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Post onset, oral rapamycin treatment delays development of mitochondrial encephalopathy only at supramaximal doses

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ABSTRACT

Mitochondrial encephalopathies are fatal, infantile neurodegenerative disorders caused by a deficit of mitochondrial functioning, for which there is urgent need to identify efficacious pharmacological treatments. Recent evidence shows that rapamycin administered both intraperitoneally or in the diet delays disease onset and enhances survival in the *Ndufs4* null mouse model of mitochondrial encephalopathy. To delineate the clinical translatability of rapamycin in treatment of mitochondrial encephalopathy, we evaluated the drug's effects on disease evolution and mitochondrial parameters adopting treatment paradigms with fixed daily, oral doses starting at symptom onset in *Ndufs4* knockout mice. Molecular mechanisms responsible for the pharmacodynamic effects of rapamycin were also evaluated. We found that rapamycin did not affect disease development at clinically-relevant doses (0.5 mg kg^{-1}). Conversely, an oral dose previously adopted for intraperitoneal administration (8 mg kg^{-1}) delayed development of neurological symptoms and increased median survival by 25%. Neurological improvement and lifespan were not further increased when the dose raised to 20 mg kg^{-1}. Notably, rapamycin at 8 mg kg^{-1} did not affect the reduced expression of respiratory complex subunits, as well as mitochondrial number and mtDNA content. This treatment regimen however significantly ameliorated architecture of mitochondria cristae in motor cortex and cerebellum. However, reduction of mTOR activity by rapamycin was not consistently found within the brain of knockout mice. Overall, data show the ability of rapamycin to improve ultrastructure of dysfunctional mitochondria and corroborate its therapeutic potential in mitochondrial disorders. The non-clinical standard doses required, however, raise concerns about its rapid and safe clinical transferability.

1. Introduction

Mitochondrial disorders are devastating childhood diseases caused by inherited mutations of nuclear or mitochondrial genes coding for proteins involved in oxidative phosphorylation, membrane transport, energetic metabolism or programmed cell death

Abbreviations: ATP5D, ATP synthase H+ transporting mitochondrial F1 complex delta subunit; Cox1, cytochrome *c* oxidase I; Cox2, cytochrome *c* oxidase subunit 2; Cox15, Cytochrome *c* oxidase assembly protein COX15 homolog; HGPS, Hutchinson-Gilford progeria syndrome; mt-Nd2, mitochondrial NADH dehydrogenase 2; mTOR, mammalian/mechanistic target of rapamycin; mTORC1/2, mammalian target of rapamycin complex ½; Ndufs4, NADH dehydrogenase [ubiquinone] iron-sulfur protein 4; Ndufv2, nuclear (NADH dehydrogenase(ubiquinone) flavoprotein 2; Opa1, mitochondrial dynamin like GTPase; OXPHOS, oxidative phosphorylation; PARP-1, poly(ADP-Ribose)polymerase 1; SDHA, succinate dehydrogenase complex, subunit A.

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(DiMauro and Mancuso, 2007; Moslemi and Darin, 2007). Oxidative phosphorylation (OXPHOS) defects are a major cause of mitochondrial disorders, with respiratory Complex I deficiency being one of the most commonly identified in childhood. Neurological symptoms occur at the age of ~4 months and the majority of patients die within the first 5 years of life (DiMauro and Mancuso, 2007; Moslemi and Darin, 2007).

Thanks to recent advancements of modern molecular diagnostics, the primary genetic defects underlying the mitochondrial dysfunction can now be identified. Unfortunately, however, the exact pathogenetic mechanisms through which gene mutations cause functional derangements of mitochondria still wait to be unequivocally deciphered. This is mainly due to the complex cascade of detrimental signals stemming from the primary genetic mutations and leading to cell demise and clinical symptoms. Unfortunately, given the difficulty in finding a cure for these genetic disorders, supportive care by means of symptomatic treatments are the only available therapeutic strategy (DiMauro and Mancuso, 2007; Moslemi and Darin, 2007). Molecules and signaling pathways contributing to evolution of mitochondrial encephalopathy are therefore intensively investigated.

A major difficulty in finding efficient therapeutic strategy for patients with mitochondrial disorders is also the scarcity of experimental models that closely resemble disease pathogenesis in humans. In this regard, the recently developed *Ndufs4* knockout mouse model (Kruse et al., 2008) reproduces some of the neurological symptoms of children with Leigh syndrome, a prototypical infantile mitochondrial encephalopathy. These mice lack the Ndufs4 subunits of respiratory complex I that assists complex assembly and activity. Akin to patients with Leigh syndrome, *Ndufs4* knockout mice develop severe, progressive encephalopathy inexorably leading to the exitus within 50–60 days of life because of respiratory failure (Quintana et al., 2010).

We recently reported that pharmacological inhibition of the nuclear enzyme PARP-1 delays disease development in *Ndufs4* knockout mice but has no effects on animal lifespan (Felici et al., 2014). In contrast with this, however, two reports demonstrate that inhibition of mTOR, a kinase with key roles in metabolism (Kennedy and Lamming, 2016), by rapamycin not only improves neurological symptoms but also prolongs lifespan of *Ndufs4* null mice (Johnson et al., 2013, 2015). These findings are of remarkable relevance because on the one hand identify mTOR as kinase involved in the pathogenesis of mitochondrial diseases, and on the other suggest that rapamycin, a drug routinely used to prevent organ rejection, can be readily translated for the treatment of patients with Leigh syndrome and similar disorders.

Thus, in light of the remarkable clinical implications of the therapeutic efficacy of rapamycin in the *Ndufs4* knockout mouse model, we attempted to confirm these findings, also evaluating whether therapeutic effects were achievable by means of different oral treatments including doses adopted in transplanted patients. Also, to unravel the mechanisms by which rapamycin ameliorates symptoms in this mouse model, we investigated the drug's effects on key parameters of mitochondrial biology such as respiratory complex subunit expression as well as organelle number and morphology.

2. Methods

2.1. Animals and drug treatment

All animal manipulations were performed in accordance with European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive 86/609/EEC) and approved by the Committee for Animal Care and Experimental Use of the University of Florence. All experiments involving animals are reported in accordance with the ARRIVE guidelines. *Ndufs4* knockout mice represent a well-established model of mitochondrial complex I deficiency (Kruse et al., 2008) and were used for the experiments. *Ndufs*4 heterozygous mice were bred to produce *Ndfus*4 knockout mice, and genotypes were determined by PCR. Both male and female animals were randomly assigned to different groups for treatment with vehicle or different doses of rapamycin starting from sign onset (P30). Rapamycin was administrated daily per os until death.

2.2. Rapamycin blood measurements

Whole-blood samples were collected in EDTA tubes by cardiac puncture immediately following euthanasia, after 5 days of treatment with rapamycin (0.5, 8 or 20 mg kg^{-1}). Samples were immediately brought to the General Laboratory Unit at the Careggi University Hospital of Florence for analysis by liquid chromatography electrospray tandem mass spectrometry (LC/ESI-MS/MS).

Sample aliquots of 100 μl were pipetted into a standard Eppendorf tube and 200 μl precipitation reagent (Methanolic Zinc Sulphate 80:20), containing deuterated rapamycin as internal standard, was added. The combined liquids were mixed for 15 s and centrifuged at high speed; the resulting clear supernatants were transferred into glass autosampler inserts and injected into the LC-MS system. The samples were cleaned on-line by a perfusion column (POROS R1/20, 2.1 × 30 mm, 20 μm particle sizes, Applied Biosystems, Darmstadt, Germany), using methanol: water (50:50) as loading phase. The analytes were eluted on Phenyl Hexyl RP column (Phenomenex Luna 5 μm particle size, 2 × 50 mm, Aschaffenburg, Germany) maintained at 60 °C and the HPLC system was set to a solvent flow of 0.3 ml/min using a mobile phase of methanol with 2% water, 1% ammonium acetate 1 M and 0.1% acetic acid.

An AB SCIEX QTRAP® 4000 LC/MS/MS system with Turbo V™ source and Electrospray Ionization (ESI) probe in positive ion mode was used. The system was operated in MRM mode for the duration of the analysis, using target fragment ions m/z 931.6 -> 864.5 for rapamycin and 934.6 -> 867.5 for deuterated rapamycin. The calibration curve ranged from 0.5 to 50 μg/L ($r2 = 0.99857$), the limit of quantification (LOQ) was 1.5 μg/L and the procedure was controlled using four levels of quality control material (2.76, 9.89, 19.5 and 39.3 μg/L). The Laboratory assured quality of analysis through participation in the International Proficiency Testing Scheme for rapamycin (IPTS).

2.3. Neuroscore analysis

The scoring system used for evaluating neurological parameters in *Ndufs4* knockout treated with vehicle or different doses of rapamycin was previously described in details (Felici et al., 2014). Briefly, a 5-point scale was used to measure different locomotor functions/impairment (ataxia, balance, hindlimb clasping and limb tone) by two blinded operators every two days. Maximum score (5 points) has been assigned to moribund or dead animals.

2.4. Evaluation of mtDNA content and respiratory subunits expression levels

Snap-frozen mice tissues were processed for genomic DNA, total RNA and protein extraction by using the NucleoSpin TriPrep kit (Macherey-Nagel, Duren, Germany) according to manufacturer's instructions. Real time PCR was performed as previously reported (Lapucci et al., 2011). The following primers were used: for *Cox1* forward 5′-TATCAATGGGAGCAGTGTTTG-3′ and reverse 5′-