with training and classes that involve tissue processing, exercise testing, research ethics, statistical analysis, and exercise biochemistry. Christopher Bossart, MD and Jacob Christensen, MD are the two on-call physicians available who work directly with the lab as needed. In the unlikely case of a medical incident, all research personnel are certified to perform CPR, as well as administration of an AED. There are AEDs and supplemental oxygen located at the Exercise Physiology Lab and hypobaric chamber. The physiology laboratory is located next door to the Student Health Center and the hypobaric chamber is less than a five-minute walk away. At the health center, there are doctors available to come to the lab if needed. The nearest fire station is right around the corner, and if 911 is called, they arrive within 5-8 minutes.

EXPECTED RISKS/BENEFITS

Potential Risks

There is risk of COVID-19 exposure for both participants and the research team as the data collection involves face-to-face interaction. In order to minimize the risk, several strategies will be implemented. A discussion with research personnel will take place before the study continues this second phase of data collection of the possible risk of COVID-19 exposure as it pertains to working with participants on the research study. Only those research team members who agree (without undue influence or coercion) will be involved. Participants will

be provided with written information (in the consent form) about the possible risk of COVID-19 exposure as it pertains to their participation in the research study.

Considering blood draws and skeletal muscle biopsy: SARS CoV-2 is not a known bloodborne pathogen[21]. Therefore, the risk of COVID-19 virus transmission will not be exacerbated by these procedures beyond the required researcher – participant proximity.

All research personnel have been trained through UNM Learning Central before commencing data collection on any new procedures adopted to prevent exposure to COVID-19 and handling biohazards. The minimum number of research personnel (no more than 2, including faculty and graduate students) required to conduct research will be allowed. Research personnel will be trained in the proper use of all Personal Protective Equipment (PPE) and room capacities by the PI and that they will only be there voluntarily. Before going to the lab, research personnel will self-screen for COVID symptoms using the UNM online COVID-19 symptoms checklist. Research personnel and participants will have the appropriate PPE including disposable gloves, googles, lab coats, and masks. Participants will always wear a face mask.

The following strategies will be implemented to minimize the risk of COVID19 transmission:

a. All surfaces and equipment used or touched during the trial will be disinfected with a 70% ethanol solution. Mouthpieces used during visit 1 will be disinfected with CIDEX. Disinfection procedures will be performed before and immediately after each trial.

b. Research staff and participants will maintain a 6-foot distance except for necessary data collection. This includes when equipment is placed on the participant and when muscle biopsies are being performed.

c. Study visits between or within participants will be schedules at least 24 hours apart to allow for disinfection.

The risks associated with a muscle biopsy include momentary discomfort or moderate pain during the time the biopsy needle is inserted, the possible appearance of a scar, and potential bruising and/or soreness at the site of sampling. To limit the potential risks, only trained technicians approved by the OIRB using sterilized instruments will perform the biopsy procedure. Additionally, the sampling site will be sterilized prior to the procedure. Participants will likely experience a small pinching sensation while the numbing agent (Lidocaine) is injected. A minimal amount of muscle tissue (20 mg) will be extracted from the participant for each biopsy. To reduce the risk of dizziness and fainting, all procedures will be performed while the participant is lying in the supine position. Once the local anesthesia wears off, the leg may feel tight and participants may feel a sensation of a deep bruise or “Charlie Horse”. The tightness in the muscle typically dissipates within 2 days and participants may begin exercising immediately, and routinely begin exercising at normal capacity within 2 days. However, most participants may be able to engage in normal physical activity the same day[20]. Participants will be given instructions for the proper care of the biopsy site (i.e. biopsy care sheet).

There are risks associated with maximal graded exercise test including the following: muscle soreness, fatigue, nausea, or dizziness during or after completion of exercise. The incidence of risk of fatal and nonfatal events during maximal exercise testing in apparently healthy individuals are approximately <0.8 per 10,000 tests or 1 per 10,000 hours of testing[22]. These risks will be minimized by screening subjects for excluded medical conditions and risk factors and by performing exercise testing in accordance with the guidelines of the American College of Sports Medicine's (ACSM) Guidelines (Pescatello, 2014).

There is very small risk of developing acute mountain sickness (AMS) when exercising in hypobaric hypoxia, especially at maximal effort. The symptoms of AMS vary from person to person but most often; headache, nausea, dizziness and general malaise. These symptoms are similar to those experienced by some after maximal exercise and thus the combination of exercise and hypoxia may exacerbate symptoms associated with each of these factors alone[23]. However, acute bouts of exercise in moderate hypoxia have been well tolerated by healthy individuals in our previous investigations[24,25]. If a participant is having symptoms of AMS or their oxygen saturation (SaO_2) drops below 70%, the exercise will immediately be terminated, the mouthpiece and nose-clip removed, and the altitude chamber will be adjusted to bring the pressure back to Albuquerque's altitude. Blood pressure, SaO_2 and heart rate will be monitored until the participant feels better. Supplemental oxygen (100%) will be available in the altitude chamber in the in the unlikely event that it would be needed. Though the word "gene" is in the title, will **not** be doing any genetic (DNA) testing related to disease, rather we are looking at genes related to HIF-1alpha signaling only. Further, no subject-related genomic analysis is planned except what is described in this document at this time. Deidentified samples will no longer carry a link to any identifiable information (only data such as sex, age, VO2 and body composition results will be retained) after data collection for the study is complete. If subjects check the box on the consent that their samples may be used in the future, samples from those participants may be kept, but will be anonymized.

Experienced personnel will be monitoring participants throughout exercise testing sessions and exercise bouts to ensure participant safety. All exercise testing will be performed under the supervision of individuals trained in and experienced with exercise testing. Termination of the exercise test or exercise bouts will occur if the participants ask, or if the researcher feels it is unsafe to continue. Participants will be required to fill out a health history questionnaire that addresses risk factors such as smoking, family history of heart disease, obesity and cardiovascular, pulmonary, and metabolic disease as well as any injury that would preclude safe exercise testing. In the unlikely case of an emergency, standard procedures for the Exercise Physiology Laboratories will be followed: these include calling 911 and the Student Health Center located next door and assessing and monitoring the participant. All investigators are certified in CPR and AED use. The Exercise Physiology Laboratories Medical Directors, Christopher Bossart MD and Jacob Christensen, MD, also would be notified immediately. The average time it takes for ambulance services to reach the Laboratory is approximately 5-8minutes, and for a physician from the Student Health Center, less than 5 min. Research team members have previous exercise testing experience and are aware of the signs and symptoms that are associated with possible adverse reactions during exercise testing

Benefits

There is no direct benefit to subjects for their participation. Subjects will have a VO_{2max} test performed and body composition assessment. Subjects will be given the results and an interpretation of these tests at the completion of their participation. Knowledge of VO_{2max} and body composition can be of benefit in that it is indicative of health, risk for a number of chronic degenerative diseases, and level of physical fitness.

We are confident that the results of this study will elucidate some underlying mechanisms of exercise adaptation. This will benefit the scientific community by confirming previous findings from animal models on humans. Clinicians may use this information to optimize exercise recommendations by prescribing exercise modalities that maximize the benefits which are confirmed by the understanding of the mechanisms that mediate beneficial adaptations.

Privacy of Participants

Privacy will be maintained by screening and testing subjects in private rooms in the Exercise Physiology lab and hypobaric chamber with no access to anyone but the study team. All subjects will sign an informed consent and fill out health history and physical activity questionnaires. All subjects will be assigned a random number for confidentiality of participant data. Only approved research team members and the PI will have access to subjects during interactions required for the study. Participant information will be held confidential through a password protected computer, with hard copies stored in a locked file cabinet.

Unanticipated Problems/Adverse Events

Muscle biopsies will be done, and maximal exercise testing as well as a moderate intensity and high intensity exercise will be performed, with some trials taking place at low pressure (high altitude). The latest recommendations of the ACSM allows exercise testing of all ages by exercise physiologists as long as subjects are screened for risk factors and excluded medical conditions. The PIs will ensure the safety of the subjects by carefully screening the participants for medical conditions, using accepted protocols for muscle microbiopsy and exercise testing at both moderate (1600m- Albuquerque) and high-altitude conditions. The study team will be trained by the PI and all team members will experienced in all aspects of study procedures, including ethical human research considerations, gathering of muscle tissue via microbiopsy and exercise testing as well as safety procedures. For the Data Safety Monitoring Plan (DSMP), the PI will be present for all procedures (maximal graded exercise testing, all exercise testing in the altitude chamber and in the lab and during the performance of microbiopsies.) Participants will be monitored (heart rate, oxygen saturation in the altitude chamber, metabolic data) continuously. If the PI decides that it is prudent for safety purposes, one of the lab physicians may be present during the exercise testing, per the ACSM Exercise Testing Guidelines. The DSMP includes the review by the PI of data related to the subjects' response to the maximal exercise testing and biopsy site after each trial. Any unanticipated problems or adverse events will be reported to the OIRB within within 7 calendar days. Though unlikely, if there were to be any serious unanticipated problems or medical events such as sudden cardiac event, serious musculoskeletal injury, excess bleeding

at biopsy sites or any other serious study-related injury to the participant, the entire study would be terminated and the OIRB would be notified. Termination of any exercise test will occur if the participants ask, or if the researcher(s) feel it is unsafe to continue. If any serious medical problem arises in the altitude chamber, the chamber will be pressurized at the standard rate of 1000 ft/min. If it is an emergency, the chamber will be pressurized faster (more than 1000ft/min), which could cause pain or injury to the eardrums. The PI and study team will follow all OIRB guidelines and will report any serious event right away if during normal business hours, or on the next business day- both verbally and using an Event Form.

If the study has to stop on short notice in the event that the university stops all on-campus operations and research again due to public health concerns related to COVID-19, research personnel and participants who have not yet started or have not completed all visits will be immediately notified to stop coming to the lab until further notice.

If a research team member or a participant reports exposure to, develops symptoms possibly associated with, or tests positive for COVID-19 within 14 days of a data collection event, the study will be paused immediately. The individual should contact the PI, Dr. Christine Mermier and report symptoms or diagnosis to UNM at http://www.unm.edu.coronavirus immediately. Any research personnel and/or participants who have had close contact with that individual will start a self-quarantine for 14 days and test for COVID-19. A "contact log" will be maintained to document which research team members were exposed to which participants, including all interactions (both < and > 6 ft.). After 14 days, only research personnel and/or participants showing no symptoms and have tested negative for COVID-19 will be able to resume data collection.

Participant Complaints

Participants who have any complaints and/or requests will have multiple mediums of communication to express this to the research team, including the principal investigator in person, over the phone, or via email. Upon reception of a complaint and/or request, the leading members of the research team will respond and address the concern and the principal investigator will be made immediately aware, if the concern was not initially directed to them. Written documentation of the concern will be made as will all subsequent communication and action. Any additional information regarding study procedures will be presented if requested. All communications and documentation between participant and research team members will be kept confidential. If the participant wants to talk with someone outside the research team, they will be directed to Dr. Christina Perry, chair of the PI's department, or to the staff at the OIRB.

PROJECT DATA

Data Management Procedures and Confidentiality

All data will be kept under a lock and key in a filing cabinet located with the Exercise Physiology Laboratory. The filing cabinets where the data will be located will be locked and the filing cabinet will be contained within the Exercise Physiology Laboratory, which also has a locking door. There will be no participant identifiers, as all subjects will be assigned a number to be matched with all data collected including tissue specimens in order to preserve participant confidentiality. However, there will be a key that links names with participant

number needed for scheduling the laboratory testing sessions. This key will be kept on the PI's password protected computer inside a locked file cabinet within the PI's office (Johnson Center, room B151), and will be deleted once all data collection is completed. The informed consents form which has participant names will be stored separately from any data sheets or questionnaires. Only the investigators approved by the OIRB for this study will be able to access the data. The data will be destroyed after manuscript publication or in five years, whichever comes first. Only the investigators will be able to access the data. No names or other identifying information will be used in any presentation or publication based on these data. Data collection sheets will be transported from the hypobaric chamber to the locked file cabinet located at the Exercise Physiology Lab immediately after each trial conducted at the hypobaric chamber.

Data Analysis/Statistical Considerations

Based on power analysis using G*Power software the number of participants needed for the study to have a power of 0.80 is 10. Since attrition is expected, and if the collected samples are not adequate for analysis, the maximum number of participants to be enrolled will be 26, so that at least 20 participants will complete all testing conditions. The effect sizes were determined *a priori* based on the magnitude (effect size) of exercise-induced HIF-1 in human muscle tissue gathered from the findings of Ameln et al (2005)[12], Slot et al. (2014)[16] and Van Thienen et al. (2017)[15]. These researchers reported effect sizes of 1, 0.83, and 1.5, respectively. The effect of each condition (combination of exercise type and environment) on exercise-induced protein and gene expression will be assessed via two-way repeated measures ANOVA. Where there is a main effect of time, condition or an interaction effect between time and condition, Bonferroni's post-hoc test will be used for multiple comparisons. The threshold for statistical significance will be set *a priori* at a p-value of $\leq$ 0.05 for all tests.

Participant Withdrawal

Any participant, at his or her desire, may stop the test or withdraw from the study at any time. The investigators also reserve the right to stop the any testing due to participant discomfort, any physiological concerns that arise from testing, failure to comply to exercise testing protocols, or if investigators feel it would be unsafe for the participants to continue.

PRIOR APPROVALS/REVIEWED AT OTHER IRBS

This project is not being reviewed by any other IRB.

REFERENCES

1 O'Gorman DJ, Karlsson HKR, McQuaid S, et al. Exercise training increases insulin-stimulated glucose disposal and GLUT4 (SLC2A4) protein content in patients with type 2 diabetes. *Diabetologia* 2006; 49(12):2983–2992. Doi: 10.1007/s00125-006-0457-3.

2 Murakami T, Shimomura Y, Yoshimura A, et al. Induction of nuclear respiratory factor-1 expression by an acute bout of exercise in rat muscle. *Biochim Biophys Acta - Gen Subj* 1998; 1381(1):113–122. Doi: 10.1016/S0304-4165(98)00018-X.

3 Coffey VG. Interaction of contractile activity and training history on mRNA abundance in skeletal muscle from trained athletes. *AJP Endocrinol Metab* 2006; 290(5):E849–E855. Doi: 10.1152/ajpendo.00299.2005.
4 Schwandt HJ, Heyduck B, Gunga HC, et al. Applied Physiology on the erythropoietin concentration in blood. *Eur J Appl Physiol* 1991; (63):463–466.
5 Richardson RS, Wagner H, Mudaliar SRD, et al. Human VEGF gene expression in skeletal muscle : effect of acute normoxic and hypoxic exercise. *Am Physiol Soc* 1999; 277:H2247–H2252.
6 Holloszy JO, Coyle EF. Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. *J Appl Physiol* 1984; 56(4):831–838. Doi: 10.1152/jappl.1984.56.4.831.
7 Egan B, Zierath JR. Review Exercise Metabolism and the Molecular Regulation of Skeletal Muscle Adaptation. *Cell Metab* 2012; 17(2):162–184. Doi: 10.1016/j.cmet.2012.12.012.
8 Liu W, Shen SM, Zhao XY, et al. Targeted genes and interacting proteins of hypoxia inducible factor-1. *Int J Biochem Mol Biol* 2012; 3(2):165–178. Doi: 10.1039/c1cs15147a.
9 Yu AY, Frid MG, Shimoda LA, et al. Temporal , spatial , and oxygen-regulated expression of hypoxia-inducible factor-1 in the lung. *Am J Physiol* 2018; (26).
10 Semenza GL. Hydroxylation of HIF-1: Oxygen Sensing at the Molecular Level. *Physiology* 2004; 19(4):176–182. Doi: 10.1152/physiol.00001.2004.
11 Vogt M, Hoppeler H. Is hypoxia training good for muscles and exercise performance? *Prog Cardiovasc Dis* 2010; 52(6):525–533. Doi: 10.1016/j.pcad.2010.02.013.
12 Ameln H. Physiological activation of hypoxia inducible factor-1 in human skeletal muscle. *FASEB J* 2005:1009–1011. Doi: 10.1096/fj.04-2304fje.
13 Kopp R, Koblitz L, Egg M, et al. HIF signaling and overall gene expression changes during hypoxia and prolonged exercise differ considerably. *Physiol Genomics* 2011; 43(9):506–516. Doi: 10.1152/physiolgenomics.00250.2010.
14 Richardson RS Noyszewski EA, Kendrick KF, Leigh JS, Wagner PD. Myoglobin O2 desaturation during exercise. Evidence of limited O2 transport. . *J Clin Invest 96(4)1916-26* 1995. Doi: 10.1172/JCI118237.
15 Van Thienen R, Masschelein E, D'Hulst G, et al. Twin resemblance in muscle HIF-1α responses to hypoxia and exercise. *Front Physiol* 2017; 7(JAN):1–11. Doi: 10.3389/fphys.2016.00676.
16 Slot IGM, Van Den Borst B, Hellwig VACV, et al. The muscle oxidative regulatory response to acute exercise is not impaired in less advanced COPD despite a decreased oxidative phenotype. *PLoS One* 2014; 9(2):1–9. Doi: 10.1371/journal.pone.0090150.
17 Molé PA, Chung Y, Tran TK, et al. Myoglobin Desaturation with Exercise Intensity in Human Gastrocnemius Muscle. *Med Sci Sport Exerc* 1999; 31(Supplement):S275. Doi: 10.1097/00005768-199905001-01341.
18 Millet GP, Faiss R, Pialoux V. Evidence for differences between hypobaric and normobaric hypoxia is conclusive. *Exerc Sport Sci Rev* 2013:133. Doi: 10.1097/JES.0b013e318271a5e1.
19 Pescatello LS, Arena R, Riebe D, et al. *ACSM's Guidelines for Exercise Testing and Prescription*. 2014.

20 Hayot M, Michaud A, Koechlin C, et al. Skeletal muscle microbiopsy: A validation study of a minimally invasive technique. *Eur Respir J* 2005. Doi: 10.1183/09031936.05.00053404.
21 Gupta MK, Lipner SR. Personal protective equipment recommendations based on COVID-19 route of transmission. *J Am Acad Dermatol* 2020. Doi: 10.1016/j.jaad.2020.04.068.
22 Goodman J. Evidence-based risk assessment and recommendations for exercise testing and physical activity clearance in apparently healthy individuals. *Appl Physiol Nutr Metab* 2011. Doi: 10.1139/H11-048.
23 Roach RC, Maes D, Sandoval D, et al. EXERCISE EXACERBATES ACUTE MOUNTAIN SICKNESS AT SIMULATED HIGH ALTITUDE. *Med Sci Sport Exerc* 1999. Doi: 10.1097/00005768-199905001-00813.
24 Carriker CR, Mermier CM, Van Dusseldorp TA, et al. Effect of acute dietary nitrate consumption on oxygen consumption during submaximal exercise in hypobaric hypoxia. *Int J Sport Nutr Exerc Metab* 2016. Doi: 10.1123/ijsnem.2015-0144.
25 White AC, Salgado RM, Astorino TA, et al. The effect of 10 days of heat acclimation on exercise performance in acute hypobaric hypoxia (4350 m). *Temperature* 2016; 3(1):176–185. Doi: 10.1080/23328940.2015.1072659.

Appendix C: Recruitment Flyers

Volunteer for a Research Study

High Altitude Exercise Study

Looking for well-trained cyclists

Free VO_2max test

You may be eligible to participate if you:

- Are a healthy 18-45 yr old
- Cycle 10-12+ hours/week
- Willing to perform high intensity exercise on a bike

- Participation includes 3 sessions of exercise for a total of 4 hours over 3 weeks
- 6 muscle microbiopsies
- Participants will be compensated up to $100.

For more information,
contact Roberto at:
(575) 202-9038 or 277-2658
rnavabjj@unm.edu

Volunteer for a Research Study

High Altitude Exercise Study

Looking for resistance trained or untrained individuals

Free VO_2max test

You may be eligible to participate if you:

- Are a healthy 18-45 yr old
- Not currently engaging in endurance exercise
- Willing to perform high intensity exercise on a bike

- Participation includes 3 sessions of exercise for a total of 4 hours over 3 weeks
- 6 muscle microbiopsies
- Participants will be compensated up to $100.

For more information,
contact Roberto at:
(575) 202-9038 or 277-2658
rnavabjj@unm.edu

Appendix D: Health History Questionnaire

HEALTH & PHYSICAL ACTIVITY QUESTIONNAIRE

Family history questions are included because certain conditions of your first degree relatives can incur risk to you during maximal exercise.

Subject #______________________________ Date___/___/___

Phone (H or cell)____________________

Age____ Sex_____ Ethnicity_______

Emergency contact (name, phone #)___________________________________
♦♦♦

MEDICAL HISTORY

Physical

injuries:____

__

Limitations____

____

Have you ever had any of the following cardiovascular problems? Please check all that apply.

Heart attack/Myocardial Infarction____ Heart surgery ____ Valve problems _____

Chest pain or pressure ____ Swollen ankles ____ Dizziness _____

Arrhythmias/Palpitations ____ Heart murmur ____ Shortness of breath _____ Congestive heart failure ____

Have you ever had any of the following? Please check all that apply.

High blood pressure _____

Asthma	_____	Total cholesterol >200 mg/dl	_____
Diabetes (specify type)	_____	HDL cholesterol <35 mg/dl	_____
Emphysema	_____		
Stroke	_____	LDL cholesterol >135 mg/dl	_____
		Triglycerides>150 mg/dl	_____

Do immediate blood relatives (biological parents & siblings **only**) have any of the conditions listed above? If yes, list the problem, and family member age at diagnosis.

_____

_____

Do you currently have any other medical condition not listed?

Details_____

_____

_____

_____

Indicate level of your overall health. Excellent ____ Good ____ Fair ____

Poor_____

Are you taking any medications, vitamins or dietary supplements now? Y N

If yes, what are they?____

Are you allergic to latex? Y N

Have you ever experienced any adverse effects during or after exercise (fainting, vomiting, shock, palpitations, hyperventilation)? Y N If yes, elaborate.____

____

♦♦

LIFESTYLE FACTORS

Do you now or have you ever used tobacco? Y N If yes: type ________________

How long?______ Quantity____/day Years since quitting______________

♦♦

EXERCISE HISTORY

Endurance training

Days per week (circle one): <3 3-5 6-7

Minutes per day (circle one): 30-60 60-240 240-360 >360

Hours per week (circle one): 1-2 3-5 6-8 >8

Training background (in years) (circle one): <1 1-3 4-5 6-15 >15

Race days/yr (circle one): 0-10 10-20 20-100 >100

Exercise mode (i.e. bike, run, etc)_____

Resistance training

Times per week (circle one): <3 3-5 6-7

Minutes per day (circle one): 30-60 60-240 240-360 >360

Hours per week (circle one): 1-2 3-5 6-8 >8

Training background (in years) (circle one): <1 1-3 4-5 6-15 >15

Training mode _____

(strength 2-6 reps, heavy load; hypertrophy 6-15 reps, moderate-heavy load)

Do you participate in other sports? Yes No (circle one)

If so, how often? (describe) _____