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Visualization of defective mitochondrial function in skeletal muscle fibers of patients with sporadic amyotrophic lateral sclerosis

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Abstract

The mitochondrial function in skeletal muscle was investigated in skeletal muscle biopsies of 26 patients with sporadic amyotrophic lateral sclerosis (ALS) and compared with investigations of 28 age-matched control muscle samples and biopsies of 6 patients with spinal muscular atrophy (SMA) and two patients with Tay-Sachs disease. In comparison to the control, SMA and Tay-Sachs biopsies, we observed in the ALS samples a significant about two-fold lower activity of complex I of mitochondrial respiratory chain. To visualise the distribution of the mitochondrial defect in skeletal muscle fibers we applied confocal laser-scanning microscopy and video fluorescence microscopy of NAD(P)H and fluorescent flavoproteins. The redox change of mitochondrial NAD(P)H and flavoproteins on addition of mitochondrial substrates, ADP, or cyanide were determined by measurement of fluorescence intensities with dual-photon UV-excitation and single-photon blue excitation. In skeletal muscle fibers of ALS patients with abnormalities of mitochondrial DNA (multiple deletions, $n=1$, or lower mtDNA levels, $n=14$) we observed a heterogeneous distribution of the mitochondrial defects among individual fibers and even within single fibers. In some patients ($n=3$) a mitochondrial defect was also detectable in cultivated skin fibroblasts. These findings support the viewpoint that the observed impairment of mitochondrial function in muscle of certain ALS patients is caused by an intrinsic mitochondrial defect which may be of pathophysiological significance in the etiology of this neurodegenerative disease.

Keywords: Amyotrophic lateral sclerosis; Mitochondrial function; Oxidative phosphorylation; Confocal laser scanning microscopy

1. Introduction

Amyotrophic lateral sclerosis is a rather common motoric disease (incidence 2–3 in 100 000 [1]) resulting in a progressive degeneration of the anterior horn cells of the spinal cord and cortical motor neurons. The primary cause for the neuronal death in amyotrophic lateral sclerosis (ALS) remains unclear. Present concepts relate the neurodegenerative process to glutamate-induced excitotoxicity [2–4], but the proposed direct involvement of glutamate transport is still under dispute [5,6].

There seems to be compelling evidence for increased oxygen radical damage in brain tissue of patient with ALS [7,8]. According to this concept it was demonstrated, that some patients with autosomal-dominant familiar ALS (FALS) have point mutations in the Cu/Zn superoxide dismutase (SOD1) gene [9]. Additionally, it was shown that mice models which carry these mutations develop severe motor neuron disease [10,11]. The most obvious ultrastructural abnormality in these mice models is the presence of vacuoles in axons and dendrites which appear to be derived from degenerating mitochondria [10,12]. Moreover, a remarkable paucity of mitochondria in degenerating axons was observed [13]. In anterior horn neurons of patients with sporadic ALS not related to point mutations in the SOD gene conglomerates of dark abnormal mitochondria were detected [14]. These findings suggest a possible involvement of mitochondria in the

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process of degeneration of motor neurons. Interestingly, Comi et al. [15] detected recently a cytochrome c oxidase (COX) subunit I microdeletion causing a severe COX deficiency in skeletal muscle of a patient with motor neuron disease. We observed a severe deficiency of NADH: CoQ oxidoreductase in skeletal muscle biopsies of 14 patients with sporadic ALS [16]. Here we describe the heterogeneous distribution of the mitochondrial defect in single saponin-permeabilized skeletal muscle fibers of ALS patients applying functional imaging techniques of mitochondria (according to Kuznetsov et al. [17]) and conventional electron microscopy.

2. Materials and methods

2.1. Patients

We studied diagnostic biopsies from musculus vastus lateralis in 26 patients with definite or probable sporadic amyotrophic lateral sclerosis (12 female, 14 male, age range 32–66 years) as defined by the El Escorial criteria [18] and a group of 6 patients with spinal muscular atrophy (5 female, 1 male, age range 6–62 years) and two adult patients with Tay-Sachs disease (hexosaminidase A deficiency; 38-year-old female, 44-year-old male). The neuropathological investigation of all biopsies indicated neurogenic changes in skeletal muscle. Skeletal muscle samples from diagnostic biopsies of 28 patients (age range 30–72 years) with slight myopatic EMG abnormalities but no biopsy evidence for a manifest myopathy were used as controls. All patients gave prior to biopsy written informed consent.

2.2. Solutions

The relaxing solution contained 10 mM EGTA/Ca-EGTA (ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N′*,*N′*-tetraacetate) buffer, free concentration of calcium 0.1 μM, 20 mM imidazole, 20 mM taurine, 49 mM K-MES, 3 mM KH_2PO_4, 9.5 mM $MgCl_2$, 5 mM ATP, 15 mM phosphocreatine, pH 7.1. The measurements were performed in a medium consisting of 110 mM mannitol, 60 mM KCl, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 0.5 mM Na_2EDTA and 60 mM Tris–HCl, pH = 7.4.

2.3. Preparation of muscle fibers

About 50 mg of biopsy tissue (M. vastus lateralis) was used for isolation of saponin-permeabilized fibers. Bundles of muscle fibers containing usually 2–4 single fibers were isolated by mechanical dissection. The saponin treatment was performed by incubation of the fiber bundles in relaxing solution (composition see above) containing 50 μg/ml saponin as described in [19].

2.4. Cell culture conditions

Human skin fibroblasts from control individuals and ALS patients were grown in a sandwich culture from a skin biopsy sample. The cells were cultured in DMEM medium supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 10 μg/ml tylosin at 37°C in a humidified atmosphere with 6.5% CO_2. After reaching confluence, the cells were harvested between passages 4 and 10. For the experiments 10×10^6 cells/ml were suspended in phosphate-buffered saline and stored on ice to avoid attachment.

2.5. Respiration measurements

The oxygen consumption of digitonin-treated cultivated skin fibroblasts was measured at 37°C using a high resolution Oroboros oxygraph (Anton Paar, Graz) according to Kunz et al. [20].

2.6. Enzyme activities

All enzyme activities were determined at 30°C by standard methods as described by Bergmeier [21].

2.7. Fluorescence microscopy

Isolated single fibers were fixed at both ends in a Heraeus flexiperm chamber and incubated in 300 μl medium for measurements. The video images were obtained with an Olympus IX-70 microscope equipped with a Kappa CF 8/1 DXC CCD camera. The NAD(P)H fluorescence image was acquired using 366 nm excitation and 450 nm long path emission (NU filter combination), the flavoprotein fluorescence image was obtained using 436 nm excitation and 525 nm emission (NIBA filter combination). The digital ratio images were calculated using the LSM software (Carl Zeiss).

2.8. Confocal microscopy

The confocal NAD(P)H fluorescence and flavoprotein fluorescence images were acquired using a Biorad MRC1024. For dual-photon excitation of NAD(P)H fluorescence a 10 W solid state laser (Millenia, Spectra Physics) was used to pump a Ti:Sa Laser (Tsunami, Spectra Physics, 82 MHz) tuned to 720 nm. Pulse width behind the laser was 50 fs. Emitted fluorescence was detected between 450 and 650 nm. It has been shown previously that this technique allows excitation of NAD(P)H in live tissue [22]. Flavoproteins were excited with the blue line of an Argon-Krypton laser (488 nm) and the fluorescence was detected with a high pass filter set at 520 nm.

2.9. Southern blots

Total DNA was isolated from 10 to 40 mg liquid nitrogen-frozen muscle samples by standard methods and Southern blots were performed with 1 μg DNA digested either by *Pvu*II or the combination of *Pvu*II and *BAM H*I. Human mtDNA isolated from human skeletal muscle mitochondria and a cloned fragment of the human 18S rRNA gene (a kind gift of Dr. C. McMillan, Montreal, Canada) were labelled with digoxygenin by the Klenow reaction. For the quantitative determination of the mtDNA/18S rDNA ratio ^{32}P-labelled probes were used. The measurement of the amounts of hybridized probes was performed applying a phosphoimager (Fujix BAS-1000, Fuji PhotoFilm Co., Tokyo, Japan).

2.10. Statistical analysis

The results are presented as mean±SD. Significant changes were assessed by two-tailed Student's *t*-test. A value of $P<0.05$ was accepted as the level of significance.

3. Results

In Table 1 the results of the enzymatic analysis of the muscle biopsy samples from 26 ALS patients, 6 patients with spinal muscular atrophy (SMA) and two patients with hexosaminidase A-deficiency (Tay-Sachs disease) are summarized in comparison to control biopsies. In comparison to the control group and to motor neuron involvement in SMA and Tay-Sachs disease we observed in accordance to our earlier work which included a smaller group of patients [16] in the ALS biopsies approximately two-fold lower activities of rotenone-sensitive NADH:cytochrome *c* reductase. Additionally, in individual biopsies of several ALS patients also low activities of cytochrome *c* oxidase were detected. On the other hand no statistical significant differences in the activities of other cytosolic and mitochondrial enzymes like lactate dehydrogenase, adenylate kinase, creatine kinase, aspartate aminotransferase, citrate synthase and succinate:cytochrome *c* reductase were detectable. These findings point to an impairment of mitochondrial respiratory chain in skeletal muscle of patients with sporadic amyotrophic lateral sclerosis which seems to be not related to denervation-associated changes in muscle since mitochondrial function was normal in chronic diseases affecting severely the peripheral motor neurons like spinal muscular atrophy or hexosaminidase A deficiency (Tay-Sachs disease). The analysis of mitochondrial DNA in ALS skeletal muscle applying Southern blot techniques revealed multiple deletions in one ALS patient and low mtDNA/18S rDNA ratios in 14 patients (average ratio of ALS patients – 1.4±1.0 of controls – 2.6±1.2 ($P<0.01$)).

In further experiments we investigated the distribution of defective mitochondria in skeletal muscle of ALS patients applying an imaging method of mitochondrial function [17]. For this autofluorescence images of mitochondrial NAD(P)H or fluorescent flavoproteins were acquired in different functional states of the permeabilized muscle fibers. Due to the fact that NAD(P)H is fluorescent in its reduced state, but the fluorescent flavoproteins in the oxidized state, the ratio of both autofluorescence signals is a sensitive indicator of the redox state of the mitochondrial NAD-system [23]. We determined digital ratio images of flavoprotein autofluorescence/NAD(P)H autofluorescence of a saponin-permeabilized fiber bundle, consisting of two single muscle fibers, in the endogenous oxidized state (Fig. 1, left image), in the state after addition of 1 mM octanoylcarnitine and 5 mM malate (Fig. 1, second image from the left), in the state after addition of 1 mM ADP (Fig. 1, third image from the left) and in the state after the addition of 4 mM cyanide (Fig. 1, right image). In the endogenous oxidized state of muscle fibers a bright ratio image was detected for both fibers due to the high flavoprotein fluorescence and the low NAD(P)H fluorescence. Substrate addition led in the case of the left fiber to a darker ratio image than for the right fiber. The change in image brightness is due to the decrease in flavoprotein fluorescence and increase of NAD(P)H fluorescence as result of the reduction of the mitochondrial NAD-system. ADP addition, which stimulated oxidative phosphorylation and therefore the efflux of reducing equivalents from the mitochondrial NADH pool, caused in the right fiber a

Table 1
Enzyme pattern in the skeletal muscle homogenate of patients with SMA, Tay-Sachs syndrome and ALS[a]

Enzyme	Controls ($n=28$)	SMA ($n=6$)	Tay-Sachs syndrome ($n=2$)	ALS ($n=26$)
Lactate dehydrogenase	286±163	241±181 (NS)	88±8	297±139 (NS)
Adenylate kinase	213±90	270±188 (NS)	93±15	286±85 (NS)
Creatine kinase	1512±398	1971±834 (NS)	1199±158	1799±626 (NS)
Aspartate aminotransferase	39.3±10	58.5±14.3 (NS)	51.1±16.3	41.3±14.4 (NS)
Citrate synthase	11.0±2.2	13.8±2.7 (NS)	16.2±0.9	11.4±3.1 (NS)
Cytochrome *c* oxidase	3.0±1.5	3.5±1.7 (NS)	6.8±3.5	2.2±1.1 (NS)
Succinate:cytochrome *c* reductase	1.7±0.7	1.8±0.6 (NS)	1.6±0.6	1.5±0.7 (NS)
NADH:cytochrome *c* reductase	3.5±1.7	2.8±0.9 (NS)	2.9±0.8	2.1±0.9 ($P<0.01$)

[a] The enzyme activities (in U/g wwt) were measured as described in Section 2. *n* – number of different patients. NS – non-significant.

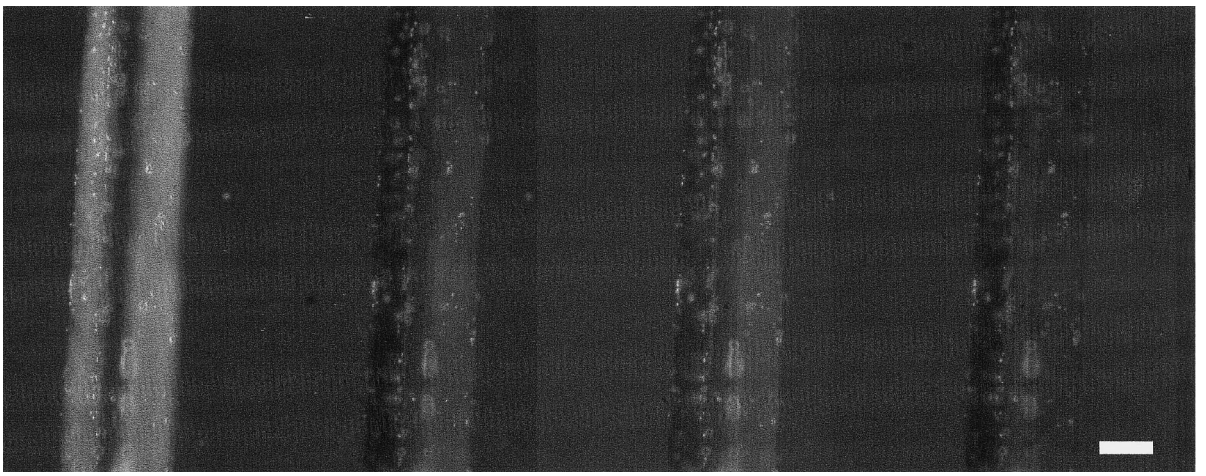

Fig. 1. Flavoprotein/NAD(P)H fluorescence ratio images of skeletal muscle fibers of a patient with amyotrophic lateral sclerosis having severe mtDNA depletion. The mtDNA/18 S rDNA ratio in skeletal muscle of this ALS patient was 0.57 (controls 2.6±1.3). Left image: oxidized state; second image from left: 1 mM octanoylcarnitine+5 mM malate; third image from left: 1 mM ADP; right image: 10 mM glutamate+4 mM KCN. Bar, 50 μm.

brighter ratio image indicating a reoxidation of the mitochondrial NAD-system due to respiratory chain stimulation. On the opposite, the left fiber remained nearly as dark as after substrate addition (the tiny bright spots are artifacts caused by lipofuscin). This is an indication of the inhibition of mitochondrial respiratory chain in the left fiber. To prove the effect of an artificial block of respiratory chain we added the inhibitor of cytochrome *c* oxidase cyanide. In both fibers a complete dark ratio image was observed (Fig. 1, right image). These results indicate a fiber specific impairment of mitochondrial function.

For further investigations of the distribution of defective mitochondria within individual muscle fibers we applied single- and dual-photon confocal microscopy. In Fig. 2 one

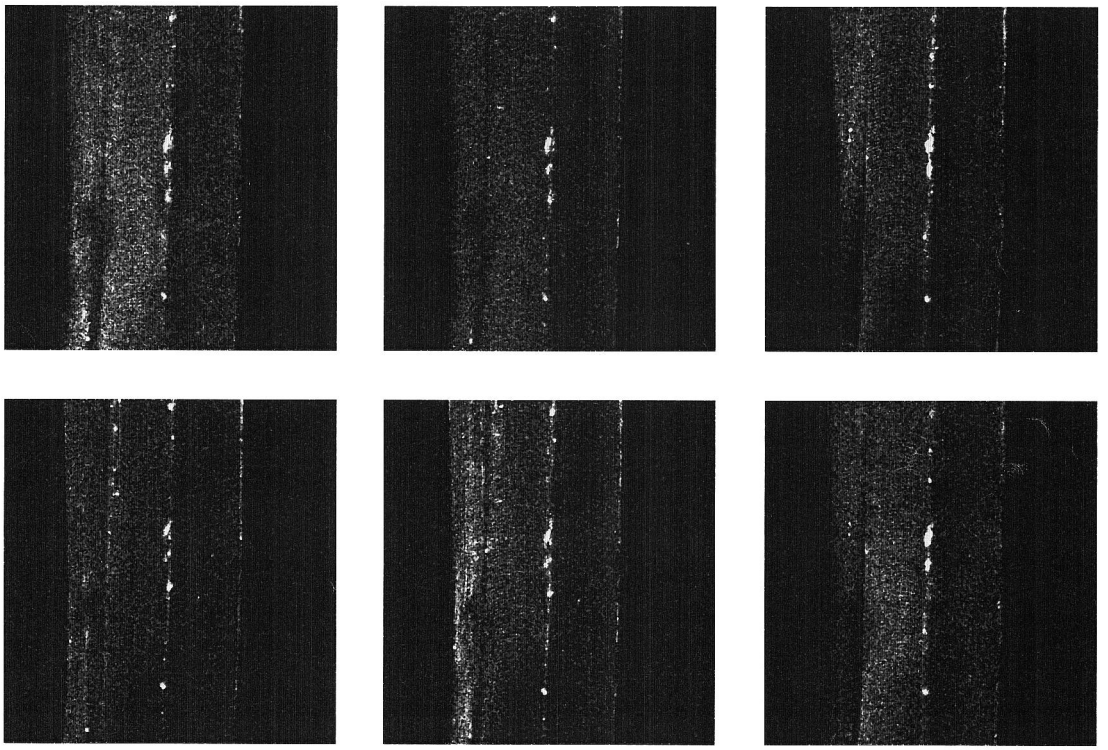

Fig. 2. Mitochondrial function in skeletal muscle of a ALS patient having multiple deletions of mtDNA visualized with CLSM of fluorescent flavoproteins (upper figures) and two-photon excitation CLSM of NAD(P)H (lower figures). Left images: Oxidized state; central images: 10 mM glutamate+5 mM malate and 1 mM ADP; right images: 4 mM KCN. In the Southern blot of *Pvu*II/*Bam*HI cleaved muscle DNA from this patient hybridized with a human mtDNA probe six additional low molecular weight bands were detected (multiple deletions).

confocal plane across a bundle of three muscle fibers of a ALS patient in three different functional states of mitochondria is shown. The upper images were obtained using the argon-krypton laser excitation of 488 nm and represent flavoprotein-caused autofluorescence changes. The lower images were acquired using the two-photon excitation of mitochondrial NAD(P)H at 720 nm. In accordance with the fluorescence properties of flavoproteins and NAD(P)H in the endogenous oxidized state (left images) the flavoprotein image was bright while the NAD(P)H image got brighter only upon reduction. After the addition of 10 mM glutamate, 5 mM malate and 1 mM ADP a bright NAD(P)H image was seen for the left fiber while the central fiber was bright in its upper part but dark in its lower part (central lower image). The opposite was valid for the flavoprotein image (central upper image). Upon addition of 4 mM KCN the left fiber even got slightly darker in the NAD(P)H image, while the central fiber became brighter in its lower part and slightly darker in its upper part (left lower image). These data are in line with the heterogeneous distribution of defective mitochondria within the ALS muscle. Even within different parts of

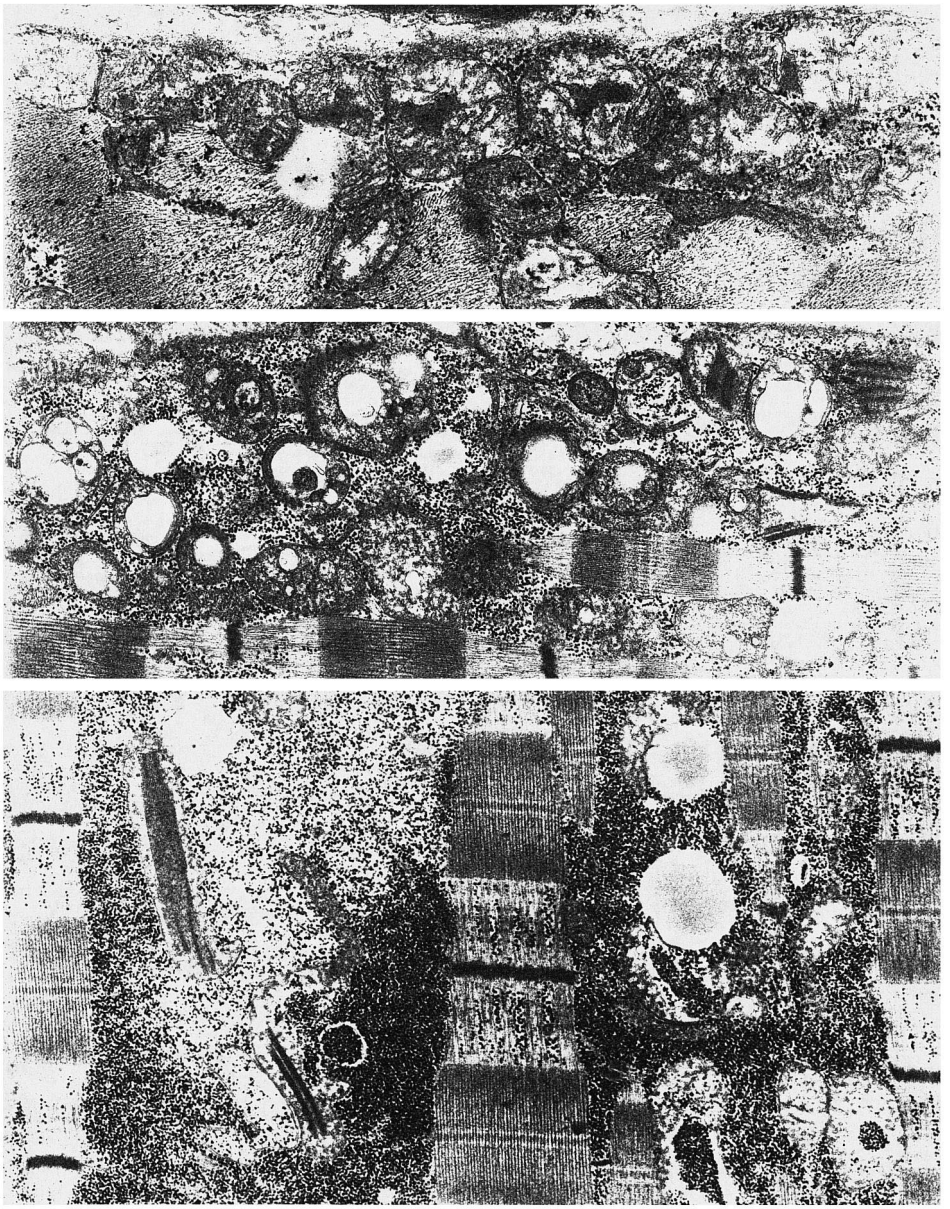

Fig. 3. Electron micrographs of skeletal muscle of a ALS patient having multiple deletions of mitochondrial DNA. Upper figure: Abnormal, partially swollen mitochondria with paracrystalline inclusions in the subsarcolemmal region. Magnification 31 000-fold. Central figure: Mitochondria in the subsarcolemmal region containing vacuoles and glycogen granula. Magnification 20 000-fold. Lower figure: Intermyofibrillar region containing mitochondria with paracrystalline inclusions and glycogen granula. Magnification 18 400-fold.

Table 2
Mitochondrial function in cultivated skin fibroblasts of patients with ALS[a]

	Controls ($n=10$)	ALS ($n=3$)
Citrate synthase (mU/mg)	86.9±12.8	64.0±16.3
NADH:CoQ1 reductase (mU/mg)	9.1±2.7	11.2±3.1
Cytochrome *c* oxidase (mU/mg)	20.9±9.6	6.9±2.8
V (glu+mal, nmol O_2/min/mg)	3.3±1.2	3.8±0.7
V (succ+rot, nmol O_2/min/mg)	12.0±1.5	6.0±0.6

[a] The rates of respiration of digitonin-treated skin fibroblasts are expressed in nmol O_2/min/mg protein at 37°C determined in the presence of 10 μg digitonin/10^6 cells, 1 mM ADP and different combinations of substrates: 10 mM glutamate+5 mM malate, or 10 mM succinate+10 μM rotenone. *n* – number of different patients.

single muscle fibers (cf. central fiber) opposite fluorescence changes of NAD(P)H and fluorescent flavoproteins were observed.

In further experiments we investigated possible structural changes of mitochondria in ALS muscle applying electron microscopy. In Fig. 3 electron micrographs from the skeletal muscle of the ALS patient investigated in Fig. 2 is shown. In accordance with the abnormal redox behavior of NAD(P)H and fluorescent flavoproteins morphologically altered mitochondria with ultrastructural abnormalities (partially swollen, with paracrystalline inclusions or vacuoles) were observed in the subsarcolemmal region (upper and central figure). In the intermyofibrillar region (lower figure) also mitochondrial abnormalities were seen. Similar ultrastructural changes of mitochondria are usually seen in mitochondrial myopathies.

To elucidate the tissue distribution of the mitochondrial defect in amyotrophic lateral sclerosis we studied mitochondrial function in cultured skin fibroblasts. The results of determinations of fibroblast enzyme activities and of maximal rates of oxygen consumption of digitonin-permeabilized fibroblasts from three patients are summarized in Table 2. It is remarkable that in the investigated ALS fibroblasts decreased activities of cytochrome *c* oxidase were observed. This result is further supported by the lower rates of fibroblast respiration with succinate and rotenone.

4. Discussion

We observed a statistically significant diminished activity of NADH:CoQ oxidoreductase in skeletal muscle biopsy samples of patients with sporadic ALS. Moreover, applying autofluorescence imaging of NAD(P)H and fluorescent flavoproteins of ALS fiber bundles we detected elevated redox states of the mitochondrial NAD-system in individual muscle fibers pointing to the presence of a respiratory chain defect. Applying confocal microscopy we observed a heterogeneous distribution of defective mitochondria even within single muscle fibers. The functional abnormalities of mitochondria in ALS muscle are in accordance with the observed ultrastructural changes seen in electron micrographs of selected cases. The approximately two-fold lower activity of complex I of respiratory chain seems to be related to the pathogenic mechanism of this disease rather than to an unspecific effect of a neurogenic change in muscular enzyme pattern since it was not observed in biopsies of patients with spinal muscular atrophy or Tay-Sachs disease. This viewpoint is further supported by investigations of mitochondrial function in cultivated skin fibroblasts of selected patients. Our interpretation is in line with the reported appearance of degenerating mitochondria in anterior horn cells of patients with ALS [14] and of mice models carrying FALS mutations [10–12].

However, the involvement of mitochondria in the process of degeneration of motor neurons can be rather complex. So it remains to discuss why motor neurons are the primary targets in the neurodegenerative process in ALS. Elevated levels of semiquinones or flavoquinones occurring as result of a partial inhibition of respiratory chain tend to react directly with oxygen forming reactive oxygen species as it is observed in intact hearts subjected to ischemia and reflow [24]. Obviously, cells being not adequately protected by defense reactions should be the primary targets of an attack of these oxygen radicals. In addition, the tissue specific mosaic expression of the mitochondrial lesion is a well known phenomenon of several mtDNA diseases causing neurodegeneration (cf. LHON [25]). Therefore, it is very likely that the observed mitochondrial defect is expressed in different tissues to a different extent.

The observed low levels of mitochondrial DNA in 14 ALS patients and the presence of multiple mitochondrial DNA deletions in the skeletal muscle of one ALS patient indicate a possible radical damage of mitochondrial DNA by a preexisting imbalance of prooxidants and antioxidants in the tissue. Under these circumstances the mitochondrial defect would further increase the radical production creating a vicious circle. The high amount of mitochondrially encoded subunits of complex 1 (7 genes) could explain why we observed a rather selective defect of NADH:CoQ oxidoreductase. The decline in activity of cytochrome *c* oxidase (being severe in some patients, but not statistically significant for all patients) in the homogenates from the ALS patients seems to be in line with this explanation.

Summarizing, our data show the presence of a mitochondrial defect in skeletal muscle of patients with sporadic ALS. This defect seems to be not related to the changes in skeletal muscle caused by denervation as it was not observed in biopsies from patients with spinal muscular atrophy or Tay-Sachs disease. Moreover, in cultured fibroblasts of some ALS patients defective mitochondria can be detected. Therefore, our data support a possible direct involvement of mitochondria in the degeneration process of motor neurons in a subgroup of patients with amyotrophic lateral sclerosis.

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