Effect of rapamycin on mitochondria and lysosomes in fibroblasts from patients with mtDNA mutations

Running head: Rapamycin treatment in mtDNA patient fibroblasts

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Abstract

Maintaining mitochondrial function and dynamics is crucial for cellular health. In muscle, defects in mitochondria result in severe myopathies where accumulation of damaged mitochondria causes deterioration and dysfunction. Importantly, understanding the role of mitochondria in disease is a necessity to determine future therapeutics. One of the most common myopathies is **m**itochondrial **e**ncephalopathy **l**actic **a**cidosis **s**troke-like episodes (MELAS), which has no current treatment. Recently, MELAS patients treated with rapamycin exhibited improved clinical outcomes. However, the cellular mechanisms of rapamycin effects in MELAS patients are currently unknown. In this study, we used cultured skin fibroblasts as a window into the mitochondrial dysfunction evident in MELAS cells, as well as to study the mechanisms of rapamycin action, compared to control, healthy individuals. We observed that mitochondria from patients were fragmented, had a 3-fold decline in the average speed of motility, a 2-fold reduced mitochondrial membrane potential and a 1.5–2-fold decline in basal respiration. Despite the reduction in mitochondrial function, mitochondrial import protein Tim23 was elevated in patient cell lines. MELAS fibroblasts had increased MnSOD, p62 and lysosomal function when compared to healthy controls. Treatment of MELAS fibroblasts with rapamycin for 24 hrs resulted in increased mitochondrial respiration compared to control cells, a higher lysosome content, and a greater localization of mitochondria to lysosomes. Our studies suggest that rapamycin has the potential to improve cellular health even in the presence of mtDNA defects, primarily via an increase in lysosomal content.

Introduction

Rapamycin, an autophagy inducer and a pharmacological inhibitor of mammalian target of rapamycin (mTOR), extends the lifespan of mice and delays the onset of age-associated diseases in model organisms by rejuvenating cellular health (1). Treatment with rapamycin has ameliorated adverse phenotypes and improved endurance exercise and mitochondrial function. The beneficial effects are due to an increase in autophagic flux, an upregulation of lysosomal biogenesis, a higher abundance of Lamp1 protein and the localization of TFEB, the master regulator of lysosomal biogenesis, to the nucleus (2-4). Lysosomes play an integral role in maintaining mitochondrial health by degrading damaged mitochondria in the process of mitophagy. Recent studies have suggested that mTOR has an additional role in mitophagy(5), wherein global autophagy induction can trigger the activation of mitophagic pathways in the presence of dysfunctional mitochondria (6, 7).

Mitochondrial myopathies are a group of neuromuscular disorders with prominent organelle dysfunction due to genetic abnormalities. Symptoms exhibited by myopathy patients include muscle weakness, exercise intolerance, mobility impairments, heart failure, stroke-like episodes, seizures, deafness, blindness and dementia. Common mitochondrial myopathies include Kearns-Sayre syndrome (KSS), myoclonus epilepsy with ragged-red fibers (MERRF), and mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS). MELAS is one of the most common maternally-inherited mitochondrial disorder, with accumulations of point mutations in the mtDNA (8, 9) and a prevalence rate estimated to be 6 per 10,000 people (10). As mitochondria are essential to nearly every cell in the body, the disease affects the brain, heart, muscles, gastrointestinal tract, endocrine function, pulmonary and renal systems (11, 12). Due to such a wide range of symptoms and genetic features, conducting controlled clinical trials with therapeutic agents is challenging (12-16). No treatments have

69   been consistently associated with an improvement in clinical outcomes (14). Hence, the lack of a

70   uniform treatment strategy provides a strong need for research in a potential therapeutic for MELAS.

71   Until recently, rapamycin was only widely used in genetic and mammalian models of disease.

72   Currently however, multiple clinical trials are now underway to test the efficacy of rapamycin in various

73   human diseases (17, 18). Treatment of patients with MELAS resulted in improved health progression,

74   and rapamycin rescued some mitochondrial defects in isolated cultured fibroblasts (17). However, the

75   cellular mechanisms of how rapamycin acts to improve cellular health in the presence of mtDNA defects

76   are currently unknown. Thus, in this study we sought to investigate the extent of mitochondrial

77   dysfunction in fibroblasts isolated from MELAS patients, as well as the effect of rapamycin treatment on

78   mitochondria and lysosomes, since the function of these organelles is inextricably tied (19).

79   **Methods**

80   *Cell culture*- Human skin fibroblasts from the forearm were obtained with informed consent from family

81   members for diagnostic purposes, with approved methodologies established at the Hospital for Sick

82   Children, Toronto, Canada. Cells cultures were established from 4 patients as well as 3 controls and

83   were maintained as frozen stocks until used for analyses. The genetic mutation and patient

84   demographics are reported in Table 1. The cells were thawed and grown at 5% CO_2 at 37° C in AMEM

85   supplemented with 10% Fetal Bovine Serum and 1% Penicillin/ Streptomycin until they reached 80-

86   90% confluency, as done previously (20, 21). Cells were treated with 100 nM rapamycin (Life

87   Technologies, Burlington, ON, CA) or vehicle (DMSO) for 24hrs. To visualize mitochondria,

88   fibroblasts were transfected with the plasmid mito-dsRED2, red fluorescent protein fused with the

89   mitochondrial targeting sequence from cytochrome c oxidase subunit VIII (Clontech, Mountain View,

90   CA) using Lipofectamine 2000 (Life Technologies, Burlington, ON, CA).

91 *Live cell imaging-* To visualize nuclei, mitochondria and lysosomes, fibroblasts were incubated with

92 Hoechst 33342 (ThermoFischer, MA, USA), 10 nM mitotracker green (Invitrogen, MA, USA) and 10

93 nM lysotracker red (Invitrogen, MA, USA) for 15 mins at 5% CO_2 at 37° C. Stained and mtDsRed

94 transfected cells were visualized using an inverted Nikon Eclipse TE-2000 confocal microscope

95 equipped with a ×60/1.5 oil objective lens and a custom-designed chamber for live cell imaging that

96 maintained a constant temperature of 37°C with 5% CO_2. Live cell time lapse imaging of mitochondrial

97 dynamics was captured at 2 second intervals for a total time of 5 mins, as done previously (22).

98 *Analysis of mitochondrial motility and morphology-* Analyses were performed using NIS Element AR

99 3.1 software (Nikon Inc., USA). Mitochondrial motility was calculated by kymographs for each

100 fibroblast, using 7-11 cells per cell line. The average motility for each fibroblast is reported by the

101 measuring speed of 8-15 mitochondria per cell. Kymographs were generated from peripheral regions of

102 each cell as individual mitochondria could be resolved for analysis. Mitochondria in tubular networks,

103 often surrounding the nucleus, were not included for analysis. Velocity measurements were exported

104 from NIS software. Calculations of the percent fused and fragmented mitochondria were made using the

105 General Analysis program in NIS Elements where the minimum size of mitochondria was set at 0.2 μm.

106 *Lysosomal number and colocalization coefficient-* Both lysosomal and nuclear number were determined

107 from confocal images from each cell line (10/20x images) and analyzed in ImageJ (NIH, USA) via the

108 count tool. Lysosomal number was normalized to the number of nuclei. Pearson coefficient of

109 lysotracker red and mitotracker green stained fibroblasts was obtained from NIS Elements. For each cell

110 line, one coverslip was stained, and three images were taken at different locations of the coverslip. For

111 each image, the colocalization coefficient value was obtained (n=3 for each cell line). Vehicle-DMSO

112 and rapamycin treated cells were imaged on the same day.

113 *Lysosomal function*- To determine lysosomal function, fibroblasts were incubated with a fluorogenic

114 substrate for proteases, DQ Red BSA (Invitrogen, MA, USA). The dye conjugated to BSA is non-

115 fluorescent when the protein is in its native state. When cleaved by proteases in the lysosome, the

116 fluorescent quenching effect is relieved and single peptides emit red fluorescence. Fluorescence was

117 quantitated by flow cytometry, as described below.

118 *Flow Cytometry*- The fluorescent geomean intensities of mitotracker green, lysotracker red, DQ Red

119 BSA and JC-1 were determined using a BD FACSCaliber flow cytometer (BD Biosciences, CA, USA)

120 equipped with FL-1 (green), FL-2 (red) and FL-3 (deeper red) detectors.

121 *JC-1 Fluorescence*- Fibroblasts were grown to 80% confluency. Cells were washed with PBS and

122 incubated with 1.5 μM of JC-1 at 37 °C in the dark for 30 mins. After incubation, cells were washed

123 with warm PBS and harvested. Pellets were resuspended in phenol red-free media and analyzed in the

124 flow cytometer.

125 *DCF Fluorescence*- Fibroblasts were seeded at 20,000 cells in a 96-well optical bottom plate. After 24

126 hrs, cells were incubated with 2'7' dichlorofluorescin diacetate (DCF) (Invitrogen, USA) at 37°C for 30

127 min. Fluorescence (excitation 480nm, emission 520 nm) was measured using a Synergy HT microplate

128 reader (BioTek Instruments,VT, USA). After incubation, cells were washed with PBS and incubated

129 with DAPI stain at 37°C for 15 min. Fluorescence was measured using the microplate reader and data

130 were normalized to DAPI staining per well.

131 *Mitochondrial Respiration*- Assays were performed using a Seahorse XF96 Cell Mito Stress Test Kit

132 (Agilent Biosciences, USA). Briefly, fibroblasts were seeded at a density of 20,000 cells per well in a

133 96-well Seahorse cell culture plate and incubated overnight. Each fibroblast cell line was seeded in 12

134 replicate wells. In experiments involving drug treatments, 6 wells per cell line were treated with DMSO

135   or rapamycin. After 24 h, the Seahorse XF96 Extracellular Flux Analyzer was used to measure the

136   oxygen consumption rate (OCR) of each well. After the OCR was measured, the seeded plate was

137   washed twice with PBS. Cell lysates were extracted from each well and Bradford reagent was added to

138   measure protein absorbance per well. The assays were analyzed using XF Wave software and all OCR

139   measurements were normalized to protein absorbance.

140   *Western blotting-* Cell protein extracts were prepared and separated in polyacrylamide SDS-PAGE gels.

141   Proteins were then transferred onto nitrocellulose membranes and blocked in 5%-10% milk in TBS-T.

142   Subsequently, membranes were incubated with the appropriate concentration of primary antibodies

143   overnight at 4°C, and then with HRP-conjugated secondary anti-mouse (Cell Signaling 7076S) or anti-

144   rabbit antibodies (Cell Signaling 7074S) for 1hr at room temperature. Membranes were then visualized

145   with enhanced chemiluminesence using a Carestream Imaging system. Primary antibodies used were

146   anti-Tim23 (BD Biosciences BD611222), anti-α smooth muscle Actin (Abcam ab5694), anti-p62

147   (Abcam ab56416), anti-B1/2 V-ATPase (SantaCruz SC-55544), anti-Kif5B (SantaCruz SC 28538), or

148   anti-MnSOD (Upstate 06-984). All antibodies were diluted 1:1000. The blots were cut into four parts.

149   The loading control was obtained from the same blot for all the antibodies. The p62 immunoblot was

150   reprobed for V-ATPase and the Tim23 immunoblot was stripped and reprobed for MnSOD, which

151   migrate at different molecular weights.

152   *Statistical analysis.* Data are presented as means +/- SD for all control and patient fibroblast lines. All

153   statistical analysis was performed using GRAPHPAD PRISM version 9.00 for Windows (GraphPad

154   Software, San Diego, CA, USA). Unpaired t-tests comparing average control to average patient were

155   performed in Figures 1-3. In Figure 4, a paired t-test (denoted as #) was used for analysis when

156   comparing vehicle vs rapamycin treatment of respective cell lines. An unpaired t-test, denoted as *, was

157   used for comparison of average vehicle vs average rapamycin treatment. In Figure 5, a one-way

158   ANOVA with a Tukey post-hoc test was performed for analyses. In all statistical analyses, a p-value <

159   0.05 was considered significant.

Results

161   *Mitochondria are fragmented and motility is reduced in MELAS fibroblasts.* To assess the

162   morphological integrity of mitochondria in fibroblasts isolated from healthy individuals and MELAS

163   patients, cells were transfected with mtDsRed, a fluorescent mitochondrially-targeted protein, and

164   visualized by confocal microscopy. A representative cell from control and MELAS fibroblast lines is

165   shown in Figure 1A, illustrating the degree of mitochondrial network fragmentation in patient cells. A

166   higher percent of fragmentation was observed in all three MELAS fibroblast lines when compared to the

167   controls, with an average 1.5-fold increase in MELAS fibroblasts (Fig.1B). To determine whether this

168   fragmentation had an impact on mitochondrial motility, 5-min time lapse videos were generated by live

169   cell imaging of mtDsRed transfected cells. Motility was calculated by generating kymographs for each

170   fibroblast. The average motility is reported by measuring the speed of 8-15 mitochondria for each

171   fibroblast. All MELAS fibroblasts lines had reduced average (Fig. 1C) mitochondrial speed, with a

172   reduction greater than 2-fold when compared to the control fibroblast lines. To assess whether this effect

173   could be due to the expression of motor proteins responsible for organelle motility along microtubules,

174   we measured the level of the kinesin motor protein isoform Kif5B by western blots in control and

175   patient fibroblasts. A decline (p=0.05) was observed in patient cell lines (Fig. 1D), which could

176   contribute to this reduction in motility.

177   *MELAS fibroblasts exhibit reductions in mitochondrial membrane potential and respiration, but no*

178   *change in mitochondrial content.* To verify changes in mitochondrial function in MELAS cells, we first

179   determined the mitochondrial membrane potential of control and MELAS fibroblast lines. JC-1 is a

180   fluorescent probe that remains in the cytosol as a monomer and emits green fluorescence when

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181   mitochondria have lost their membrane potential. The dye aggregates within mitochondria to emit red

182   fluorescence where there is an intact membrane potential. Thus, the ratio of red to green fluorescence is

183   an indicator of mitochondrial health within the cell. MELAS fibroblast lines exhibited a significant

184   decline in the JC-1 ratio, indicating the loss of mitochondrial membrane potential in comparison to the

185   control cells (Fig. 2A). To assess mitochondrial respiration, we measured the oxygen consumption rate

186   (OCR) of our fibroblast lines using Seahorse. A typical OCR profile, along with the drug additions and

187   resulting data is shown in Fig. 2B. There was a significant decline in basal (Fig. 2C) and proton linked

188   (Fig. 2D) mitochondrial respiration in all MELAS cell lines. Maximal respiration, which is an indication

189   of total mitochondria in the cell, did not differ between control and MELAS cells (Fig. 2C). Patients

190   displayed a significantly increased Tim23 protein expression relative to controls (Fig 2E) suggesting a

191   compensatory adaptation in the import pathway, to correct for the mitochondrial dysfunction observed.

192   *MELAS fibroblasts have increased protease function and oxidative stress.* Lysosomes play an

193   integral part in maintaining cellular health by degrading dysfunctional organelles, like mitochondria. We

194   determined lysosomal content in fibroblasts using flow cytometry following incubation with the

195   fluorescent dye, lysotracker red. No differences were detected between control and MELAS fibroblast

196   cell lines (Fig. 3A). This corresponded to the lack of difference observed between control and patients in

197   the lysosomal protein marker B1/2 V-ATPase (Fig. 3C, D). Lysosomal function of live cells was also

198   assessed using the fluorescence intensity of a proteolytically cleaved fragment generated in the lysosome

199   (Fig. 3B). Despite individual differences, two out of the three MELAS cell lines exhibited clearly

200   elevated lysosomal protease function compared to control fibroblasts (Fig. 3B). In order to match this

201   with estimates of autophagy, p62 was used as a surrogate indicator of autophagy flux in these cells. The

202   content of p62 was elevated in the patients by about 30% ($p<0.05$; Fig. 3C, D), suggesting that while

203   lysosomal function was enhanced, uptake or delivery of autophagic substrates into the lysosome could

204   be impaired in patient cells. Since ROS can act as a signal for the activation of autophagy, we used DCF

205   fluorescence as an indicator of ROS emission, and MnSOD protein as a measure of anti-oxidant capacity

206   in these cells. Our data indicate that these patient cells exhibited a marked 7-8-fold increase (P<0.05;

207   Fig. 3E) in DCF fluorescence compared to controls, an increase similar to previous reports (21), which

208   was only accompanied by a 2-fold higher level (P<0.05; Fig. 3D) of MnSOD protein.

209   *Rapamycin rescues basal and proton leak mitochondrial respiration in MELAS fibroblasts*. We then

210   assessed mitochondrial function following treatment of the cells with rapamycin for 24 hours.   Upon

211   addition of rapamycin, control fibroblasts displayed 2-9-fold increases in DCF fluorescence. However,

212   DCF fluorescence in MELAS fibroblasts did not increase in response to rapamycin (Fig. 3F).   In

213   addition, while rapamycin treatment had very little effect on OCR parameters measured in control cells,

214   apart from a minor reduction in basal respiration (Fig. 4A, B), rapamycin treatment significantly

215   improved the respiratory function of MELAS fibroblasts, by increasing basal (60%, P<0.05), as well as

216   proton leak (100%, P<0.05; Fig. 4C,D) respiration.

217   *The beneficial effect of rapamycin on mitochondria could be mediated by increased lysosomal number.*

218   It has been known for some time that rapamycin treatment leads to an elevation in lysosome content (2-

219   4). To evaluate this in patient and control cells, we stained fibroblasts with lysosomal and nuclear

220   markers (Fig. 5A). We observed that rapamycin induced a 4-5-fold increase (P<0.05) in lysosomal

221   number in both control and patient cells when compared to vehicle treatment (Figs. 5B). Rapamycin had

222   no significant effect on lysosomal function in either control or patient cell lines (Fig. 5C). Our data also

223   show that, upon rapamycin treatment, lysosomes colocalize to a much greater extent with mitochondria

224   in MELAS fibroblasts (Fig. 5D). This increase in colocalization was not evident in control cells. These

225   data suggest that the rapamycin-induced increase in lysosome content may help traffic dysfunctional

226   mitochondria to the organelle for clearance. This would serve to reduce the pool of dysfunctional

227 organelles and improve mitochondrial function in MELAS patient cells, as evident from better

228 respiratory rates and a lack of increase in ROS emission, when treated with the drug.

229

Discussion

Mitochondrial defects can cause severe energy deficits and cell death. To preserve health, the maintenance of mitochondrial integrity and function is critical. Potential therapeutic strategies for mitochondrial disorders are being investigated, however there is currently no effective intervention. Rapamycin has shown benefits for muscle myopathies (2, 17, 23) but the mechanism of action is not fully known. Our data suggest that as an autophagy inducer, rapamycin may improve mitochondrial dysfunction, since we observed that mitochondrial respiration in MELAS cells was, in part, rescued by rapamycin treatment.

Mitochondrial dysfunction is a common feature of mitochondrial myopathies such as MELAS (23-25). We observed a ~50% reduction in membrane potential in patient cells, similar to other reported literature in MELAS fibroblasts (23). Along with lower mitochondrial membrane potential, mitochondrial respiration was reduced with no detectable changes in mitochondrial content (26). Another recent study (27) has reported no changes in ATP production in MELAS fibroblasts. Similarly, we detected no differences in ATP-linked respiration in our cell lines. An indirect measure of mitochondrial content in a cell is maximal respiratory capacity, which is not different between our control and patient fibroblasts. Studies have generally reported no changes in total mitochondrial mass in MELAS fibroblasts (21, 26) and have observed an upregulation of mitochondrial import machinery (20). We noted an increase in Tim23, a mitochondrial protein encoded by the nuclear genome, similar to previous studies in mtDNA disease patients (20). Higher levels of Tim23 might be an indication of a compensatory retrograde signaling pathway activated in the presence of mitochondrial dysfunction. In the analysis of multiple MELAS patients, individual differences in pathophysiology have been observed, whereby some patients exhibit an extreme phenotype of reduced mitochondrial potential, respiration and mitochondrial proteins, whereas others have less drastic changes, with no differences detected (23). This

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253 is likely due to variability in patient symptoms, age of onset and manifestations, and this could clearly

254 account for some of the variability observed between patient cells in the current study. Other limitations

255 that could have introduced variability among patient and control cells include different passage numbers,

256 as well as cell proliferation rates.

257   Mitochondrial motility is necessary to maintain a healthy pool of the organelle. Cytoskeletal and

258 motor proteins allow for the transport of mitochondria to diverse cellular regions in an effort to maintain

259 reticular, network-like structures. We have previously shown that situations of high oxidative stress led

260 to mitochondria becoming stationery and fragmented (22). Reduced mitochondrial motility has been

261 reported in a variety of metabolic, neurodegenerative and mitochondrial diseases (28). Our data indicate

262 that MELAS fibroblasts exhibit fragmented mitochondria, as reported by others (23), and have a reduced

263 average motility. This is likely due in part to lower levels of the kinesin isoform Kif5B, as well as

264 elevated antioxidant enzymes, suggestive of increased oxidative stress. Fragmented, dysfunctional

265 mitochondria are ideal substrates for the mitophagy pathway, leading to their subsequent degradation

266 within lysosomes.

267   Earlier studies using electron microscopy and flow cytometry have documented an increase in

268 lysosomal number in MELAS cells (26). More recently, investigations have identified the localization of

269 mitochondria to autophagosomes, suggesting mitochondrial degradation (23). While we did not observe

270 an increase in lysosomal content, either visualized using lysotracker staining, or a well-established

271 marker of lysosome content, B1/2 V-ATPase, our data do indicate that protease function within

272 lysosomes was elevated in MELAS fibroblasts. This would suggest an enhanced capacity for cargo

273 degradation in MELAS cells. However, p62 levels were increased in the patients, and this is most often

274 interpreted as an indicator of reduced autophagy flux. This diminished autophagy is supported by the

275 literature which shows that patient cells have elevated levels of autophagy markers such as ATG12-

276 ATG5 and LC3, as well as an accumulation of autophagosomes (23, 24).

277   The mammalian target of rapamycin mTOR has a well-established role in regulating autophagy.

278 This may also extend to mitophagy (5). The activation of autophagy using rapamycin-induced mTOR

279 inhibition can trigger the activation of mitophagic pathways when there are dysfunctional mitochondria,

280 in a dose- and duration-dependent manner (6, 7). Chronic treatment with rapamycin improved disease

281 phenotype of cells from patients with Leber's hereditary optic neuropathy (LHON), a mitochondrial

282 disorder. Rapamycin treatment induced autophagy as detected by autophagosome and mitochondrial

283 specific markers co-localization. Furthermore, the threshold of pathogenic mtDNA mutations decreased,

284 suggesting that mitophagy selectively degraded dysfunctional mitochondria (6). We observed a similar

285 increase in lysosomal number in control and patient cell lines with rapamycin treatment. However, in

286 patient cells, lysosomes appeared to be larger in size, had increased staining intensity, and the index of

287 colocalization with mitochondria was significantly elevated in patient cells as a result of rapamycin

288 treatment. This increased colocalization may have resulted in mitochondrial repair, as we observed an

289 increase in organelle function upon rapamycin treatment, but only in patient fibroblasts. This was

290 evident from some improvements in mitochondrial respiration. In addition, notwithstanding the

291 limitations of using DCF as an indicator of ROS (17), the lack of increase in DCF fluorescence in

292 response to rapamycin suggests the induction of a more efficient electron transport chain (29) and a

293 healthier antioxidant status within the MELAS cells (30).

294   In addition to studies involving cells in culture, the benefits of rapamycin treatment have been

295 observed in *ex vivo* and *in vivo* models. In mice exhibiting extreme myopathy, rapamycin treatment

296 improved the adverse phenotype. Treated mice exhibited a reversal of mitochondrial structural

297 abnormalities, mitochondrial dysfunction and an improvement in muscle function. Rapamycin increased

298   autophagic flux in mice and induced TFEB translocation to the nucleus, which results in lysosomal

299   biogenesis (2). In a recent clinical trial, MELAS patients who were switched to rapamycin treatment

300   exhibited improved metabolic function with less oxidative stress. Furthermore, rapamycin activated

301   autophagy and attenuated the decline in mitochondrial membrane potential and fragmentation of

302   mitochondria (17).

303   The patients' cells in the current study have the same mitochondrial content as control cells, but

304   possess mitochondria that are fragmented and poorly motile, with indices of reduced membrane

305   potential, higher ROS emission which outstrips antioxidant capacity, and lower basal respiration. These

306   mitochondria should be recycled and removed to enhance the quality of the organelle pool. Clearance

307   would be achieved with an enhanced rate of mitophagy directed to the lysosome. In MELAS patients,

308   lysosome number is similar as in control cells, but these lysosomes have modestly enhanced lysosome

309   proteolytic function, which seems insufficient to reduce mitochondrial dysfunction. In addition, the lack

310   of mitochondrial motility, perhaps as a result of reduced cytoskeletal transport and enhanced oxidative

311   stress (22), impairs mitochondrial trafficking to the lysosome. Evidence for impaired delivery is found

312   in the increased level of p62, an autophagy substrate. Attainment of a healthier mitochondrial pool could

313   be achieved by providing the cell with a greater number of lysosomes, thereby reducing the traffic jam.

314   Rapamycin treatment clearly increased lysosome content, providing a significantly improved destination

315   for the removal of impaired mitochondria, evident by the co-localization index. This greater lysosomal

316   degradation of dysfunctional mitochondria likely contributes to the better respiration rates back toward

317   healthy control cells, an enhancement that might be amplified with a longer rapamycin treatment time.

318   We speculate (Fig. 6) that the maintenance of a constant mitochondrial content between patient and

319   control cells also obligates a higher degree of retrograde signaling in the presence of rapamycin from

320   mitochondria to the nucleus in patient cells, directing gene expression toward enhanced biogenesis in the

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321 face of increased mitochondrial degradation in lysosomes. This is evident in non-treated cells by the

322 higher expression of Tim23, and has been documented previously (20) in MELAS cells. Thus, in

323 summary, our pre-clinical data suggest that rapamycin may have some benefits for MELAS patients

324 with the potential to rescue mitochondrial deficits, primarily via enhanced lysosomal content.

325

326 **Acknowledgments**

329

330 **Table 1. Age, sex and mutation identified from forearm samples in four MELAS patients.**

	Patient ID Number	**Age**	**Sex**	**Mutation**	**Heteroplasmy**
P1	5509	6 months	M	G13513A	70%
P2	13801	>18 years	F	T8993C	72 or 82%
P3	21124	2 years	F	T8993C	>95%
P4	20551	2 years	F	G13513A	unknown

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336 **Figure Legends**

337 **Figure 1**. Mitochondrial motility and morphology in fibroblasts isolated from human control individuals

338 and MELAS patients. A) Mitochondrial network visualized by mtDsRed in control and patient

339 representative fibroblast. Live cell imaging was performed with a confocal microscope equipped with a

340 heated stage and CO_2 chamber. B) Percent of fragmented mitochondria in cells from each cell line,

341 control and patient (n=7-12 cells per cell line for control and patient). All cell lines were included in the

342 average control (Cont) and patient (Pat). Average percent of fragmentation is shown for all

343 control/patient cell line. Control represented by black bar and patient cell lines by grey bar. Motility of

344 mitochondria was measured via kymographs to determine (C) average speed. Kymographs were

345 generated from time lapse videos of 2 sec intervals for 5mins duration. N= 6-12 cells per cell line and 8-

346 15 mitochondria per cell. The average control had n=24 and average patient n=21 cells. D) Kif-5B

347 protein level in three control and three patient cell lines. Unpaired t-test was performed where * p-value

348 < 0.05 and *** p-value is <0.0001.

349 **Figure 2.** Mitochondrial function and amount in healthy and MELAS fibroblasts. A) Mitochondrial

350 membrane potential as measured by fluorescent probe JC-1. Aggregated form of JC-1 emits a red

351 fluorescence in mitochondria with an intact membrane potential whereas a monomer JC-1 fluoresces

352 green when present in the cytosol. Fluorescent intensities were measured in the flow cytometer for

353 control (Cont) and patient (Pat). B) A diagram of an annotated seahorse XF Analyzer graph with

354 different measured parameters labeled for Mitostress assay C) Average basal, spare respiratory capacity

355 and maximal respiration in three control and three MELAS fibroblast cell lines, n=7. D) Average Proton

356 leak dependant respiration and ATP Production in three control and three MELAS fibroblast cell lines,

357 n=7. E) Tim23 levels three control and three patient cell lines. Smooth muscle actin, α-SMA, used as

358   loading control. Unpaired t-test was performed where * p-value < 0.05, ** p-value < 0.01 and *** p-

359   value is <0.0001.

360   **Figure 3.** Lysosomal function and oxidative stress in healthy and MELAS fibroblasts. A) Fluorescent

361   intensity for lysotracker red and B) lysosomal function via DQ Red BSA in control and patient

362   fibroblasts, n=7. All cell lines were included in the average control (Cont) and patient (Pat). No

363   significance (n.s) when comparing lysosomal function between average control and patient fibroblasts.

364   C) B1/2 v-ATPase, p62, MnSOD protein expression in three control and three patient cell lines. Smooth

365   muscle actin, α-SMA, used as loading control. D) Normalized protein expression in control and patient

366   cell lines (n=3). E) ROS measured with DCF fluorescent probe in control and patients. F) Fold change

367   of ROS levels upon rapamycin treatment. n=5 for each cell line treated with DMSO or rapamycin.

368   Unpaired t-test was performed where * p-value < 0.05 and *** p-value is <0.0001 patient vs control.

369   **Figure 4**. Rapamycin effects mitochondrial respiration in fibroblasts. A-B) Average basal respiration,

370   spare respiratory capacity, proton leak and ATP production for all control fibroblast lines with and

371   without rapamycin treatment. C-D) Basal, spare respiratory capacity, proton leak and ATP production

372   for all MELAS lines with and without rapamycin, n= 4-6 for each cell line treated with DMSO or

373   rapamycin. All cell lines were included in the average control (C - / + rapamycin) or patient (P - / +

374   rapamycin) for a n=11. Paired t-test was performed where # p-value < 0.05 rapamycin treatment (+rapa)

375   vs vehicle-DMSO treatment (-rapa) of respective cell line. Unpaired t-test was performed where * p-

376   value < 0.05 and ** p-value <0.01 of average vehicle vs average rapamycin treatment.

377   **Figure 5.** Rapamycin effect mediated through lysosomal alterations. A) Representative confocal image

378   of hoechst (H), mitotracker green (MTG) and lysotracker red (LTR) staining from one control and

379   patient treated with DMSO or rapamycin. B) Quantification of lysosomal number from DMSO and

380   rapamycin treatment in control and patient cell lines (n=3). Rapamycin treatment increased lysosomal

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381 number per nucleus in control fibroblast lines. C) Rapamycin effect on lysosomal function in control and

382 MELAS fibroblast lines, n=7. All cell lines were included in the average control (Cont) and patient

383 (Pat). D) LTR signal colocalizes with MTG with a higher colocalization coefficient in MELAS lines

384 treated with rapamycin. Images were taken from different locations on a coverslip, n=3 images for each

385 cell lines. 1-way ANOVA was performed where * p-value < 0.05 of average rapamycin treated vs

386 average vehicle-DMSO treatment.

387 **Figure 6.** Illustration of the maintenance of mitochondrial health in control and patients with mtDNA

388 mutations. In comparison to healthy controls, mtDNA patients exhibit a similar total mitochondrial

389 content, but these organelles are fragmented with elevated ROS emission, lower membrane potential and

390 basal respiration, and poor cellular motility. To compensate for loss of mitochondrial function, patients

391 have an increased mitochondrial retrograde signaling the nucleus, altering gene expression, as shown

392 previously (20). Lysosome content is similar in control and patient cells, and the addition of rapamycin

393 increases lysosomes in both cell types equally. This effect improves mitochondrial quality in patient

394 cells, promoting mitophagy as evident from greater colocalization of lysosomes and mitochondria, and

395 improved organelle function.

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Table 1. Age, sex and mutation identified from forearm samples in four MELAS patients.

	Patient ID Number	Age	Sex	Mutation	Heteroplasmy
P1	5509	6 months	M	G13513A	70%
P2	13801	>18 years	F	T8993C	72 or 82%
P3	21124	2 years	F	T8993C	>95%
P4	20551	2 years	F	G13513A	unknown

A

Control

Patient

10μm

B

Percent Fragmented Mitochondria

*

C1 C2 C3 P1 P3 P4 X Cont X Pat

C

Average Mitochondrial Speed
μm/s x 1000

***

C1 C2 C3 P1 P3 P4 X Cont X Pat

D

C1 C2 C3 P2 P3 P4

Kif5B — 100kDa

Ponceau — 50kDa

— 37kDa

Kif5B / Ponceau Ratio

P = 0.05

Cont Pat

A

B

C

D

E

A

LTR Fluorescence — bar graph with categories: C1, C2, C3, P1, P2, P3, X Cont, X Pat

B

DQ Red BSA Fluorescence — bar graph with categories: C1, C2, C3, P1, P2, P3, X Cont, X Pat
***
***

C

C1 C2 C3 P2 P3 P4

V-ATPase -50kDa
         -75kDa
p62
         -25kDa
MnSOD
α SMA   -37kDa

D

Protein / αSMA Ratio — bar graph
Control (black), Patient (gray)
V-ATPase, p62 *, MnSOD *

E

DCF Fluorescence / DAPI — bar graph with categories: C1, C2, C3, P1, P2, P3, X Cont, X Pat
*

F

DCF Fluorescence Fold Change (AvgRapa /AvgCont) — bar graph with categories: C1, C2, C3, P1, P2, P3

A

B

C

D

A

	DMSO	Rapamycin
Control		
Patient		

B

C

D