Pyruvate induces mitochondrial biogenesis by a PGC-1α independent mechanism.

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Abstract

Oxidative cells increase mitochondrial mass in response to stimuli such as changes in energy demand or cellular differentiation. This plasticity enables the cell to adapt dynamically in order to achieve necessary oxidative capacity. However, the pathways involved in triggering mitochondrial biogenesis are poorly defined. The present study examines the impact of altering energy provision on mitochondrial biogenesis in muscle cells. C2C12 myoblasts were chronically treated with supraphysiological levels of sodium pyruvate for 72 hr. Treated cells exhibited increased mitochondrial protein expression, basal respiratory rate and maximal oxidative capacity. The increase in mitochondrial biogenesis was independent of increases in PGC-1α and PGC-1β mRNA expression. To further assess whether PGC-1α expression was necessary for pyruvate action, cells were infected with adenovirus containing shRNA for PGC-1α prior to treatment with pyruvate. Despite a 70% reduction in PGC-1α mRNA the effect of pyruvate was preserved. Furthermore, pyruvate induced mitochondrial biogenesis in primary myoblasts from PGC-1α null mice. These data suggest that regulation of mitochondrial biogenesis by pyruvate in myoblasts is independent of PGC-1α, suggesting the existence of a novel energy-sensing pathway regulating oxidative capacity.

Keywords:
Oxidative metabolism, PGC1a, mitochondria, muscle
**Introduction**

Adaptive alterations in mitochondrial mass and function are a key feature of skeletal muscle. Mitochondrial biogenesis is increased in this cell type under conditions such as: differentiation of myoblasts into mature myotubes (16); in the presence of increasing concentrations of extracellular pyruvate (2); and when energy demand exceeds oxidative capacity, such as in endurance training (6). Conversely, cessation of exercise training and aging are conditions that can dramatically reduce mitochondrial capacity in this tissue (22; 27).

Despite a number of stimuli being known, the signaling pathways controlling mitochondrial biogenesis in skeletal muscle are not clearly defined. An emerging regulator of mitochondrial replication is the peroxisomal proliferator activator receptor gamma co-activator 1 alpha (PGC-1α), which acts through coactivation of a number of transcription factors (20; 28). For example, PGC-1α coactivates the peroxisome proliferator activated receptors (PPARs) to upregulate fatty acid oxidation while it induces the transcription of oxidative phosphorylation genes by coactivating nuclear respiratory factor (NRF) 1 and 2. A close homologue of PGC-1α, PGC-1β, may share a similar role in mitochondrial metabolism (12; 24).

In the present study, we explore pyruvate-induced mitochondrial biogenesis in muscle myoblasts and the role of PGC-1α in mediating this effect. We report that supraphysiological concentrations of pyruvate increase mitochondrial mass and functionality as determined by a comprehensive array of mitochondrial measures.
Furthermore, we establish that this action does not increase PGC-1α, or β mRNA expression and, in fact, is not dependent on PGC-1α.
Materials and Methods

Cell culture. C2C12 cells (American Type Culture Collection, Manassas, VA) were seeded in either basal media or test media. Basal media was Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 1% antibiotics and 4 mM L-glutamine. Pyruvate test media was DMEM with no sodium pyruvate (Invitrogen) supplemented with 10% fetal bovine serum, 1% antibiotics, 4 mM L-glutamine and 50 mM sodium pyruvate (Sigma, St. Louis, MO). Sodium chloride or sodium bicarbonate test media was basal media supplemented with either an additional 50 mM sodium chloride or sodium bicarbonate, respectively. Cells were incubated for 72 hr prior to manipulation. Sodium chloride and pyruvate-free DMEM was obtained as a customized reagent (Invitrogen). All studies were performed on myoblasts i.e. undifferentiated proliferating muscle progenitors.

Flow cytometry. Cells were trypsinized, centrifuged at 1000 rpm for 5 min, resuspended and counted. Cells were incubated with MitoTracker Green FM (Molecular Probes, Eugene, OR) at a final concentration of 100 nM for 30 min at 37°C. After incubation, cells were centrifuged at 1000 rpm for 5 min. Media was removed and cells were resuspended in PBS at a concentration of 1×10^6 cells/ml. The forward scatter versus side scatter area was used to analyze a homogeneous population of live cells after doublet exclusion using a FacsAria Flow Cytometer (BD Biosciences, San Diego, CA).
**Confocal Microscopy.** Following treatment, media was removed and cells were incubated with 100 nM MitoTracker Green FM for 30 min at 37°C, then with 2 µg/ml Hoechst 33342 dye for a further 10 min. Phenol red-free DMEM (Invitrogen) was added and the cells were visualized on a Zeiss 510 Meta Confocal Microscope at 63X magnification (Carl Zeiss, Inc., Thornwood, NY).

**Protein expression studies.** Cytochrome c protein level was measured using a commercially available enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. For Western blot analyses, cells were washed three times with phosphate buffered solution (PBS), then solubilized with PBS containing 0.5% Triton X-100 and protease inhibitors for 10 min at 4°C. Extracts were briefly sonicated and the protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce). 10 µg of cell lysate was mixed with LDS sample buffer and reducing reagent (Invitrogen), vortexed and heated at 70°C for 10 min. Samples were loaded and resolved on a 4-12% Bis-Tris mini gel (Invitrogen). Following transfer, the nitrocellulose membranes were incubated with indicated primary antibodies. Immunoreactive bands were detected using ECL Western blotting detection reagents (Amersham Biosource). Anti-mitochondrial protein antibodies were purchased from MitoSciences (Eugene, OR). Anti-Sir2 (silent information regulator 2) antibodies were purchased from Upstate (Lake Placid, NY).

**Cellular respiration assay.** Cells were washed with phosphate-buffered saline (PBS), trypsinized and resuspended in respiration medium (PBS without Ca^2+/Mg^2+, 25 mM...
HEPES pH 7.0, 5.55 mM glucose, 1% BSA, fatty acid-free). 300,000 cells in 2 ml were transferred to a Clark-type oxygen electrode chamber that was connected to a circulating water bath at 37°C. Basal respiration was measured and then non-phosphorylating (uncoupled) respiration was determined in the presence of the ATP synthase inhibitor, oligomycin (2 µg/ml). Carbonylcyanide-3-chlorophenylhydrazone (CCCP), a chemical uncoupler, was titrated into the cell solution to determine oxidative capacity (maximal respiration). Data obtained from a background (blank) measurement of oxygen flux using heavy gas titration was subtracted from sample data to control for instrumental influence.

**Adenoviral studies.** A recombinant adenoviral vector expressing a shRNA targeted to mouse PGC-1α was kindly provided by Dr. Marc Montminy, Salk Institute for Biological Studies and was generated as described by Koo et al (9). C2C12 myoblasts were plated on 6-well plates in basal media at a density of 3×10⁴ cells per well (control) or 8×10⁴ cells per well (treated). Cells were infected 6 hours after seeding with either Ad-sh-PGC-1α (3.0×10¹² particle/ml) or Ad-sh-scrambled (3.0×10¹² particle/ml) at 1.25×10⁵ particle/cell in basal media. The media was changed 24 hr later to either basal media or test media. Virus at 1×10⁵ particle/cell was added to each well 24 hr after the media was changed and cells were harvested 48 hr after second infection.

**Real-time quantitative PCR analysis.** Following the isolation of total RNA and preparation of cDNA, the expression profile of PGC-1α and PGC-1β genes (Accession #: NM_008904 and NM_133249, respectively) were measured using real-time PCR.
Quantitative RT-PCR was performed on an ABI Prism 7900HT Sequence Detection System and analyzed using SDS 2.0 software (Applied Biosystems, Foster City, CA). The expression of each target gene was normalized by the endogenous control 18S rRNA (Applied Biosystems). An Assay-on-Demand 20X mix containing primers and probe specific for each target gene (ID #: PGC-1α / Mm00447183_m1 and PGC-1β / Mm00504720_m1) was obtained from Applied Biosystems. Data were determined in duplicate from 3 wells per treatment and expressed as the mean ± SEM. Differences were considered statistically significant at P<0.05 by One-way ANOVA with Dunnett's Multiple Comparison Test.

Gene Chip analysis. The GeneChip experiment was conducted in the Genomics Factory, Novartis PHARMA AG, Basel, Switzerland on a Gene Chip Mouse Genome 430 2.0 Expression Array (Affymetrix Inc.; Santa Clara, CA). The oligonucleotide probes were 25mers and 11 probe pairs per sequence were used. The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE5497 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE5497). Gene Set Enrichment Analysis was performed as previously described (15).

PGC-1α null myoblast studies. Myoblasts derived from PGC-1α null mice and wild-type littermates were seeded in Ham’s F-10 medium containing 20% FBS, 1% penicillin-streptomycin (Invitrogen) and 2.5 ng/ml basic fibroblast growth factor (13). Once the
cells attached, the media was removed and cells were incubated in either fresh basal media or pyruvate (50 mM) test media for 72 hr at 37°C.

*Statistical analyses.* Experiments were repeated on at least two independent days and a representative figure provided. Within one experiment, n=3 refers to 3 different culture plates. Statistical analyses were performed on the data presented using one-way ANOVA and Tukey’s multiple comparison test, except where noted. P<0.05 was considered significant.
Results

C2C12 myoblasts were incubated in either basal media that contains 1 mM sodium pyruvate or test media supplemented with 50 mM sodium pyruvate (SP) for 72 hr. The cells were then stained with MitoTracker Green FM, a mitochondrial specific fluorescent dye, and the mitochondrial mass was assessed using fluorescence activated cell sorting (FACS) (Figure 1a). FACS analyses revealed significantly greater mitochondrial staining in SP treated cells than control cells. The increase in MitoTracker fluorescence was not a result of increased cell size as determined by size-gating analysis (Figure 1b). Further evidence of increased mitochondrial content was demonstrated using confocal microscopy and MitoTracker Green FM staining (Figure 1c).

To further establish the effect of pyruvate on mitochondrial biogenesis, we analyzed several different mitochondrial specific markers. Cytochrome c, a mitochondrial electron transporter, protein expression increased following 72 hr pyruvate treatment (Figure 2a). This effect was significant at 24 hr of treatment and maximal at 48 hr (data not shown). Cells were also incubated in media supplemented with 50 mM sodium chloride (NaCl) or 50 mM sodium bicarbonate (NaHCO₃), to control for changes in the osmolarity. Neither NaCl nor NaHCO₃ significantly increased cytochrome c expression indicating that the observed increase in mitochondrial biogenesis was a pyruvate specific effect. As an additional control, pyruvate treatments were performed at normal osmotic pressure using a medium with reduced salt content. Under these conditions, pyruvate still increased cytochrome c expression compared to control (data not shown).
Other mitochondrial markers were assessed in lysates from control and SP treated cells (Figure 2b). There was a significant increase in expression of core 2 protein, a component of complex III, and of 20 kDa subunit, a component of complex I, in SP treated cells. Interestingly, there was no increase in the expression level of F1α, a component of ATP synthase or of 30 kDa Ip subunit, a component of complex II.

We also measured cellular respiration to evaluate if functional changes accompanied the apparent increase in mitochondrial mass (Figure 2c). Using a Clark-type oxygen electrode we measured oxygen consumption in intact cells. SP treated cells had a significantly higher basal respiration rate than control cells. Maximal oxidative capacity, in the presence of CCCP, was approximately twice that of control cells, whereas, no significant difference in uncoupling was apparent. These data suggest that pyruvate treatment results in increased oxidative capacity and not increased uncoupling.

PGC-1α has been implicated as a key regulator of mitochondrial biogenesis, however, it is expressed at very low levels in C2C12 myoblasts (24; 28). Using quantitative RT-PCR, we analyzed PGC-1α mRNA expression levels in control and SP treated C2C12 cells and found that pyruvate treatment did not upregulate PGC-1α expression (Figure 3a). Interestingly, PGC-1β mRNA levels were significantly reduced following pyruvate treatment (Figure 3b). Despite low PGC-1α expression in C2C12 myoblasts we employed shRNA technology to further knockdown PGC-1α in these cells. Cells were infected with either PGC-1α or scrambled shRNA adenoviruses and then incubated in
either basal or SP test media for 72 hr. Cytochrome c protein expression was increased in SP treated cells compared to control cells despite a further 70% decrease in PGC-1α mRNA expression (Figure 3c). Cells treated with control virus were not significantly different to untreated cells (data not shown). Also, protein levels of Sirtuin 1 (SIRT1), a regulator of PGC-1α activity, were unchanged over a 24 hr period of pyruvate treatment (Figure 3d).

To further explore the role of PGC-1α in pyruvate-induced mitochondrial biogenesis we treated primary mouse myoblasts from PGC-1α null mice and wild-type littermates with pyruvate and measured both mitochondrial mass (Figure 4a) and cytochrome c protein expression levels (Figure 4b). Using FACS analysis, we found that in both PGC-1α null and wildtype myoblasts treated with pyruvate there was significantly more mitochondrial staining than in control cells. Furthermore, cytochrome c levels were significantly increased in response to pyruvate treatment in both the PGC-1α null and wild-type myoblasts. These data suggest that pyruvate induces mitochondrial biogenesis independently of PGC-1α.

We performed gene array profiling on basal and pyruvate treated cells. Using Gene Set Enrichment Analysis (a technique that collectively examines changes in pathway or functionally related genes), we observed a significant upregulation of genes associated with free radical scavenging (Figure 5, Table 1). Interestingly, genes associated with myocellular differentiation were significantly down regulated.
Discussion

Mitochondrial dysfunction in skeletal muscle has been implicated in a number of diseases, such as type 2 diabetes, obesity and some inherited myopathies. A potential therapeutic approach to these disorders is to enhance oxidative capacity by increasing mitochondrial mass, however, the control mechanisms governing mitochondrial replication are not fully elucidated. The present study examines mitochondrial adaptation in response to high pyruvate concentrations, a circumstance where oxidative energy supply exceeds energy demand.

A previous study examined the action of high pyruvate concentrations in L6 myoblasts. The authors reported increased mitochondrial content as indicated by nonyl acridine orange (NAO) staining and an increase in mitochondrial membrane potential with pyruvate treatment (2). Some controversy exists over the use of NAO in estimating mitochondrial content, particularly when there are changes in mitochondrial membrane potential (7; 8). Therefore, we sought to corroborate these findings with a comprehensive panel of mitochondrial markers and then examine the mechanistic basis for pyruvate action.

In agreement with the previous study, treatment of myoblasts with high pyruvate concentrations induced mitochondrial biogenesis, as determined by cytochrome c expression, mitochondrial staining [using MitoTracker Green FM, a mitochondrial dye insensitive to changes in membrane potential (19)], expression of complexes I and III and
basal and uncoupled respiration. It is somewhat surprising that pyruvate did not increase all electron transport chain proteins, however, it is conceivable that in this setting of increased mitochondrial biogenesis these proteins are not rate limiting. Importantly, these effects were not a result of changes in osmolarity, a parameter that was not controlled in the previous study.

Differentiation of myoblast precursor cells to more metabolically active myotubes results in an increased mitochondrial mass. Therefore, it is important to establish whether agents that increase mitochondrial biogenesis are doing so by inducing cellular differentiation. Pyruvate treatment did not induce myoblast fusion or genes associated with muscle differentiation. In fact, using Gene Set Enrichment Analysis, it was apparent that the gene program associated with myocellular determination was down-regulated. Interestingly, using the same analysis, genes associated with free radical scavenging, a key auxiliary requirement to mitochondrial activity, were up-regulated. Increased production of reactive oxygen species following pyruvate treatment has been observed in a number of cellular systems (18; 29) and likely stimulates upregulation of anti-oxidative machinery. This provides some support of the observation that pyruvate has anti-oxidative properties (1). In addition, it is possible that increased free radical production could in some way induce mitochondrial replication.

The nuclear receptor coactivator PGC-1α is a key regulator of mitochondrial biogenesis and gluconeogenesis in muscle and liver, respectively. Ectopic expression of PGC-1α, or a close homologue, PGC-1β, results in increases in mitochondrial mass (10; 24; 28). The
extent to which these factors act independently and/or redundantly in controlling oxidative capacity is seemingly dependent on tissue type and stimulus. For example, unlike PGC-1α, PGC-1β is not up-regulated during exercise or cold stress (14). Moreover, PGC-1α null mice have reduced exercise tolerance and other metabolic derangements associated with diminished mitochondrial capacity (11).

PGC-1α mRNA was expressed at low levels in C2C12 myoblasts under basal conditions and did not increase with pyruvate treatment. However, we could not exclude the possibility that post-translational modification of existing PGC-1α protein could lead to increased activity, independently of up-regulation of the gene. Therefore, we treated cells with shRNA targeted to PGC-1α, resulting in a further reduction in target mRNA levels. Even with this reduction, pyruvate induction of the mitochondrial marker cytochrome c was unaffected. Interestingly, PGC-1β expression was decreased with pyruvate treatment and unaffected by PGC-1α knockdown. Importantly, primary myoblasts from the PGC-1α null mouse (13) also responded to pyruvate treatment.

One potential fate of acetyl CoA generated from pyruvate entry into the mitochondria is the formation of acetyl carnitine. In addition to liberating mitochondrial CoA, acetyl carnitine can pass out of the mitochondria and cell, providing an overflow mechanism for excess energy supply when the cell is energy replete. Intriguingly, a number of reports have demonstrated that supplementing diets with acetyl carnitine can help maintain or improve mitochondrial function in rats (4; 5). It is also noteworthy that, administration of pyruvate reduced the reaccumulation of weight gain following diet-induced weight loss
in humans (25; 26). The present data imply that pyruvate-induced mitochondrial biogenesis and increased oxidative capacity may have contributed to this phenotype.

A second fate of excess pyruvate is the production of lactate with the concomitant oxidation of NADH to NAD\(^+\). The NAD\(^+\)-dependent histone deacetylase SIRT1 interacts with PGC-1\(\alpha\) (17) and regulates PGC-1\(\alpha\)-dependent gene expression in a nutrient sensitive manner in hepatocytes (21). Intriguingly, increased SIRT1 activity has also been linked to downregulation of muscle cell differentiation (3), a function that is similarly affected by pyruvate treatment in the present study. We were unable to detect increases in Sir2 protein levels with pyruvate treatment, however, we cannot exclude the possibility that SIRT1 activity levels are increased and can function independently of PGC-1\(\alpha\). An alternate possibility is that the decrease in myogenicity is a direct result of increased mitochondrial content, as suggested by Seyer et al (23).

Combined, our results indicate that cell autonomous regulation of mitochondrial mass and function is sensitive to three-carbon fuel availability but which does not invoke the participation of the transcriptional coactivators PGC-1\(\alpha\) and PGC-1\(\beta\). Given that lower organisms (e.g., yeast, fruit flies) lack PGC-1\(\alpha\), our data support the view that additional control elements governing mitochondrial replication may play a role in tissues exposed to high flux of oxidative substrates, including pyruvate.
Acknowledgments:

We wish to thank: Bruce Spiegelman for the kind gift of primary PGC-1α null and wild-type myoblasts; Mark Montminy for the shRNA PGC-1α adenovirus; Deborah Ahern-Ridlon and Akos Szilvasi for their technical assistance with the confocal microscopy and FACS analyses; Nanguneri Nirmala and Joseph Szustakowski for the GeneChip data analysis; Daniel Kemp for the myogenic gene set; and Thomas Hughes for his critical review of this manuscript.
Figure Legends:

Figure 1. Mitochondrial mass in muscle cells is increased with pyruvate treatment.
C2C12 myoblasts were incubated in either basal or SP test media for 72 hr. Cells were trypsinized and stained with 100 nM MitoTracker Green FM. FACS analysis of the total cell population (a) and size-gated populations (Green-A indicates MitoTracker staining) (b) was performed (n=3 ± SEM). Cells were gated according to size as follows: blue-smaller cells, gray-medium sized cells, red-larger cells. Fluorescence is represented on the x-axis and cell number on the y-axis. A right-shift in distribution indicates an increase in fluorescence. In (c), stained cells were visualized using confocal microscopy. Nuclei were stained with 2 µg/ml Hoechst stain.

Figure 2. Pyruvate stimulates mitochondrial protein expression and cellular respiration in C2C12 myoblasts.
Cytochrome c protein levels in total cellular lysates from cells incubated in basal or test media were measured by an ELISA (a). Test media was basal media supplemented with either an additional 50 mM SP, 50 mM NaCl or 50 mM NaHCO₃. (n=6 ± SEM). In (b), extracts from basal or SP treated cells were subjected to Western blot analysis. Resulting blots were probed for: F1α (ATP synthase), core 2 (Complex III), 30 kDa Ip subunit (Complex II) and 20 kDa subunit (Complex I). In (c), basal, uncoupled and maximum respiration of cells was determined in cells incubated in basal or SP treated (n=3 ± SEM).
**Figure 3. Pyruvate induced mitochondrial biogenesis is not dependent on PGC-1α.**

C2C12 myoblasts incubated in basal media or SP test media following infection with either PGC-1α or scrambled shRNA. Total RNA was extracted for quantitative real-time PCR analysis of PGC-1α (a) and PGC-1β (b) expression levels (n=3 ± SEM). Cells were also extracted for cytochrome c protein determination (c) (n=9 ± SEM). In (d), total cellular lysates were made from C2C12 myoblasts incubated in basal or SP test media at indicated time points. SIRT1 protein expression was analyzed by Western blot analysis and normalized against actin (n=6 ± SEM).

**Figure 4. Mitochondrial biogenesis is induced by pyruvate in myoblasts from PGC-1α null mice.**

Primary myoblasts from PGC-1α null mice and wild-type littermates were incubated in either basal or SP test media for 48 hours. (a) Cells were incubated with 100 nM MitoTracker Green FM for 30 minutes and the mitochondrial mass was assessed using FACS analysis (n=6±SEM). (b) Cytochrome c protein levels in total cellular lysates were measured using an ELISA (n=4 ± SEM).

**Figure 5. Gene Set Enrichment Analysis (GSEA) of pyruvate treated C2C12 myoblasts.**

Cells were incubated in either basal or 50 mM pyruvate (hs) test media for 72 hr. Total RNA extracted from cells was subjected to GeneChip array analysis. Relative gene expression in the two conditions was plotted in scatter. GSEA was performed to assess
pathway gene expression levels. Two representative probe sets are highlighted in blue (n=3 ± SEM).

Reference List


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26. Stanko RT, Reynolds HR, Hoyson R, Janosky JE, and Wolf R. Pyruvate supplementation of a low-cholesterol, low-fat diet: effects on plasma lipid


Table 1. *Gene expression analysis of control and pyruvate treated muscle cells*

<table>
<thead>
<tr>
<th>Down-regulated genes</th>
<th>Ratio</th>
<th>Up-regulated genes</th>
<th>Ratio</th>
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<td>troponin C2, fast</td>
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<td>superoxide dismutase 3, extracellular</td>
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<td>RIKEN cDNA C030006K11 gene</td>
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The 10 genes most affected with treatment from each probe set shown in Figure 4 are listed. The ratio is calculated by dividing the gene expression level of treated samples by the expression level of untreated samples.
**Figure 1**
Figure 2
Figure 3
Figure 4

(a) Mitotracker staining (fold over untreated cells) for WT cells and PGC-1α null cells under Basal and 50 mM SP conditions.

(b) Cytochrome c protein (ng/mg extract) for WT cells and PGC-1α null cells under Basal and 50 mM SP conditions.
Figure 5