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-1α 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 1α (PGC-1α), a major player in the regulation of skeletal muscle mitochondrial metabolism. However, it is currently unknown whether PGC-1α, besides stimulating mitochondrial proliferation, also affects the functional capacity per mitochondrion. Therefore, we here tested whether PGC-1α overexpression, besides increasing mitochondrial content, also leads to intrinsic mitochondrial adaptations. Skeletal muscle mitochondria from 10 male, muscle-specific PGC-1α overexpressing mice (PGC-1α 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-1α 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-1α Tg mitochondria (P = 0.02) and this was accompanied by a blunted reduction in mitochondrial ROS production upon FAs in PGC-1α Tg versus WT mitochondria (P = 0.04). Uncoupling protein 3 (UCP3) levels were markedly reduced in PGC-1α Tg mitochondria (P < 0.001). Taken together, in addition to stimulating mitochondrial proliferation in skeletal muscle, we show here that overexpression of PGC-1α leads to intrinsic mitochondrial adaptations that seem restricted to fat metabolism.


Peroxisome proliferator-activated receptor (PPAR) γ co-activator 1α (PGC-1α) 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-1α protein levels were increased upon contractile activity, both in rat skeletal muscle in vivo and in skeletal muscle cells in culture (Ircher et al., 2003). Additionally, several transgenic animal models revealed the importance of PGC-1α in the regulation of mitochondrial metabolism. Thus, skeletal muscle tissue from PGC-1α 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-1α 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-1α in regulating mitochondrial metabolism is clear-cut, it is currently unknown whether induction of PGC-1α simply enhances the proliferation of skeletal muscle mitochondria or whether elevated PGC-1α levels also lead to functionally distinctive mitochondria. In other words, does PGC-1α 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...
mitochondria from PGC-1α 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 (Houlistis et al., 2006; Bonnard et al., 2008; Figueiredo et al., 2008; Puente-Maestu et al., 2009) and successful therapeutic strategies include exercise training and/or an active lifestyle (Meex et al., 2010; Prolla, 2009) and successful therapeutic strategies include mitochondria-derived reactive oxygen species (ROS) production in PGC-1α overexpressing mice by electron spin resonance (ESR) spectroscopy. Our results indicate that PGC-1α, 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-1α overexpressing mice (PGC-1αTg), 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® m/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 anesthesia was induced with isoflurane (Fluram®, Fluka, Zwijndrecht, 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.

Western blot analyses

Western blotting was performed with equal amounts of mitochondrial protein. Blots were blocked with Licor-blocking buffer (Westburg, Leusden, The Netherlands) and incubated overnight (RT) with the primary antibody against UCP3 (Hoek et al., 2003) and adenine nucleotide translocase 1 (ANT1) (MitoSciences, Eugene, OR, USA). After incubation with the appropriate secondary antibodies, specific protein bands were detected with the Fluorchem system (FluorChem, FluorChem, 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 protease inhibitor cocktail (Complete Mini, Roche) for additional analyses (see below).

Electron spin resonance spectroscopy (ESR)

ESR spectroscopy was performed principally as described before (Hoek 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 (MitoSciences, Eugene, OR, USA) to 0.4 mg mitochondrial protein in the presence of 5 mM pyruvate as a substrate and oligomycin (1 μg/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 pyruvate and FCCP/oligomycin, which were dissolved in 50% and 96% ethanol, respectively.

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 μM 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 μM of ADP. State 4 respiration was measured as the residual respiration following addition of 1 μg/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 μM, 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 mitochondrial protein in the presence of 5 mM pyruvate as a substrate and oligomycin (1 μg/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.

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

**Mitochondrial superoxide production**

A representative example of a superoxide anion radical derived DMOPO-OH* spectrum is shown in Figure 3A. Mitochondrial superoxide production under state 4 conditions was similar in PGC-1αTg 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-1αTg 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-1αTg mice and averaged only ~29% of the levels observed in WT mice (P < 0.001, Fig. 4A). In contrast, ANT1 was significantly elevated in mitochondria from PGC-1αTg 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-1αTg 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-1αTg 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-1α is known to have profound effects on skeletal muscle mitochondrial metabolism and mitochondrial density in particular. It remains to be established however, whether PGC-1α 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-1α overexpressing mice and found that induction of PGC-1α, 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-1αTg 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-1αTg 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-1α 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-1αTg mice, Zechner et al. (2010) observed substantial reductions in respiration rates in PGC-1α knockout mice upon pyruvate. Apparently, PGC-1α 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-1α Tg mice (n = 7–8). B,C: Representative recordings from oxygen consumption measurements of skeletal muscle mitochondria isolated from WT (thin lines) or PGC-1α 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-1α Tg mice (n = 8–10). WT mitochondria are depicted by the white bars while the black bars represent the PGC-1α Tg mitochondria. The values represent the means ± 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-1α 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-1α Tg mice, as compared to WT. Thus, although mitochondrial respiration upon the carbohydrate-derived substrate pyruvate tended to be reduced by PGC-1α overexpression, PGC-1α 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-1α is key in causing the abovementioned changes it is interesting to note that the subcellular localization of PGC-1α may also be of importance for its regulatory role in mitochondrial metabolism. Thus, it was recently shown that PGC-1α 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-1α (Safdar et al., 2011), indicating that relocation of PGC-1α is of importance for the adaptive response to exercise.

In the present study, PGC-1α 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
mitochondrion. Interestingly however, UCP3 was profoundly reduced indicating that the overexpression of PGC-1α 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-1α Tg mice, oligomycin-insensitive (state 4o) respiration, indicative for mitochondrial proton leak, was higher rather than lower in PGC-1α Tg mice. Furthermore, we did not observe differences in mitochondrial superoxide production in PGC-1α 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-1α Tg and WT mitochondria, despite a reported increase in mitochondrial superoxide dismutase 2 (SOD2) in PGC-1α Tg mice (Wenz et al., 2009). It cannot be excluded however, that the intramitochondrial superoxide production is increased in PGC-1α Tg mice, which is normalized by enhanced mitochondrial anti-oxidant defense mechanisms.

Although the reduced UCP3 protein content in PGC-1α 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-1α Tg overexpressing mice, as evidenced by a significantly elevated EC50 value (Fig. 2B,C). Interestingly however, the maximal uncoupling capacity (Vmax) that could be induced by FAs tended to be increased, rather than decreased, in PGC-1α Tg mice as compared to WT animals (Fig. 2C). These changes were also accompanied by an elevated level of ANT1, the mitochondrial ADP/ATP anti-porter that has also been implicated in FA-induced uncoupling (Skulachev, 1991).

Taken together, PGC-1α 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
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-1α 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-1α 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-1α Tg mice. Despite these changes in UCP3, basal mitochondrial uncoupling was however unchanged whereas the capacity for FA-induced uncoupled was diminished in PGC-1α Tg mice. Our results show that PGC-1α does not merely increase the formation of new skeletal mitochondria but clearly leads to intrinsic mitochondrial adaptations that seem restricted to FA metabolism.

![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-1α Tg mitochondria. Values are expressed as mean ± SEM. *P < 0.05.](image)

![Fig. 5. A: Citrate synthase (CS) and (B) β-hydroxylacyl dehydrogenase (β-HAD) activity in isolated skeletal muscle mitochondria from WT (white bars) and PGC-1α Tg (black bars) mice. Values are expressed as mean ± SEM. *P < 0.05.](image)
TABLE 1. Total mitochondrial ADP phosphorylation capacity in skeletal muscle

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Substrate</th>
<th>WT</th>
<th>PGC-1αTg</th>
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<tr>
<td></td>
<td>Pyruvate</td>
<td>2.103 ± 0.280</td>
<td>8.417 ± 0.562</td>
</tr>
<tr>
<td></td>
<td>Carnitine + Palmitoyl-CoA</td>
<td>549 ± 80</td>
<td>4,658 ± 374</td>
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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 number of mitochondria in the same animal. Values represent the mean ± SEM of seven to eight separate experiments. *P < 0.05.

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Literature Cited