PGC-1α regulates a HIF2α-dependent switch in skeletal muscle fiber types

Kyle A. Rasbach, Rana K. Gupta, Jorge L. Ruas, Jun Wu, Elnaz Naseri, Jennifer L. Estall, and Bruce M. Spiegelman[1]

The Dana-Farber Cancer Institute and Department of Cell Biology, Harvard Medical School, Boston, MA 02115

Contributed by Bruce M. Spiegelman, October 28, 2010 (sent for review August 28, 2010)

The coactivator peroxisome proliferator-activated receptor-gamma coactivator 1 α (PGC-1α) coordinates a broad set of transcriptional programs that regulate the response of skeletal muscle to exercise. However, the complete transcriptional network controlled by PGC-1α has not been described. In this study, we used a qPCR-based screen of all known transcriptional components (Quanttrx) to identify transcription factors that are quantitatively regulated by PGC-1α in cultured skeletal muscle cells. This analysis identified hypoxia-inducible factor 2 α (HIF2α) as a major PGC-1α target in skeletal muscle that is positively regulated by both exercise and β-adrenergic signaling. This transcriptional regulation of HIF2α is completely dependent on the PGC-1α/ERRα complex and is further modulated by the action of SIRT1. Transcriptional profiling of HIF2α target genes in primary myotubes suggested an unexpected role for HIF2α in the regulation of muscle fiber types, specifically enhancing the expression of a slow twitch gene program. The PGC-1α–mediated switch to slow, oxidative fibers in vitro is dependent on HIF2α, and mice with a muscle-specific knockout of HIF2α increase the expression of genes and proteins characteristic of a fast-twitch fiber-type switch. These data indicate that HIF2α acts downstream of PGC-1α as a key regulator of a muscle fiber-type program and the adaptive response to exercise.

Peroxisome proliferator-activated receptor-gamma coactivator 1 α (PGC-1α) regulates a number of metabolic programs in skeletal muscle that control the basal expression of a number of metabolic gene programs and at least partially regulates muscle's response to exercise (1, 2). Notably, increased expression of PGC-1α in response to exercise and other stimuli promotes mitochondrial biogenesis, increases fatty acid oxidation, increases GLUT-4 expression and glucose utilization, stimulates the expression of genes of the neuromuscular junction, and promotes a fiber-type switch toward oxidative, slow fibers (3–6). We also recently demonstrated that PGC-1α regulates the expression of VEGF and other angiogenic factors in response to hypoxia and nutrient deprivation, and this pathway seems central to exercise-induced angiogenesis (7). Taken together, it is apparent that PGC-1α orchestrates and coordinates the broad adaptive response of skeletal muscle to physical activity and exercise training.

PGC-1α regulates these metabolic programs by binding to and activating a variety of nuclear receptors and other transcription factors, to form active transcriptional complexes (1, 8, 9). For example, PGC-1α binding to ERRα promotes programs of mitochondrial biogenesis and angiogenesis, whereas GAPBA/PGC-1α binding drives transcription of the neuromuscular junction gene program (6, 7, 10, 11). Interestingly, PGC-1α often regulates the expression of transcription factors that it coactivates, leading to a feed-forward switch (1). For example, PGC-1α dramatically increases PPARα expression in various cell types and also coactivates PPARα to increase the rates of fatty acid oxidation (12). Similar patterns of coactivation and regulation of expression by PGC-1α have also been shown for ERRα, ERRγ, NRF1, MEF2, and GABP (1).

The hypoxia inducible factors (HIFs) are members of the Per-ARNT-Sim-bHLH family of transcription factors that regulate the cellular response to hypoxic conditions (13, 14). HIFα isoforms (HIF1α and EPAS1/HIF2α) are constitutively hydroxylated under normoxic conditions by a family of prolyl hydroxylase enzymes, PHDs 1, 2, and 3 (13–16). The prolyl hydroxylation of HIFα allows for binding of the E3 ligase VHL, resulting in the rapid ubiquitination and proteasomal degradation of HIFs during normoxia (17, 18). During hypoxia, the PHDs are inactivated, allowing for the stabilization and accumulation of HIFα isoforms, which then bind CBP/p300, dimerize with their requisite binding partner HIF1β/ARNT, and drive transcription of hypoxia-responsive genes (13, 19–21). HIF1α is the best characterized member of this family and is a potent regulator of glycolytic and angiogenic gene programs (22). Although both HIF1α and HIF2α are known to bind to similar consensus sequences (HREs) and regulate overlapping gene sets, evidence is emerging that HIF2α may regulate the expression of some different genes than HIF1α (22, 23). Recent studies suggest that hepatic erythropoietin and SOD2 are HIF2α-specific targets in the liver (24, 25). Additionally, HIF1α and HIF2α play antagonistic roles regarding the regulation of nitric oxide synthesis in cytokine-stimulated macrophages, whereas global deletion of HIF2α on a pure BL6 background is embryonically lethal, suggesting that HIF1α and HIF2α are not completely redundant in function (26, 27).

Whereas roles for the regulation of and roles of HIF1α in muscle's response to exercise and hypoxia have begun to be defined, a role for HIF2α has not been explored (28, 29). Recent reports suggest that HIF2α expression is modulated during high-intensity exercise in humans, suggesting a role for HIF2α in the adaptive response to exercise (30, 31). Here we demonstrate that PGC-1α and ERRα are potent regulators of HIF2α transcription in skeletal muscle and that HIF2α is a critical mediator of the PGC-1α–dependent fiber-type switch in skeletal muscle.

Results

PGC-1α Regulates Expression of HIF2α. Before this study, the entire spectrum of transcription factors that are regulated by PGC-1α in any particular tissue had not been described. To elucidate the composition of the PGC-1α transcriptome in skeletal muscle, we expressed GFP or PGC-1α in differentiated myotubes and compared the expression of all known transcription factors, coactivators, and repressors (or proteins containing a motif associated with these functions) in a high-throughput qPCR-based assay we recently described (Quanttrx) (28). Of the ~1,800 genes represented in this database, >300 factors were increased in response to PGC-1α expression; HIF2α showed one of the greatest quantitative changes (~300-fold; Dataset S1). Although many known targets of PGC1α action were also increased, such as ERRα and PPARα, the RNA expression of HIF1α was not affected (Fig. 1*A*). This result is in agreement with earlier work (6). The PGC-1α–mediated increase in HIF2α mRNA expression did not cause a detectable increase in HIF2α protein under normoxic conditions (21% O_2); however, upon PHD inactivation and HIF stabilization with 2,2 dipyridyl, HIF2α protein was markedly elevated in the

Author contributions: K.A.R., R.K.G., and B.M.S. designed research; K.A.R., J.L.R., E.N., J.W., and J.L.E. performed research; K.A.R., R.K.G., J.L.R., J.W., and J.L.E. contributed new reagents/analytic tools; K.A.R., J.L.R., E.N., and B.M.S. analyzed data; and K.A.R., J.W., and B.M.S. wrote the paper.

The authors declare no conflict of interest.

[1]To whom correspondence should be addressed. E-mail: Bruce_Spiegelman@dfci.harvard.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1016089107/-/DCSupplemental.

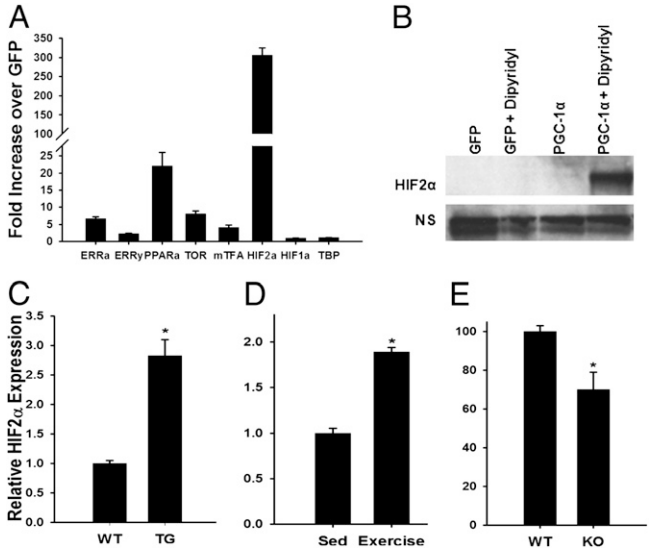

Fig. 1. PGC-1α is a powerful regulator of HIF2α Expression. (*A*) Expression of Quantrrx identified PGC-1α–regulated transcription, including HIF2α, was validated by qPCR. (*B*) HIF2α expression was measured by immunoblot in myotubes overexpressing GFP of PGC-1α in the presence or absence of the HIFα stabilizer 2,2 dipyridyl (100 μM) for 6 h. HIF2α expression response to a number of conditions that modulate PGC-1α expression is shown, including muscle-specific PGC-1α transgenesis (*C*), voluntary overnight wheel running (*D*), and muscle-specific deletion of PGC-1α (*E*). Data are expressed as means ± SE of $n = 3–9$; $^*P < 0.05$ compared with relevant controls.

PGC-1α–expressing myotubes, compared with GFP controls (Fig. 1*B*). This result suggests that HIF2α activity may not be dramatically altered by elevated PGC-1α alone and may require a secondary stabilization event to display maximal transcriptional activity. However, because intramuscular oxygen tensions are closer to 6–7% even in the sedentary state, this likely provides a more permissive environment for HIFα stabilization.

To determine whether PGC-1α can regulate HIF2α expression in vivo, we used several genetic gain- and loss-of-function models and also challenged mice with a number of stimuli known to induce PGC-1α. HIF2α mRNA expression is increased 2.5-fold in the gastrocnemius of mice with a muscle-specific transgenic expression of PGC-1α (Fig. 1*C*) (32). Additionally, overnight wheel running, an exercise protocol known to induce PGC-1α, increased the expression of HIF2α approximately twofold, whereas it induced PGC1α approximately sevenfold (Fig. 1*D*). Conversely, HIF2α expression is decreased 27% in the gastrocnemius of muscle-specific PGC-1α knockout (MKO) animals (Fig. 1*E*). PGC-1α is also known to be potently regulated by β-adrenergic receptor activation during exercise and this effect can be mimicked by injection of the β2-receptor agonist clenbuterol (33). Intraperitoneal administration of clenbuterol promoted a rapid induction of PGC-1α (Fig. 2*A*) and also stimulated an elevation of HIF2α expression; this effect was completely abolished in the PGC-1α MKO mice (Fig. 2*A*). Taken together, these data indicate that HIF2α mRNA expression is induced in response to pharmacological and physiological stimuli in muscle and that this inducible regulation is completely dependent on PGC-1α.

PGC-1α Regulation of HIF2α Is Partially Muscle Selective. PGC-1α is highly expressed in tissues with greatest energy demands, such as brown fat, skeletal muscle, heart, and kidney (Fig. S1). Within skeletal muscle, PGC-1α is most abundant within oxidative muscle, such as the soleus, and is expressed at lower levels in "whiter" muscles such as the gastrocnemius and quadriceps (Fig. S1). Consistent with this, HIF2α expression correlates positively with the expression of PGC-1α in a majority of tissues, being most abundantly expressed in highly metabolic tissues including brown fat, soleus, heart, and kidney (Fig. S1). Somewhat paradoxically, HIF2α mRNA expression is highest in the lung, a tissue with high oxygen tensions that is unlikely to allow for HIF2α stabilization (Fig. S1).

Because HIF2α expression is correlated with the expression of PGC-1α in many tissues, we asked whether PGC-1α could regulate the expression of HIF2α in a variety of cell types. As previously shown, PGC-1α robustly up-regulated HIF2α expression in primary myotubes and significantly increased HIF2α expression in immortalized C2C12 myotubes (fivefold) (Fig. 2*B*). Forced expression of PGC-1α in primary hepatocytes, primary renal proximal tubular cells (RPTCs), and primary brown adipocytes resulted in very modest increases HIF2α expression (two- to threefold), whereas HIF2α expression remained unchanged in 3T3-L1 adipocytes (Fig. 2*B*). These data suggest that robust regulation of HIF2α by PGC1α is at least partially muscle-cell selective.

ERRα Is Required for the PGC-1α–Mediated Increase in HIF2α Expression. PGC-1α is a key mediator of mitochondrial biogenesis and angiogenesis in response to exercise in skeletal muscle; both the mitochondrial energetics and angiogenic response to PGC-1α are completely dependent on PGC-1α binding to ERRα (7, 34). We investigated whether ERRα may also play a role in the PGC-1α–dependent regulation of HIF2α. Primary ERRα KO myotubes show a dramatic reduction in the expression of HIF2α in the basal state, suggesting that ERRα may indeed regulate the expression of HIF2α (Fig. 3*A*). Additionally, adenoviral expression of ERRα results in a dramatic increase in HIF2α expression, whereas the forced expression of ERRγ has a much more mild effect (Fig. 3*B*) (8-fold vs. 36-fold).

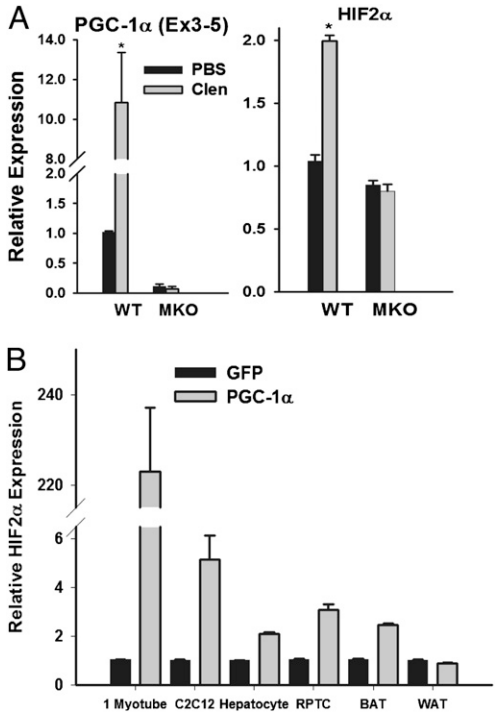

Fig. 2. Regulation of HIF2α by PGC-1α is a muscle-selective effect. (*A*) WT or PGC-1α muscle-specific knockouts (MKOs) were i.p. injected with clenbuterol (1 mg/kg) and PGC-1α and HIF2α expression was measured 6 h later. (*B*) GFP or PGC-1α was overexpressed for 48 h using adenovirus in primary myotubes, C2C12 myotubes, primary hepatocytes, primary renal proximal tubular cells (RPTC), primary brown adipose cells (BAT), or 3T3 L1 adipocytes (WAT) and HIF2α expression was measured by qPCR. Data are expressed as means ± SE of $n = 3$; $^*P < 0.05$ compared with GFP controls.

PHYSIOLOGY

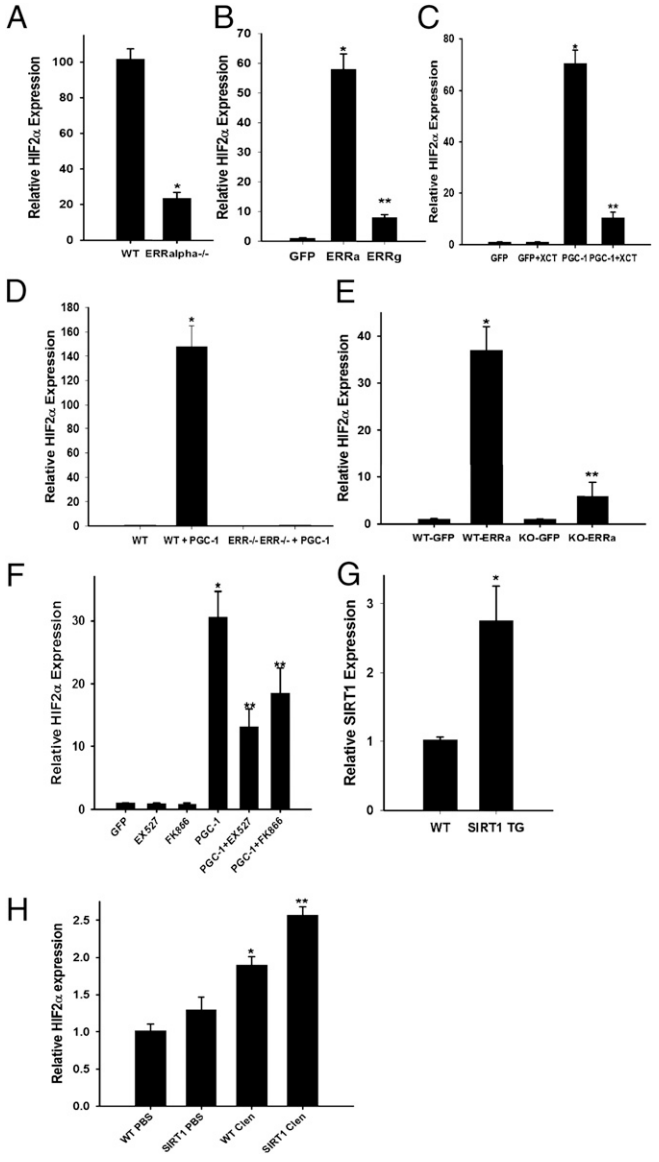

Fig. 3. Expression of HIF2α is dependent on PGC-1α coactivation of ERRα and modulated by SIRT1. (*A*) Expression of HIF2α was compared in WT and ERRα KO myotubes by qPCR. (*B*) Expression of HIF2α was measured by qPCR in response to 48 h adenoviral overexpression of either ERRα or ERRγ. HIF2α expression in response to the adenoviral overexpression of PGC-1α (48 h) was compared in WT myotubes in the presence or absence of the ERRα inverse agonist XCT-790 (*C*) or in WT and ERRα KO myotubes (*D*). (*E*) HIF2α expression was also measured in response to adenoviral overexpression of ERRα in WT and PGC-1α KO myotubes. (*F*) Myotubes were pretreated with EX527 (1 μM) or FK866 (20 nM) and subsequently overexpressed PGC-1α for 12 h at which time HIF2α expression was measured by qPCR. WT and SIRT1 transgenic mice (*G*) were treated with clenbuterol (1 mg/kg) and HIF2α expression in gastrocnemius was measured 6 h later by qPCR (*H*). Data are expressed as means ± SE of n = 3–6; different subscripts are statistically different from each other, $P < 0.05$.

To determine whether the PGC-1α–mediated induction of HIF2α requires ERRα, we first used an ERRα-specific inverse agonist, XCT-790. Pretreating myotubes with XCT-790 (10 μM) before infection with PGC-1α blocked the up-regulation of HIF2α mRNA (Fig. 3*C*). In addition, the genetic loss of ERRα completely abolished the PGC-1α–mediated increases in HIF2α expression (Fig. 3*D*). This result demonstrates an absolute requirement for ERRα in this process, at least in primary cell culture. Interestingly, the increase in HIF2α in response to the forced expression of ERRα was significantly blunted in PGC-1α$^{-/-}$ myotubes, suggesting that ERRα also requires coactivation by PGC-1α to influence the expression of HIF2α (Fig. 3*E*).

PGC-1α Regulation of HIF2α Is Modulated by SIRT1. SIRT1 is a NAD$^+$-dependent protein deacetylase that has been implicated in regulating whole body metabolism (35). SIRT1 has been shown to deacetylate and active PGC-1α to enhance the regulation of PGC-1α gene programs (36). In addition, SIRT1 has also recently been shown to associate with, deacetylate, and activate HIF2α (37). To examine the involvement of SIRT1 in the PGC1α/HIF2α axis, we pretreated myotubes with a specific SIRT1 inhibitor, EX-527, or a noncompetitive inhibitor of NAMPT, FK-866, known to reduce intracellular concentrations of NAD$^+$ and reduce SIRT1 activity (38, 39). Pretreatment with either EX-527 or FK-866 blocked the PGC-1α–mediated up-regulation of HIF2α (Fig. 3*F*). Conversely, we explored whether elevated SIRT1 expression and activity in vivo was sufficient to enhance the PGC-1α regulation of HIF2α mRNA. Wild-type littermate controls and whole-body SIRT1 transgenic mice (approximately threefold overexpression, Fig. 3*G*) were injected i.p. with clenbuterol to promote a PGC-1α–dependent increase in HIF2α in the gastrocnemius (Fig. 3*H*). Up-regulation of SIRT1 resulted in a marked potentiation of HIF2α expression in response to clenbuterol (Fig. 3*H*). Taken together, these data suggest that SIRT1 enhances the activity of PGC-1α and the subsequent expression and activity of HIF2α.

HIF2α Coordinates a Slow-Type Muscle Fiber Switch. Because HIF2α is a robust transcriptional target of PGC-1α and ERRα, we next sought to explore the function of HIF2α in skeletal muscle. Because a role for HIF2α has not been described in skeletal muscle, we took an unbiased approach to characterizing HIF2α-regulated gene programs. Primary myotubes were virally transduced with either a GFP or a HIF2α protein, in which key proline residues have been mutated to alanine, allowing for HIF2α stabilization and accumulation in normoxia (~2,000-fold overexpression of HIF2α) (40). RNAs were then isolated and subjected to Affimetrix analysis (Dataset S2). All genes that were altered >1.5-fold were included in an unbiased Ingenuity pathway analysis (Fig. S2). Interestingly, one of the most significant biological pathways identified by this analysis was skeletal muscle system development and function (Fig. S2). Gene sets involved in cardiovascular disease and skeletal and muscular disorders were scored as significant, suggesting that HIF2α may indeed play an important role in skeletal muscle development and function (Fig. S2). Further analysis of gene sets involved in skeletal muscle function and development revealed a substantially overrepresented set of myosin heavy chain family members that are dramatically altered by HIF2α (Fig. 4*A*). Interestingly, MyoVIIa (MyoHCI), a key part of slow twitch type I muscle fiber function, was dramatically increased by HIF2α. The second most down-regulated gene on the array was MyoIV (MyoHCIIb), a gene whose expression is characteristic of fast-twitch type IIb fibers (Fig. 4*A* and Dataset S2). These data suggest HIF2α may be a potential regulator of slow fiber-type determination, by increasing the slow twitch myosin heavy chain muscle program and suppressing the expression of fast twitch markers.

Regulation of a Slow Twitch Muscle Fiber Gene Program Is HIF2α Dependent. Because HIF1α and HIF2α regulate a wide overlapping spectrum of genes, we profiled muscle fiber-type genes in response to normoxia-stabile mutants of HIF1α and HIF2α (Fig. 4*B*). Both HIFα isoforms robustly activated the angiogenic program, as exemplified by increased VEGF-A expression; HIF1α demonstrated a greater potency than HIF2α in these experiments (Fig. 4*B*). Importantly, expression of genes characteristic of skeletal muscle fiber-type switching appears to be a *specific* response to HIF2α. mRNAs for several genes characteristic of slow-twitch muscle fibers such as MyoHCI, Myoglobin, Cal-

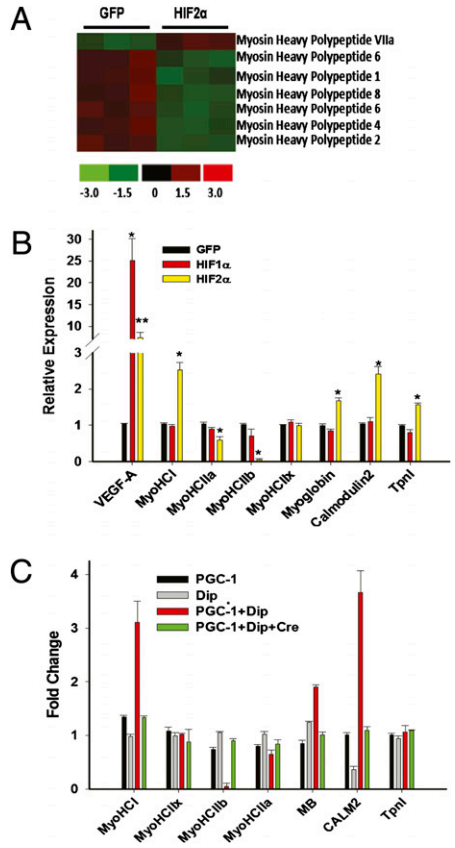

Fig. 4. HIF2α controls a skeletal muscle function and development pathway that includes the regulation of a slow-type muscle fiber switch. (*A*) Primary myotubes overexpressing either GFP or a stable HIF2α mutant were subjected to dCHIP analysis of representative myosin heavy chain isoforms from Affimetrix 2.0 gene array data (Fig. S3). (*B*) Primary myotubes were infected by adenovirus with stable forms of either HIF1α or HIF2α for 48 h and representative gene expression was measured by qPCR. (*C*) Primary myotubes were isolated from homozygous floxed EPAS/HIF2α mice with or without adenoviral CRE recombinase (12 h before GFP or PGC-1α) to delete HIF2α and GFP or PGC-1α (48 h) in the presence or absence of 2,2 dipyridyl (12 h) and gene expression was measured by qPCR.

modulin2, and Troponin I are increased at the mRNA level by HIF2α, but are largely unchanged in response to HIF1α (Fig. 4*B*). Additionally, HIF2α but not HIF1α dramatically represses the expression of MyoHCIIb, a gene whose expression serves as a marker of ultrafast twitch (type IIb) muscle fibers (Fig. 4*B*). HIF2α also caused a mild repression of mRNA for the intermediate fiber-type marker, MyoHCIIa, but has no affect MyoHCIIx expression (Fig. 4*B*). These data suggest that the gene program of the slow-twitch fiber type is specifically regulated by HIF2α and remains largely unaffected by HIF1α. This result defines an important function for HIF2α.

We next asked whether the well-studied effect of PGC-1α in causing a switch to the oxidative type I and type IIa fibers required HIF2α. To determine HIF2α dependence, primary myoblasts were isolated and cultured from floxed HIF2α mice ($HIF2\alpha^{fl/fl}$); the HIF2α gene was excised using an adenoviral Cre-recombinase and $HIF2\alpha^{fl/fl}$ myotubes or $HIF2\alpha^{fl/fl}$-CRE myotubes were treated with or without adenoviral PGC-1α in the presence of absence of 2,2 dipyridyl and a subquent analysis of fiber-type genes was performed by qPCR (Fig. 4*C*). Under conditions where HIF2α had been stabilized, the forced expression of PGC-1α stimulated mRNA levels for MyoHCI, Myoglobin, and Calmodulin2 and suppressed MyoHCIIb. Importantly, this response was essentially abrogated in myotubes lacking HIF2α, suggesting that the gene program of PGC-1α/ERRα–mediated slow-twitch fiber-type transition is absolutely dependent on the presence of functional HIF2α (Fig. 4*C*). Additional HIF2α target genes induced by PGC-1α and 2,2 dipyridyl were also regulated in a similar manner. Interestingly, the expression of HIF2α does not appear to be directly regulated by ERRα as there are no ERR concensus sites in HIF2α promoter regions. Unlike many transcription factors that are regulated by PGC-1α, HIF2 is not coactivated by PGC-1α (Fig. S3).

HIF2α Regulates Fiber-Type Determination in Vivo. To determine the relative contribution of HIF2α to fiber-type determination in vivo, we created a muscle-specific knockout of HIF2α by excising both alleles of HIF2α with a myogenin-driven Cre-recombinase (HIF2α-MKO). An analysis of the gene program of fiber types was subsequently performed by qPCR on muscles from 12-wk-old mice. Genetic ablation of HIF2α (~90% reduction in soleus, Fig. 5*A*), promoted a shift toward fast twitch myosin heavy chain gene expression as exemplified by marked increases in the expression of MyoHCIIb and reductions in MyoHCI, calmodulin2, and TpnI (Fig. 5*B*). As anticipated, we also observed a significant decrease in the expression of angiogenic genes such as VEGF-A and genes associated with the antioxidant defense system such as SOD2 (Fig. 5*B*). To further examine the role of HIF2α in the regulation of physiological fiber-type determination, a histological fiber-type analysis was performed. Mice lacking HIF2α showed dramatic reduction in the histological presence of the slow-twitch, oxidative myosin heavy chain MyHCI, accompanied by an up-regulation of the ultrafast-twitch MyHCIIb in the soleus (Fig. 5*C*). Consistent with the gene expression data, no change in the expression of MyHCIIa was noted (Fig. 5*C*). Taken together, these data suggest an important role for HIF2α in the determination of muscle fiber type in vivo and provide additional clues regarding the function of HIF2α in skeletal muscle (Fig. S4).

Discussion

PGC1α is known to mediate many of the benefits of exercise on skeletal muscle, including mitochondrial biogenesis, angiogenesis, fiber-type switching, and resistance to muscular atrophy/dystrophy (1). The transcriptional pathways by which these dif-

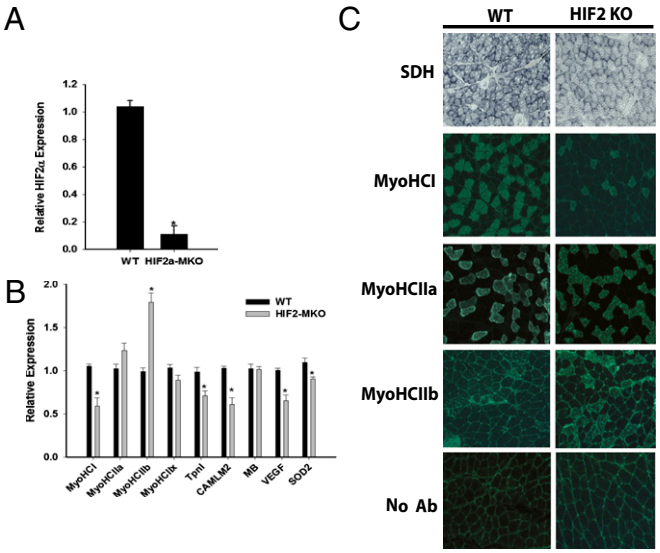

Fig. 5. Skeletal muscle fiber-type switching is regulated by HIF2α in vivo. (*A* and *B*) Gene expression from soleus of HIF2α muscle-specific knockout mice (HIF2-MKO) was measured by qPCR. (*C*) Expression of myosin heavy chain isoform (MyHCI, MyHCIIa, and MyHCIIb) is measured by immunohistochemistry in the soleus of HIF2-MKO mice and WT controls. Data are expressed as means ± SE of $n = 3–8$; $^*P < 0.05$ compared with relevant controls.

PHYSIOLOGY

ferent programs are executed have been well studied in some cases and are less understood in others. The switching of muscle fibers from type IIb to types IIa and I (typically due to physical training) is interesting because this switch gives muscle more endurance, and oxidative fibers are more resistant to muscular atrophy due to aging or denervation, compared with type IIb fibers. Hence, it is medically important to understand PGC1α-mediated fiber-type switching in greater mechanistic detail than we do presently.

In this article we show that HIF2α is a key mediator of a PGC-1α–dependent muscle fiber-type switch. In addition, we demonstrate a mechanism by which HIF2α is regulated at the level of transcription in the context of oxidative biology. The PGC-1α–dependent transcription of HIF2α requires ERRα and is also modulated by the action of SIRT1. Our data suggest that HIF2α expression and activation by PGC-1α occur downstream of exercise and β-adrenergic signaling.

HIF2α-MKO mice show changes in gene expression that are indicative of a switch to fast-type muscle fibers, as demonstrated by decreases in gene expression patterns that are characteristic of type I muscle fibers and an increase in expression characteristic of type IIb fibers (Fig. 5*C*). In addition, HIF2α regulates calmodulin2 gene expression (Fig. 5*B*), which could contribute to the mechanisms by which HIF2α affects fiber types. Calmodulin activation through binding to calcium promotes an increase in calcineurin A activity, which has been shown to result in the exclusion of NFAT from the nucleus, to subsequently increase the activity of MEF2 and drive a slow-type fiber switch. PGC-1α has also been shown to coactivate MEF2 to promote a transition toward oxidative fiber types, a process that is thus likely to be potentiated through the PGC-1α–dependent induction of HIF2α (41–47).

The very potent regulation of HIF2α by PGC-1α appears to be at least a partially muscle-selective effect. Interestingly, a critical mediator of this response, ERRα, is well expressed in the liver, kidney, and adipose tissue, but PGC-1α does not strongly induce HIF2α in these tissues (48). There are also no obvious ERR consensus sites within the HIF2α promoter sequence and PGC-1α does not directly coactivate HIF2α (Fig. S3) (49). Further studies of the upstream events that regulate HIF2α expression through PGC-1α are warranted and could lead to new therapeutic targets affecting fiber-type determination.

While both PGC-1α and HIF2α are now implicated in promoting slow-type muscle fiber twitch, it has been argued that switching to type I muscle fibers cannot occur in response to physiological stimuli and that type I fiber-type determination is a developmental process (50). Thus, we cannot exclude the possibility that PGC-1α may regulate HIF2α in the context of muscle development and be an early factor that regulates the fiber-type determination program. Additionally, our genetic models do not exclude the possibility that the contribution of HIF2α to fiber-type determination is completely a developmental effect, but because we also show HIF2α to regulate angiogenesis and SOD2 downstream of PGC-1α in vitro and in vivo, we suggest that the PGC-1α–mediated increases in HIF2α under physiological conditions may contribute to exercise-induced angiogenesis and control of antioxidant defense mechanisms. Thus, HIF2α regulation by PGC-1α may play divergent roles in the adaptive response to exercise and development.

In conclusion, this work places HIF2α as a key mediator in oxidative biology and fiber-type determination. Additional insight into the mechanisms by which HIF2α regulates the development of slow-twitch muscle fibers may ultimately lead to new targets and mechanisms that can be exploited to combat muscle atrophy, wasting, and disease such as Duchenne muscular dystrophy and age-related sarcopenia.

Materials and Methods

Reagents. All primers were purchased from Integrated DNA Technologies. Antibodies toward HIF2α were purchased from Novus Bio (NB100-122), whereas IHC antibodies for fiber-type analysis were purchased from the Developmental Studies Hybridoma Antibody Core at the University of Iowa (SC-71, BF-F3, and A4.840). EX-527 and FK-866 were purchased from Tocris. All other reagents were purchased from Sigma.

Animal Experimentation. Muscle-specific PGC-1α transgenic and knockout mice were generated and maintained as previously described (6, 7, 32, 51, 52). Generation of HIF2α muscle-specific knockout mice was accomplished by crossing mice with a floxed HIF2α allele (24) with animals transgenically expressing Cre-recombinase under the control of the myogenin promoter and the MEF2C enhancer (a generous gift from R. Bassel-Duby and E. N. Olson, University of Texas Southwestern, Dallas, TX) (53). All experimental cohorts were compared with littermate controls and experiments were performed on mice between 12 and 14 wk of age. For exercise experiments, adult C57/bl6 mice (Jackson Laboratories) (6 wk old) were housed individually and subjected to one bout ad libitum running on in-cage voluntary running wheels (Mini Mitter, a Respironics company). The mice ran at least 8 km on the basis of data collected through electronic monitoring and processed with the VitalView Data Acquisition System. Clenbuterol-injected animals were injected i.p. with a dose of 1 mg/kg. Animals were euthanized and tissue samples were collected 6 h after clenbuterol injection. All experiments were performed in accordance with the Animal Facility Institutional Animal Care and Use Committee regulations.

Quanttrx Analysis. Genome-wide transcription factor profiling (Quanttrx) was performed according to the method of Gupta et al., where 10 μg of RNA was isolated and reverse transcribed for each treatment group. Experiments were performed in triplicate and analyzed by the ΔΔ*Ct* method and normalized to several housekeeping genes (54).

Cell Culture. Primary cell culture was performed as previously described. $ERR^{-/-}$ myotubes were a generous gift from Zhidan Wu, Novartis Pharmaceuticals (Cambridge, MA). $EPAS1^{fl/fl}$ myotubes were isolated from 7-d-old litters from EPAS1fl/fl homozygotes from Jackson Laboratories. PGC-1α adenovirus is as previously described. The ERRα and ERRγ adenoviruses were a generous gift from Don McDonell at Duke University (Durham, NC). The stable HIF2α adenovirus was a generous gift from Stephen Lee at University of Ottawa (Ottawa, ON, Canada).

Gene Expression Analysis. Total RNA was isolated from tissues using the TRIzol reagent (Invitrogen) and prepped using the Qiagen RNeasy kit according to the manufacturer's instructions. RNA was reverse transcribed and analyzed using an Applied Biosystems Real-Time PCR System 7300, using the ΔΔ*Ct* method. Relative gene expression was normalized to 18S RNA levels. Affimetrix experiments were performed by the Dana-Farber microarray core facility and subsequent data analysis was conducted with dChip and Ingenuity pathway analysis software suites.

Statistical Analysis. All results are expressed as means, and error bars depict SEs. A two-tailed Student's *t* test was used to determine *P* values. Mulivariate statistics were analyzed using one-way ANOVA with Student Newman–Keuls post hoc analysis. Statistical significance was defined as $P < 0.05$. For all cell-based experiments, at least $n = 3$ was reached. For animal experiments, at least four mice per group were used.

ACKNOWLEDGMENTS. This work was supported by Grants DK54477 and DK61562 (to B.M.S., Harvard Medical School, Boston). J.W. was supported by a postdoctoral fellowship from the American Heart Association (Founders Affiliate 09POST2010078).

1. Handschin C, Spiegelman BM (2006) Peroxisome proliferator-activated receptor gamma coactivator 1 coactivators, energy homeostasis, and metabolism. *Endocr Rev* 27:728–735.
2. Olesen J, Kiilerich K, Pilegaard H (2010) PGC-1alpha-mediated adaptations in skeletal muscle. *Pflugers Arch* 460:153–162.
3. Wu Z, et al. (1999) Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* 98:115–124.
4. Michael LF, et al. (2001) Restoration of insulin-sensitive glucose transporter (GLUT4) gene expression in muscle cells by the transcriptional coactivator PGC-1. *Proc Natl Acad Sci USA* 98:3820–3825.
5. Lin J, et al. (2002) Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. *Nature* 418:797–801.
6. Handschin C, et al. (2007) PGC-1alpha regulates the neuromuscular junction program and ameliorates Duchenne muscular dystrophy. *Genes Dev* 21:770–783.

7. Arany Z, et al. (2008) HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1alpha. *Nature* 451:1008–1012.
8. Puigserver P, et al. (1998) A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* 92:829–839.
9. Puigserver P, et al. (1999) Activation of PPARgamma coactivator-1 through transcription factor docking. *Science* 286:1368–1371.
10. Huss JM, Kopp RP, Kelly DP (2002) Peroxisome proliferator-activated receptor coactivator-1alpha (PGC-1alpha) coactivates the cardiac-enriched nuclear receptors estrogen-related receptor-alpha and -gamma. Identification of novel leucine-rich interaction motif within PGC-1alpha. *J Biol Chem* 277:40265–40274.
11. Herzog B, et al. (2006) Estrogen-related receptor alpha is a repressor of phosphoenolpyruvate carboxykinase gene transcription. *J Biol Chem* 281:99–106.
12. Vega RB, Huss JM, Kelly DP (2000) The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor alpha in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. *Mol Cell Biol* 20:1868–1876.
13. Wang GL, Jiang BH, Rue EA, Semenza GL (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proc Natl Acad Sci USA* 92:5510–5514.
14. Wang GL, Semenza GL (1993) Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia. *J Biol Chem* 268:21513–21518.
15. Epstein AC, et al. (2001) C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* 107:43–54.
16. Bruick RK, McKnight SL (2001) A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* 294:1337–1340.
17. Maxwell PH, et al. (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* 399:271–275.
18. Cockman ME, et al. (2000) Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. *J Biol Chem* 275:25733–25741.
19. Arany Z, et al. (1996) An essential role for p300/CBP in the cellular response to hypoxia. *Proc Natl Acad Sci USA* 93:12969–12973.
20. Jiang BH, Rue E, Wang GL, Roe R, Semenza GL (1996) Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. *J Biol Chem* 271:17771–17778.
21. Wood SM, Gleadle JM, Pugh CW, Hankinson O, Ratcliffe PJ (1996) The role of the aryl hydrocarbon receptor nuclear translocator (ARNT) in hypoxic induction of gene expression. Studies in ARNT-deficient cells. *J Biol Chem* 271:15117–15123.
22. Hu CJ, Wang LY, Chodosh LA, Keith B, Simon MC (2003) Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. *Mol Cell Biol* 23:9361–9374.
23. Hu CJ, Sataur A, Wang L, Chen H, Simon MC (2007) The N-terminal transactivation domain confers target gene specificity of hypoxia-inducible factors HIF-1alpha and HIF-2alpha. *Mol Biol Cell* 18:4528–4542.
24. Rankin EB, et al. (2007) Hypoxia-inducible factor-2 (HIF-2) regulates hepatic erythropoietin in vivo. *J Clin Invest* 117:1068–1077.
25. Oktay Y, et al. (2007) Hypoxia-inducible factor 2alpha regulates expression of the mitochondrial aconitase chaperone protein frataxin. *J Biol Chem* 282:11750–11756.
26. Takeda N, et al. (2010) Differential activation and antagonistic function of HIF-alpha isoforms in macrophages are essential for NO homeostasis. *Genes Dev* 24:491–501.
27. Huang LE, Bunn HF (2003) Hypoxia-inducible factor and its biomedical relevance. *J Biol Chem* 278:19575–19578.
28. Mason S, Johnson RS (2007) The role of HIF-1 in hypoxic response in the skeletal muscle. *Adv Exp Med Biol* 618:229–244.
29. Mason SD, et al. (2007) HIF-1alpha in endurance training: Suppression of oxidative metabolism. *Am J Physiol Regul Integr Comp Physiol* 293:R2059–R2069.
30. Henderson J, et al. (2005) The EPAS1 gene influences the aerobic-anaerobic contribution in elite endurance athletes. *Hum Genet* 118:416–423.
31. Nordsborg NB, Lundby C, Leick L, Pilegaard H (2010) Relative workload determines exercise induced increases in PGC-1alpha mRNA. *Med Sci Sports Exerc* 42:1477–1484.
32. Sandri M, et al. (2006) PGC-1alpha protects skeletal muscle from atrophy by suppressing FoxO3 action and atrophy-specific gene transcription. *Proc Natl Acad Sci USA* 103:16260–16265.
33. Chinsomboon J, et al. (2009) The transcriptional coactivator PGC-1alpha mediates exercise-induced angiogenesis in skeletal muscle. *Proc Natl Acad Sci USA* 106: 21401–21406.
34. Mootha VK, et al. (2004) Erralpha and Gabpa/b specify PGC-1alpha-dependent oxidative phosphorylation gene expression that is altered in diabetic muscle. *Proc Natl Acad Sci USA* 101:6570–6575.
35. Banks AS, et al. (2008) SirT1 gain of function increases energy efficiency and prevents diabetes in mice. *Cell Metab* 8:333–341.
36. Rodgers JT, Lerin C, Gerhart-Hines Z, Puigserver P (2008) Metabolic adaptations through the PGC-1 alpha and SIRT1 pathways. *FEBS Lett* 582:46–53.
37. Dioum EM, et al. (2009) Regulation of hypoxia-inducible factor 2alpha signaling by the stress-responsive deacetylase sirtuin 1. *Science* 324:1289–1293.
38. Peck B, et al. (2010) SIRT inhibitors induce cell death and p53 acetylation through targeting both SIRT1 and SIRT2. *Mol Cancer Ther* 9:844–855.
39. Hasmann M, Schemainda I (2003) FK866, a highly specific noncompetitive inhibitor of nicotinamide phosphoribosyltransferase, represents a novel mechanism for induction of tumor cell apoptosis. *Cancer Res* 63:7436–7442.
40. Franovic A, et al. (2007) Translational up-regulation of the EGFR by tumor hypoxia provides a nonmutational explanation for its overexpression in human cancer. *Proc Natl Acad Sci USA* 104:13092–13097.
41. Calabria E, et al. (2009) NFAT isoforms control activity-dependent muscle fiber type specification. *Proc Natl Acad Sci USA* 106:13335–13340.
42. Chin ER, et al. (1998) A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. *Genes Dev* 12:2499–2509.
43. McCullagh KJ, et al. (2004) NFAT is a nerve activity sensor in skeletal muscle and controls activity-dependent myosin switching. *Proc Natl Acad Sci USA* 101:10590–10595.
44. Mu X, Brown LD, Liu Y, Schneider MF (2007) Roles of the calcineurin and CaMK signaling pathways in fast-to-slow fiber type transformation of cultured adult mouse skeletal muscle fibers. *Physiol Genomics* 30:300–312.
45. Naya FJ, et al. (2000) Stimulation of slow skeletal muscle fiber gene expression by calcineurin in vivo. *J Biol Chem* 275:4545–4548.
46. Serrano AL, et al. (2001) Calcineurin controls nerve activity-dependent specification of slow skeletal muscle fibers but not muscle growth. *Proc Natl Acad Sci USA* 98: 13108–13113.
47. Swoap SJ, et al. (2000) The calcineurin-NFAT pathway and muscle fiber-type gene expression. *Am J Physiol Cell Physiol* 279:C915–C924.
48. Sladek R, Bader JA, Giguère V (1997) The orphan nuclear receptor estrogen-related receptor alpha is a transcriptional regulator of the human medium-chain acyl coenzyme A dehydrogenase gene. *Mol Cell Biol* 17:5400–5409.
49. Barry JB, Laganiere J, Giguere V (2006) A single nucleotide in an estrogen-related receptor alpha site can dictate mode of binding and peroxisome proliferator-activated receptor gamma coactivator 1alpha activation of target promoters. *Mol Endocrinol* 20:302–310.
50. Handschin C, Summermatter S, LeBrasseur NK, Lin J, Spiegelman BM (2010) For a pragmatic approach to exercise studies. *J Appl Physiol*, 108:223–233; author reply 226.
51. Wenz T, Rossi SG, Rotundo RL, Spiegelman BM, Moraes CT (2009) Increased muscle PGC-1alpha expression protects from sarcopenia and metabolic disease during aging. *Proc Natl Acad Sci USA* 106:20405–20410.
52. Calvo JA, et al. (2008) Muscle-specific expression of PPARgamma coactivator-1alpha improves exercise performance and increases peak oxygen uptake. *J Appl Physiol* 104: 1304–1312.
53. Li S, et al. (2005) Requirement for serum response factor for skeletal muscle growth and maturation revealed by tissue-specific gene deletion in mice. *Proc Natl Acad Sci USA* 102:1082–1087.
54. Gupta RK, et al. (2010) Transcriptional control of preadipocyte determination by Zfp423. *Nature* 464:619–623.