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Bioenergetic Analysis of Peroxisome Proliferator-activated Receptor γ Coactivators 1α and 1β (PGC-1α and PGC-1β) in Muscle Cells∗

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Peroxisome proliferator-activated receptor γ coactivator (PGC)-1α is a coactivator of nuclear receptors and other transcription factors that regulates several components of energy metabolism, particularly certain aspects of adaptive thermogenesis in brown fat and skeletal muscle, hepatic gluconeogenesis, and fiber type switching in skeletal muscle. PGC-1α has been shown to induce mitochondrial biogenesis when expressed in muscle cells, and preliminary analysis has suggested that this molecule may specifically increase the fraction of uncoupled versus coupled respiration. In this paper, we have performed detailed bioenergetic analyses of the function of PGC-1α and its homolog PGC-1β in muscle cells by monitoring simultaneously oxygen consumption and membrane potential. Cells expressing PGC-1α or PGC-1β display higher proton leak rates at any given membrane potential than control cells. However, cells expressing PGC-1α have a higher proportion of their mitochondrial respiration linked to proton leak than cells expressing PGC-1β. Although these two proteins cause a similar increase in the expression of many mitochondrial genes, PGC-1β preferentially induces certain genes involved in the removal of reactive oxygen species, recently recognized as activators of uncoupling proteins. Together, these data indicate that PGC-1α and PGC-1β profoundly alter mitochondrial metabolism and suggest that these proteins are likely to play different physiological functions.

Mitochondria play a central role in metabolism by coupling cellular respiration to the production of ATP. However, this coupling is not perfectly tight. Indeed, it is estimated that approximately 20% of the standard metabolic rate in mammals is due to a leak of protons across the mitochondrial inner membrane in a manner that uncouples cellular respiration from ATP production, thereby generating heat (1). This cycle is called basal proton leak. In addition to this basal leak, there is an inducible leak of protons catalyzed by uncoupling protein 1 (UCP1) in brown fat. Two close homologs of this protein have been discovered, UCP2 and UCP3 (2–6). Although the function of these homologs is not clear, recent work suggests that they might have an important role in the protection against reactive oxygen species (ROS) (7, 8) and the modulation of cellular ATP levels, especially in insulin-secreting β cells (9, 10). Interestingly, none of the genetic studies using either knockout mice or mice overexpressing moderate levels of UCP2 and UCP3 show a significant effect of these proteins in determining standard metabolic rate by uncoupling cellular respiration (11, 12).

Many changes in the cellular environment result in modulation of mitochondrial metabolism. Basal proton leak rate changes in response to hormonal status and metabolic depression (13–19). Also, small mammals with high standard metabolic rates have higher proton leak rates than large mammals with low standard metabolic rates (20, 21). Furthermore, proton leak rates differ between phylogenetic groups; it is higher in endotherms than in ectotherms (22). The fact that mitochondrial functions can be altered in response to environmental stimuli is due to the execution of a coordinated program of genes expression. PPARγ coactivator-1α (PGC-1α) has been shown to be a powerful regulator of multiple aspects of mitochondrial gene expression. Indeed, PGC-1α is cold-induced in brown fat and muscle where it plays a role in adaptive thermogenesis by regulating a complex program of increased mitochondrial biogenesis and uncoupled respiration via coactivation of peroxisome proliferator-activated receptors (PPARs), nuclear respiratory factor 1, and perhaps other transcription factors (23, 24). Furthermore, transgenic mice expressing physiological levels of PGC-1α protein in skeletal muscle display an increased content of oxidative type I fibers compared with their wild-type counterparts (25).

Homologs of PGC-1α have been discovered, namely PGC-1β (26, 27) and PGC-1-related coactivator (28). The expression of PGC-1β and PGC-1-related coactivator is not cold-inducible in brown fat and skeletal muscle, suggesting that they might have distinct roles from PGC-1α (26, 28). However, both PGC-1β and PGC-1-related coactivator coactivate nuclear respiratory factor 1, implying that they may also have a role in mitochondrial metabolism (26, 28).

To date, there are no bioenergetic mechanisms that might explain how PGC-1α increases uncoupled respiration, nor any
studies examining a role for its closest homolog PGC-1β in mitochondrial metabolism. In this report, we measure the respiration and proton leak kinetics of C2C12 muscle cells expressing either PGC-1α or PGC-1β. We report that PGC-1β, like PGC-1α, increases mitochondrial metabolism. Although both PGC-1α and PGC-1β increase proton leak, cells expressing PGC-1α have a higher proportion of their mitochondrial respiration linked to proton leak than those expressing PGC-1β.

EXPERIMENTAL PROCEDURES

Cell Culture—C2C12 myoblasts were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum at 37 °C, 5% CO2. The differentiation of myoblasts into myotubes took 3–5 days. The cells were then infected with adenoviruses expressing GFP, PGC-1α, or PGC-1β. The GFP and PGC-1α viruses were made using the adenovirus expression system (Clontech). For PGC-1β, a recombinant adenovirus containing the full-length cDNA encoding PGC-1β was prepared by subcloning of the cDNA into the pAdCMV-lacZ vector and cotransfection of the recombinant plasmid with plasmid pJM17 into 293 cells using previously described methods (29). Two days after infection of myotubes, the cells were harvested to determine mitochondrial membrane potential, respiration rate, mitochondrial volume density, and cristae surface density.

The cells were isolated by washing with phosphate-buffered saline and trypanosimizing for 5 min. Dulbecco’s modified Eagle’s medium with 2% horse serum. The differentiation into myotubes took 3–5 days medium with 2% horse serum. The differentiation into myotubes took 3–5 days medium with 2% horse serum. The differentiation into myotubes took 3–5 days medium with 2% horse serum. The differentiation into myotubes took 3–5 days medium with 2% horse serum.

Electron Microscopy—Electron microscopy was carried out as described in Ref. 32. Mitochondria were incubated (0.3 mg/ml in assay medium containing 120 mM KCl, 5 mM KH2PO4, 3 mM Hepes, and 1 mg EGTa (pH 7.2 at room temperature) in a chamber thermostatted at 37 °C using a recirculating water bath. The oxygen consumption rates, measured with a Clark-type electrode, and the mitochondrial membrane potential values, determined using a TPPM electrode, were recorded simultaneously. Rotenone (5 μm), oligomycin (1 μg/ml mitochondrial protein), and valinomycin (50 μm) were present at the beginning of each run. TPPM was added up to 1.3 μm for calibration. Mitochondria were fed succinate (4 mM), and the inhibitor malonate was gradually added up to 1.3 mM to inhibit mitochondrial respiration and membrane potential. Finally, p-trifluoro- methoxyphenylhydrazine (0.15 μM) was added to determine the drift of the TPPM electrode, if any. The oxygen solubility of the medium was considered 406 nmol of oxygen/ml (33), and the TPPM binding correction was considered 0.4 (μM of mitochondrial protein) (33).

Proton Leak Kinetics in Isolated Mitochondria—The proton leak kinetics in isolated mitochondria was determined as described in Ref. 30. Mitochondria were incubated (0.3 mg/ml) in assay medium containing 120 mM KCl, 5 mM KH2PO4, 3 mM Hepes, and 1 mg EGTa (pH 7.2 at room temperature) in a chamber thermostatted at 37 °C using a recirculating water bath. The oxygen consumption rates, measured with a Clark-type electrode, and the mitochondrial membrane potential values, determined using a TPPM electrode, were recorded simultaneously. Rotenone (5 μm), oligomycin (1 μg/ml mitochondrial protein), and valinomycin (50 μm) were present at the beginning of each run. TPPM was added up to 1.3 μm for calibration. Mitochondria were fed succinate (4 mM), and the inhibitor malonate was gradually added up to 1.3 mM to inhibit mitochondrial respiration and membrane potential. Finally, p-trifluoromethoxyphenylhydrazine (0.15 μM) was added to determine the drift of the TPPM electrode, if any. The oxygen solubility of the medium was considered 406 nmol of oxygen/ml (33), and the TPPM binding correction was considered 0.4 (μM of mitochondrial protein) (33).

Isolation of Mitochondria from Transgenic Mice—Wild-type and transgenic mice expressing GFP-1α from the muscle creatine kinase promoter in the muscle tissues (transgenic line 31) were housed on a 12D:12N photoperiod cycle and fed ad libitum (25). The mice were sacrificed, and their whole leg muscle mass was excised and minced with razor blades. The minced tissue was diluted 1/10 (w/v) in isolation buffer containing 100 mM KCl, 50 mM Tris-HCl, 2 mM EGTA, 0.5% bovine serum albumin, pH 7.4, at 4 °C and homogenized in a mortar with six passes using a medium tight pestle. The homogenate was centrifuged at 2000 × g at 4 °C for 5 min. The supernatant was collected and centrifuged at 10,000 × g for 10 min. The supernatant was discarded, and the pellet was resuspended in isolation medium and centrifuged again at 10,000 × g for 10 min. The supernatant was discarded, and the pellet was resuspended in 500 μl of isolation medium. The protein concentration of the mitochondrial suspensions was determined using the bicinchoninic acid kit with bovine serum albumin as a standard.

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Statistical Analyses—All of the statistical analyses were performed using Sigma Stat 2.0. Comparisons of the contribution of ATP turnover and proton leak to mitochondrial respiration between cells infected with GFP and PGC-1α or with GFP and PGC-1β were carried out with paired Student t test. Comparisons of mitochondrial volume density and cristae surface density values between GFP, PGC-1α-, and PGC-1β-expressing cells were done with one-way analysis of variance and the a posteriori Tukey test.

RESULTS

Proton Leak Kinetics of Muscle Cells Expressing PGC-1α or PGC-1β—To study the functions of PGC-1α and PGC-1β, these coactivators were expressed with adenoviral vectors in C2C12 muscle cells. These cells were used because skeletal muscle in vivo expresses both PGClα, but the cultured cells express much less of these proteins, making them an attractive system for studying PGC-1 function. As shown below, these infections achieved roughly equivalent levels of PGC-1α and PGC-1β mRNA. In addition, mRNAs for several well established target genes of PGC-1α (cytochrome c and UCP2) are activated equivalently in cells infected with PGC-1α or PGC-1β.

We first measured the proton conductance in these cells, as well as cells expressing a control GFP. Proton conductance can be defined as the flow of protons (proton leak) across the mitochondrial membrane at a given membrane potential and represents the fraction of mitochondrial respiration that is not coupled to ATP production. Respiration, the consumption of oxygen, is used as a surrogate for proton leak (30). The highest point of a proton leak curve represents the respiration rate and membrane potential of cells/mitochondria in the presence of oligomycin, an inhibitor of the F1F0-ATP synthase (30). Then respiration and membrane potential are inhibited gradually by adding increasing amounts of myoxthiazol, a specific inhibitor of the electron transport chain (30). As shown in Fig. 1 (A and B), the respiration rate of PGC-1α- and PGC-1β-expressing
cells at any given membrane potential was substantially higher than GFP controls. For example, the respiration rate at 190 mV of PGC-1α cells and the paired GFP control was ~7 and 3 nmol of oxygen/min/10^6 cells, respectively, an increase of 2.5-fold (Fig. 1A). The respiration rate of PGC-1β cells and the paired GFP control at 172 mV was ~3 and 0.5 nmol of oxygen/min/10^6 cells, respectively, a 6-fold increase (Fig. 1B).

To compare the proton leak kinetics of the cells expressing PGC-1α and PGC-1β, we expressed the respiration rates and membrane potentials of these cells as percentages of the initial respiration rate and membrane potential of paired GFP controls (Fig. 1C). The proton conductance of cells expressing PGC-1β was approximately three times higher than those expressing PGC-1α (Fig. 1C). Indeed, the respiration rate of PGC-1β, when normalized to its paired GFP control, was at least twice that of PGC-1α cells at any given membrane potential (Fig. 1C).

**Control of Mitochondrial Respiration by PGC-1α and PGC-1β**

Mitochondrial respiration is divided into two blocks: ATP turnover and proton leak. ATP turnover is the fraction of mitochondrial respiration devoted to ATP production and is sensitive to oligomycin. Proton leak is the fraction of mitochondrial respiration not coupled to ATP production and is insensitive to oligomycin. Metabolic efficiency represents the balance between the ATP turnover and proton leak blocks. Therefore, when total mitochondrial respiration is altered, metabolic efficiency can only be preserved if ATP turnover and proton leak are affected in a similar way. For example, if mitochondrial respiration doubles, metabolic efficiency will be preserved if both ATP turnover and proton leak double. We determined the proportion of mitochondrial respiration devoted to ATP turnover and proton leak in cells expressing PGC-1α, PGC-1β, and GFP. We used two different approaches to carry out this analysis; because each approach has its own limitation, together they provide a more reliable overview of the metabolic organization of these cells.

In the first calculation, we expressed the respiration rate of PGC-1α, PGC-1β, and GFP cells in the presence of oligomycin (from Fig. 1) as a fraction of their mitochondrial respiration (data not shown) without taking into account membrane potential values. The potential problem with this approach is actually that it does not take into account differing membrane potential values of the different cells. By this method, PGC-1α-expressing cells devote 37% of their mitochondrial respiration to proton leak, compared with 23% for paired GFP controls (Fig. 2A). In other words, PGC-1α increases uncoupled respiration more than coupled respiration. This is in agreement with the initial study by Wu et al. (24) using C2C12 myotubes infected with retroviruses reporting that PGC-1α increases uncoupled respiration more than coupled respiration. In contrast, cells expressing PGC-1β display a fraction of mitochondrial respiration caused by proton leak similar to that of the GFP controls (Fig. 2B). Indeed, the ATP turnover and proton leak blocks increased coordinately in PGC-1β-expressing cells so that they are almost as coupled as GFP control cells (Fig. 2B).

As a second approach, we took into account the membrane potential values and calculated the proportion of mitochondrial respiration devoted to proton leak at the resting membrane potential of PGC-1α, PGC-1β, and GFP cells. This is the membrane potential in the absence of oligomycin (data not shown). The membrane potential value of these cells was slightly higher in the resting state than in the presence of oligomycin, which is the highest point of the proton leak curves presented in Fig. 1. To obtain the predicted proton leak rate at the resting membrane potential of these cells, we fitted a linear curve through the proton leak points of PGC-1α, PGC-1β cells, and paired GFP controls from Fig. 1 (A and B) (19). A caveat with

![Proton leak kinetics in C2C12 myotubes](image)
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### DISCUSSION

The coactivator PGC-1α has been shown to induce mitochondrial biogenesis in cultured adipocytes as well as skeletal and cardiac myocytes (23, 24, 34). Initial studies using the inhibitor oligomycin suggested that PGC-1α might specifically induce an increased proton leak in skeletal muscle cells, but these studies had to be considered suggestive because a bona fide asertiment of alterations in proton leak can only be determined by examining experimental and control cells at different mitochondrial membrane potentials. In this study, we have performed a much more complete and mechanistic analysis of the effects of PGC-1α and the related coactivator PGC-1β on the bioenergetics of mitochondria.

One basic question addressed here is whether PGC-1β has the capacity to induce mitochondrial biogenesis and increase respiration. As shown in Figs. 1B, 3A, and 4A, PGC-1β powerfully stimulates mitochondrial biogenesis and respiration. Indeed, it is more potent in this regard than PGC-1α at similar levels of mRNA, even though several genes are induced by both coactivators to a similar extent.

Another key issue addressed in this study is the fraction of mitochondrial respiration coupled or uncoupled in cells expressing PGC-1α or PGC-1β. There are several possible explanations for the increased fraction of uncoupled respiration observed in the presence of PGC-1α versus PGC-1β. First, it is necessary to examine how coupled and uncoupled respiration in particular can be regulated. A key factor that needs consideration with respect to respiration rate relates to the elevated mitochondrial volume density of these cells. We can account for the differences in mitochondrial volume density between cells expressing PGC-1α or PGC-1β and control cells simply by dividing the respiration rates of these cells by their respective mitochondrial volume density value. This calculation gives an indication of the respiration rate of mitochondria inside the cells. After correcting for differences in mitochondrial volume density, the total respiration rate of cells expressing PGC-1α or PGC-1β is similar to control cells. However, the proton leak rate (highest point of the proton leak curves in Fig. 1) of cells expressing PGC-1α remains higher than that of cells expressing PGC-1β or control cells, both of which have similar proton leak rates after correction. These data suggest the proton leak rate of individual mitochondria is higher in the presence of PGC-1α than PGC-1β and that mitochondrial volume density is not the only explanation for the higher fraction of leak-related respiration in cells expressing PGC-1α.

Modifications in the intrinsic properties of mitochondria could explain higher proton leak rates in cells expressing PGC-1α versus PGC-1β. Cells expressing PGC-1α, but not PGC-1β, displayed a statistically significant increase of 10% in cristae surface density (Figs. 3B and 4B), which would be expected to augment their proton leak by increasing the area of membrane across which protons can re-enter the matrix compartment. In support of the idea that elevated PGC-1α levels increase the proton leak of individual mitochondria, a separate experiment using mitochondria isolated from the skeletal muscle of mice expressing PGC-1α at the level found in type I
isolated mitochondria and is called the state 4 point. The represent the resting respiration rate and membrane potential of "Experimental Procedures." and membrane potential. The experimental details are described under amounts of malonate were added to gradually inhibit respiration rate. The mitochondria were fed succinate as substrate, and increasing incubated in the presence of oligomycin, nigericin, rotenone, and TPMP. to contribute to leak in these experiments. The mitochondria were binds fatty acids, known activators of the UCPs. The UCPs are unlikelyments were carried out in the presence of bovine serum albumin, whichopen squares represent mitochondria from wild-type mice; therepresent mitochondria from mice expressing PGC-1 muscle fibers (Fig. 4). Intriguingly, only cells expressing PGC-1 heavy subunit, both involved in the removal/degradation of ROS (Fig. 6B). It is conceivable, or even likely, that both PGC-1a and PGC-1b action result in the generation of ROS via the activation of mitochondrial metabolism, thereby activating UCP2 and/or UCP3. However, it is also possible that PGC-1b is better at the removal of these ROS because mRNAs for two cytoplasmic enzymes playing a role in metabolism of ROS are induced by this coactivator but not by PGC-1a. Whether regulation of ROS in the cytoplasm can influence superoxide on the matrix side of the inner mitochondrial membrane and regulate UCP2 or UCP3 is an open question (35). The relative ability of PGC-1a and PGC-1b to produce ROS and activate UCPs via this mechanism remains to be determined. Another important aspect to consider in this analysis concerns ATP turnover, the fraction of mitochondrial respiration providing ATP for the ATP consumers. There are important differences regarding ATP turnover between cells expressing PGC-1a and PGC-1b. Indeed, 75% of the increase in mitochondrial respiration in cells expressing PGC-1b was due to an increase in ATP turnover, and 25% was explained by an in-
ever, it is important to appreciate that PGC-1α (present study), is not cold-inducible in these tissues (26). How-
not lead to a particularly inefficient mitochondrial metabolism
particularly interesting in light of the fact that PGC-1α
muscular work, will slightly decrease membrane potentials
trast, high ATP demand, expected upon the performance of
potential, which will in turn increase proton leak. In stark con-
resulting in a tighter coupling of metabolism because proton leak decreases rapidly as membrane potential is lowered. Said another way, PGC-1α, by setting up a relatively uncoupled state at rest, probably provides a system where rapid ATP turnover, such as occurs during the performance of exercise, will effectively slow down excessive proton leak. PGC-1β, in contrast, drives relatively less proton leak at rest, so it will provide more ATP when this is needed but cannot otherwise provide metabolic flexibility. Because both PGC-1α and PGC-1β are present in many tissues, the relative expression levels of these coactivators likely will play an important role in setting metabolic efficiency.

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Bioenergetic Analysis of Peroxisome Proliferator-activated Receptor γ Coactivators 1α and 1β (PGC-1α and PGC-1β) in Muscle Cells

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