Mitochondrial bioenergetics in ocular fibroblasts of two myasthenia gravis cases

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Abstract

Myasthenia gravis (MG) is a rare, treatable, antibody-mediated disease characterized by fatigable muscle weakness of extraocular muscles (EOMs) and non-ocular skeletal muscles. The antibodies are directed against muscle-endplate proteins, most frequently the acetylcholine receptor (AChR) alpha-subunit. Although most MG patients respond to immunosuppressive treatment, some individuals, frequently with African-genetic ancestry, develop treatment-resistant ophthalmoplegia (OP-MG). Although the underlying pathogenetic mechanisms of OP-MG remain unknown, experimental rodent models of MG showed upregulation of genes involved in oxidative metabolism in muscles. EOMs are highly dependent on oxidative metabolism. We opportunistically sampled EOM-tendons of two rare OP-MG patients (and non-MG controls) undergoing re-alignment surgery, and established ocular fibroblast cultures. Metabolic assays were performed on these live cells to assess real-time differences in energy metabolism. To study the cellular bioenergetic profiles in the context of MG, we exposed the cultures to homologous 5% MG sera for 24 h, vs. growth media, from two independent MG patients (with circulating AChR-antibodies) and five controls without MG, and estimated the fold change in oxygen consumption rates in response to three compounds which inhibit different mitochondrial chain complexes. Quantitative PCR (qPCR) was performed in cells before and after MG sera exposure, to assess transcript levels of mitochondrial genes, PDK4, ANGPTL4 and UCP3, which were altered in experimental MG. In response to the mitochondrial stressors, basal oxidative metabolism parameters were similar between OP-MG and control fibroblasts (p = 0.81). However, after exposure to MG sera, bioenergetic parameters (oxygen consumption rate as an indicator of oxidative phosphorylation; extracellular acidification rate as an indicator of glycolysis), were induced to higher levels in OP-MG fibroblasts compared to controls (2.6-fold vs 1.5-fold; p = 0.031) without evidence of mitochondrial insufficiency in the OP-MG ocular fibroblasts. In support of the bioenergetic responses to the same MG sera, gene transcripts of PDK4 and ANGPTL4 in ocular fibroblasts also showed significant upregulation (p < 0.041), but similarly in OP-MG and control cases. Taken together we showed similar basal and metabolic adaptive responses after exposure to mitochondrial inhibitors in ocular fibroblasts derived from OP-MG cases and controls, although the OP-MG cells showed greater activation in response to MG conditions. These pilot results in orbital-derived tissues provide support for myasthenic-induced changes in cellular metabolism and evidence that orbital fibroblasts may be useful for dynamic bioenergetic assessments.

1. Introduction

Myasthenia gravis (MG) is a rare, but treatable antibody-mediated disease which is characterized by fatigable muscle weakness. The pathogenic antibodies are directed against specific muscle-endplate proteins, most frequently the acetylcholine receptor (AChR) alpha-

Abbreviations: AChR, acetylcholine receptor; EOM, extraocular muscle; MG, myasthenia gravis; OP-MG, ophthalmoplegic MG; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; ECAR, extracellular acidification rate; OCR, oxygen consumption rate; max resp, maximal respiration; SRC, spare respiratory capacity; NMOC, non-mitochondrial oxygen consumption; SMA, smooth muscle actin; Cq, quantitation cycle; SEM, standard error of the mean; SD, standard deviation; PL, proton leak; EAMG, experimental autoimmune myasthenia gravis.

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subunit. MG occurs with similar frequency world-wide (Mombaur et al., 2015). The extraocular muscles (EOMs) frequently manifest weakness first in MG, but typically respond to immunosuppressive treatment much like the non-ocular skeletal muscles. However, some individuals with MG, frequently with African-genetic ancestry, develop treatment-resistant ophthalmoplegia (OP-MG) (Heckmann et al., 2007; Europa et al., 2018). In the most severe cases, all EOMs and lid elevators remain paralysed and patients are left with substantial morbidity (Findlay et al., 2016). The pathogenesis of the OP-MG subphenotype remains unknown.

Mitochondrial function is particularly critical in EOMs because they require a constant energy supply to sustain high firing rates. There is histopathological evidence that the EOMs and skeletal muscles of MG cases may be subject to mitochondrial stress although it is unclear whether this is a primary disease mechanism or secondary to poor contractility (Europa et al., 2019). In experimental autoimmune MG (EAMG), genes from pathways involved in oxidative metabolism were found to be upregulated in muscles, including EOMs (Zhou et al., 2014; Kaminski et al., 2016). Genetic association studies and gene-profiling in patient-derived myocytes, point to mitochondrial metabolism pathways being involved in OP-MG pathogenesis (Nel et al., 2019a; Nel et al., 2019b). As the EOMs are highly dependent on oxidative metabolism (Porter and Baker, 1996), they may be particularly vulnerable to alterations of oxidative metabolism that may occur in MG.

The EOMs comprise a unique muscle allotype (Porter et al., 2006). Similarly, the perimysial ocular fibroblasts of EOMs have a phenotype that is distinct from fibroblasts associated with limb muscle, and play homeostatic roles in the orbital microenvironment (Kusner et al., 2010). Therefore, we opportunistically sampled EOM myotendons from individuals undergoing ocular re-alignment surgeries and used these to establish ocular fibroblast cultures. As skin-derived fibroblasts were used to infer altered bioenergetics in neurodegenerative disorders (Sonntag et al., 2017), we postulated that biochemical assays of ocular fibroblast cultures, with/without MG sera, may provide useful information regarding mitochondrial function in the understanding of OP-MG pathogenesis. We performed metabolic assays on these ocular fibroblasts in real time. Quantitative expression analysis of three genes, which were previously induced in the muscles of rodents during EAMG, was performed to assess the effect of MG sera on the transcriptome of ocular fibroblasts in vitro.

2. Materials and Methods

2.1. Patients

In highly selected MG cases with treatment-resistant ophthalmoplegia and diplopia, ocular re-alignment surgery may be considered (Heckmann and Nel, 2018; Rautenbach et al., 2017). We acquired resected EOM-myotendons from two OP-MG patients with acetylcholine receptor (AChR) antibody positive generalized MG; a female aged 27, on azathioprine and low dose prednisone and a female, aged 42 years on methotrexate weekly. For comparison, specimens from newly-diagnosed AChR-antibody positive MG patients (range 14–65 years), with EOM involvement at the time of sampling and without comorbid disease or infections, who had not yet been treated with immune therapies. The sera were stored at −20 °C before long-term preservation in liquid nitrogen (12–18 months). Cells were used up to passage 9.

2.3. MG stimulus in vitro

We exposed the cultures to growth medium supplemented with 5% MG sera for 24 h. These homologous sera samples had previously been obtained from newly-diagnosed AChR-antibody positive MG patients (range 14–65 years), with EOM involvement at the time of sampling and without comorbid disease or infections, who had not yet been treated with immune therapies. The sera were stored at −20 °C (Nel et al., 2019a).

2.4. Phenotyping ocular fibroblasts

Flow cytometry (BD Accuri C6 flow cytometer; Becton Dickinson Biosciences) was used to quantify fibroblast and myofibroblast populations using immuno labelling with vimentin and α-smooth muscle actin (α-SMA) antibodies respectively. Data was analysed using FlowJo (Tree Star Inc). Gating excluded dead cells, debris and doublets.

2.5. Analysis of mitochondrial respiration and glycolysis

The Seahorse XF96 Analyzer (Seahorse Bioscience, Agilent technologies) was used to perform bioenergetic assays by measuring extracellular flux to calculate the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in adherent cells (Divakaruni et al., 2014). This was performed at basal conditions and after incubation in growth medium supplemented with 5% MG sera for 24 h.

All biological samples of ocular fibroblast cultures (passage 6–8) were grown and harvested using 0.25% trypsin-EDTA. Ocular fibroblasts were seeded in 96-well microwells at a cell density of 40 × 10^3/100 μl in triplicate wells per condition (with/without MG sera) for each independent experiment. All cells (with/without MG sera) were all plated on the same plate as recommended (Yepez et al., 2018). The cells were allowed to settle for 24 h and then inspected to insure a confluent monolayer before continuing with the experiment. On day 2, the cells were treated with fresh growth medium or 5% MG sera. After 24 h the cells were washed and replaced with 200 μl/well Seahorse XF RPMI base medium supplemented with 1% glucose (1.0 M), 1% pyruvate (100 mM) and L-glutamine (200 mM) per the manufacturer’s guidelines. The fibroblast cultures were then incubated for 1 h in a non-CO₂
The day prior to each experiment, the cartridge was prepared with calibrant and incubated in a non-CO\(_2\) incubator. On the day of the experiment, the compounds (oligomycin, FCCP \(\pm\) rotenone/antimycin A) were reconstituted using Seahorse XF base medium as specified in the manufacturer’s guidelines and systematically loaded into the cartridge injection ports (20 \(\mu\)L/port). The FCCP concentration had been optimised using skin fibroblasts, and although ocular fibroblasts were used in these experiments, similar responses to FCCP were observed and were consistent between repeated experiments.

The Phenotype-test is an overall qualitative assessment of the metabolic state of the cell by comparing OCR and ECAR measurements before and after the simultaneous injection of a ‘mix’ of oligomycin (1.0 \(\mu\)M) and carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (1.5 \(\mu\)M) (Leipnitz et al., 2018). Together, these stressors inhibit ATP synthase and uncouple respiration resulting in maximal OCR and ECAR rates which is referred to as the “stressed phenotype”. A metabolic shift from quiescence to aerobic, energetic or glycolytic phenotypes indicates increased requirements for ATP for processes such as protein synthesis (Divakaruni et al., 2014).

The Mitostress test was independently performed, comprising sequential injection of ‘stressors’ compounds (oligomycin, FCCP, and Rotenone/Antimycin A) to the Seahorse XF cartridge. The basal respiration of the cells was first measured. After the sequential injection of the ‘stressor compounds’ oligomycin (1.0 \(\mu\)M), FCCP (1.5 \(\mu\)M) and Rotenone/Antimycin A (0.5 \(\mu\)M) the maximal respiration, spare respiratory capacity (SRC), ATP respiration, proton leak and non-mitochondrial oxygen consumption rate of the cells were measured. Addition of oligomycin (inhbits adenosine ATP-synthase), allows for measurement of ATP-linked oxygen consumption and proton leak. Maximal respiration is calculated after FCCP which uncouples respiration from ATP production and is used to calculate the spare respiratory capacity, a marker of respiratory reserve SRC (Brand and Nicholls, 2011). A combination of rotenone and Antimycin A inhibits complexes I and III of the electron transport chain respectively, and allows the calculation of non-mitochondrial oxygen consumption (Divakaruni et al., 2014). To measure mitochondrial sufficiency in live-cell metabolic assays, we calculated the cell respiratory control ratio (CRCR) as proposed by Brand and Nicholls. The CRCR is calculated as the ratio of maximal respiration to proton leak (Brand and Nicholls, 2011).

2.6. Quantitative PCR

In order to confirm that MG sera influences the intracellular environment of cultured ocular fibroblasts, we studied its effect on the expression of genes related to mitochondrial pathways known to be altered in EAMG (Kaminski et al., 2016), and which previously showed altered responses in myocytes in response to MG sera (Nel et al., 2019a).

Using qPCR, we profiled three genes in duplicate samples, before and after exposure to MG sera (5% \(\times\)24 hours) using sera from three homologous donors as described in 2.3. RNA was extracted using the Roche High Pure RNA kit. RNA concentrations were > 80 ng/\(\mu\)L (spectrophotometric ratios of A\(_{260}/A_{280}\) >1.8; A\(_{260}/A_{230}\) >1.7, Nanodrop v.1000). The RNA integrity values were 7–9 (Agilent Bioanalyzer). For each sample, 300 ng RNA was reverse transcribed using Promega ImProm-II Reverse Transcriptase. Quantitative PCR was performed using the KAPA SYBR FAST qPCR Master Mix (Sigma-Aldrich) and the Lightcycler 2.0 (Roche Life Science) using QuantiTect primer assays (Qiagen). As previously described, we normalized using the average quantitation cycle (C\(_{q}\)) values of RPLP0 and CSNK2A2 as they were found to be stable by geNorm (Etschmann, Wilcken et al. 2006) and Bestkeeper (Pfaffl, Tichopad et al. 2004) methods across all cultures (including with MG sera (\(p > 0.61\)) (Nel et al., 2019a). Data analysis was performed by the \(\Delta\Delta\text{Cq}\) method (Schmittgen and Livak, 2008).

2.7. Data analysis

Metabolic assays using 2 OP-MG samples and 5 control samples were performed in triplicate. The Seahorse XF Report Generator was used to generate the OCR and ECAR values from the raw data. Absolute OCR and ECAR values (without and with MG sera) were compared between OP-MG and control ocular fibroblast by unpaired t-tests. Responses of cell cultures to MG sera were calculated as fold change of MG sera vs untreated values for each experiment and examined with a t-test. The gene expression levels in response to MG sera (fold change of average values) were analysed with the Wilcoxon matched-pairs for non-parametric data, and log-transformed fold changes shown for graphical representation. P values ≤ 0.05 (2-tailed) were considered significant. Graphs were produced using Prism GraphPad 8.

3. Results

3.1. Cell cultures comprised ocular fibroblasts

Flow cytometry showed 98% (SD=2%) of cell were vimentin positive cells (not shown). Although a large proportion of the cells were also immunoreactive to α-SMA (mean 45%; SD=35%), indicating differentiation into myofibroblasts, the proportions were similar between OP-MG and control cultures (\(p = 0.38\)) and therefore distinction between metabolic responses of fibroblasts vs myofibroblasts were not interrogated in this study.

3.2. Mitochondrial bioenergetic function in ocular fibroblasts

In the Phenotype test under basal conditions (Fig. 1A), all the ocular fibroblasts showed similar states of quiescence (or resting states) in three independent experiments. After the addition of oligomycin/FCCP, a similar increase in OCR and ECAR was seen in both OP-MG and control ocular fibroblasts, again showing similar responses to the stressors.

3.3. MG sera induced relatively higher bioenergetic responses in OP-MG ocular fibroblasts

Experiments were performed to assess the cell cultures’ bioenergetic responses to the homologous MG sera: Phenotype tests with the addition of the “stressor mix” (Fig. 1B) and Mitostress tests in which the stressors were given sequentially (Fig. 2A). In response to 5% MG sera for 24 h compared to untreated cells (normal medium), the Phenotype tests showed that the OP-MG ocular fibroblasts became relatively more “energetic” than the controls with increased estimates of OCR and ECAR. The OCR measurement in response to MG sera were ≥ 2.6-fold increased in OP-MG fibroblasts compared to 1.5-fold in control (\(p = 0.031\)) (Fig. 1C). The ECAR showed a trend towards increased responses in OP-MG (1.8-fold) compared to controls (1.4-fold, \(p = 0.054\)) (Fig. 1D).

Similarly, the Mitostress assays showed an increase in cellular bioenergetics responses to MG sera exposure in the OP-MG ocular fibroblasts compared to controls (Fig. 2B). Although the control cell cultures’ responses were similar, the two independently-derived OP-MG fibroblasts cultures varied widely in their responsivity, with only ATP-linked respiration showing a consistent and significant ≥ 2 fold greater response (\(p = 0.034\)) to MG exposure, and non-mitochondrial oxygen consumption showed a trend towards consistently higher responses compared to controls (\(p < 0.129\)). The calculated cell respiratory control ratio i.e. maximal respiration/proton leak, which is an indicator of mitochondrial insufficiency (Brand and Nicholls, 2011), was similar in OP-MG and control fibroblasts at basal conditions and after MG sera exposure (\(p > 0.43\)) (Fig. 3).

Taken together, the results suggest that MG sera induced higher levels of oxidative metabolism and glycolysis and therefore an altered bioenergetic phenotype in OP-MG ocular fibroblasts compared to controls using three different MG sera.
3.4. MG sera induced gene expression in ocular fibroblasts

As MG sera induced dynamic changes in oxidative metabolism in the ocular fibroblasts, we used qPCR to confirm that MG sera affected gene transcript regulation of three genes regulating oxidative metabolism and known to be MG-responsive in EAMG muscle (PDK4, ANGPTL4 and UCP3). The basal expression levels of these genes were similar in all ocular fibroblast cultures, irrespective of their phenotype. In response to the same three MG sera used in the metabolic assays, we found a ~2-fold upregulation of PDK4 (p = 0.016) and ANGPTL4 (p = 0.031) transcripts compared to basal levels in all ocular fibroblasts, irrespective of their phenotype, while UCP3 expression levels remained unchanged. Although the primary pathogenic effect of MG sera is directed at the muscle endplate, these results showed that the MG sera may also influence the cellular genetic environment in ocular fibroblasts.

Fig. 1. The Phenotype test: MG sera induced a more energetic metabolic phenotype in OP-MG cell cultures. A. OP-MG and control ocular fibroblasts have similar metabolic phenotypes in plain growth medium. B. OP-MG ocular fibroblasts become more “energetic” after exposure to 5% MG sera. “Stressed” values are indicated by crosses. Graphs C and D show the fold change for averaged OCR (p ≤ 0.043) and ECAR values (p ≤ 0.054) in response to myasthenia gravis (MG) sera (5% for 24 h) from untreated OCR levels in two independent experiments (plated in triplicate). The average values from 3 independent experiments (performed in triplicate) for OP-MG (n = 2) and controls (n = 5) are shown. Error bars show mean and standard error of the mean.

Fig. 2. The Mito-stress test: MG sera induced change in measures of oxygen consumption rate. A. Illustrative graph showing the different parameters of oxygen consumption rate (OCR) presented in B. B. Each data point represents the fold change in OCR for each ocular fibroblast culture (OP-MG=2; controls=5) in response to myasthenia gravis (MG) sera (5% for 24 h) from untreated OCR levels in two independent experiments (plated in triplicate). Respiration (resp); non-mitochondrial oxygen consumption (NMOC).
MG sera. This supports observations in the EAMG rodent model, that MG induces an adaptive response in the oxidative metabolism of cells.

Although there was no evidence of impairment in the mitochondrial bioenergetics of ocular fibroblasts from 2 OP-MG cases in the presence of MG sera, we observed relatively more energetic responses compared to that in the control cultures in five independent experiments. In support of the induced bioenergetic responses to MG sera in ocular fibroblasts, we report significant gene transcript changes in ocular fibroblasts of two key mitochondrial pathway genes, in response to the same MG sera. Although the mitochondrial uncoupling protein UCP3 is expressed in dermal fibroblasts (Mori et al., 2008), it is far more abundantly expressed in skeletal muscle (Boss et al., 2000). Muscle UCP3 levels were altered in a rodent EAMG model (Kaminski et al., 2016), but the UCP3 transcript levels in ocular fibroblasts remained unaffected by the exposure to MG sera similar to our previous results in dermal-derived transdifferentiated myoblasts (Nel et al., 2019b), likely due to tissue-specific responses of this gene in muscle vs fibroblasts.

We acknowledge that few samples is a substantial limitation, but this is dependent on opportunistic sampling of rare patients undergoing surgery which involves cutting orbital muscle tendons. Although we have not included control sera in these experiments, previous reports found that in contrast to 1–10% MG sera (AChR-antibody positive and negative), control sera did not induce any morphological changes in cultured myoblasts or lymphocytes after 24 h (Luckman et al., 2006, Auret et al., 2014). Bioenergetic analyses of live cells may be impacted by many factors including cell density, and we have attempted to mitigate at least some of these effects by analysing each cell line’s responsivity to MG sera compared to the response to normal medium.

5. Conclusion

These preliminary experiments using rare, patient-derived perimysial ocular fibroblasts, provide support for the myasthenic-induced shifts in oxidative metabolism previously observed in animal models of experimentally-induced myasthenia gravis. The differential responses induced by MG sera in these EOM supportive cells from OP-MG cases, compared to controls, may suggest altered regulation of bioenergetic adaptive pathways under certain conditions, but requires additional validation.

Ethics comment

We have read and abided by all the ethical standards for manuscripts submitted to IBRO reports.

CRediT authorship contribution statement

Tarin Europa, Melissa Nel: data analysis, Tarin Europa, Maribanyana Lebeko, Melissa Nel, Jeannine Heckmann: Methodology, Conceptualization, Jeannine Heckmann: Project administration, Funding acquisition.

Conflict of interests

None.

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