Exploring the role of PGC-1 alpha in defining myonuclear domain size

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Running head: PGC1-α and Myonuclear organisation

Keywords:
- PGC1-α
- Myonuclear organisation
- Myonuclear domain
- Myofibre

Total number of Tables: 3
Total number of Figures: 1

Contract grant sponsor: Medical Research Council UK
Contract grant number: MR/N002768/1
ABSTRACT

Myofibres are multinucleated cells, with each nucleus controlling the protein synthesis in a finite volume of cytoplasm termed myonuclear domain (MND). What determines MND size remains unclear. In the present study, we aimed to test the hypothesis that the level of expression of the transcriptional coactivator PGC-1α and subsequent activation of the mitochondrial biogenesis are major contributors. Hence, we used two transgenic mouse models with varying expressions of PGC-1α in skeletal muscles. We then isolated myofibres, membrane-permeabilised them and analysed the 3D spatial arrangements of myonuclei. Our results showed, when PGC-1α is over-expressed, MND volume decreases; whereas, when PGC-1α is lacking no change occurs. This directly demonstrates that PGC-1α and mitochondrial number regulates the size of MNDs in myofibres. This also suggests that PGC-1α facilitates the addition of new myonuclei in order to reach MND volumes that optimise the regulation of gene products related to mitochondria-related proteins.
INTRODUCTION

Skeletal muscle is a complex but highly-ordered structure composed of myofibres that can be several centimetres long and a hundred micrometres wide. Such long and large myofibres cannot be supported by only one myonucleus (Edgerton and Roy, 1991). Individual myofibres can encompass several hundred myonuclei, with each myonucleus controlling the gene products in a finite volume of cytoplasm termed myonuclear domain (MND) (Hall and Ralston, 1989; Ralston and Hall, 1992). MND sizes are constant during growth or senescence (Gundersen and Bruusgaard, 2008). However, average MND volumes tend to vary between myofibres expressing distinct myosin heavy chain isoforms (Bruusgaard et al., 2003; Bruusgaard et al., 2006). MNDs are smaller in type I myofibres than in type II muscle cells. Despite this clear difference, it remains unclear whether this phenomenon is directly related to the myosin heavy chain isoform composition or to other closely related parameters such as oxidative capacity and mitochondrial content (Tseng et al., 1994). As type I myofibres contain a much higher concentration of mitochondria than type II muscle cells, and as all myonuclei produce mRNA at the same rate, Moyes & LeMoine have suggested that smaller MNDs might be a direct consequence of an increased demand and production of bioenergetic enzymes per se (Moyes and LeMoine, 2005) rather than different myosin heavy chain expression. In the present study, we aimed to experimentally confirm this statement.

Mitochondrial biogenesis and production is controlled by the transcriptional coactivator, peroxisome-proliferator-activated receptor-γ coactivator 1-α (PGC-1α) (Arany et al., 2005; Lin et al., 2005). Indeed, skeletal muscle-specific PGC-1α knockout mice (MKO) have decreased number and function of mitochondria (Perez-Schindler et al., 2013) concomitant with a shift from oxidative toward glycolytic myofibres (Handschin et al., 2007) but without any clear shift of the myosin heavy chain isoform composition at the protein level (Perez-Schindler et al., 2013). On the other hand, muscle-specific PGC-1α over-expression mice (MCK) have stimulated activation of mitochondrial genes, increased mitochondrial density and oxidative capacity but no major transition in myosin heavy chain expression at the protein level (Perez-Schindler et al., 2013). Hence, in the present study, we tested the hypothesis that PGC-1α expression and subsequent modulation of mitochondrial number are key regulators of myonuclear organisation. In other words, we suggested that MND size would be increased in MKO mice and decreased in MCK rodents.
MATERIALS AND METHODS

Animals
Peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) muscle-specific transgenic mice (MCK) and PGC-1α muscle-specific knockout mice (MKO) were generated as previously described (Perez-Schindler et al., 2013). Age-matched MCK, MKO and wild-type (WT) mice were sacrificed by CO₂ inhalation and Extensor Digitorum Longus (EDL) and diaphragm (DIA) muscles were dissected. The Animal Experimentation Ethics Committee of The University of Basel approved all animal procedures. It should be noted that four 7-month old animals were used per “genotype”.

Myofibre Permeabilisation
Muscle samples were placed in relaxing solution at 4°C. Bundles of approximately 50 myofibres were dissected free and then tied with surgical silk to glass capillary tubes at slightly stretched lengths. They were then treated with skinning solution (relaxing solution containing glycerol; 50:50 v/v) for 24 hours at 4°C, after which they were transferred to -20°C (Frontera and Larsson, 1997).

Myonuclear organisation of single myofibres
On the day of experiment (within two weeks after the permeabilisation procedure), bundles were detached from the capillary tubes, transferred to a relaxing solution, and single myofibres were dissected. Arrays of approximately nine myofibres were prepared at room temperature (RT). For each myofibre, both ends were clamped to half-split copper meshes designed for electron microscopy (SPI G100 2010C-XA, width, 3 mm), which had been glued to cover slips (Menzel-Gläser, 22 x 50 mm, thickness 0.13-0.16 mm). Myofibres were mounted at a fixed sarcomere length of ≈2.20 μm. This was a prerequisite for exact determination of myonuclear spatial organization as it allowed accurate comparisons between myofibres (Cristea et al., 2010; Qaisar et al., 2012).

At RT, arrays were subsequently subjected to actin staining (Alexa Fluor Phalloidin 488, Molecular Probes, A12379) and myonuclear staining (DAPI, Molecular Probes, D3571). Images were taken using a confocal microscope (Zeiss Axiovert 200, objective x20). To visualise myofibres in 3D, stacks of 100 images were acquired (1 μm z increments) and analysed with a custom-made Matlab programme.
Solution
Relaxing solution contained 4 mM Mg-ATP, 1 mM free Mg\(^{2+}\), 20 mM imidazole, 7 mM EGTA, 14.5 mM creatine phosphate, and KCl to adjust the ionic strength to 180 mM and pH to 7.0. The concentration of free Ca\(^{2+}\) was \(10^{-9.00}\) M.

Fluorescent labelling
Further staining was achieved using Pax7 (primary antibody: DSHB, AB 528428; secondary antibody: Alexa Fluor® 488, Novex, A-11001).

Statistical analysis
Data are presented as mean ± standard error of the means (SEMs). A total of 252 myofibres were isolated and tested (Table 1 and figure 1). Because many myofibres were studied for each muscle, animal and genotype, a specific model was used to statistically analyse the data (Ochala et al., 2011). This model is based on an analysis of variance including the following factors: “genotype”, “muscle” and “animal” (where “animal” was nested within “genotype”). The only interaction terms that were judged to be of importance and therefore included were that between “genotype” and “muscle”. The SAS JMP software was used for the generation of such model.
RESULTS

The most common method used to count the number of myonuclei and estimate the MND size is muscle cross-sections. This method is accompanied by a number of technical limitations questioning the reliability of 2D data (Qaisar and Larsson, 2014). Here, we applied a 3D approach to precisely define the spatial arrangement of myonuclei.

Increase in myonuclei number and decrease in MND size when PGC-1α is over-expressed

We counted the number of myonuclei per fibre length and observed that, in the EDL muscle, the overall number of nuclei per mm fibre length was significantly greater in MCK and MKO than in WT (Table 2). On the contrary, in the diaphragm muscle, no difference was detected (Table 2).

As previously observed (Qaisar et al., 2012), a linear correlation existed between the volume of MNDs and myofibre cross-sectional area (CSA) in all muscles and genotypes (Figure 1). To account for this, the MND to myofibre CSA ratio was calculated. Thus, in the EDL muscle, at any given CSA, MND was significantly smaller in MCK than in WT (Table 2). In the diaphragm muscle, we did not notice any difference (Table 2).

The average MND provides valuable information on the average volume controlled by each myonucleus; however, it does not estimate the spatial arrangement of myonuclei. To precisely define the latter, we calculated nearest neighbour distances (NNs) using the 3D coordinates of individual myonuclei within each single myofibre. At any given CSA, in the EDL muscle, NN was significantly smaller in MCK than in WT (Table 2). In the diaphragm muscle, we did not observe any difference (Table 2). To evaluate the variability in the spatial arrangement of myonuclei, we used the standard deviation related to NNs within each single myofibre (SD-NN) (Qaisar et al., 2012). In the present study, SD-NN was not affected by the various genotypes (Table 2).

Change in myonuclear shape when PGC-1α is over-expressed or lacking

We measured a number of morphological parameters (Figure 1). In the EDL muscle, the aspect ratio was significantly smaller in MCK than in WT indicating that nuclei were rounder (Table 3). In the diaphragm muscle, on the other hand, the aspect ratio and area were
significantly greater in MKO than in WT suggesting that nuclei were bigger and longer (Table 3).
DISCUSSION

In the present study, in order to investigate whether PGC-1α directly regulates MND volume in myofibres, we used two transgenic mouse models with varying skeletal muscle expressions of the transcriptional coactivator PGC-1α known to be a key mediator of mitochondrial biogenesis and number (Handschin et al., 2007; Perez-Schindler et al., 2013). Our results demonstrate that a relation between these two entities exists but is more complex than initially hypothesised.

PGC-1α regulates MND size

In EDL muscle where PGC-1α is over-expressed, we observed rounder nuclei (Table 3) as well as an increase in the amount of myonuclei and subsequent decreases in MND volume and NN (Table 2). Hence, this confirms what others have suggested without any experimental evidence (Moyes and LeMoine, 2005), that is, PGC-1α and mitochondrial number defines MND size independently of myosin heavy chain composition. Interestingly, the incorporation of new myonuclei, to reach MND volumes that would optimise the regulation of gene products related to mitochondria-related proteins, was not associated with any significant myonuclear disorganisation (SD-NN, Table 2). This probably means that inter-nuclear communication and coordination of bioenergetic and contractile protein expressions is not deteriorated (Table 3).

In diaphragm muscle, on the other hand, PGC-1α over-expression did not lead to any noticeable changes (Tables 2 and 3). The reasons are unclear. One potential explanation lies in the design of the transgenic line overexpressing PGC-1α. This was driven by the creatine kinase promoter, which is more potent in glycolytic (e.g. EDL) compared to oxidative (e.g. diaphragm) muscles (Arnold et al., 2014; Lin et al., 2002). Another potential explanation could originate from EDL and diaphragm having distinct morphological, metabolic and contractile properties. Indeed, because of various functional demands (Schiaffino and Reggiani, 2011), respiratory myofibres have a greater overall mitochondria volume density (Gamboa and Andrade, 2010) and smaller average MND size (Verheul et al., 2004) when compared with limb muscle fibres. What causes such MND size difference? Redshaw and co-workers have shown that satellite cell proliferation and fusion rates differ between diaphragm and limb muscles of mammals (Redshaw et al., 2010). Satellite cells originating from the
diaphragm proliferate less but differentiate more (Redshaw et al., 2010). This may allow a better incorporation of new myonuclei in adult respiratory muscle. The volume of MNDs may then be optimal to cover for the high demands in contractile and bioenergetic proteins. Increasing PGC-1α content in the diaphragm of MCK mice may not have the power to further activate the same downstream transcription factors that would further reduce MND size.

**Clinical implications**

Across species, PGC-1α content and mitochondrial biogenesis has been shown to decrease in response to the ageing process or to various disease states (Dillon et al., 2012). Over-expressing PGC-1α confers therapeutic benefits by notably improving mitochondrial biogenesis and number in various tissues and cells (Dillon et al., 2012). However, according to our current findings, such positive effect would be limited to limb muscles and less obvious for respiratory muscles known to be also altered during biological ageing and serious muscle disorders such as muscular dystrophies and congenital myopathies (Lindqvist et al., 2013).
ACKNOWLEDGMENTS

The authors have no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

CH and JO contributed to the conception and design of the work
JR, AP, YL, BC, CH and JO did the acquisition, analysis, and interpretation of data
JR, AP, YL, BC, CH and JO drafted the work and revised it critically
JR, AP, YL, BC, CH and JO approved the final version to be published
JR, AP, YL, BC, CH and JO agreed on all aspects of the work
References


TABLES

Table 1: Single myofibres studied

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<th></th>
<th>WT</th>
<th>MCK</th>
<th>MKO</th>
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<tbody>
<tr>
<td></td>
<td>DIA</td>
<td>EDL</td>
<td>DIA</td>
</tr>
<tr>
<td>Number of animals</td>
<td>4</td>
<td>4</td>
<td>4</td>
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<tr>
<td>Number of myofibres</td>
<td>29</td>
<td>49</td>
<td>22</td>
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Age-matched MCK (PGC-1α muscle-specific over-expression), MKO (PGC-1α muscle-specific knockout) and WT (wild-type) mice were killed. EDL (Extensor Digitorum Longus) and DIA (diaphragm) muscles were dissected. Single myofibres were isolated and tested.
Table 2: Myonuclear organisation

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>MCK</th>
<th>MKO</th>
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<tbody>
<tr>
<td></td>
<td>DIA</td>
<td>EDL</td>
<td>DIA</td>
</tr>
<tr>
<td>Nuclei number (count mm⁻¹)</td>
<td>34.00 ± 2.00</td>
<td>43.00 ± 2.00</td>
<td>* 32.00 ± 3.00</td>
</tr>
<tr>
<td>MND volume (µm³ x 10³)</td>
<td>21.13 ± 1.94</td>
<td>54.77 ± 3.42</td>
<td>* 20.79 ± 2.68</td>
</tr>
<tr>
<td>MND:CSA (A.U.)</td>
<td>35.26 ± 3.22</td>
<td>25.92 ± 1.24</td>
<td>* 42.13 ± 5.40</td>
</tr>
<tr>
<td>NN (µm)</td>
<td>30.00 ± 2.24</td>
<td>36.60 ± 1.00</td>
<td>* 30.12 ± 3.24</td>
</tr>
<tr>
<td>NN:CSA (A.U.)</td>
<td>0.057 ± 0.008</td>
<td>0.019 ± 0.001</td>
<td>0.075 ± 0.018</td>
</tr>
<tr>
<td>SD-NN (µm)</td>
<td>19.10 ± 3.34</td>
<td>10.40 ± 0.60</td>
<td>15.73 ± 3.63</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEMs. Age-matched MCK (PGC-1α muscle-specific over-expression), MKO (PGC-1α muscle-specific knockout) and WT (wild-type) mice were killed. EDL (Extensor Digitorum Longus) and DIA (diaphragm) muscles were dissected. Single myofibres were mounted and a broad range of 3D structural parameters were analysed. MND=myonuclear domain; NN=distance to nearest neighbour; SD-NN=standard deviation of the distance to nearest neighbour. * means that a significant difference (p<0.05) was observed in comparison to WT for the same muscle. + represents a significant difference (p<0.05) with diaphragm for the same genotype.
Table 3: Myonuclear morphology

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>MCK</th>
<th>MKO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DIA</td>
<td>EDL</td>
<td>DIA</td>
</tr>
<tr>
<td>Aspect ratio (A.U.)</td>
<td>1.92 ± 0.22</td>
<td>2.87 ± 0.32 *</td>
<td>1.93 ± 0.23</td>
</tr>
<tr>
<td>Area (µm²)</td>
<td>60.65 ± 5.41</td>
<td>68.00 ± 6.12</td>
<td>73.00 ± 5.70</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEMs. Age-matched MCK (PGC-1α muscle-specific over-expression), MKO (PGC-1α muscle-specific knockout) and WT (wild-type) mice were killed. EDL (Extensor Digitorum Longus) and DIA (diaphragm) muscles were dissected. Single myofibres were mounted and a broad range of 3D structural parameters were analysed. * means that a significant difference (p<0.05) was observed in comparison to WT for the same muscle. + represents a significant difference (p<0.05) with diaphragm for the same genotype.
FIGURE LEGENDS

**Figure 1: Typical myonuclei and isolated myofibres**
Single myofibres were isolated from age-matched MCK (PGC-1α muscle-specific over-expression), MKO (PGC-1α muscle-specific knockout) and WT (wild-type) rodents. They were then stained for actin (Alexa Fluor Phalloidin 488, green) and myonuclei (DAPI, blue). Scatterplots of MND volume versus myofibre CSA for cells isolated from MCK (red, PGC-1α muscle-specific over-expression), MKO (green, PGC-1α muscle-specific knockout) and WT (blue, wild-type) rodents. All regression lines demonstrated a statistically significant correlation (p<0.05).