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

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Abstract

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.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s).

Author Contributions

Author contributions: M.E.L., L.P., T.G., C.J.S., and H.R. conception and design of research; M.E.L., H.F., and H.R. performed experiments; M.E.L., H.F., and H.R. analyzed data; M.E.L., H.F., and H.R. interpreted results of experiments; M.E.L. and H.R. prepared figures; M.E.L. and H.R. drafted manuscript; M.E.L., R.S.J., T.G., C.J.S., and H.R. edited and revised manuscript; M.E.L., H.F., L.P., R.S.J., T.G., C.J.S., and H.R. approved final version of manuscript.

Keywords

aerobic exercise; human skeletal muscle; gene regulation; mitochondria; 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, those 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-1 α hydroxylation (5, 10). PHD regulation modulates HIF-1 activity both in vivo and in vitro (2, 29), with PHD2 being the primary HIF-1 α 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 on the observations that HIF-1 effects on skeletal muscle metabolism are in some ways opposite to those of long-term endurance training together with that the exercise response of HIF-1-regulated genes after training is attenuated, we hypothesize that training activates negative regulators of HIF.

We explore the regulators of the HIF system in two relevant human experiments: a cross-sectional study comparing moderately active individuals with elite athletes, and in a longitudinal study comparing a separate group of individuals before and after a 6-wk training period. The importance of the descriptive findings in the human studies was verified in vitro.

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 $\pm$ 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 $\pm$ SE $\dot{V}O_{2\text{peak}}$ 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 μ kat/g dry muscle for EA and 0.22 ± 0.1 μ kat/g dry muscle for MA (45).

Experimental design for the 6-wk training study

Twenty-four young, moderately active male subjects (means $\pm$ SE age 23 ± 1 yr, weight 78.6 ± 2.7 kg, height 182 ± 2 cm, and $\dot{V}O_{2\text{peak}}$ 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 $\dot{V}O_{2\text{peak}}$ as previously described (42). The mean improvement in $\dot{V}O_{2\text{peak}}$ 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 MgCl₂, 100 mM NaCl, 1 mM Na₃VO₄, 2 mM dithiothreitol (DTT), and 0.4 mM phenylmethylsulfonyl 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 & Schnell, 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, Little Chalfont, UK) or SuperSignal 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% CO₂. At 80% confluency the cells were differentiated in medium containing 2% fetal calf serum for 3 days. The cells were subsequently treated with 1 mM dimethyloxallyl glycine (DMOG) (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% CO₂, 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. 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 nmol; 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 C_T value was obtained by subtracting 18S rRNA C_T values from the respective target C_T values. The expression of each target was then evaluated by 2^{-C_T}. 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 training-adapted elite athletes (national top level cyclists and triathletes, $\dot{V}O_{2\text{peak}}$ of 75 ml·kg⁻¹·min⁻¹) and a matched control group of moderately active individuals ($\dot{V}O_{2\text{peak}}$ of 47 ml·kg⁻¹·min⁻¹), we compared skeletal muscle biopsies from the vastus lateralis muscle of each group. 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 $\mu\text{kat/g}$ dry muscle, respectively. Expression analysis of all three major prolyl hydroxylases (PHD1–3) showed that PHD2 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-1 α 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 seen in the serum of submaximally exercising HIF-1 α knockout mice (24) could be due to an increased PDHc activity as part of the phenotype. Taken that the HIF-1 α knockout model also shows features not generally associated with human exercise response, such as postexercise locomotor muscle damage, we emphasize that the suggested moderation of HIF activation acts in concert with additional and previously described adaptations of the skeletal muscle.

Activation of HIF-1 in the early phase of adaptation (1, 24) is said to be crucial for VEGF-driven, exercise-induced skeletal muscle angiogenesis (38). The present findings offer a tentative explanation for the observation that an increase in capillarity seems to precede increases in mitochondrial enzyme activity in several models of exercise (8, 35). It has been speculated that improvements in oxygen delivery, through increases in vascularity, may be involved in the subsequent adaptive increases in aerobic-oxidative potential (8, 9).

To acquire the full metabolic benefit of endurance training may take up to 6 months or longer. In this study, we observe a trend toward an activation of the HIF-1 negative regulators already after 6 wk of endurance training, suggestive of activation in the late part of exercise adaptation. It can be speculated that the subsequent inhibition of HIF-1 activity (Fig. 1) is part of the switch from angiogenic to metabolic adaptation. In line with this, 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 PDK-1 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.

Apparently, 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.

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Fig. 1.

Schematic model of hypoxia-inducible factor (HIF-1) regulation. During normoxia, HIF-1 is hydroxylated by prolyl hydroxylase (PHD) followed by ubiquitinylation and subsequent degradation. During hypoxia, PHDs are inhibited. HIF-1 is thus stabilized, enters the nucleus, and dimerizes with aryl hydrocarbon receptor nuclear translocator (ARNT). The transcriptional activity of HIF can be modulated through hydroxylation of an asparagine residue, which inhibits binding to the coactivator CBP/p300, or by sirtuin 6 (SIRT6), which deacetylates HIF-1 target genes.

Fig. 2.

PHD expression is higher in skeletal muscle from elite athletes. *A*: mRNA expression of three HIF-1-specific prolyl hydroxylases (PHD1–3) in skeletal muscle tissue of endurance-trained athletes (EA) ($n = 12$) compared with moderately active (MA) men ($n = 9$). 18S rRNA was used as an endogenous control. *B, left*: representative immunoblots of PHD1–3 in skeletal muscle biopsies obtained from three MA compared with three EA. *Right*: PHD1–3 protein levels in 6 moderately MA compared with 6 EA, related to the level of α -actinin.

*Significant differences between endurance-trained and control subjects at the $P < 0.05$ level. All data are expressed as means $\pm$ SE.

Fig. 3.

Expression levels of FIH and SIRT6, negative regulators of HIF-1 transcriptional activity, are higher in EA compared with MA individuals. *A*: skeletal muscle FIH mRNA levels in MA ($n = 9$) compared with EA ($n = 7$). *B*: skeletal muscle SIRT6 mRNA levels in MA ($n = 8$) compared with EA ($n = 7$). *C*: representative immunoblots of FIH and SIRT6 in skeletal muscle biopsies obtained from three MA men compared with three EA. *A*: FIH protein levels in MA ($n = 6$) compared with EA ($n = 6$). *Bottom right*: SIRT6 protein levels in MA ($n = 6$) compared with EA ($n = 7$). Protein levels are related to α -actinin. *Significant differences at the $P < 0.05$ level. 18S rRNA was used as an endogenous control for the mRNA data. All data are expressed as means $\pm$ SE.

Fig. 4.

Six weeks of endurance training of untrained individuals induces the expression of the negative regulators of HIF-1 activity. *A*: PHD2 mRNA (*left*, $n = 22$) and protein (*right*, $n = 9$) levels before and after 6 wk of endurance training. *B*: PHD3 mRNA (*left*, $n = 22$) and protein (*right*, $n = 9$) levels before and after 6 wk of endurance training. *C*: FIH mRNA (*left*, $n = 22$) and protein (*right*, $n = 9$) levels before and after 6 wk of endurance training. *D*: SIRT6 mRNA (*left*, $n = 22$) and protein (*right*, $n = 9$) levels before and after 6 wk of

endurance training.*Significant differences between pre- and posttraining at the $P < 0.05$ level.

Fig. 5.

PHD activity in human skeletal muscle is coupled to increased activity of the Krebs cycle enzyme citrate synthase (CS), through the HIF-1 target gene PDK-1. *A*: real-time PCR analyses of PDK-1 mRNA levels in skeletal muscle tissue of MA men ($n = 7$) compared with EA ($n = 10$). 18S rRNA was used as an endogenous control. *B*: human satellite cell PDK-1 mRNA levels after 6 h exposure to the PHD inhibitor dimethyloxallyl glycine (DMOG) ($n = 4$) compared with control (ctrl) ($n = 4$). *C*: CS activity at 6, 48, and 96 h in differentiated skeletal muscle C2C12 cells treated with the PHD inhibitor DMOG ($n = 4$) or small interfering RNA (siRNA; $n = 4$) against PDK-1. Data are presented as fold change relative to negative controls; vehicle (DMSO = Ctrl) and scramble siRNA (Neg ctrl), respectively. *D*: Lactate accumulation at 6, 48, and 96 h in media from differentiated skeletal muscle C2C12 cells treated with DMOG ($n = 5$) or vehicle (DMSO = Ctrl). Data are presented as fold change relative to negative controls. *E*: immunoblot of HIF-1 protein in the cells presented in *D*. *Significant differences at the $P < 0.05$ level.