

Enhanced lipid-but not carbohydrate-supported mitochondrial respiration in skeletal muscle of PGC-1 alpha overexpressing mice

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Enhanced Lipid—But Not Carbohydrate—Supported Mitochondrial Respiration in Skeletal Muscle of PGC-1 α Overexpressing Mice

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Skeletal muscle mitochondrial dysfunction has been linked to several disease states as well as the process of aging. A possible factor involved is the peroxisome proliferator-activated receptor (PPAR) γ co-activator 1 α (PGC-1 α), a major player in the regulation of skeletal muscle mitochondrial metabolism. However, it is currently unknown whether PGC-1 α , besides stimulating mitochondrial proliferation, also affects the functional capacity per mitochondrion. Therefore, we here tested whether PGC-1 α overexpression, besides increasing mitochondrial content, also leads to intrinsic mitochondrial adaptations. Skeletal muscle mitochondria from 10 male, muscle-specific PGC-1 α overexpressing mice (PGC-1 α Tg) and 8 wild-type (WT) mice were isolated. Equal mitochondrial quantities were then analyzed for their oxidative capacity by high-resolution respirometry, fuelled by a carbohydrate-derived (pyruvate) and a lipid (palmitoyl-CoA plus carnitine) substrate. Additionally, mitochondria were tested for reactive oxygen species (superoxide) production and fatty acid (FA)-induced uncoupling. PGC-1 α Tg mitochondria were characterized by an improved intrinsic mitochondrial fat oxidative capacity as evidenced by pronounced increase in ADP-stimulated respiration ($P < 0.001$) and maximal uncoupled respiration ($P < 0.001$) upon palmitoyl-CoA plus carnitine. Interestingly, intrinsic mitochondrial capacity on a carbohydrate-derived substrate tended to be reduced. Furthermore, the sensitivity to FA-induced uncoupling was diminished in PGC-1 α Tg mitochondria ($P = 0.02$) and this was accompanied by a blunted reduction in mitochondrial ROS production upon FAs in PGC-1 α Tg versus WT mitochondria ($P = 0.04$). Uncoupling protein 3 (UCP3) levels were markedly reduced in PGC-1 α Tg mitochondria ($P < 0.001$). Taken together, in addition to stimulating mitochondrial proliferation in skeletal muscle, we show here that overexpression of PGC-1 α leads to intrinsic mitochondrial adaptations that seem restricted to fat metabolism.

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Peroxisome proliferator-activated receptor (PPAR) γ co-activator 1 α (PGC-1 α) is a major player in the regulation of mitochondrial metabolism in oxidative tissues such as skeletal muscle. This transcriptional co-factor controls a broad range of oxidative genes, thereby possessing the ability to coordinate the enhanced mitochondrial biogenesis seen in skeletal muscle in response to, for example, exercise training. Indeed, it was shown that PGC-1 α protein levels were increased upon contractile activity, both in rat skeletal muscle *in vivo* and in skeletal muscle cells in culture (Irrcher et al., 2003). Additionally, several transgenic animal models revealed the importance of PGC-1 α in the regulation of mitochondrial metabolism. Thus, skeletal muscle tissue from PGC-1 α knockout mice is characterized by reduced levels of mitochondrial respiratory chain proteins, a lower mitochondrial yield, and a diminished mitochondrial respiration in permeabilized muscle fibers (Leone et al., 2005). In contrast, muscle-specific overexpression of PGC-1 α resulted in a profound increase the expression of oxidative genes and a very apparent fiber type shift towards more oxidative type I muscle fibers (Lin et al., 2002).

Although the role of PGC-1 α in regulating mitochondrial metabolism is clear-cut, it is currently unknown whether induction of PGC-1 α simply enhances the proliferation of

skeletal muscle mitochondria or whether elevated PGC-1 α levels also lead to functionally distinctive mitochondria. In other words, does PGC-1 α also regulate the intrinsic mitochondrial capacity, that is, the capacity per mitochondrion? This is a relevant question, given the fact that diseases like type 2

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diabetes mellitus, chronic obstructive pulmonary disease (COPD) but also the aging process are characterized by mitochondrial dysfunction in skeletal muscle tissue (Mogensen et al., 2007; Figueiredo et al., 2008; Phielix et al., 2008; Naimi et al., 2011). Furthermore, this mitochondrial dysfunction is associated with a reduced expression of PGC-1 α (Mootha et al., 2003; Patti et al., 2003; Remels et al., 2007; Anderson and Prolla, 2009) and successful therapeutic strategies include exercise training and/or an active lifestyle (Meex et al., 2010; Safdar et al., 2011), known to positively affect PGC-1 α levels.

In this context, the first aim of the present study was to perform a detailed characterization of isolated skeletal muscle mitochondria from PGC-1 α overexpressing mice. To this purpose, we used high-resolution respirometry to determine maximal ADP-stimulated (state 3) respiration, oligomycin-insensitive (state 4) respiration (reflecting mitochondrial proton leak), and maximally uncoupled (state uncoupled) respiration (reflecting the maximal capacity of the electron transport chain), upon both a lipid- and a carbohydrate-derived substrate.

Furthermore, since the mitochondrial dysfunction that was observed in type 2 diabetes, COPD and aging is possibly caused by elevated oxidative stress (Houstis et al., 2006; Bonnard et al., 2008; Figueiredo et al., 2008; Puente-Maestu et al., 2009) and since overexpression of PGC-1 α has been shown to alleviate oxidative stress (Kong et al., 2010), the secondary aim of the present study was to determine mitochondrial-derived reactive oxygen species (ROS) production in PGC-1 α overexpressing mice by electron spin resonance (ESR) spectroscopy.

Our results indicate that PGC-1 α , next to the positive effects on mitochondrial density, also regulates intrinsic mitochondrial adaptations that seem restricted to fat metabolism.

Materials and Methods

Animals

Ten male, muscle-specific PGC-1 α overexpressing mice (PGC-1 α Tg), generated as described previously (Lin et al., 2002) were used in the present study. Eight male C57Bl/6 mice (Charles River, Maastricht, The Netherlands) were used as controls (WT). The mice were housed on a 12:12 h light–dark cycle (light from 7:00 a.m. to 7:00 p.m.), at 21–22°C and had free access to standard chow diet (ssniff[®] r/m-h 10 mm, Bio Services, Uden, The Netherlands) and tap water. All experiments were approved by the Animal Care and Use Committee of the Maastricht University and complied with the principles of laboratory animal care.

Tissue collection and mitochondrial isolation

Mice were sedated by a mixture of 79% CO₂ and 21% O₂ and sacrificed by decapitation. Skeletal muscle tissue of both hind limbs was rapidly dissected and placed into ice-cold mitochondrial isolation buffer (~15 ml) containing 100 mM sucrose, 50 mM KCl, 20 mM K⁺-TES, 1 mM EDTA and 0.2% (w/v) fatty acid (FA)-free BSA. The muscle tissue was freed of white adipose and connective tissue, weighed and used for isolation of skeletal muscle mitochondria as described previously (Shabalina et al., 2010), with slight modifications. Thus, tissues were finely minced with pre-cooled scissors and homogenized in a potter homogenizer with a teflon pestle in the presence of ~8 units/g of tissue proteinase Subtilisin (Sigma–Aldrich, St. Louis, MO). Tissue homogenates were centrifuged at 8,500g for 10 min at 4°C using a Beckman J2-MC centrifuge and the resulting pellets were resuspended and subsequently centrifuged at 800g for 10 min. Then, the supernatants were centrifuged at 8,500g for 10 min after which the final mitochondrial pellets were resuspended by hand-homogenization in a small glass homogenizer in isolation medium. Mitochondrial protein concentration was measured using fluorescamine (Fluram[®], Fluka, Zwijndrecht, The Netherlands)

with BSA as a standard (Udenfriend et al., 1972) and the remaining mitochondria were stored at –80°C in the presence of a proteinase inhibitor cocktail (Complete Mini, Roche) for additional analyses (see below).

Oxygen consumption in isolated mitochondria

Mitochondrial respiratory rates of freshly isolated mitochondria were measured at 37°C by polarographic oxygen sensors in a two-chamber Oxygraph (OROBOROS[®] Instruments, Innsbruck, Austria). Mitochondrial function was characterized using a carbohydrate-derived substrate (5 mM pyruvate) and a FA-derived substrate (50 μ M palmitoyl-CoA plus 2 mM carnitine). For this purpose, mitochondria (0.2 mg for pyruvate and 0.5 mg for palmitoyl-CoA plus carnitine) were incubated in a medium consisting of 100 mM sucrose, 20 mM K⁺-Tes (pH 7.2), 50 mM KCl, 2 mM MgCl₂, 1 mM EDTA, 4 mM KH₂PO₄, 3 mM malate and 0.1% of FA-free BSA. Maximal coupled (state 3) respiration was initiated by addition of 450 μ M of ADP. State 4 respiration was measured as the residual respiration following addition of 1 μ g/ml oligomycin. Maximal oxygen flux rates (state uncoupled) were obtained by titration of the chemical uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) in a concentration of 0.75–2.0 μ M, dependent on the amount necessary to induce maximal mitochondrial respiration.

Fatty acid (FA)-induced respiration uncoupled from ATP synthesis was determined as a marker for FA-induced uncoupling. Thus, increasing amounts of palmitate were automatically titrated (OROBOROS[®] Instruments, Innsbruck, Austria) to 0.4 mg mitochondria in the presence of 5 mM pyruvate as a substrate and oligomycin (1 μ g/ml) to block ATP synthesis (state 4o respiration) while respiration was monitored. The free concentrations of FAs were calculated using the equation described in (Richieri et al., 1999) for the binding of FA to BSA at 37°C. Data for FA concentration-response curves were analyzed with the four parameter logistic curve fit option of the Sigmaplot 8.0 application.

All substrates were dissolved in double distilled water except palmitate and FCCP/oligomycin, which were dissolved in 50% and 96% ethanol, respectively.

Electron spin resonance spectroscopy (ESR)

ESR spectroscopy was performed principally as described before (Hoeks et al., 2008), to analyze mitochondrial superoxide anion radical production in freshly isolated mitochondria under state 4 conditions. Briefly, mitochondria were diluted in the same medium as for the respiration measurements (0.1 mg/ml) and incubated for 5 min at 37°C. Immediately after incubation, 100 mM activated charcoal purified 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) (Sigma–Aldrich) and substrate (5 mM pyruvate) was added. To study the effects of FA-induced uncoupling on mitochondrial superoxide production, mitochondria were also analyzed in the presence of 70 μ M palmitate (before correction for binding to BSA) with pyruvate as a substrate.

Superoxide anion radical derived DMPO-OH[•] signals, previously shown to be completely inhibited by superoxide dismutase (SOD) (Nabben et al., 2008), were measured on a Bruker EMX 1273. Quantification of the spectra was performed by peak height measurements using the WIN-EPR spectrum program (Bruker, Rheinstetten, Germany).

Western blot analyses

Western blotting was performed with equal amounts of mitochondrial protein. Blots were blocked with Licor-blockingbuffer (Westburg, Leusden, The Netherlands) and incubated overnight (RT) with the primary antibody against UCP3 (Hoeks et al., 2003) and adenine nucleotide translocase 1 (ANT1) (MitoSciences, Eugene, OR, USA). After incubation with the appropriate secondary antibodies, specific protein bands were

analyzed in arbitrary units (AU) with the Odyssey Near Infrared Imager (Licor, Westburg, Leusden, The Netherlands).

Citrate synthase and β -hydroxyacyl CoA dehydrogenase activity

Equal amounts of mitochondrial protein were analyzed spectrophotometrically for citrate synthase (CS) and β -hydroxyacyl dehydrogenase (β -HAD) activity as previously described (Shephard and Garland, 1969; Bergmeyer et al., 1974).

Statistical analysis

Results are presented as mean \pm SEM. Statistical analyses were performed with SPSS 16.0 for Mac software (SPSS Inc., Chicago, IL). Differences between groups were determined with two-sided unpaired Student's *t*-tests. Outcomes were considered statistically significant if $P < 0.05$.

Results

Mitochondrial yield

The wet weights of the combined skeletal muscles excised from both hind limbs were similar between genotypes (1.67 ± 0.21 g vs. 1.77 ± 0.14 g in WT vs. PGC-1 α Tg, respectively, $P = 0.94$). As anticipated, the mitochondrial yield (Fig. 1A) from skeletal muscle tissue was ~ 4.5 -fold higher in PGC-1 α Tg mice, as compared to WT (8.9 ± 1.6 mg vs. 40.8 ± 3.4 mg mitochondrial protein/g muscle tissue, $P < 0.001$).

Intrinsic mitochondrial respiration

Mitochondrial oxygen consumption rates in isolated mitochondria on the carbohydrate-derived substrate pyruvate and the lipid-derived substrate palmitoyl-CoA (in the presence of carnitine) are displayed in Figure 1B,C, respectively.

Interestingly, ADP-stimulated respiration fuelled by pyruvate tended to be lower in PGC-1 α Tg mice (Fig. 1B,D, $P = 0.08$) whereas oligomycin-insensitive (state 4o) respiration, reflecting mitochondrial proton leak, was significantly higher in PGC-1 α Tg mice as compared to the WT control group (13.7 ± 0.5 nmol O₂/min/mg vs. 17.4 ± 0.8 nmol O₂/min/mg in WT vs. PGC-1 α Tg, respectively, $P = 0.002$). Maximal uncoupled respiration upon pyruvate, reflecting the maximal capacity of the electron transport chain on the given substrate, was unaffected in PGC-1 α Tg mice (382.4 ± 17.1 nmol O₂/min/mg vs. 396.5 ± 18.7 nmol O₂/min/mg in WT vs. PGC-1 α Tg, respectively, $P = 0.60$).

In contrast, maximal ADP-stimulated respiration fuelled by the lipid substrate carnitine plus palmitoyl-CoA was enhanced considerably in PGC-1 α Tg mice as compared to WT mice (Fig. 1C,E, $P < 0.001$). Also state 4o respiration (13.2 ± 0.4 nmol O₂/min/mg vs. 20.7 ± 1.1 nmol O₂/min/mg in WT vs. PGC-1 α Tg, respectively, $P < 0.001$) and maximal uncoupled respiration (103.8 ± 3.6 nmol O₂/min/mg vs. 159.4 ± 6.2 nmol O₂/min/mg in WT vs. PGC-1 α Tg, respectively, $P < 0.001$) on the lipid substrate were significantly higher in PGC-1 α Tg mice.

FA-induced uncoupling

As anticipated, FA titration stimulated mitochondrial respiration that is not coupled to ATP production (i.e., FA-induced uncoupling) in both genotypes in a dose-dependent manner (Fig. 2A,B). However, mitochondria from PGC-1 α Tg mice displayed a reduced sensitivity for the uncoupling effects of FAs, as evidenced by a significantly elevated EC₅₀ value ($P = 0.02$, Fig. 2C), that is, a higher FA concentration is needed in order to induce 50% of maximal uncoupling. At the same time, PGC-1 α Tg mitochondria tended to reach a higher level of maximal uncoupling, indicated by an increased V_{\max} value ($P = 0.07$, Fig. 2C).

Mitochondrial superoxide production

A representative example of a superoxide anion radical derived DMPO-OH^{*} spectrum is shown in Figure 3A. Mitochondrial superoxide production under state 4 conditions was similar in PGC-1 α Tg versus WT mice (Fig. 3C, $P = 0.45$). Incubation with palmitate, leading to FA-induced uncoupling, significantly decreased mitochondrial superoxide production in WT mice ($P < 0.001$, Fig. 3B). Although incubation with FA also reduced superoxide production in PGC-1 α Tg mitochondria, this decrease was significantly less pronounced (Fig. 3C, $P = 0.04$).

Mitochondrial proteins and enzyme activities

Mitochondrial UCP3 levels were markedly reduced in PGC-1 α Tg mice and averaged only $\sim 29\%$ of the levels observed in WT mice ($P < 0.001$, Fig. 4A). In contrast, ANT1 was significantly elevated in mitochondria from PGC-1 α Tg mice as compared to WT mitochondria ($P = 0.02$, Fig. 4B).

We also determined CS and β -HAD activity per mitochondrion, that is, expressed per mg of mitochondrial protein (Fig. 5). This analysis revealed that both CS ($P < 0.001$, Fig. 5A) and β -HAD ($P = 0.01$, Fig. 5B) activity were significantly enhanced in isolated mitochondria from PGC-1 α Tg mice, by ~ 1.6 - and ~ 1.5 -fold, respectively.

Total ATP production capacity

The results on oxygen consumption rates in isolated skeletal muscle mitochondria presented above, reflect the intrinsic mitochondrial capacity (i.e., per mitochondrion) fuelled by pyruvate and palmitoyl-CoA + carnitine and were expressed per mg mitochondrial protein. To extrapolate these findings to whole muscle, we estimated the total ATP production capacity per gram of muscle (Table 1) by multiplying the mitochondrial yield by the ATP synthesis rate. This analysis revealed that the total ATP production rate is significantly elevated in PGC-1 α Tg mice as compared to WT mice, both on pyruvate (~ 4 -fold, $P < 0.001$) and on carnitine + palmitoyl-CoA (~ 8.5 -fold, $P < 0.001$).

Discussion

The transcriptional co-activator PGC-1 α is known to have profound effects on skeletal muscle mitochondrial metabolism and mitochondrial density in particular. It remains to be established however, whether PGC-1 α also regulates the intrinsic capacity of skeletal muscle mitochondria, that is, the functional capacity per mitochondrion. In this context, we here extensively characterized isolated mitochondria from PGC-1 α overexpressing mice and found that induction of PGC-1 α , next to increasing mitochondrial mass, also leads to intrinsic mitochondrial adaptations that specifically relate to mitochondrial fat metabolism.

The present study shows that mitochondrial respiration in PGC-1 α Tg mice is drastically enhanced upon a FA substrate (palmitoyl-CoA + carnitine) but not upon the carbohydrate-derived substrate pyruvate. Thus, both ADP-stimulated respiration and maximal uncoupled respiration were significantly enhanced in PGC-1 α Tg mice upon palmitoyl-CoA + carnitine, indicating an enhanced capacity for fat oxidation, electron transport and of the phosphorylation system. These findings are in line with a recent study showing that mitochondrial respiration rates in PGC-1 α knockout mice were dramatically reduced when fuelled with palmitoyl-carnitine as a substrate (Zechner et al., 2010). However, whereas the pyruvate-supported mitochondrial respiration in the present study was similar between WT and PGC-1 α Tg mice, Zechner et al. (2010) observed substantial reductions in respiration rates in PGC-1 α knockout mice upon pyruvate. Apparently, PGC-1 α is required to render a normal capacity for

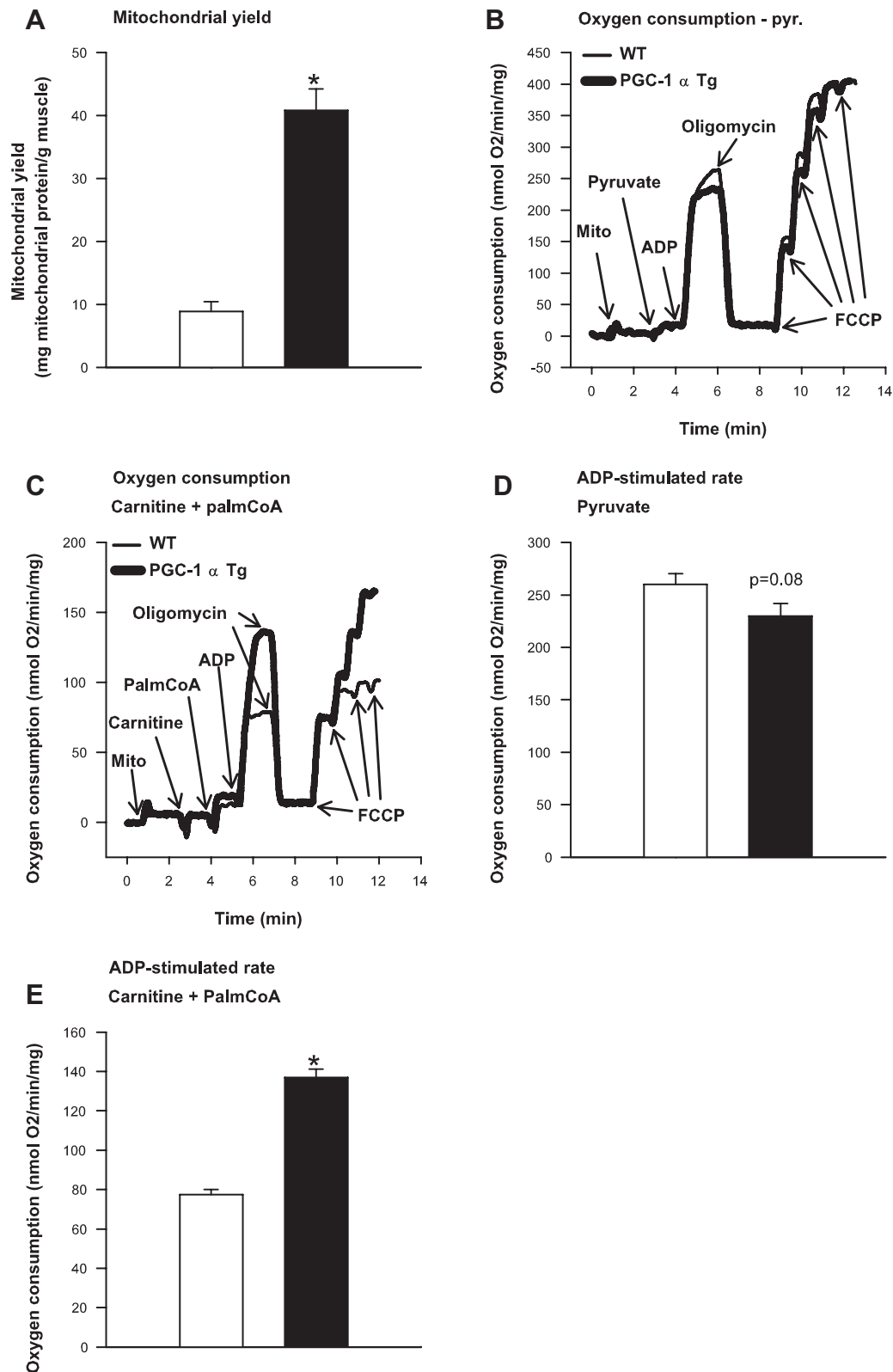


Fig. 1. **A:** Mitochondrial yield from combined skeletal muscle from both hind limbs of WT versus PGC-1 α Tg mice ($n = 7-8$). **B,C:** Representative recordings from oxygen consumption measurements of skeletal muscle mitochondria isolated from WT (thin lines) or PGC-1 α Tg (thick lines) mice, fuelled by either pyruvate (**B**) or carnitine + palmitoyl-CoA (**C**). **D,E:** Mitochondrial ADP-stimulated respiration upon (**D**) pyruvate and (**E**) carnitine + palmitoyl-CoA in WT versus PGC-1 α Tg mice ($n = 8-10$). WT mitochondria are depicted by the white bars while the black bars represent the PGC-1 α Tg mitochondria. The values represent the means \pm SEM. * $P < 0.05$.

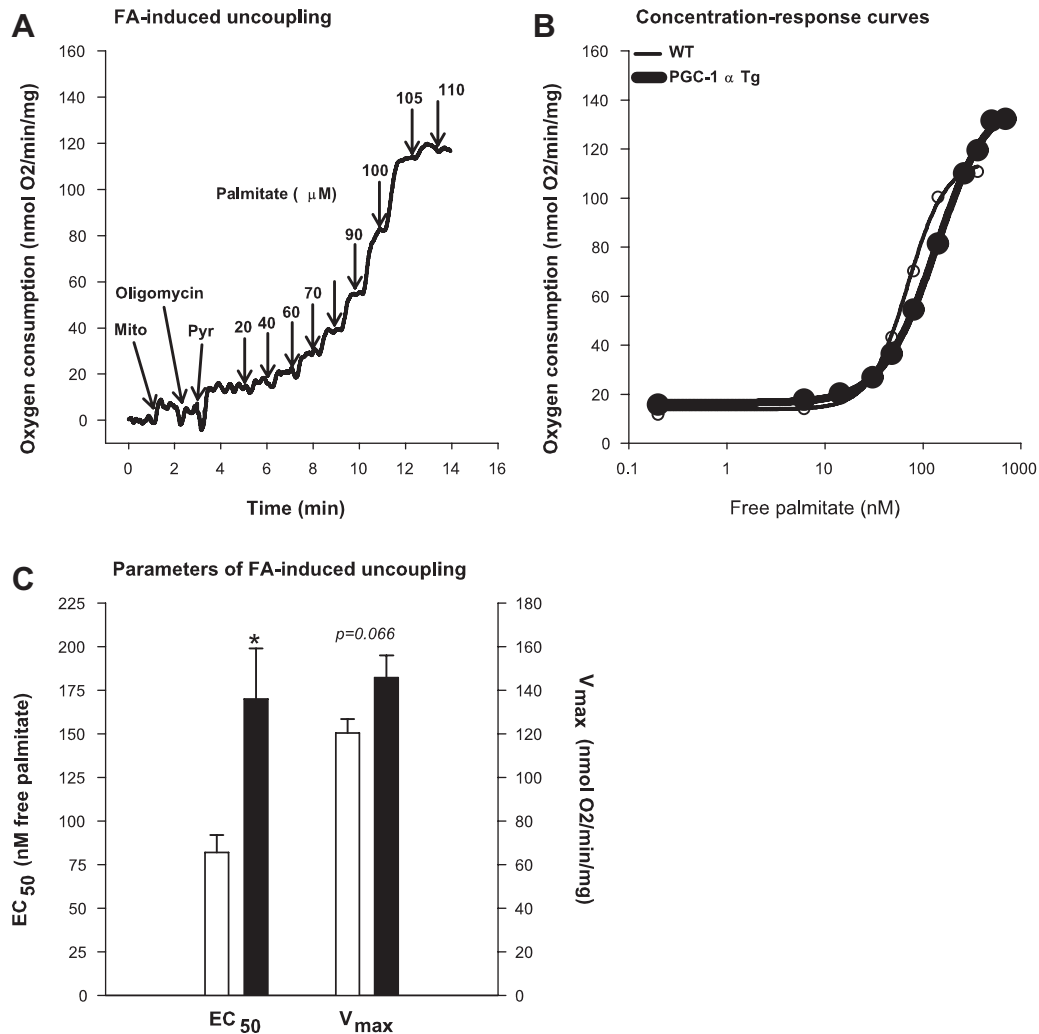


Fig. 2. **A:** Typical experiment assessing fatty acid-induced uncoupling, that is, monitoring oxygen consumption while titrating mitochondria with palmitate with pyruvate as a substrate and in the presence of oligomycin to block ATP synthesis. **B:** Palmitate concentration—oxygen consumption response curves in skeletal muscle mitochondria from WT versus PGC-1 α Tg mice ($n = 8-10$). The open symbols and thin line represent WT mitochondria, while filled symbols and thick line indicate mitochondria isolated from PGC-1 α Tg mice. **C:** Parameters of palmitate concentration-response curves of skeletal muscle mitochondria from WT versus PGC-1 α Tg mice ($n = 8-10$). PGC-1 α Tg mitochondria (black bars) show a decreased sensitivity for FA-induced uncoupling, evidenced by a significantly increased EC₅₀ value as compared to WT mitochondria (white bars). PGC-1 α Tg mitochondria also tolerate higher concentrations of fatty acids as reflected by higher absolute levels of maximal FA-induced uncoupling (V_{max}). The values represent the means \pm SEM. * $P < 0.05$.

pyruvate-supported mitochondrial respiration but overexpression of the co-activator does not further enhance the mitochondrial capacity upon this carbohydrate-derived substrate. This in contrast to the mitochondrial capacity for FA oxidation, which can be enhanced further by overexpressing PGC-1 α over basal levels.

In agreement with previous studies (Lin et al., 2002; Choi et al., 2008; Wenz et al., 2009) we also found that the mitochondrial yield from 1 g of combined muscle tissue is about 4.5-fold higher in PGC-1 α Tg mice, as compared to WT. Thus, although mitochondrial respiration upon the carbohydrate-derived substrate pyruvate tended to be reduced by PGC-1 α overexpression, PGC-1 α Tg mice display an enhanced capacity to produce ATP when expressed per gram of muscle tissue, both on carbohydrate and lipid substrates (Table 1). For carbohydrates, this is however fully accounted for by the increase in mitochondrial mass, whereas for lipids, the elevated ATP production capacity results from an increased intrinsic

mitochondrial capacity in combination with an augmented mitochondrial density.

Although the genetic overexpression of PGC-1 α is key in causing the abovementioned changes it is interesting to note that the subcellular localization of PGC-1 α may also be of importance for its regulatory role in mitochondrial metabolism. Thus, it was recently shown that PGC-1 α is also localized inside mitochondria possibly mediating the cross talk between the nuclear and the mitochondrial genome to promote mitochondrial biogenesis (Aquilano et al., 2010). In this context, it was also shown that an acute bout of endurance exercise in mice increased both the nuclear and the mitochondrial abundance of PGC-1 α (Safdar et al., 2011), indicating that relocation of PGC-1 α is of importance for the adaptive response to exercise.

In the present study, PGC-1 α Tg mice also showed an increased mitochondrial CS and β -HAD activity, indicative of an enhanced capacity of both the TCA cycle and β -oxidation per

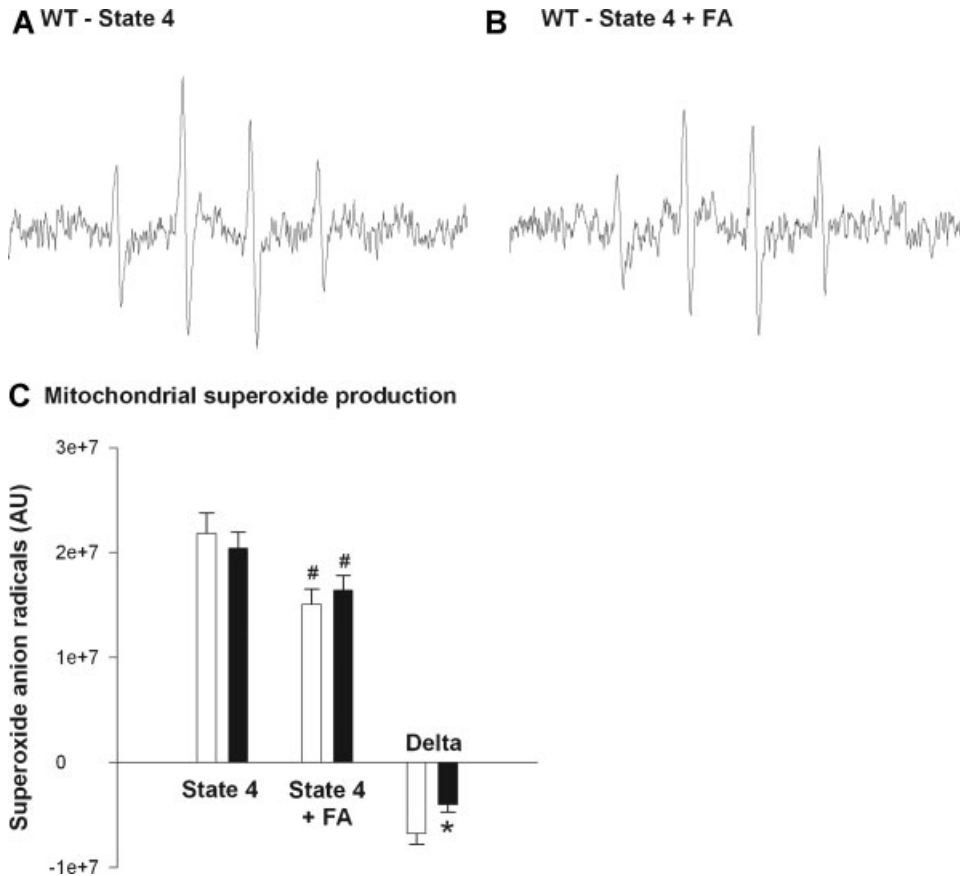


Fig. 3. **A:** Representative ESR spectrum of WT skeletal muscle mitochondria (0.1 mg/ml) at 37°C in the presence of 100 mM DMPO, reflecting mitochondrial superoxide anion radical production in WT mitochondria under state 4 conditions. **B:** Similar to **A** but in the presence of 70 μ M palmitate to stimulate FA-induced uncoupling. FA-induced uncoupling clearly diminishes mitochondrial superoxide anion radical production, directly visible as a reduction of the peak heights. **C:** Quantification of mitochondrial superoxide anion radical production under state 4 conditions in the absence and presence of palmitate for both WT (white bars) and PGC-1 α Tg (black bars) mice ($n = 8-9$). The decrease in superoxide anion radical production upon palmitate (“delta”) was calculated by subtracting the superoxide anion radical production under state 4 conditions in the presence of FA from the superoxide radical signal under (basal) state 4 conditions (i.e., in the absence of FA). The reduced sensitivity for FA-induced uncoupling (see Fig. 2) in PGC-1 α Tg mitochondria is reflected in a blunted reduction of mitochondrial superoxide anion radical production upon palmitate. The values represent the means \pm SEM. # $P < 0.05$ for state 4 + FA versus state 4, * $P < 0.05$ for WT versus PGC-1 α Tg.

mitochondrion. Interestingly however, UCP3 was profoundly reduced indicating that the overexpression of PGC-1 α does not merely enhance all proteins related to mitochondrial metabolism. UCP3 has been implicated in the regulation of mitochondrial ROS production via mild uncoupling, that is, a lowering of the mitochondrial proton gradient that is not coupled to ATP synthesis (Echtay et al., 2002; Echtay et al., 2003; Brand et al., 2004). However, despite clearly lowered UCP3 protein levels in PGC-1 α Tg mice, oligomycin-insensitive (state 4o) respiration, indicative for mitochondrial proton leak, was higher rather than lower in PGC-1 α Tg mice. Furthermore, we did not observe differences in mitochondrial superoxide production in PGC-1 α Tg versus WT mice under state 4 conditions. In other words, the net amount of mitochondrial superoxide that leaves the mitochondria under these conditions is similar between PGC-1 α Tg and WT mitochondria, despite a reported increase in mitochondrial superoxide dismutase 2 (SOD2) in PGC-1 α Tg mice (Wenz et al., 2009). It cannot be excluded however, that the intra-mitochondrial superoxide production is increased in PGC-1 α Tg mice, which is normalized by enhanced mitochondrial anti-oxidant defense mechanisms.

Although the reduced UCP3 protein content in PGC-1 α Tg mitochondria did not affect basal state 4 respiration and/or superoxide producing capacity, it has been suggested that UCP3 needs to be activated by FAs, in order to display its uncoupling and ROS-lowering properties (Zackova and Jezek, 2002; Esteves and Brand, 2005). Therefore, we tested the capacity for FA-induced uncoupling and indeed found that—consistent with lower UCP3 levels—this was reduced in mitochondria from PGC-1 α overexpressing mice, as evidenced by a significantly elevated EC_{50} value (Fig. 2B,C). Interestingly however, the maximal uncoupling capacity (V_{max}) that could be induced by FAs tended to be increased, rather than decreased, in PGC-1 α Tg mice as compared to WT animals (Fig. 2C). These changes were also accompanied by an elevated level of ANTI1, the mitochondrial ADP/ATP anti-porter that has also been implicated in FA-induced uncoupling (Skulachev, 1991).

Taken together, PGC-1 α Tg mitochondria are less sensitive to the uncoupling effects of FAs but can tolerate higher concentrations of FAs leading to higher absolute levels of maximal FA-induced uncoupling. Given the potential ROS-lowering effect of mitochondrial uncoupling we then tested whether the observed differences in FA-induced uncoupling

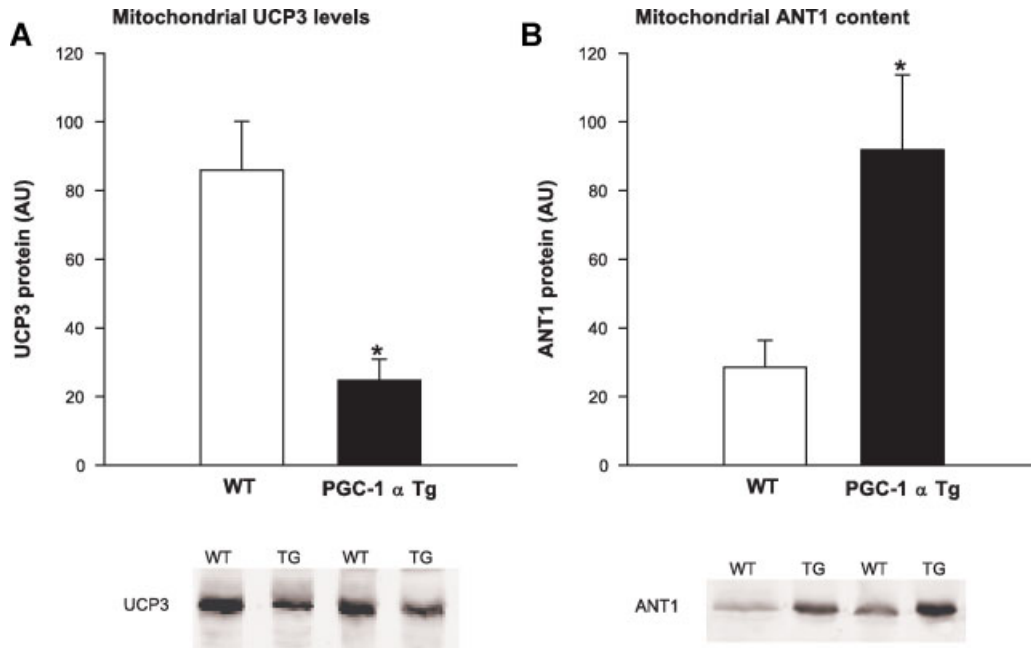


Fig. 4. Quantification of Western blot analyses for (A) mitochondrial uncoupling protein 3 (UCP3) and (B) adenine nucleotide translocator (ANT1) in skeletal muscle mitochondria, including representative immunoblots. WT mitochondria are depicted by the white bars while the black bars represent the PGC-1 α Tg mitochondria. Values are expressed as mean \pm SEM. * $P < 0.05$.

were reflected in differences in mitochondrial ROS production upon FA loading.

Indeed, our data show that FA-induced uncoupling significantly reduced mitochondrial superoxide production under state 4 conditions in both genotypes. Furthermore, the reduced capacity for FA-induced uncoupling in PGC-1 α Tg mice was also reflected in a significantly less pronounced reduction in mitochondrial superoxide production upon FAs under these conditions (Fig. 3C).

In summary, mitochondria isolated from PGC-1 α Tg mice clearly favor a FA- over a carbohydrate-derived substrate.

Additionally, CS (citric acid cycle) and β -HAD (β -oxidation) activity were both significantly elevated when expressed per mitochondrion, whereas skeletal muscle UCP3 content was lowered considerably in PGC-1 α Tg mice. Despite these changes in UCP3, basal mitochondrial uncoupling was however unchanged whereas the capacity for FA-induced uncoupled was diminished in PGC-1 α Tg mice. Our results show that PGC-1 α does not merely increase the formation of new skeletal mitochondria but clearly leads to intrinsic mitochondrial adaptations that seem restricted to FA metabolism.

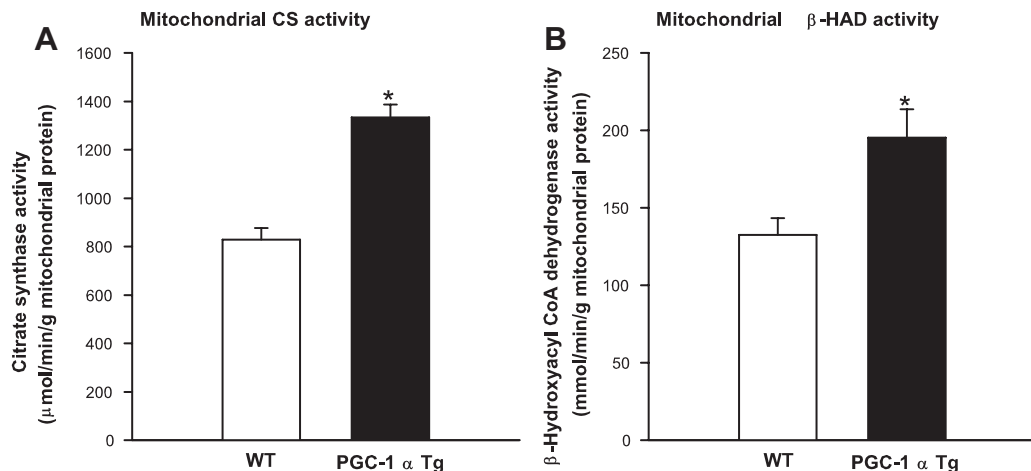


Fig. 5. A: Citrate synthase (CS) and (B) β -hydroxyacyl dehydrogenase (β -HAD) activity in isolated skeletal muscle mitochondria from WT (white bars) and PGC-1 α Tg (black bars) mice. Values are expressed as mean \pm SEM. * $P < 0.05$.

TABLE 1. Total mitochondrial ADP phosphorylation capacity in skeletal muscle

Genotype	Substrate	
	Pyruvate	Carnitine + Palmitoyl-CoA
WT	2,103 \pm 280	549 \pm 80
PGC-1 α Tg	8,417 \pm 562*	4,658 \pm 374*

Total ADP phosphorylation capacity was estimated as the respiratory response (in nmol O₂/min/mg mitochondrial protein) to ADP (state 3 respiration) minus oligomycin-insensitive oxygen consumption (state 4 respiration) and multiplied with the mitochondrial yield from the same animal. Values represent the means \pm SEM of seven to eight separate experiments. *P < 0.05.

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