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The Transcriptional Coactivator PGC-1β Drives the Formation of Oxidative Type IIX Fibers in Skeletal Muscle

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SUMMARY

Skeletal muscle must perform different kinds of work, and distinct fiber types have evolved to accommodate these. Previous work had shown that the transcriptional coactivator PGC-1α drives the formation of type I and IIA muscle fibers, which are "slow-twitch" and highly oxidative. We show here that transgenic expression of PGC-1β, a coactivator functionally similar to but distinct from PGC-1α, causes a marked induction of IIX fibers, which are oxidative but have "fast-twitch" biophysical properties. PGC-1β coactivates the MEF2 family of transcription factors to stimulate the type IIX myosin heavy chain (MHC) promoter. PGC-1β transgenic muscle fibers are rich in mitochondria and are highly oxidative, at least in part due to coactivation by PGC-1β of ERRα and PPARα. Consequently, these transgenic animals can run for longer and at higher work loads than wild-type animals. Together, these data indicate that PGC-1β drives the formation of highly oxidative fibers containing type IIX MHC.

INTRODUCTION

Skeletal muscle converts chemical energy into motion and force, ranging from rapid and sudden bursts of intense activity to continuous low-intensity work (Berchtold et al., 2000; Hood, 2001; Pette, 2002). At one functional extreme, muscles such as the soleus perform slow but steady activities such as postural tension. At the other extreme, muscles such as the quadriceps and extensor digitorum longus (EDL) typically perform intense and rapid activities. Optimal efficiency of these disparate roles in different fibers is achieved by both specific myofibrillar protein composition and a metabolic capacity that is best suited for that composition. Most muscles in mammals contain a mixture of fiber types, but some muscle beds are enriched in particular fiber types. For example, the soleus muscle is rich in type I and IIA myosin heavy chains (MHCs), whose biophysical attributes confer upon the soleus a "slow-twitch" phenotype. Type I and IIA fibers are also rich in mitochondria, allowing for continuous activity with less fatigue. These attributes are ideal for the slow but constant role of maintaining postural tension. In contrast, muscles like the EDL and quadriceps contain more of the faster type IIB MHCs and lack rich mitochondrial networks, attributes more amenable to rapid and sudden activity of shorter duration.

Adult skeletal muscle fibers also contain a fourth MHC in abundance, type IIX (Schiaffino and Reggiani, 1994). The specific role of IIX-containing fibers is still poorly understood, but it appears to fall outside of the simple paradigm of slow, oxidative fibers versus fast, glycolytic fibers. Type IIX fibers are often oxidative, like I and IIA fibers (Larsson et al., 1991). Consistent with this, they often reside deeper in muscles (Larsson et al., 1991), where fibers are likely to contribute more to baseline muscle tone. However, IIX-containing fibers have "faster twitch" attributes, intermediate between IIA and IIB fibers (Bottinelli et al., 1994; Larsson et al., 1991). IIX-containing fibers can also be recruited transiently during fiber type conversion from IIB to IIA (Jacobs-El et al., 1993) and appear to increase in frequency during old age (Larsson et al., 1993). Hence, a spectrum of fiber types exists, ranging from I through IIA, IIX, and IIB, with a concordant decrease in oxidative capacity. How these coordinated changes in fiber type and mitochondrial content are determined is only beginning to be understood.

Recent work has implicated the transcriptional coactivator PGC-1α in muscle fiber type switching and determination (Lin et al., 2002b). PGC-1α and β are transcriptional coactivators that interact directly with transcriptional factors, chromatin-modifying enzymes, the basal transcriptional machinery, and the splicing machinery to effect powerful induction of transcriptional activity (reviewed in Knutti and Kralli, 2001; Lin et al., 2005a; Puigserver and Spiegelman, 2003; Scarpulla, 2002). PGC-1α was originally identified as a coactivator for PPARγ that was induced by cold exposure in brown fat (Puigserver

et al., 1998). PGC-1β was later identified by homology to PGC-1α (Kressler et al., 2002; Lin et al., 2002a). It has now become apparent that these proteins can also coactivate a myriad of other transcription factors, both inside and outside the nuclear receptor family. Most notably, PGC-1α plays a dominant role in activating the full program of mitochondrial biogenesis and respiration by docking on transcription factors such as ERRα, NRF-2, and NRF-1 (Mootha et al., 2004; St-Pierre et al., 2003; Wu et al., 1999). PGC-1β appears equally potent in this function in isolated cells (St-Pierre et al., 2003), although in vivo evidence for this is still lacking. In addition, these coactivators perform quite different functions in specific tissues. In the liver, PGC-1α is strongly induced by fasting and activates the program of gluconeogenesis via docking on HNF-4, FOXO1, GR, and other transcription factors (Puigserver et al., 2003; Yoon et al., 2001). PGC-1β, on the other hand, does not induce gluconeogenic gene expression effectively. In contrast, a high-fat diet given to mice leads to the induction of PGC-1β in liver, leading to induction of fatty-acid synthesis and export via docking of PGC-1β on LXR, SREBP1c, and FOXA2 (Lin et al., 2005b; Wolfrum and Stoffel, 2006). PGC-1α can coactivate LXR, but not SREBP1c or FOXA2. The differing roles of PGC-1α and PGC-1β in tissues other than liver are not clear.

Fiber composition in skeletal muscle is dictated by a combination of developmental decisions and physiological cues, most notably patterns of innervation (Pette, 2002). These cues are transduced via secondary pathways, such as calcineurin and CaM kinase. Transgenic expression of PGC-1α in skeletal muscle leads to activation of genes typical of slow type I and IIA fibers (Lin et al., 2002b). At the same time, as in most other tissues, PGC-1α also induces a broad genetic program of mitochondrial biology (Lin et al., 2002b). In this way, PGC-1α activates multiple transcriptional programs and induces a coordinated conversion to fibers having characteristics of slow, oxidative type I and IIA fibers. It is noteworthy that PGC-1α is clearly not the sole determinant of type I and IIA fibers since *PGC-1α*$^{-/-}$ mice still contain apparently normal amounts of these fibers (Arany et al., 2005). The role of PGC-1β in fiber type determination has not been studied to date. Transgenic expression of PGC-1β in all tissues at once leads to hypermetabolism and resistance to diabetes (Kamei et al., 2003), but the skeletal muscle phenotype and functional adaptations of these animals had not been reported. Moreover, the role of PGC-1α and β in regulating other fiber types, such as IIB and IIX, had also not been examined. To address these issues, we report here our analysis of mice with transgenic expression of PGC-1β specifically in skeletal muscle. We show that PGC-1β induces fibers with characteristics of type IIX fibers without increasing type I fibers. It does this at least in part via coactivation of the MEF2D transcription factor. Simultaneously, PGC-1β powerfully activates the mitochondrial program. These changes yield mice with greatly increased capacity to sustain physical activity.

RESULTS

Endogenous and Transgenic Expression of PGC-1β in Skeletal Muscle

To determine the relative expression of PGC-1α and β in different skeletal muscle beds, RNA was isolated from various muscles of wild-type FVB mice, and quantitative real-time PCR was performed. The relative expression levels of MHC isotypes were also measured, as MHC expression is typically used as an indicator of the composition of different fiber types in muscle beds. As expected (Lin et al., 2002b), PGC-1α was expressed at highest levels in the type I-enriched soleus muscle and at relatively lower levels in the "fast-twitch" beds like quadriceps and extensor digitorum longus (see Figure S1 in the Supplemental Data available with this article online). In contrast, although PGC-1β expression was relatively high in the soleus, it was highest in the EDL muscle (Figure 1A; Figure S1). A similar increase of PGC-1β in the EDL muscle, compared to other fast-twitch muscles, has been noted in the rat (Koves et al., 2005). As has been shown elsewhere, MHC I and IIA were expressed most strongly in the soleus muscle, and much less so in the EDL (Figure S1). MHC IIX, on the other hand, was expressed most highly in EDL, and expression of MHC IIX showed a good correlation with that of PGC-1β, with the exception of the specialized soleus (Figure 1A). Hence, the expression pattern of PGC-1β most closely resembles the expression of MHC IIX.

To evaluate the relationship between PGC-1β and MHC IIX levels in greater detail, cross-sections from gastrocnemius muscle were used for in situ immunohistochemistry using an affinity-purified polyclonal antibody recognizing PGC-1β and compared to in situ mRNA hybridization using a probe unique for MHC IIX. Gastrocnemius was chosen because it contains the widest variety of fiber types. As shown in Figure 1B, staining of PGC-1β (brown arrows) was almost exclusively nuclear, consistent with its role as a transcriptional coactivator. Importantly, not every nucleus stained positive for PGC-1β (brown versus blue arrows), and some nuclei stained much more prominently than others. Similarly, a subset of fibers contained prominent amounts of MHC IIX mRNA (black dot), while the rest had little or none. Moreover, as with staining for PGC-1β, fibers containing MHC IIX were smaller in size, and more IIX fibers were found in the deeper, more oxidative portions of the gastrocnemius (Figure S2). Importantly, of the fibers that contained nuclei clearly staining with PGC-1β antibody, 20% showed labeling for IIX, while only 7% of the remaining fibers were labeled for IIX (Figure 1B, right panel). Hence, these data illustrate that PGC-1β protein levels were significantly enriched in IIX fibers.

We next evaluated the role of PGC-1β in skeletal muscle biology by generating a transgenic mouse expressing PGC-1β in skeletal muscle. The complete PGC-1β cDNA was cloned 3′ to 4.8 kb of the promoter of muscle creatine kinase (MCK), which is expressed most strongly in skeletal muscle, and less so in other striated muscles. The human

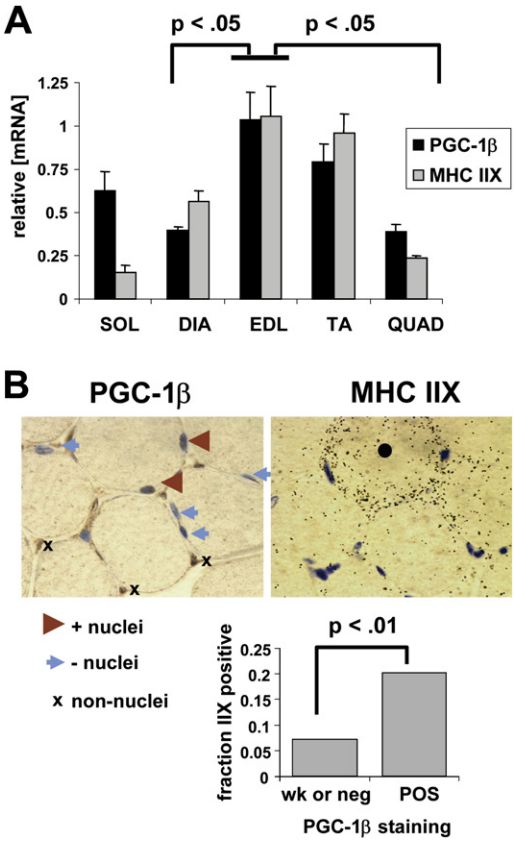


Figure 1. Endogenous Expression of PGC-1β in Skeletal Muscle

(A) Relative mRNA expression levels of PGC-1β MHC IIX in the indicated muscles (SOL, soleus; DIA, diaphragm; EDL, extensor digitorum longus; TA, tibialis anterior; QUAD, quadriceps). Muscles were isolated from 5-month-old wild-type male FVB mice, and mRNA expression was evaluated by quantitative real-time PCR. n = 6 for each muscle. In this and all other figures, error bars represent ±SEM.

(B) 100× magnification images of immunohistochemical staining with affinity-purified antibody against PGC-1β (left panel) and in situ mRNA hybridization with a probe specific for MHC IIX (right panel) from adjacent cross-sections of gastrocnemius muscle of wild-type male FVB mice. Brown arrows indicate nuclei that are deeply stained for PGC-1β protein, while blue arrows indicate nuclei that did not stain. The black dot indicates a fiber containing abundant MHC IIX mRNA. The graph below shows the fraction of fibers that clearly contain nuclei staining for PGC-1β and that also contain abundant MHC IIX mRNA (right bar), as compared to the fraction of the remaining fibers that contain MHC IIX mRNA (left bar). >400 fibers from two WT and two transgenic animals were scored.

growth hormone polyadenylation site was inserted 3′ of the cDNA (Figure S3). Mouse oocytes were injected with this construct, and three mouse lines that expressed the transgene were isolated. Line T9 expressed approximately 100-fold more PGC-1β mRNA in quadriceps muscle than in nontransgenic wild-type controls, as assessed by quantitative real-time PCR; expression of PGC-1β in line T34 was increased about 25-fold; and expression in line T37 was increased about 10-fold (Figure 2A). Most subsequent experiments were conducted on the lowest expresser, T37. Expression of the transgene in various tissues and different muscle beds was evaluated (Figure 2B). As expected from using the MCK promoter, the transgene was expressed only in muscle. Moreover, consistent with the expression of endogenous MCK, expression of the transgene was highest in "white," glycolytic muscle such as quadriceps, EDL, and gastrocnemius and was lower in "red," oxidative muscle such as soleus or heart (Figure 2B). Western blotting confirmed that the mRNA from the transgene was translated into protein, with PGC-1β protein levels in the gastrocnemius and tibialis muscles from the T37 line approximately 10-fold and 3-fold greater than in nontransgenic muscle, respectively (Figure 2C). In situ immunohistochemistry revealed that nearly every fiber in muscle from transgenic mice contained nuclei that were deeply stained, as compared with the paler staining of nuclei from wild-type fibers (Figure 2D). Of note, even though almost all fibers from the transgenic animals contained at least one strongly staining nucleus, some nuclei remained unstained; these nuclei likely reflect satellite or endothelial cells, where the transgene would not be expected to be expressed.

PGC-1β Converts Muscle Fibers to a IIX Type

The muscle from the PGC-1β transgenic mice was strikingly redder in appearance than wild-type controls (Figure 2E). This was equally true in all three mouse lines, despite the differences in expression levels of the transgene. H&E stains revealed that fibers in the transgenic animals tended to be smaller in size (Figure S4), while trichrome stains showed no evidence of fibrosis in the transgenic animals. To determine whether the change in muscle color was due to fiber type switching like that seen in PGC-1α transgenic mice (Lin et al., 2002b), ATPase chromatographic stains were used to identify fibers containing type I and IIA MHCs. Surprisingly, no induction of type I or IIA fibers was seen in the PGC-1β transgenic mice (Figure S5). This suggests that PGC-1β, unlike PGC-1α, does not induce the formation of classical slow type I or IIA fibers.

To evaluate the composition of fibers in PGC-1β transgenic mice more specifically, the expression of each of the four adult MHC transcripts was measured in skeletal muscle from WT and transgenic mice. As shown in Figure 3A, mRNAs encoding MHCs I, IIA, and IIB were all repressed in the transgenic mice compared to wild-type littermates. However, there was a striking induction of MHC IIX mRNA in the transgenic animals, by as much as 5-fold in the gastrocnemius muscle. This was true in all skeletal muscles examined (Figure 3A), except the type I-rich soleus, where the transgene is poorly expressed (Figure S6). Interestingly, the levels of PGC-1α mRNA in PGC-1β transgenic animals were substantially reduced (Figure S7). To determine whether the induction of the MHC IIX mRNA transcript resulted in increased MHC IIX protein, western blotting was performed on whole-cell extracts using an antibody specific for the IIX isotype (6H1, a kind gift from L.A. Leinwand). As shown

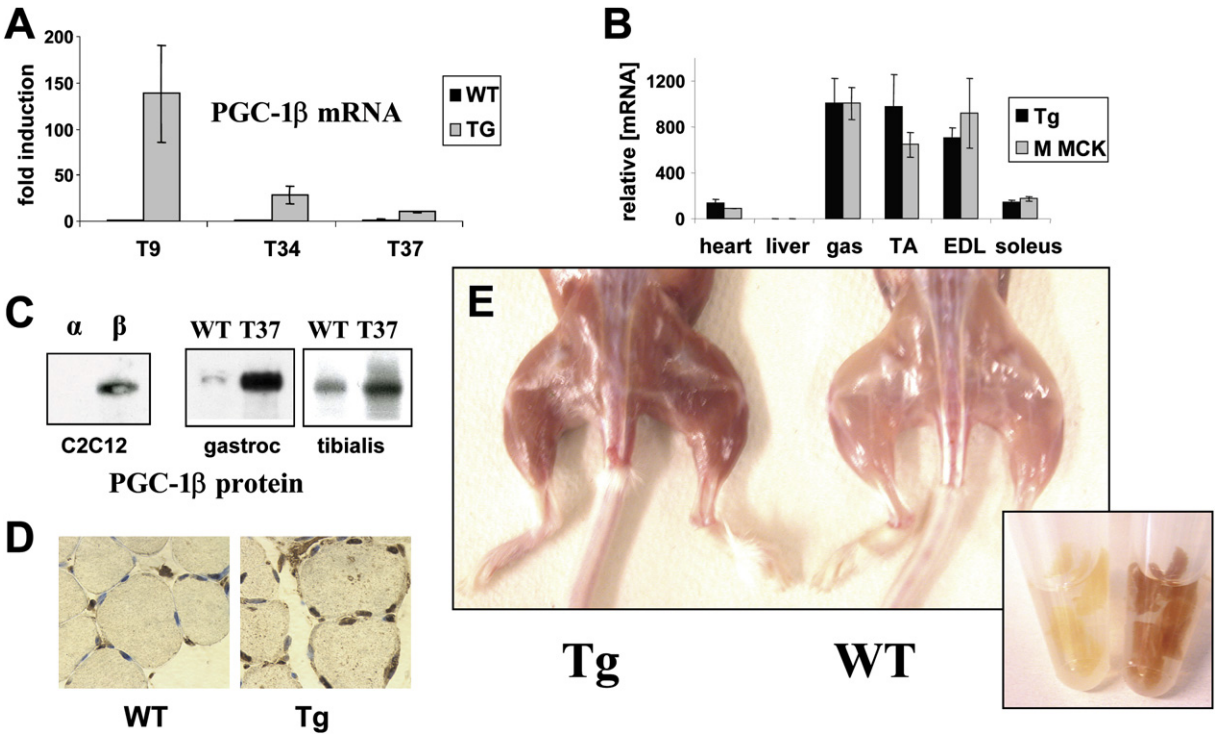


Figure 2. Transgenic Expression of PGC-1β in Skeletal Muscle

(A) Relative mRNA expression levels of PGC-1β in quadriceps muscle from each of three independent lines of PGC-1β transgenic mice, compared to wild-type littermate control. TG = transgenic.

(B) Relative mRNA expression levels of the transgenic PGC-1β (Tg) and endogenous muscle creatine kinase (M MCK) in the indicated tissues taken from transgenic mice of the T37 line. n = 3 for each sample.

(C) PGC-1β protein levels in gastrocnemius and tibialis anterior muscles from transgenic mice of the T37 line versus wild-type animals (right panels), visualized by western blotting with affinity-purified rabbit polyclonal antibody against PGC-1β. As control for the antibody, extracts from C2C12 myoblasts transfected with plasmid expressing either PGC-1α or β were used (left panel).

(D) Immunohistochemical staining of gastrocnemius fibers from WT or transgenic animals with affinity-purified antibody against PGC-1β. 100× magnification.

(E) A transgenic mouse from the T37 line and a wild-type littermate were skinned after euthanasia and photographed. The inset shows quadriceps from the same animals, bathed in saline solution.

in Figure 3B, a marked induction of MHC IIX protein was detected in PGC-1β transgenic animals. MHC proteins can also be detected by Coomassie staining of cell extracts separated by SDS-PAGE. Under these conditions, MHC IIA and IIX proteins nearly comigrate in a single band, while MHC IIB and MHC I migrate with smaller molecular weights. As shown in Figure 3C, the band containing MHC IIA and IIX was markedly induced in extracts from PGC-1β transgenic mice compared to wild-type littermates, while MHC IIB was repressed.

To investigate what proportion of fibers in the transgenic animals had increased MHC IIX expression, in situ immunohistochemistry using the MHC IIX-specific antibody and in situ mRNA hybridization using the probe unique for MHC IIX were performed on muscles from WT and transgenic animals. As shown in Figures 3D and 3E and Figures S8 and S9, nearly 100% of fibers in the transgenic animals contained abundant MHC IIX mRNA and protein, as compared to only 15%–20% in WT animals (again with the exception of the soleus muscle; Figure S8). Hence, forced expression of PGC-1β in skeletal muscle drives nearly all fibers to express MHC IIX.

Various transcription factors, including NFAT, MEF2, MyoD, and myogenin (Allen et al., 2005; Beylkin et al., 2006), have been implicated in the regulation of the MHC genes. The mechanisms that specify expression of each MHC in different fiber types, however, remain poorly understood. To determine whether PGC-1β regulates the MHC IIX promoter, a 2 kb fragment of DNA surrounding the MHC IIX transcriptional start site was cloned 5′ to a luciferase reporter gene. Transfection of this plasmid into 10T1/2 fibroblast cells yielded little luciferase activity (Figure 4A), consistent with the muscle-specific expression of MHC IIX. The MEF2 transcription factors are thought to be master regulators of skeletal muscle determination (McKinsey et al., 2002; Naya and Olson, 1999), and the phosphatase calcineurin is also known to contribute significantly to regulation of skeletal muscle-specific transcription by transducing Ca^{2+} and other signals (Bassel-Duby and Olson, 2003, 2006). Cotransfection of

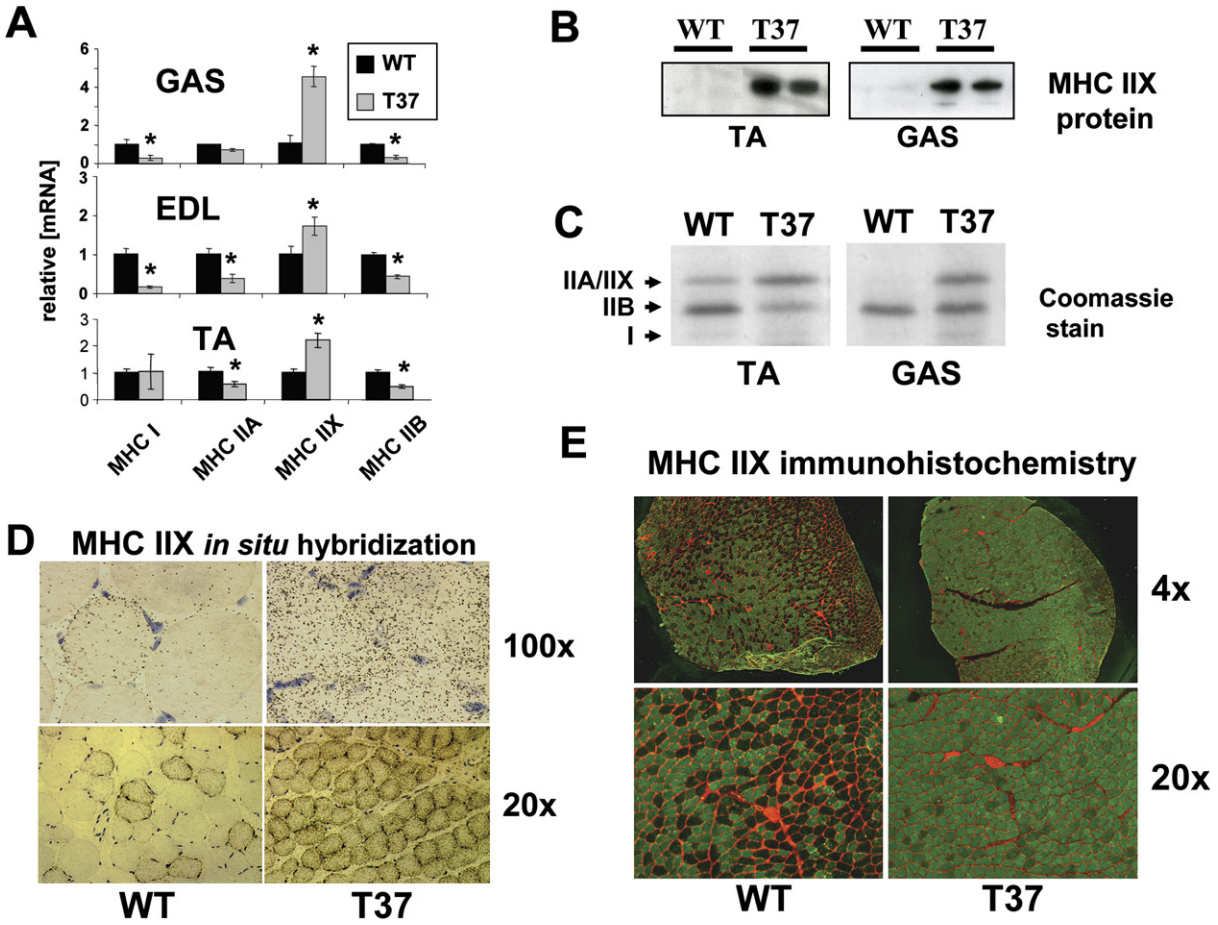


Figure 3. PGC-1β Shifts Muscle Fibers to a IIX Type

(A) Relative mRNA expression levels of the four adult MHC isotypes in the indicated muscles, isolated from 3-month-old T37 transgenic mice or wild-type littermates. n = 3 for each muscle. *$p < 0.05$.

(B) Western blotting of extracts from gastrocnemius of WT and T37 animals using the MHC IIX-specific monoclonal antibody 6H1.

(C) Coomassie staining of whole-cell extracts, separated by SDS-PAGE, isolated from the indicated muscles from T37 transgenic or wild-type mice.

(D) In situ mRNA hybridization of tibialis anterior muscle cross-sections from WT and transgenic (T37) mice with a probe specific for MHC IIX.

(E) Immunohistochemical analysis of tibialis anterior muscle from WT and T37 animals using antibodies against MHC IIX (6H1, green) and laminin (red). DAPI staining of nuclei is in blue.

the MHC IIX luciferase construct with plasmids encoding for MEF2A, C, and D as well as constitutively active calcineurin was sufficient to activate the MHC IIX promoter in these cells (Figure 4A). Significantly, simultaneous expression of PGC-1β stimulated transcription from the promoter 5-fold further (Figure 4A). PGC-1β had no effect on the promoter in the absence of MEF2 transcription factors (data not shown). Transfection of the three MEF2s individually indicated that activation of the MHC IIX promoter was almost entirely due to coactivation of MEF2D by PGC-1β (Figure 4B). Interestingly, the MHC IIA and MHC IIB promoters were less responsive to MEF2s and calcineurin than the MHC IIX promoter, and, importantly, PGC-1β had no additional effect on these promoters (Figure S10). Also of note, the abundance of MEF2 proteins in muscle from transgenic animals was not increased (Figure S11). These data strongly suggest that PGC-1β activates the MHC IIX promoter at least in part via coactivation of MEF2D.

These transfection studies also showed that PGC-1α also strongly stimulated the MHC IIX promoter via MEF2D (Figure 4A). We have previously described transgenic mice encoding for PGC-1α under the control of the MCK promoter, similar to the PGC-1β transgenic mice described here, but we did not study the IIX fiber content of these mice. To determine whether PGC-1α also activated the MHC IIX promoter in vivo, levels of mRNA for each adult MHC were evaluated in quadriceps muscle from PGC-1α transgenic mice. As shown in Figures 4C and 4D, MHC IIX mRNA was induced from about 2-fold in the tibialis anterior to about 6-fold in the quadriceps of transgenic mice compared to wild-type littermate controls. However, unlike in the PGC-1β transgenic mice, type I and IIA MHCs were also induced, rather than

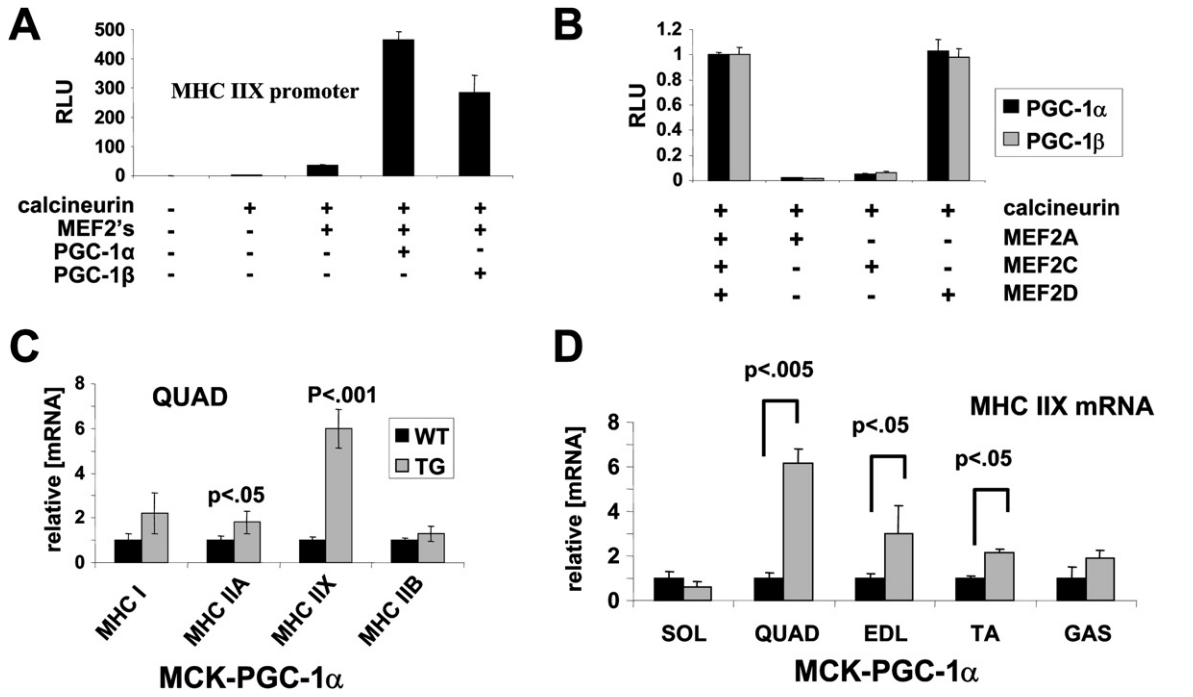


Figure 4. PGC-1β Coactivates MEF2 to Stimulate the MHC IIX Promoter

(A) Luciferase activity in 10T1/2 cells transfected with the MHC IIX promoter-luciferase construct plus plasmids encoding for the indicated proteins. Samples were performed in triplicate. MEF2s = equal amounts of plasmids encoding MEF2A, C, and D.

(B) Luciferase activity as in (A).

(C) Relative mRNA expression levels of the four adult MHC isotypes in quadriceps muscles isolated from MCK-PGC-1α transgenic mice or wild-type littermates. n = 8 for each muscle.

(D) Relative mRNA expression levels of MHC IIX in the indicated muscles isolated from MCK-PGC-1α transgenic mice or wild-type littermates. n = 4 for each muscle.

repressed (Figure 4C). This is consistent with the induction of type I and IIA fibers seen in these animals (Lin et al., 2002b). Hence, both PGC-1 proteins drive skeletal muscle fibers in vivo to express MHC IIX at least in part via docking on MEF2D. However, PGC-1α is distinguished by its ability to also drive type I and IIA MHCs, while PGC-1β represses the expression of these MHCs.

PGC-1β Increases Transcription of Genes of Mitochondrial Function In Vivo

PGC-1α and β are both potent inducers of mitochondrial biogenesis in isolated cells (Meirhaeghe et al., 2003; St-Pierre et al., 2003), and PGC-1α can also increase mitochondrial biogenesis in vivo (Lehman et al., 2000). To determine whether PGC-1β induces expression of genes of mitochondrial function in vivo, RNA was isolated from quadriceps muscle from PGC-1β transgenic mice and evaluated by quantitative PCR. As shown in Figure 5A (left panels), a number of genes encoding for members of the electron transport chain (*cycs*, somatic cyctochrome *c*; *nduf5*, a member of the NADH dehydrogenase complex; *atp5o*, a member of the ATPase complex; and *cox5b*, a member of the cytochrome oxidase complex) were induced up to 5-fold in transgenic mice compared to wild-type littermates. The induction was even more potent in the T9 transgenic line that expresses more PGC-1β (Figure 5A, lower left panel). Similarly, genes encoding for proteins of fatty-acid metabolism (CD36, fatty-acid transport protein; MCAD, medium-chain fatty-acid dehydrogenase; and CPT1, carnitine transferase 1), which occurs inside mitochondria, were also markedly elevated in both the T37 and T9 transgenic lines (Figure 5A, right panels). To evaluate these changes in a more systematic fashion, microarray analyses were performed with RNA from wild-type and transgenic animals. As shown in Figure 5B, each of the 25 most highly expressed genes of mitochondrial function was induced in the transgenic mice. To determine whether these changes in gene expression resulted in higher protein concentrations, extracts from wild-type and transgenic muscles were evaluated by western blotting with specific antibodies. As shown in Figure 5C, all of the mitochondrial proteins measured (members of electron transport chain complexes I and III) were induced in the transgenic mice. These included proteins encoded by both nuclear (e.g., *nduf*) and mitochondrial (e.g., ND6) genes.

We next investigated the transcription factor (or factors) through which PGC-1β functions in vivo. To do this, we used the unbiased informatics approach developed by Mootha et al. (2004). This program examines the

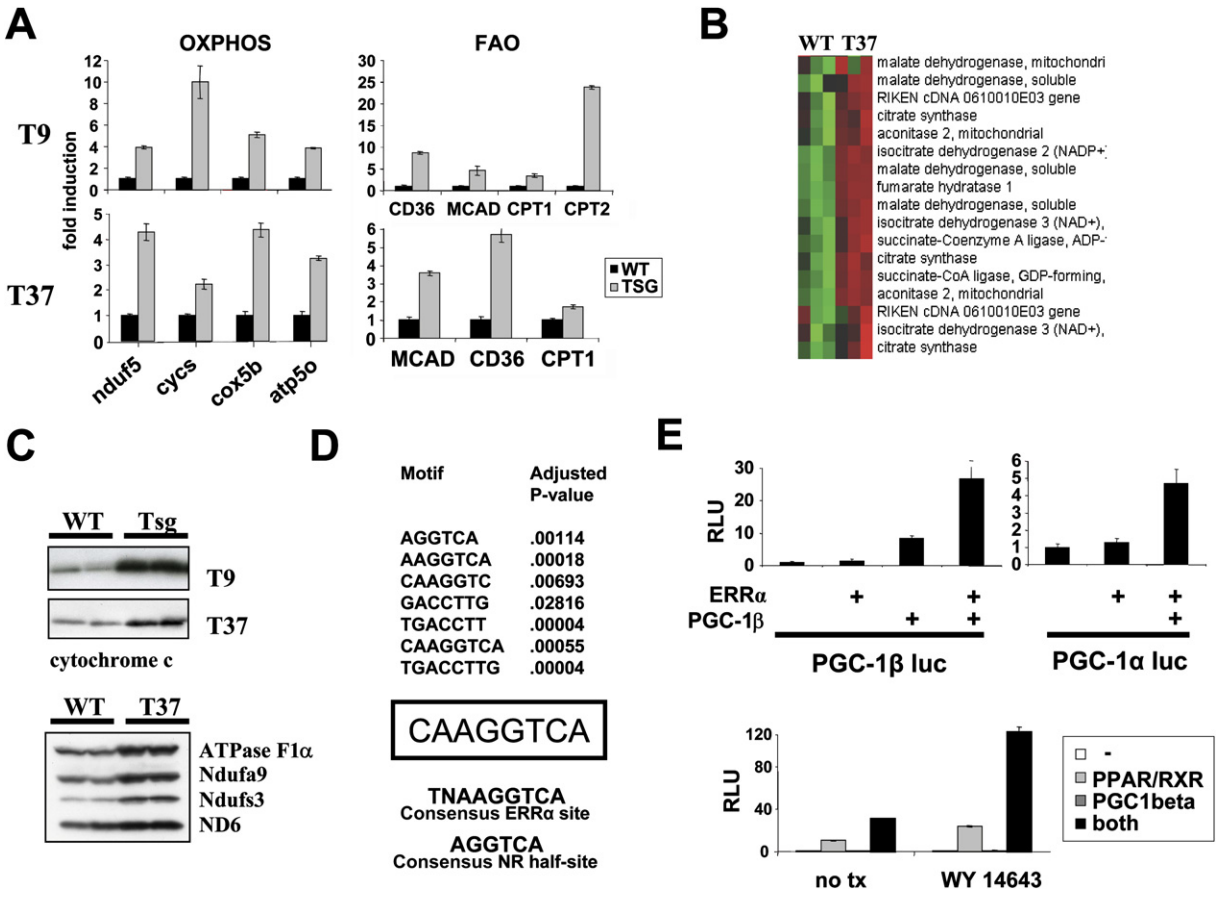


Figure 5. PGC-1β Stimulates Genes of Oxidative Phosphorylation and Fatty-Acid Oxidation In Vivo

(A) Relative mRNA expression levels of the indicated genes in quadriceps muscles isolated from 3-month-old T9 (top panels) or T37 (bottom panels) transgenic mice versus wild-type littermates. OXPHOS, genes of oxidative phosphorylation; FAO, genes of fatty-acid oxidation and transport. n = 3 for each muscle.

(B) Microarray analysis of RNAs from T37 quadriceps and wild-type littermates. The mitochondrial genes that are most highly expressed are shown. Green and red indicate lower and higher expression, respectively.

(C) Western blots of extracts, separated by SDS-PAGE, made from gastrocnemius muscles of the indicated transgenic lines and wild-type littermates.

(D) motifADE analysis of the microarray data described in (B). Shown are 6-, 7-, and 8-mers that are present statistically more frequently in the promoter regions of genes that are more highly expressed in transgenic animals. A consensus motif is shown in the box and is compared to known consensus motifs for ERRα binding and nuclear receptor binding.

(E) PGC-1β coactivates ERRα to stimulate expression of the PGC-1α and β promoters (top panels) and coactivates PPARα to stimulate expression of a nuclear receptor consensus binding site. 10T1/2 cells were transfected with luciferase (luc) reporter plasmids and plasmids encoding for ERRα and/or PGC-1β (top panels) or PPARα + RXR and/or PGC-1β (bottom panels). Cells were simultaneously treated with the PPARα agonist WY14848 where indicated. Luciferase activity was assayed 48 hr later.

expression data in entire Affymetrix-based microarrays and compares it to known gene promoter sequences. Short DNA sequences are then identified that are disproportionately represented in promoter regions of genes either increased or decreased by a given intervention. The sensitivity of the program is such that it favors the detection of relatively large coordinated gene sets, such as those genes involved in mitochondrial biogenesis. We successfully used this originally to identify the orphan nuclear receptor ERRα as an early target of PGC-1α in mitochondrial biogenesis (Mootha et al., 2004). A similar analysis had not been performed with PGC-1β. When the gene sets from wild-type and PGC-1β transgenic animals were compared, a number of sequences were found with significantly higher frequency in genes more highly expressed in the transgenic mice (Figure 5D). All of these sequences were homologous to the consensus sequence for binding to nuclear receptors, most closely to that of ERRα (Figure 5D).

Both PGC-1 coactivators have previously been shown to coactivate ERRα in cultured cells (Huss et al., 2002; Kamei et al., 2003; Mootha et al., 2004). Moreover, PGC-1α has been shown to act, in part via ERRα, in a positive feedback loop on its own promoter (Handschin et al., 2003; Mootha et al., 2004). To determine whether PGC-1β participates in a similar feedback loop, 10T1/2 cells were

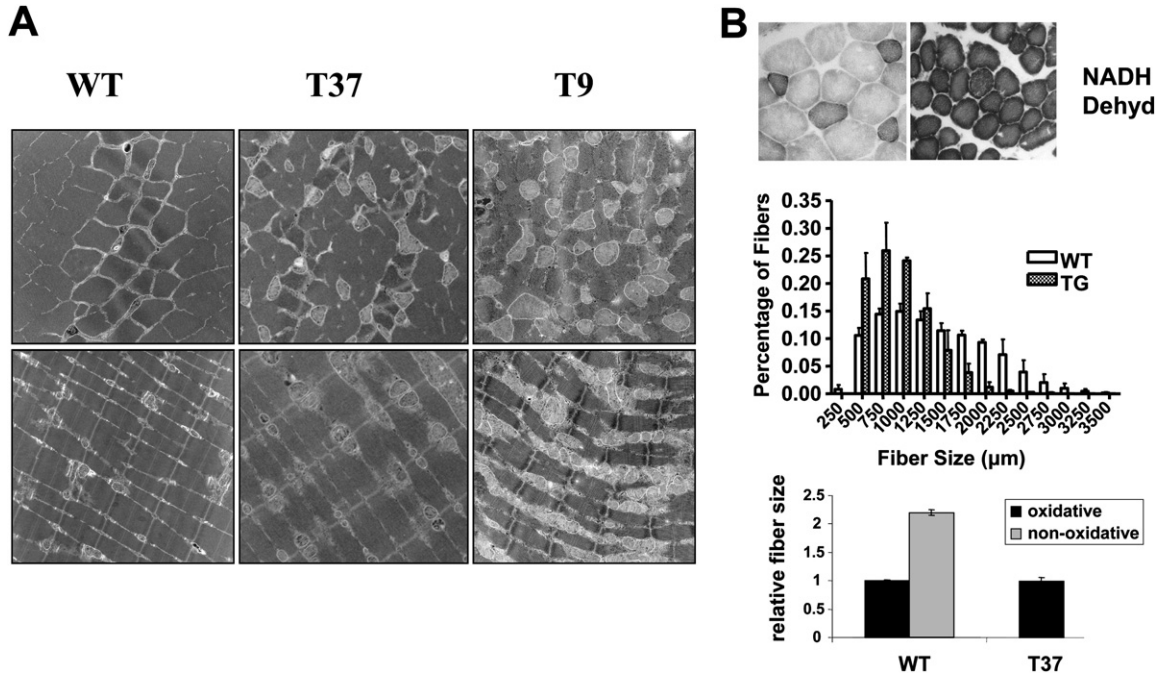


Figure 6. PGC-1β Stimulates Mitochondrial Biogenesis and Formation of Oxidative Fibers In Vivo

(A) Transmission electron micrographs from cross- (top panels) and longitudinal (bottom panels) sections taken from tibialis anterior muscles from T37 and T9 transgenic and wild-type animals.

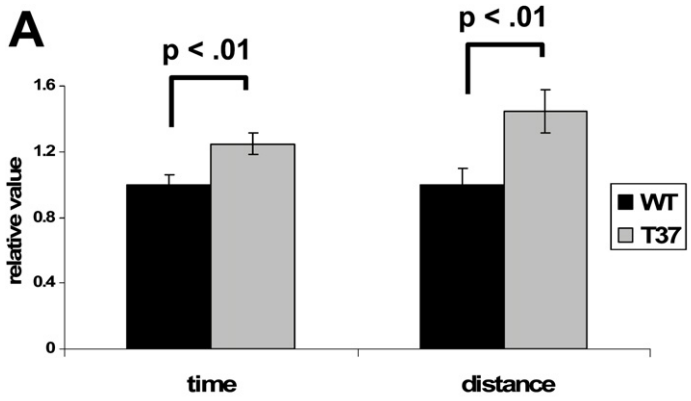
(B) Top panels show NADH staining of cross-sections from tibialis anterior muscles from T37 and wild-type littermate animals. Middle panel shows average fiber area (in cross-section) in tibialis anterior of wild-type and transgenic animals. n = 6 for each group. Bottom panel shows average fiber size in the same samples as above, separated according to oxidative capacity as determined by NADH staining.

transfected with a PGC-1β promoter-luciferase construct and plasmids encoding for PGC-1β and ERRα. As shown in Figure 5E, PGC-1β and ERRα acted synergistically to activate the PGC-1β promoter. Similarly, PGC-1β and ERRα also acted synergistically on the PGC-1α promoter (Figure 5E, right panel). Hence, PGC-1β efficiently coactivates ERRα to participate in a positive feedback loop with both itself and PGC-1α. The gene set induced in the muscles from PGC-1β transgenic mice also contains a number of genes known to be targets of the nuclear receptor PPARα, such as genes of fatty-acid import and oxidation (Kelly and Scarpulla, 2004). Figure 5E also shows that PGC-1β coactivates PPARα on a reporter containing repeated nuclear binding sites. Together, these data strongly suggest that PGC-1β drives genes of mitochondrial function in vivo, including genes of fatty-acid metabolism and oxidative phosphorylation, at least in part via coactivation of nuclear receptors such as ERRα and PPARα.

PGC-1β Drives the Formation of Oxidative Fibers

Figure 6A shows electron micrographs of transverse sections from wild-type mice and two lines of MCK-PGC-1β transgenic mice. In both transgenic mice, the presence of mitochondria is markedly induced. In the high PGC-1β expressor (T9), the mitochondrial biogenesis is so extreme as to displace and disorganize the myofibrillar apparatus (Figure 6A, right panels). The lower PGC-1β expressor (T37) induced mitochondria to a lesser extent (middle panels), and myofibrillar architecture is preserved. Hence, PGC-1β not only activates the genes of mitochondrial function but also activates the full program of mitochondrial biogenesis in vivo.

Normally, skeletal muscle displays a patchwork of glycolytic and oxidative fibers. Certain muscles such as the soleus and deeper sections of muscles such as the gastrocnemius tend to contain more oxidative fibers, while muscles such as the tibialis anterior contain mostly glycolytic fibers interspersed with a few oxidative fibers. To address whether this pattern is disturbed in PGC-1β transgenic mice, sections from muscles were stained in situ for the activity of NADH dehydrogenase, the first complex of the electron transport chain and a marker of oxidative fibers. Strikingly, in the PGC-1β transgenic mice, every fiber in the tibialis anterior was oxidative (Figure 6B, top panel). This was also true in all other muscles examined (data not shown). Oxidative fibers are typically smaller in diameter than glycolytic fibers. As shown in Figure 6B (middle and lower graphs), tibialis anterior from wild-type mice contained a mix of large, glycolytic fibers and smaller, oxidative fibers, while the same muscle from transgenic mice contained only the latter fibers. Consistent with this, muscles from transgenic mice were slightly smaller (data not shown). Taken together, these data demonstrate that PGC-1β coordinately induces in vivo fibers with type IIX characteristics, including the



Stage (10%incline)	Time (min)	Speed (m/min)	Duration (min)
1	0-5	10	5
2	5-7	12	2
3+	+2	+2	2

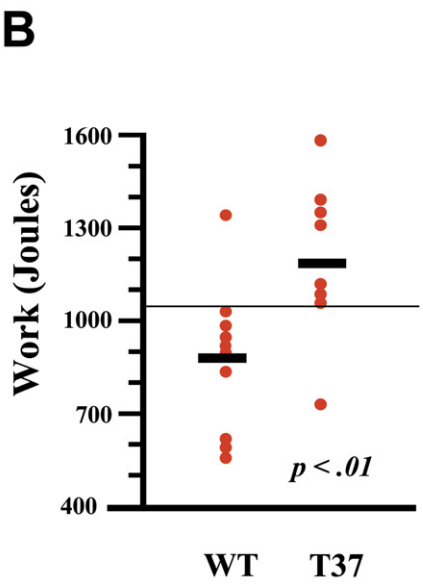


Figure 7. PGC-1β Increases Functional Capacity In Vivo

(A) Transgenic (T37) and wild-type littermate controls were made to run on a treadmill set at a 10% incline, following the protocol outlined in the lower panel: after a 5 minute run-in period at 10 meters/minute, the speed was increased by 2 meters/minute every 2 minutes. The time that the mice remained on the treadmill before exhaustion is indicated on the left bars, and the distance run during that time is indicated on the right. All experiments were performed blinded to the genotype of the animals. n = 9 each group.

(B) Work achieved by wild-type and transgenic animals in (A) was calculated as outlined in methods.

induction of MHC IIX protein and a coordinated increase in oxidative capacity.

PGC-1β Transgenic Mice Have Increased Capacity for Oxidative Work

Oxidative phosphorylation is by far the most efficient way to generate ATP. For this reason, endurance exercise relies largely on oxidative fibers in skeletal muscle. The oxidative capacity in the skeletal muscle of PGC-1β mice therefore suggested that these mice might have an altered capacity for aerobic exercise. To test this hypothesis, transgenic mice and wild-type littermate controls were subjected to treadmill exercise. The treadmill was set to a constant 10% incline and the speed was increased by 2 m/min every 2 min, and mice were forced to run to exhaustion. Exhaustion closely follows the attainment of the anaerobic threshold, which in turn closely correlates with oxidative capacity. Transgenic mice were able to run, on average, for 32.5 min to exhaustion, compared to 26 min for wild-type littermates ($p = 0.01$), which reflects a distance run of 746 meters, versus 516 meters with wild-type animals (Figure 7A). This indicated that PGC-1β transgenic mice could perform significantly more work than the wild-type controls, calculated as 1174 joules versus 886 joules (Figure 7B). Hence, increasing PGC-1β in skeletal muscle is sufficient to increase the capacity to perform work under oxidative conditions in vivo.

DISCUSSION

Skeletal muscles perform a variety of different kinds of work. In response to these requirements, animals have evolved different muscle fibers, with different energetic capacities and biophysical properties. At one extreme, type I fibers are highly oxidative, with the highest content of mitochondria of any muscle fibers, and rich in slow-twitch type I MHC. At the other extreme, type IIB fibers have an MHC with rapid and powerful contraction properties, are poor in mitochondria, and rely mainly on glycolytic metabolism. Type IIX fibers are the least understood of the skeletal muscle fibers, partly because there is no muscle bed that is dramatically enriched in these fibers. Whereas soleus muscle can be studied as a model of type I fibers, because these fibers make up as much as 70% of the soleus, there is no equivalent model for IIX fibers. Type IIX fibers are also unusual in that their MHC is "faster twitch" than type I or IIA fibers, but they are also generally more oxidative and mitochondria rich than IIB fibers. Fibers with such attributes are likely important since the requirements for muscle action do not always fall precisely into the stereotypic dichotomy of slow, high-endurance actions versus fast, low-endurance actions.

Our data show that PGC-1β strongly regulates the development of type IIX fibers and present a valuable animal model appropriate to study the physiology of type IIX-rich muscle in vivo. While skeletal muscle with

elevated PGC-1α shows an increase in fibers of all oxidative types, we show here that expression of PGC-1β drives only the development of type IIX fibers. Importantly, the transgenic mice show both an increase in the expression of type IIX MHC and a large increase in mitochondrial content. Furthermore, the expression of transgenic PGC-1β actually suppresses the expression of MHC I and IIA, in contrast to the induction seen with transgenic expression of PGC-1α. That the effects described here of PGC-1β on fiber types are likely to be of physiological relevance is strongly suggested by the fact that levels of PGC-1β correlate well with those of MHC IIX gene expression. It is important to note that PGC-1β is unlikely to be the sole determinant of IIX fibers. In particular, PGC-1β expression is high in the soleus, but this muscle contains almost no IIX fibers. Clearly, other factors must also be involved in this fiber type decision.

PGC-1α mRNA is repressed in PGC-1β transgenic animals, and this may contribute to the observed reduction in MHC I and IIA expression. It is unlikely, however, that repression of PGC-1α contributes to the induction of IIX fibers since PGC-1α seems to promote IIX formation (Figure 4) and since complete absence of PGC-1α in −/− animals does not lead to increased IIX fibers (data not shown). It is also formally possible that some of the effects seen in the PGC-1β transgenic animals stem from altered expression of PGC-1β during development.

Having mice with a greatly increased content of type IIX fibers allowed us to analyze the actual performance of muscles enriched in these fibers in a way that has not been possible before. We show here that elevated oxidative IIX fiber content increases the ''athletic'' performance of mice and allows them to run further and do more work before exhaustion. Since the transgene is expressed only in muscle, we can reasonably ascribe these changes to the muscles themselves. However, whether this increased performance in running is due to alteration in the contractile apparatus or altered fuel homeostasis will require further study.

A key aspect of transcriptional control in development and homeostasis is the coordinated induction of multiple genetic programs. For example, the determination of skeletal muscle fiber type requires the coordinated activation of specific myofibrillar proteins and the appropriate metabolic program. Transcriptional coactivators are well suited to play a role in such coordinated regulation by superimposing their regulatory activity on underlying core programs that are controlled by transcription factors. The data presented here provide a strong example of such a mechanism: PGC-1β coordinates the coactivation of at least MEF2, ERRα, and PPARα, and likely a number of other transcription factors, to activate both MHC IIX expression and the full program of mitochondrial biogenesis.

Previous work showed that PGC-1α can drive the formation of type I and IIA fibers in skeletal muscle in vivo. In sharp contrast, PGC-1β in fact repressed expression of MHC type I and IIA. Since PGC-1α also binds to and coactivates MEF2D, it is not immediately apparent why PGC-1β drives only type IIX MHC in vivo. The simplest interpretation of these data is that while coactivation of MEF2 is important, there are likely to be other transcriptional components in the muscle fiber type systems that distinguish the actions of PGC-1α and β. Indeed, studies in liver have already shown that these highly related coactivators can nevertheless bind to and coactivate overlapping but nonidentical transcription factors.

What stimuli alter the content of type IIX fibers and how PGC-1β is regulated in muscle are poorly understood. PGC-1β expression appears less sensitive to acute exercise than PGC-1α, but how PGC-1β is altered during chronic exercise, when fiber type switching is expected to occur, is not known. As stated earlier, type IIX fibers also appear to be an important transition stage when fibers switch from IIB to IIA. It will therefore be of interest to evaluate the role of PGC-1β during these transition periods. PGC-1β is also repressed in diabetic muscle, along with PGC-1α and a significant part of the repertoire of mitochondrial genes. However, whether this relates to the content of fiber types in diabetic muscle, and whether type IIX fibers have different metabolic profiles that may be protective against diabetes, awaits further investigation.

Finally, these data have potential importance for the therapy of a number of muscular and neuromuscular diseases in humans. Many conditions accompanied by loss of physical mobility, including paraplegia, prolonged bed rest, and muscular dystrophies, involve a loss of oxidative fibers and their replacement with glycolytic fibers. This, in turn, results in a further loss of resistance to fatigue, exacerbating the patient's condition in a downward spiral. The identification of PGC-1β as a potential mediator of the development of oxidative type IIX fibers suggests new ways to modulate muscle fiber type in health and disease.

EXPERIMENTAL PROCEDURES

Animals

All animal experiments were performed according to procedures approved by the Dana-Farber Cancer Institute's Institutional Animal Care and Use Committee. Mice were maintained on a standard rodent chow diet with 12 hr light and dark cycles. To generate transgenic mice, the complete PGC-1β cDNA was cloned 3′ to 4.8 kb of the promoter of muscle creatine kinase (MCK) (Johnson et al., 1989), and the human growth hormone polyadenylation site was inserted 3′ to the cDNA. Mouse oocytes were injected with this construct by the Dana-Farber Cancer Institute Core Facility. Exercise tolerance was measured by performing the above described protocol on a motor-driven treadmill (Columbus Instruments) following 3 days of acclimation at 14 m/min and 0% grade for 5 min. MCK-PGC-1α mice have been described previously (Lin et al., 2002b).

Cells and Reagents

C2C12 and 10T1/2 cells were from ATCC and were grown in DMEM 10% FBS. Antibodies against cytochrome *c* and electron transport chain proteins were from Pharmingen and MitoSciences, respectively. The affinity-purified rabbit antibody against PGC-1β has been described previously (Lin et al., 2005b). The MHC IIX-specific monoclonal antibody 6H1 was a kind gift of L.A. Leinwand. The probe specific for MHC IIX mRNA, used for in situ mRNA hybridization, was created by PCR amplification of cDNA from skeletal muscle using primers 5′-GGGAGAAGTGAGCTTCAACC-3′ and 5′-CGAGAGGAG

CAGGCTGAGCCAGAC-3′. The PGC-1α and β promoter-luciferase plasmids; the DR1-luciferase plasmid; and plasmids encoding for PGC-1α, PGC-1β, ERRα, and PPARα have been described previously (Handschin et al., 2003; Lin et al., 2005b). The MHC IIX promoter-luciferase plasmid was generated by amplifying a 2 kb fragment of DNA encompassing 1.7 kb 5′ to 300 bases 3′ of the MHC IIX start site and subcloning into the pGL3-Basic plasmid (Promega). The MHC IIA promoter-luciferase plasmid was a kind gift of D.L. Allen. Transfections were done using Superfect (QIAGEN) according to the manufacturer's instructions. Wy14848 was from Wyeth.

Histological Analyses

Electron microscopy was performed at the Brigham and Women's Core Facility. For all other histologies, tissues were snap frozen in liquid nitrogen immediately after harvesting. Fiber typing was performed with cryostat sections using metachromatic dye-ATPase methods as described previously (Ogilvie and Feeback, 1990). The cross-sectional area of fibers was determined using MetaMorph software after immunocytochemical labeling of cryostat sections with anti-laminin antibody. Five separate slides from five separate animals were used for each group.

Protein Studies

Protein lysates were prepared in a buffer containing PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and a cocktail of protease inhibitors; separated on 4%–12% NuPAGE gels (Invitrogen); and immunoblotted with the indicated antibodies in the presence of 5% Blotto. Separation of proteins for determination of MHC composition was performed by SDS-PAGE as described (Talmadge and Roy, 1993), followed by Coomassie staining.

Gene Expression Studies

Total RNAs were isolated from mouse tissue or cultured cells using the Trizol method (Invitrogen). Samples for real-time PCR analyses were reverse transcribed (Invitrogen), and quantitative real-time PCR reactions were performed on the cDNAs in the presence of fluorescent dye (SYBR Green, Bio-Rad). DNA product of the expected size was confirmed for each primer pair. All results are expressed as means ± SEM. Two-tailed independent Student's t tests were used to determine all p values. Microarrays were performed by the Dana-Farber Cancer Institute Core Facility using Affymetrix MOE430A chips. motifADE (Mootha et al., 2004) was applied to the comparison between expression arrays derived from three wild-type versus three transgenic quadriceps muscles. All 6-, 7-, and 8-mers with a modified p value < 0.05 were sought.

Supplemental Data

Supplemental Data include eleven figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/5/1/35/DC1/.

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Accession Numbers

Array data are available at ArrayExpress with the accession number E-MEXP-939.