Activated mTOR signaling pathway in myofibers with inherited metabolic defect might be an evidence for mTOR inhibition therapies

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Abstract

Background: Abnormally activated mechanistic target of rapamycin (mTOR) pathway has been reported in several model animals with inherited metabolic myopathies (IMMs). However, the profiles of mTOR pathway in skeletal muscles from patients are still unknown. This study aimed to analyze the activity of mTOR pathway in IMMs muscles.

Methods: We collected muscle samples from 25 patients with mitochondrial myopathy (MM), lipid storage disease (LSD) or Pompe disease (PD). To evaluate the activity of mTOR pathway in muscle specimens, phosphorylation of S6 ribosomal protein (p-S6) and p70S6 kinase (p-p70S6K) were analyzed by Western blotting and immunohistochemistry.

Results: Western blotting results showed that p-p70S6K/p70S6K in muscles from LSD and MM was up-regulated when compared with normal controls (NC) (NC *vs.* LSD, $U=2.000$, $P=0.024$; NC *vs.* MM: $U=6.000$, $P=0.043$). Likewise, p-S6/S6 was also up-regulated in muscles from all three subgroups of IMMs (NC *vs.* LSD, $U=0.000$, $P=0.006$; NC *vs.* PD, $U=0.000$, $P=0.006$; NC *vs.* MM, $U=1.000$, $P=0.007$). Immunohistochemical study revealed that p-S6 was mainly expressed in fibers with metabolic defect. In MM muscles, most p-S6 positive fibers showed cytochrome C oxidase (COX) deficiency ($U=5.000$, $P=0.001$). In LSD and PD muscles, p-S6 was mainly overexpressed in fibers with intramuscular vacuoles containing lipid droplets ($U=0.000$, $P=0.002$) or basophilic materials ($U=0.000$, $P=0.002$).

Conclusion: The mTOR pathway might be activated in myofibers with various metabolic defects, which might provide evidence for mTOR inhibition therapy in human IMMs.

Keywords: mTOR pathway; Mitochondrial myopathy; Lipid storage disease; Pompe disease

Introduction

Inherited metabolic myopathies (IMMs) are a diverse group of rare genetic diseases in which a defect in cellular metabolism leads to muscle dysfunction ranging from exercise intolerance to muscle weakness and muscle atrophy. IMMs are generally categorized into three major types: disorders in glycogen metabolism, lipid metabolism, and mitochondrial function. To date, there is still a lack of effective treatment for these disorders and therapy is primarily palliative, in some instances, may include changes in diet and exercise.

Mechanistic target of rapamycin protein (mTOR) is a highly conserved serine/threonine kinase that nucleates two multi-protein complexes, rapamycin-sensitive mTOR complex I (mTORC1) and rapamycin-insensitive mTOR complex II (mTORC2). The mTORC1 is a major regulator of cell metabolism and energy homeostasis, and when activated, changes the cell metabolism from catabolic to anabolic program, thus stimulating synthesis of proteins, nucleotides, and lipids while repressing autophagy.[1,2] Interventions of mTOR pathway have been widely used in clinical practice, such as transplant medicine[3] and oncology.[4] Many studies have also reported that mTOR inhibition can extend lifespan in both invertebrates[5-7] and mammalian species.[8]

In the last ten years, abnormally enhanced mTOR signaling was reported in several model animals with IMMs and inhibition of mTOR function has shown great potential as a novel therapy for various types of IMMs. Rapamycin, a specific mTORC1 inhibitor, robustly attenuates disease progression in mitochondrial encephalopathy mouse

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models.[9-11] It is also reported that rapamycin therapy is beneficial in cellular or animal models of glycogen storage disease (GSD) Type Ia,[12] Type II (Pompe disease)[13] and Type III.[14] Additionally, rapamycin treatment shows effective in the zebrafish model of multiple Acyl-CoA dehydrogenation deficiency (MADD),[15] a subtype of lipid storage disease (LSD). Although these studies have reported the therapeutic effect of mTOR inhibitors in model animals or cells, the profiles of mTOR pathway in patients with inherited metabolic diseases are still unknown. Here, we investigated the activity of mTOR pathway in biopsied muscle samples from patients with glycogenosis, lipid metabolism, and mitochondrial diseases.

Methods

Ethical approval

This study was approved by the Human Ethics Board of Qilu Hospital, Shandong University, and written informed consent was obtained from all the patients and controls.

Patients and muscle biopsy

Muscle specimens of biceps brachii from 25 patients including seven with LSD, seven with Pompe disease (PD) and 11 with primary mitochondrial myopathy (MM) were obtained at the Neuromuscular Institute of Qilu Hospital, Shandong University. The profiles of all patients are summarized in Table 1. Eight patients including four with neurogenic damages (ND) in muscle pathology and four with myotonic dystrophy (MD) were taken as disease controls (DC). All the diagnoses were confirmed base on clinical features, electrophysiological study, muscle biopsies, blood acyl-carnitine/urine organic acid analysis, acid alpha-glucosidase (GAA) activity assay, and gene analysis. Four muscle biopsies from subjects who were ultimately deemed to be free of neuromuscular diseases were selected as the normal controls (NC). The profiles of DC and NC are summarized in Supplementary Table 1, http://links.lww.com/CM9/A20. Muscle specimens from patients and controls were frozen in isopentane that had already been precooled in liquid nitrogen and stored at −80°C.

Western blotting

Western blotting was performed on muscle homogenates. Aliquots containing 20-μg protein were loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel for electrophoresis. Proteins were transferred to polyvinylidene fluoride (PVDF) (Bio-Rad, USA) for 1.5 h at 100 V, 4°C. All the total and phosphorylated antibodies, such as S6 and p70S6K, were obtained from Cell Signaling

Table 1: The clinical characteristics of 25 patients with inherited metabolic myopathies.

Patient	Age of onset (years)	Age of biopsy (years)	Gender (M/F)	Clinical diagnosis	Gene diagnosis	CK (U/L)	Biopsied muscle	MMT of biopsied muscle*
L1	65	67	F	LSD	*ETFDH* c.770A>G; c.1227A>C; c.−75A>G	102	BB	4−
L2	50	51	F	LSD	*ETFDH* c.1205C>T; c.1411A>G	342	BB	5
L3	35	37	M	LSD	*ETFDH* c.770A>G	2389	BB	4+
L4	28	29	M	LSD	*ETFDH* c.1157G>T	236	BB	5
L5	3	3	F	LSD	*PNPLA2* c.516C>G; c.918delG	1480	BB	5
L6	24	25	M	LSD	*ETFDH* c.829_830insG	970	BB	5−
L7	31	31	F	LSD	*ETFDH* c.389A>T; c.1395T>G	206	BB	5
P1	26	30	F	PD	*GAA* c.2105G>A; c.2238G>C	185	BB	5−
P2	20	22	F	PD	*GAA* c.1726G>A; c.1735G>A,	704	BB	4
P3	Birth	24	F	PD	*GAA* c.1082C>T; c.1082C>T	150	BB	4+
P4	22	29	M	PD	*GAA* c.2238G>C; c.2297A>C	217	BB	5
P5	1	8	M	PD	*GAA* c.2237G>A; c.2238G>C	399	BB	5−
P6	21	22	F	PD	*GAA* c.2238G>C; c.2662G>T	1731	BB	5
P7	14	19	M	PD	*GAA* c.837G>C; c.2238G>C	1710	BB	5
M1	14	17	F	MM	mtDNA deletion	106	BB	5
M2	20	31	M	MM	mtDNA deletion	136	BB	5
M3	20	20	M	MM	m.3243A>G	1243	BB	5
M4	58	58	F	MM	m.3243A>G	53	BB	5
M5	35	38	M	MM	m.3243A>G	22,120	BB	4
M6	21	42	M	MM	mtDNA deletion	101	BB	4−
M7	11	47	F	MM	mtDNA deletion	61	BB	4
M8	60	60	M	MM	m.3243A>G	104	BB	4
M9	35	35	M	MM	m.3243A>G	202	BB	5
M10	10	10	F	MM	m.3243A>G	147	BB	5
M11	34	44	M	MM	m.8344A>G	340	BB	4

* Muscle strength was evaluated by the ordinal six-point 0 to 5 of manual muscle testing. M: Male; F: Female; BB: Biceps brachii; CK: Creatine kinase; *ETFDH*: Electron transfer flavoprotein dehydrogenase; GAA: Acid alpha-glucosidase; LSD: Lipid storage disease; MM: Mitochondrial myopathy; MMT: Manual muscle testing; mtDNA: Mitochondrial DNA; *PNPLA*: Patatin-like phospholipase domain-containing protein; PD: Pompe disease.

Technology (USA), glyceraldehyde-3-phosphate dehydrogenase was used as internal standard control. The signal was detected by enhanced chemiluminescence using a Western blotting luminol reagent (Merck Millipore, Germany). The mean gray values of protein bands were measured by ImageJ 1.46r software (National Institutes of Health, USA).

Muscle pathology

Frozen muscle samples were sliced into 6-μm-thick serial sections. For histological examination, serial sections were stained with hematoxylin and eosin (HE), succinate reductase (SDH) and cytochrome C oxidase (COX) (S/C), oil red O (ORO) and periodic acid Schiff (PAS). Immunohistochemical study with phosphorylated S6 ribosomal protein (p-S6) rabbit monoclonal antibody (diluted in 1:200) were performed with routine protocol. The p-S6 positive fibers, blue fibers (S/C double stain), basophilic vacuolated fibers, and fibers with increased lipid droplets were counted under light microscopy (magnification × 200).

Statistical analysis

All data were presented as the mean ± standard error (SE). Mann Whitney *U* test was used to compare the results between the two groups. Statistical analyses were performed using the GraphPad Prism 5.02 software (GraphPad Software Inc., USA). A *P* value of <0.05 was considered statistically significant.

Results

The mTOR signaling pathway was up-regulated in muscle with IMM

As shown in Figure 1, we evaluated the activity of mTOR signaling pathway in muscles by investigating the phosphorylation of S6 and p70S6K, downstream targets of mTOR.[16] The p70S6K is a direct mTORC1 substrate, the ratio of phosphorylated p70S6K/total p70S6K was significantly increased in muscles with LSD and MM compared with normal controls (NC *vs.* LSD, $U=2.000$, $P=0.024$; NC *vs.* MM: $U=6.000$, $P=0.043$). The phosphorylation level of p70S6K also trended higher in muscles with PD despite no significant difference was observed (NC *vs.* PD, $U=8.000$, $P=0.315$).

Activated p70S6K phosphorylates the downstream S6 ribosomal protein. The ratio of p-S6/total S6 was higher in muscles with IMM than that in normal controls (NC *vs.* LSD, $U=0.000$, $P=0.006$; NC *vs.* PD, $U=0.000$, $P=0.006$; NC *vs.* MM, $U=1.000$, $P=0.007$) and disease controls (ND *vs.* LSD, $U=0.000$, $P=0.006$; ND *vs.* PD, $U=0.000$, $P=0.006$; ND *vs.* MM, $U=0.000$, $P=0.005$; MD *vs.* LSD, $U=0.000$, $P=0.006$; MD *vs.* PD, $U=0.000$, $P=0.006$; MD *vs.* MM, $U=0.000$, $P=0.005$). There was no significant difference of phosphorylated S6 and p70S6K between disease controls and normal controls. These results highly indicated that the mTOR pathway was activated in muscles from patients with IMMs.

Activated mTOR signaling pathway was correlated with respiratory chain deficiency and lipid/glycogen storage

As shown in Figure 2A, serial sections showed that the induction of p-S6 in muscles of metabolic myopathies was robust but regional: In MM muscles, p-S6 were prominent in blue fibers (with advanced respiratory chain deficiency). More than 30% COX deficiency fibers (blue fibers) are p-S6 positive, while among COX positive myofibers, less than 3% fibers showed p-S6 positive [Figure 2B] (COX deficiency fibers *vs.* COX positive fibers, $U=5.000$, $P=0.001$).

ORO and PAS staining were performed for labeling the lipid droplets or glycogen particles in LSD or PD muscles respectively. More than 90% of vacuolated fibers

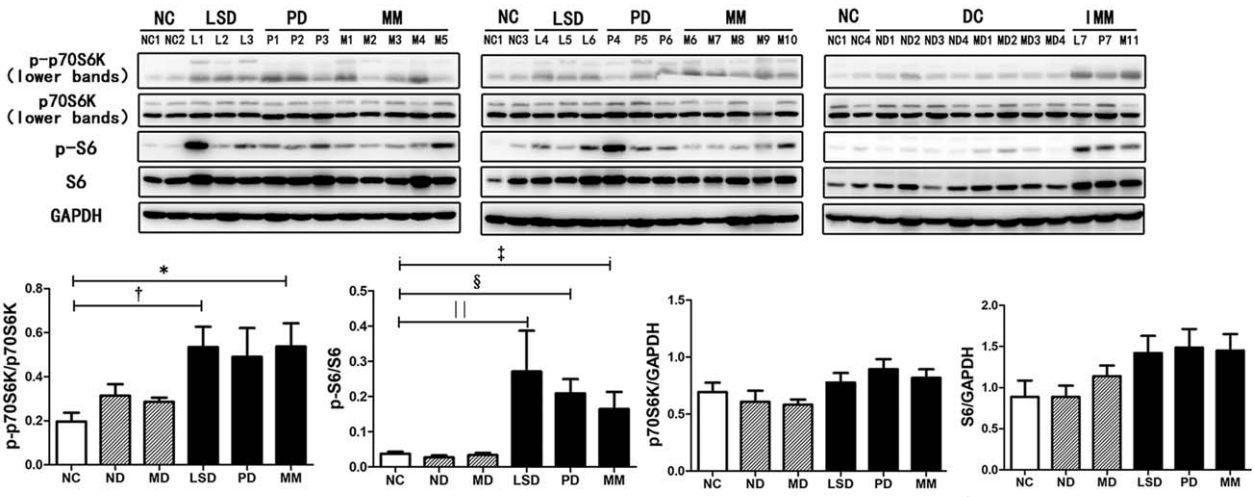

Figure 1: Western blotting analysis of phosphorylation of p70S6K and S6 in the muscle of inherited metabolic myopathies patients and controls. *NC *vs.* MM: $U=6.000$, $P=0.043$; †NC *vs.* LSD, $U=2.000$, $P=0.024$; ‡NC *vs.* MM: $U=1.000$, $P=0.007$; §NC *vs.* PD: $U=0.000$, $P=0.006$; ||NC *vs.* LSD: $U=0.000$, $P=0.006$. DC: Disease control; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase glyceraldehyde-3-phosphate dehydrogenase; IMM: Inherited metabolic myopathy; LSD: Lipid storage disease; MD: Myotonic dystrophy; MM: Mitochondrial myopathy; NC: Normal control; ND: Neurogenic damage; PD: Pompe disease; p-p70S6K: Phosphorylation of p70S6 kinase; p-S6: Phosphorylation of S6 ribosomal protein; S6: S6 ribosomal protein.

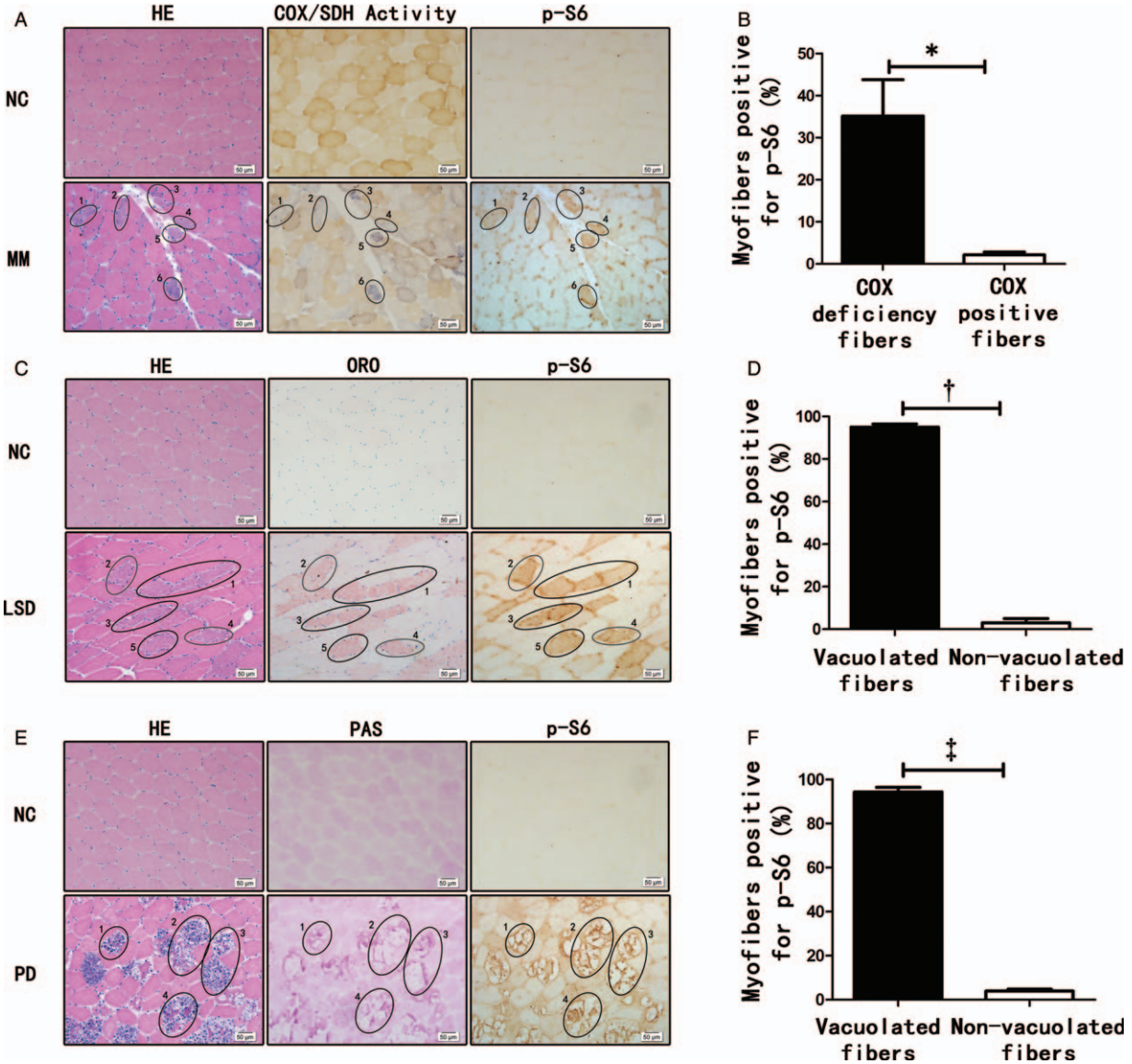

Figure 2: Expression of p-S6 in muscle sections from inherited metabolic myopathies patients and controls. (A) Respiratory chain activity and S6-phosphorylation in muscle from one normal control and one patient with mitochondrial myopathy. Histochemical activity analysis of cytochrome C oxidase (COX; brown) and succinate dehydrogenase (SDH; blue) and p-S6 immunostaining in consecutive sections. (B) Amount of p-S6 positive muscle fibers in COX negative fibers (blue fibers) and COX positive fibers from muscle sections of mitochondrial myopathy. (C) Lipid storage and S6-phosphorylation in muscle from one normal control and one patient with lipid storage disease. Analysis of HE, ORO staining and p-S6 immunostaining in consecutive sections. (D) Amount of p-S6 positive muscle fibers in vacuolated fibers and non-vacuolated fibers from muscle sections of lipid storage disease. (E) glycogen storage and S6-phosphorylation in muscle from one normal control and one patient with lipid storage disease. Analysis of HE, PAS staining and p-S6 immunostaining in consecutive sections. (F) Amount of p-S6 positive muscle fibers in vacuolated fibers and non-vacuolated fibers from muscle sections of Pompe disease. Original magnification, × 200; three microscopic field of consecutive sections were measured for each patient. Same fibers in consecutive sections were circled and marked with number. *COX deficiency fibers *vs.* COX positive fibers: $U=5.000$, $P=0.001$; †Vacuolated fibers *vs.* Non-vacuolated fibers: $U=0.000$, $P=0.002$; ‡Vacuolated fibers *vs.* Non-vacuolated fibers: $U=0.000$, $P=0.002$. COX: Cytochrome C oxidase; HE: Hematoxylin-eosin; LSD: Lipid storage disease; MM: Mitochondrial myopathy; NC: Normal control; ORO: Oil red O; PAS: Periodic acid Schiff; PD: Pompe disease; p-S6: Phosphorylation of S6 ribosomal protein; SDH: Succinate reductase.

containing increased lipid droplet were positive for p-S6 while most non-vacuolated fibers were p-S6 negative (vacuolated fibers *vs.* non-vacuolated fibers, $U=0.000$, $P=0.002$) [Figure 2C and 2D]. Likewise, in patients with PD, Most basophilic vacuolated fibers were p-S6 positive, while non-vacuolated fibers were p-S6 negative [Figure 2E and 2F] (vacuolated fibers *vs.* non-vacuolated fibers, $U=0.000$, $P=0.002$). These findings demonstrated that the activation of mTOR pathway was paralleled with metabolic defects in affected myofibers.

Discussion

For a long time, mTOR has been considered as an important component in the energy and nutrient pathways, all its biological functions are essential to guarantee body survival and homeostasis. The mTOR signaling pathway plays an important role in regulating mitochondrial oxidative function, lipid metabolism[17] and autophagy. The present report is the first study on mTOR pathway in skeletal muscles from patients with IMMs.

Phosphorylation of p70S6K or S6 ribosomal protein was a useful biomarker for evaluating the activity of mTOR pathway. For the first time, we found a significantly increased level of phosphorylated S6 ribosomal protein and p70S6K in IMM muscles compared with disease and normal controls, indicating an activated mTOR signaling pathway in patients with glycogen and lipid metabolic defect as well as mitochondrial dysfunction. Since the phosphorylation of S6 ribosomal protein and p70S6K were unchanged in disease controls, the up-regulation of mTOR signaling in IMMs muscles was considered to be specific. Moreover, immunohistochemical staining of serial sections showed that mTOR pathway was induced in a mosaic pattern: positive p-S6 staining mainly located in myofibers with metabolic defects, including COX deficiency fibers and fibers with lipid or glycogen storage. The p-S6 staining intensity was paralleled with the ORO or PAS staining intensity, indicating that there may be some correlations between the mTOR signaling pathway and severity of metabolic defects.

Our findings suggested that the mTOR pathway was activated in MM and LSD patients, which is in line with most previous studies in animal models. Increased mTOR activity was observed respectively in brain and muscle from mice with mitochondrial encephalopathy[9] or myopathy.[18] Besides, the activated mTOR pathway was observed in brain, liver, and kidney of homozygotes zebrafish of LSD.[15]

Interestingly, we also found the phosphorylation of S6 and p70S6K were up-regulated in PD muscles. These findings are inconsistent with some previous studies in which mTOR pathway was suppressed in primary skin fibroblast from patients with PD[19] and myotubes from acid alpha-glucosidase (GAA) knock-out mouse, a murine model of PD.[20] The authors suggested that the disturbed mTOR pathway may result in abnormally enhanced autophagy in PD, and mTOR pathway agonist may ameliorate disease symptoms. In contrast, several other studies reported that mTOR inhibition could benefit GAA knock-out mouse and GSD III canine model via suppressing glycogen synthesis in skeletal muscle.[13,14] Taken together, we speculated that the role of mTOR pathway in GSD may be very sophisticated, the contradictory results might be caused by evaluation in different stages of the disease course.

Although the pathogenesis is varied, upregulated mTOR activity in muscles from patients with respiratory chain disorders and lipid/glycogen dysmetabolism was found an up-regulated mTOR activity coincidentally in our study. Combined with previous reports,[21,22] we assumed that the enhanced mTOR signaling might be a feedback response to severe or persistent oxidative stress load: Increased reactive oxygen species (ROS) has been well documented in several studies of cells from patients with mitochondrial encephalomyopathy,[23,24] LSD,[25] and PD.[26] Accumulation of pathological levels of ROS may induce a metabolic reprogramming and reduce ROS production, allowing cells to up-regulate oxygen-independent glycolysis instead of oxygen-dependent oxidative phosphorylation (OXPHOS), which is known as Warburg switching.[27] Also, it is reported that mTOR plays an important role in modulating Warburg switching by activates hypoxia-inducible factor-1α (HIF-1α).[28-30] Taken together, we hypothesized that elevated mTOR signaling in IMM muscles may be involved in modulating ROS induced metabolic reprogramming. Besides, activated mTOR pathway may also be related to other stress response,[18] including the transcription of metabolic cytokines, mitochondria unfolded protein response.

In conclusion, we found that the mTOR pathway was activated in myofibers with various metabolic defects, indicating that enhanced mTOR signaling may be a feedback response to severe or persistent metabolic stress load. This study may provide evidence for mTOR inhibition therapy in human IMMs.

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Conflicts of interest

None.

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