Expression profiling following local muscle inactivity in humans provides new perspective on diabetes-related genes

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Abstract

Physical activity enhances muscle mitochondrial gene expression, while inactivity and mitochondrial dysfunction are both risk factors for developing diabetes. Defective activation of the transcriptional coactivator PGC-1α may contribute to the gene expression pattern observed in diabetic and insulin-resistant skeletal muscle. We proposed that greater insight into the mitochondrial component of skeletal muscle “diabetes” would be possible if the clinical transcriptome data were contrasted with local muscle inactivity-induced modulation of mitochondrial genes in otherwise healthy subjects. We studied PPARGC1A (PGC-1α), PPARGC1B (PGC-1β), NRF1, and a variety of mitochondrial DNA (mtDNA) and nuclear-encoded mitochondrial genes critical for oxidative phosphorylation in soleus muscle biopsies obtained from six healthy men and women before and after 5 weeks of local muscle inactivity. Muscle inactivity resulted in a coordinated down-regulation of PGC-1α and genes involved with mitochondrial metabolism, including muscle substrate delivery genes. Decreased expression of the mtDNA helicase Twinkle was related to the decline in mitochondrial RNA polymerase (r = 0.83, p < 0.04), suggesting that mtDNA transcription and replication are coregulated in human muscle tissue. In contrast to the situation in diabetes, PGC-1β expression was not significantly altered, while NRF1 expression was actually up-regulated following muscle inactivity. We can conclude that reduced PGC-1α expression described in Type 2 diabetes may be partly explained by muscle inactivity. Further, although diabetes patients are typically inactive, our analysis indicates that local muscle inactivity may not be expected to contribute to the decreased NRF1 and PGC-1β expression noted in insulin-resistant and Type 2 diabetes patients, suggesting these changes may be more disease specific.

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From epidemiological studies, physical inactivity has been identified as being one of the most important risk factors for the development of cardiovascular and metabolic disease in humans [5,17,18,30]. There is now emerging evidence from large-scale gene expression studies that specific mitochondrial defects could represent an important factor for the development of insulin resistance and Type 2 diabetes [13,14,16,20,21,30]. Defective activation of the nuclear hormone receptor coactivator PPARGC1A (PGC-1α), a master regulator of mitochondrial gene expression, has been proposed to contribute directly to the development of Type 2 diabetes mellitus and insulin resistance [13,15,16,20]. Specifically, it has been shown that PGC-1α, PPARGC1B (PGC-1β), and NRF1 expression and the expression of PGC-1α-regulated oxidative phosphorylation (oxphos) genes are down-regulated in the skeletal muscle of diabetic and prediabetic insulin-resistant subjects [13,16,20]. It has also been demonstrated that a prevalent polymorphism in PGC-1α’s role in coordinating mitochondrial function has accumulated with a variety of mechanistic studies [11,15,31] demonstrating a central role for this coactivator in mammalian energy metabolism [24]. Indeed, PGC-1α also appears to regulate mitochondrial gene expression in response to exercise [22,24,25], potentially
coordinating the general increase in oxphos genes with physical activity [29]. This substantial volume of evidence has positioned PGC-1α as playing a central role in the development of Type 2 diabetes [14] and led to the premature conclusion that PGC-1α activation is a logical objective for a diabetes drug development program [4,13,19].

It is very important to consider that patients with diabetes are generally physically inactive and thus it is important to establish how “diabetes” interacts with muscle inactivity, to facilitate the overall interpretation of muscle gene expression changes. To interpret the available clinical data, we decided to establish the impact of local muscle inactivity on healthy skeletal muscle gene expression and then contrast this with changes observed in patients. Indeed, it was recently concluded [14] that it was an important objective to determine if down-regulation of PGC-1α signaling was a primary or secondary event in the pathogenesis of Type 2 diabetes. Our primary objective was to establish if the PGC-1α-related transcript response in patients [13,16,20] could be partly explained by muscle inactivity. In addition, by contrasting the two transcript analysis strategies we should be able to identify more clearly “diabetes”-specific alterations. We utilized a model of skeletal muscle disuse [1,2,6] to impose 5 weeks of muscle inactivity on only one leg of healthy volunteers. Muscle biopsies were obtained before and after this intervention period, from the soleus, and RNA was prepared to profile PGC-1α, PGC-1β, mitochondrial substrate delivery genes, nuclear-and mitochondrial DNA (mtDNA)-encoded oxphos genes, and regulators of mitochondrial transcription and replication. This represents the first analysis of the impact of muscle inactivity on human mitochondrial transcript abundance.

Results

Coordinated down-regulation of substrate delivery genes

Oxidative ATP regeneration is a sequential process requiring substrate delivery to the cell (carbon units and oxygen), including acetyl group delivery to the mitochondria and subsequent reducing equivalent production. It was therefore important to characterize key genes responsible for mitochondrial substrate delivery. We demonstrated a substantial reduction in genes responsible for muscle Kreb-cycle function (citrate synthase (CS), $p = 0.03$), acetyl group production (pyruvate decarboxylase (PDH1A), $p = 0.008$), lipid transport (carnitine palmitoyl transferase Type 1 (CPT1B), $p = 0.002$) and glucose uptake (GLUT4 (SLC2A4), $p = 0.004$) (Fig. 1). Interestingly, these four substrate delivery genes were expressed in a coordinated manner, irrespective of tissue status, indicating close physiological regulation of both lipid-and carbohydrate-related substrate delivery genes (Fig. 2).

Nuclear-encoded oxphos genes are down-regulated by inactivity

The extent to which nuclear DNA-and mtDNA-encoded gene expression is coregulated at the transcript level is still unclear in human cells. We demonstrated that the nuclear-encoded oxphos genes cytochrome $c$ oxidase subunit 4 (COX4I1 (COXIV); $p = 0.007$) and succinate dehydrogenase (SDHA; $p = 0.003$) were down-regulated following 5 weeks of inactivity (Fig. 3), while uncoupling protein 3 (UCP3) expression was not significant altered ($p = 0.1$). UCP3 expression was, overall, not tightly coordinated with the other oxphos genes. There was greater intersubject variation for the mtDNA-encoded transcript expression and as a consequence none of the individual mtDNA-encoded transcripts demonstrated a statistically significant reduction (cytochrome $b$ of complex III (MT-CYB; $p = 0.2$), NADH dehydrogenase subunit 4 (MT-ND4; $p = 0.1$), and cytochrome $c$ oxidase subunit I (MT-CO1, $p = 0.07$)) (Fig. 3, $F = 4.49, p = 0.04$). The expression of MT-CYB, MT-ND4, and MT-CO1 was, as expected, highly linearly correlated ($r > 0.9$).

Down-regulation of mtDNA replication and transcriptional regulatory genes by inactivity

The apparent coregulation between some nuclear-encoded and some mtDNA-encoded genes suggests that mtDNA transcription was partially reduced as clearly several nuclear-encoded transcripts were (Figs. 1 and 3). To provide evidence of a coordinated reduction in the transcript abundance of oxphos genes, we profiled key regulators of mtDNA replication and transcription (Figs. 4A and 4B). The mtDNA helicase PEO1 (known as “Twinkle”) and the mitochondrial RNA polymerase (POLRMT) were down-regulated following 5 weeks of inactivity ($p < 0.05$). Furthermore, the magnitude of the reduction in Twinkle correlated with the magnitude of the reduction in POLRMT ($r = 0.83, p = 0.04$). In contrast, mitochondrial transcription factor A (TFAM), transcription factor B1 mitochondrial (TFB1M), transcription factor B2 mitochondrial (TFB2M), mitochondrial transcription termination factor (MTERF), DNA polymerase γ subunit (POLG), and mitochondrial single-
stranded DNA-binding protein (SSBP1) were not altered by 5 weeks inactivity in human soleus muscle (Figs. 4A and 4B).

PGC-1α transcript expression is substantially down-regulated by inactivity

To determine if changes in PGC-1α expression matched those observed in Type 2 diabetes, we profiled PGC-1α mRNA using primers that avoid any of the common polymorphisms or known splice variants. In addition, PGC-1α is thought to regulate tissue levels of PGC-1β (also altered in diabetes patients) and thus we examined the relationship between PGC-1α and PGC-1β transcript abundance across 12 human skeletal muscle samples. As demonstrated in Fig. 5A, PGC-1α was significantly down-regulated following 5 weeks of muscle inactivity ($p = 0.03$), while PGC-1β expression was not significantly altered. Furthermore, we found a surprising increase in NRF1 expression ($p = 0.007$), providing a clear contrast to previous findings from patients with diabetes (NRF2α and NRF2β expression was unchanged; data not shown).

Fig. 2. The interrelationship between the mRNA levels of substrate delivery genes in human skeletal muscle. Total RNA was prepared and analyzed for CS, PDHA1, CPT1B, and Glut 4 mRNA expression as described for Fig. 1. The linear correlations were established by regression analysis of the Ct value for the individual genes normalized to 18S and plotted in the six possible combinations. The square symbols represent the values from post-muscle unloading, while the diamonds represent the baseline values.
shown). We found evidence that PGC-1α and PGC-1δ transcript abundance was coregulated across all human muscle samples (Fig. 5B). For PGC-1α to be responsible for human skeletal muscle mitochondrial gene expression it would be logical for PGC-1α mRNA abundance to be related to the transcript abundance of a number of oxphos-related genes in human skeletal muscle. In the present analysis we were able to demonstrate that PGC-1α transcript abundance was related to nine nuclear-and mtDNA-encoded genes (Table 1), supporting the idea that PGC-1α regulates oxphos gene expression in human skeletal muscle.

Discussion

In the present study we demonstrate that a coordinated down-regulation of oxphos genes, including regulators of mitochondrial DNA transcription and replication, occurs in human skeletal muscle following a 5-week period of muscle inactivity. This response is generally consistent with the coordinated down-regulation of mitochondrial genes previously reported in Type 2 diabetes patients [16,20]. Our data would suggest that physical inactivity may contribute to the decreased PGC-1α expression in patients with diabetes [13,16,20], while reduced NRF1 and PGC-1δ (which were up-regulated and unchanged, respectively, in the present study) appear more disease specific. Although inactivity in humans had previously been associated with reduced mitochondrial enzyme capacity, this is the first study to identify which components of the mitochondrial biogenesis pathway are specifically regulated in response to muscle inactivity in humans.

Two large transcriptome analysis studies [16,20] followed on from epidemiological evidence that genetic variation in PGC-1α is related to the development of Type 2 diabetes [3]. Utilizing qPCR and microarrays the investigators proposed that skeletal muscle insulin resistance involved a coordinated down-regulation of oxphos genes as a consequence of disrupted PGC-1α and NRF1 signaling [16,20] and potentially of reduced PGC-1δ [13]. Numerous in vitro and transgenic studies from the same laboratories have strongly reinforced this view [4,7,12] and led to the proposal that PGC-1α plays a
primary role in the development of diabetes [19] and therefore represents a therapeutic target for drug development [4,15]. Importantly, the metabolic consequences of PGC-1α and PGC-1β expression are distinct, with PGC-1α promoting a more uncoupled phenotype, while PGC-1β appears to promote gene expression that favors coupling of oxphos in muscle cells [26] such that selective modulators are presumably required.

In the present study we produced the first comprehensive analysis of key regulators of mitochondrial biogenesis in human skeletal muscle in response to muscle activation levels. In general we provide evidence that in humans PGC-1α is related to the expression of many oxphos and mitochondrial substrate delivery genes (Table 1). This supports the idea that PGC-1α expression is a key physiological regulator of tissue oxidative capacity in human skeletal muscle (or that a common factor regulates all the aforementioned genes). While PGC-1α and PGC-1β mRNA content was regulated in a consistent manner across all the muscle samples, PGC-1β was not down-regulated by inactivity alone. This implies that the reduction in PGC-1α expression in diabetes patients may partly reflect lack of physical activity as these patients are historically known to be inactive. In contrast it is clear that muscle inactivity per se has distinct effects on NRF1 and PGC-1β expression and that lack of physical activity is not a compelling explanation for the diabetes-related changes observed for NRF1 and PGC-1β. Loss of PGC-1β expression in diabetes patients may, based on muscle cell studies, promote energy conservation potentially contributing to obesity [26].

The genuine molecular nature of the proposed mitochondrial defect in insulin resistance and diabetes has eluded clear identification in humans. What is clear is that the nature of the “mitochondrial defect” differs across different organs. For example, it transpires that increased PGC-1α activity may contribute to the failure of insulin to suppress postprandial hepatic glucose output [11], while PGC-1α expression also appears to be elevated, not suppressed, in glucose-insensitive pancreatic β cells—actively contributing to impaired insulin secretion [31]. It has recently been demonstrated that in two separate European populations, no relationship between PGC-1α Gly482Ser polymorphism and diabetes exists [27], which makes sense if the PGC-1α role varies, in opposite directions, in the various insulin-sensitive organs. If PGC-1α changes in skeletal muscle are secondary to inactivity then perhaps altered expression of NRF1 and/or PGC-1β contributes to the abnormal muscle mitochondrial metabolism and lipid storage observed in diabetes. Furthermore, it is not unusual to observe an increase in mitochondrial gene transcripts when a primary mitochondrial defect exists within the mitochondrial respiratory chain [8] as the cell attempts to compensate for the “primary” defect. Indeed, we believe that it is unlikely that a single mitochondrial-related defect underlies the mitochondrial phenotype observed in diabetes patients. This would also lead one to conclude that the most effective method for selectively stimulating mitochondrial growth in skeletal muscle may be a program of physical therapy, which, depending on the intensity, frequency, and timing of the posttraining glucose tolerance test, reduces skeletal muscle insulin resistance in humans [10,23].

![Figure 5](image-url)

**Fig. 5.** (A) The effects of 5 weeks of inactivity on the mRNA levels of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and PGC-1β in human skeletal muscle. Total RNA was prepared and analyzed for PGC-1α and PGC-1β mRNA expression together with 18S as an internal standard, using real-time PCR with an ABI 7700 system. Target genes were normalized to 18S through subtraction (ΔΔCt, methods). Data are presented as means ± SEM, given as fold change from baseline (n = 6). *p < 0.05. (B) The interrelationship between the mRNA levels of PGC-1α and PGC-1β. Total RNA was prepared and analyzed for PGC-1α and for PGC-1β mRNA expression as described for panel A. The linear correlations were established by regression analysis of the Ct value for the individual gene normalized to 18S and plotted. The square symbols represent the values from post-muscle unloading, while the diamonds represent the baseline values.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Correlation coefficient</th>
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<tr>
<td>CS</td>
<td>0.59</td>
</tr>
<tr>
<td>PDHA1</td>
<td>0.65</td>
</tr>
<tr>
<td>CPT1B</td>
<td>0.73</td>
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<tr>
<td>GLUT4</td>
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<td>MT-CYB</td>
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<tr>
<td>COXIV</td>
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<tr>
<td>SDHA</td>
<td>0.41</td>
</tr>
<tr>
<td>UCP3</td>
<td>0.76</td>
</tr>
</tbody>
</table>

**Table 1**

**Linear relationship between PGC-1α and oxphos gene expression**

Skeletal muscle expression of the gene transcripts citrate synthase (CS), pyruvate decarboxylase (PDHA1), carnitine palmitoyltransferase Type 1 (CPT1B), glucose transporter 4 (GLUT4), cytochrome b of complex III (MT-CYB), NADH dehydrogenase subunit 4 (MT-ND4), cytochrome c oxidase subunit 1 (MT-CO1), cytochrome c oxidase subunit 4 (COXIV), succinate dehydrogenase (SDHA), and uncoupling protein 3 (UCP3) were measured and values were corrected to 18S. Relationships were profiled using linear regression (n = 12).
contrast, developing a muscle-specific “activator” of the PGC-1α pathway may be extremely challenging, as PGC-1α does not belong to a protein class that is easily amenable to pharmaceutical manipulation by a drug-like molecule [9], and ultimately exercise therapy is probably a simpler option, with greater overall benefits.

In conclusion, we provide the first evidence that in humans, inactivity results in a coordinated down-regulation of PGC-1α–related oxphos genes and components of the mitochondrial replication system at the transcript level. We also demonstrate a surprising increase in NRF1 following muscle inactivity, contrasting with the decreased NRF1 observed in diabetes patients, and relatively unchanged expression of PGC-1β (again contrasted with a decline in diabetes patients). This suggests that reduced PGC-1α expression in the skeletal muscle of Type 2 diabetes could be secondary to inactivity. Clearly our initial study is limited by both sample size and the lack of characterization of local muscle insulin sensitivity (whole body glucose tolerance should have remained unchanged). Future studies should focus on examining muscle inactivity-induced changes in the mitochondrial proteome and contrast this with observations made from insulin-resistant and diabetic patients linking changes to directly assessed muscle insulin sensitivity. When doing so it is probably important to keep in mind that “insulin resistance” is more than impaired glucose uptake and several biochemical markers of insulin’s action should be measured. Finally, we would argue that quantitative assessment of patient characteristics is essential for future transcriptome characterization of muscle phenotype in diabetes patients, especially as “new” targets are introduced as having therapeutic merit. Indeed, when considering environmental and genetic contributions to metabolic disease [13] it is definitely worth considering that the tendency to participate in physical activity may well be a component of both the environmental and the familial influences on mitochondrial gene expression in human skeletal muscle.

Research design and methods

Inactivity protocol and subject characteristics

Six healthy adult subjects, four men and two women, volunteered for this study. At the time of being recruited the subjects did not participate in any regular training programs. A written consent was given after the procedures and risks associated with participation in the study had been explained. The experimental protocol was approved by the Institutional Review Board at the University of Arkansas for Medical Sciences (Little Rock, AR, USA) and the study was conducted in accordance with the Declaration of Helsinki as revised in 2000. The subjects were between 30 and 56 years of age, with a body fat range of 9–25% (calculated from biceps, triceps, suprailliac, and front thigh skinfolds, using a Harpenden caliper). The subjects had a body mass range of 57–91 kg and a height range of 165–185 cm. The body mass and calculated percentage body fat did not change during the study period. Volunteers were subjected to unilateral unloading of the lower limb for 5 weeks as previously described [1,2,6], refraining from any physical activity. The local muscle inactivity resulted in an ~12% reduction in muscle cross-sectional area over the 5-week period. The physiological results and global changes in muscle volume from this study have been reported elsewhere [28]. Subjects lived at home and carried out their normal work tasks throughout the experimental period. To ensure compliance all subjects were interviewed by one of the investigators on a daily basis. Skin temperature and circumference were measured midcalf at least twice weekly during scheduled visits to the laboratory. The unloaded calf consistently demonstrated a 2-to 3-cm greater circumference than the weight-bearing limb, while calf temperature was ~2–3°C lower (p < 0.05) in the unloaded leg compared with the weight-bearing leg during the study, indicating good compliance [28]. As prophylaxis to venous thromboembolism, subjects received a daily dose of 325 mg aspirin. In addition, they wore knee-length, medical-graded (Class 2) compression stockings (BSN-Jobst, Inc., Rutherford College, NC, USA) during any ambulatory activity.

Quantitative real-time RT-PCR

Total RNA was isolated by the use of TRIZol (Invitrogen); however, the RNA remained contaminated with DNA and this created a problem when measuring mitochondrial mRNA transcripts. To resolve this we treated each RNA sample with a DNase treatment using the RNeasy Mini Kit column procedure (Qiagen), followed by quantification using a spectrophotometer. Two micrograms of RNA was reverse transcribed by reverse transcription reagents (Applied Biosystems) using random hexamer primers, in a total volume of 60 μl. Amplification aliquots contained 5 μl of the sample cDNA, 2× TaqMan Universal PCR Master Mix, and an optimized concentration of each primer, added to a final volume of 25 μl, and were measured in triplicate. The mix for 18S rRNA was prepared according to the manufacturer’s recommendation (Applied Biosystems) and run in triplicate at 1:1000 dilution. Thermal cycling conditions included 2 min at 50°C, 10 min at 95°C, and then 45 cycles each of 15 s at 95°C and 1 min at 65°C. Oligonucleotide primers were designed using a primer design center (http://www.probelibrary.com) and synthesized by Invitrogen (Stockholm, Sweden). The primer sequences will be provided on request. The primers were designed to amplify across exon–exon boundaries to avoid amplification of genomic DNA. This was not possible for mtDNA-encoded genes or CS as they are intronless. The 18S rRNA was selected as an endogenous control to correct for potential variations in RNA loading since there are no established low-abundance housekeeping genes for this experimental paradigm. The ΔΔCt method [30] was used to calculate relative changes in mRNA abundance. The threshold cycle (Ct) for 18S was subtracted from the Ct for the target gene—to adjust for variations in mRNA/cDNA generation efficacy. The preintervention values reflect baseline gene expression levels and were subtracted from the postexercise value to calculate the increase or decrease in mRNA abundance. The changes are described using the “fold changes” convention, in which a
twofold increase is a doubling and a twofold decrease is a reduction by half. All data are means ± SEM. Following ANOVA for global changes in gene expression, individual gene expression data were compared using a paired-test, with precise p values being presented. For the purpose of discussion a p < 0.05 was considered significant. Linear regression was utilized to determine coexpression relationships across genes.

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References


