UNIVERSITY OF CALIFORNIA, SAN DIEGO

Hypoxia Inducible Factor-1alpha in the Skeletal Muscle During Exercise and Endurance Training

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by

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The dissertation of Steven D. Mason is approved, and it is acceptable in quality and form for publication on microfilm.

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To my family, and especially my wife, Becky, without whom none of this would have happened.

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ABSTRACT OF THE DISSERTATION

Hypoxia Inducible Factor 1alpha in the Skeletal Muscle

During Exercise and Endurance Training

by

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Exercise forces skeletal muscle to deal with severe oxygen stress, causing the muscle to have to balance oxygen demand with oxygen availability. Endurance training further challenges the muscle by repeatedly exposing it to lowered oxygen levels. The muscle's ability to cope with this oxygen stress is essential for its function in the body. The primary response pathway for hypoxia runs through the transcription factor Hypoxia Inducible Factor-1α (HIF-1α), a transcription factor which upregulates glycolysis and angiogenesis in response to hypoxia.

To study the role of HIF-1α in untrained and endurance-trained muscle, we have created a mouse lacking HIF-1α specifically in the skeletal muscle. Although structurally normal, loss of HIF-1α results in muscles that are unable to maintain glycolytic flux in response to exertion. As a result, the muscles undergo an adaptive compensatory response resulting in increased oxidative capacity. This response makes

the HIF-1α null mice more suited for endurance exercise, as they have greater sub-maximal endurance relative to control littermates. The endurance advantage comes at a cost, though, as HIF-1α null muscles experience severe muscle damage in response to exhaustive exercise.

Endurance training of the mice shows that both control and HIF-1α null mice are able to improve overall endurance due to an increase in hexokinase activity. However, only control mice can improve oxidative capacity, as HIF-1α null mice were unable to improve on already elevated levels of citrate synthase and β-hydroxyacyl-CoA dehydrogenase, and elevated capillary to fiber ratios. Examination of HIF-1α null muscles reveals that they have constitutively active AMP-activated protein kinase (AMPK). This activation of AMPK correlates with the adaptive response of the HIF-1α null muscle, making it a likely cause of the increase in oxidative capacity.

In conclusion, HIF-1α in the skeletal muscle is important for maintaining glycolytic flux, but is not essential for the muscular response to endurance training. Removal of HIF-1α from the muscle results in the muscle being more suited for endurance exercise and training.

I. GENERAL INTRODUCTION

The greatest challenge facing skeletal muscle is the need to match ATP production with energy demand during exercise. As exercise becomes intensity rises, the demand for ATP increases, and more rapid and efficient ways of producing ATP are required. The pathways leading to ATP production during exercise can be divided into two major categories: aerobic (oxygen requiring) and anaerobic (oxygen independent). During exercise, a muscle must balance the input of both aerobic and anaerobic metabolism to meet energy demands. The balance between the two is determined by the type, intensity, and duration of exercise (Brooks, 1998). Endurance exercise relies primarily on aerobic metabolism for ATP generation, meaning the muscle must the available oxygen to produce much-needed ATP. The difficulty of this task is compounded by the availability of oxygen to the muscle, which can change greatly from rest to exercise. During exercise in normoxia, the partial pressure of oxygen in the muscle has been measured at 3.1 mm Hg, even though oxygen in the inspired air has a partial pressure of 160 mm Hg, and oxygen in the capillaries in the muscle has a partial pressure of 38 mm Hg (Richardson et al., 1995). This low level of oxygen during exercise necessitates a mechanism to enable the muscle to maintain optimum performance.

The cellular hypoxic response and HIF-1α

The primary oxygen response factors within a cell are the transcription factors of the Hypoxia Inducible Factor (HIF) family, HIF-1, HIF-2 and HIF-3. Only two of these members, HIF-1 and HIF-2, have been characterized appreciably. Of those two, HIF-1 is the more ubiquitous isoform (Stroka et al., 2001), as the induction of HIF-2

protein under hypoxia is limited to certain cell types within tissues (Wiesener et al., 2003).

First purified and sequenced in 1995, HIF-1 is a heterodimeric protein composed of two basic helix-loop-helix-PAS transcription factors: the aryl hydrocarbon nuclear receptor (ARNT, also referred to as Hypoxia Inducible Factor-1β), and HIF-1α (Wang et al., 1995; Wang and Semenza, 1995). While HIF-1α and ARNT are each constitutively expressed and translated, ARNT protein levels are relatively stable but HIF-1α protein levels are regulated primarily by the availability of oxygen to the cell. Under normoxic conditions, HIF-1α protein is hydroxylated by members of a family of prolyl hydroxylases on two conserved proline residues in its oxygen-dependent degradation domain (ODD) (Bruick and McKnight, 2001; Epstein et al., 2001). This hydroxylation enables recognition of HIF-1α by an E3 ubiquitin ligase complex, of which the von Hippel Lindau (VHL) protein is the primary factor responsible for recognizing and binding to hydroxylated HIF-1α (Ivan et al., 2001; Jaakkola et al., 2001). The hydroxylation of HIF-1α at its proline residues is essential for this interaction as their mutation results in less binding of VHL with HIF-1α (Epstein et al., 2001). Further verification of the importance of the proline residues comes from other studies looking at manipulation of the ODD. Wholesale deletion of the ODD results in a stable HIF-1α protein and HIF-1 target gene activation, and fusion of the ODD to a normally oxygen-insensitive protein makes that protein oxygen sensitive (Huang et al., 1998). The interaction of HIF-1α with VHL results in ubiquitylation of HIF-1α, and targeting of HIF-1α to the 26S proteasome for

degradation (Cockman et al., 2000). This regulation of HIF-1α protein through hydroxylation is quite strict; the half-life of new HIF-1α protein under normoxia has been demonstrated to be as short as five minutes (Huang et al., 1998).

When oxygen concentration drops, and cells and tissues become hypoxic, the hydroxylation of HIF-1α is blocked, resulting in decreased interaction between HIF-1α and VHL (Jaakkola et al., 2001). As a result, HIF-1α protein is stabilized, allowing it to dimerize with ARNT and turn on transcription of target genes. The oxygen sensing machinery that so tightly regulates HIF-1α under normoxia is also quite sensitive to inhibition by hypoxia; hypoxic cells begin accumulating HIF-1α protein within 2 minutes of hypoxia (Jewell et al., 2001). In vivo, the sensitivity of cells to hypoxia is tissue-specific. In work with mice exposed to normobaric hypoxia, Stroka et al. (Stroka et al., 2001) saw that brain tissue begins accumulating HIF-1α protein when inspired oxygen is dropped to 18%, while kidney and liver only respond to more severe hypoxia. Additionally, the authors found stable HIF-1α protein under normoxia in skeletal muscle, showing that some tissues have the ability, and need, to accumulate HIF-1α protein independently of hypoxia. This finding was recently repeated by Pisani and Dechesne (Pisani and Dechesne, 2005), who also showed that normoxic HIF-1α stability in the muscle is dependent on fiber type. Muscles that are composed primarily of type II fast twitch fibers have a higher level of HIF-1α protein at rest in normoxia than muscles with a higher proportion of type I fibers.

Once HIF-1α is stabilized, it interacts with ARNT, forming the HIF-1 complex. This enables HIF-1 to recognize hypoxia responsive elements (HRE) in the

promoters and/or enhancers of genes in the nucleus. The HRE is a short consensus sequence that HIF-1 binds to in order to upregulate transcription of target genes (Madan and Curtin, 1993; Wang and Semenza, 1993). Once activated, the transcriptional response of HIF-1α to hypoxia enables cells to cope with oxygen stress while working to increase oxygen delivery (Semenza, 2001). To help cells and tissues survive oxygen stress, HIF-1α upregulates transcription of genes that amplify glycolysis and glucose transport into the cell. Genes in this category include glucose transporters 1 and 3 (GLUT1, GLUT3), as well as the glycolytic genes hexokinase I and II (HKI, HKII), phosphoglycerate kinase 1 (PGK1), and lactate dehydrogenase A (LDHA), among others (Semenza, 2002). In order to increase oxygen availability, HIF-1α coordinates a response that increases oxygen delivery to the hypoxic region. Two key transcriptional targets for this function are vascular endothelial growth factor (VEGF), and erythropoietin (EPO) (Semenza, 2002). Other HIF-1α target genes include genes involved in cell cycle and apoptosis signaling, however, the role of HIF-1α in the cellular proliferation/survival response is not completely understood. In addition to the multitude of genes identified as having HREs in their promoters (meaning they can be directly regulated by HIF-1α), many more genes have been shown to have expression patterns correlating with HIF-1α activity, indicating that they are also directly or indirectly regulated by HIF-1α. GLUT4, the primary muscle glucose transporter, falls into this category as it has not yet been shown to have an HRE, but it does have expression patterns that correlate with HIF-1α activity and

expression (Silva et al., 2005). New HIF-1α targets are continually being discovered as the understanding of how HIF-1α helps cells and tissue respond to hypoxia grows.

Loss of HIF-1α can have profound effects on cells and tissues. A primary result of the loss of HIF-1α is that cells are unable to upregulate HIF target genes in response to hypoxia. This leads to a failure to upregulate glucose transport and glycolysis, resulting in decreased ATP levels during hypoxia (Seagroves et al., 2001). Surprisingly, this failure extends to normoxia as well for some cell types, as macrophages lacking HIF-1α have as little as 15-20% of the ATP content as control macrophages in normoxia (Cramer et al., 2003). HIF-1α is also essential for development, where local hypoxia results from the lack of an established vascular system. In evidence of this, mice lacking HIF-1α in their germ line die *in utero* due to defects in cephalic vascular formation and defective neural fold formation (Ryan et al., 1998).

Another important role for HIF-1α has been found in tumor growth and development. Solid tumors become hypoxic as they grow larger, and tumors forming following inactivation of the VHL tumor suppressor protein are aggressive and well vascularized (Kondo and Kaelin, 2001), leading to the hypothesis that HIF-1α is a positive factor in tumor development. To that end, it has been shown that solid tumors lacking HIF-1α do not grow as rapidly as normal tumors, indicating that this is indeed the case (Ryan et al., 2000).

Tissue and cell type-specific deletion of HIF-1α has shown HIF-1 signaling to be integral in many different places in the body. Deletion of HIF-1α in chondrocytes

results in bone deformities and abnormalities in the trachea due to increased chondrocyte growth (Schipani et al., 2001), while in myeloid cells, loss of HIF-1α reduces their mobility and invasiveness, and their ability to kill bacteria (Cramer et al., 2003). Combining the results of these studies shows that the hypoxic response through HIF-1α plays an important role in development, disease, and homeostasis.

Muscular response to acute endurance exercise

As mentioned earlier, skeletal muscle experiences a drop in intramuscular oxygen during exercise, leading to a hypothesis for a possible role for HIF-1α in the muscle during and following exercise. Surprisingly, however, little research has been done looking directly at HIF-1α function in the muscle.

In the muscular response to exercise, several changes occur that are likely mediated by HIF-1α. Due to the increased demand for oxygen in the muscle, both the body and the skeletal muscle undergo several acute performance-oriented changes. These changes have the goal of increasing oxygen delivery to the muscle and improving its metabolic capabilities. Since acute exercise bout is too short of a time period to allow for vascular remodeling or a significant increase in red blood cell content, one of the primary ways exercising skeletal muscle receives greater oxygen delivery during exercise is through increased blood flow to the skeletal muscle. This is accomplished through two main pathways: a decrease in blood flow to non-exercising tissues (i.e., the kidney and spleen) and increased blood flow to the skeletal muscle itself (Rowell, 1974). In addition to increased oxygen delivery, the greater

blood flow also allows for increased metabolite delivery to and waste clearance from the exercising muscle.

The metabolic changes in exercising muscle serve to increase ATP production while minimizing the impact of non-essential ATP consuming pathways. A key protein that helps the muscle accomplish this is the AMP-activated protein kinase (AMPK). Exercise, and the resulting increase in ATP consumption, causes an increase in the AMP to ATP ratio. AMP then binds with AMPK, making AMPK a better substrate for phosphorylation and activation by an upstream kinase (Hawley et al., 1995). Once activated, AMPK phosphorylates targets leading to increased glucose transport, glycolysis, and fatty acid oxidation, as well as decreased ATP consumption (Winder, 2001). Two key phosphorylation targets are the GLUT4 Enhancer Factor (GEF) and Acetyl-CoA Carboxylase (ACC). Phosphorylation of GEF by AMPK results in an increase in GLUT4 expression and, eventually, increased GLUT4 protein accumulation (Holmes et al., 2005; Zheng et al., 2001), while phosphorylation of ACC inactivates it and causes a decrease in malonyl-CoA levels (Kaushik et al., 2001; Winder et al., 1997). Malonyl-CoA, is inhibitor of carnitine palmitoyltransferase (CPT), which catalyzes a rate-limiting step of fatty-acid β-oxidation (Winder et al., 1989). The AMPK-caused decrease in malonyl-CoA allows for an increase in CPT activity, thus increasing β-oxidation during exercise. Loss of AMPK in the skeletal muscle, through the use of a dominant negative form of AMPK's catalytic α subunit, results in the muscles being more sensitive to, and slower to recover from, fatigue (Mu et al., 2003), and demonstrates the importance of AMPK during exercise,

Additional changes in the muscle during exercise directly affect glycolytic flux, an area that may be mediated by HIF-1α activity. Glucose uptake by the muscle increases dramatically during exercise (Kjaer et al., 1991), which is likely a result of increased glucose transporter 4 (GLUT4) translocation to the cell surface (Thorell et al., 1999). Additionally, glycolytic flux is constant and integral during aerobic and anaerobic exercise, and leads to lactate accumulation during both (Kemper et al., 2001).

As can be expected, mutations that block or inhibit steps in these important metabolic pathways can have dramatic phenotypes. Several myopathies have been characterized that result from a blockage in carbohydrate metabolism, and are collectively referred to as glycogen storage diseases (GSD). Two of these diseases are GSD V, muscle glycogen phosphorylase deficiency, and GSD VII, muscle phosphofructokinase deficiency, also known as McArdle's Disease and PFKD, respectively. Patients with either myopathy have decreased carbohydrate utilization resulting in increased glycogen storage, decreased lactate accumulation during exercise, exercise intolerance, and muscle damage following intense exercise (DiMauro et al., 1984). As a result of the decreased carbohydrate metabolism, the myopathic muscles frequently have a compensatory response, resulting in their relying more on phosphocreatine and/or aerobic metabolism for ATP production during exertion (Argov et al., 1987; Vissing et al., 1996). Another compensatory response, especially in patients with McArdle's Disease, is the Second Wind phenomenon. In this case, normally exercise intolerant patients perform an initial exercise with difficulty, rest briefly, and can then exercise for a much longer period of time with

much less discomfort. The cause of this phenomenon is not fully understood, but is likely due to compensation from blood glucose and increased fatty acid oxidation (Haller and Vissing, 2002).

In addition to maintaining ATP levels, another main challenge of skeletal muscle is resisting fatigue. Muscle fibers, and therefore muscles with differing fiber composition, vary in their resistance to fatigue. Type I fibers, which are slow twitch and highly oxidative, are highly fatigue resistant. On the other hand, fast-twitch type II fibers are more susceptible and fatigue quite rapidly. The mechanisms leading to fatigue sensation are not completely understood yet, but to a large degree, are thought to involve lactate signaling. As exercise continues, serum lactate levels increase, and lactate has been shown to correlate well with fatigue sensation. One classic experiment by Fitts and Holloszy (Fitts and Holloszy, 1976) demonstrated that muscle contractile force decreases as lactate levels increase. Additionally, administration of dichloroacetate, an activator of pyruvate dehydrogenase (PDH) through inhibition of pyruvate dehydrogenase kinase, decreases lactate accumulation and increases endurance capacity in untrained subjects (Ludvik et al., 1993). However, as this process involves PDH activation, and thus will increase oxidative metabolism, the decreased lactate accumulation may merely be correlative to the increase in endurance rather than causative. Other causes of fatigue may be intracellular changes, such as changes in pH, decreased ATP levels, or a failure to regulate Ca^{2+} release or reuptake (Dalakas et al., 1998). Since the HIF-1α mediated increase in glycolysis also results in increased lactate production, modulation of HIF-1α in the muscle may have an impact on endurance and fatigue.

Gene transcription in the skeletal muscle is greatly affected both during exercise and recovery following exercise. Expression of interleukin 6, a cytokine that has been proposed to have a large role in fatigue sensation, is markedly increased during exercise (Keller et al., 2001). The transcription of several important metabolic genes is affected by exercise. In a study looking at gene expression immediately following a four hour cycling exercise in untrained patients, Pilegaard et al. (Pilegaard et al., 2000) saw elevated expression of heme oxygenase-1 (HO-1) and pyruvate dehydrogenase kinase 4 (PDK4). During the recovery from exercise, muscles further increased PDK4 expression, and also upregulated hexokinase II (HKII), lipoprotein lipase (LPL), and uncoupling protein 3 (UCP3). In a different study, and of specific relation to HIF-1α, expression of VEGF, and its receptor Flt-1 were seen to be upregulated following exercise in rats (Olfert et al., 2001). Additionally, untrained skeletal muscle has a marked upregulation of HIF-1α, HIF-2α, and EPO mRNA during recovery from exercise (Ameln et al., 2005; Lundby et al., 2005). These transcriptional changes show a coordinated effort by the muscle to adapt to the stress of exercise and become better suited for endurance activities, and give further evidence for an important role for HIF-1α function in the muscle.

The role of HIF-1 in untrained muscle and during acute exercise has been studied, although its function is not yet completely understood. As mentioned above, resting untrained skeletal muscle has stable HIF-1α protein, suggesting that HIF-1 has an important role in maintaining homeostasis in the muscle. This hypothesis was strengthened by the findings of Ameln et al. (Ameln et al., 2005), who recently showed that acute exercise leads to increased stabilization of HIF-1α protein, perhaps

giving the mechanism for the increase in expression of HIF-1 target genes following exercise. However, it is not yet clear exactly what role HIF-1 does play in the way muscles respond during exercise.

Muscular response to endurance training

The ability of the skeletal muscle to acclimate to repeated exertion is central to its role in the body. This ability to acclimate enables it to become better suited and prepared for exercise, something muscle can achieve rather quickly. Endurance training studies have been carried out extensively in humans as well as animal models to understand how muscles undergo this acclimation to exercise. Two main categories that the changes fall under are morphological changes and enzymatic changes, resulting in a change in the profile of the muscle. The end result of endurance training is that the skeletal muscle has improved delivery and utilization of its available oxygen, leading to enhanced performance and endurance. Given that oxygen is central to these changes, it is very likely that the primary hypoxia responsive factor, HIF-1, has a large role in helping the muscle to acclimate to repeated exercise.

The most significant change seen in the muscle as a result of endurance training is increased endurance. However, there are other markers of improved muscle capability beyond just endurance. Two of the more prominent ones are the respiratory exchange ratio (RER) and VO_2max. A measure of fuel utilization, the RER generally has a downward shift following training, indicating an increase in fatty acid oxidation relative to carbohydrate metabolism. VO_2max is the maximal oxygen consumption achievable by the subject, and is closely linked to aerobic metabolic

capacity. Like overall endurance, this parameter also usually increases following endurance training, indicating an increase in oxidative capacity by the subject.

Morphologically, there are two main adaptations a muscle undergoes during endurance training – an increase in capillary density and a shift in fiber type composition. The advantage of increased capillary density is obvious as it allows for increased oxygen and metabolite delivery to the exercising muscle, thus increasing aerobic capacity. Increased capillary density can occur after only six to eight weeks of endurance training; this short of a period has been shown to lead to a 30% increase in capillary density (Hoppeler et al., 1985). The HIF-1α target, VEGF, is of critical importance here as deletion of VEGF in the muscle following development results in a dramatic drop in muscle capillary density and capillary to fiber ratio (Tang et al., 2004).

The shift in fiber type composition allows the skeletal muscle to better take advantage of this increase in oxygen delivery, and also contributes to the changes in VO_2max and RER that are seen in trained patients and animals. In addition to the two main categories (type II fast twitch and type I slow twitch), muscle fibers can be classified according to their metabolic preferences. Type I fibers are oxidative, and rely heavily on aerobic metabolism, while type II fibers can be broken into two major categories: type IIA and type IIB. Type IIB fibers are largely glycolytic, while type IIA fibers are largely oxidative despite being fast-twitch. Endurance training has been shown to cause a shift toward slow twitch fibers in humans (reviewed in Fluck and Hoppeler, 2003). Additionally, trained muscles have a greater percentage of type IIA fibers versus type IIB, indicating an increase in oxidative capacity (Holloszy and

Coyle, 1984). This shift toward an oxidative profile enables a trained muscle to take full advantage of the increased capillary density.

In addition to morphological changes, there are numerous metabolic changes in trained muscle relative to untrained muscle. Generally, these changes increase the muscle's ability to rapidly produce ATP during exercise, especially from the beta-oxidation of fatty acids. Improvements in ATP production generally come in the form of upregulated metabolic enzymes and the resulting increased capacity for oxidative phosphorylation. Increased oxidative phosphorylation is a result of elevated mitochondrial density in the muscle, and upregulation levels of the metabolic enzymes contained therein. In previous studies, endurance training has resulted in an increase of 40% in mitochondrial volume in the skeletal muscle, and significant increases have also been seen in the aerobic metabolic enzymes citrate synthase, β-hydroxyacyl-CoA dehydrogenase, and carnitine palmitoyl transferase (Berthon et al., 1998; Harms and Hickson, 1983; Hoppeler et al., 1985; Schantz et al., 1983).

Oxidative phosphorylation is not the only metabolic pathway upregulated as a result of training. Activity of hexokinase, a HIF-1 target, also increases as a result of endurance training, indicating improved carbohydrate metabolism (Taylor et al., 2005). The benefit of this increase for the muscle is two-fold. First, as the initial enzyme in glycolysis, an increase in hexokinase activity will allow for greater flux into glycolysis, allowing for greater pyruvate and ATP production. Secondly, since muscle lacks glucose-6-phosphatase, any glucose that enters the muscle will be phosphorylated by hexokinase and remain in the muscle to either be metabolized

immediately or stored as glycogen for later use. An increase in hexokinase will thus help ensure there will be enough carbohydrate fuel for the muscle during exercise.

A third metabolic consequence of endurance training is an increase in glycogen storage in the muscle. This is not only a result of the increased in hexokinase activity, but also a result of increased glycogen synthase (Christ-Roberts et al., 2004), and is another way in which a trained muscle is better prepared for exertion. Additionally, endurance-trained muscle is slower to deplete its glycogen stores than untrained muscles, a change which enables muscles to perform longer since they can spare glycogen for when it is absolutely needed (Holloszy and Coyle, 1984).

Although not yet completely understood, the mechanism underlying the acclimation of skeletal muscle to endurance training is coming to light, and some of the key factors regulating the response to endurance training have been identified. Surprisingly, despite the preponderance of HIF-1α targets following exercise, and the importance of angiogenesis to the training response, much of the research into factors regulating the endurance training response has focused on other genes. Two important transcription factors that have a role in upregulating oxidative metabolism are the nuclear respiratory factors 1 and 2 (NRF-1 and 2). NRF-1 and 2 bind to specific response elements of target genes such as mitochondrial transcription factor A (TFAM), cytochrome c, and succinate dehydrogenase subunit B (Scarpulla, 2002). Highlighting the importance of NRF-1, endurance exercise has been shown to increase NRF-1 protein, and a mouse constitutively overexpressing NRF-1 has increased oxidative capacity, as well as increased GLUT4 expression (Baar et al., 2003). However, the NRF-1 transgenic mouse does not have elevated citrate synthase,

cyclooxygenase-IV, or succinate:ubiquinol oxidoreductase, indicating that NRF-1 by itself is not sufficient to cause the training-induced changes. Very little research has been done on a connection between HIF-1 and NRF-1, although the two have parallel expression patterns in postnatal hearts (Nau et al., 2002).

Members of the peroxisome proliferator-activated receptor (PPAR) family have also has been hypothesized to have a role in the muscular response to training. One of them, PPARα, has been shown to upregulate mitochondrial genes in charge of fatty acid oxidation, leading to increased oxidation (Gulick et al., 1994; Vega et al., 2000). The primary member of the PPAR family in the skeletal muscle is PPARδ, which has been shown to have an important role in determining muscle oxidative capacity. In work with a PPARδ transgenic mouse, Wang et al. (Wang et al., 2004) showed that overexpression of PPARδ in the skeletal muscle results in a mouse with a greater proportion of type I oxidative fibers, leading to a mitochondrial content, resistance to obesity, and dramatically increased endurance. Intriguingly, hypoxia has been shown to down-regulate PPARα, and this down-regulation appears to be HIF-1 dependent (Narravula and Colgan, 2001). It is not currently known if this down-regulation extends to PPARδ as well, but the HIF-1 regulated gene DEC1/Stra13 has been shown to inhibit PPARγ-2 (Yun et al., 2002). These findings make it interesting to speculate as to whether HIF-1α has a similar interplay with other members of the PPAR family, in particularly PPARδ.

Another gene that has been shown to possibly have a role regulating the muscle response to endurance training is PPARγ coactivator-1α (PGC-1α), which stimulates the expression of NRF-1 and NRF-2, among other genes (Wu et al., 1999).

In the same study, Wu, et al. also saw that PGC-1α binds with NRF-1 and coactivates it at the TFAM promoter, leading to increased mitochondrial biogenesis. In the skeletal muscle, PGC-1α is normally expressed in type I fibers, and constitutive expression of PGC-1α in the muscle at normal physiological levels results in a transition of type II fibers to being more like type I fibers. This results in the fibers becoming more fatigue resistant in isolated stimulation assessments (Lin et al., 2002).

These three families of genes, the NRFs, PPARs, and PGCs, all have the potential to regulate the changes seen in the muscle. They all increase oxidative capacity and improve muscle performance. Interestingly, not much research has been done on any connections between them and HIF-1, even though the demands of exercise, which induce HIF-1α, are also what lead to their activation, either at the protein level or through transcription (Murakami et al., 1998; Russell et al., 2005; Terada et al., 2002).

In part because of the repeated oxygen stress placed on skeletal muscle during exercise, a role for HIF-1α in the muscular response to exercise and training has been proposed (Hoppeler and Fluck, 2002). Some of the responses seen from muscle during training further corroborate this hypothesis. As hexokinase II and VEGF are two prominent HIF-1α targets, and since an increase in hexokinase and angiogenesis are two common changes following training, a role for HIF-1α can be proposed. Additionally, training under ischemic conditions results in greater citrate synthase activity than exercise with normal blood flow (Esbjornsson et al., 1993; Kaijser et al., 1990). Finally, as mentioned before, transcription of several HIF-1α targets is increased in the muscle following exercise, and transcription of HIF-1α itself is

upregulated following repeated hypoxic exercise (Vogt et al., 2001). Thus it can be hypothesized that HIF-1α has a role in the muscular training response to exercise, although no research has directly addressed this to date.

Experimental Approach

A complete understanding of the role of HIF-1 in skeletal muscle will require a combination of physiological, molecular, and genetic techniques. Through the use of a skeletal muscle-specific knockout of HIF-1α, the direct contributions of HIF-1α to acute exercise and endurance training in the skeletal muscle can be discerned.

Skeletal-muscle-specific deletion of HIF-1α can be accomplished through the LoxP/Cre method for targeted gene deletion (Gu et al., 1994). The LoxP/Cre method involves flanking ("floxing") a specific region of a target gene with the LoxP DNA sequence, which is the recognition site for the Cre recombinase transgene. Upon encountering the LoxP sequence, Cre recombinase makes incisions in the DNA, resulting in DNA between the LoxP sequences being excised and deleted from the genome. Mice with two floxed copies of a gene are referred to as "double-floxed", and can have that gene specifically and completely deleted from the genome of a given tissue. Control of Cre recombinase expression allows for the tissue-specific deletion of the target gene(s). Different gene promoters can be utilized to limit Cre expression to specific tissues or time points.

Studying the role of HIF-1α in the skeletal muscle will be done using mice that are homozygous for an allele of HIF-1α in which exon 2 has been flanked by the LoxP sequence in the genomic DNA (Ryan et al., 2000). Under normal conditions (absence

of the Cre transgene), HIF-1α expression and activity are unaffected by the floxing. The presence of Cre recombinase leads to excision of exon 2, which removes the helix-loop-helix motif from the HIF-1α protein and prevents it from dimerizing with ARNT and turning on transcription of HIF-1 target genes. This effectively deletes HIF-1α from the proteome of the tissue being studied. Cre recombinase expression can be directed to the skeletal muscle by coupling the Cre transgene with the muscle creatine kinase (MCK) promoter (Bruning et al., 1998). The mouse with double-floxed HIF-1α has already been created in the Randall Johnson laboratory at UCSD. The Ronald Kahn laboratory at the Joslin Diabetes Center in the Harvard Medical School created and has generously provided us with the MCK-Cre mouse. Crossing the HIF-1α double-floxed mice with the MCK-Cre mice will create a mouse lacking HIF-1α specifically in the skeletal muscle, and enable the study of the role of HIF-1α specifically in the skeletal muscle.

The second chapter of this thesis deals studies the function of HIF-1α in untrained skeletal muscle. Once the HIF-1α/MCK-Cre mouse has been created, various physiological and molecular techniques can be employed to determine the role of HIF-1α in the skeletal muscle at rest and during acute exercise. These techniques will include basic histology, endurance assessments, enzyme activity assays, and gene expression measurements.

The third chapter of this thesis details the role of HIF-1α in the muscular response to repeated exercise. Endurance training of HIF-1α/MCK-Cre mice will allow insight into HIF-1α activity during the performance-oriented changes that take

place in the muscle. Analysis of the muscle following training will reveal what role, if any, HIF-1α had during training.

The final chapter of this thesis will place the findings of these studies in context with other current research in the field. Ongoing collaborations will be highlighted, as well as future directions for the study of HIF-1α in the skeletal muscle.

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II. LOSS OF SKELETAL MUSCLE HIF-1α

RESULTS IN ALTERED EXERCISE ENDURANCE

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Loss of Skeletal Muscle HIF-1α Results in Altered Exercise Endurance

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The physiological flux of oxygen is extreme in exercising skeletal muscle. Hypoxia is thus a critical parameter in muscle function, influencing production of ATP, utilization of energy-producing substrates, and manufacture of exhaustion-inducing metabolites. Glycolysis is the central source of anaerobic energy in animals, and this metabolic pathway is regulated under low-oxygen conditions by the transcription factor hypoxia-inducible factor 1α (HIF-1α). To determine the role of HIF-1α in regulating skeletal muscle function, we tissue-specifically deleted the gene encoding the factor in skeletal muscle. Significant exercise-induced changes in expression of genes are decreased or absent in the skeletal-muscle HIF-1α knockout mice (HIF-1α KOs); changes in activities of glycolytic enzymes are seen as well. There is an increase in activity of rate-limiting enzymes of the mitochondria in the muscles of HIF-1α KOs, indicating that the citric acid cycle and increased fatty acid oxidation may be compensating for decreased flow through the glycolytic pathway. This is corroborated by a finding of no significant decreases in muscle ATP, but significantly decreased amounts of lactate in the serum of exercising HIF-1α KOs. This metabolic shift away from glycolysis and toward oxidation has the consequence of increasing exercise times in the HIF-1α KOs. However, repeated exercise trials give rise to extensive muscle damage in HIF-1α KOs, ultimately resulting in greatly reduced exercise times relative to wild-type animals. The muscle damage seen is similar to that detected in humans in diseases caused by deficiencies in skeletal muscle glycogenolysis and glycolysis. Thus, these results demonstrate an important role for the HIF-1 pathway in the metabolic control of muscle function.

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Introduction

During exercise in normoxia, the partial pressure of oxygen in muscle tissue has been shown to dip to as low as 3.1 mm Hg, whereas in the capillary, it remains at 38 mm Hg (Hoppeler et al. 2003). In order to maintain effort, skeletal muscle exertion must be able to rely on pathways designed to help the tissue cope with oxygen stress after oxygen delivery capacity is exceeded. A switch between aerobic and non-aerobic metabolism during strenuous exertion requires mechanisms to adjust metabolic function, and this need is acute in extended exertion in skeletal muscle. It is clear that the transcription factor hypoxia-inducible factor 1α (HIF-1α) is an essential factor in maintenance of ATP levels in cells (Seagroves et al. 2001). In fact, although HIF-1α is typically thought of as acting only during hypoxia, its loss has an effect on both normoxic and hypoxic ATP levels in a number of tissue types (Seagroves et al. 2001; Cramer et al. 2003), and this implicates the factor in regulation of metabolic function even during conditions of normal physiologic oxygenation.

In skeletal muscle, signaling of fatigue has been studied extensively, and signaling of exhaustion involves, to some degree, elevated systemic lactic acid, a by-product of the glycolytic pathway of metabolism (Myers and Ashley 1997). Thus, the glycolytic pathway is intrinsically involved in muscle function and fatigue, and this in turn is linked to the response to hypoxia. To understand how the primary hypoxia-responsive transcription factor controls skeletal muscle function, we targeted mouse skeletal muscle for tissue-specific deletion of *HIF-1α* via the use of a conditionally targeted allele of the gene (Ryan et al. 2000; Schipani et al. 2001). This mouse strain was crossed into a strain transgenic for the skeletal-muscle-specific muscle creatine kinase (MCK) promoter, which drives expression of the *cre* recombinase gene (Bruning et al. 1998; Sauer 1998). We found that loss of the regulation of hypoxic response in muscle has a profound effect on the function of the muscle during exertion, with effects that mimic human metabolic myopathies.

Results/Discussion

In 4-mo–old mice with the skeletal-muscle *HIF-1α* gene knocked out (HIF-1α KOs), the frequency of excision was evaluated through real-time PCR techniques. We saw deletion

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Abbreviations: B-HAD, beta-hydroxyacyl CoA dehydrogenase; CS, citrate synthase; GLUT4, glucose transporter 4; HIF-1α, hypoxia-inducible factor 1α; HIF-1α KO, skeletal-muscle HIF-1α knockout mouse; HMP, hexose monophosphate; LDH-A, lactate dehydrogenase-A; MCK, muscle creatine kinase; PAS, periodic acid–Schiff; PCNA, proliferating cellular nuclear antigen; PCr, phosphocreatine; PFKD, phosphofructokinase disease; PFK-M, muscle-specific form of phosphofructokinase; PGK, phosphoglycerate kinase; PK, pyruvate kinase; RER, respiratory exchange ratio; VEGF, vascular endothelial growth factor; WT, wild-type

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Table 1. Excision of HIF-1α in Various Tissues

Tissue	Average Percent Deletion
Gastrocnemius	54.9 ± 4.9
Heart	41.6 ± 14
Uterus	1.00
Liver	2.00

Deletion levels are the average percent of HIF-1α deleted ± SE.
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frequencies consistent with those described previously for this *cre* recombinase transgene (Bruning et al. 1998) with some variation in penetration; mean frequency of deletion was 54.9%, with the highest frequency of muscle-specific deletion of HIF-1α being 72% in the gastrocnemius of 4-mo–old mice homozygous for the *loxP*-flanked allele (Table 1). This transgene is expressed at a lower level in cardiac tissue, and cardiac deletion was detected (Table 1); however, none of the phenotypes described below were seen in cardiac myocyte-specific deletions of HIF-1α (Figure 1A). Gross muscle sections were evaluated histologically to evaluate both vascularization and fiber type (Tables 2 and 3), and ultrastructurally to determine number of mitochondria (Figure 1B). No changes were detected in any of these features in HIF-1α KOs, except for a slight but statistically significant decrease in type IIA fibers in the soleus muscles (Table 3). Similar hematocrit and blood hemoglobin levels were seen in HIF-1α KOs and wild-type (WT) mice (Figure 2).

As can be seen in Table 4, significant changes in HIF-1α–dependent gene expression occur in muscle during exercise, including changes in genes involved in glucose transport and metabolism. Vascular endothelial growth factor (VEGF), which increases vascular permeability, and glucose transporter 4 (GLUT4), the muscle-specific glucose transporter, show increased levels in exercise and likely increase the availability of glucose to the muscle. The muscle-specific form of phosphofructokinase (PFK-M), phosphoglycerate kinase (PGK), and lactate dehydrogenase-A (LDH-A) are also up-regulated at the mRNA level by exercise, and this up-regulation is inhibited by the loss of HIF-1α, further demonstrating that HIF-1α is important for transcriptional response during skeletal muscle activity.

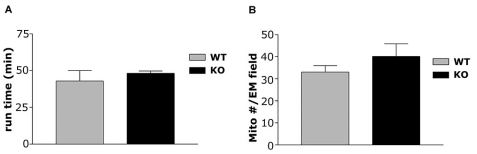

Figure 1. Exercise Capacity of Cardiac HIF-1α KOs and HIF-1α/MCK/*cre* Mitochondrial Density

(A) Mice lacking cardiac HIF-1α perform no differently in endurance running trials than WT mice, showing that the increase in exercise capacity seen in MCK/Cre mice is due to deletion of HIF-1α in skeletal muscle, not cardiac tissue.
(B) Mice lacking skeletal muscle HIF-1α have a slight but non-significant increase in mitochondrial density as measured by the number of mitochondria per electron microscope field of view.
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In Table 5, we show the changes in enzymatic activity in a number of key glycolytic enzymes affected by deletion of HIF-1α. As can be seen from the data, several of the enzymes assayed showed a decrease in activity in response to exercise. In particular, the activity of one of the key rate-limiting enzymes, PFK, was significantly lower following exercise in HIF-1α KOs compared to WT mice, indicating that HIF-1α KOs may have difficulty maintaining optimal PFK activity. The responses of other glycolytic enzymes to exercise were fairly similar between WT mice and HIF-1α KOs. These include no significant changes in phosphoglucose isomerase activity and significant, yet similar, decreases in aldolase, glyceraldehyde 3-phosphate dehydrogenase, and PGK activities. An exception to this is that WT muscles were able to significantly increase pyruvate kinase (PK) activity (see Table 4; $p < 0.05$). LDH activity was also increased in the WT mice, although the level did not reach statistical significance. Activities of both PK and LDH were not significantly changed in HIF-1α KO muscles following exercise. Increased activities of PK, and subsequently LDH, could be expected to lead to increased levels of lactate in the WT mice relative to HIF-1α KOs.In Figure 3A, it can be seen that the decrease in PFK activity in the HIF-1α KOs is correlated with a trend approaching significance ($p = 0.10$) toward an increased amount of hexose monophosphates (HMPs), which are pre-PFK glycolytic metabolites, following stimulation of the HIF-1α KO muscle. This increase was not due to differences in glucose uptake, since animals of both genotypes were able to

Table 2. Fiber Typing of Gastrocnemius Muscle

Gastrocnemius	WT Superficial	Deep	HIF-1α KO Superficial	Deep
Fiber type I (%)	0.0 ± 0.0	7.8 ± 3.1	0.0 ± 0.0	10.2 ± 3.6
Fiber type IIA (%)	94.4 ± 3.1	44.1 ± 8.6	90.8 ± 4.2	42.9 ± 9.8
Fiber type IIB (%)	5.6 ± 3.1	48.0 ± 6.3	9.2 ± 4.2	47.0 ± 7.9
Fiber size (μm^2)	3929 ± 46	2670 ± 147	4354 ± 387	3247 ± 129
Capillary density (number/mm^2)	274 ± 16	645 ± 50	232 ± 22	500 ± 19
Capillary/fiber ratio	1.04 ± 0.06	1.62 ± 0.08	1.08 ± 0.07	1.55 ± 0.02

Values are percent ± SE.
DOI: 10.1371/journal.pbio.0020288.t002

Table 3. Fiber Typing of Soleus Muscle

Soleus	WT	HIF-1α KO
Fiber type I (%)	64.1 ± 1.5	68.2 ± 0.8
Fiber type IIA (%)	30.1 ± 1.5*	25.2 ± 0.7*
Fiber type IIB (%)	5.8 ± 0.9	6.5 ± 0.6

Values are percent ± SE.
* $p < 0.05$, WT vs. KO.
DOI: 10.1371/journal.pbio.0020288.t003

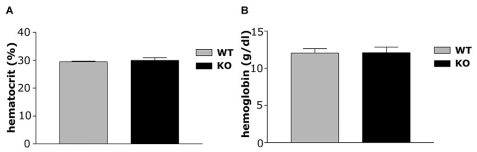

Figure 2. Hematocrit and Hemoglobin Levels in HIF-1α KOs and WT Mice
(A) Hematocrit levels are virtually identical in both HIF-1α KOs (n = 3) and WT (n = 4) mice, indicating that loss of HIF-1α in skeletal muscle does not affect oxygen carrying capacity of the blood.
(B) In addition to similar hematocrit levels, WT mice and HIF-1α KOs have very close blood hemoglobin levels.
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significantly increase intramuscular glucose to a similar degree (Figure 3B). Consistent with decreased flow through the glycolytic pathway, however, the increased amount of HMPs was correlated with increased muscle glycogenolysis (Figure 3C) and increased depletion of phosphocreatine (PCr) (Figure 3D), with a resultant decrease in the PCr/ATP ratio in HIF-1α KO muscle (Figure 3E), although there was only a nonsignificant drop in overall muscle ATP concentrations (Figure 3F). Intramuscular levels of lactate did increase in both HIF-1α KOs and WT mice during stimulation, although lactate accumulation did not differ significantly between them (Figure 3G). In order to evaluate whether these changes had any effect on overall muscle force, we measured force and calcium release in isolated single fibers; as can be seen in Figure 4A and 4B, there were no significant changes in these parameters, indicating that the muscle can compensate at this level for the metabolic changes induced by loss of HIF-1α.

Given altered levels of glycolytic throughput without significant changes in intramuscular ATP levels, it is likely that there is increased activity of oxidative pathways in the HIF-1α KO muscle. Increased muscle oxidative activity is typical in patients with myopathies involving muscle glycolysis or glycogenolysis, including phosphofructokinase disease

Table 4. Relative Gene Expression Levels

Enzyme	WT Resting	Postexercise	Percent Increase	HIF-1α KO Resting	Postexercise	Percent Increase
VEGF	1.00 ± 0.11	1.73 ± 0.58	72.7	1.21 ± 0.24	1.36 ± 0.64	12.7
GLUT4	1.00 ± 0.09*	1.67 ± 0.32*	66.5	1.25 ± 0.24	1.50 ± 0.40	20.2
PFK-M	1.00 ± 0.08*	1.54 ± 0.22*	54.1	1.18 ± 0.18	1.59 ± 0.37	34.7
PGK	1.00 ± 0.06**	1.93 ± 0.35**	93.4	1.34 ± 0.22	2.30 ± 0.64	71.9
LDH-A	1.00 ± 0.05**	1.69 ± 0.21**	68.6	1.19 ± 0.14	1.60 ± 0.24	34.7

Expression levels are means relative to resting WT for each gene ± SE.
Percent increase indicates percent increase of postexercise average gene expression over resting average.
*$p < 0.05$, rest vs. postexercise; **$p < 0.01$, rest vs. postexercise.
DOI: 10.1371/journal.pbio.0020288.t004

Table 5. Glycolytic Enzyme Activity Levels from Gastrocnemius Muscles

Enzyme	WT Resting	Postexercise	HIF-1α KO Resting	Postexercise
PGI	1.46 ± 0.03	1.52 ± 0.02	1.50 ± 0.03	1.47 ± 0.02
PFK	1.26 ± 0.09	1.17 ± 0.04 *	1.40 ± 0.05 **	1.06 ± 0.02 */**
Aldolase	1.13 ± 0.04 **	0.89 ± 0.04 **	1.07 ± 0.03 **	0.95 ± 0.01 **
GAPDH	2.37 ± 0.06 **	1.81 ± 0.06 **	2.37 ± 0.08 **	1.86 ± 0.04 **
PGK	1.38 ± 0.02 **	1.20 ± 0.03 **	1.41 ± 0.04 **	1.18 ± 0.02 **
PK	1.47 ± 0.03 **	1.58 ± 0.03 **	1.48 ± 0.03	1.52 ± 0.03
LDH	3.34 ± 0.10	3.91 ± 0.38	3.54 ± 0.10	3.56 ± 0.07

Activities are in U/mg protein ± SE.
GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PGI, phosphoglucose isomerase.
*$p < 0.05$, WT vs. HIF-1α KO for given exercised or resting state; **$p < 0.05$, rest vs. postexercise within given genotype.
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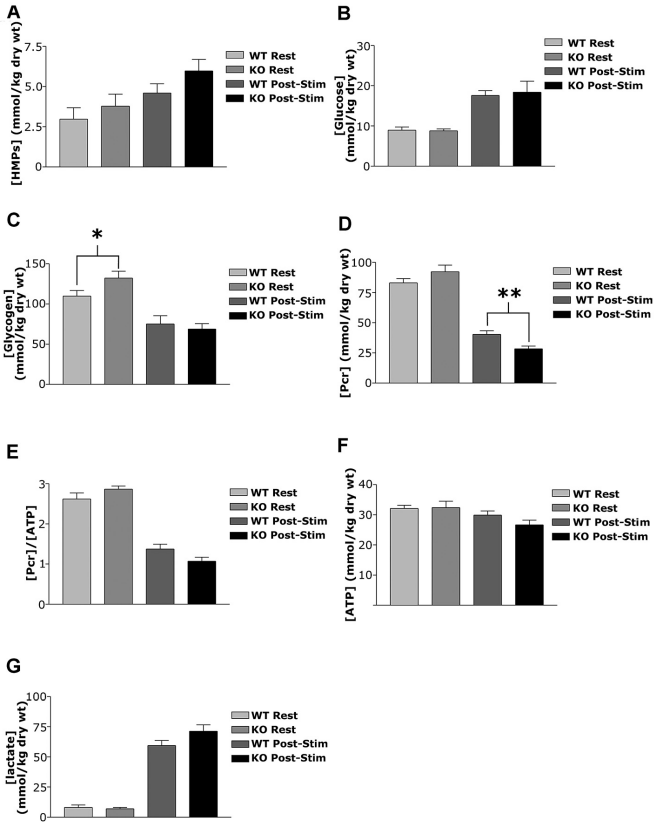

Figure 3. Intramuscular Metabolite Levels at Rest and Following Stimulation

(A) Glycolytic intermediates were measured from gastrocnemius muscles following the isolated stimulation protocol. Resting values represent levels in the unstimulated gastrocnemius from the same animals. HIF-1α KOs had a trend toward greater accumulated levels of HMPs during the stimulation protocol, although the difference did not reach statistical significance ($p = 0.10$). This difference could be indicative of a blockage in the glycolytic pathway at PFK.

(B) No significant differences were seen between HIF-1α KOs and WT intramuscular glucose levels at rest or following stimulation. Both HIF-1α KO and WT muscles were able to significantly increase glucose uptake, leading to greater levels of intramuscular glucose in response to stimulation (WT, $p < 0.001$; KO, $p < 0.05$).

(C) HIF-1α KOs have more stored glycogen than do WT mice. Glycogen levels were measured following the same stimulation protocol as in (B). The change in glycogen from rest to poststimulation was also greater in the HIF-1α KOs, indicating that they metabolized more glycogen in response to stimulation ($p < 0.01$; $*p < 0.05$, WT at rest vs. KO at rest).

(D) HIF-1α KOs utilize more PCr in response to stimulation than do WT mice. Similar levels of PCr were seen at rest, but HIF-1α KOs metabolized significantly more during stimulation ($p < 0.05$) and had much lower levels following the protocol ($**p < 0.01$, WT poststimulation vs. KO poststimulation).

(E) A trend toward lower PCr/ATP concentration ratios was seen in HIF-1α KOs relative to WT mice following stimulation, although the difference did not quite reach statistical significance ($p < 0.10$). A trend toward a greater drop from rest to poststimulation in the PCr/ATP ratio was also seen in HIF-1α KOs following stimulation ($p < 0.10$), indicating that they had to rely more heavily on PCr for ATP generation.

(F) Slight but nonsignificant differences were seen in whole-muscle ATP levels at rest or following stimulation. Although HIF-1α KOs exhibited altered substrate utilization, they were able to meet their ATP demands during the protocol.

(G) Both HIF-1α KOs and WT animals produced significant intramuscular lactate during the stimulation protocol; however, there was no significant difference in the amount produced by either genotype. Resting intramuscular lactate levels were also similar for WTs and HIF-1α KOs.

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(PFKD) and McArdle's disease (Vissing et al. 1996). We analyzed the activity of citrate synthase (CS), a key allosteric enzyme of the citric acid cycle, in WT and HIF-1α KO muscle (Figure 5A), and found that it was up-regulated in HIF-1α KOs. CS is a mitochondrial enzyme that responds to decreases in ATP concentration allosterically, allowing for increased oxidative activity in the mitochondria. In addition, significant up-regulation of the mitochondrial enzyme beta-hydroxyacyl CoA dehydrogenase (B-HAD) was seen in HIF-1α KO muscle (Figure 5B). B-HAD is also affected by energy levels in the cell, and decreases in NADH/NAD^+ concentration ratios cause the enzyme to increase mitochondrial oxidation of fatty acids (Nelson and Cox 2000). Increased activity of oxidative pathways in the muscle should result in more rapid lactate clearance, as in fact occurs in PFKD patients during exercise; this phenomenon gives rise to a "second wind" in these patients, and under some circumstances allows for an increase in exercise endurance (Vissing et al. 1996; Haller and Vissing 2002), although this was disputed in one recent study (Haller and Vissing 2004). This decreased lactate accumulation postexercise clearly occurs in the HIF-1α KOs, as can be seen in Figure 5C. This systemically lower level of lactate postexercise indicates that there may be a shift toward a more oxidative metabolism in skeletal muscle.

As mentioned above, patients with muscle glycolytic deficiencies demonstrate both increased exercise-induced muscle damage and a "second wind"; the latter phenomenon allows them to exercise for extended periods of time at submaximal levels. This is thought to be due to an increase in rates of oxidative ATP production, and a decreased utilization of and need for muscle glycogen (Vissing et al. 1996; Haller and Vissing 2002). To assess whether this is also the

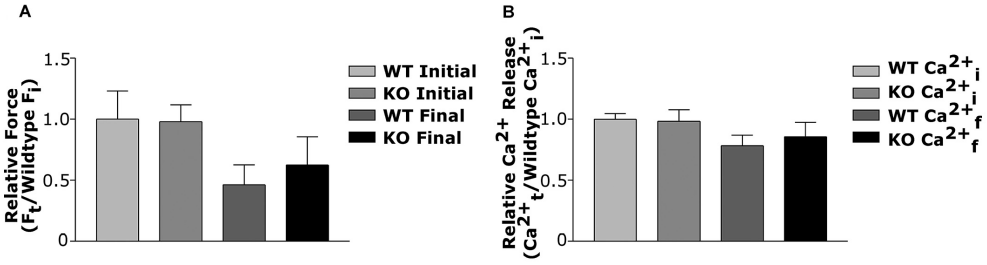

Figure 4. Force Generation and Ca^{2+} Release in Isolated Muscle Fibers during Stimulation

(A) No differences were seen in total force generation in isolated muscle fibers. Mechanically dissected fibers from the flexor brevis muscle were subjected to a fatiguing protocol. Neither initial nor final forces differed between HIF-1α KO and WT fibers.

(B) Ca^{2+} release and reuptake in HIF-1α KO and WT fibers was not different during the stimulation protocol. Ca^{2+} levels were measured in individual fibers through use of fura-2 Ca^{2+} indicator. The altered substrate utilization did not affect the ability of the fibers to maintain proper Ca^{2+} flux.

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case in the HIF-1α KOs, both WT mice and HIF-1α KOs were subjected to endurance tests to assess muscle function. To first determine whether HIF-1α KOs were capable of extended activity during exercise, the animals were given a swimming endurance test. As can be seen in Figure 6A, HIF-1α KOs were capable of significantly longer-duration swimming activity when compared to matched WT controls ($p < 0.05$).

Further testing was done to determine the parameters of this increased endurance. HIF-1α KOs were run on an enclosed treadmill, with a 5° incline and an initial velocity of 10 m/min, with an increase in velocity every 5 min. In their first runs, HIF-1α KOs again had significantly greater endurance, as shown by their consistently longer run times compared to WT controls ($p < 0.01$, Figure 6B).

As it has been shown that muscle groups and fibers respond differently to eccentric exercise (i.e., downhill running) than to concentric exercise (i.e., uphill running) (Nardone and Schieppati 1988), mice from both genotypes were run on a 10° decline with the same velocity and time parameters as in the uphill runs. Eccentric exercises have been shown to recruit primarily fast-twitch glycolytic fibers for contraction, as opposed to the traditional recruitment of slower, smaller, oxidative motor units in concentric contraction, where animals with an increased capacity for muscle oxidation would be at an advantage (Nardone and Schieppati 1988). Now, the trend from swimming and uphill running tests was reversed, with WT mice able to run for a significantly longer time than HIF-1α KOs ($p < 0.05$, Figure 6C). Within genotypes, WT mice ran for significantly longer times downhill than uphill ($p < 0.01$); HIF-1α KOs did the reverse, and ran for significantly shorter times downhill than uphill ($p < 0.05$). Substrate utilization confirms the shift toward glycolytic fibers in downhill running; both genotypes had higher average respiratory exchange ratio (RER) values when running downhill compared with running uphill (Figure 6D and 6E).

PFKD and McArdle's disease demonstrate significant myopathic effects in muscle, including soreness and cramping induced by bouts of exercise. After 1 d of recovery from endurance testing, HIF-1α KOs had increased levels of the MM isoform of creatine kinase in their serum (unpublished

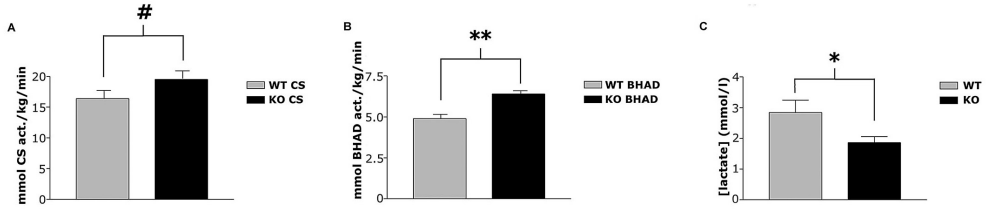

Figure 5. Oxidative Metabolism and Serum Lactate Production in HIF-1α KOs and WT Mice

(A) HIF-1α KOs have higher resting levels of CS activity. CS is an enzyme in the Krebs cycle that can be regulated allosterically by ATP levels. Increased CS activity is indicative of increased muscle oxidative capacity, which is common in patients with glycogenolytic or glycolytic myopathies ($^{\#}p < 0.10$, KO vs. WT).

(B) HIF-1α KOs have higher resting levels of B-HAD activity, which is indicative of a greater ability to oxidize fatty acids ($^{**}p < 0.01$, WT vs. KO).

(C) Lower serum lactate levels were seen in HIF-1α KOs following a timed 25-minute run ($^{*}p < 0.05$, WT vs. KO).

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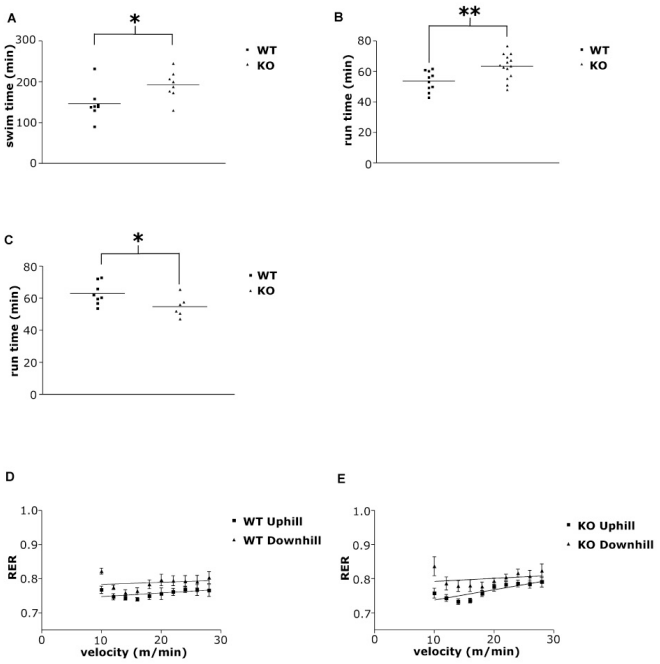

Figure 6. Endurance Capabilities of Untrained Mice

(A) HIF-1α KOs have greater endurance in swimming tests as shown by their ability to swim on average more than 45 min longer than WT (*$p < 0.05$, WT vs. KO).

(B) HIF-1α KOs have greater endurance than WT mice in uphill running tests. Although only a 10-min difference is seen between run times, it is to be noted that because of the protocol, this 10 min included two velocity increases (**$p < 0.01$, WT vs. KO).

(C) HIF-1α KOs have less endurance than WT mice in downhill running tests. The same protocol was used as in Figure 4A, except the mice were run on a 10° decline (*$p < 0.05$, WT vs. KO).

(D) RER uphill vs. downhill in WT mice. As would be expected from eccentric exercises relying more heavily on glycolytic fibers, the RER values are higher in mice running downhill than in those running uphill.

(E) RER uphill vs. downhill in HIF-1α KOs. Once again, higher RER values are observed for mice running downhill than those running uphill.

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data), indicative of skeletal muscle damage. To further investigate this finding, mice were run on a treadmill daily for 4 d. By the second day, the trend for increased endurance in the HIF-1α KOs was absent, and by the final day, HIF-1α KOs were running for significantly shorter times than they had on the first day ($p < 0.01$, Figure 7A). In addition, a repeated measures ANOVA performed on run times showed that the response of the HIF-1α KOs to the protocol was significantly different than that of the WT mice ($p < 0.05$). Histological examination of gastrocnemius tissue following 1 d of recovery revealed significantly greater amounts of muscle damage in HIF-1α KO tissue than WT tissue (Figure 7B). Staining of the tissue for proliferating cellular nuclear antigen (PCNA) and counts of positive nuclei (Olive et al. 1995) also revealed more cell division in HIF-1α KOs than in WTs, another indication that HIF-1α KOs had been subject to greater tissue damage (Figure 7C and 7D).

As noted above, both PFKD and McArdle's disease are marked by increased resting intramuscular levels of glycogen, a failure of serum lactate to rise during exertion, an exercise-induced "second wind," and signs of muscle damage following exertion, including elevated levels of creatine kinase in the serum (Tarui et al. 1965; Layzer et al. 1967). In addition, PFKD is characterized by elevated levels of HMPs (Tarui et al. 1965; Layzer et al. 1967; Argov et al. 1987; Grehl et al. 1998) and greater PCr utilization during contraction (Argov et al. 1987; Grehl et al. 1998). We see many of these hallmarks of muscle deficiencies in glycolytic processing in HIF-1α KOs. The effects are not likely due to glucose uptake, as WT and HIF-1α KO intramuscular glucose levels were not different at rest or following stimulation (see Figure 3B), and both types of mice responded similarly to a glucose tolerance test (Figure 8A). Periodic acid–Schiff (PAS) staining of tissue from mice of both genotypes gave further demonstration of increased glycogen levels in resting muscles from HIF-1α KOs (Figure 8B).

Given the differences in performance observed in the HIF-1α KOs in eccentric and concentric exercise, it is clear that the HIF-1 pathway and hypoxic response have a central role in determining the capacity for work and endurance through regulation of glycolysis. It is also clear that these mice will provide an important model system to investigate the physiology of muscle response during work and oxygen depletion, and may be useful as a model for a group of very debilitating myopathic syndromes in humans.

Materials and Methods

Mouse strains and crosses. Mice were generated from *HIF-1α loxP*-flanked allele mouse stocks backcrossed into a C57Bl6/J background. These were crossed into a C57Bl6/J strain containing the *MCK/cre* transgene. Controls were in all cases littermates that were genotyped as containing only the *loxP*-flanked *HIF-1α* allele or only the *MCK/cre* transgene. No phenotypic differences were seen in the two controls, so they were considered interchangeably as WT control animals.

Genotyping and real-time PCR for HIF-1α deletion. Mice from the above crosses were genotyped using DNA extracted from tail sections.

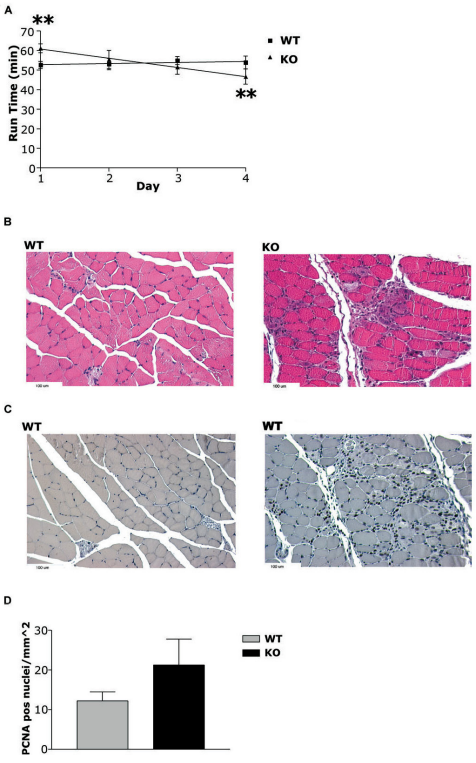

Figure 7. Increased Muscle Damage in HIF-1α KOs Following Repeated Exercise

(A) WT mice and HIF-1α KOs underwent a 4-d endurance test, in which animals were run to exhaustion on each of four successive days with a minimum of 22 h rest between trials. HIF-1α KOs demonstrated initially greater endurance under the protocol; however, by the second day, their endurance advantage was eliminated, and by the fourth day, HIF-1α KOs were running for a significantly shorter time (**$p < 0.01$) than on the first day, while WT animals were running for approximately similar times as on the first day. Repeated measures ANOVA revealed that the decrease in performance on each successive day was unique to HIF-1α KOs ($p < 0.05$).

(B) Example of hematoxylin and eosin staining of gastrocnemius muscles after 1 d of recovery by mice after the 4-d endurance test. Evidence of greater damage can be seen in HIF-1α KO muscles compared to WT muscles.

(C) Example of PCNA staining of gastrocnemius muscles from exercised mice, demonstrating increased levels of muscle regeneration in HIF-1α KOs.

(D) Number of PCNA-positive nuclei per square millimeter in gastrocnemius muscles of WT mice ($n = 5$) and HIF-1α KOs ($n = 7$) that ran repeatedly for 4 d. Although HIF-1α KOs have almost twice as many PCNA-positive nuclei per square millimeter, the difference is not significant, because of wild variations in that population. F-test analysis of the data reveals that the variance is much greater in the HIF-1α KO population than the WT population ($p < 0.05$).

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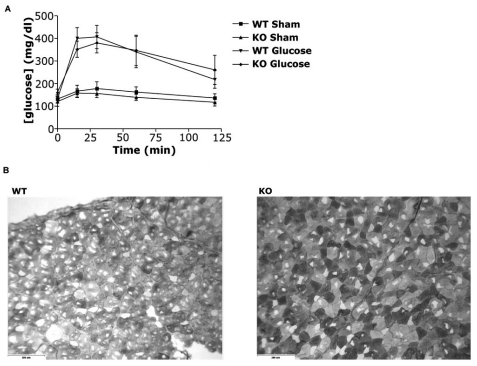

Figure 8. Glucose Tolerance and Glycogen Storage

(A) No significant differences were seen in resting blood glucose levels in HIF-1α KOs or WT mice. Following injection of glucose at a dosage of 2 g/kg, no differences were seen in the maximum levels of blood glucose or the rate of glucose disappearance in either genotype.

(B) Representative PAS staining of gastrocnemius muscle from WT mice and HIF-1α KOs. HIF-1α KOs demonstrate darker staining, indicating more stored glycogen.

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DNA was then extracted from the gastrocnemius, heart, liver, and uterus of eight 4-mo–old, *loxP*-flanked *HIF-1α*–positive and *MCK/cre*–positive mice. *HIF-1α* levels were measured by real-time PCR analysis using the Universal PCR Master Mix Kit (Applied Biosystems, Foster City, California, United States) and the ABI Prism 7700 Sequence Detector (Applied Biosystems). Conditions for the PCR were one 10-min incubation at 95 °C (polymerase activation), followed by 40 cycles of 15 s at 95 °C (denaturation) and 1 min at 60 °C (anneal/extend). The degree of excision was calculated by comparing *HIF-1α* DNA levels to *c-Jun* DNA levels. *HIF-1α* real-time PCR primers and probe were as follows: forward primer, HIFLOX501/F 5′-CTATGGAGGCCAGAAGAGGGTAT-3′; reverse primer, HIFLOX574/R 5′-CCCACATCAGGTGGCTCATAA-3′; probe, HIFLOX/P 5′-(6FAM)AGATCCCTTGAAGCTAG(MGBNFQ)-3′.

Muscle histology and electron microscopy. Paraffined gastrocnemius sections were deparaffinized and stained with Gill II hematoxylin. Sections were then washed successively in water, a bluing agent, water again, and 95% ethanol, and restained with eosin. Hematoxylin and eosin staining was performed by the University of California at San Diego (UCSD) Cancer Center Histology Resource (La Jolla, California, United States). Imaging was performed on sections mounted on slides using Cytoseal 60 (VWR, West Chester, Pennsylvania, United States). Electron microscopy was performed by standard methods on gastrocnemius muscle. Briefly, fixation was by 25.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). Postfix was in 1% osmium tetroxide. The section was stained in 2% uranyl acetate in sodium maleate buffer (pH 5.2), then placed in Epon resin (VWR, West Chester, Pennsylvania, United States), and cured overnight at 60 °C. Fiber typing was performed using the metachromatic dye ATPase method (Ogilvie and Feeback 1990). PAS staining was performed as has been described (Bancroft and Stevens 1996).

Assessment of exhaustion. Untrained, age-matched WT mice and HIF-1α KOs (WT, $n = 10$; KO, $n = 14$) were run either on an Omnipacer treadmill (Columbus Instruments, Columbus, Ohio, United States) or on an enclosed-chamber modular treadmill (Columbus Instruments) with a 5° incline at an initial velocity of 10 m/min. Velocity was increased by 2 m/min every 5 min during the assessment. Exhaustion was determined to be the point at which the animal would not resume running when provoked through a low-voltage power grid. Gas flow (O_2 and CO_2) into and out of the enclosed chamber treadmill was monitored using the Paramax O_2 sensor and a CO_2 sensor (Columbus Instruments) and analyzed using Oxymax software (Columbus Instruments) to determine metabolic parameters. The downhill running assessment (WT, $n = 8$; KO, $n = 6$)

was carried out in the enclosed-chamber modular treadmill at a 10° decline using the same protocol as above.

In the swimming exhaustion assessment, a second group of WT and HIF-1α KOs ($n = 8$ for each class) was placed in a 30 °C water bath with mild turbulence. Exhaustion was determined to be the point at which the animal experienced three successive periods below the surface of more than 3 s.

Isolated stimulation and metabolic analysis. The Achilles tendon was surgically freed from live, anesthetized mice (WT, $n = 8$; KO, $n = 6$) and attached to a force transducer to record contractile force. Muscles were electrically stimulated through excitation of the sciatic nerve. Stimulation was in the form of 8–10-V direct titanic contractions using 200-ms trains at 70 Hz with 0.2 ms duration. Initial frequency of tetanic contraction was one every 8 s and was increased every 2 minutes to one every 4 s and one every 3 s, up to the end point of 6 min. Isolated muscles were then immediately harvested and snap-frozen for ATP, lactate, phosphocreatine, and glycogen analyses. Samples were freeze-dried and analyzed by enzymatic assay as has been previously described (Bergmeyer 1974). The unstimulated gastrocnemius muscle from each mouse was used as a resting control.

Real-time PCR measurement of gene expression. For basal gene expression levels, total RNA was isolated from gastrocnemius tissue from seven WT and five HIF-1α KOs using RNA-Bee (Tel-Test, Friendswood, Texas, United States). Reverse transcription was performed using the Superscript First Stand Synthesis System for RT-PCR (Invitrogen, Carlsbad, California, United States). Amplification was performed using the ABIPrism 7700 as described above. Reverse transcription real-time PCR primers and probes were as follows. For PGK-1: reverse primer, PGK/R 5′-CAGGACCATTC-CAAACAATCTG-3′; forward primer, PGK/F 5′-CTGTGGTACTGA-GAGCAGCAAGA-3′; probe, PGK/P 5′-(6~FAM)TAGCTCGACCCA-CAGCCTCGGCATAT(TAMRA)-(phosphate)-3′. For VEGF-A: reverse primer, VEGF/R 5′-ATCCGCATGATCTGCATGG-3′; forward primer, VEGF/F 5′-AGTCCCATGAAGTGATCAAGTTCA-3; probe, VEGF/P (6~FAM)TGCCCACGTCAGAGAGCAACATCAC(BHQ~6~FAM). For GLUT4: reverse primer, GLUT-4/R 5′-CCCATGCCGACAATGAAGTT-3′; forward primer, GLUT-4/F 5′-TGTGGCCTTCTTTGAGATTGG-3′; probe, GLUT-4/P 5′(6-FAM)TGGCCCCATTCC-CTGGTTCATT(BHQ1-Q)-3′. For PFK-M: reverse primer, PFK-M/R 5′-AAGTCGTGCAGATGGTGTTCAG-3′; forward primer, PFK-M/F 5′-GCCACGGTTTCCAATAACGT-3′; probe, PFK-M/P 5′-(6-FAM)CCTGGGTCAGACTTCAG-CATCGGG(BHQ1-Q)-3′. For LDH-A: reverse primer, LDH-A/R 5′-ATGCACCCGCCTAAGGTTCTT-3′; forward primer, LDH-A/F 5′-TGCCTACGAGGTGATCAAGCT-3′; probe, LDH-A/P 5′-(6-FAM)TGGCAGACTTGGCTGAGAGCAT(BHQ1-Q)-3′.

For changes in gene expression due to exercise, age-matched male mice (WT, $n = 5$; KO, $n = 6$) were run on a treadmill at 25 m/min for 30 min. Following the run, mice were euthanized and RNA was isolated and analyzed as described above.

Analysis of enzyme activity levels. For changes in enzyme activity levels with exercise, mice (WT, $n = 5$; KO, $n = 12$) were run on a treadmill using the same protocol as for the gene expression analysis. Tissue was harvested after the run and from resting mice (WT, $n = 6$; KO, $n = 10$), and enzymes were extracted and analyzed spectrophotometrically as has been described (Reichmann et al. 1983), with the exception that fructose 1,6-bisphosphate was replaced with fructose 2,6-bisphosphate for stabilization of PFK. Units of activity were normalized to milligrams of total protein using a BCA protein quantification kit (Pierce Biotechnology, Rockford, Illinois, United States).

Creatine kinase, serum lactate, hematocrit, and hemoglobin levels. Creatine kinase levels were analyzed from serum from WT mice and HIF-1α KOs 24 h after running-induced exhaustion using a kit from Sigma (St. Louis, Missouri, United States). Creatine kinase isoforms were analyzed enzymatically and then fractionated by gel electrophoresis. Serum lactate levels were analyzed by the UCSD Comparative Neuromuscular Laboratory from blood obtained by cardiac puncture from six WT mice and six HIF-1α KOs following 25 min running time on the treadmill ramp at 25 m/min. Hematocrit and hemoglobin levels were measured from resting mice (WT, $n = 6$; KO, $n = 4$) by the UCSD Animal Care Program Diagnostic Laboratory.

Glucose tolerance curve. Animals were assigned into either a sham (WT, $n = 5$; KO, $n = 4$) or glucose tolerance group (WT, $n = 8$; KO, $n = 8$). Experimental animals were injected with 0.3 g/ml glucose in PBS to achieve a dosage of 2 g/kg. Sham animals were injected with an equivalent amount of PBS. Blood was drawn from the tail at time intervals of 0, 15, 30, 60, and 120 min. Samples were then centrifuged to isolate plasma. Plasma blood glucose was quantified using the Infinity Glucose Kit (Sigma).

Calcium uptake measurements. Intact individual muscle fibers (WT, $n = 6$; KO, $n = 4$) were mechanically dissected from the flexor brevis muscle and loaded with fura-2. Fibers were then stimulated while force generation and Ca^{2+} release were monitored.

Four-day endurance test. Endurance was tested by running 24 animals (WT, $n = 10$; KO, $n = 14$) on the Omnipacer Treadmill or the enclosed-chamber modular treadmill using the same exhaustion protocol described above. Mice ran according to this protocol every day for 4 d with a minimum of 22 h of rest between trials. Following the fourth trial, mice were given 24 h of rest and then euthanized. Tissue was harvested and stained using hematoxylin-eosin (as described above) and α-PCNA (Pharmingen, San Diego, California, United States) combined with a DAB Kit (Vector Labs, Burlingame, California, United States).

Statistical analysis. Statistical analyses (unpaired Student's t-test, Mann-Whitney test, ANOVA) were carried out using StatView software (SAS Institute, Cary, North Carolina, United States) or Prism software (GraphPad Software, San Diego, California, United States).

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III. HIF-1α IN ENDURANCE TRAINING:

SUPPRESSION OF OXIDATIVE METABOLISM

Summary

During endurance training, exercising skeletal muscle experiences severe and repetitive oxygen stress, and the muscle's ability to cope with and improve its function through that stress is central to its role in the body. The primary transcriptional response factor for hypoxic adaptation is hypoxia inducible factor-1α (HIF-1α), which upregulates glycolysis and angiogenesis in response to low levels of tissue oxygenation. We have created mice specifically lacking skeletal muscle HIF-1α and subjected them to an endurance training protocol. We found that only wild type mice improve their oxidative capacity, as measured by their respiratory exchange ratio; surprisingly, we found that HIF-1α null mice have already upregulated this parameter without training. Furthermore, untrained HIF-1α null mice have increased capillary density, and elevated oxidative enzyme activities. These changes are correlated with a constitutively activated AMP-activated protein kinase in the HIF-1α null muscles. This data demonstrates that removal of HIF-1α causes an adaptive response in skeletal muscle akin to endurance training, and provides evidence for the suppression of mitochondrial biogenesis by HIF-1α in normal tissue.

Introduction

One of the greatest challenges facing skeletal muscle is the need to match energy production with energy demand during exercise. The need to maintain optimal metabolic flux within the muscle is of such great importance that it is the most plastic aspect of muscle physiology – muscles have the ability to adopt different metabolic profiles depending on the type of exercise they are most frequently called upon to

perform. The ability of the skeletal muscle to keep up with increased energy demand can be greatly limited by the availability of oxygen to the tissue. In fact, exercising muscle tissue exhibits very low oxygen tensions relative to what is available in inspired air and circulating blood, indicating that exercising muscle is subject to severe and repeated oxygen stress. The primary response mechanism to low oxygen concentrations in a cell is the transcription factor hypoxia inducible factor 1 (HIF-1), a heterodimeric, basic helix-loop-helix, PAS domain containing transcription factor. The regulated subunit of HIF-1, HIF-1α, is controlled by oxygen levels within the cell; it is hydroxylated and degraded under normoxia, but is stable and translocates to the nucleus under hypoxia (Jaakkola et al., 2001). Activated HIF-1α turns on transcription of genes; this in turn aids cells and tissues in coping with oxygen stress. Targets include genes responsible for glycolysis, glucose uptake, and angiogenesis (Semenza, 1999). Recent studies have examined the role of HIF-1α in the skeletal muscle. In one study, Pisani and Dechesne found elevated levels of HIF-1α protein at rest under normoxia in the skeletal muscles of untrained mice, in a fiber-type dependent manner (Pisani and Dechesne, 2005). Ameln and colleagues demonstrated that acute exercise leads to stabilized HIF-1α protein (Ameln et al., 2005). Finally, we have previously shown that deletion of HIF-1α in the skeletal muscle of mice leads to mice with impaired glycolytic flux, enhanced aerobic metabolism, and increased submaximal endurance (Mason et al., 2004). These studies have highlighted the importance of HIF-1 signaling in the skeletal muscle in the untrained state. A muscle's ability to improve through repeated exercise is essential to its role. The primary way through which endurance athletes improve their performance is by regular aerobic exercise, with an

end result that muscles adapt through increased oxygen delivery and more efficient energy utilization.

Some of the major physiological changes seen as a result of endurance training are increased muscular capillary density (Prior et al., 2004), increased mitochondrial volume (Hoppeler et al., 1985), increased reliance on fatty acid metabolism for energy, a shift in fiber type toward oxidative fibers, decreased lactate production (Holloszy and Coyle, 1984), and increased hexokinase enzyme activity (Taylor et al., 2005). Since several of these changes are potentially regulated by HIF-1, we have investigated the hypothesis that HIF-1α is a necessary factor in the skeletal muscle's response to endurance training. We have trained mice lacking HIF-1α in their skeletal muscle, and found that contrary to being unable to train, their muscles have already undergone an adaptive response that better prepares the muscles for endurance exercise. These changes are only matched by wild type (WT) mice following endurance training, and are linked to a constitutively activated AMP-activated protein kinase response.

Results

Response of WT and HIF-1α null mice to endurance training. The creation and initial characterization of the skeletal muscle specific HIF-1α null mouse has been described previously (Mason et al., 2004). In order to study the role of HIF-1α in the response of skeletal muscle to endurance training, WT and MCK-Cre expressing mice were trained on a treadmill. The training protocol consisted of running on a 5° incline at 18 m/min for 30 minutes, five days per week for 6 weeks. Following training,

control and HIF-1α null mice were given an endurance test, during which mice ran on the inclined treadmill for 5 minutes each at 12, 16, 20, and 24 m/min, and then ran at 28m/min until exhaustion. Surprisingly, as can be seen in Figure 9A, both WT and HIF-1α null mice demonstrated a dramatic improvement in running ability following training, as compared to their untrained littermates. Untrained WT mice were able to run for an average of 41.13 ± 5.03 minutes before exhaustion, and untrained HIF-1α null mice ran for an average of 44.42 ± 5.59 minutes. Intriguingly, improvement following training was similar in mice of both genotypes. Trained control mice were able to run for 67.16 ± 9.85 minutes, and trained HIF-1α null mice for 78.08 ± 10.65 minutes, improvements of 63.30% and 75.79% respectively (Fig. 9B). These results clearly indicate that HIF-1α is not required for improvement following endurance training. Despite the similar improvements in overall running ability, metabolic monitoring of mice during the endurance test revealed that WT and HIF-1α null mice responded differently to the training protocol. As mentioned earlier, a common result of endurance training is an increase in the ability of the muscles to utilize fatty acids for energy. Values for respiratory exchange ratio (RER) in WT mice during the endurance test show that at lower velocities, trained WT mice have a lower RER relative to their untrained littermates, indicating that they are indeed able to shift toward a more oxidative metabolic profile (Fig. 9C). Conversely, this shift was absent in the trained HIF-1α null mice; their RER values after training were nearly identical to their untrained values at all velocities (Fig. 9D). Comparison of RER values from untrained WT and HIF-1α null mice show that loss of HIF-1α has caused an adaptive response in the null muscles. As shown in Fig. 9E, untrained HIF-1α null mice already

have a decreased RER at lower velocities, relative to untrained WT mice. This finding indicates that loss of HIF-1α gives rise to a phenotype mimicking exercise training.

No changes in blood oxygen carrying capacity following training. Following training, whole blood was extracted for analysis from trained mice and their untrained littermates via cardiac puncture. As shown in Table 6, there were no initial differences in red blood cell count, hemoglobin concentration, or hematocrit between untrained WT and HIF-1α null mice. Additionally, none of these parameters changed following training in mice of either genotype, indicating that the differences in RER and VO_2 were due to changes in the skeletal muscle and not due to changes in the blood oxygen carrying capacity.

Training-induced changes in muscle morphology. Following the findings of increased endurance in all mice following training, we set out to determine what caused the similar level of improvement between the two genotypes. Additionally, we looked to see what caused the RER change in the untrained HIF-1α null mice, and how the trained WT mice were able to match those parameters. As one common result of endurance training is an increase in intramuscular metabolite storage (Ranallo and Rhodes, 1998; Reynolds et al., 1995; Simi et al., 1991), we stained gastrocnemius samples for stored glycogen using a PAS stain, and stored triglycerides using an Oil Red O stain. No effects of training were seen on intramuscular glycogen or triglyceride storage in either WT or HIF-1α null mice (data not shown). Therefore, the improvements in mice from both genotypes following training were not a result of increased intramuscular metabolite storage. Endurance training has been shown to increase the abundance of nicotinic acetylcholine receptors at the neuromuscular

junction (Desaulniers et al., 1998), however, α-bungarotoxin staining of gastrocnemius sections from mice involved in this study showed no changes in this parameter, either (data not shown).

One of the most pronounced changes in skeletal muscle morphology as a result of endurance training is a shift in fiber type profile: from glycolytic fibers to more aerobic fibers (Holloszy and Coyle, 1984). Representative fields of myofibrillar ATPase staining from deep within the gastrocnemius muscle are shown in Figs. 10A-D, and show that WT mice had a typical training-induced shift in fiber type. Muscle from WT mice had a significant increase of 36.4% in type I (slow-twitch oxidative) fibers and a significant 17.2% decrease in type IIB (fast-twitch glycolytic) fibers from deep sections of gastrocnemius muscle (Figs. 10E-G). Interestingly, no significant changes were seen in the fiber type composition of trained HIF-1α null muscles. However, trained WT mice did have a significantly greater prevalence of type IIA (fast-twitch oxidative) fibers and lower abundance of type IIB fibers relative to trained HIF-1α null mice. No changes were seen in fiber type distribution of superficial portions of the gastrocnemius muscle (Table 7). These findings indicate that while the WT muscles responded as expected in this parameter, HIF-1α null muscles were deficient in the major morphological changes typically thought necessary to achieve improvement following training.

Increases in capillary density of gastrocnemius muscle. Another common way muscles change to meet the demands of endurance training is to increase capillary density. To measure this parameter in our mice, we stained gastrocnemius sections for the endothelial cell-specific marker CD31. Representative pictures of CD31stained

deep gastrocnemius sections from untrained and trained mice are shown in Figs. 11A-D. In superficial gastrocnemius sections, WT muscles demonstrated a trend ($p<0.10$) toward an increased capillary to fiber ratio. This increase was much more pronounced in the deep portions of the gastrocnemius muscle, as WT mice had a significant ($p<0.05$) increase from 1.26 ± 0.04 capillaries/fiber in the untrained state to 1.46 ± 0.04 capillaries/fiber following training (Fig. 11E, Table 7). There were no training-induced increases in the capillary/fiber ratio in HIF-1α null muscles; however the deep portions of the untrained HIF-1α null muscles actually already have elevated capillary/fiber ratios relative to untrained WT muscles. This finding indicates that loss of HIF-1α in the untrained skeletal muscle has caused an adaptive angiogenic response similar to that of endurance training, and the training protocol was unable to further improve upon that adaptation.

Metabolic profile of trained skeletal muscle. As mentioned before, a hallmark of endurance training is an increase in the oxidative capacity of the skeletal muscle. An increase in oxidative capacity would also represented by increases in mitochondrial metabolic enzymes. We measured the activities of β-hydroxyacyl-CoA dehydrogenase (βHAD) and citrate synthase (CS), two key aerobic metabolic enzymes localized to the mitochondria. βHAD catalyzes a rate-limiting step of fatty acid β-oxidation, and endurance training caused a significant upregulation of its activity in the gastrocnemius muscles of exercised WT mice (Fig. 12A). Interestingly, as seen before in these mice (Mason et al., 2004), untrained HIF-1α null muscles already had elevated βHAD activity relative to untrained WT muscles, and did not increase it as a result of training. As the gatekeeper to the citric acid cycle, CS catalyzes the addition

of acetyl-CoA to oxaloacetate, producing citrate in a tightly regulated reaction. Similar to βHAD, activity of this enzyme increased as a result of endurance training in exercised WT muscles (Fig. 12B), and was already elevated in the muscles of untrained HIF-1α null mice. Training did not produce any further increase in CS activity in HIF-1α null mice. In order to determine if the muscles of the trained mice increased their mitochondrial content, we measured mitochondrial DNA (mtDNA) from quadriceps muscle via real-time PCR. Training induced a dramatic increase of 65.1% in muscles from WT mice, but HIF-1α null muscles only demonstrated a non-significant increase of less than 20% (Figs. 12C-D). However, post-training levels of WT and HIF-1α null mtDNA were not significantly different, indicating that once again, the HIF-1α null muscles did not need to increase them significantly during endurance training. In fact, the difference in mtDNA from muscles of trained WT mice and untrained HIF-1α null mice is not statistically significant ($p>0.05$) either. Thus, the training stimulus was not able to provoke any increase in mtDNA in the HIF-null muscles in order for them to handle the demands of repeated exertion. Here also the data indicates that an adaptive event has geared the muscles of HIF-1α null mice toward endurance exercise, and endurance training has been unable to improve upon that adaptation.

Another enzyme that has been demonstrated to be upregulated in response to endurance training is hexokinase (Taylor et al., 2005). In the skeletal muscle, hexokinase has a dual role: catalyzing the first step of glycolysis as well as phosphorylating glucose that has entered the muscle fiber, resulting in its being unable to leave the muscle and leading to the glucose being either metabolized, or stored as

glycogen. Measured levels of hexokinase in both trained WT, and trained HIF-1α null exercised muscles increased significantly over that seen in untrained muscles of the same genotype (Fig. 12E). Additionally, both genotypes increased hexokinase activity to the same degree, indicating that this change likely is responsible for their similar increases in endurance.

Changes in gene expression following training. RNA was harvested from quadriceps muscle taken at rest and immediately following the endurance test from both trained and untrained mice. Real time PCR was then used to measure changes in gene expression in WT and HIF-1α null mice. No significant effects of training were seen on VEGF or myoglobin mRNA (data not shown). Expression of mitochondrial transcription factor A (TFAM), a key regulator of mtDNA copy number and expression of its encoded genes, was surprisingly unchanged in response to training in WT muscles (Fig. 13A). However, HIF-1α null quadriceps exhibited a trend toward increased TFAM expression in the trained state, which correlates with a significant increase in TFAM mRNA seen from the gastrocnemius muscle of untrained HIF-1α null mice (data not shown). Expression of PPAR-γ coactivator-1α (PGC-1α) was not different between WT and HIF-1α null mice in resting muscle, either before or after training (Fig 13B). Training did result in PGC-1α expression being more responsive to exercise as both trained WT and HIF-1α null muscles demonstrated a trend toward increased PGC-1α mRNA immediately after exercise. Glucose transporter 4 (Glut4) expression, the primary glucose transporter in the muscle, increased significantly following exercise in WT muscle, but was already greatly increased in HIF-1α null

muscle, revealing another way in which the HIF-1α null muscles are already adapted for endurance exercise (Fig 13C). Training did not have any significant impact on Glut4 expression in either WT or HIF-1α null muscles. Untrained WT muscles were able to significantly increase expression of hexokinase II (HKII), the predominant hexokinase isoform in the skeletal muscle, in response to exercise. However, at rest, untrained HIF-1α null muscles had elevated HKII relative to WT muscles, but did not increase this further following exercise (Fig 13D). No increases in HKII expression were seen in either trained WT or HIF-1α null muscles, indicating that the training induced changes leading to increase hexokinase enzyme activity in the muscles were post-transcriptional.

Response of AMPK to HIF-1α deletion and exercise. AMP activated protein kinase (AMPK) acts in the cell to turn on ATP producing pathways, and turn off ATP consuming pathways in times of metabolic need (Hardie, 2003). It can be activated by increased cellular AMP as well as through phosphorylation of threonine residue 172 of its alpha subunit, and exercise has been shown to increase AMPK activity (Winder and Hardie, 1996). It has been linked to increases in many of the parameters described above, and could be linked in turn to an energy-deficient state induced by loss of HIF-1α, since HIF-1α is linked to decreased ATP production during hypoxia (Seagroves et al., 2001). To see if the response of AMPK was affected in the muscles of our HIF-1α null mice due to HIF-1α-dependent alterations in ATP production, we performed western blots on extracts from quadriceps muscles of untrained WT, and HIF-1α null mice at rest, and immediately following a timed 30-minute run at 24m/min. A representative blot for phosphorylated AMPK is shown in Fig. 13E. As expected,

exercise caused a 3-fold increase in the level of phospho-AMPK in WT muscles. However, resting HIF-1α null muscles already have a 3-fold greater level of phospho-AMPK relative to WT muscles, and do not further increase this during exercise (Figs. 13E-F). The increases in phospho-AMPK were not a function of increased total AMPK as those levels were unaffected by either loss of HIF-1α or exercise (Figs 13G-H). The increase in phospho-AMPK is further evidence that muscles lacking HIF-1α exist in a "pretrained" state (Frosig et al., 2004); they further indicate that the mechanism for this pre-adaptation is a HIF-dependent, ATP flux-regulated increase in constitutive AMPK phosphorylation.

Discussion

Exercising muscles experience severe oxygen deprivation (Richardson et al., 1995), and thus it is easy to imagine a central role for the primary hypoxia response factor, HIF-1α, during the muscular response to endurance training. The primary training response is better delivery and utilization of oxygen in the muscle, which further strengthens this hypothesis arguing for the centrality of the HIF response to training. Surprisingly, our data does not support this hypothesis. Loss of HIF-1α did not have any visible negative impact on endurance training in the HIF-1α null mice. In fact, these mice have actually undergone an adaptive process prior to training, leading to their being better suited for endurance exercise; endurance training is unable to further improve upon this adaptation. As a result, they neither increased oxidative capacity following training, nor increased fatty acid oxidation; primarily because these parameters had already been elevated as a result of HIF-1α deletion. The main factor

leading to the common increase in endurance in both WT and HIF-1α null mice is likely an increase in hexokinase activity, an enzyme whose activity increased to the same degree following training in both genotypes. As an entry point enzyme in glycolysis, hexokinase is of great importance to endurance. Since muscle lacks glucose-6-phosphatase, hexokinase activity ensures that glucose, once it enters the muscle fiber, cannot leave and will thus provide energy either immediately, or later, as stored glycogen. It has recently been demonstrated that knockdown of hexokinase decreases muscle glycogen stores, and hexokinase activity correlates very strongly with endurance capacity (Fueger et al., 2005). These findings corroborate the conclusion that an increase in hexokinase activity is a key factor leading to the training-induced endurance increase in both genotypes studied here. Our previous work demonstrated an increased endurance in the untrained HIF-1α null mice not shown in this study. This is due to differences in protocols used during the run tests here, where the mice spent the majority of their run at higher velocities, which in turn provides a more intense exercise stimulus. More intense endurance exercise require increased carbohydrate metabolism, while less intense exercises allow muscles to derive a greater portion of their energy from fatty acid oxidation (Brooks, 1998). In our previous study, the majority of the run time was at a lower velocity, thereby allowing the muscles of the HIF-1α null mice to take full advantage of their adaptation for better fatty acid metabolism, leading to their observed increased endurance in those protocols. The most intriguing finding of this study was the degree to which loss of HIF-1α caused an endurance training-like adaptation of the skeletal muscle. Without any prior training, HIF-1α null muscles had elevated CS and βHAD activities,

increased capillary to fiber ratios, upregulated resting Glut4 expression, and phosphorylated AMPK. No fiber type composition changes were observed in the HIF-1α null muscles, indicating that no changes were needed in order to keep up with the demands of the training protocol. The adaptations had the effect of lowering the RER of HIF-1α null mice during low-intensity exercise, and led to the increased endurance under the sub-maximal exercise protocol we described previously (Mason et al., 2004). We have found evidence that loss of HIF-1α in skeletal muscle leads to a constitutively elevated level of phospho-AMPK; this in turn can lead to the adaptive changes which otherwise occur during training. Activated AMPK has recently been shown to phosphorylate Glut4 Enhancer Factor (GEF), causing GEF to translocate to the nucleus and bind to the Glut4 promoter (Holmes et al., 2005). Additionally, several studies have demonstrated an AMPK-dependant increase in citrate synthase activity (Winder et al., 2000), and mitochondrial DNA and biogenesis (Bergeron et al., 2001; Kukidome et al., 2006). In fact, Kukidome, et al., saw a 22% increase in mtDNA content through constant activation of AMPK via the drug AICAR (Kukidome et al., 2006). This is very similar to the non-significant increase of 24% in mtDNA found in the loss of HIF-1α in the mice described in this study. Finally, constant activation of AMPK has been seen to lead to increased skeletal muscle angiogenesis (Ouchi et al., 2005); this may also explain the increased vascularization seen in the mutants described here. Coupling these previous results with our findings strongly indicates that increased levels of phospho-AMPK are the source of the adaptations seen as a result of the loss of HIF-1α in the skeletal muscle of these mice. Our data unequivocally shows that HIF-1α signaling is not essential for the muscular response

to endurance training. Recent findings lead to questions about whether HIF-1α signaling would be beneficial to endurance training, and have shown a potential for HIF-1α to have a strongly negative effect on mitochondrial adaptation. In work with a pheochromocytoma line, Dahia et al., have shown that constitutively active HIF-1α leads to a sharp decrease in succinate dehydrogenase B protein, indicating a likely drop in electron transport chain activity (Dahia et al., 2005). Additionally, Papandreou, et al. (Papandreou et al., 2006), and Kim, et al. (Kim et al., 2006), have recently demonstrated that HIF-1 can directly downregulate mitochondrial oxygen consumption, through increased expression of pyruvate dehydrogenase kinase I, an inhibitor of pyruvate dehydrogenase. This work indicates that HIF-1 signaling may not be of benefit to endurance training, since it would lead to a blockade of oxidative metabolism. Therefore, training may well involve suppression of the HIF-1 signaling pathway, and, in turn, increased levels of phospho-AMPK caused by hypoxia-driven ATP flux. In support of that hypothesis, Lundby, et al., have recently demonstrated a decrease in the induction of HIF-1α and HIF-2α in endurance-trained muscles following exercise (Lundby et al., 2005). These recent results, when combined with the findings described above, support a key role for the suppression of HIF-1 signaling during training, ultimately leading to an adaptive response through AMPK causing increased oxidative capacity in skeletal muscle.

Materials and Methods

Mouse strains and crosses. Skeletal muscle specific HIF-1α null mice were generated from a cross of a C57Bl6/J strain containing the MCK/cre transgene

(described previously (Bruning et al., 1998) with a C57Bl6/J strain homozygous for the HIF-1α loxPflanked allele. WT mice were littermates that were homozygous for the loxP-flanked HIF-1α allele but did not carry the MCK-Cre transgene.

Endurance training and endurance assessment. WT (n = 15) and HIF-1α null (n = 13) mice were trained on an enclosed chamber modular treadmill (Columbus Instruments, Columbus, Ohio) held at a constant 5¡incline. Mice trained by running for 30 minutes at 18m/min 5 days per week for 6 weeks. Following completion of the training protocol, mice were given two days to recover before an endurance test. During the endurance test trained (WT n = 7, HIF-1α null n = 7) and untrained (WT n = 11, HIF-1α null n = 6) mice ran on the treadmill at a 5¡incline with an initial velocity of 12m/min. After 5 minutes, velocity was increased to 16m/min for 5 minutes with 4m/min increases every 5 minutes until the mice reached 28m/min. Velocity was then held constant at 28m/min until exhaustion, which was determined as the point in which mice no longer responded to a low voltage electrical grid at the back of the treadmill. Metabolic monitoring during the run was carried out by measuring O_2 and CO_2 levels in gas going into and leaving the treadmill using the Paramax O_2 sensor and a CO2 sensor (Columbus Instruments). Gas exchange data was analyzed using Oxymax software (Columbus Instruments).

Whole blood analysis. Whole blood was collected from trained (WT n = 11, HIF-1α null n = 11) and untrained (WT n = 10, HIF-1α null n = 8) mice at rest and immediately following exercise via cardiac puncture. Blood was stored in EDTA

citrate tubes (Terumo Medical Corp., Elkton, MD) until analysis by the UCSD Animal Care Program Diagnostic Laboratory (La Jolla, CA).

Histological analysis of skeletal muscle. Gastrocnemius sections were harvested from untrained and trained mice of both genotypes and frozen in Tissue-Tek OCT compound (Sakura Finetek USA, Torrance, CA). Sections were cut 10μm thick for histological analysis. PAS, Oil Red O, and myosin ATPase stainings were all performed out as has been described previously (Bancroft and Stevens, 1996; Koopman et al., 2001; Ogilvie and Feeback, 1990). Neuromuscular junction staining was carried out using a FITC-conjugated α-bungarotoxin probed (Molecular Probes, Inc., Eugene, OR) according to a previously published protocol (Barbier et al., 2004). CD31 staining was carried out using a rat anti-mouse CD31 primary antibody (BD Biosciences, San Jose, CA) and a FITC-conjugated goat anti-rat secondary antibody (Pierce Biotechnology, Inc., Rockford, IL). Briefly, slides were fixed for 10 minutes in cold (-20°C) acetone before a 10-minute rinse in PBS with 0.1% tween-20 (PBST). Blocking was for 1 hour in PBS-T supplemented with 3% bovine serum albumin and 10% normal goat serum (Sigma, St. Louis, MO) before a 1-hour room temperature primary antibody incubation in blocking solution. Slides were then rinsed 3 times for 5 minutes in PBS-T before a 1-hour room temperature incubation with the secondary antibody in the blocking solution. Following the secondary antibody, slides were rinsed 3 times for 5 minutes in PBS-T, incubated for 1 minute in 1μg/ml DAPI (Sigma), rinsed once more in PBS-T for 5 minutes, and mounted in Vectashield mounting medium for fluorescence (Vector Laboratories, Burlingame, CA).

Measurement of mitochondrial DNA and gene expression. Mitochondrial DNA (mtDNA) was extracted from quadriceps muscle of untrained and trained mice (n = 8 for all genotypes and conditions) using a DNEasy kit for extraction of DNA from tissue (Qiagen, Valencia, CA). DNA concentration was determined by spectrophotometry and mtDNA was then quantified via real-time PCR using previously described primers and protocol (Shen et al., 2004). For gene expression, total RNA was isolated from quadriceps muscle from untrained and trained rested and exercised mice (minimum n = 4) using Trizol reagent (Invitrogen, Carlsbad, CA), treated with DNase I (Invitrogen) to remove DNA, and then reverse transcribed using the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen). Primer sequences were as have previously been described. Real-time PCR was carried out on an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) using Platinum quantitative-PCR supermixes for SYBR green or taqman real-time PCR (Invitrogen). Conditions for the PCR were 40 cycles of 95°C for 15 s (denaturing) and 60°C for 1-min (anneal/extend). Primers and probes were synthesized according to previously published sequences (Jorgensen et al., 2005; Mason et al., 2004; van den Bosch et al., 2005; van Weel et al., 2004).

Analysis of enzyme activity. Measurements of enzyme activity levels in untrained and trained mice were made from gastrocnemius muscles taken immediately after the endurance test. Enzymes were extracted and analyzed spectrophotometrically according to previously described protocols (Houle-Leroy et al., 2000; Reichmann et al., 1983). Units of enzyme activity were normalized to total protein concentration using a BCA protein quantification kit (Pierce Biotechnology, Inc).

Western blot analysis of AMPK. Homogenates were made from quadriceps muscle from untrained resting (WT n = 6, HIF-1α null n = 5) and exercised (WT n = 6, HIF-1α null n = 6) mice. Exercise consisted of a timed run on an enclosed modular treadmill for 30 minutes at 24m/min. Mice were sacrificed and tissue harvested immediately after the run to prevent any time effects on AMPK phosphorylation. Muscles were homogenized in a buffer containing 20mM HEPES, 1mM EDTA, 1mM Na_3VO_4, 2mM EGTA, 10mM $MgCl_2$, 50mM β-glycerophosphate, 1mM DTT, 1mM PMSF, 1% Triton X-100, 10% glycerol, and 1x EDTA-free Complete protease inhibitor (Roche Diagnostics, Indianapolis, IN). Following homogenization with a Polytron tissue homogenizer, samples were centrifuged for 30 minutes at 15,000xg. The supernatants were then western-blotted for phosphorylated and total AMPK using antibodies from Cell Signaling Technologies (Danvers, MA). Statistical Analysis. Statistical analyses (unpaired student t-test and Mann-Whitney test) were carried out using Prism software (Graphpad Software Inc, San Diego, CA).

This chapter, in full, is a reprint of the material as it has been submitted to PLoS Biology, Mason, S.D., Ameln, H., Duh, R., McNulty, W.J., Howlett, R.A., Olfert, I.M., Sundberg, C.J., Poellinger, L., and Johnson, R.S. PLoS Biol. (In submission). The dissertation author was the primary investigator and author of this paper.

Table 6. Red blood cell count, hemoglobin, and hematocrit in trained and untrained mice.
Analysis of whole blood from untrained and trained mice shows no differences in red blood cell count (RBC), hemoglobin concentration (Hgb), and hematocrit as a result of HIF-1α deletion or training. Numbers are mean ± SE.

	WT Untrained	HIF-null Untrained	WT Trained	HIF-null Untrained
n	10	8	11	11
RBC (x10^6)	8.60±0.70	9.15±0.29	9.35±0.31	9.02±0.17
Hgb (g/dl)	12.93±1.12	13.56±0.51	13.93±0.54	13.55±0.29
Hematocrit	43.90±1.44	44.28±1.42	43.95±1.59	43.43±0.84

Table 7. Fiber type and capillary density analysis of gastrocnemius muscles.
Fiber typing of superficial sections from gastrocnemius muscles of trained and untrained mice shows no training-induced changes in fiber type distribution. Deep sections of muscles from WT mice showed a significant increase in type I (slow twitch oxidative) fibers and a significant decrease in type IIB (fast-twitch glycolytic) fibers after training, changes which were absent in HIF-1α null mice. Additionally, trained WT mice significantly increased capillary density of deep gastrocnemius sections, and had a trend toward increased capillarity of superficial sections. Deep sections of HIF-1α null muscles already have elevated capillarity, which does not increase during training. Numbers are mean ± SE, # - $p<0.10$, * - $p<0.05$ trained vs. untrained within genotype; ^ - $p<0.05$ WT vs. HIF-null.

	WT Untrained		**HIF-null Untrained**		**WT Trained**		**HIF-null Trained**	
	Superficial	Deep	Superficial	Deep	Superficial	Deep	Superficial	Deep
n	9	9	5	5	10	8	9	9
I	0.56±0.20	7.44±0.78*	0.95±0.46	8.75±1.23	1.05±0.37	10.15±0.96*	1.47±0.49	10.02±0.57
IIA	89.88±1.07	45.78±2.27	87.68±2.83	49.74±3.30	87.35±1.74	51.10±2.23^	88.59±1.54	43.05±1.73^
IIB	9.55±1.00	46.78±2.07*	11.36±3.06	41.51±3.87	11.60±1.45	38.75±2.81*^	9.95±1.34	46.93±2.13^
Cap. Dens. (n)	0.81±0.04# (11)	1.26±0.04 (11)	0.86±0.05 (8)	1.47±0.07* (8)	0.92±0.04# (12)	1.44±0.07* (12)	0.92±0.05 (8)	1.46±0.04 (8)

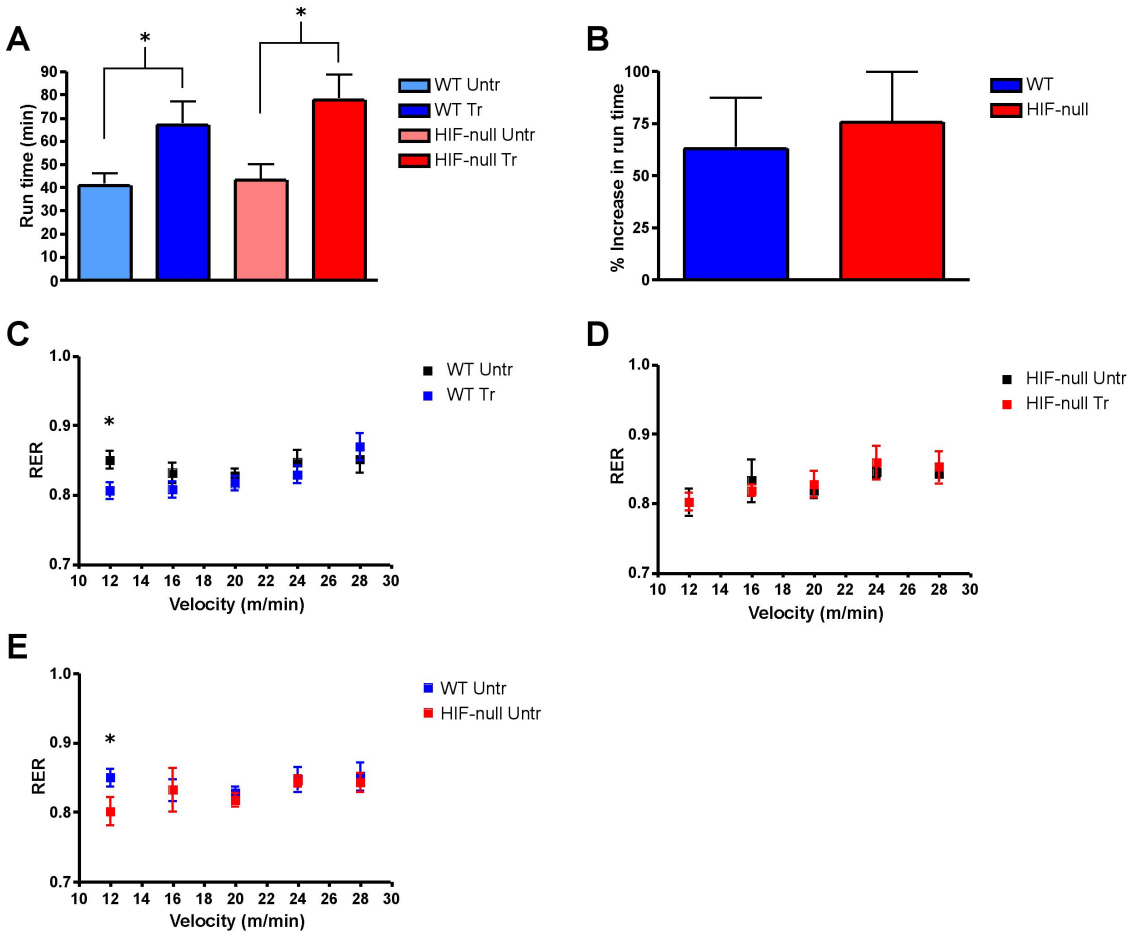


Figure 9. Endurance and physiological changes in WT and HIF-null mice.
A. HIF-1α null and WT mice both improve their exercise capacity following endurance training. Untrained WT mice were able to run for an average of 41.13 ± 5.03 minutes before exhaustion; untrained HIF-1α null mice ran for an average of 44.42 ± 5.59 minutes. Both genotypes of trained mice demonstrated dramatic increases in endurance, with WT mice running for and average of 67.16 ± 9.85 minutes, and trained HIF-1α null mice for 78.08 ± 10.65 minutes.
B. The total increase in running time is similar for trained mice of both genotypes. Following training, WT and HIF-1α null mice had improvements of 63.30% and 75.79%, respectively.
C. WT mice decrease their respiratory exchange ratio (RER) during lower intensity exercise following endurance training, indicating they have been able to upregulate their aerobic metabolism.
D. No changes in RER are seen in HIF-1α null mice following training.
E. Untrained HIF-1α null mice have a lower RER during less intense exercise, indicating that they have a greater capacity for oxidative metabolism without training.
Graphs show averages ± SE; * - $p<0.05$.

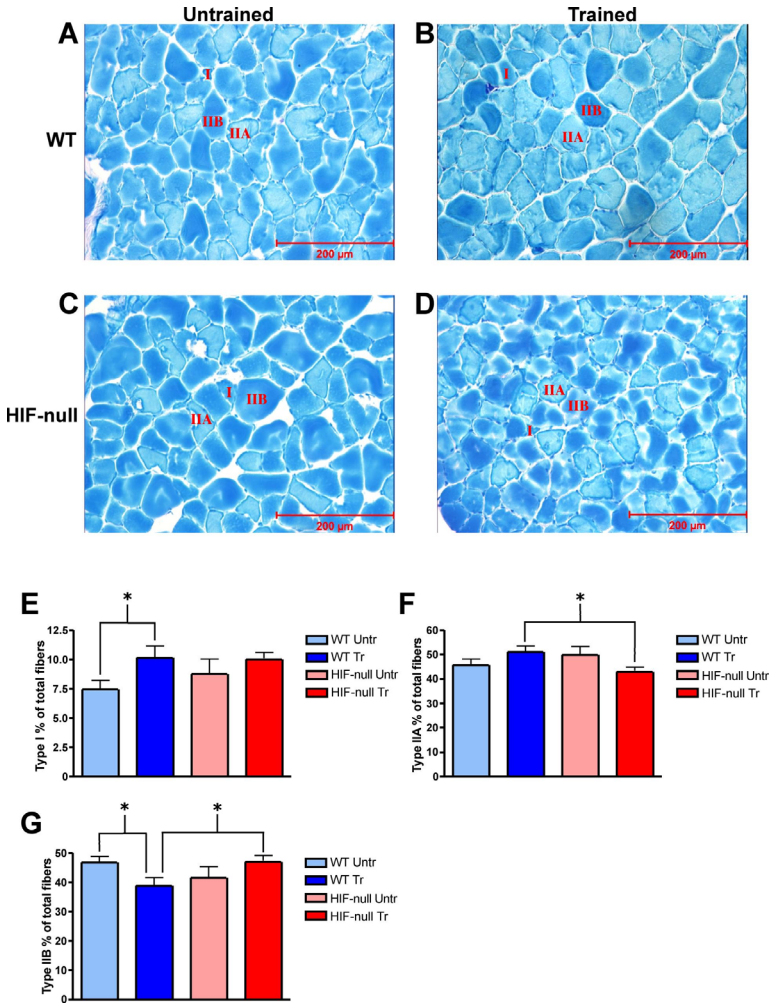


Figure 10. WT mice exhibit a shift in muscle fiber type composition following training.

A-D. Representative 20x fields from deep sections of untrained (panels A and C) and trained (panels B and D) WT (WT, panels A and B) and HIF-1α null (HIF-null, panels C and D) gastrocnemius muscle stained for myosin ATPase to determine fiber type distribution. Type I, IIA, and IIB fiber types are indicated in each panel. Note the greater proportion of oxidative fibers in the trained WT muscle.

E. Deep sections of WT gastrocnemius muscles have a significant increase in their percentage of type I fibers following training, a trend that is absent in trained HIF-1α null muscles.

F. Trained WT gastrocnemius muscles have a greater proportion of type IIA fast twitch oxidative fibers than trained HIF-1α null muscles.

G. Following training, WT muscles also have a significantly smaller percentage of type IIB fast-twitch glycolytic fibers than untrained WT muscles, which is also less than the percentage of type IIB fibers in trained HIF-1α null muscles. Graphs show averages ± SE; * - $p<0.05$.

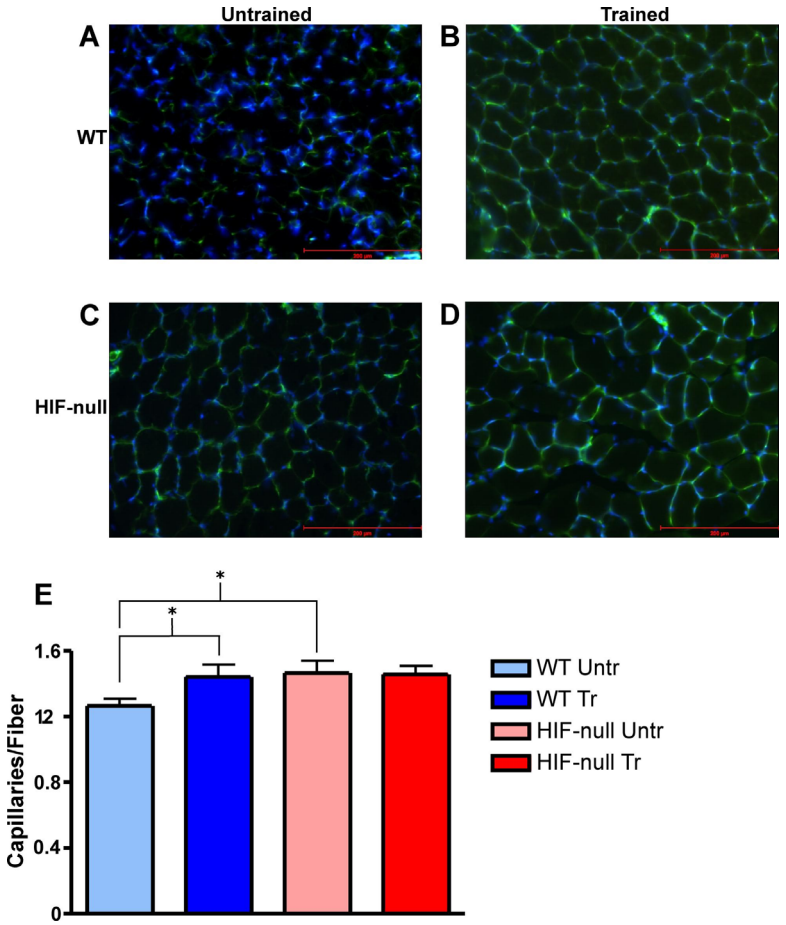


Figure 11. Capillary density changes as a result of HIF-1α deletion and endurance training.

A-D. Representative 20x fields from deep sections of untrained (panels A and C) and trained (panels B and D) WT (WT, panels A and B) and HIF-1α null (HIF-null, panels C and D) gastrocnemius muscle stained for the endothelial cell-specific marker CD31 (green; blue = DAPI) to show capillaries. Note the training-dependent increase in capillary density in WT muscle, and the adaptive increase in capillary density in untrained HIF-1α null muscles.

E. Endurance training results in an increase from 1.26 ± 0.04 capillaries/fiber to 1.46 ± 0.04 capillaries/fiber in deep sections of WT mouse gastrocnemius. HIF-1α null gastrocnemius already has elevated capillary density in the untrained state, which does further increase during training. Graph shows averages ± SE; * - $p<0.05$.

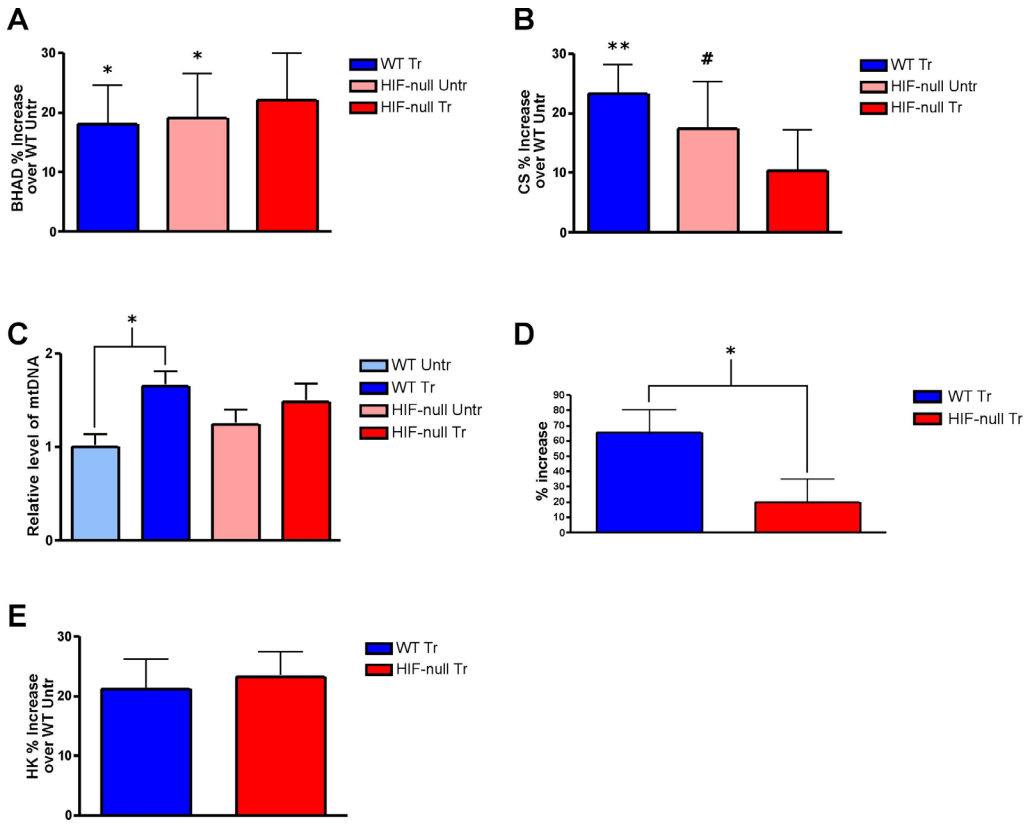
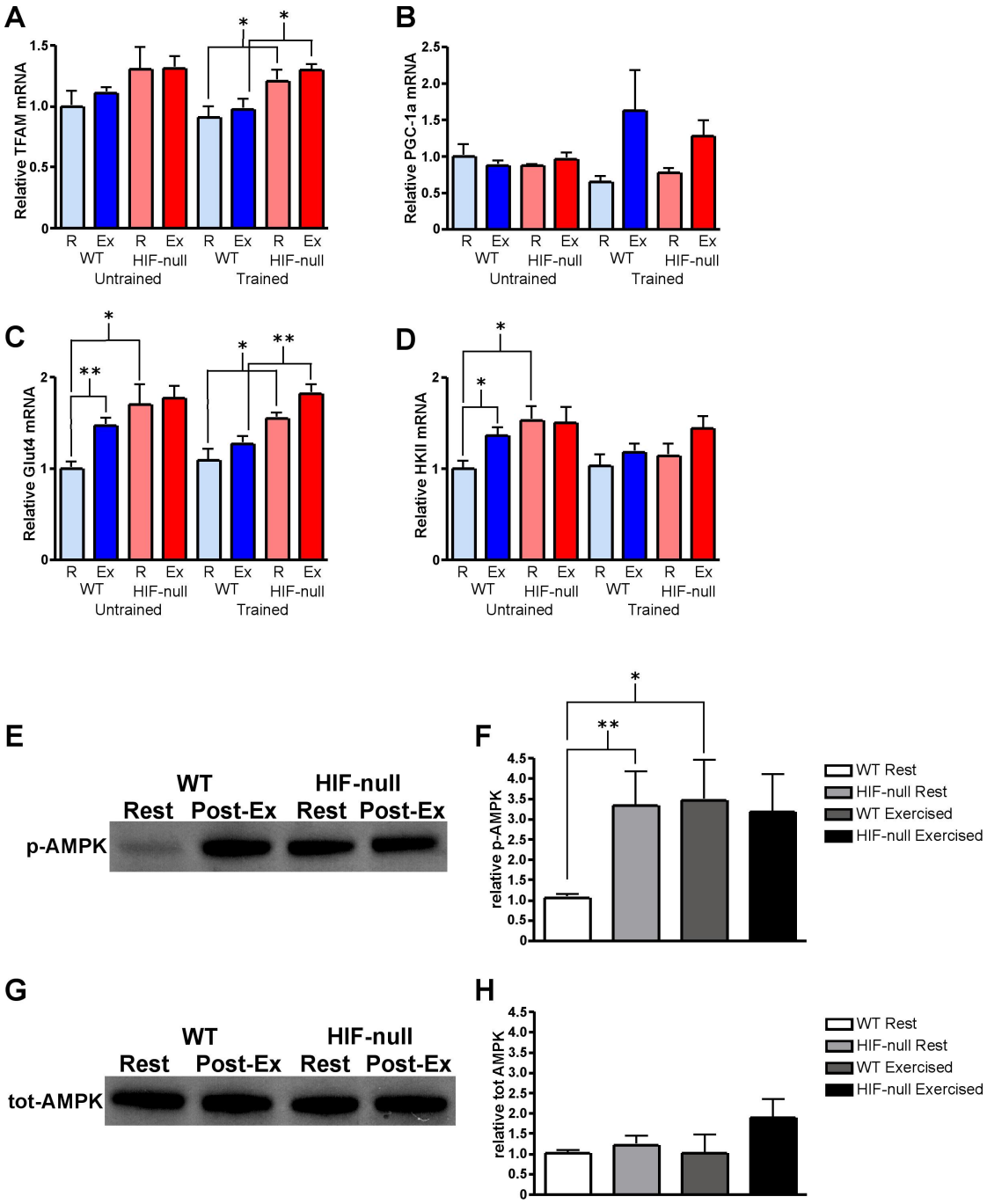


Figure 12. Response of key metabolic factors to endurance training.
A. HIF-1α null mice are unable to increase β-hydroxylacyl-CoA dehydrogenase activity (βHAD) due to elevated levels prior to training. WT mice were able to increase this parameter, showing that they were able to increase their oxidative capacity during training. Note that the post-activity level of βHAD in the WT mice just matches what HIF-1α null mice had prior to training. (* - $p<0.05$ vs. WT untrained βHAD activity)
B. Training led to a significant increase in citrate synthase (CS) activity in gastrocnemius muscles from WT mice. This increase was absent from HIF-1α null mice as they already had elevated CS levels prior to training. (# - $p<0.10$, ** - $p<0.01$ vs. WT untrained CS activity)
C,D. WT mice, but not HIF-1α null mice, dramatically increase their skeletal muscle mitochondrial DNA (mtDNA) levels during endurance training. Following training, WT mice have 65% more mtDNA than before training, while HIF-1α null mice only have a non-significant increase of less than 20%. (* - $p<0.05$)
E. Hexokinase activity, the first enzyme of glycolysis, was increased in both WT and HIF-1α null gastrocnemius muscles in a HIF-1α independent manner. Similar to the increases in endurance, the upregulation of hexokinase is to the same degree in both genotypes. (* - $p<0.05$ within genotype untrained vs. trained hexokinase activity). Graphs show averages ± SE.

Figure 13. Gene expression and AMPK activation in resting and exercised WT and HIF-null mice.
A. Untrained HIF-1α null muscles show a non-significant trend toward increased mitochondrial transcription factor A (TFAM) expression over WT muscles. This trend becomes significant following training.
B. PPAR-γ coactivator-1α (PGC-1α) expression is not different between WT and HIF-1α null muscles. Endurance training results in PGC-1α expression becoming more responsive to exercise, with exercise-induced increases detected immediately following exercise.
C. Although it is exercise-inducible in WT muscle, mRNA levels of Glucose transporter 4 (Glut4) are elevated in resting HIF-1α null muscles. Endurance training does not have a significant impact on Glut4 mRNA in either WT or HIF-1α null muscles.
D. Similar to Glut4, hexokinase II (HKII) expression is exercise-inducible in WT muscles, but already elevated in HIF-1α null muscles. Following training, HKII expression is not elevated; therefore the increased hexokinase enzyme activity seen from training is through a post-translational mechanism.
E. Representative western blot for phosphorylated AMP-activated protein kinase (AMPK) from resting and exercised quadriceps muscles. Note the increase in phospho-AMPK in exercised WT muscle, as well as resting HIF-1α null muscle.
F. Density quantification of phospho-AMPK western blots shows that both exercise and HIF-1α deletion result in a 3-fold increase in AMPK activation. Exercised HIF-1α null muscles do not increase phospho-AMPK levels.
E. Representative western blots for total AMPK showing no differences among the conditions tested.
F. Density quantification of western blots for total AMPK reveals that the increases in phospho-AMPK were not a function of increased AMPK protein levels. Graphs show averages ± SE; * - $p<0.05$, ** - $p<0.01$.


A
Relative TFAM mRNA
B
Relative PGC-1a mRNA
C
Relative Glut4 mRNA
D
Relative HKII mRNA
R Ex R Ex R Ex R Ex
WT HIF-null WT HIF-null
Untrained Trained
E
WT HIF-null
Rest Post-Ex Rest Post-Ex
p-AMPK
F
relative p-AMPK
WT Rest
HIF-null Rest
WT Exercised
HIF-null Exercised
G
WT HIF-null
Rest Post-Ex Rest Post-Ex
tot-AMPK
H
relative tot AMPK
WT Rest
HIF-null Rest
WT Exercised
HIF-null Exercised

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IV. CONCLUSIONS AND GENERAL DISCUSSION

This thesis presents experiments performed to gain a better understanding of the role of HIF-1α in skeletal muscle homeostasis, function, and adaptation to endurance training. It also seeks to paint a clearer picture of HIF-1α function in the body. This chapter will give a brief summary of some of the major findings of these studies and place them in the context of recent findings in the field. It will close by looking at current and future studies being undertaken to better understand HIF-1α activity in the skeletal muscle.

Chapter two of this thesis describes the characterization of the skeletal muscle-specific HIF-1α null mouse. Prior to this study, numerous findings had indicated the likelihood of a role for HIF-1α in skeletal muscle function. As mentioned in the introduction, several studies have shown an exercise-induced increase in transcription of HIF-1 target genes. Additionally, stable HIF-1α protein had been seen in resting, normoxic skeletal muscle from mice (Pisani and Dechesne, 2005). Shortly after the study described in this chapter was first published, another study showed that acute exercise leads to an increase in HIF-1α protein in the skeletal muscle (Ameln et al., 2005), giving further evidence that HIF-1α is indeed present and active within the muscle as a result of exercise. What were not known were the specific functions of HIF-1α in this context.

We created the skeletal muscle-specific HIF-1α-null mouse by crossing mice expressing the Cre recombinase transgene under the control of the muscle creatine kinase promoter with mice that were homozygous for a floxed form of HIF-1α. Expression of Cre in the skeletal muscles results in excision of exon two of HIF-1α,

making a non-functional gene product. This effectively deletes HIF-1α from the proteome of the skeletal muscles. Comparison of the muscles from these mice with muscles from wild type (WT) mice allowed for insight into the role of HIF-1α in normal skeletal muscle.

In the absence of HIF-1α, the skeletal muscle appeared to be unchanged by the loss of HIF-1α at the histological level. Fiber type size and distribution were the same in HIF-1α null and WT animals, and there were no significant differences in mitochondrial density or distribution as observed by electron microscopy. However, the similarities ended there. Due to the loss of HIF-1α, the muscles of the HIF-1α null mice had decreased glycolytic flux as evidenced by their failure to maintain PFK activity during exercise, an increase in pre-PFK glycolytic intermediates in an isolated stimulation protocol, and a decrease in serum lactate accumulation during a timed run. Additionally, the null muscles have increased glycogen storage, indicating that they may not be able to take full advantage of carbohydrate metabolism. As a result, the muscles had to increase their reliance on alternative metabolic pathways. The compensatory response took the form of an increased reliance on phosphocreatine for ATP production during the isolated stimulation protocol, and an increase in aerobic metabolic capacity, namely elevated levels of citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (βHAD). Interestingly, the compensatory response of the HIF-1α null muscles had the result of making them better suited for endurance exercise, as HIF-1α null mice both ran uphill and swam for longer times than control mice. However, their inability to upregulate glycolysis was manifested when the HIF-

1α null mice were unable to maintain their endurance edge while running downhill, an exercise that forces muscles to rely more heavily on glycolytic metabolism.

Perhaps the most surprising finding in this study was the severe muscle damage which HIF-1α null muscle experienced following endurance exercise. In repeated exercise tests, the HIF-1α null mice were unable to maintain their endurance edge over control mice, and had increased muscle damage post-exercise as evidenced by histology and creatine kinase in their serum. This damage was likely a cause of the decreased glycolytic capacity, as it correlates well with several known human metabolic myopathies in which carbohydrate metabolism is impaired (DiMauro et al., 1984). The exact mechanism connecting impaired glycolysis and muscle damage is currently unknown, both in the HIF-1α-null mice and in the human myopathies. Two leading possibilities are inadequate ATP production due to decreased glycolysis or increased ROS production due to an increased reliance on oxidative phosphorylation. This is an area we are still investigating in the HIF-1α null mice.

From the initial characterization of the HIF-1α knockout mouse, we can see that HIF-1α is critical for maintaining glycolytic flux in the muscle. This is important for proper balance of substrate utilization during exercise. Surprisingly, the muscles can compensate by upregulating alternative metabolic pathways, which leads to their being able to perform better in a single endurance assessment. Although the muscles are able to compensate for the loss of HIF-1α, they cannot fully recover, and are thus at a disadvantage when it comes to handling endurance exercise.

The third chapter of this thesis focuses on the role of HIF-1α in the skeletal muscle during endurance training. Previous studies have shown that endurance

training leads to increases in capillary density and oxidative capacity, which contribute to increased endurance (Hoppeler et al., 1985). Since many of these changes revolve around improving oxygen delivery and utilization, researchers have hypothesized that HIF-1α is crucial to the endurance training response (Hoppeler and Fluck, 2002; Lundby et al., 2005). Many of the changes seen in the muscles of the HIF-1α-null mice are similar to those seen in a muscle after training, which lead us to question the ability of the HIF-1α null muscles to improve under an endurance training protocol.

Interestingly, both WT and HIF-1α null mice had significant increases in endurance capacity as a result of training, however; only WT mice exhibited a shift toward increased oxidative metabolism, as evidenced by their decreased RER at lower intensity exercises. In contrast, the HIF-1α null mice already had a lowered RER, indicating that their adaptive response following the loss of HIF-1α had caused a shift toward better fatty acid oxidation. A closer look at the muscles of the trained and untrained mice bore this out, as untrained HIF-1α null mice already had an elevated capillary to fiber ratio, and increased CS and βHAD activities, that were not improved by training. Alternatively, these improvements were seen in the WT mice. Underscoring the importance of carbohydrate metabolism to endurance, hexokinase activity increased to the same degree in both WT and HIF-1α null muscles. Hexokinase has been shown to be integral to endurance capacity in mice (Fueger et al., 2005), and the similar increases in hexokinase activity correlate well with the similar increases in endurance in mice of both genotypes, showing that hexokinase is a driving force behind the improvements.

As it became apparent that the loss of HIF-1α had led to muscles that were predisposed for endurance training, the goal of the project became to determine the factors that lead to the adaptations in the HIF-1α null muscles. AMP-activated protein kinase is a protein that is activated by phosphorylation as cellular energy levels drop (Hardie, 2003). Once activated, it upregulates energy producing pathways while inhibiting unnecessary energy consuming pathways. HIF-1α null muscles were found to have high levels of phospho-AMPK at rest, a finding that is consistent with endurance trained muscles (Frosig et al., 2004). Additionally, activation of AMPK in the skeletal muscle has been shown to lead to increased oxidative capacity, GLUT4 expression, and angiogenesis (Bergeron et al., 2001; Holmes et al., 2005; Ouchi et al., 2005); things that were all seen in the untrained HIF-1α null mice. The finding of constitutively active AMPK in the HIF-1α null muscles strongly indicates that it is behind the training-like adaptive changes.

The other primary member of the HIF family, HIF-2α, has been shown to have many similar targets as HIF-1α (Wang et al., 2005). Therefore, it is possible that some compensation for loss of HIF-1α by HIF-2α exists. However, HIF-1α and HIF-2α do have somewhat distinct roles; HIF-1α has more metabolic effects (in particular, upregulation of glycolysis) than have been seen from HIF-2α (Wang et al., 2005), making it less likely that HIF-2α is masking many phenotypes from being seen in the HIF-1α null mouse. It is possible, though, that HIF-2α activity could be behind the increased vascularity of the HIF-1α null muscle. Very few studies have examined the role of HIF-2α in the skeletal muscle, and studying this may lead to significant

insights into HIF and muscle biology. To that end, we are creating HIF-1α and HIF-2α muscle-specific double knockout mice using HIF-2α floxed mice generously provide by the Celeste Simon laboratory at the University of Penn. Crossing these mice into the HIF-1α/MCK-Cre line will create a HIF-1α/HIF-2α muscle-specific double knockout, which we will study using a similar approach as described in this thesis.

From this training study, it can be seen that, far from being needed for endurance training, loss of HIF-1α has better prepared the muscle for endurance training. Instead, other factors, such as AMPK, are important for the changes seen in the skeletal muscle as a result of endurance training. At this point, we can safely conclude that HIF-1α is not necessary for the endurance training response. Other recent data is corroborating this conclusion.

Given that the loss of HIF-1α leads to muscles (and mice) that are better suited for endurance activity, it is tempting to speculate that a result of endurance training is to remove HIF-1 signaling. Indeed, several recent results give credence to this hypothesis. The first of these is from Dahia (Dahia et al., 2005), et al., who saw in a pheochromocytoma cell line that constitutive activation of HIF-1α leads to a dramatic drop in succinate dehydrogenase subunit B (SDHB) protein. As SDH is a part of both the Krebs cycle and the electron transport chain, this drop in SDHB would cause a sharp decrease in oxidative phosphorylation. Similarly, the hypoxia mimic cobalt chloride has just been shown to inhibit cyctochrome c oxidase subunit 4 processing, which would lead to a decrease in COX4 protein and impaired oxidative phosphorylation (Hervouet et al., 2006). The HIF-1α-dependent mechanism leading

to the drop in oxidative capacity in these studies has not been completely determined yet, but may be a result of decreased flow of acetyl-CoA into the Krebs cycle from pyruvate dehydrogenase (PDH). This connection was recently elucidated by Kim, et al., and Papandreou, et al. (Kim et al., 2006; Papandreou et al., 2006), who discovered that HIF-1 directly upregulates pyruvate dehydrogenase kinase I (PDKI), and inhibitor of PDH. This inhibition results in decreased oxygen consumption in cells with activated HIF-1. We have recently begun a collaborative project with the Nicholas Denko laboratory at Stanford to see if this connection extends to myoblasts.

The hypothesis that endurance training diminishes HIF-1α signaling also gains support from a study by Lundby et al. (Lundby et al., 2005), which showed that endurance trained muscles do not upregulate HIF-1α or HIF-2α mRNA as much as untrained muscles following exercise. The authors do see induction of HIF-1α and HIF-2α mRNA in untrained muscle following exercise, which leads them to conclude that the HIF pathways are important factors in the early adaptive response to endurance training. As our HIF-1α-null mice have no difficulty increasing their performance as a result of endurance training, combining our data with their findings indicates that there is, in fact, no part of the training response for which HIF-1α is necessary, and a result of endurance training may indeed be to down-regulate HIF-1α signaling.

Given the previously mentioned findings, it is also intriguing to speculate that HIF-1α may be inhibitory to endurance training. Constant activation of PDKI, as well as activation of the pathway that leads to decreased SDHB and COX4, would only lead to decreased oxidative capacity, which is the exact opposite of the training

response. Additionally, hypoxia has been shown to inhibit PPARα in a HIF-1α dependent manner (Narravula and Colgan, 2001). This inhibition could potentially extend to PPARδ and γ, two members of the PPAR family that have been shown to be important for the response of skeletal muscle to endurance training. Activation of HIF-1 could prevent these factors from enacting their essential changes. To further study the possibility of HIF-1α being inhibitory toward endurance training, we are creating a mouse with a constitutively active form of HIF-1α expressed specifically in the skeletal muscle. Characterization of this mouse will continue to give insight into the function of HIF-1α in the muscle.

The work described in this thesis shows that while HIF-1α is important in the untrained skeletal muscle, the muscle is able to overcome its absence. Additionally, loss of HIF-1α has no negative impact on endurance training. Instead, it is becoming apparent that diminishing the HIF-1α response is actually quite beneficial, and possibly necessary, for endurance training. The collaborations and follow-up studies described here will be useful in the efforts to better understand HIF-1α function and muscle biology, both in conjunction with each other and separately.

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