Mitochondrial changes in skeletal muscle in amyotrophic lateral sclerosis and other neurogenic atrophies

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Previous findings suggested specific mitochondrial dysfunction in skeletal muscle of patients with amyotrophic lateral sclerosis (ALS). To answer the question of whether the dysfunction is specific, we investigated the histochemical distribution of mitochondrial marker activities, the ratio of mitochondrial (mt) versus nuclear (n) DNA, and the activities of citrate synthase (CS) and respiratory chain enzymes in muscle biopsies of 24 patients with sporadic ALS. The data were compared with those in 23 patients with other neurogenic atrophies (NAs), and 21 healthy controls. Muscle histology revealed similar signs of focally diminished mitochondrial oxidation activity in muscle fibres in both diseased groups. There was only minimal decline of mt/nDNA ratios in ALS and NA patients in comparison with healthy controls. The specific activities of mitochondrial markers CS and succinate dehydrogenase were significantly increased in both ALS and NA patients. The specific activities of respiratory chain enzymes were not significantly different in all three groups. It is concluded that the histochemical, biochemical and molecular mitochondrial changes in muscle are not specific for ALS, but accompany other NAs as well.

Keywords: amyotrophic lateral sclerosis; neurogenic atrophies; mitochondria

Abbreviations: ALS = amyotrophic lateral sclerosis; COX = cytochrome c oxidase; CPEO = chronic progressive external ophthalomoplegia; CS = citrate synthase; HC = healthy control; mt = mitochondrial; n = nuclear; NA = neurogenic atrophy; NCP = non-collagen protein; PGI = phosphoglucose isomerase; SALS = sporadic amyotrophic lateral sclerosis; SDH = succinate dehydrogenase

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Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder affecting both upper and lower motor neurons, but not primarily the skeletal muscle. The cause of the disease is unknown. It has been hypothesized that the balance between formation of reactive oxygen species and its detoxification in the CNS may be impaired in ALS as well as in other neurodegenerative diseases (Beal, 1995). There is substantial evidence implicating mitochondrial dysfunction in sporadic ALS (SALS). Mitochondrial abnormalities could be detected not only in the CNS (Bowling et al., 1993; Fujita et al., 1996; Borthwick et al., 1999; Wiedemann et al., 2002) but also in liver cells (Nakano et al., 1987), peripheral blood lymphocytes (Curti et al., 1996) and skeletal muscle of SALS patients.

However, the data on mitochondrial dysfunction in skeletal muscle in SALS are controversial. Severely reduced cytochrome c oxidase (COX) activity in muscle due to a microdeletion of the mitochondrially encoded subunit I of COX was detected in a single patient with motor neuron disease but atypical clinical findings (Comi et al., 1998). Another study established a biochemically diminished activity of NADH:CoQ oxidoreductase in muscle of ALS patients (Wiedemann et al., 1998). The bicycle exercise test revealed an abnormal increase of serum lactate in 11 ALS patients investigated
A diminished level of mtDNA, biochemical respiratory chain defects and histochemical focal loss of mitochondrial activity were observed in muscle of 11 of 17 ALS patients (Vielhaber et al., 1999, 2000). Nevertheless, another study did not find significant differences in mitochondrial function respirometrically assessed in skinned muscle fibres of ALS patients in comparison with controls (Echaniz-Laguna et al., 2002).

We investigated the histological and histochemical distribution of mitochondrial marker activities, the ratio of mitochondrial versus nuclear (mt/n) DNA, and the activities of citrate synthase (CS) and of respiratory chain enzymes in muscle biopsies of SALS patients. Results were compared with those in patients with other neurogenic atrophies (NAs) and with healthy persons.

**Patients and methods**

**Patients**

Twenty-four patients suffering from SALS were included in this study. The diagnostic open muscle biopsy was taken from a clinically minimally affected muscle (Mm. biceps brachii, deltoideus, tibialis anterior or vastus lateralis). No autopic samples were used. In the further course of the disease, a definite ALS in 20 and probable ALS in four patients could be established according to El Escorial criteria (Brooks, 1994). Demographic data are summarized in Table 1. The age of onset was 38–78 years (median 58), and the duration of the disease was 1–4 years (mean 1.5).

Skeletal muscle samples from diagnostic biopsies of 23 patients with NAs (Table 1) with a similar degree of atrophy were also investigated. In this group, 12 patients had polyneuropathies (PNPs), six had neuropathies and radiculopathies, and five suffered from adult spinal muscle atrophy (SMA).

Control muscle specimens were obtained from 21 patients with myalgia (Table 1), who underwent muscle biopsy for exclusion of a muscular disease. These patients were deemed to be ‘healthy controls’ (HCs) if they were ultimately found to have no muscle disease or another neurological illness by combined clinical, radiological, electromyographic, histological and biochemical criteria.

**Histology/histochemistry**

Serial cryostat sections (8 and 10 μm) of fresh frozen tissue were stained with haematoxylin–eosin, Gomori’s trichrome, periodic acid–Schiff, Sudan black, acid phosphatase and muscular ATPase at different pHs. Mitochondrial activity was demonstrated by NADH reaction. Slices were dried in air for 1–3 days and incubated for 45 min in a medium containing 0.2 M Tris pH 7.5, 1.22 mM nitroblue-tetrazoliumchloride, 1.13 mM NADH. After rinsing with water, the slices were embedded with glycerine gelatine (Merck).

Identification and differentiation of myopathological signs of focally diminished mitochondrial oxidation activity in muscle fibres were performed according to Carpenter and Karpati (2002). The target was defined as a three-zoned appearance with a pale centre, darker intermediate zone and the normal-appearing outer portion of the fibre (Fig. 1A). If the intermediate zone was lacking, the term ‘targetoid’ was used. Core was defined as a pale central area with a darker border zone within the muscle fibre (Fig. 1B) (Carpenter and Karpati, 2002). If only a pale central or eccentric area without a darker border zone was found, the additional term ‘core-like lesion’ was used. Sequential demonstration of COX and SDH activities was performed on 10 μm slices of cryostat sections of muscle biopsies with an adapted protocol according to Borthwick et al. (1999). COX activity was demonstrated by using a medium containing 4 mM 3,3-diaminobenzidine tetrahydrochloride and 100 μM cytochrome c, 10 U/l catalase in 50 mM phosphate buffer, pH 7.4 at 37°C for 60 min. After rinsing in 50 mM phosphate buffer, SDH activity was stained by using 1.5 mM nitroblue tetrazolium, 0.2 mM phenazine methosulphate and 1 mM sodium azide in 50 mM phosphate buffer,

![Table 1](https://example.com/table1.png)

**Fig. 1** (A) Target in a single muscle fibre (NADH-reaction). (B) Cores in muscle fibres are marked by asterisks (NADH-reaction).
Molecular genetic investigations
Total DNA was extracted by standard methods from muscle biopsies and was digested with BamH I prior to Southern blotting. DNA was hybridized to an mtDNA and an nDNA probe. The mtDNA probe was amplified by polymerase chain reaction (PCR) located in the 16S rRNA gene of the mitochondrial genome (position 5002–5349), and the nDNA probe by PCR located in the nuclear 18S rRNA gene (position 2199–2596). PCR amplification was performed using the PCR DIG Probe Synthesis Kit (Roche, Germany) to label the probes with digoxigenin-11-2'-dUTP. After hybridization with Dig Easy Hyb solution (Roche, Germany) at 50°C, DNA was labelled with anti-digoxigenin-POD (Roche, Germany) and finally incubated in enhanced chemiluminescence (ECL) solution (Amersham, USA) before chemiluminescence was detected by autoradiography (Hyperfilm, Amersham, USA) (Fig. 2). Intensities of the mtDNA band (16.5 kb) and the nDNA band (5 kb) were measured using Image Quant Software (Molecular Dynamics, USA). The relative amount of mtDNA was calculated as the ratio of the signal of the mitochondrial band to that of the nuclear band (mt/nDNA ratio).

Enzyme and protein determinations
Activities of mitochondrial respiratory chain complexes, CS and phosphoglucone isomerase (PGI) in muscle biopsies from ALS and NA patients were compared with those from HCs. Small pieces of frozen tissue were homogenized (1/30 w/v) in a solution containing 50 mM Tris buffer (pH 7.5), 100 mM KCl, 5 mM MgCl₂ and 1 mM EDTA using a glass/glass homogenizer (Kontes Glass Co., Vineland, NJ). Enzyme activities were assayed as described in previous studies (Zierz et al., 1990; Gellerich et al., 2002, 2005). Enzymatic measurements were performed at 30°C using a DU 640 photometer (Beckman Instruments, Palo Alto, CA). Assays were run in duplicate using two different volumes of homogenate. The mitochondrial marker enzyme CS was measured according to Shepard and Garland (1969). The activity of rotenone-sensitive NADH:CoQ1 oxidoreductase (complex I) was measured according to Estornell et al. (1993).

SDH and succinate:cytochrome c oxidoreductase (complex II + III) were measured as described by King (1967). Ubiquinol:cytochrome c oxidoreductase (complex III) was measured according to Krahenbühl et al. (1994). The activity of cytochrome c oxidase (COX, complex IV) was estimated by oxidation of reduced beef heart cytochrome c as described by Wharton and Tzagoloff (1967). The activity of PGI was measured according to Bergmeyer (1983).

Since it was reported previously that the activities of cytochrome oxidase strongly depend on the storage conditions (Pach and Reichmann, 1990), the data of activities of respiratory chain complexes were only analysed from those biopsies that have been stored continuously in liquid nitrogen at −196°C. The data of 16 ALS and 10 NA biopsies which have been stored for a longer time in a freezer at −80°C were excluded.

Non-collagen protein (NCP) was determined by the bicinchonic acid assay (Wichelmann et al., 1988) after digestion of the homogenate with sodium hydroxide (50 mM) and pelleting the insoluble collagen protein by centrifugation (12 000 g for 10 min) as described previously (Zierz et al., 1990). Bovin serum albumin was used as a standard.

Statistics
The mean values and SDs were calculated using the SIGMASTAT 3.1 software (Systat Software Inc., Point Richmond, CA). Significances were tested with the SS20 software package using analysis of variance (ANOVA) on ranks according to Kruskal–Wallis. In cases where ANOVA revealed significant differences, a post-test analysis was performed using the Mann–Whitney test. A P value of <0.05 was considered to be statistically significant.

Results
Histology/histochemistry
Histological examination revealed fibres with signs of locally diminished mitochondrial oxidation activity such as cores, core-like lesions, targets and targetoids in 18 out of 24 (75%) of ALS patients, 17 out of 23 (73%) of NA patients and in none of the HCs. The total number of the fibres with these histological changes was very similar in both diseased groups (Table 2). Few COX-negative fibres could be found in 67% of ALS patients and in 41% of NA patients, but also in 62% of HCs. The number of COX-negative fibres was not significantly increased in biopsies from patients with ALS and NAs compared with HCs (Table 2). A single ragged red fibre was detected in one HC.

Quantification of mtDNA
Southern blot analysis did not reveal deletions of mtDNA in any patient. The relative content of mtDNA expressed as the
mt/nDNA ratio was slightly decreased in ALS and NA patients (both ~8%) in comparison with HCs (Table 3).

### Activities of CS, SDH and PGI

Relative to sample weight, the CS activities were slightly but not significantly increased in ALS and NA patients (Table 3). Since the NCP in both diseased groups was significantly decreased by 11 and 17%, respectively, specific activities of CS and SDH were significantly increased in both ALS and NA patients (Tables 3 and 4). Wet weight-related and specific activity of the non-mitochondrial glycolytic enzyme PGI was not significantly different in all three groups (Table 3).

### Activities of respiratory chain complexes

Enzyme activities were analysed relative to sample wet weight and as specific activities relative to NCP. Enzyme activities relative to sample wet weight and specific activities of respiratory chain complexes in both diseased groups were not significantly changed in comparison with HCs (Table 4).

### Discussion

In contrast to all previous studies, the results of muscle biopsies from ALS patients were compared with those from a similar number of similarly aged NA patients and HCs.

### Histochemical signs of focally diminished mitochondrial oxidation activities

Histological signs of mitochondrial staining in ALS patients seem not to be specific for the following reasons. (i) There were similarly increased amount of cores, core-like lesions, targets and targetoids in the muscle fibres of ALS and NA patients (Table 2). This is consistent with the well-established interpretation of these changes as signs of denervation (Dubowitz and Brooke, 1973; Vielhaber et al., 2000). (ii) No significant increase of COX-negative fibres was found in both diseased groups. In NA patients, the number of COX-negative fibres was even slightly higher than in the ALS group (Table 2). The similar number of COX-negative fibres in three groups of similarly advanced age is plausible, because this histological phenomenon is a well-known sign of muscle ageing (Del Bo et al., 2003). In our ALS and NA patients, COX-negative fibres were

#### Table 2 Histochemical findings in muscle biopsies of patients with ALS and other neurogenic atrophies in comparison with healthy controls

<table>
<thead>
<tr>
<th></th>
<th>HC (n = 21)</th>
<th>ALS (n = 24)</th>
<th>NA (n = 23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-negative fibres (fibres %)</td>
<td>0.14 ± 0.17</td>
<td>0.22 ± 0.25</td>
<td>0.37 ± 1.28</td>
</tr>
<tr>
<td>Affected individuals (%)</td>
<td>62</td>
<td>67</td>
<td>41</td>
</tr>
<tr>
<td>Ragged red fibres (fibres %)</td>
<td>0.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Affected individuals (%)</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cores (fibres %)</td>
<td>0</td>
<td>0.96 ± 2.01</td>
<td>0.96 ± 0.41</td>
</tr>
<tr>
<td>Affected individuals (%)</td>
<td>0</td>
<td>38</td>
<td>35</td>
</tr>
<tr>
<td>Core-like lesions (fibres %)</td>
<td>0</td>
<td>4.70 ± 7.18</td>
<td>4.83 ± 1.70</td>
</tr>
<tr>
<td>Affected individuals (%)</td>
<td>75</td>
<td>61</td>
<td>61</td>
</tr>
<tr>
<td>Targets (fibres %)</td>
<td>0</td>
<td>0.09 ± 0.29</td>
<td>0.19 ± 0.13</td>
</tr>
<tr>
<td>Affected individuals (%)</td>
<td>0</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Targetoids (fibres %)</td>
<td>0</td>
<td>0.22 ± 0.60</td>
<td>0.34 ± 0.07</td>
</tr>
<tr>
<td>Affected individuals (%)</td>
<td>0</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Fibres with core, core-like, target or targetoid changes (%)</td>
<td>0</td>
<td>5.96 ± 8.56</td>
<td>5.96 ± 9.36</td>
</tr>
<tr>
<td>Affected individuals (%)</td>
<td>0</td>
<td>75</td>
<td>73</td>
</tr>
</tbody>
</table>

Values are means ± SD.

#### Table 3 Enzyme activities and relative amounts of mtDNA in biopsies of patients with ALS and other neurogenic atrophies in comparison with healthy controls

<table>
<thead>
<tr>
<th></th>
<th>HC (n = 21)</th>
<th>ALS (n = 24)</th>
<th>NA (n = 23)</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCP (mg/gww)</td>
<td>127 ± 21</td>
<td>114 ± 14*</td>
<td>105 ± 16.3*</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>CS (U/gww)</td>
<td>8.7 ± 2</td>
<td>9.4 ± 2.4</td>
<td>10.5 ± 3.2</td>
<td>NS</td>
</tr>
<tr>
<td>CS (U/g NCP)</td>
<td>71 ± 20</td>
<td>84 ± 15*</td>
<td>99 ± 33*</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>PGI (U/gww)</td>
<td>179.8 ± 38.2</td>
<td>174.8 ± 50.3</td>
<td>144.3 ± 46.9</td>
<td>NS</td>
</tr>
<tr>
<td>PGI (U/g NCP)</td>
<td>1433 ± 261</td>
<td>1335 ± 325</td>
<td>1293 ± 273</td>
<td>NS</td>
</tr>
<tr>
<td>mt/nDNA</td>
<td>5.4 ± 1.8</td>
<td>5.0 ± 2.0</td>
<td>5.0 ± 1.9</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SD. CS = citrate synthase; PGI = phosphoglucose isomerase; mt = mitochondrial; n = nuclear; NCP = non-collagen protein; NS = not significant; gww = g wet weight; U = units; *in post-test analysis significantly different from HCs using the Mann–Whitney test (P < 0.05).
10 times less frequent than in the study by Vielhaber et al. (2000) which detected these changes in 1–2% of fibres in 11 out of 17 ALS patients. In that study, however, the prevalence of COX-negative fibres in ALS muscle was only compared with five HCs and not with patients who suffered from other NAs (Vielhaber et al., 2000).

**Molecular mitochondrial changes**

Whereas in the previous study (Vielhaber et al., 2000), multiple mtDNA deletions were found in one of 17 patients, no mtDNA deletions could be found in our 24 patients. There was a slight and not significant (~8%) decline of mt/nDNA ratios in both ALS and NA patients in comparison with HCs (Table 3). These data seem to be in line with the findings of Vielhaber et al. (2000) that demonstrated a significantly diminished mt/nDNA ratio in the ALS patients compared with HCs. However, in the previous study, the diminished levels of mtDNA were interpreted as ALS specific on the basis of comparison of the molecular data from 17 ALS patients aged 40–66 years with those from only two NA (SMA) patients aged 6 and 18 years (Vielhaber et al., 2000). Our data indicate that the decline of mt/nDNA ratios in ALS muscle is an unspecific neurogenic phenomenon.

**Mitochondrial marker enzymes**

CS activity per muscle wet weight was normal in 17 ALS patients in a previous study (Vielhaber et al., 2000). A subsequent study of the same authors showed a moderate increase of CS activity per wet weight in 11 ALS patients compared with 12 age-matched healthy controls (Vielhaber et al., 2003). However, both studies lacked comparison with adequate neurogenic controls.

In the present study, CS activity calculated per wet weight as well as per NCP was increased in ALS patients. Nevertheless, this could not be established as ALS specific, because a significant increase of CS activity per wet weight and a still more pronounced increase of specific CS activity were also found in the NA group (Table 3). In line with the increase of CS activity, the specific activity of SDH—another nuclear encoded mitochondrial marker—was significantly increased in both ALS and NA muscle (Table 4).

The unchanged activities of the non-mitochondrial enzyme PGI in ALS and NA patients and the increase of mitochondrial markers CS and SDH in both diseased groups (Tables 3 and 4) suggest different changes of the cytosolic and mitochondrial compartments along with NA including ALS.

**Respiratory chain enzyme activities**

Diminished activity of respiratory chain enzymes in ALS patients compared with HCs was observed previously (Vielhaber et al., 2000, 2003; Wiedemann et al., 2002). Because of the lack of relevant neurogenic controls, these studies failed to establish whether the changes were really ALS specific. In another study on 14 early-stage ALS patients compared with seven HCs, no significant differences of mitochondrial respiration using the skinned fibres technique was found, but again no other NAs were investigated (Echaniz-Laguna et al., 2002).

In the present study, enzyme activities relative to sample wet weight and specific activities of respiratory chain enzymes were not significantly different in all three groups (Table 4).

Thus, the respiratory chain enzyme activities in ALS muscle seem to be similar to those in other NAs.

**Comparison with primary mitochondrial diseases**

It has been suggested that by studying a pool of muscular mitochondria, only the presence or the absence of major mitochondrial defects in muscle can be identified.

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**Table 4 Activities of respiratory chain complexes in muscle biopsies of patients with ALS and other neurogenic atrophies in comparison with healthy controls**

<table>
<thead>
<tr>
<th></th>
<th>HC (n = 21)</th>
<th>ALS (n = 8)</th>
<th>NA (n = 13)</th>
<th>ANOVA</th>
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<tbody>
<tr>
<td>SDH</td>
<td>4.25 ± 1.09</td>
<td>4.81 ± 0.95</td>
<td>5.30 ± 2.26</td>
<td>NS</td>
</tr>
<tr>
<td>I</td>
<td>1.75 ± 0.58</td>
<td>1.77 ± 1.13</td>
<td>1.60 ± 1.10</td>
<td></td>
</tr>
<tr>
<td>II + III</td>
<td>2.52 ± 0.63</td>
<td>2.52 ± 0.90</td>
<td>2.47 ± 1.03</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>14.6 ± 3.99</td>
<td>13.9 ± 4.61</td>
<td>15.1 ± 3.71</td>
<td>NS</td>
</tr>
<tr>
<td>COX</td>
<td>10.4 ± 2.55</td>
<td>10.7 ± 2.72</td>
<td>11.1 ± 5.63</td>
<td>NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Specific activities (U/g NCP)</th>
<th>SDH</th>
<th>I</th>
<th>II + III</th>
<th>III</th>
<th>COX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>34 ± 9</td>
<td>14 ± 8</td>
<td>21 ± 7</td>
<td>115 ± 38</td>
<td>88 ± 19</td>
</tr>
<tr>
<td></td>
<td>41 ± 8*</td>
<td>14 ± 9</td>
<td>22 ± 8</td>
<td>136 ± 33</td>
<td>100 ± 55</td>
</tr>
</tbody>
</table>

Values are means ± SD. SDH = succinate dehydrogenase; U = units; gww = g wet weight; NS = not significant; NCP = non-collagen protein; COX = cytochrome c oxidase; *in post-test analysis significantly different from HCs using the Mann–Whitney test (P < 0.05).
In order to give an estimation of the meanings of ‘moderate’ and ‘major’ changes, our data were compared with those from 21 patients with chronic progressive external ophthalmoplegia (CPEO) due to deletions of mtDNA investigated in our laboratory with the identical methods and conditions (Gellerich et al., 2002). The increase of specific CS activity in ALS and NA muscle (Table 3) was clearly less than the almost 2-fold increase of CS in CPEO muscle compared with HCs (Gellerich et al., 2002). In order to eliminate effects of different NCP contents in atrophic ALS and NA muscle, and non-atrophic CPEO muscle, the respiratory chain enzyme activities were normalized by CS activity. Activities of complexes I, II + III and III, and COX normalized by CS were decreased in muscle biopsies of both ALS and NA patients (Fig. 3). However, the changes of the CS-scaled respiratory chain enzyme activities were clearly larger in CPEO muscle than in ALS and NA muscle (Fig. 3). Thus, the reductions of CS-scaled respiratory chain enzyme activities in ALS and NA patients in the present study could be classified as moderate rather than major in comparison with primary mitochondrial myopathies.

The mt/nDNA ratios in the CPEO patients (5.28 ± 1.32) were similar to those in ALS, NA and HC groups of the present study. Although biochemical changes in mitochondrial CPEO and NAs including ALS were comparable, the histological changes were clearly different. The focally diminished mitochondrial staining in the ALS and NA groups could not be observed in CPEO. The clearly increased amount of COX-negative fibres was found in CPEO patients, but not in ALS and NA muscle. The significant number of typical ragged red fibres detected in CPEO patients could be diagnosed in ALS patients neither in the present nor in the previous study (Vielhaber et al., 2000).

### Conclusion

The qualitatively and quantitatively similar histochemical, biochemical and molecular mitochondrial changes in ALS and NA muscle could be established as signs of neurogenic transformation of skeletal muscle, which are not specific for ALS, but accompany other NAs as well. The changes of muscle mitochondria function in ALS and other NAs could be considered as moderate in comparison with those caused by classical mtDNA defects.

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