Muscle choline kinase beta defect causes mitochondrial dysfunction and increased mitophagy

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Choline kinase is the first step enzyme for phosphatidylcholine (PC) de novo biosynthesis. Loss of choline kinase activity in muscle causes rostrocaudal muscular dystrophy (rmd) in mouse and congenital muscular dystrophy in human, characterized by distinct mitochondrial morphological abnormalities. We performed biochemical and pathological analyses on skeletal muscle mitochondria from rmd mice. No mitochondria were found in the center of muscle fibers, while those located at the periphery of the fibers were significantly enlarged. Muscle mitochondria in rmd mice exhibited significantly decreased PC levels, impaired respiratory chain enzyme activities, decreased mitochondrial ATP synthesis, decreased coenzyme Q and increased superoxide production. Electron microscopy showed the selective autophagic elimination of mitochondria in rmd muscle. Molecular markers of mitophagy, including Parkin, PINK1, LC3, polyubiquitin and p62, were localized to mitochondria of rmd muscle. Quantitative analysis shows that the number of mitochondria in muscle fibers and mitochondrial DNA copy number were decreased. We demonstrated that the genetic defect in choline kinase in muscle results in mitochondrial dysfunction and subsequent mitochondrial loss through enhanced activation of mitophagy. These findings provide a first evidence for a pathomechanistic link between de novo PC biosynthesis and mitochondrial abnormality.

INTRODUCTION

Phosphatidylcholine (PC) is the major phospholipid in eukaryotic cell membranes. Disruption of PC synthesis by loss-of-function mutations in CHKB (GenBank Gene ID 1120), which encodes the primary choline kinase isoform in muscle, causes autosomal recessive congenital muscular dystrophy with mitochondrial structural abnormalities in human (1). Loss-of-function mutation in the murine ortholog, Chkb, is reported to cause rostrocaudal muscular dystrophy (rmd) in the laboratory mouse (2). Rmd is so-named because of a gradient of severity of muscle damage—hindlimbs (caudal) are affected more severely than forelimbs (rostral). The most outstanding feature of the muscle pathology in both human patients and rmd mice is a peculiar mitochondrial abnormality—mitochondria are greatly enlarged at the periphery of the fiber and absent from the center.

Mitochondria have a variety of cellular functions from energy production to triggering apoptotic cell death (3,4). Inhibition of mitochondrial respiration [chemically or by mitochondrial DNA (mDNA) mutations], disruption of inner membrane potential, senescence and enhanced reactive oxygen species (ROS) production are all known to cause mitochondrial morphological abnormalities (5–8). Conversely,
primary mitochondrial morphological changes can subsequently cause mitochondrial and cellular dysfunction. Mitochondria are dynamic organelles, which continuously fuse and divide. Disequilibrium of mitochondrial fusion and fission can cause alterations of mitochondrial morphology with mitochondrial dysfunction (9,10). Thus, mitochondrial function and morphology are tightly linked.

It has been reported that mitochondria in rmd show decreased membrane potential (11). However, there have been no further studies about mitochondrial functional abnormalities in rmd, although its morphology is the most distinct feature compared with other myopathies. In addition, there has been no study about mitochondrial function when PC synthesis is blocked in vivo, although mitochondrial respiratory enzyme activities are dependent on membrane phospholipids (12). We hypothesized that the abnormal mitochondrial morphology in rmd skeletal muscles indicates the presence of a bioenergetic dysfunction caused by mitochondrial membrane phospholipid alteration.

In this study, we demonstrate that mitochondria in rmd mouse muscle show reduced PC level, bioenergetic dysfunction and increased ROS production are ubiquitinated and eliminated via mitophagy, leading to the peculiar mitochondrial loss in the skeletal muscle. These findings provide further evidence that mitochondrial dysfunction is related to phospholipid metabolism and may play a role in the pathogenesis of muscle disease.

RESULTS

Light microscopic examination of H&E-stained samples from 8-week-old homozygous rmd mutant mice and littermate controls confirmed dystrophic muscle pathology, especially in hindlimb muscles, as previously described (2) (Fig. 1A–D). NADH-TR and immunohistochemistry for mitochondrial outer membrane protein Tom20 and EM (M–P) show abnormal mitochondria. Mitochondria in rmd muscle fibers are enlarged and prominent at the periphery, but sparse in the center (I–L). z, Z line; m, M line; i, I band.

Figure 1. Muscle histopathology. H&E staining of triceps or quadriceps femoris muscles in 8-week-old homozygous rmd mutant mice and unaffected (+/rmd or +/+ littermate controls (A–D) shows dystrophic changes including variation in fiber size, necrosis and regeneration of individual fibers and interstitial fibrosis. NADH-TR staining (E–H), immunostaining of mitochondrial outer membrane protein Tom20 and EM (M–P) show abnormal mitochondria. Mitochondria in rmd muscle fibers are enlarged and prominent at the periphery, but sparse in the center (I–L). z, Z line; m, M line; i, I band.
synthesis in isolated mitochondria in *rmd* muscle. Compared with healthy littermates, only mitochondrial respiratory Complex III activity was significantly decreased in mitochondria from *rmd* forelimb muscles, while Complex I, III and IV activities were significantly decreased in *rmd* hindlimb muscles (Fig. 3A). Mitochondrial ATP synthesis was severely decreased, especially in hindlimb muscles (Fig. 3B), and coenzyme Q9 was moderately decreased in *rmd* compared with littermates (Fig. 3C).

In-gel activity staining on native PAGE showed decreased Complex III activity, especially in hindlimb (Fig. 4A), although normal protein levels of the Complex III were detected by western blot followed by Native PAGE (Fig. 4B). There was no difference in mobility of Complex III in *rmd* and littermate. Furthermore, respiratory chain supercomplex formation, which is important for effective electron transport (24), was not altered in *rmd* (Supplementary Material, Fig. S1).

Mitochondria are a major site of ROS production under normal circumstances and the production of ROS is enhanced when respiration is blocked. To determine whether the identified respiratory defects lead to elevated ROS, we measured superoxide levels from isolated mitochondria. Superoxide production was significantly increased in *rmd* muscle mitochondria, especially in those isolated from the hindlimbs (Fig. 5A). Moreover, the MDA level (Fig. 5B) and 4-hydroxynonenal adducts (Fig. 5C) were increased in *rmd* muscles indicating that oxidative stress is increased in *rmd* muscle.

Interestingly, examination of muscle sections by EM revealed autophagosomes selectively engulfing an entire mitochondrion, without cytoplasm, suggesting that mitophagy is activated in *rmd* skeletal muscles (Fig. 6A). Western blots of isolated mitochondria from muscle showed significantly increased levels of the autophagosome marker LC3 in *rmd* (Fig. 6B). In addition, polyubiquitinated proteins and p62/SQSTM1, which connects ubiquitination and autophagic machineries, were also increased in isolated mitochondria (Fig. 6B). These data suggest that mitochondria are polyubiquitinated and p62 is recruited to mitochondria. We also analyzed PINK1 and the E3 ubiquitin ligase Parkin, which are known to contribute to ubiquitination and mitophagy of damaged mitochondria (25,26). PINK1 and Parkin levels were increased in *rmd* isolated muscle mitochondria (Fig. 6B), suggesting that they were recruited to mitochondria to promote mitophagy. Immunohistochemical analyses demonstrated the colocalization of p62, polyubiquitin and LC3 with mitochondria (Fig. 6C).

We quantified mitochondrial numbers in muscle fibers, mitochondria occupying-area relative to muscle cross-sectional area and mean mitochondrial area in cross-section by morphometric analysis in EM. In *rmd*, the average number of mitochondria per fiber was profoundly decreased (Fig. 7A). However, the average area occupied by mitochondria in each muscle fiber was comparable with littermates (Fig. 7A). This was due to increased mean mitochondrial area in *rmd* (Fig. 7A).

We quantified mtDNA copy number relative to nuclear DNA. In *rmd*, mtDNA was decreased both in forelimb and...
hindlimb muscles compared with littermate controls (Fig. 7B),
which was in agreement with the number of mitochondria
decrease. The mtDNA copy number in liver is preserved in
rmd, and reduction in muscle is progressive in age.

**DISCUSSION**

In the rmd mouse, we observed greater superoxide production
and more significant Complex III and ATP synthesis
deficiencies in hindlimb than in forelimb muscles, correlating
with the more severe caudal phenotype. PC was decreased in
isolated rmd muscle mitochondria as a consequence of disrup-
tion of muscle PC biosynthesis because PC cannot be syn-
thesized in mitochondria. This suggests that muscle damage
in the rmd mouse is primarily due to mitochondrial dysfunc-
tion possibly caused by the impaired PC biosynthesis.

Why then are mitochondrial functions altered when PC is
decreased? Mitochondria produce energy mainly via oxidative
phosphorylation, which transfers electrons by a series of redox
reactions through four enzyme complexes, and pumps protons
across the mitochondrial inner membrane, producing an elec-
trochemical proton gradient that enables ATP synthesis (3).
Here, we demonstrate for the first time a Complex III activity
decrease without the loss of the enzyme protein complex in
rmd muscle mitochondria, suggesting a link between
decreased PC content and Complex III activity. One possible
explanation is that mitochondrial PC alterations may directly
impair Complex III function by affecting lipid–protein
interactions (27). PC is a component of the yeast respiratory
enzyme complex, as revealed by X-ray crystallography, and
thus may regulate enzyme function (28). Alteration of fatty
acid composition in PC has been shown to change enzymatic
activity in Complexes I, III and IV in a mouse model (29). In
this model, Complex III activity is profoundly increased when
n-3 fatty acid is increased. In rmd, it is reported that docosa-
hexaenoic acid containing PC, the major n-3 fatty acid in
muscle PC, is profoundly decreased in muscle and isolated
mitochondria (1). This suggests a possible association between
phospholipid composition alterations and respiratory chain
enzymatic activities due to the choline kinase defect in rmd
muscle.

Through the oxidative phosphorylation process, ROS are
also generated as byproducts even in normal cellular states,
but especially when respiration is inhibited (30,31). In rmd
mouse muscle, ROS production from isolated mitochondria
was increased, which may be related to the respiratory chain
defect caused by PC reduction in mitochondria. Interestingly,
selenium-deficient myopathy is associated with muscle pathol-
ogy showing similar enlarged and sparse mitochondrial
morphological abnormalities to the rmd mice and the human
congenital muscular dystrophy caused by CHKB
mutations (32). As selenium is a cofactor of glutathione peroxidase,
selenium deficiency is thought to cause oxidative stress (33,34).
Morphological similarity between choline kinase beta
deficiency and selenium deficiency suggests that ROS may
play a key role in the formation of the mitochondrial
abnormalities in rmd myopathy. In another model, depletion of glutathione, which provides cells with a reducing environment and detoxifies the ROS, is reported to cause mitochondria enlargement in muscle, also suggesting the possible link between mitochondrial enlargement and ROS in skeletal muscle (35).

In addition, as a major site of ROS production, mitochondria themselves are prone to ROS damage (36). Recent studies have shown that damaged mitochondria are eliminated by selective autophagy, called mitophagy, most likely as a quality control mechanism to protect the cells (37,38). In addition to mitochondrial enlargement, we observed large areas devoid of mitochondria. Mitochondrial depolarization can trigger mitophagy in cell culture models (26). PINK1 and Parkin interactions promote ubiquitination of mitochondrial outer membrane proteins, and induce mitophagy. This process is mediated by p62, an adaptor molecule, which interacts directly with ubiquitin and LC3 (25,39). ROS generated from mitochondria are also important for mitophagy (39).

Interestingly, we found increased mitophagy in rmd, accompanied by mitochondrial ubiquitination and recruitment of p62 and LC3. Enhanced PINK1 and Parkin expression in mitochondria likely reflects the process of elimination of damaged mitochondria as a consequence of mitochondrial dysfunction and ROS production. These findings were similar to those in cells treated with the protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) or respiratory chain inhibitors (25,26). In rmd, decreased membrane potential (11), as a consequence of respiratory chain insufficiency and ROS production, may trigger mitophagy and thus increased mitochondrial clearance, which may lead to energy crisis and result in cell death and muscular dystrophy.

We observed progressive loss of mtDNA with age, together with progressive loss of mitochondria. We suggest that mtDNA depletion in this case results from increased mitophagy, because mtDNA is known to be degraded by mitophagy in cultured hepatocytes (40) and because the pathological features of CHKB-deficient myopathy are clearly distinct from those
observed in ‘primary’ mtDNA depletion syndromes, usually associated with defective mtDNA synthesis, in which muscle fiber mitochondria are increased both in number and size, causing the ‘ragged-red fiber’ appearance (41).

In summary, we have demonstrated for the first time a pathogenic mechanism that links PC reduction in the mitochondrial membranes of rmd muscle to mitochondrial morphological and functional abnormalities and the induction of mitophagy as a response to structural and functional damage by ROS generation or impaired bioenergetics. These findings indicate the importance of PC de novo synthesis pathway and phospholipid composition of mitochondrial membrane in the maintenance of mitochondria and muscle.

MATERIALS AND METHODS

Rmd mice

Eight-week-old rmd mice (2) were used for all analysis and were compared with healthy littermates. The Ethical Review Committee on the Care and Use of Rodents in the National Institute of Neuroscience, National Center of Neurology and Psychiatry approved all mouse experiments.

Histological analyses

The quadriceps femoris muscles were freeze-fixed in liquid-nitrogen-cooled isopentane and stored at −80°C. Serial transverse sections of 10 μm thickness were stained with a series of histochemical methods, including hematoxylin and eosin (H&E) and nicotine amide adenine dinucleotide-tetrazolium reductase (NADH-TR), as previously described (13), and were observed by light microscopy.

Immunohistochemical analyses were performed as previously described (13). Briefly, 6 μm thick frozen muscle sections were fixed in cold acetone for 5 min. After blocking with 5% normal goat serum, sections were incubated with primary antibodies for 2 h at 37°C. After rinses with phosphate-buffered saline, sections were incubated with secondary Alexa Fluor 488- or Alexa Fluor 568-labeled goat anti-mouse

Figure 5. Mitochondrial superoxide production is increased and oxidative stress is increased in muscle tissue in rmd. (A) Mitochondrial superoxide production is enhanced in rmd, especially in hindlimb muscle mitochondria. Data are shown as the mean ± SD of seven experiments. *P < 0.001. (B) MDA levels are increased in muscle tissue. **P < 0.0005. Data are shown as the mean ± SD (n = 4 for rmd and n = 5 for littermate controls). (C) HNE4-modified proteins are increased in rmd hindlimb muscle. Coomassie brilliant blue staining is shown as a loading control. Representative data of six samples.

Figure 6. Mitochondrial degeneration in rmd. (A) EM of extensor digitorum longus muscle. In rmd, mitochondria are degraded by mitophagy. Scale bar = 0.5 μm. (B) Western blot of isolated muscle mitochondria immunodetected for Parkin, polyubiquitin, p62/SQSTM1 and LC3. TOM20, a mitochondrial outer membrane protein is used as loading control. Hindlimb mitochondria in rmd show significantly increased expression level in these mitophagy markers. (C) p62 and TOM20 immunohistochemistry of hindlimb muscle section. Note that mitochondria are significantly enlarged and sparse in rmd. p62 colocalizes with the mitochondrial outer membrane protein TOM20. Polyubiquitin and mitochondrial protein cytochrome c oxidase (COX) colocalize. LC3 and TOM20 colocalize. Polyubiquitin and p62 colocalize. Scale bar = 10 μm.
or rabbit antibodies at room temperature for 45 min. Confocal images were obtained with FLUOVIEW FV500 systems (Olympus) using a ×100 objective.

For observation by electron microscopy (EM), muscle samples were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Specimens were post-fixed in 1% osmium tetroxide in the same buffer, dehydrated with graded series of ethanol and embedded in epon, as previously described (13). Ultrathin sections were stained with uranyl acetate and lead citrated, and were analyzed by a FEI Tecnai Spirit at 120 kV.

**Isolation of skeletal muscle mitochondria**

Mitochondria from skeletal muscle of whole forelimb and hindlimbs were isolated by differential centrifugation. Fresh muscle was minced and homogenized using a motor-driven Teflon pestle homogenizer with ice-cold mitochondrial isolation buffer [10 mM Tris–HCl pH 7.2, 320 mM sucrose, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 1 mg/ml bovine serum albumin (BSA)] and centrifuged at 1500g for 5 min. Supernatant fraction was centrifuged at 15 000g for 20 min, and the pellet was resuspended in mitochondrial isolation buffer. The centrifugation was repeated twice. Protein concentration was determined by the Bradford method using Bio-Rad Protein Assay (Bio-Rad Laboratories), according to the manufacturer’s protocol.

**Lipid extraction, phospholipid separation and determination**

PC, phosphatidylethanolamine (PE) and cardiolipin (CL) were extracted from isolated mitochondria of forelimb and hindlimb muscles, separated by one-dimensional thin layer chromatography (TLC) and amount of each phospholipid was measured by phosphorus analysis (14,15). Briefly, total lipids in frozen muscle biopsy samples were extracted according to the method of Bligh and Dyer (14). Each extract was evaporated to dryness under nitrogen, and the residues were then dissolved in a small amount of a 2:1 v/v mixture of chloroform and methanol and applied to a TLC plate (Merck, Silica Gel 60). The plate was developed with a medium of chloroform: methanol:formic acid:acetic acid = 100:100:9:9 (v/v/v/v). The products and standards were visualized with primulin reagent, and the products identified by comparison with chromatographic standards. PC and PE were then scraped from the TLC plate for quantification. Phospholipids were quantified according to the method of Rouser et al. (15). Briefly, the lipids were digested by heating for 1 h at 200°C with 70% perchloric acid. After cooling, ammonium molybdate and ascorbic acid solution were added in that order. Color was developed after heating for 5 min in a boiling water bath. Absorbance was determined at 820 nm by spectrophotometer. Phospholipid levels were corrected by the total protein amount in isolated mitochondria.
Respiratory enzyme activity and ATP synthesis

Mitochondrial respiratory enzyme activities were measured as previously described, using colorimetric assays in isolated mitochondria (16,17). Complex I (NADH-ubiquinone oxidoreductase) activity was measured by the reduction of 10 μM decylubiquinone (DB) in the presence of 2 mM potassium cyanide (KCN), 50 μg/ml antimycin and 50 μM NADH at 272 nm. Complex II (succinate-ubiquinone oxidoreductase) activity was measured by the reduction of 50 μM 2,6-dichlorophenolindophenol in the presence of 20 mM succinate, 2.5 μg/ml rotenone, 2.5 μg/ml antimycin, 2 mM KCN and 50 μM DB at 600 nm. Complex III (ubiquinol-ferricytochrome c oxidoreductase) activity was measured by the reduction of 50 μM cytochrome c at 550 nm in the presence of 50 μM reduced DB and 2 mM KCN. Complex IV (ferrocytochrome c-oxigen oxidoreductase) activity was measured by the oxidation of 2.5 μM reduced cytochrome c at 550 nm. The activity was calculated using an extinction coefficient of 8 μM⁻¹ cm⁻¹, 19.1 μM⁻¹ cm⁻¹, 19.0 μM⁻¹ cm⁻¹ and 19.0 μM⁻¹ cm⁻¹ for Complexes I, II, III and IV, respectively. The specific activity of the enzymes was expressed as nmol of each substrate oxidized or reduced/min/mg of mitochondrial protein.

Mitochondrial ATP synthesis was measured by the method of Manfredi and colleagues (18). Briefly, isolated mitochondria were resuspended in 0.25 m sucrose, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM MgCl₂, 1 mM ethylene glycol tetraacetic acid (EGTA) and 10 mM KH₂PO₄, pH 7.4. Then 0.15 mM P₁,P₅-di(adenosine) pentaphosphate, 1 mM malate, 1 mM pyruvate, luciferin and luciferase and 0.1 mM adenosine triphosphate (ATP) were added, and light emission was recorded by luminometer. For each sample, 1 mM oligomycin-added sample was used to obtain the baseline luminescence corresponding to non-mitochondrial ATP production.

CoQ9 determination

Total CoQ9 contents in isolated mitochondria were analyzed with high performance liquid chromatography (HPLC) by electrochemical detection according to the standard procedure described by Tang et al. (19). Briefly, isolated muscle mitochondria pellet were lysed with 2-propanol, vortexed for 1 min and centrifuged at 2000g for 10 min and then clear supernatant was applied for HPLC Coul Array Detector Model 5600A (ESA BIOSCIENCES, Inc.) with Capcell Pak C18 MG 100 column (3.2 I.D × 150 mm length; ESA BIOSCIENCES, Inc.). The mobile phase was degassed methanol containing 0.4% sodium acetate, 1.5% acetic acid, 1% 2-propanol and 8% n-hexane. Chromatographic data were analyzed with CoulArray Data Station 3.00 (ESA BIOSciences). Standard curves were created with both oxidized and reduced CoQ9. Total CoQ9 level was determined according to the standard curve and corrected by the total protein level in isolated mitochondria as measured by the Bradford method.

High-resolution clear native PAGE

High-resolution clear native polyacrylamide gel electrophoresis (PAGE) was performed by the method of Wittig et al. (20). Briefly, isolated mitochondria were solubilized with Native PAGE Sample buffer (Invitrogen) containing 0.3% n-dodecyl-β-D-maltoside (Dojindo). Twenty micrograms of protein were applied to 3–12% NativePAGE Bis-Tris gel (Invitrogen). Native PAGE buffer (Invitrogen) was used for anode buffer and Native PAGE buffer containing 0.02% n-dodecyl-β-D-maltoside and 0.05% deoxycholate was used for cathode buffer.

For in-gel catalytic activity assays, gels were incubated in the following solutions: Complex I, 5 mM Tris–HCl pH 7.4, 140 μM NADH and 3 mM nitro tetrazolium blue (NTB); Complex II, 5 mM Tris–HCl pH 7.4, 20 mM succinate, 3 mM NTB and 200 μM phenazine methosulfate; Complex III, 50 mM sodium phosphate buffer pH 7.2 and 0.5 mg/ml diaminobenzidine (DAB); Complex IV, 50 mM sodium phosphate buffer (pH 7.2), 0.5 mg/ml DAB and 5 mM cytochrome c.

For immunoblotting, gels were incubated for 20 min in 300 mM Tris, 100 mM acetic acid, 1% sodium dodecyl sulfate (SDS), pH 8.6 and then electrophorrted to polyvinylidene fluoride (PVDF) membrane (Millipore). Complexes II and III were detected with monoclonal antibodies against the 70 kDa subunit (Abcam) and core 2 subunit (Invitrogen), respectively.

Measurement of mitochondrial superoxide (O₂⁻) production

Mitochondrial superoxide production was measured by dehydrothidium (DHE) (Molecular Probes), as described previously (21). Isolated mitochondria were incubated with 200 mM mannitol, 70 mM sucrose, 2 mM HEPES pH 7.4, 0.5 mM EGTA and 0.1% BSA. Reagents were added in the following order: 1 mM glutamate, 1 mM malate, 1 μM DHE, 0.25 mM ADP and 5 mM KH₂PO₄. Fluorescence was measured by Cytofluor 4000 (Applied biosystems) at excitation/emission = 530/620 nm.

Measurement of malondialdehyde in muscle

Malondialdehyde (MDA) levels were measured in muscle homogenates using an LPO-485 assay kit (BIOXYTEC), according to the manufacturer’s protocol.

Western blot analysis for muscle tissue and isolated mitochondria

Proteins were extracted from quadriceps femoris muscles or mitochondria isolated from forelimb and hindlimb muscles and suspended in SDS sample buffer; 125 mM Tris–HCl pH 6.8, 5% β-mercaptoethanol, 2% SDS and 10% glycerol. Extracted proteins were separated on acrylamide gels, and then transferred onto PVDF membranes (Millipore). Blocking solution of 5% skim milk was used. ImageQuant LAS 4000 Mini Biomolecular Imager (GE Healthcare) was used for evaluating bands.

Quantification of mtDNA by real-time PCR

Total DNA was isolated from triceps and quadriceps femoris and liver by proteinase K digestion and standard phenol–chloroform extraction.
DNA−calculated using the following formula: mtDNA forward primer, ATGGAAAGCCTGCCATCATG; reverse primer, GAGGCTGTTGCTTGTGTGAC; forward primer, CCTATCACCCTTGCCATCAT; ND1 genome copy number. We used the following primers: ND1 forward primer, ATGGAAAGCCTGCCATCATG; ND1 reverse primer, GAGGCTGTTGCTTGTGTGAC; pcam1 DNA forward primer, ATGGAAGCCTGCCATCATG; pcam1 DNA reverse primer, TCTTTGTTGTCAGCATCAC.

The amount of mtDNA relative to nuclear DNA was calculated using the following formula: mtDNA/nuclear DNA = 2−(CtmtDNA−CtnuclearDNA) where Ct is the threshold cycle (22).

Morphometrical analysis of mitochondria

Cross-sectional EM image of extensor digitorum longus (EDL) muscle from rmd and littersmates was analyzed by Image J software (23). Total areas of all mitochondria in 20 muscle fibers were calculated and compared with cross-sectional fiber areas. Total number of mitochondria per muscle fiber was counted.

Antibodies

Primary antibodies used were: mouse anti-4-hydroxy-2-nonenal (4-HNE) modified protein antibody (HNEJ-2, JaliCA), rabbit anti-PINK1 antibody (BC100-494, Novus Biologicals), mouse anti-Parkin antibody (4211, Cell Signaling), rabbit anti-p62/SQSTM1 antibody (PWS860, Biomol), rabbit anti-poly-ubiquitin antibody (FK1, Biomol), rabbit anti-TOM20 antibody (FL-145, Santa Cruz), mouse anti-COX subunit 1 antibody (Invitrogen) and mouse anti-VDAC antibody (20B12, Santa Cruz). Second antibodies used were: horse radish peroxidase-labeled goat anti-mouse (Beckman Coulter) or rabbit antibodies (Cell Signaling). Alexa Fluor 488- and Alexa Fluor 568-labeled goat anti-mouse or rabbit antibodies (Invitrogen).

Statistical analysis

Data are presented as mean ± SD. Mean differences were compared with the analysis of t-test using R software version 2.11.0 (http://www.r-project.org/).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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