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High-intensity interval training-induced metabolic adaptation coupled with an increase in Hif-1α and glycolytic protein expression

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Abe T, Kitaoka Y, Kikuchi DM, Takeda K, Numata O, Takemasa T. High-intensity interval training-induced metabolic adaptation coupled with an increase in Hif-1α and glycolytic protein expression. *J Appl Physiol* 119: 1297–1302, 2015. First published October 1, 2015; doi:10.1152/japplphysiol.00499.2015.—It is known that repeated bouts of high-intensity interval training (HIIT) lead to enhanced levels of glycolysis, glycogenesis, and lactate transport proteins in skeletal muscle; however, little is known about the molecular mechanisms underlying these adaptations. To decipher the mechanism leading to improvement of skeletal muscle glycolytic capacity associated with HIIT, we examined the role of hypoxia-inducible factor-1α (Hif-1α), the major transcription factor regulating the expression of genes related to anaerobic metabolism, in the adaptation to HIIT. First, we induced Hif-1α accumulation using ethyl 3,4-dihydroxybenzoate (EDHB) to assess the potential role of Hif-1α in skeletal muscle. Treatment with EDHB significantly increased the protein levels of Hif-1α in gastrocnemius muscles, accompanied by elevated expression of genes related to glycolysis, glycogenesis, and lactate transport. Daily administration of EDHB for 1 wk resulted in elevated glycolytic enzyme activity in gastrocnemius muscles. Second, we examined whether a single bout of HIIT could induce Hif-1α protein accumulation and subsequent increase in the expression of genes related to anaerobic metabolism in skeletal muscle. We observed that the protein levels of Hif-1α and expression of the target genes were elevated 3 h after an acute bout of HIIT in gastrocnemius muscles. Last, we examined the effects of long-term HIIT. We found that long-term HIIT increased the basal levels of Hif-1α as well as the glycolytic capacity in gastrocnemius muscles. Our results suggest that Hif-1α is a key regulator in the metabolic adaptation to high-intensity training.

anaerobic metabolism; gene regulation; Hif-1α; high-intensity training; skeletal muscle introduction

HIGH-INTENSITY INTERVAL TRAINING (HIIT) is a type of physical training, consisting of repeated high-intensity exercise alternated with rest periods. There is a growing understanding that HIIT, which is performed at higher intensity but for shorter time period compared with traditional endurance training, induces more time-efficient stimulus to increase aerobic capacity in skeletal muscle (2, 6).

Previous studies have reported that HIIT activates molecular signaling pathways linked to peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), which is well known as a master regulator of mitochondrial biogenesis, in both rodents (31) and humans (2, 15). Skeletal muscle glycolytic capacity could also be altered with HIIT, as a consequence of the increase in the enzymes involved in muscle glycolysis and glycogenesis, such as phosphofructokinase (Pfk) (10, 14), lactate dehydrogenase (Ldh) (14), and glycogen synthase (Gys) (3). Recently, the protein levels of monocarboxylate transporter 4 (Mct4), which facilitates lactate release from muscle cells, were shown to increase with HIIT (9, 20). However, although previous studies have shown HIIT leads to enhanced levels of the proteins involved with anaerobic metabolism, the molecular mechanism underlying these adaptations is unknown.

Recent studies indicate that hypoxia-inducible factor-1α (Hif-1α) is involved in the fast muscle phenotype. Hif-1α was initially identified as an important protein for hypoxia adaptation (23). Hif-1α is mainly degradated in normoxia by the prolyl hydroxylase (Phd) pathway, while Hif-1α accumulates in hypoxia with the decline of Phd activity. Inhibition of Phd with ethyl 3,4-dihydroxybenzoate (EDHB) induced Hif-1α protein accumulation in at least liver and kidney, and whole body hypoxic tolerance (11). A previous study reported that EDHB treatment increased vascular endothelial growth factor (VEGF) expression in rat skeletal muscle (28). Under hypoxic conditions, the energy metabolism of the cell shifts from aerobic to anaerobic, and Hif-1α contributes to this shift via upregulation of glycogen metabolism gene transcription (25). The glycolytic enzyme genes Pfk and Ldh are the main targets of Hif-1α (33), although glycogenesis enzyme genes and the lactate transporter gene are also regulated by Hif-1α (21, 32).

In skeletal muscle cells, the protein levels of Hif-1α are observed even under normoxia condition and higher in fast muscles than in slow muscles (22). In addition, upregulation of Hif-1α induces a transition from slow to fast muscle phenotype (17). Previous studies have shown that an acute bout of strength training, considered to improve the muscle anaerobic capacity similar to HIIT, increases Hif-1α expression in humans (1) and mice (17). These results imply that Hif-1α has a pivotal role in glycolytic capacity enhancement through HIIT. Therefore, in the current study, we examined whether Hif-1α affects the expression of genes related to glycolysis, glycogenesis, and lactate transport and whether an acute bout of HIIT alters the protein levels of Hif-1α. Further, we investigated the effect of long-term HIIT on the basal levels of Hif-1α.

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Table 1. *Primer sequences for qRT-PCR*

Target Gene	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)
Actb	ACAACGGCTCCGGCATGTGCAAA	ACCCATTCCCACCATCACACCCTGG
Gys1	GTCCTCGCTTCCAGGATTGG	GTGTAGATGCCACCCACCTTG
Hk2	GGCTAGGAGCTACCACACAC	AACTCGCCATGTTCTGTCCC
Ldh-a	TGTTGGGGTTGGTGCTGTTGGCAT	AACAAGGGCAAGCTCATCCGCCAA
Mct4	GGCGGACAGAGGCAGATACA	GCTTTCACCAAGAACTGAGCTG
Pfkm	ACAATCTGCAAGAAAGCAGCG	TACCTTGGGCATCTCCACCA
Pygm	GTACAAGAACCCAAGAGAGTGGA	CGAGAAGGTTCAACACCCCA
Tbp	CTGCCACACCAGCTTCTGA	TGCAGCAAATCGGCTTGGG

MATERIALS AND METHODS

Animals. All experimental procedures performed in this study were approved by the Institutional Animal Experiment Committee of the University of Tsukuba. Male 7- to 8-wk-old ICR (Institute of Cancer Research) mice (Tokyo Laboratory Animals Science, Japan) were used in this study. The mice were housed in temperature (23°C ± 2°C)- and humidity (55% ± 5%)-controlled holding facilities under a 12:12-h light:dark cycle and had ad libitum access to food and water. Upon completion of experimental treatments, the mice were killed by cervical dislocation; their lower limb muscles were dissected, quickly frozen in liquid nitrogen, and stored at −80°C until analysis.

Intraperitoneal ethyl 3,4-dihydroxybenzoate (EDHB) administration. In this study, we performed experiments using a single dose and a repeated dose of EDHB. DMSO was used as a solvent for EDHB. All EDHB injection groups were compared with vehicle injection groups. In the single-dose experiment, the mice received an intraperitoneal injection of 250 mg/kg EDHB, and lower limb muscles were sampled 4 h after injection. The repeated-dose experiment was performed for 1 wk. Mice received 100 mg/kg EDHB every day. The dose of EDHB was selected according to previous studies (11, 28). To avoid the effect of the last day of EDHB treatment, muscle excision was performed 24 h after the last intraperitoneal injection.

HIIT protocol. HIIT was based on the protocol in rats described by Terada previously (30, 31), with minor modifications. Mice performed a 20-s swimming exercise at most 20 times or until the mouse reached exhaustion, with a weight equivalent to 10% of their body weight. The weight was tied near the base of their tail. Between exercise bouts, mice were landed from water, and a 10-s rest period was allowed. Exhaustion was defined as when mice could not rise to surface more than 3 s. A barrel filled with water to a depth of 60 cm was used for the swimming exercise, and water temperature was maintained at 35°C during the exercise. Immediately after exercise, blood samples were collected from the tail, and blood lactate was measured using portable blood lactate analyzer (Lactate Pro 2, Arkray, Japan). The mice were exercised at the same time of day, and then randomly assigned to 3 groups: 0 h (immediately), 3 h, and 6 h after the swimming exercise. The animals were slaughtered following a timed sequence at the end of the exercise. Mice in the Pre group were slaughtered just before the exercise of other groups. All the mice including the Pre group had performed a practice swimming exercise for 2 days before the HIIT experiment.

Long-term training protocol. Training mice were randomly assigned to HIIT and Sedentary (Sed) groups. HIIT protocol was the same as above, and mice were trained for 6 wk. To control load, the weight of each mouse was measured one time per week. HIIT was performed once per day in the morning (from 9:00 AM to 11:00 AM) and 5 days/wk. Each rest day was after 2 or 3 consecutive training days. Muscle samples were taken 24 h after the last training to avoid the effect of the last bout of exercise. To evaluate exercise performance, another six mice per group performed the exhaustion swimming test (29). The mice swam to exhaustion with a load of 10% body weight, and the time to reach exhaustion was measured. These mice were not used for any other analysis. The mice swam to exhaustion with a load of 10% body weight, and the time to reach exhaustion was measured. The definition of exhaustion was the same in *HIIT protocol*.

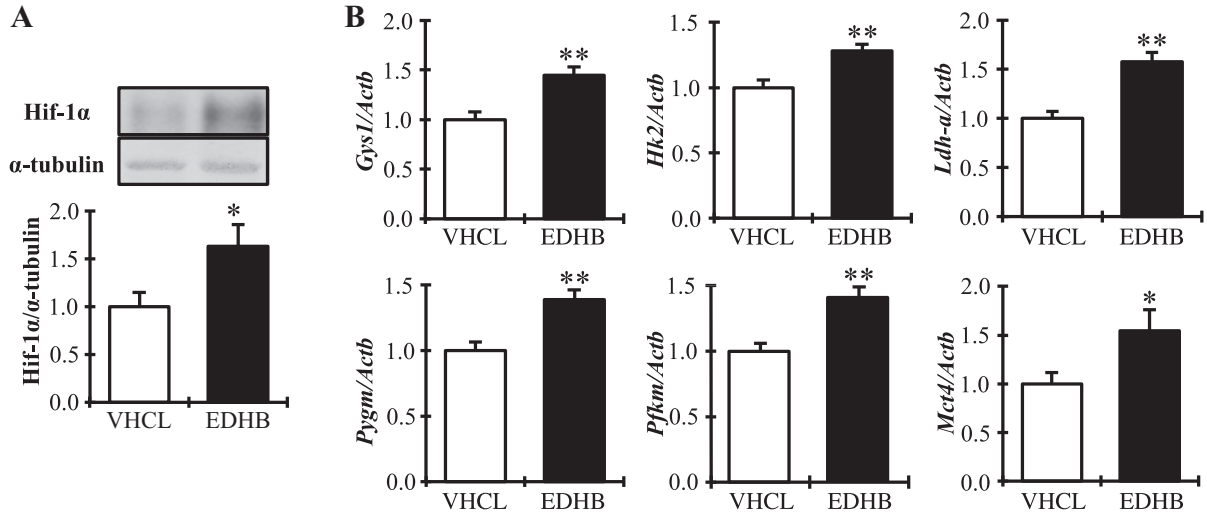

Fig. 1. Hypoxia inducible factor-1α (Hif-1α) accumulation following administration of ethyl 3,4-dihydroxybenzoate (EDHB) leads to an increase in the expression of genes related to glycolytic, glycogenesis, and lactate transport. *A*: protein levels of Hif-1α in gastrocnemius muscle of the EDHB group (EDHB) ($n = 8$) compared with the vehicle (VHCL) group. α-Tubulin was used as an endogenous control. *B*: mRNA expression of genes related to glycolysis (*Pygm*, *Pfkm*, and *Ldh-a*), glycogenesis (*Gys1* and *Hk2*), and lactate transport (*Mct4*) in the muscle tissues of the EDHB group compared with the VHCL group. *Actb* was used as an endogenous control. $^*P < 0.05$, $^{**}P < 0.01$ vs. VHCL. All data are expressed as means + SE; $n = 10$ per group.

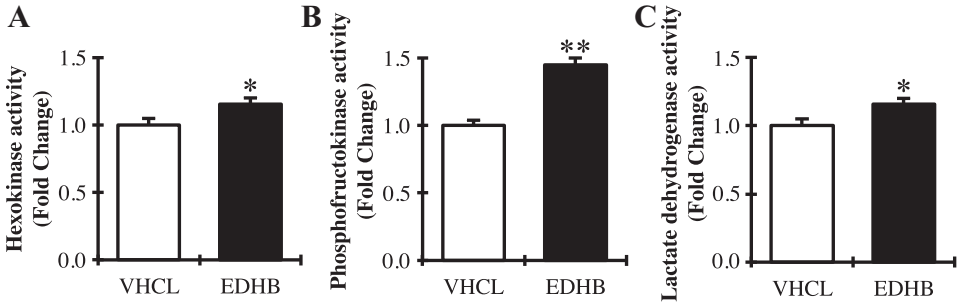

Fig. 2. Daily administration of EDHB for 1 wk enhances the activities of glycolytic enzyme. *A*: hexokinase (Hk) activity compared between EDHB and VHCL groups. *B*: phosphofructokinase (Pfk) activity compared between EDHB and VHCL groups. *C*: lactate dehydrogenase (Ldh) activity compared between EDHB and VHCL groups. $*P < 0.05$, $**P < 0.01$ vs. VHCL. All data are expressed as means + SE; $n = 10$ per group.

Protein isolation and quantitation. Protein fractions were isolated from gastrocnemius muscles, and frozen in lysis buffer [50 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM EDTA, 10 mM NaF, 10 mM $Na_4P_2O_7$, 2 mM $NaVO_4$, 1% sodium deoxycholate, 1% NP-40, and 0.2% sodium dodecyl sulfate] with a protease inhibitor mix (Nakarai Tesque, Japan) on ice. The homogenates were then centrifuged at 15,000 rpm for 10 min at 4°C, and the supernatants were collected. Protein levels in the homogenate samples were measured using a BCA protein assay kit (Nakarai Tesque, Japan). Hif-1α protein was detected by the monoclonal antibody NB100-105 (Novus Biologicals) at a dilution of 1:1,000. Purity of the protein fractions was validated using an antibody against α-tubulin (cat. no. 2144, Cell Signaling) diluted 1:1,000. Horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgGs were used as secondary antibodies at a dilution of 1:5,000. The bands were visualized by a chemiluminescence detection system according to the manufacturer's instructions (Nakarai Tesque, Japan). Images for each membrane were analyzed using ImageJ software.

RNA isolation and quantitative RT-PCR (qRT-PCR analyses). mRNA was isolated from frozen gastrocnemius muscles using the Trizol reagent (Invitrogen). The quantity and quality of RNA were validated with Nanodrop (Thermo Scientific). cDNA synthesis was performed using the PrimeScript RT Master Mix (Takara, Japan). qRT-PCR was performed with the Thermal Cycler Dice Real-Time System using SYBR Premix Ex taq II (Takara, Japan). The PCR protocol was as follows: denaturation for 15 s at 95°C and

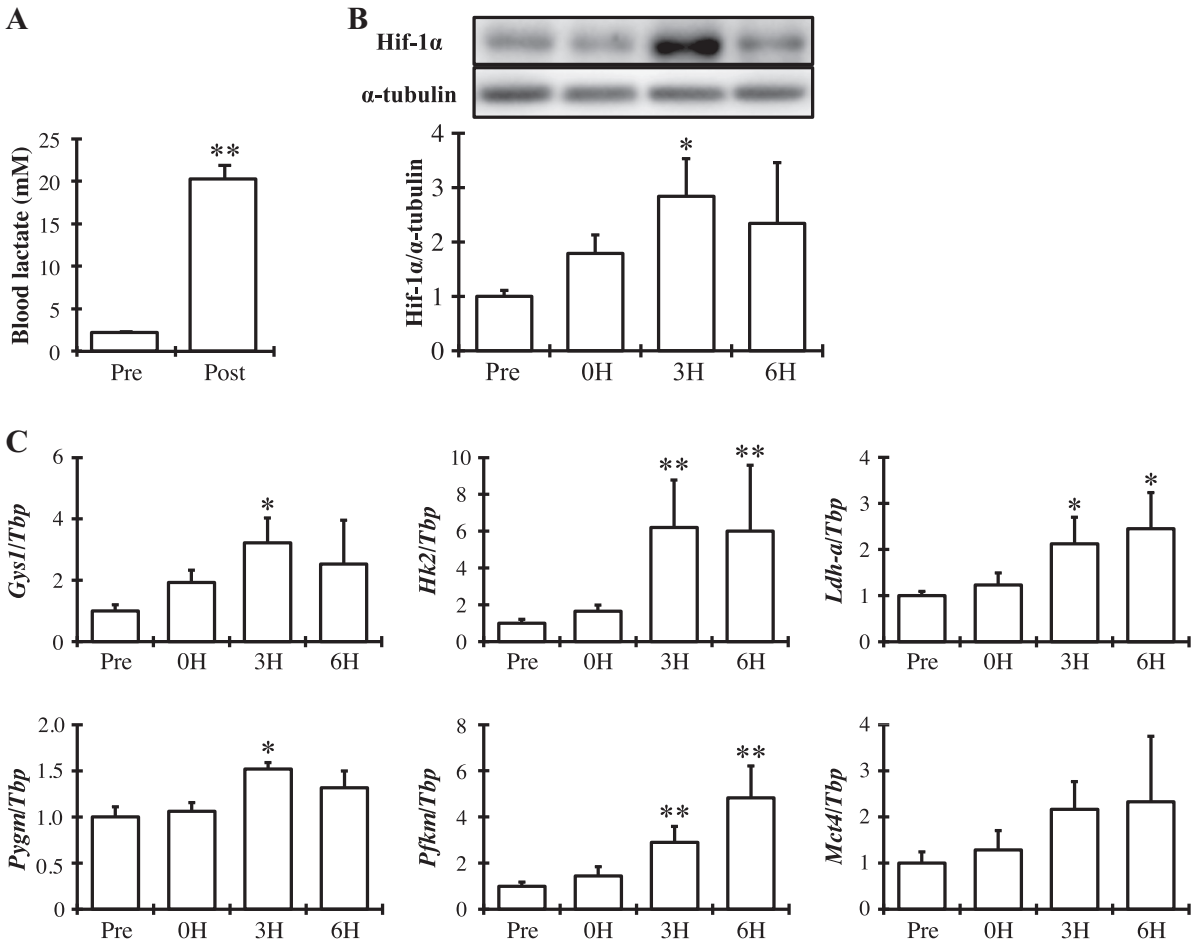

Fig. 3. A single bout of high-intensity interval training (HIIT) increases the protein levels of Hif-1α and the expression of the target genes. *A*: comparison of blood lactate levels before (Pre) and immediately after (Post) a bout of HIIT. $**P < 0.01$ vs. Pre. *B*: comparison of the protein levels of Hif-1α in gastrocnemius muscle before (Pre) and immediately (0H), 3 h (3H), and 6 h (6H) after swimming. *C*: mRNA expression of Hif-1α target genes at each time point. *Tbp* was used as an endogenous control. $*P < 0.05$, $**P < 0.01$ vs. Pre. All data are expressed as means + SE; $n = 8$–9 per group.

annealing and extension for 40 s at 60°C (40 cycles). The dissociation curve for each sample was analyzed to verify the specificity of each reaction. The relative mRNA expression levels of the target genes were determined by the delta-delta Ct method and normalized to the expression of β-*actin* (*Actb*) or *TATAbox binding protein* (*Tbp*). In a single bout of HIIT experiment, *Tbp* was used as an endogenous control, because it has been shown that *Actb* is not a good endogenous control in exercise experiments (18). The primer sequences are shown in Table 1.

Enzyme activity. The activities of Pfk, Hk, and Ldh were measured to assess the muscle's glycolytic capacity. Pfk and Hk activities were determined using the standard procedures described by Shonk and Boxer (27), and Ldh activity was determined using the standard procedure described by Chi et al. (4). The samples of HIIT and Sedentary group were analyzed alternately.

Muscle glycogen measurement. The glycogen content in muscle was measured as described previously (16). Aliquots of frozen tibialis anterior muscle were added to microcentrifuge tubes containing 30% KOH and 3% Na_2SO_4 and then heated at 60°C for 10 min. Glycogen was precipitated by adding 100% ethanol and centrifuged at 6,000 rpm for 30 min at 4°C. After the supernatants were collected, the precipitates were desiccated. The glycogen pellets were hydrolyzed and then treated with 500 μl of H_2SO_4 and 100 μl of 5% phenol. The mixture was allowed to stand for 30 min at room temperature. Thereafter, the samples were measured spectrophotometrically at 490 nm. The results were expressed as milligrams of glycogen per gram of tissue.

Statistical analysis. All data were tested for normality using the Kolmogorov-Smirnov test, and were found to be normally distributed. The data of the EDHB dosing experiment were analyzed using an unpaired *t*-test for comparison between the vehicle (VHCL) and EDHB groups. Differences in blood lactate expression between the preexercise group and postexercise group were also tested using an unpaired *t*-test. Multigroup comparisons were performed by one-way ANOVA, followed by a post hoc test (Dunnett's test) for multiple comparisons. For all comparisons, statistical significance was defined as $P < 0.05$. Data were expressed as means ± SE.

RESULTS

Effect of EDHB administration on Hif-1α accumulation. To determine whether Hif-1α has a potential role in regulating the genes related to glycolysis, glycogenesis, and lactate transport in skeletal muscle, we investigated the gene expression after exposure to EDHB, a chemical facilitating Hif-1α accumulation (11). In the current study, the protein levels of Hif-1α in gastrocnemius muscle of the mice increased by ~50% 4 h after treatment with EDHB (Fig. 1). The expression of genes associated with glycolysis (*Pygm*, *Pfkm*, and *Ldh-a*) encoding glycogen phosphorylase (Pyg), Pfk, and Ldh, were significantly higher in the EDHB group. The mRNA expression levels of *Gys1*, *Hk2*, and *Mct4*, encoding Gys, Hk, and Mct4, were also significantly increased (Fig. 1). To confirm the effect of continuous Hif-1α protein accumulation on glycolytic capacity, the mice were treated with EDHB every day for 1 wk. Continuous daily administration of EDHB increased the enzyme activity of Pfk, Ldh, and Hk (Fig. 2). These results indicate that Hif-1α has a potential role in facilitating anaerobic metabolism in skeletal muscle.

Effect of an acute bout of HIIT on Hif-1α and anaerobic metabolism gene expression. Although strength training induces an increase in the protein levels of Hif-1α, little is known about the effect of HIIT on the protein levels of Hif-1α and the expression of the target genes. In this study, we used forcible swimming exercise as a HIIT model, because it is hard to reach an extreme high-intensity load in mice with treadmill running exercises. After a single bout of HIIT, the blood lactate levels of mice increased to >20 mM. The protein levels of Hif-1α increased significantly 3 h after HIIT (Fig. 3). The mRNA expression of *Gys1, Hk2, Pygm, Pfkm*, and *Ldh-a* was significantly increased 3 h after HIIT, and the expression of *Hk2*,

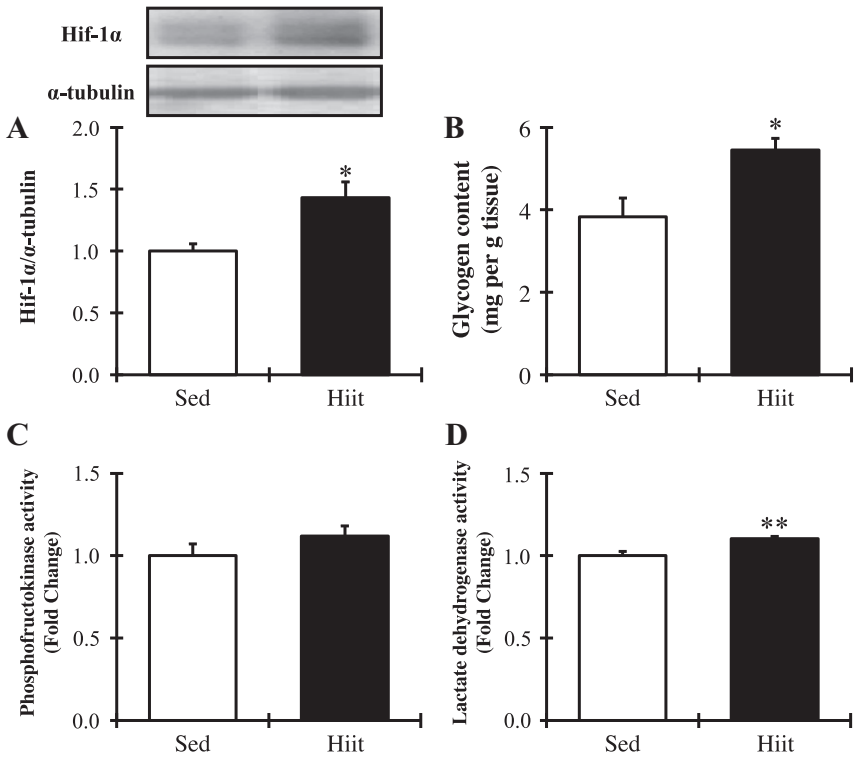

Fig. 4. Long-term HIIT elevates the basal protein levels of Hif-1α and glycolytic capacity. *A*: comparison of the basal levels of Hif-1α in gastrocnemius muscle between the sedentary (Sed) and HIIT groups. α-Tubulin was used as an endogenous control. *B*: comparison of glycogen levels in tibialis anterior muscle between the Sed and HIIT groups. *C* and *D*: comparison of glycolytic enzyme activity in gastrocnemius muscle between the Sed and HIIT groups. *$P < 0.05$, **$P < 0.01$ vs. Sed. All data are expressed as means + SE; $n = 8$–10 per group.

Pfkm, and *Ldh-a* was increased even after 6 h (Fig. 3). There was no significant increase in *Mct4* expression.

Training adaptation of Hif-1α and glycolytic capacity. Next, we examined whether long-term HIIT changes the basal protein levels of Hif-1α and the activities of Ldh and Pfk, which are widely used markers of glycolytic capacity. The mice underwent 6 wk of training and were compared with untrained mice. The basal levels of Hif-1α were significantly elevated in the HIIT group compared with Sed group (Fig. 4). In the HIIT group, Ldh activity was significantly increased, whereas there was no change in Pfk activity (Fig. 4). Glycogen is a key energy source during high-intensity exercise and represents an important storage form of energy for anaerobic performance. We found that 6 wk of HIIT induced a significant increase in muscle glycogen content (Fig. 4). We also measured the exercise performance of the HIIT and Sed group by exhaustion swimming test. HIIT group swam significant longer (735 ± 64 s) than the Sed group (289 ± 49 s) in the test.

DISCUSSION

In high-intensity exercise, skeletal muscle mainly utilizes glycogen as a substrate for energy resynthesis. Therefore, it is considered that muscle glycolytic capacity and glycogen storage are important factors for exercise performance in high-intensity exercise, as well as mitochondrial capacity. However, although several studies have investigated exercise-induced changes in signaling cascades related to aerobic metabolism, little is known about the molecular mechanism underlying the changes in anaerobic metabolism. In this study, we showed that increased protein levels of Hif-1α following EDHB treatment induced the mRNA expression of proteins involved in anaerobic metabolism, as reported in the case of cancer cells (25). We also showed that HIIT increased the protein levels of Hif-1α, accompanied by a concurrent increase in the gene expression of glycolytic and glycogenesis enzymes. Our results suggest that the enhanced expression of genes related to glycolytic capacity after HIIT results from increased levels of Hif-1α, which is known as a major transcription factor regulating glycolytic metabolism. In recent studies, strength training increased the protein levels of Hif-1α in humans and mice models (1, 17). Taken together, Hif-1α may play an important role in improvement of glycolytic capacity in sprint training as well as strength training, and possibly contribute performance of high-intensity exercise.

The protein levels of Hif-1α are regulated by various pathways (24); however, there is little information about the pathways involved in Hif-1α protein regulation associated with exercise. In general, Hif-1α is regulated by an oxygen-dependent mechanism, and a correlation between the state of microvascular P_{O_2} in muscles and the protein levels of Hif-1α has been shown (17, 19). However, apart from oxygen-dependent mechanisms, other mechanisms also regulate the protein levels of Hif-1α. Prolyl hydroxylase 2 (Phd2) is a major negative regulator of Hif-1α, and this protein is expressed at higher levels in elite endurance athletes than in moderately active individuals (13). Furthermore, the mRNA levels of *factor inhibiting Hif (Fih)* and *Phd3* are also higher in elite endurance athletes (13). Thus elevation of basal Hif-1α levels after long-term HIIT may be mediated by these factors. Hif-1α synthesis is regulated by the mammalian target of rapamycin (mTOR) pathway, an essential pathway for load-induced skeletal muscle hypertrophy (5). Strength training affects both the mTOR pathway and Hif-1α expression (1, 17). Taken together, the mTOR pathway may be involved in exercise-induced increase in Hif-1α.

Some studies have proposed other transcription factor candidates to be involved in the training-induced enhancement of glycolytic capacity. c-myc is a major regulator of anaerobic metabolism, similar to Hif-1α, and is considered to induce glycolysis during exercise (7). c-myc is possibly related to the training-induced transcriptional shift, because conserved binding sites for Hif-1α are found overrepresented near the conserved Myc E boxes in glycolytic genes (12). However, the c-myc protein is very unstable (5), and it was impossible to detect this protein. Receptor-interacting protein 140 (Rip140) is another possible regulator of glycolysis in skeletal muscle (26). Clenbuterol, a β2-adrenergic agonist that induces transitions from slow to fast muscle phenotypes, increases the protein levels of Rip140 and the activity of glycolysis enzymes (8). However, since HIIT does not affect the protein levels of Rip140 (9), Rip140 is not likely to be involved in the adaptation to HIIT.

Last, the use of EDHB for inducing Hif-1α accumulation may be considered a study limitation. Since EDHB inhibits all Phds, it is possible that other Hifs or other pathways regulated by Phds may also be affected. Another study limitation is that our forcible swimming exercise protocol of HIIT may be a stressful conditions for mice. Future studies are needed to confirm HIIT-induced anaerobic adaptations in humans or using other kinds of physical training such as treadmill running in rodents.

In summary, we have shown that *1*) Hif-1α induced the expressions of anaerobic metabolic genes, and *2*) Hif-1α protein content was increased after an acute bout of HIIT and long-term HIIT in mouse skeletal muscle. Therefore, it is possible that Hif-1α is one of the key regulators in the HIIT-induced adaptations of anaerobic metabolism in skeletal muscle. Our data could be a cue to reveal the molecular mechanisms underlying the adaptation of anaerobic metabolism associated with high-intensity training.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: T.A., Y.K., O.N., and T.T. conception and design of research; T.A., Y.K., D.M.K., and K.T. performed experiments; T.A., Y.K., and T.T. analyzed data; T.A., Y.K., and T.T. interpreted results of experiments; T.A. prepared figures; T.A. and Y.K. drafted manuscript; T.A., Y.K., and T.T. edited and revised manuscript; T.A., Y.K., D.K., K.T., O.N., and T.T approved final version of manuscript.

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