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Disconnecting Mitochondrial Content from Respiratory Chain Capacity in PGC-1-Deficient Skeletal Muscle

Glenn C. Rowe,[1,2] Ian S. Patten,[1,2] Zsuzsanna K. Zsengeller,[3] Riyad El-Khoury,[4,5] Mitsuharu Okutsu,[6] Sophia Bampoh,[1,2] Nicole Koulisis,[1,2] Caitlin Farrell,[1,2] Michael F. Hirshman,[7] Zhen Yan,[6] Laurie J. Goodyear,[7] Pierre Rustin,[4,5] and Zolt Arany[1,2,*]
[1]Cardiovascular Institute
[2]Center for Vascular Biology Research
[3]Department of Pathology
Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA 02215, USA
[4]INSERM, UMR 676, Hôpital Robert Debré, 75019 Paris, France
[5]Faculté de Médecine Denis Diderot, Université Paris 7, 75005 Paris, France
[6]Center for Skeletal Muscle Research, Robert M. Berne Cardiovascular Research Center, University of Virginia School of Medicine, Charlottesville, VA 22908, USA
[7]Joslin Diabetes Center, Boston, MA 02215, USA
*Correspondence: zarany@bidmc.harvard.edu
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SUMMARY

The transcriptional coactivators PGC-1α and PGC-1β are widely thought to be required for mitochondrial biogenesis and fiber typing in skeletal muscle. Here, we show that mice lacking both PGC-1s in myocytes do indeed have profoundly deficient mitochondrial respiration but, surprisingly, have preserved mitochondrial content, isolated muscle contraction capacity, fiber-type composition, in-cage ambulation, and voluntary running capacity. Most of these findings are recapitulated in cell culture and, thus, are cell autonomous. Functional electron microscopy reveals normal cristae density with decreased cytochrome oxidase activity. These data lead to the following surprising conclusions: (1) PGC-1s are in fact dispensable for baseline muscle function, mitochondrial content, and fiber typing, (2) endurance fatigue at low workloads is not limited by muscle mitochondrial capacity, and (3) mitochondrial content and cristae density can be dissociated from respiratory capacity.

INTRODUCTION

Mitochondria in skeletal muscle serve diverse functions and are critical for health. Human mutations in mitochondrial DNA (mtDNA) often lead to muscle dystrophy (Seneca et al., 2001; Wallace, 2005). Aberrant mitochondrial function has been proposed to contribute to insulin resistance (Hoeks and Schrauwen, 2012; Szendroedi et al., 2012). Boosting mitochondrial activity in rodents improves exercise capacity (Arany et al., 2007; Calvo et al., 2008; Narkar et al., 2008) and organismal health in numerous models of disease ranging from muscle dystrophy to amyotrophic lateral sclerosis (Da Cruz et al., 2012; Handschin et al., 2007c).

PGC-1α and PGC-1β have emerged as potent transcriptional coactivators that regulate broad programs of mitochondrial biology (Finck and Kelly, 2006; Rowe et al., 2010; Scarpulla, 2008). The PGC-1s also coordinately regulate various ancillary programs relevant to mitochondria. In muscle, the PGC-1s coregulate fatty acid transport and angiogenesis—two processes that are critical for bringing fuel and oxygen to mitochondria (Arany et al., 2008; Madrazo and Kelly, 2008; Rowe et al., 2011; Vega et al., 2000). The PGC-1s have also been implicated in regulating genes of the neuromuscular junction (Handschin et al., 2007c) and muscle-fiber typing (Arany et al., 2007; Lin et al., 2002), both of which are relevant to mitochondrial biology in skeletal muscle.

There has been controversy, however, regarding the extent to which PGC-1α and PGC-1β normally contribute to these mitochondrial and ancillary programs. Although gain-of-function models have been compelling, as outlined above, loss-of-function experiments have been less conclusive. The extensive redundancy that exists between PGC-1α and PGC-1β complicates interpretations (Lai et al., 2008; St-Pierre et al., 2003). Deletion of either PGC-1α or PGC-1β alone, in the whole body or in skeletal muscle specifically, only mildly affects muscle mitochondria and function (Handschin et al., 2007a, 2007b; Lai et al., 2008; Leone et al., 2005; Lin et al., 2004; Sonoda et al., 2007; Zechner et al., 2010), whereas whole-body deletion of both coactivators leads to perinatal lethality due to heart failure (Lai et al., 2008). Evaluation of the role of the PGC-1s in skeletal muscle thus requires deletion of both coactivators specifically in this tissue. Zechner et al. (2010) recently generated mice in which PGC-1β is deleted in skeletal muscle and a hypomorphic allele of PGC-1α is present in all tissues. The authors demonstrated a dramatic loss of electron transport chain (ETC) activity in

muscle from these mice, but unchanged fiber types and glucose handling. However, whole-body PGC-1α deficiency has profound effects on ambulatory activity, metabolism, and diurnal behavior (Leone et al., 2005; Lin et al., 2004; Liu et al., 2007), all of which are significant confounders in skeletal muscle studies. Moreover, the PGC-1α allele used in the experiments by Zechner et al. (2010) retains intact exons 1–5 (Leone et al., 2005), which encode the transactivation and nuclear-receptor-binding domains of PGC-1α, and encode for almost all of NT-PGC-1α and PGC-1α4, a naturally occurring splice variant of PGC-1α that is known to retain significant activity (Chang et al., 2010, 2012; Ruas et al., 2012; Zhang et al., 2009). Therefore, the mice likely retained some PGC-1 activity in skeletal muscle. These findings have thus led to controversy (Handschin and Spiegelman, 2011; Zechner et al., 2011).

In light of these controversies, we used floxed alleles of both PGC-1α and PGC-1β (Lai et al., 2008; Lin et al., 2004) to generate animals that lack both coactivators specifically in skeletal muscle and retain intact expression of both coactivators in other tissues. We find that ETC activity is severely blunted in these animals. Surprisingly, however, other aspects of mitochondrial biology, including mitochondrial density itself, are intact in both animals and isolated cells. In fact, surprisingly, baseline muscle function is normal, both in vivo and ex vivo. Similarly, fiber types are unchanged. Only forced exercise capacity is impaired. Thus, the data resolve prior controversies and indicate that mitochondrial content and ETC capacity are dissociable, that most of ETC capacity in muscle is dispensable for normal function, and that PGC-1 coactivators likely serve to "boost" the system and enable it to withstand stress beyond ordinary activities.

RESULTS

PGC-1α and PGC-1β Are Critical for ETC Capacity

Mice lacking PGC-1α and PGC-1β strictly in skeletal muscle (double-knockout [DKO] mice) were generated by Cre/Lox recombination. The PGC-1α and PGC-1β floxed alleles were kindly provided by Dr. Bruce Spiegelman (Lin et al., 2004) and Dr. Daniel Kelly (Lai et al., 2008), respectively. Both alleles are complete nulls after Cre/Lox recombination. Cre recombinase was driven by a modified myogenin promoter containing the MEF2 enhancer (Li et al., 2005), an allele widely used for muscle-specific recombination. The DKO mice were born in Mendelian ratios and appeared grossly indistinguishable from their littermate controls. The messenger RNA (mRNA) expression of both PGC-1α and PGC-1β was reduced by 60% in quadriceps from DKO animals (Figure 1A). The residual expression of the PGC-1s likely reflects expression in nonmyocytes, in which the Cre recombinase is not expressed. PGC-1-related coactivator (PRC) is a coactivator with weak homology to the PGC-1s (Rowe et al., 2010; Scarpulla, 2008). The expression of PRC was not altered in the DKO animals (Figure 1A). Hematoxylin and eosin staining of transverse sections from the muscles showed no obvious abnormalities (Figure S1A).

The mRNA expressions of nuclear genes encoding components of the ETC and mitochondrial ATPase were severely repressed by 60% in quadriceps from DKO animals (Figure 1B). The RNA expression of genes encoded on the mitochondrial genome was similarly repressed (Figure 1C). Soleus and extensor digitorum longus (EDL) muscles revealed similar findings (Figures S1B and S1C). Western blotting revealed dramatic decreases in proteins of the ETC and oxidative phosphorylation (Figures 1D and 1E). The activities of all four complexes of the ETC and the ATPase complex, measured directly in intact quadriceps muscle via state-of-the-art enzymatic spectrophotometric assays (Bénit et al., 2006; Rowe et al., 2012; Rustin et al., 1994), were severely depressed, by as much as >60% (Figures 1F and S1D). These marked respiratory defects suggested that the mice would have blunted exercise capacity. Indeed, DKO mice were severely limited in their ability to carry out a treadmill-based exercise stress test (Figures 1G–1J). Together, these data indicate that DKO mice have profoundly decreased ETC capacity, which is reflected in marked defects in forced exercise capacity.

ETC Capacity Is Dispensable for Baseline Activity and Muscle Function

Surprisingly, spontaneous in-cage locomotor activity was intact in DKO mice (Figure 2A). However, mice are normally much more active than a standard cage allows them to be. To provide more opportunity for physical activity, we placed voluntary running wheels in the cages and monitored them electronically. Both DKO and control animals voluntarily ran nightly, running ~10 hr/day for an approximately calculated total distance of 60–80 km over an 11-day period (Figures 2B and 2C). DKO mice spent the same amount of time on the wheels (Figure 2D) as the control animals, and revealed only a mild, statistically nonsignificant decrease in total running distance (Figure 2E) or average revolutions/minute (Figure 2F). The mice appeared healthy at all times (not shown).

To test muscle function in the absence of systemic organismal effects, we explanted the EDL muscles and tested them ex vivo. The DKO and control muscles achieved a nearly indistinguishable maximum tetanic force of ~200 mM/mm^2 (Figure 2G), isometric twitch force of ~15 mN/mm^2 (Figure 2H), and rate of fatigability (Figure 2I). Maximum titanic force and fatigability were similarly preserved in soleus muscles from DKO animals (Figure S2A–S2C). Muscle contractile function and fatigability are dependent, to some extent, on the relative makeup of fiber types. However, the fiber-type compositions of DKO and control animals were nearly identical in both plantaris and soleus muscles (Figures 2J, 2K, and S2D). Thus, together, these data indicate that, surprisingly, DKO mice retain nearly intact fiber-type composition, baseline muscle activity, and fatigability both in vivo and ex vivo.

Mitochondrial Content and ETC Capacity Are Separable

In light of the remarkable loss of respiratory chain capacity in DKO mice (Figure 1), we performed electron microscopy (EM) to evaluate mitochondria in muscle from DKO animals. Surprisingly, the DKO mitochondria appeared entirely normal in number, morphology, and overall content (Figures 3A and 3B). The content of mtDNA was also unperturbed in DKO muscles (Figure 3C). Regular intermyofibrillar and subsarcolemmal mitochondria were seen. No differences in mitochondrial size or cristae density and morphology were appreciated. Blinded

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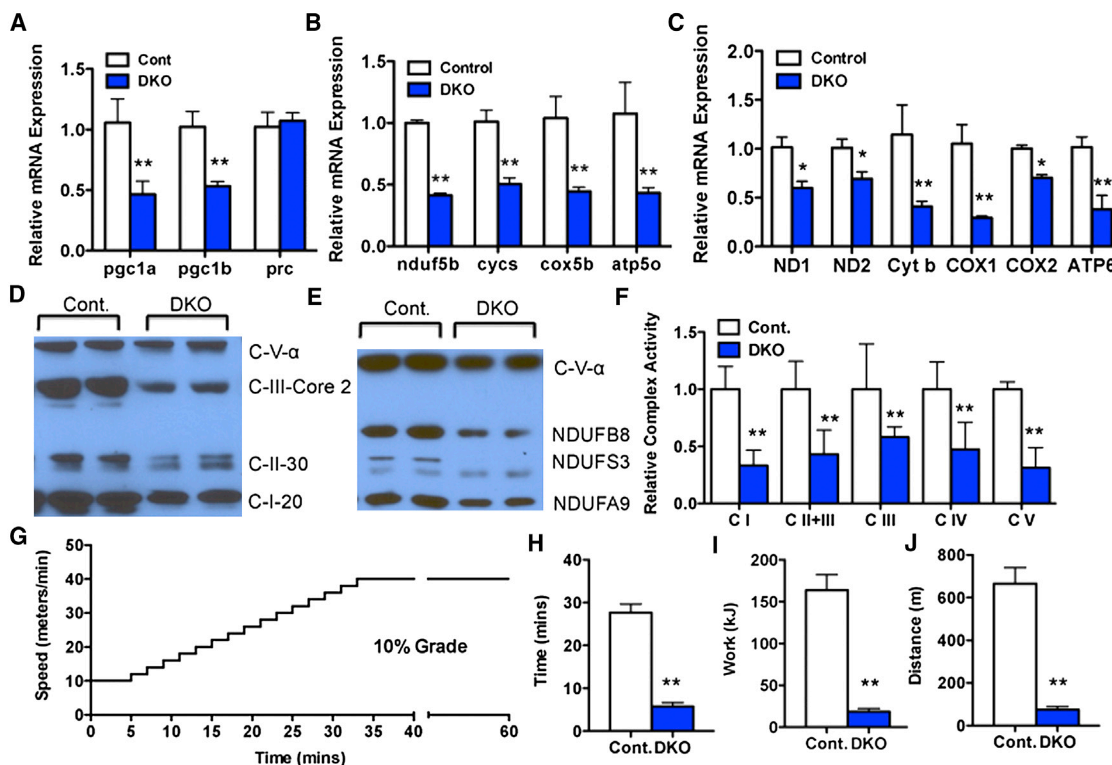

Figure 1. PGC-1α and PGC-1β Are Critical for ETC Capacity

(A–C) Relative mRNA expression of the indicated genes from quadriceps (quads) of Myo-DKO mice (blue bars) and littermate controls (white bars): PGC-1 isoforms (A), nuclear-encoded ETC genes (B), and mitochondrial-encoded ETC genes (C).

(D and E) Western blot analyses of indicated ETC proteins from quads of Myo-DKO mice.

(F) Relative activity of the indicated ETC complexes in quad muscles from DKO and control animals.

(G) Schematic of the exercise exhaustion protocol.

(H–J) Maximal time (H), work (I), and distance (J) achieved on running treadmills prior to exhaustion. Error bars indicate SEM; $n > 3$ per group in all panels; *, $p < 0.05$. N.S., not statistically significant.

See also Figure S1.

experts in mitochondrial morphology were unable to distinguish between the two genotypes.

These surprising findings indicated that mitochondrial biogenesis is separable from assembly of the respiratory chain. Consistent with this notion and the preserved mtDNA content in DKO muscles, the expression of genes involved in replication of mtDNA was not reduced in DKO animals (Figure 3D). Expression of most genes involved in synthesis of cardiolipin, the predominant structural mitochondrial lipid, was similarly unaffected, as were, for the most part, genes involved in other mitochondrial functions, such as apoptosis, proteolysis, and fusion/fission (Figure S3A–S3C).

Cell-Autonomous Separation of Mitochondrial Content and ETC Activity

The observed DKO phenotypes may have had a developmental origin or may have been affected by cues outside the muscle. To investigate the above findings in a cell-autonomous setting, primary muscle myoblasts were isolated from PGC-1α/β homozygous double-floxed animals, exposed to adenoviruses expressing Cre recombinase (or LacZ as a control), and then differentiated into myotubes in cell culture. The efficiency of PGC-1α and PGC-1β deletion was >95% (Figure S3D). Differentiated myotubes appeared grossly normal (Figure S3E) and showed normal temporal expression of differentiation markers (Figure S3F), indicating that, interestingly, PGC-1α and PGC-1β are dispensable for myoblast-to-myotube differentiation in cell culture.

The mRNA expression of nuclear and mitochondrial respiratory chain components was markedly decreased in DKO myotubes (Figure 3E), as was the content of Complex I-V proteins (Figure 3F), recapitulating the findings in vivo. The maximal oxygen consumption rate (OCR) was reduced by 60% in DKO myotubes, reflecting the marked decreases in components of the ETC (Figure 3G). On the other hand, basal OCR was unaffected in DKO myotubes (Figure 3G), again indicating that PGC-1α and PGC-1β are dispensable for baseline function. OCR in the presence of palmitate as a primary fuel source was also markedly blunted in DKO myotubes (Figure S3G). State 3 and state 4o respiration (treated with oligomycin) in permeabilized myotubes provided with ADP and pyruvate/malate as substrates was also markedly reduced (Figure 3H).

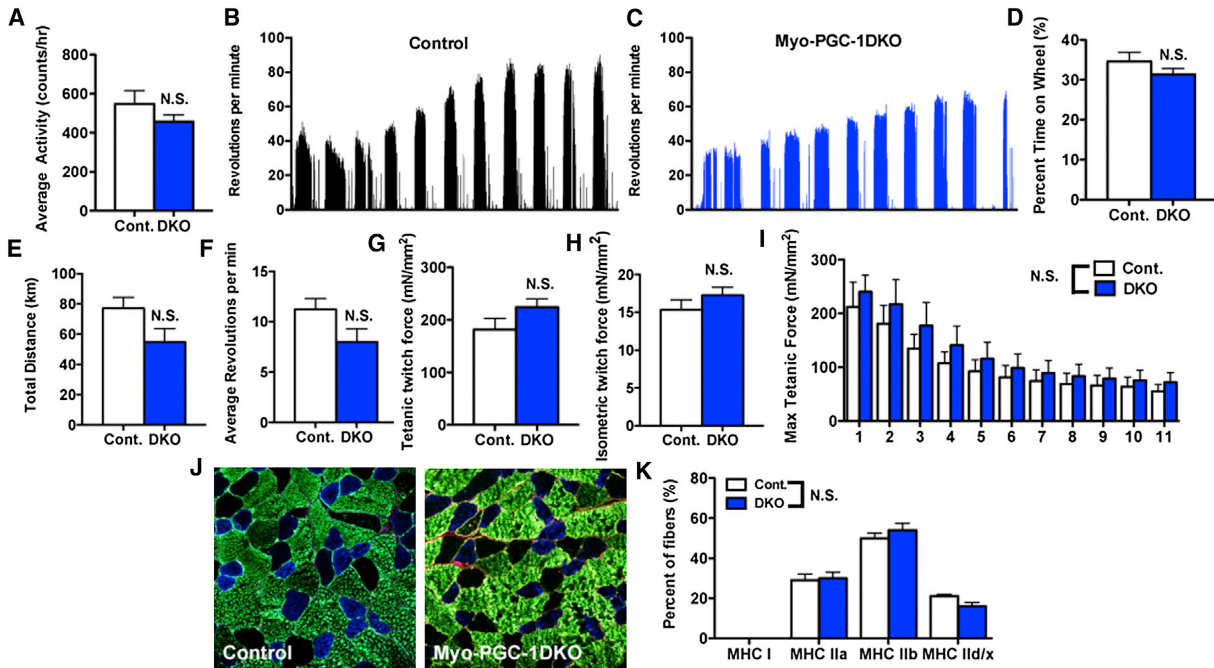

Figure 2. PGC-1s Are Dispensable for Baseline Activity and Muscle Function

(A) In-cage locomotor activity of Myo-DKO mice and control littermates during a 72 hr period.

(B and C) Samples of voluntary wheel activity during 11 days.

(D–F) Quantification of (B) and (C), represented as the percentage of time spent on wheels (D), total distance run (E), and average revolutions per minute (F).

(G–I) Maximal tetanic (G) and isometric (H) twitch forces and contraction-stimulated fatigability (I) of explanted extensor digitorum longus muscle from Myo-DKO and control animals.

(J and K) Fiber-type composition in plantaris of DKO and control animals.

(J) Representative images of immunostaining: MHC Ia (red), MHC IIa (blue), and MHC IIb (green); unstained fibers were counted as MHC IId/x.

(K) Quantification of immunostaining. Error bars indicate SEM; n > 6 per group in all panels. N.S., not statistically significant.

See also Figure S2.

Finally, transmission EM (TEM) revealed preserved mitochondrial content in DKO myotubes (Figure 3I), again analogous to the findings in vivo. Mitochondrial content, as quantified by MitoTracker dye, was identical between DKO and control cells (Figure 3J), as were the steady-state mtDNA amount (Figure 3K) and expression of genes required for mtDNA replication (Figure 3L). Altogether, these data indicate that the separation of mitochondrial content and ETC activity is cell autonomous and independent of developmental processes. Moreover, despite profound effects on respiration capacity, PGC-1α and PGC-1β are dispensable for baseline respiration and myoblast-to-myotube differentiation.

Direct Visualization of ETC Activity at Resolution of Cristae

In general, cristae density has been thought to reflect ETC capacity (Giraud et al., 2002; Strauss et al., 2008). Surprisingly, however, we found that cristae density was normal in the DKO animals (Figure 3A). We therefore sought to measure ETC activity in situ, at a magnification sufficient to resolve individual cristae. We used a recently redeveloped technique in which an electron-dense trace is generated in situ by Complex IV-mediated oxidative polymerization of 3,3′-diaminobenzidine (DAB) (Zsengeller et al., 2012; Figure 4A). The method thus directly visualizes actively respiring cristae. Images from control animals revealed uniform contrast throughout the cristae and surrounding mitochondrial membrane (presumably the inner membrane; Figure 4B), consistent with cristae that are packed with ETC components. In sharp contrast, mitochondria from DKO animals revealed numerous cristae with only occasional contrast, in a stippled pattern (Figure 4B, white arrowheads). Some cristae lacked contrast altogether. No such abnormalities were observed in the control animals. Thus, these data indicate that cristae in DKO animals are capable of forming, but have reduced cytochrome *c* oxidase (COX) capacity.

DISCUSSION

As outlined above, the precise role of the PGC-1 coactivators in skeletal muscle at baseline has been the subject of debate (Handschin and Spiegelman, 2011; Zechner et al., 2011). Here, we resolved this issue by using alleles of PGC-1α and PGC-1β that are most likely complete nulls (Handschin et al., 2007b; Lai et al., 2008), and by using a skeletal-muscle-specific Cre driver in the context of homozygous floxed alleles, thereby leaving other tissues unaffected. We conclude that (1) PGC-1α and PGC-1β are critically required for normal ETC and oxidative capacity; (2) the PGC-1s do not play a role in baseline fiber-type

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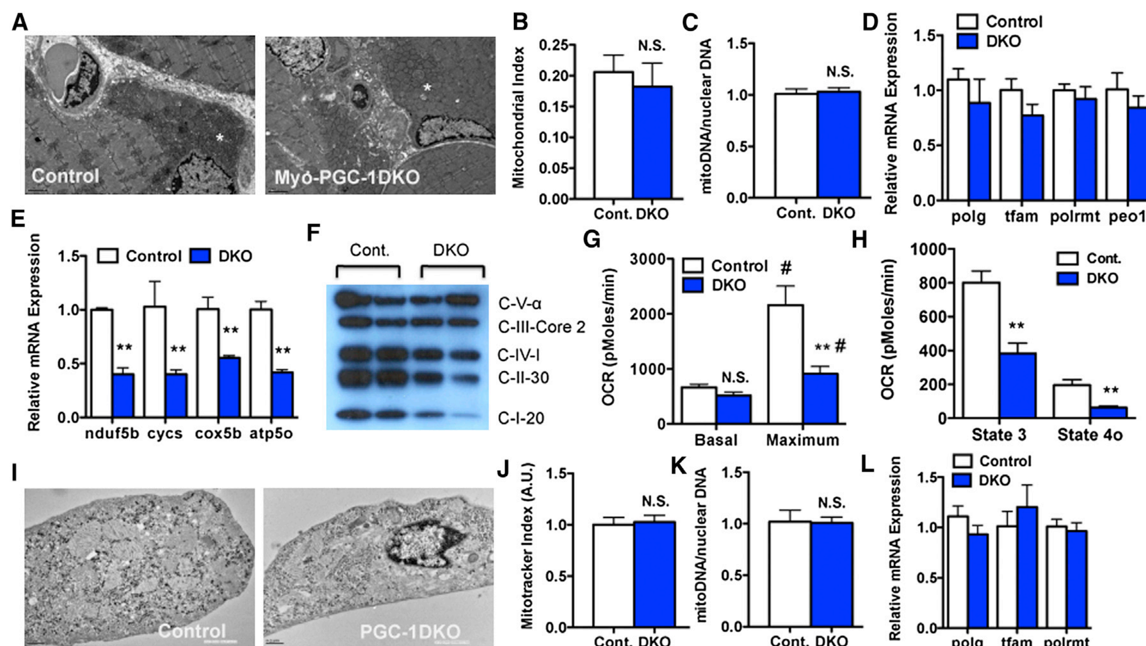

Figure 3. Mitochondrial Content and ETC Capacity Are Separable in DKO Muscle and Cultured Myotubes

(A and B) Representative images (A) and morphometric quantification of the mitochondrial density (B) of transmission electron micrographs of transverse sections of the midportion of the quads of Myo-DKO mice and control littermates. Sample mitochondria are labeled with a white asterisk.

(C) Mitochondrial-to-nuclear DNA content ratio in quad muscles from Myo-DKO and control animals.

(D) Relative expression of the indicated genes from the quads of Myo-DKO and control animals.

(E–L) Differentiated myotubes from primary myoblasts isolated from DKO (blue) versus control (white) muscles.

(E) Relative mRNA expression of the indicated genes.

(F) Western blot analysis of the indicated ETC proteins.

(G) Basal and uncoupled respiration rates of DKO and control differentiated myotubes.

(H) State 3 and state 4o respiration rates in permeabilized DKO and control differentiated myotubes.

(I) TEM of DKO and control differentiated myotubes.

(J and K) Quantification of mitochondrial content by MitoTracker (J) and mitochondrial-to-nuclear DNA content ratio (K) in DKO and control cells.

(L) Relative expression of the indicated genes in primary DKO and control myotubes (n > 4 fields from 6 animals per group). Error bars indicate SEM; n > 3 per group in all panels; *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with basal state; N.S., not statistically significant.

See also Figure S3.

composition; (3) the PGC-1s are dispensable for baseline muscle contractile force and fatigability, thus dissociating these processes from oxidative capacity in the muscle; and (4) the PGC-1s are also dispensable for baseline mitochondrial density per se, thus dissociating this process from oxidative capacity.

Our data suggest that PGC-1 coactivators are dispensable for most of the developmental and hormonal cues that determine muscle differentiation and fiber-type composition (Schiaffino and Reggiani, 2011). Consistent with this notion, deletion of the PGC-1s did not affect myoblast-to-myotube differentiation in cell culture (Figure S3). On the other hand, gain-of-function models convincingly demonstrated the ability of PGC-1s to drive fiber-type conversion (Arany et al., 2007; Lin et al., 2002). These observations suggest that the PGC-1s primarily transduce postnatal physiological signals to affect fiber-type composition. Exercise, for example, is known to induce PGC-1α and changes in fiber types. Muscle PGC-1α alone is dispensable for exercise-induced fiber-type transformation and mitochondrial biogenesis (Geng et al., 2010; Rowe et al., 2012), suggesting that redundancy with PGC-1β may be important in this context. If so, then PGC-1β may be modified by exercise at the posttranslational level, since, in contrast to PGC-1α, levels of PGC-1β transcripts are typically not affected by exercise. Consistent with this notion, PGC-1β is stabilized by cyclic AMP signaling (Shoag et al., 2013).

It is striking that mice lacking >60% of muscle oxidative capacity remain capable of normal locomotion, and indeed of voluntarily running up to 10 km per night (Figure 2). The dissociation of these processes demonstrates that oxidative capacity in skeletal muscle is not primarily rate limiting for most baseline activities. The data also indicate that the "comfort zone" rate at which mice voluntarily run on in-cage wheels is determined by factors other than oxidative capacity. Fatigue, be it experienced or physiological, is a significant burden faced by patients with myopathies and has a large impact on their perceived health status. The low workload fatigue experienced by these patients is likely mechanistically different from that experienced by higher-intensity work such as endurance running. The data presented here suggest that mitochondrial insufficiency in these patients may not account for their fatigue.

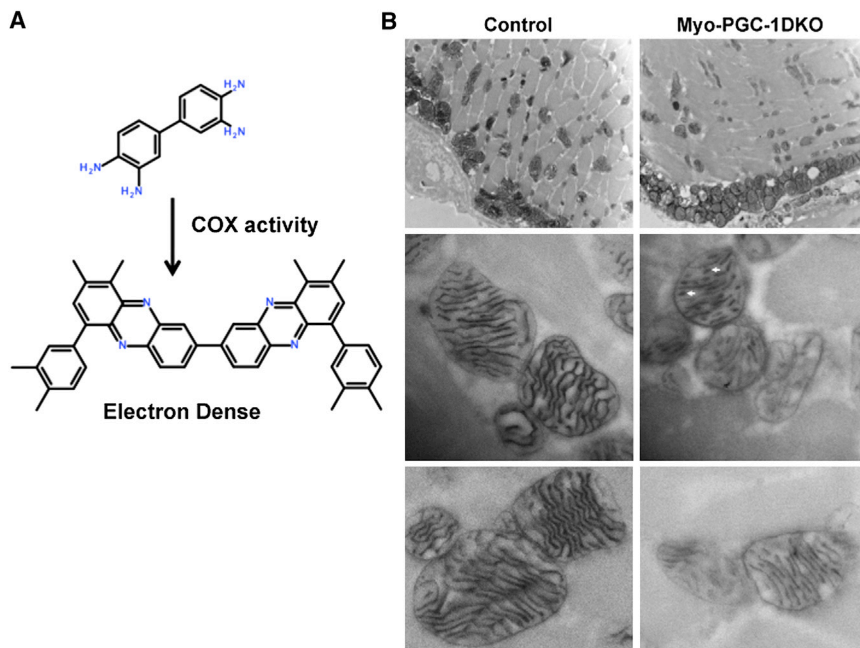

Figure 4. Decreased Ultrastructural ETC Activity in Myo-PGC-1DKO Animals

(A) Schematic of DAB polymerization.

(B) TEM ultrastructural analysis of mitochondrial COX enzymatic activity in quadriceps muscle of Myo-DKO animals and control littermates. Punctate mitochondrial cristae are labeled with a white arrowhead.

DKO cells and muscles contained normal amounts of mitochondria and cristae, despite profound decreases in ETC capacity. The regulation of these two processes is thus separable. mtDNA content was also normal in DKO animals and cells, as was the expression of genes involved in mitochondrial genome replication (Figure 3D). Thus, the PGC-1s are not rate limiting for mtDNA content in skeletal muscle. Coordinated regulation of genes involved in cardiolipin biosynthesis was also unaltered in DKO animals and cells. It thus appears that a different threshold of required PGC-1 activity exists for genes of the ETC compared with other mitochondrial functions. It will be of interest in the future to determine whether this involves binding to different transcription factors and/or altered post-translational modifications. PRC, a coactivator with weak homology to the PGC-1s, may play a role, although we did not observe compensatory upregulation of PRC in the DKO animals.

High-resolution visualization of cytochrome oxidase activity intriguingly revealed a stippled pattern of activity along cristae in DKO animals, in contrast to the continuous activity seen in control animals. Assembly of ETC complexes is known to be tightly regulated and exquisitely stoichiometric (Lenaz and Genova, 2010; Papa et al., 2012). The discontinuous pattern of staining on EM thus suggests that ETC complexes form normally at some sites on the cristae, but not at all at other sites. This pattern may be favored because less dense packing of ETC components along the cristae may not allow proper substrate shuttling (Giraud et al., 2002; Strauss et al., 2008).

In summary, the present study resolves important controversies concerning the role of PGC-1α and PGC-1β in skeletal muscle, and in the process also demonstrates that oxidative capacity in muscle is surprisingly dissociable from mitochondrial content, from isolated muscle contractility, and from normal organismal activity and fatigability.

EXPERIMENTAL PROCEDURES

All animal experiments were performed according to procedures approved by the Beth Israel Deaconess Medical Center IACUC. PGC-1α floxed mice (Lin et al., 2004), PGC-1β floxed mice (Lai et al., 2008), and Myogenin/MEF2-Cre driver (Li et al., 2005) have been described previously. Respiratory chain complex activity was measured as previously described (Bénit et al., 2006; Rustin et al., 1994). EM was performed by the BIDMC Electron Micrograph Core. Functional COX EM was performed as previously described (Zsengeller et al., 2012). OCRs (pmol/min) were assessed with the use of an XF Flux Analyzer (Seahorse Biosciences). Data are presented as means ± SEM. Statistical analysis was performed with Student's t test for all in vitro experiments, and ANOVAs were performed for all in vivo experiments; p values of <0.05 were considered statistically significant.

For more details regarding the materials and methods used in this work, see the Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.04.023.

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