Transcriptional coactivator PGC-1α controls the energy state and contractile function of cardiac muscle

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Summary

Skeletal and cardiac muscle depend on high turnover of ATP made by mitochondria in order to contract efficiently. The transcriptional coactivator PGC-1α has been shown to function as a major regulator of mitochondrial biogenesis and respiration in both skeletal and cardiac muscle, but this has been based only on gain-of-function studies. Using genetic knockout mice, we show here that, while PGC-1α KO mice appear to retain normal mitochondrial volume in both muscle beds, expression of genes of oxidative phosphorylation is markedly blunted. Hearts from these mice have reduced mitochondrial enzymatic activities and decreased levels of ATP. Importantly, isolated hearts lacking PGC-1α have a diminished ability to increase work output in response to chemical or electrical stimulation. As mice lacking PGC-1α age, cardiac dysfunction becomes evident in vivo. These data indicate that PGC-1α is vital for the heart to meet increased demands for ATP and work in response to physiological stimuli.

Introduction

Living organisms must convert chemical energy into mechanical work. Muscle is a specialized tissue devoted primarily to this task. The energy needs in muscle are quite high and must be precisely regulated. Nowhere is this more true than in the heart, where work is generated unfailingly for decades and where energy consumption is higher than in any other organ. The availability of energy in skeletal and cardiac muscle can be significantly altered in both health and disease. Chronic exercise in skeletal muscle, for example, stimulates a switching from predominantly glycolytic to more oxidative fibers, which contain more mitochondria and are resistant to fatigue (Booth and Thomason, 1991). Conversely, aberrations in energy production are seen in such diverse muscular diseases as muscular dystrophies, mitochondrial myopathies (Kelly and Strauss, 1994; Wallace, 1992), and chronic congestive heart failure (Ingwall, 2002; Ingwall and Weiss, 2004). How these defects come about remains incompletely understood, in particular at the level of gene expression control.

PPARγ coactivator-1α (PGC-1α) and -β are potent transcriptional coactivators of nuclear receptors and other transcription factors (Knutti and Kralli, 2001; Puigserver and Spiegelman, 2003; Scarpulla, 2002). They can control specific metabolic pathways, especially oxidative metabolism, in a variety of tissues. PGC-1α targets promoters by interacting directly with numerous DNA binding transcription factors and then coordinating several biochemical events, including recruitment of chromatin modifying enzymes such as p300/CBP and SRC-1 (Puigserver et al., 1999), interaction with the basal transcription machinery (Wallberg et al., 2003), and linking of transcription to RNA splicing (Monsalve et al., 2000). The outcome is robust activation of gene expression in coherent metabolic pathways. PGC-1β, identified by virtue of sequence homology to PGC-1α, has a similar tissue distribution as PGC-1α and coactivates an overlapping but not identical repertoire of transcription factors (Kressler et al., 2002; Lin et al., 2002a; Lin et al., 2003). In the liver, for instance, PGC-1α docks HNF4α and FOXO1 (Lin et al., 2003) and activates gluconeogenesis, while PGC-1β activates lipid biosynthesis by docking SREBP (Lin et al., 2005).

One of the primary effects of PGC-1α is the activation of mitochondrial biogenesis and oxidative phosphorylation. In brown fat, cold exposure induces PGC-1α expression, which leads to mitochondrial proliferation, uncoupling of oxidative phosphorylation through increased expression of UCP-1, and energy dissipation in the form of heat (Lin et al., 2004; Puigserver et al., 1998). In skeletal and cardiac muscle cells, forced expression of PGC-1α in vitro activates mitochondrial biogenesis, oxidative phosphorylation, and respiration (Lehman et al., 2000; St-Pierre et al., 2003). Forced expression of PGC-1α in type II skeletal muscle fibers in vivo leads to mitochondrial pro-

liferation and switching to more oxidative (type IIa and I) fibers that resist fatigue from repeated electrical stimulation (Lin et al., 2002b). Marked overexpression of PGC-1α in cardiac muscle in vivo leads to mitochondrial proliferation to such an extent that the myofibrillar contractile apparatus is displaced, leading to cardiomyopathy and congestive heart failure (Lehman et al., 2000). Temporally limiting PGC-1α overexpression to the adult heart results in reversible contractile dysfunction through unclear mechanisms (Russell et al., 2004). Taken together, these gain-of-function experiments have demonstrated the capacity of PGC-1α to activate the full programs of mitochondrial biogenesis and oxidative phosphorylation in both skeletal and cardiac muscle beds.

Expression of PGC-1α in muscle beds is itself highly modulated, consistent with a role in cellular adaptation to environmental stimuli. Chronic exercise leads to increased PGC-1α mRNA in skeletal muscle, followed by an increased mitochondrial content, resistance to fatigue, and presence of more oxidative fibers (Baar et al., 2002; Russell et al., 2003; Terada et al., 2002; Wu et al., 2002). Conversely, levels of PGC-1α and genes of mitochondrial metabolism are decreased in skeletal muscle of diabetic patients, as well as prediabetic family members, arguing for an early and perhaps causal role of this pathway in the pathogenesis of type 2 diabetes (Mootha et al., 2003; Patti et al., 2003). In the heart, PGC-1α expression is induced perinatally and then in the adult heart, which has led to the hypothesis that PGC-1α regulates the burst of mitochondrial biogenesis also observed perinatally (Lehman and Kelly, 2002b). Pathologically hypertrophied hearts, modeled by surgical constriction of the aorta, have decreased expression of both PGC-1α and its target genes of fatty acid oxidation and oxidative phosphorylation (Barger et al., 2000; Garnier et al., 2003; Lehman and Kelly, 2002b). Similarly, overexpression of either HDAC5 (Czubryt et al., 2003) or cyclinT1 (Sano et al., 2004) in the heart leads to PGC-1α downregulation, mitochondrial dysfunction, and profound heart failure, suggesting a role for diminished PGC-1α in the development of heart failure. These experiments have suggested that PGC-1α in different muscle beds can coordinate levels of energy production with mechanical energy needs and that aberration of that balance can lead to dysfunction and disease.

Here, we investigate whether PGC-1α is required for proper mitochondrial function, energy generation, and contractile function in skeletal and cardiac muscle. Using PGC-1α knockout (KO) mice, we show that, while PGC-1α is required neither for mitochondrial biogenesis per se nor for differentiation of oxidative skeletal muscle fibers, the absence of PGC-1α leads to reduced mitochondrial function and profound defects in the ability of the heart to respond to increased demand.

Results

Normal tissue structure and mitochondrial biogenesis in the absence of PGC-1α

PGC-1α KO mice were generated by homologous recombination (Lin et al., 2004). The mice are very cold sensitive, consistent with a role for PGC-1α in adaptive thermogenesis. They are also lean and hyperactive, associated with defects in the striatal part of the brain (Lin et al., 2004).

At 3 months, ratios of heart weight to tibial length, often increased in the presence of cardiac structural abnormality, were slightly lower in KO than in wt mice (Figure 1A). Hearts from KO mice showed no histological evidence of hypertrophy or dilatation on low-power views, nor of increased fibrosis on high magnification views of Trichrome stainings (Figures 1B and 1C), both common cardiac mechanisms of compensation in disease. Isolated cardiomyocytes also did not show evidence of hypertrophy (see Figure S1A in the Supplemental Data available with this article online). In skeletal muscle, fibers were of normal size and revealed no gross structural abnormalities (Figure 2A). Hence, at 3 months, PGC-1α KO mice show no overt histological signs of abnormalities in these muscle beds.

PGC-1α can activate the full program of mitochondrial biogenesis in vitro and in vivo (Lehman et al., 2000; Puigserver et al., 1998; St-Pierre et al., 2003; Wu et al., 1999). Moreover, PGC-1α gene expression in the heart is sharply increased at the time of birth, coinciding with a sudden increase in mitochondrial biogenesis (Lehman et al., 2000). This has led to the hypothesis that PGC-1α dictates, at least in part, mitochondrial biogenesis in vivo. We tested this by examining mitochondria in cardiac and skeletal muscle by electron microscopy (EM). Surprisingly, only subtle differences were notable between mitochondria from KO and wt mice in either cardiac or skeletal muscle (Figures 1D and 1E, respectively). In the heart, the mitochondria appeared slightly less well packed between myofibers, and slight dilation of cristae was sometimes noted (Figure 1D and data not shown). Importantly, however, the overall volume content of mitochondria appeared unchanged in KO mice. Morphometric quantification of mitochondrial volume confirmed that approximately one third of the heart is taken up by mitochondria, and no difference was seen between wt and KO mice (Figure 1D). Thus, despite the ability of PGC-1α to activate the full program of mitochondrial biogenesis in vitro and in vivo, PGC-1α is not required for mitochondrial biogenesis in vivo in cardiac and skeletal muscle.

Skeletal muscle fiber types in the absence of PGC-1α

Mammals adjust to chronically increased mechanical energy needs (e.g., exercise) by converting some glycolytic skeletal muscle fibers into more oxidative fibers (Berchtold et al., 2000). The latter are rich in mitochondria and contain myofibrillar proteins that are more conducive to sustained work output (Hood, 2001). The mechanism by which this conversion occurs remains incompletely understood, but the current evidence suggests that it involves, at least in part, the induction by cAMP and Ca^{2+} of PGC-1α, which then activates transcriptional programs of fiber type switching and mitochondrial biogenesis (Czubryt et al., 2003; Handschin et al., 2003; Lin et al., 2002b; Wu et al., 2002). Ectopic expression of PGC-1α in vivo in glycolytic (type IIb) skeletal myofibers is sufficient to drive fiber switching to a phenotype with morphology and functional characteristics of more oxidative types (I and IIa) (Lin et al., 2002b). Whether PGC-1α is *necessary* for this process in vivo, however, is not known.

Compared to wt mice, PGC-1α KO mice are hyperactive and move more throughout the day, in effect constantly "exercising" (Lin et al., 2004). From this aspect, one might expect that skeletal muscle from KO mice would contain more oxidative fibers in response to the increased energy demands and neuronal stimulation. On the other hand, if PGC-1α were indeed necessary for differentiation to more oxidative fibers, one would predict *fewer* (if any) type I and IIa fibers in the KO mice.

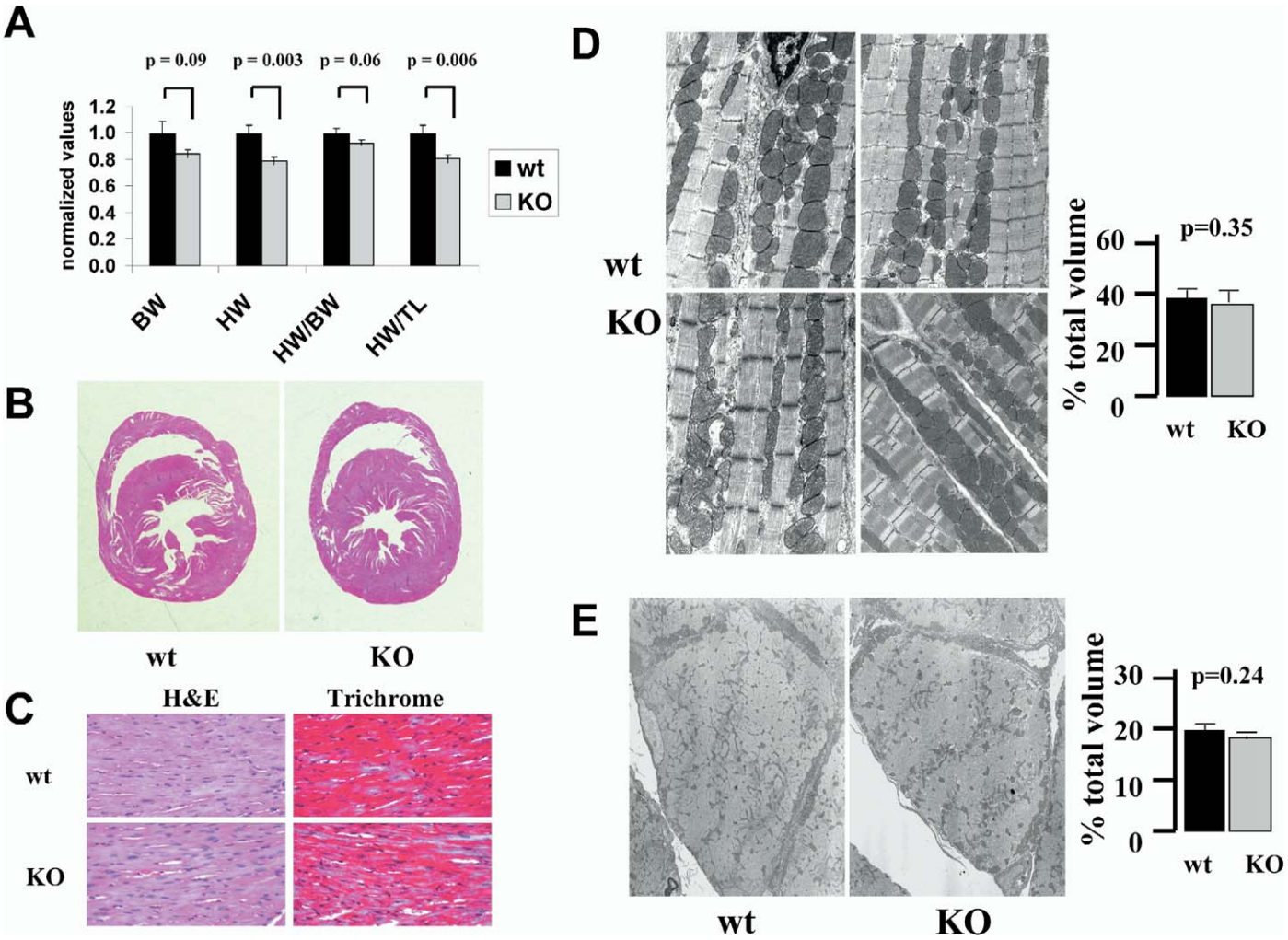

Figure 1. Normal tissue structure and mitochondrial biogenesis in the absence of PGC-1α

PGC-1α wt and KO mice were harvested at 3 months.

A) Body weight (BW), heart weight (HW), and ratios of heart weight to body weight (HW/BW) and tibial length (HW/TL). Error bars are ±SEM.

B) Low-power views after H&E staining of transverse sections from wt and KO hearts.

C) High magnification views of H&E (left panels) and Trichrome (right panels) stainings from the same sections as in (**B**).

D) Electron microscopy (8000×) photographs of longitudinal anterior sections from wt (top panels) and KO (lower panels) hearts. Morphometric quantification of mitochondrial volume from 10 wt and 15 KO electron micrographs is shown on the right.

E) Low-power electron microscopy photographs of transverse sections form wt and KO quadriceps muscle. Morphometric quantification of mitochondrial volume from samples of 11 wt and 8 KO electron micrographs is shown on the right.

To test these possibilities, fiber type composition in skeletal muscle was evaluated in PGC-1α KO mice by metachromatic staining and immunocytochemistry, which allows for the distinction of type I, IIa, and IIb fibers. As seen in Figure 2, there were no obvious differences between wt and KO mice in fiber type composition: the soleus muscle, a type I-rich (red) muscle, was of equal size (data not shown) and had equal numbers of type I, IIa, and IIb fibers in KO and wt mice. Consistent with this, expression of genes normally enriched in oxidative fibers (such as troponin I slow, SERCA2, and sarcolipin) was similar in soleus muscle from wt and KO mice (data not shown). Interestingly, expression of these genes was slightly but significantly (about 2-fold) *elevated* in KO quadriceps, a type IIb-rich muscle (Figure 2C). This is consistent with the notion that hyperactivity in KO mice leads to a slight induction of oxidative fiber type genes. Hence, despite its ability to induce type I and IIa fiber formation when expressed ectopically, PGC-1α is *not* absolutely necessary for oxidative fiber formation in vivo.

Deficient mitochondria in skeletal and cardiac muscle from PGC-1α KO mice

Having normal mitochondrial number does not necessarily indicate normal mitochondrial function. To test if there is an abnormal pattern of gene expression in KO mice, we used RNA from skeletal and cardiac muscle, combined with microarray analyses and quantitative real-time PCR. These data (Figure 3A and Figure S1B) revealed that the majority of the most highly expressed genes necessary for mitochondrial function, such as subunits of ATP synthase (e.g., *atp5i* and *atp5c1*) and cytochrome c oxidase (e.g., *cox5b* and *cox7a2*), are reduced by

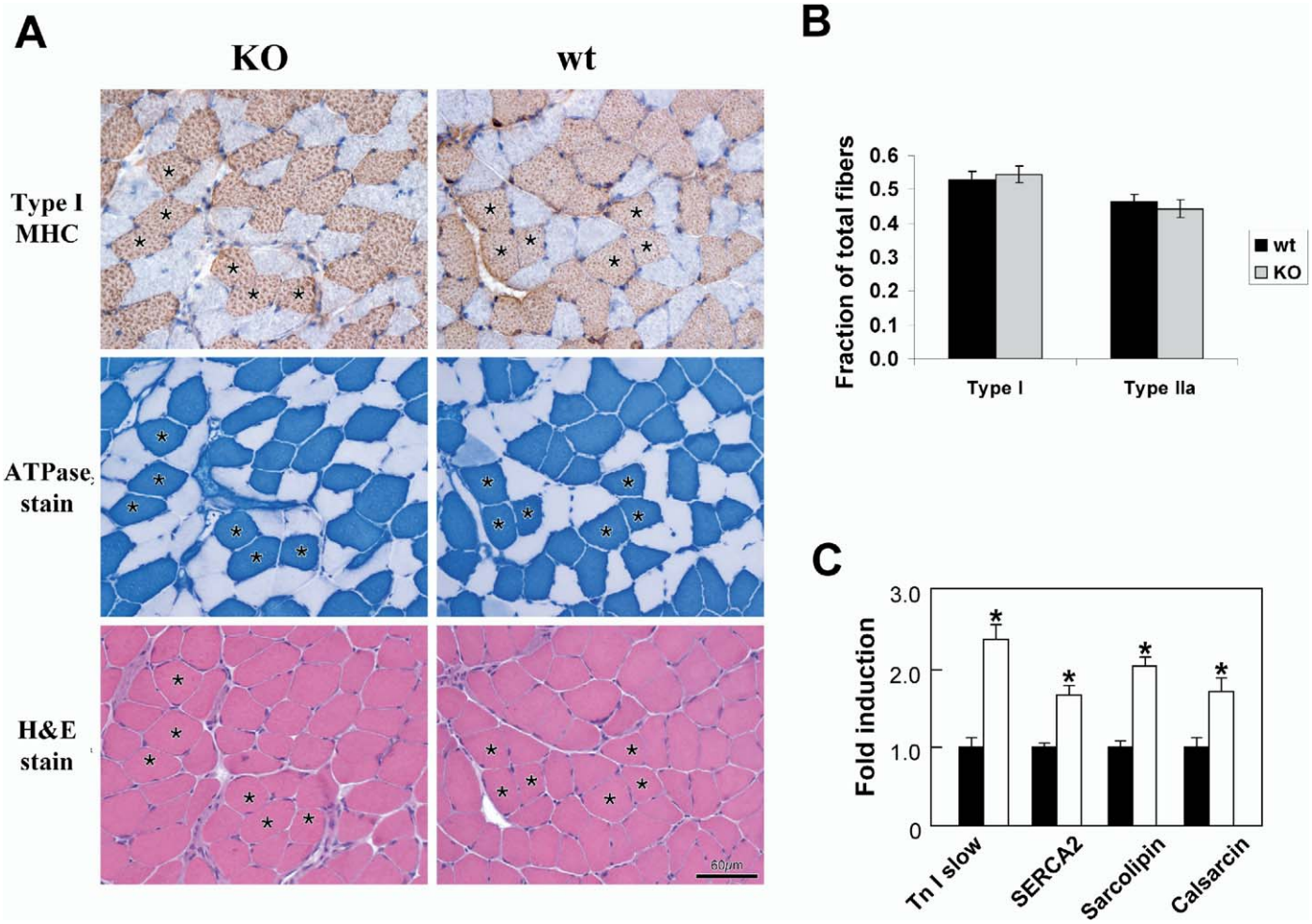

Figure 2. Skeletal muscle fiber types in the absence of PGC-1α

A) Immunocytochemical staining of type I β-myosin heavy chain (MHC, top panel), metachromatic ATPase staining (middle panel), and standard H&E staining (bottom panel) of transverse sections from wt and KO soleus muscle. Asterisks indicate examples of type I fibers.

B) Fraction of type I and IIa fibers in WT and KO soleus muscle, as determined by counting fibers in complete transverse sections from both sides of two WT and two KO mice. Error bars are ±SEM.

C) mRNA expression of genes, normally enriched in type I fibers, in quadriceps (type I-poor) muscle from three wt and three KO mice, as assayed by real-time quantitative PCR. *$p < 0.05$. Error bars are ±SEM.

20%–50% in skeletal muscle from KO mice. A comparison with cultured muscle cells ectopically expressing PGC-1α indicated that these same genes are induced by PGC-1α (Figure 3A, left column). Importantly, these changes in gene expression in the KO mice were observed in the absence of significant changes in proportion of oxidative fiber types (Figure 2).

PGC-1α KO mice have many aberrant characteristics, including hyperactivity and increased total body MVO_2 (Lin et al., 2004), which might affect the changes in gene expression observed. To test this, we examined cultured muscle cells isolated from the whole animal, thereby removing the confounding variables inherent to the in vivo setting. As shown in Figure 3B, the reduced expression of genes necessary for mitochondrial function, such as cytochrome c (*cycs*) or cytochrome c oxidase subunit 5b (*cox5b*), is still apparent in the absence of PGC-1α, even though these cells were isolated as satellite cells and differentiated in culture many days. These data strongly suggest that these decreases in gene expression in muscle are cell autonomous, i.e., do not depend on the whole animal KO environment. In contrast, the type I fiber-specific genes that were induced in whole animal skeletal muscle, such as troponin I slow (*tnis*) and calsarcin (Figure 2C), were *not* induced in primary cells from wt and KO mice (Figure 3B), consistent with the notion that the induction of these genes in vivo reflects a neurohormonal response rather than a cell-autonomous phenomenon.

Decreases in expression of genes relating to mitochondrial biology were even more marked in the heart (Figure 3C). Quantitative real-time PCR demonstrated a 30%–50% reduction in the expression of genes of oxidative phosphorylation, fatty acid oxidation, and ATP synthesis (Table 1 and data not shown). Western blotting with antibodies against products of two of these genes (cytochrome c and ND4L, a member of the NADH dehydrogenase complex) showed significant reductions in protein levels (Figure S1D). The expression of many of these same genes, such as *cycs* or *cox5b*, is reduced to a similar extent in

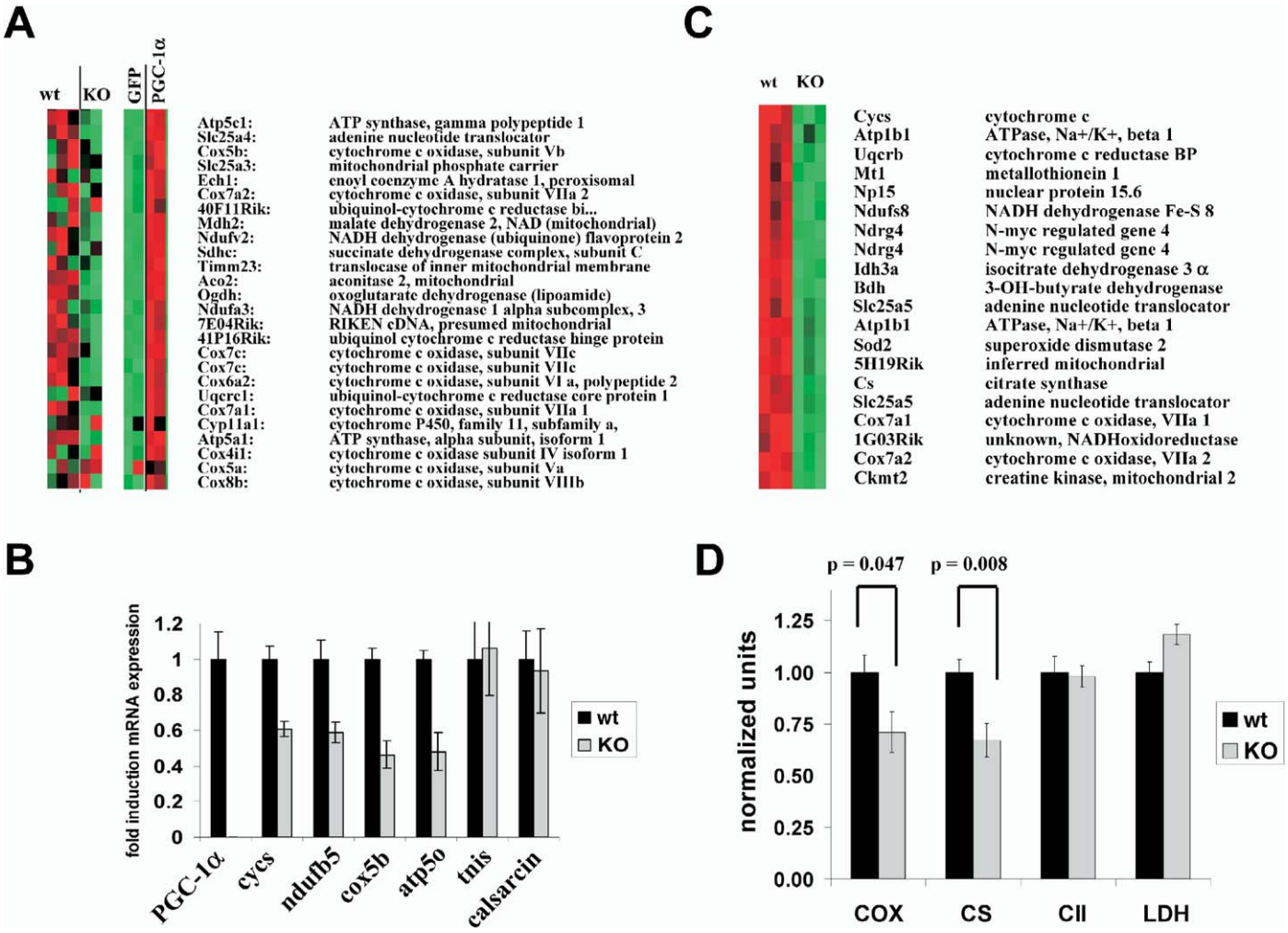

Figure 3. Deficient mitochondria in skeletal muscle from PGC-1α KO mice

A) Representative Affymetrix microarray analysis of mRNA from wt and KO quadriceps muscle (left panel) and from C2C12 cells infected with adenovirus encoding for PGC-1α or GFP (right panel). The 25 mitochondrial genes that are most highly expressed in quadriceps muscle are shown. Red and green colors indicate increased and decreased expression, respectively.

B) mRNA expression of representative genes of mitochondrial biology (*cycs*, *ndufc1*, *cox5b*, *atp5i*) and genes normally enriched in type I fibers (troponin I slow, calsarcan) in differentiated primary skeletal cells harvested from quadriceps (type I-poor) muscle from wt and KO mice. *$p < 0.05$. Error bars are ±SEM.

C) Representative Affymetrix microarray analysis of total RNA from wt and KO hearts. The 20 most highly expressed and decreased genes are shown. Color coding is as in (**A**). Statistical analysis of mitochondrial gene expression in the entire microarray is presented in Figure S1C.

D) Enzymatic activity in extracts from wt and KO hearts (n = 5 and 5). CS, citrate synthase. COX, cytochrome c oxidase. CII, electron transport chain complex II. LDH, lactate dehydrogenase. Error bars are ±SEM.

a well-established model of cardiac hypertrophy generated by constriction of the transverse aorta (Table 1), as has been shown before (Barger and Kelly, 2000; Lehman and Kelly, 2002a; Weinberg et al., 2003). In addition, the expression of a number of transcription factors known to act in a positive feedback loop with PGC-1α, including PPAR-α and ERR-α (Huss et al., 2004; Mootha et al., 2004), was also decreased (Table 1) in KO mice, likely contributing to the reduction of target gene expression. PGC-1α has also been shown to increase the expression of the transcription factor TFAM (Wu et al., 1999), which in turn translocates to the mitochondria and mediates both transcription and replication of the mitochondrial genome (Kelly and Scarpulla, 2004). As shown in Table 1, the expression of TFAM is reduced by 50% in PGC-1α KO hearts. Consistent with this, expression of genes encoded by the mitochondrial DNA (such as *cybmm* and ND5, Table 1) and the amount of mitochondrial DNA itself (Figure S1E) are both also reduced in KO hearts. Taken together, these data demonstrate that PGC-1α is required for normal expression of genes relating to mitochondrial biology in skeletal and cardiac muscle in vivo.

To test if these changes in gene expression result in corresponding functional deficiencies, select enzymatic activities in cardiac extracts were assayed. Cytochrome c oxidase (COX), a key enzyme in electron transport and oxidative phosphorylation, and citrate synthase (CS), the rate-limiting enzyme in the Krebs cycle, both showed more than 30% reduced activity in hearts from KO mice (Figure 3D). Interestingly, complex II of the electron transport chain (CII), which is made up of subunits encoded only by the nuclear genome, was not diminished in the KO hearts. As comparison, the activity of the cytoplasmic

Table 1. Gene expression in wt and PGC-1α KO hearts

	PGC-1α KO versus wt		TAC versus sham		
	Fold ind.	p value	Fold ind.	p value	
cycs	0.55	0.01	0.81	0.14	Oxidative phosphorylation (encoded by nucleus)
cox5b	0.59	0.005	0.61	0.02	
atp5o	0.48	0.005	0.64	0.06	
ndufb5	0.44	0.002	0.78	0.003	
cybmm	0.50	0.017	nd	nd	Oxidative phosphorylation (encoded by mitochondria)
ND1	0.70	0.11	nd	nd	
ND5	0.50	0.021	nd	nd	
MCAD	0.48	0.06	0.76	0.10	Fatty acid oxydation
CPT-1	0.65	0.22	0.69	0.07	
CPT-2	0.67	0.03	nd	nd	
CD36	0.71	0.20	nd	nd	
TFAM	0.55	0.047	nd	nd	Transcription regulation
ERR-α	0.72	0.07	nd	nd	
PPAR-α	0.60	0.11	nd	nd	
PPAR-γ	1.03	0.98	nd	nd	
NRF-1	0.87	0.35	nd	nd	
NRF-2a	0.90	0.34	nd	nd	
ANP	7.5	0.005	18.0	0.0002	Cardiac stress
BNP	2.1	0.06	2.2	0.23	
α-MHC	1.6	0.009	0.7	0.03	
β-MHC	2.6	0.06	11.9	0.001	
sk α-actin	13.5	0.004	7.2	0.008	
hsp70	2.8	0.58	2.6	0.09	
SERCA 2a	1.1	0.37	0.7	0.002	

Fold induction of gene expression in KO hearts (n = 3) over that in wt hearts (n = 3) from 3-month-old mice is shown in the left columns. For comparison, fold induction of the same genes in hearts from same-age mice subjected to 1 month of transverse thoracic aortic banding (TAC, n = 5) over that in sham-treated hearts (n = 5) is shown in the right columns. TAC hearts were kindly provided by Dr. Richard Lee.

enzyme lactate dehydrogenase (LDH) was slightly increased (Figure 4C), consistent with changes in LDH gene expression (data not shown).

Defective function in isolated hearts from PGC-1α KO mice

Given the high requirement of the heart for ATP and the deficiencies in gene expression and mitochondrial enzymatic activities observed, we asked whether PGC-1α KO mice have altered contractile function. To do this, isolated and perfused hearts (Langendorff preparations) were used. For these experiments, hearts are explanted and perfused ex vivo, allowing regular contractions to continue. A balloon is inserted into the left ventricle and expanded to fill the cavity, and the pressure within the balloon is continuously measured. This allows precise evaluation of pressures and rates of pressure change during each (now isovolumic) contraction. As stated earlier, KO mice are lean, hyperactive, and have a highly altered glucose/insulin axis (Lin et al., 2004). Using the well-established Langendorff preparations therefore allowed us to define the contractile properties of the hearts independent of confounding variables present in the whole-body setting, such as available substrates or neurohormonal environment.

Using this approach, hearts from 3-month-old PGC-1α KO mice revealed small but significant contractile deficiencies when allowed to spontaneously pump without further stimulus (Table S1A): both the rate of change of pressure during contraction (+dP/dt) and the maximal achieved pressure (LVSP) were reduced about 10% ($p < 0.05$). These deficiencies, however, became more pronounced under stimulation (Table S1A and Figure 4A). For example, isolated hearts can be made to beat at higher rates by apposing an electrode directly onto the heart and pacing at an increased frequency. This purely chronotropic challenge in KO hearts led to 20% less forceful contractions (+dP/dt) than in wt hearts (Table S1A). When the hearts were forced to abruptly increase work by infusion with dobutamine (a β-adrenergic agonist that leads to large increases in both heart rate and force of contraction, i.e., both a chronotropic and inotropic challenge), +dP/dt in wt hearts increased by 30% more than in KO hearts (Figure 4A and Table S1A). A similar pattern of mild baseline deficiency and worsening under stimulation was noted for the parameter that reflects measured relaxation (–dP/dt), a process that also requires energy (restoring the Ca^{2+} gradient). Interestingly, KO hearts also showed a significant inability to increase heart rate in response to dobutamine (Table S1A). Lower heart rate can increase +dP/dt; hence, the deficiency in +dP/dt seen here probably underestimates the true magnitude of deficiency in KO hearts. A better index of cardiac work, therefore, is the rate pressure product (RPP), equal to heart rate times the increase in pressure developed by each contraction (DevP). As seen in Figure 4B, wt hearts can increase RPP by nearly twice as much as can KO hearts in response to dobutamine.

In the experiments described above, hearts were perfused with modified Krebs bicarbonate buffer, which contains glucose and pyruvate as energy sources. However, the heart uses primarily free fatty acids as a carbon source in vivo (Ingwall, 2003). Moreover, PGC-1α is well-known to activate genes of fatty acid import and oxidation, and expression of these genes is decreased in PGC-1α KO hearts (Table 1). We therefore also measured the contractile function of wt and KO hearts perfused with a buffer containing physiological concentrations of

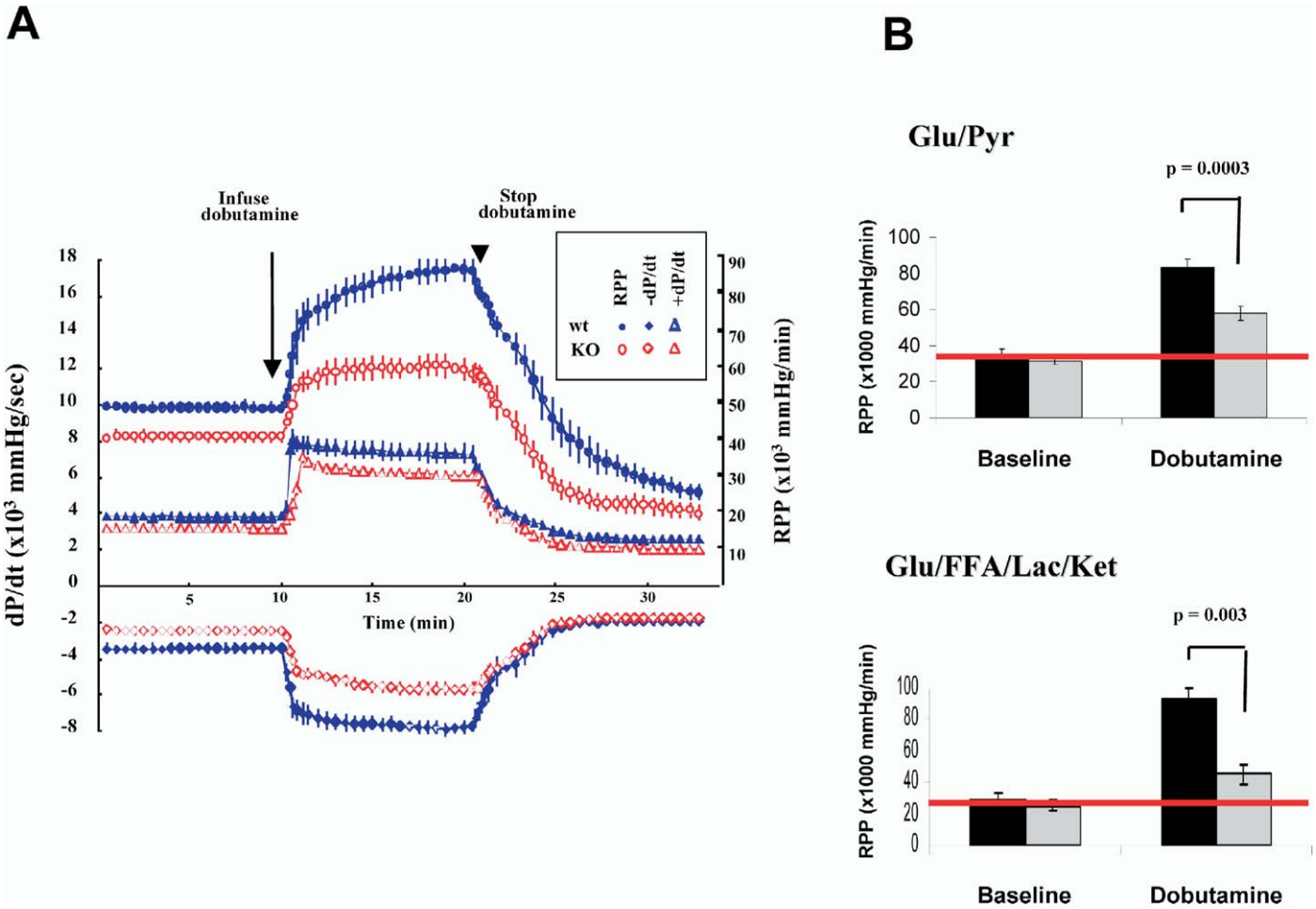

Figure 4. Defective cardiac function in PGC-1α KO mice

A) Contractile performance of hearts from PGC-1α KO (red, n = 5) compared to wt (blue, n = 5) hearts measured in Langendorff preparations. Hearts were electrically paced at 7.0 Hz. After 10 min, hearts were challenged with 300 nM dobutamine for 12 min and then allowed to recover for another 15 min. +dP/dt, positive change in pressure over time during isovolumic contraction. –dP/dt, negative change in pressure over time during isovolumic relaxation. RPP, rate pressure product = heart rate times pressure differential between the fully contracted and relaxed states. RPP models the work done by the heart in vivo.

B) Rate pressure product in wt and KO hearts at baseline and in response to dobutamine. In the top panel, hearts from wt (n = 5) and KO (n = 5) mice were perfused with modified Krebs bicarbonate buffer containing glucose and pyruvate. In the bottom panel, hearts from wt (n = 4) and KO (n = 4) mice were perfused with buffer containing physiological concentrations of free fatty acids, glucose, lactate, and ketones, as described in Experimental Procedures. Error bars are ±SEM.

all carbon sources normally used by the heart: free fatty acids, glucose, ketones, and lactate. Under these conditions, the contractile deficiencies of the KO hearts became even more pronounced (Table S1B). In response to dobutamine, for instance, the RPP increased only 30% as much in KO hearts as it did in wt hearts (Figure 4C and Table S1B). Together, these data show that hearts from PGC-1α KO mice have a profound defect in contractile reserve, suggesting that PGC-1α is vital to increasing cardiac work output in response to increased demand.

Hearts from PGC-1α KO mice are energy deficient

The KO hearts were next studied for their ability to maintain readily available sources of energy for mechanical work. The ATP content of wt and KO hearts was measured by ^{31}P nuclear magnetic resonance (NMR) using the Langendorff heart preparations (Figure 5A). From these measurements, concentrations of ATP, inorganic phosphate (Pi), and phosphocreatine (PCr) can be calculated from the area under each peak. PCr, the primary energy reserve compound in excitable tissues, is normally present in cardiac muscle at twice the concentration of ATP and is in rapid exchange with ATP; an accurate measure of available high-energy phosphate bonds thus requires evaluation of both ATP and PCr concentrations. The most powerful advantages of this NMR technique stem from its ability to noninvasively measure ATP and PCr contents in intact, beating hearts under differing conditions and while simultaneously measuring contractile performance.

Using this approach, we found that ATP levels in unstimulated PGC-1α KO hearts were reduced by approximately 20% compared with wt hearts (Figures 5A and 5B and Figure S2A). The concentration of ATP in cardiac tissue is normally tightly controlled and, in cases of terminal heart failure, is reduced by only approximately 30%. Hence, the absence of PGC-1α results in a profound energetic defect in the heart. Similarly, the concentration of PCr was also reduced by ~20% (Figures 5A

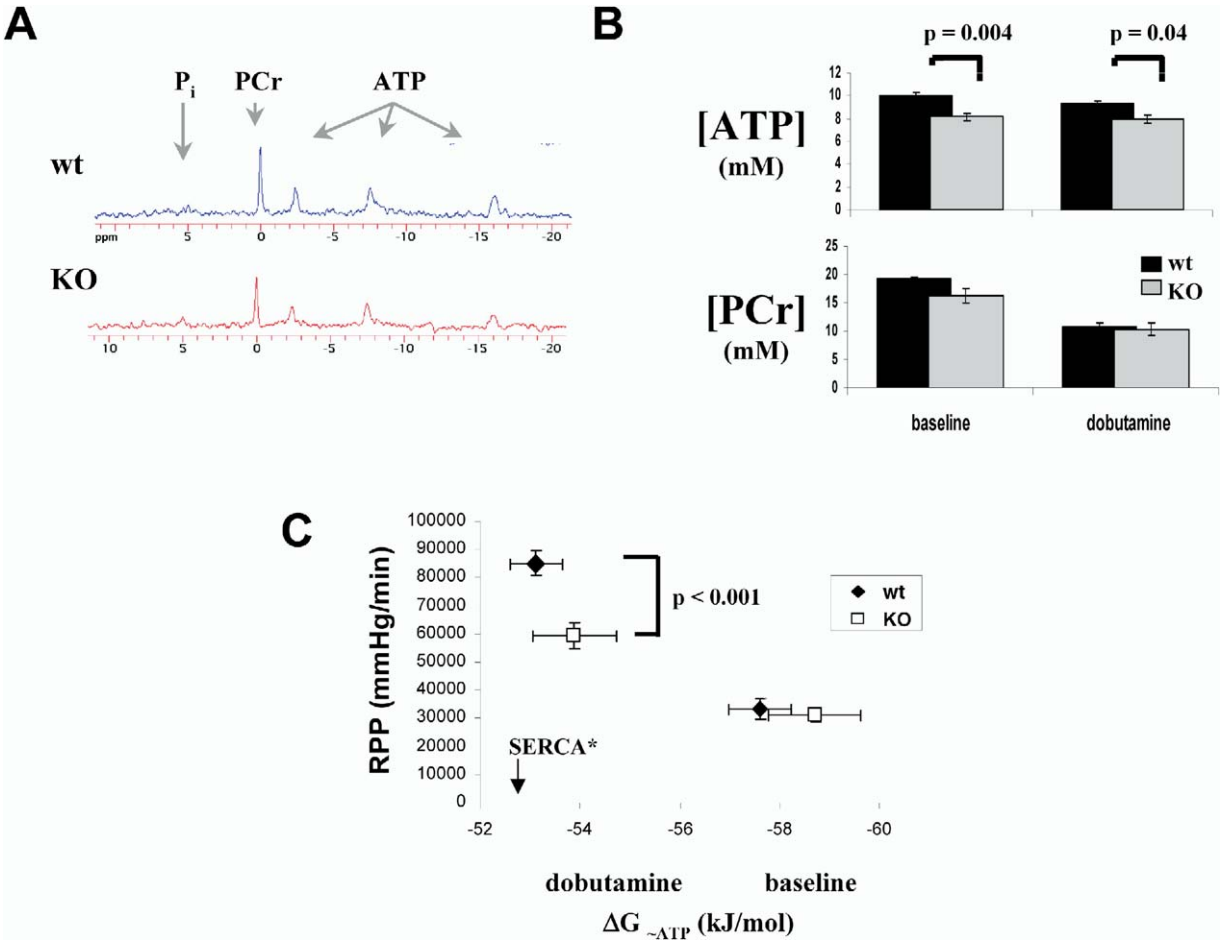

Figure 5. Hearts from PGC-1α KO mice are energy deficient

A) Representative spectra acquired from wt and KO hearts during active, unstimulated contraction. Peaks corresponding to signals from the phosphorus atoms in each of the phosphates of ATP and phosphocreatine (PCr), and free inorganic phosphate (P_i) are indicated.

B) Average ATP and PCr concentrations in wt and KO hearts that are either unstimulated or maximally stimulated with 300 nM dobutamine infusion, as assayed from NMR spectra as in (**A**). N = 5 and 5. Error bars are ±SEM.

C) Relationship, in wt and KO hearts, between the rate pressure product (RPP, a measure of cardiac work) and $\Delta G_{\sim ATP}$ (representing the driving force available from ATP hydrolysis). Note that, at baseline, both KO and wt hearts generate similar amounts of work for a similar $\Delta G_{\sim ATP}$, while, under dobutamine stimulation, KO hearts have markedly blunted work output for a similar drop in $\Delta G_{\sim ATP}$. Note also that both KO and wt hearts are maximally stimulated by dobutamine to the theoretical limit for $\Delta G_{\sim ATP}$, i.e., the chemical driving force needed to drive Ca^{2+} into the sarcoplasmic reticulum by the SERCA channel (indicated with an arrow). Error bars are ±SEM.

and 5B and Figure S2A). Interestingly, inotropic and chronotropic stimulation of the hearts by dobutamine infusion resulted in PCr depletion down to similar concentrations in both wt and KO hearts (Figure 5B and Figure S2A).

In order to perform mechanical work, the heart requires a sufficiently elevated chemical driving force ($\Delta G_{\sim ATP}$), calculated from the ratio of concentrations of ATP to ADP and P_i, the so-called phosphorylation potential ([ATP]/[ADP]*[Pi]). This ratio reflects the free energy released from ATP hydrolysis, which can then be used to overcome a coupled and unfavorable reaction. Without a sufficiently large phosphorylation potential, ATP-dependent reactions such as the actomyosin ATPase reaction and Ca^{2+} sequestration cannot occur. Thus, the heart can only increase work output until $\Delta G_{\sim ATP}$ approaches the level needed by the most unfavorable coupled reaction. In wild-type hearts, it has been estimated that this threshold potential is that needed to drive the sarcoplasmic reticulum Ca++ ATPase (SERCA), at a $\Delta G_{\sim ATP}$ of approximately 53 kJ/mol (Ingwall, 2002). As seen in Figure 5D, that threshold is reached in PGC-1α KO hearts with much less work output than in wt hearts. Thus, for a similar change in $\Delta G_{\sim ATP}$ (equivalent to about 5 kJ/mol), hearts from PGC-1α KO mice generate much less work than do wt hearts.

Cardiac dysfunction in PGC-1α KO mice in vivo

To determine if the multiple defects described above result in cardiac insufficiency in vivo, echocardiography was performed. At 3 months of age, no differences between wt and KO mice were apparent (Figure S2B). Cardiac dysfunction, however, often becomes apparent only with age, both in mice and humans. Older mice were therefore also examined. As shown in Figure 6A, by 7–8 months of age, PGC-1α KO mice displayed clear features of cardiac dysfunction in vivo. Left ventricular chamber size in KO mice was slightly more dilated, both at end

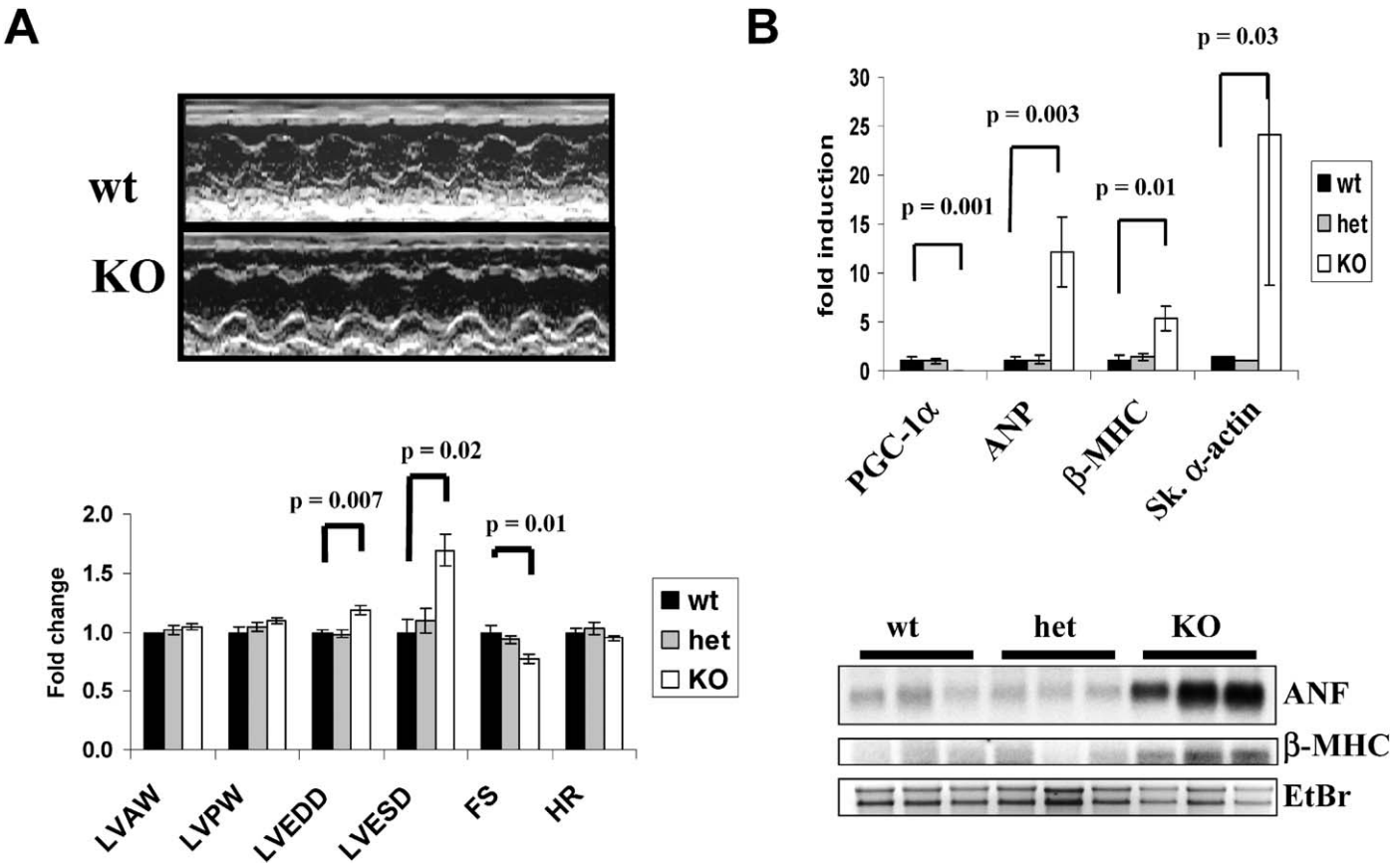

Figure 6. Cardiac dysfunction in PGC-1α KO mice in vivo

A) Cardiac echocardiograms were performed on 7- to 8-month-old wt (n = 4), heterozygote (n = 3), and KO (n = 3) female mice. The top panel shows representative M mode echocardiograms (in which a one-dimensional signal across the center of a transverse section of the heart is visualized over time) of wt and KO mice. The bottom panel shows in bar graph format the normalized left ventricular anterior wall (LVAW) and posterior wall (LVPW) thicknesses, end diastolic (LVEDD) and end systolic (LVESD) diameters, and fractional shortening (FS, calculated as LVEDD – LVESD/LVEDD). Error bars are ±SEM.

B) mRNA expression, in the same hearts as in (**A**), of the indicated genes as assayed by real-time quantitative PCR (top panel) and Northern blotting (bottom panel). EtBr, ethidium bromide stain of gel prior to transfer. Error bars are ±SEM.

diastole and end systole (Figure 6A). Most notably, KO mice showed significantly reduced fractional shortening (the difference in internal diameter between the fully dilated and fully contracted state, Figure 6A), demonstrating poor cardiac contraction in vivo in the absence of PGC-1α.

The 7- to 8-month-old KO mice also displayed molecular evidence of cardiac stress. Hearts faced with prolonged duress often undergo partial genetic reprogramming, with the induction of a number of so-called "fetal genes," such as β-MHC and skeletal α-actin (Friddle et al., 2000; Olson and Schneider, 2003). In addition, cardiac myocytes respond to stretch by inducing the genes for and secretion of atrial and brain natriuretic peptides (ANP and BNP), with the goal of reducing intravascular volume by stimulating diuresis (Cameron and Ellmers, 2003). As shown in Figure 6B, all these genes are also strongly induced in hearts of 7- to 8-month-old PGC-1α KO mice. Together, these data show that, by this later age, the defects resulting from the absence of PGC-1α lead to frank cardiac dysfunction in vivo.

Interestingly, hearts from 3-month-old mice also revealed similar molecular signs of cardiac stress, although less pronounced (Table 1 and Figure S2C). In fact, circulating levels of pro-ANP hormone were induced 2-fold in 3-month-old KO mice (Figure S2D), consistent with the induction of the ANP gene in the hearts. Hence, even at 3 months of age, PGC-1α KO hearts show clear signs of cardiac stress in vivo, consistent with the significant energetic and contractile defects observed in isolated heart preparations.

Discussion

The heart must maintain an unfailing blood circulation in states of both rest and high demand, such as that triggered by exercise. A trained athlete, for example, can rapidly increase cardiac output as much as 6-fold (Baim and Grossman, 2000). The amount of work done by the heart, therefore, must be efficient, dependable, and highly adaptable. Generating this work depends on a copious and equally adaptable supply of ATP. In a given day, the heart can consume approximately ten times its own weight in ATP (Ingwall, 2003; Ingwall and Weiss, 2004). The heart therefore needs an abundant and *ongoing* generation of ATP that can adapt to changing external or internal demands. The bulk of this occurs through efficient oxidative phosphorylation in mitochondria.

Recent data have suggested an important role for PGC-1α in the control of mitochondrial metabolism of the heart, but this has been based only on gain-of-function studies. What has remained unclear to date is the requirement for endogenous PGC-1α in the function of the intact heart. In this study, we have addressed this question by studying hearts from mice lacking PGC-1α. Measurements of cardiac gene expression and echocardiographic indices of cardiac function in vivo were combined with functional analyses of isolated heart preparations. The use of these Langendorff preparations allowed us to isolate the hearts from their nutrient and hormonal milieu, which was crucial because mice lacking PGC-1α demonstrate a profound hyperactivity and an altered glucose/insulin axis (Lin et al., 2004). We show here that the absence of PGC-1α leads to blunted mitochondrial enzymatic activity and frankly decreased levels of ATP in the heart. In the face of this energetic defect, hearts from PGC-1α KO mice are unable to normally perform the work of contraction, and KO mice eventually develop cardiac dysfunction in vivo.

The contractile defects observed in isolated heart preparations became most pronounced when hearts were forced to accomplish more work, such as that needed for exercise or "fight or flight" responses. Such situations are typically triggered by catecholamine surges and can be modeled ex vivo in Langendorff preparations by stimulation with saturating amounts of dobutamine, a potent β-adrenergic agonist. When this was done, the synthesis of ATP in PGC-1α KO hearts was insufficient to meet increased demand, and the energetic limit was reached with far less physical work achieved than in wt hearts. These data indicate that functional PGC-1α is critical for normal ATP generation and cardiac work. Moreover, the data suggest that the primary function of PGC-1α may be to serve an adaptive role, equipping the heart with the enhanced ability to respond to sudden increases in demand. This is consistent with the observation that PGC-1α expression in cardiac muscle is low before birth, when such responses are unnecessary, and then induced during the first few weeks of life (Lehman et al., 2000; Lehman and Kelly, 2002b).

It is important to note that hearts devoid of PGC-1α have blunted responses of both contraction and heart rate in response to dobutamine challenge. In fact, cardiac force is typically higher in the context of lower heart rates (Katz, 2001), so the force-generating defect in KO mice may be underestimated. Altered β-adrenergic signaling could be an obvious cause for these blunted responses, but this is unlikely for several reasons: first, dobutamine stimulation causes similar change in the chemical driving force for ATPase reaction, i.e., energy drain, in both KO and wt hearts; second, the functional defects of KO hearts are also exacerbated by pacing at elevated heart rates; third, expression of genes of β-adrenergic signaling such as the β receptors or βARK are not altered in KO mice (Figure S2E). Why the response of heart rate is blunted in PGC-1α KO hearts is unclear. Much of the regulation of heart rate in vivo is neurohormonal (Katz, 2001), but, in Langendorff preparations, the hearts are no longer under this control. Hence, PGC-1α appears to be required for maximal automaticity of pacemaker cells. This may occur due to bioenergetic insufficiency, but it is also possible that the absence of PGC-1α leads to defects in heart rate (and/or contraction) through mechanisms not involving the mitochondria. Further experimentation on this point will be needed.

That the cardiac defects observed in the Langendorff preparations have physiological relevance in vivo is indicated by the molecular changes observed in 3-month-old hearts, including induction of ANP, β-MHC, and skeletal α-actin (Table 1). Moreover, mice lacking PGC-1α go on, by 8 months of age, to develop enlargement of left ventricular chamber size and diminished contractile function (Figure 6). At the same time, no evidence of cardiac hypertrophy is seen (Figures 1 and 6, Figure S1A), suggesting that this phenotype is more akin to that of primary dilated cardiomyopathy than to dilation secondary to hypertrophic cardiomyopathy. It is likely that the phenotype will deteriorate with age. It will also be interesting to determine if the lack of PGC-1α will impair cardiac adaptation to specific long-term stressors, such as exercise or surgical constriction of the aorta. Unfortunately, PGC-1α KO mice have other phenotypes, including hyperactivity and an aberrant insulin/glucose axis (Lin et al., 2004), making it difficult to evaluate in a well-controlled fashion the cardiac response to exercise in whole mice. A critical evaluation of the role of PGC-1α in cardiac function in intact animals will thus require the cardiac-specific knockout of PGC-1α.

Physical activity induces PGC-1α expression in skeletal muscle tissue (Baar et al., 2002; Terada et al., 2002; Wu et al., 2002), and PGC-1α induces muscle fiber type switching to more oxidative fibers when expressed in transgenic mice (Lin et al., 2002b). On this basis, one might have expected a decrease of such fibers in PGC-1α KO mice. We show here, however, that in the absence of PGC-1α there is no obvious decrease in oxidative fibers in skeletal muscle (Figure 2). In fact, in quadriceps muscle, which is primarily glycolytic in nature, there is a 2- to 3-fold induction of genes specific to oxidative fibers (Figure 3B), although no frank increase in oxidative fibers was noted in primarily glycolytic muscle such as extensor digitorus longus (data not shown). This increase is not observed in isolated skeletal muscle cells from KO versus wt mice, suggesting that the difference is not cell autonomous (Figure 3B). These observations are most consistent with the notion that the hyperactivity of KO mice causes an increased motor nerve input to skeletal muscle that is sufficient to activate the expression of genes characteristic of oxidative fibers. Importantly, these data also demonstrate that mitochondria and oxidative fibers can form without the presence of PGC-1α. Since PGC-1β can also stimulate mitochondrial biogenesis and respiration when expressed ectopically (St-Pierre et al., 2003), it is plausible that PGC-1β may be compensating for the absence of PGC-1α in both mitochondrial and oxidative fiber formation in vivo. It is also possible that PGC-1α plays a more prominent role in adaptive responses to physical exercise, rather than the basal profiles of mitochondria or fiber types. Clearly, an understanding of how the absence of PGC-1α affects muscle fiber type formation in mice with comparable levels of physical movement will require skeletal muscle-specific KO of PGC-1α.

The notion that energy "starvation" is a significant contributor to the pathogenesis of cardiac failure has long been entertained (Ingwall and Weiss, 2004). The work described here lends significant support to this hypothesis: the absence of PGC-1α causes a decrease in available ATP supply and leads to significant contractile defects. Indeed, significant drops in PGC-1α transcript levels have been observed in various rodent models of cardiac failure (Czubryt et al., 2003; Feingold et al., 2004; Garnier et al., 2003; Lehman and Kelly, 2002b; Sano et

al., 2004). The results presented here thus support the idea that stimulation of PGC-1α activity represents a potential new therapeutic modality. Indeed, ectopic expression of PGC-1α in skeletal muscle can improve indices of fatiguability (Lin et al., 2002b). While massive overexpression of PGC-1α in normal hearts leads to cardiomyopathy (Lehman et al., 2000; Russell et al., 2004), increasing the expression of active PGC-1α at more physiological levels might improve contractile function in failing hearts.

Experimental procedures

Animals

All animal experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee. Mice were maintained on a standard rodent chow diet with 12 hr light and dark cycles. Frozen heart samples from 3-month-old mice 1 month after sustaining banding of their aorta were kindly provided by Dr. Richard Lee (Weinberg et al., 2003).

Cells and reagents

C2C12 cells were from ATCC and were grown in DMEM 10% FBS and differentiated for 5 days in DMEM 4% HS prior to adenoviral infection. Primary skeletal muscle cells were harvested as published (Sabourin et al., 1999) and grown and differentiated as above. Left ventricular cardiomyocytes were isolated as described (Matsui et al., 2002; Nagata et al., 2000). Cell surface was determined using NIH image. Adenovirus-expressing PGC-1α has been published (Puigserver et al., 1998). Pro-ANP assays were performed using the kit from ALPCO Diagnostics following the included protocol. Antibodies against cytochrome c and ND4L were from Pharmingen and Santa Cruz, respectively.

Histological analyses

Tissues for regular histology were frozen in liquid nitrogen immediately upon mouse sacrifice. Cardiac H&E and Trichrome stains were performed by American HistoLabs. Tissues for fiber type evaluation were embedded in gum tragacanth and OCT freezing matrix and quickly frozen in isopentane cooled in liquid nitrogen. Fiber typing was performed with cryostat sections using metachromatic dye-ATPase methods as described (Ogilvie and Feeback, 1990). Immunohistochemical staining was performed with a monoclonal antibody against skeletal slow myosin (NOQ7.5.4D, Sigma).

Genetic expression studies

Total RNAs were isolated from mouse tissue or cultured cells using the TRIzol method (Invitrogen). For microarray analyses, skeletal muscle and cardiac RNAs were evaluated with Affymetrix U74v2 and 430A chips, respectively, using the DFCI core facility. All subsequent analyses were performed with dCHIP software. For Northern analyses, 10 μg of RNA were separated on a formaldehyde gel, transferred to nylon membrane, and hybridized with probes amplified by PCR from genomic DNA. 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 (Cybergreen, Bio-Rad). DNA product of the expected size was confirmed for each primer pair.

Langendorff preparations

Mice were heparinized (100 units, IP) 15 min before being sacrificed. Hearts were isolated and perfused in the Langendorff mode as described (Chu et al., 1996). Hearts were perfused either with modified Krebs bicarbonate buffer containing pyruvate (0.5 mM) and glucose (10 mM) or with buffer containing physiological concentrations of mixed free fatty acid (0.4 mM) (palmitate, palmitoleic, linoleic, and oleic) carried in 1% BSA, glucose (5.5 mM), β-hydroxybutarate (0.19 mM), and lactate (1.0 mM). The composition of this mixture was chosen based on literature reports (Previs et al., 1999; Rofe et al., 1985) and direct measurements of substrate concentrations in the plasma of mice (data not shown). A water-filled balloon was then inserted into the left ventricle (LV) and inflated to LV end diastolic pressure of 10 mmHg. Isovolumic contractile performance data were collected online at a sampling rate of 200 Hz. Baseline measurements were made after a 20 min stabilization period (equivalent for all experiments and both genotypes). The hearts were then paced at 7.0 Hz using monophasic square wave pulses delivered from a stimulator (model S88; Grass Instrument Co., Quincy, MA) through salt bridge pacing wires consisting of PE-90 tubing filled with 4 M KCl in 2% agarose. The hearts were then challenged with 300 nM of dobutamine infused through a side tubing driven by a digital console driver (Cole-Parmer Instrument Co.) at 2% of coronary flow rate.

^{31}P NMR spectroscopy

^{31}P NMR spectra were obtained at 161.94 MHz using a GE-400 wide-bore spectrometer (Omega, General Electric, Fremont, CA). The isolated perfused hearts, in a 10 mm NMR sample tube, were inserted into a ^{1}H/^{31}P double-tuned probe situated in a 89 cm bore, 9.4 T superconducting magnet. Spectra were collected during 8 min periods and consisted of data averaged from 208 free induction decays as described (Chu et al., 1996; Spindler et al., 1998). ^{31}P NMR spectra were analyzed using 20 Hz exponential multiplication and zero and first-order phase corrections. The resonance areas corresponding to ATP, PCr, and Pi were fitted to Lorentzian functions and corrected for saturation (ATP [1.0], PCr [1.2], and Pi [1.15]). The changes in concentration of ATP, PCr, and Pi during the protocols were calculated as described (Spindler et al., 1998). The free energy released by ATP hydrolysis ($\Delta G_{\sim ATP}$) is calculated as $\Delta G_{\sim ATP} = \Delta G^{\circ} + RT\ ln$ ([ADP][Pi]/[ATP]).

Biochemical assays

Cardiac ventricular tissue was homogenized in potassium phosphate buffer containing 1 mM EDTA and 1 mM β-mercaptoethanol (pH 7.4). Aliquots were removed for assays of protein (Lowry et al., 1951) and total creatine content (Kammermeier, 1973). Triton X-100 was then added to the homogenate at a final concentration of 0.1%, and the activities of citrate synthase (Srere et al., 1963), cytochrome C oxidase (Stieglerova et al., 2000), and electron transport chain complex II (Makita and Sagara, 1990) were measured as described.

Echocardiographic analyses

Transthoracic echocardiography was performed using a 15 Mhz linear-array probe and a Sonos 4500 ultrasonograph (Hewlett Packard). The images were taken in 2K (left parasternal long and short axes) and M mode (left parasternal short axis). All echocardiograms were performed blinded to the mouse genotype. LV fractional shortening was calculated using the formula LVEDD – LVESD/LVEDD.

Statistical analysis

All results are expressed as means ±SEM. Two-tailed independent Student's t tests were used to determine all p values.

Supplemental data

Supplemental data include two figures and one table and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/1/4/259/DC1/.

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