Negative regulation of HIF in skeletal muscle of elite endurance athletes: a tentative mechanism promoting oxidative metabolism

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Lindholm ME, Fischer H, Poellinger L, Johnson RS, Gustafsson T, Sundberg CJ, Rundqvist H. Negative regulation of HIF in skeletal muscle of elite endurance athletes: a tentative mechanism promoting oxidative metabolism. Am J Physiol Regul Integr Comp Physiol 307: R248–R255, 2014. First published June 4, 2014; doi:10.1152/ajpregu.00036.2013.—The transcription factor hypoxia-inducible factor (HIF) has been suggested as a candidate for mediating training adaptation in skeletal muscle. However, recent evidence rather associates HIF attenuation with a trained phenotype. For example, a muscle-specific HIF deletion increases endurance performance, partly through decreased levels of pyruvate dehydrogenase kinase 1 (PDK-1). HIF activity is regulated on multiple levels: modulation of protein stability, transactivation capacity, and target gene availability. Prolyl hydroxylases (PHD1–3) induces HIF degradation, whereas factor-inhibiting HIF (FIH) and the histone deacetylase sirtuin-6 (SIRT6) repress its transcriptional activity. Together, these negative regulators introduce a mechanism for moderating HIF activity in vivo. We hypothesized that long-term training induces their expression. Negative regulators of HIF were explored by comparing skeletal muscle tissue from moderately active individuals (MA) with elite athletes (EA). In elite athletes, expression of the negative regulators PHD2 (MA 73.54 ± 9.54, EA 98.03 ± 6.58), FIH (MA 4.31 ± 0.25, EA 30.96 ± 7.99) and SIRT6 (MA 0.24 ± 0.07, EA 11.42 ± 2.22) were all significantly higher, whereas the response gene, PDK-1 was lower (MA 0.12 ± 0.03, EA 0.04 ± 0.01). Similar results were observed in a separate 6-wk training study. In vitro, activation of HIF in human primary muscle cell culture by PHD inactivation strongly induced PDK-1 (0.84 ± 0.12 vs 4.70 ± 0.63), providing evidence of a regulatory link between PHD activity and PDK-1 levels in a relevant model system. Citrate synthase activity, closely associated with aerobic exercise adaptation, increased upon PDK-1 silencing. We suggest that training-induced negative regulation of HIF mediates the attenuation of PDK-1 and contributes to skeletal muscle adaptation to exercise.

aerobic exercise; human skeletal muscle; gene regulation; mitochondrion; adaptation

THE TRANSCRIPTION FACTOR hypoxia-inducible factor (HIF-1) is a key mediator of cellular adaptation to hypoxia. It is continuously degraded in normoxia but stabilized and activated under hypoxic conditions. Target genes of HIF-1 increase oxygen delivery through, e.g., erythropoietin (EPO)-mediated erythropoiesis and vascular endothelial growth factor (VEGF)-induced angiogenesis, and/or alter tissue function at low oxygen availability through, e.g., increased expression of glucose transporters and glycolytic enzymes (6, 34). Exercise-induced local hypoxia and skeletal muscle HIF-1 activity have been suggested as candidates for inducing training adaptation (33, 39, 43).

HIF-1 is expressed in skeletal muscle (37), stabilized in response to sciatic nerve stimulation in rat muscle (38), and activated in response to a single bout of endurance exercise in sedentary humans as well as in mice (1, 24, 25).

Based on these observations, HIF-1 has been suggested to mediate part of the skeletal muscle adaptation to long-term training. However, after 4 wk of unilateral knee-extensor training, HIF-1 mRNA levels transiently increase only in the untrained leg when exposed to acute exercise (21). There are also studies indicating that the increase in VEGF mRNA seen in response to acute exercise is attenuated after a period of endurance training (32). Together, these studies suggest that the response of HIF-1 and its target genes to acute exercise may be blunted after a period of exercise training.

Several recent in vitro and in vivo studies support the notion that HIF-1 effects on muscle metabolism are opposite to those of long-term endurance training, implying that an attenuation of the HIF-1 response to acute exercise could be beneficial for long-term adaptation. For instance, HIF-1 acts to reduce pyruvate flux into the mitochondria through increased expression of PDK-1 (17, 30), thereby inactivating the pyruvate dehydrogenase complex (PDHc). Mice with a skeletal muscle-specific HIF-1 deletion show several of the features typically associated with trained muscle, especially with respect to mitochondrial function (24, 25). More importantly, in patients with Chuvash polycythemia, elevated HIF activity influences human muscle metabolism and leads to increased lactate accumulation and reduced muscle pH in response to exercise (12).

Attenuation of HIF-1 activity to achieve the more oxidative skeletal muscle phenotype associated with training could be done by activation of its negative regulators, which is achieved on multiple levels (Fig. 1). HIF protein levels are regulated by hydroxylation of critical proline residues (15, 16) followed by rapid degradation of the protein via the ubiquitin-proteasome pathway. Three HIF-specific prolyl hydroxylases (PHD1–3) catalyze HIF-1α hydroxylation (5, 10). PHD regulation modulates HIF-1 activity both in vivo and in vitro (2, 29), with PHD2 being the primary HIF-1α PHD under normal conditions (4). Factor-inhibiting HIF (FIH) represses transcriptional activity of HIF through hydroxylation of an asparagine residue, thereby inhibiting binding to the coactivator CBP/p300 (7, 18, 23). Sirtuin 6 (SIRT6) is a histone-3 lysine-9 deacetylase (28) and an epigenetic corepressor of HIF-1, specifically targeting glycolytic genes in favor of mitochondrial activity (47). Based
Experimental design for the 6-wk training study. Twenty-four young, moderately active male subjects (means ± SE age 23 ± 1 yr, weight 78.6 ± 2.7 kg, height 182 ± 2 cm, and VO$_2$peak 48.4 ± 1.32 ml·kg$^{-1}$·min$^{-1}$) performed a supervised 6-wk endurance training program, four 45-min sessions per week at 70% of their pretraining VO$_2$peak as previously described (42). The mean improvement in VO$_2$peak and CS activity were 13% and 26%, respectively [for further information on performance parameters see previously published data from the current study (44)]. Biopsy samples were taken from the vastus lateralis muscle before and 24 h after the last training session.

RNA isolation and reverse transcription. Total RNA from the biopsy samples and cells was extracted by the TRIZol method (Invitrogen, Carlsbad, CA). Two micrograms of total RNA was used to generate cDNA through reverse transcription by MulV reverse transcriptase (N808-0018 Applied Biosystems, Carlsbad, CA) using random hexamer primers (Roche Diagnostics, Mannheim, Germany) in a total volume of 20 μl according to the manufacturer’s specifications.

Real-time PCR analysis. Real-time PCR was used for quantification of specific mRNAs. Primer pairs and probes were supplied as Taqman Reagent kits from Applied Biosystems and used according to the manufacturer’s instructions (PHD1 Hs00363196_m1, PHD2 Hs00254392_m1, PHD3 Hs00222966_m1, FIH Hs00215495_m1, SIRT6 Hs00213036_m1, PDK-1 Hs00176853_m1). Either 18s rRNA (4310893E) or GAPDH (4326317E) was selected as an endogenous control to correct for potential variations in RNA loading (Applied Biosystems). For all genes, samples were amplified simultaneously in duplicate in one assay run as described previously (1).

Preparation of muscle homogenate. Muscle samples (∼20 mg) were homogenized using glass homogenizers in a buffer containing 20 mM HEPES (pH 7.5), 0.2 mM EDTA (pH 7.4), 1.5 mM MgCl$_2$, 100 mM NaCl, 1 mM Na$_2$VO$_4$, 2 mM dithiothreitol (DTT), and 0.4 mM phenylmethylsulfonl fluoride (PMSF). Samples were rotated at 4°C for 60 min and centrifuged at 10,000 g at 4°C for 10 min.

Immunoblot analysis. Muscle homogenate was separated on a sodium dodecyl sulfate (SDS)-polyacrylamide gel, 40 μg of protein per lane, blotted onto Protran nitrocellulose or PVDF membranes (Schleicher & Schuell, Kassel, Germany), and blocked for 1 h at room temperature with 5% dry milk or 0.5% gelatine in Tris-buffered saline containing 0.1% Tween 20 (TBS-T). The membranes were incubated with primary antibody for 90 min at room temperature or overnight in cold room (NB 100-137 PHD2/EGLN1, NB 100-303 PHD3/EGLN3, Novus Biol, Littleton, CO; FIH/HIF1AN ab63163-100 Abcam, Cambridge, UK; SIRT-6 no. 2590, Cell Signaling, Danvers, MA) and incubated with the appropriate horseradish peroxidase-linked secondary antibody (New England BioLabs, Ipswich, MA). α-Actinin (A7811, Sigma-Aldrich, St. Louis, MO) was used as a loading control antibody on the same membrane for each factor. Immunocomplex on the membrane was detected using an enhanced chemiluminescence (ECL) system (GE Healthcare, West Femto Maximum Sensitivity Substrate (Pierce).

Primary cell extracts. Primary human satellite cells were isolated from human skeletal muscle and cultured in Dulbecco’s modified Eagle’s medium (DMEM-F12) with 20% fetal calf serum (FCS) and 1% penicillin streptomycin at 37°C, 5% CO$_2$. At 80% confluency the cells were subcultured with 1 mM dimethylsulfoxyl glycine (DMSO) (Cayman Chemicals) in DMSO or with DMSO only (diluted in differentiation medium) for 6 h, scraped off the plate, and stored in TRizol (Invitrogen) at −20°C for subsequent RNA isolation and analysis.

PDK-1 and PHD inhibition in C2C12 cells. Murine skeletal muscle C2C12 cells were cultured at 37°C, 5% CO$_2$, and differentiated into myotubes in six-well plates using DMEM-F12 medium without fetal calf serum. The myotubes were transfected overnight using Lipofectamine 2000 transfection reagent (2 μl/well) with a small interfering RNA (siRNA) cocktail of four siRNAs against PDK-1 (Dharmacon) (50 pmol/siRNA-well$^{-1}$) according to the manufacturer’s instructions.

**METHODS**

Ethical approval. Before each study, the experimental protocol was explained to all subjects and informed consent was obtained. Each study was approved by the Ethics Committee of Karolinska Institutet (approval numbers: 98-413 and 99-057) and conformed to the Declaration of Helsinki.

Experimental model for the cross-sectional study. The cross-sectional study included 12 endurance-trained men (elite cyclists and triathletes) and 9 moderately active men (controls). For the endurance-trained group (EA) (means ± SE age, height, and weight were 22.3 ± 0.8 yr, 181 ± 2.0 cm, and 71.6 ± 1.8 kg, respectively; and for the control group (MA) age, height, and weight were 23.6 ± 1.0 yr, 180 ± 1.9 cm, and 71.9 ± 1.8 kg, respectively. Mean ± SE VO$_2$peak was 75 ± 1.9 ml·kg$^{-1}$·min$^{-1}$ for the EA group and 47 ± 1.5 ml·kg$^{-1}$·min$^{-1}$ for the MA group, as previously published (11). Biopsy samples were obtained at rest from the vastus lateralis muscle by the percutaneous needle technique (3). Citrate synthase (CS) activity was 0.56 ± 0.1 μkat/g dry muscle for EA and 0.22 ± 0.1 μkat/g dry muscle for MA (45).
Scramble siRNA (50 pmol/well) was used as a negative control. For inhibition of PHDs, DMOG treatment was used; medium was changed into DMEM-F12 supplemented with 4% fetal calf serum and 1% ABAM, with vehicle (DMSO) or DMSO with 1 mM DMOG. Lactate was measured on the cell medium using standard lactate strips (Roche, Basel, Switzerland), and cells were subsequently harvested in TRIzol for mRNA and in 0.1 M phosphate buffer with 0.5% bovine serum albumin (BSA, pH 7.7) for enzyme activity analysis at 6, 48, and 96 h.

Enzymatic analysis. CS activity was measured in a reagent solution [0.1 M Tris·HCl, 0.1 M EDTA, 0.1 M L-malate, malate dehydrogenase (8 ng/μl) and 0.23 mM NAD⁺]. Adding acetyl-CoA started the reaction where CS produces citrate from oxaloacetate and indirectly increases the production of NADH. NADH production was followed over time (2–12 min) using a ratio fluorometer. The activity was assessed relative to a standard curve of NADH (1.5–12 mmol; reported in mmol·kg⁻¹·min⁻¹).

Statistics. For mRNA data, control experiments revealed approximately equal efficiencies over different starting template concentrations for target genes and the endogenous control. For each individual, all samples were simultaneously analyzed in one assay run. Measurements of the relative distribution of the target genes were performed for each individual; a Ct value was obtained by subtracting 18S rRNA Ct values from the respective target Ct values. The expression of each target was then evaluated by 2⁻ΔCt. Unpaired Student’s t-test was used to compare the mRNA and protein expression levels in MA with levels in EA muscle. Paired Student’s t-test was used for the longitudinal study. The in vitro data are an average of three consecutive experiments, each consisting of two replicates. C2C12 CS activity and lactate were analyzed using a two-way ANOVA comparing changes in treatment and control groups over time. Differences were considered significant at P < 0.05. Unless otherwise stated, data are presented as means ± SE.

RESULTS

Elite athletes show significantly higher amounts of PHD2 mRNA and protein. To address the question of how the HIF regulatory system differs between extremely endurance-trained and moderately active individuals, two matched control groups of moderately active individuals (V\(^\text{O}_2\) peak of 47 ml·kg⁻¹·min⁻¹) and a matched control group of moderately active individuals (V\(^\text{O}_2\) peak of 47 ml·kg⁻¹·min⁻¹), were compared. The activity of the Krebs cycle enzyme CS was used as a measure of local aerobic adaptation. The elite and moderately active groups had an average of 0.56 and 0.22 μkat/g dry muscle, respectively. Expression analysis of all three major prolyl hydroxylases (PHD1–3) showed that PHD2 mRNA and PHD3 mRNA levels (Fig. 2A) and PHD2 protein (Fig. 2B) were significantly higher in elite athletes than in moderately active individuals.

PHD2 is the most abundant prolyl hydroxylase in human skeletal muscle. In both trained and untrained individuals, PHD2 mRNA was the most abundantly expressed of the three prolyl hydroxylases in skeletal muscle (Fig. 2A). This is consistent with the findings in mouse, where PHD2 was shown to be significantly more abundant in the skeletal muscle than PHD1 and PHD3 (20). Experiments using siRNA and biochemical purification studies have shown that PHD2 is the primary HIF-1α prolyl hydroxylase under normal conditions in most cells (4, 14).

FIH and SIRT6 levels higher in elite athletes. The transcriptional activity of HIF is regulated on multiple levels. Hydroxylation of HIF by FIH inhibits binding to the coactivator CBP/p300. SIRT6 inhibits HIF-1 transcriptional activity by deacetylation of histones of its target genes. Expression of both FIH and SIRT6 was significantly higher in skeletal muscle of elite athletes than in moderately active individuals (Fig. 3). Taken together, these data show significantly higher levels of the HIF-negative regulators of both stability and transcriptional activity.
Negative regulators of HIF increase in response to endurance exercise training. To investigate whether the differences seen between the elite athletes and moderately active individuals are mainly attributable to continuous high-intensity training and not only to constitutional differences between the groups, we studied the effect of 6 wk of bicycle endurance training on the expression of these negative regulators. Twenty-four male subjects were included in the study and biopsies were taken from the vastus lateralis muscle before (pre) and after (post) 6 wk of training. The postbiopsy was taken 24 h after the last exercise bout. During the training period the mRNA expression levels of PHD2, PHD3, and FIH increased significantly and there was a trend toward SIRT6 upregulation (Fig. 4). At the protein level, PHD2 increased significantly and there was a trend toward SIRT6 upregulation, whereas there was no change in PHD3 or FIH (Fig. 4). These data support that the negative regulators of HIF are training induced.

Expression of the HIF target PDK-1 is lower in elite athletes. PDK-1 was a natural choice as a target gene reflecting HIF-1 activity in human skeletal muscle based on the evidence that HIF-1 exerts its inhibitory effect on mitochondrial oxygen consumption through increased expression of PDK-1 (17, 30). Our data showed that PDK-1 mRNA levels were significantly lower in elite athletes than in moderately active individuals (Fig. 5A). The pronounced difference in PDK-1 levels supports the notion of a reduced HIF-1 activity in elite athletes.

Prolyl hydroxylase activity influences PDK-1 mRNA expression in human myoblasts. To confirm the association between prolyl hydroxylase activity and PDK-1 mRNA levels in skeletal muscle, human primary myoblasts were treated with the prolyl hydroxylase inhibitor DMOG for 6 h. The treatment caused a significant, fivefold increase in PDK-1 mRNA (Fig. 5B).

Silencing of PDK-1 increases muscle CS activity. CS activity is the most widely used marker of endurance-trained muscle; it reflects the activity of the Krebs cycle and the aerobic use of glucose as a source of energy. High skeletal muscle CS activity in response to endurance training is a well-known phenomenon and for both of the human studies included in the study, CS activity was significantly higher in the trained state (44, 45). Two days of PDK-1 siRNA treatment upregulated CS activity in a mouse skeletal muscle cell line. Treating the same cells with DMOG and thereby increasing the HIF-1 activity led to a reduction in CS activity (Fig. 5C). The elevated HIF-1 activity also resulted in higher lactate levels in the cell medium of DMOG-treated cells (Fig. 5D).

DISCUSSION

The main finding of the current study is the remarkably high levels of multiple HIF inhibitors in skeletal muscle of elite endurance athletes. We propose that an upregulation of these negative regulators may lead to a subsequent attenuation of the HIF response as indicated by in vitro experiments and the low levels of HIF target PDK-1 in trained muscle. We speculate that this may represent a functional switch toward a higher capacity to activate the oxidative system in response to endurance training.

The expression of prolyl hydroxylases in human skeletal muscle has not been previously described. We suggest that the lower PDK-1 levels found in the elite athletes stem from a reduced HIF-1 activity as a consequence of increased PHD2
activity induced by long-term endurance training. PHD2 activity is regulated by availability of its substrate (α-ketoglutarate) and product (succinate), both metabolites in the tricarboxylic acid cycle (TCA). PHD2 expression is regulated by hypoxia (26), probably through HIF-1 (27), providing a negative feedback loop on HIF-1 activity during prolonged hypoxia. Increased amounts of skeletal muscle TCA substrates, as well as lower oxygen levels during exercise, may be part of the adaptive stimuli.

FIH and SIRT6, inhibitors of HIF-1 transcriptional activity, show very high levels in elite athletes. In a study by Radak (31), one bout of running exercise in sedentary subjects increased the HIF coactivator p300/ CBP fivefold, while the inhibitor SIRT6 decreased. In active subjects, however, p300/
CBP decreased with exercise, supporting a training-induced downregulation of the HIF response (31). In mice, high levels of FIH and VHL have been associated with slow/oxidative muscle types, such as the soleus, with relatively lower levels of HIF (22).

Reduced exercise-induced lactate accumulation is a well-described phenotype of endurance-trained muscle. It has previously been demonstrated that PDHc is activated by skeletal muscle contraction (13, 40) and a stable upregulation of PDHc activity is seen with long-term aerobic training (19). We show that PHD2 inhibition increase muscle cell PDK-1 levels and lactate accumulation. The significantly lower amount of lactate that PHD2 inhibition increase muscle cell PDK-1 expression and contribute to the well-known activation of the HIF-1 regulatory system are present at higher levels in elite athletes than in moderately active control subjects. We suggest that this may, through repression of HIF-1 activity, attenuate the HIF-1 activity has been shown to inhibit mitochondrial biogenesis in cells (46). The reduced CS activity in response to PHD inhibition and the increase in activity with silencing of PDHc to leads to the speculation that this may also be true in skeletal muscle. However, further studies using other models are required to fully describe this.

We conclude that several of the major known components of the HIF-1 regulatory system are present at higher levels in elite athletes than in moderately active control subjects. We suggest that this may, through repression of HIF-1 activity, attenuate PDK-1 expression and contribute to the well-known activation of PDHc (the rate-limiting step of aerobic ATP synthesis) thus allowing for an increased capacity to utilize oxygen in response to exercise training.

**Perspectives and Significance**

In the future, studies are warranted that correlate protein levels of negative regulators of HIF and PDK-1 response to acute exercise in trained and untrained human subjects. The design of such studies are ongoing in our lab.

Several PHD inhibitors are currently in clinical trials, e.g., for the treatment of anemia. Although an off-target effect, skeletal muscle, as the largest organ in the body, may be widely affected by this systemic inhibition, and it can be speculated that these treatments will influence muscle functionality, with trends similar to those in individuals with Chuvash polycythemia. Exercise studies combined with PHD inhibitors as well as agonists will provide valuable information on the role of PHDs in exercise adaptation.
ATTENUATION OF THE HIF SYSTEM IN ELITE ATHLETES

Apparentley, the adaptability and complexity of organ interactions and in vivo function provides a vast advantage in contrast to the single cell system, the human body is able to balance the need for increased oxygen supply by angiogenic and hematopoietic events with the ability to allow for increased oxygen utilization. There is a constant search for ways of mimicking the health-promoting effects of exercise; the current study may provide a clue of how to go about this quest.

AUTHOR CONTRIBUTIONS


REFERENCES


