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REGULAR PAPER

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Severe depletion of mitochondrial DNA in spinal muscular atrophy

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Abstract Spinal muscular atrophy (SMA) is a neuromuscular disorder in childhood leading to a dramatic loss of muscle strength. Functional investigations with high-resolution polarography and enzyme measurements of the respiratory chain revealed lowered activities in muscle tissue of SMA patients. To gain a better understanding of this low energy supply we analyzed the amount of mitochondrial DNA (mtDNA) in skeletal muscle of 20 unrelated children with genetically proven SMA and 31 controls. Quantitative Southern blot analysis revealed a severe and homogeneous decrease in the content of muscle mtDNA in relation to nuclear DNA in SMA patients (90.3±7.8%), whereas by immunofluorescence no decrease in the number of mitochondria was detected. In addition, a two- to threefold reduction of the nuclear-encoded complex II (succinate dehydrogenase) activity was detected in SMA muscle tissue. Western blot analysis showed a significant reduction of both mitochondrial- and nuclear-encoded cytochrome c oxidase subunits. Our results indicate that mtDNA depletion in SMA is a consequence of severe atrophy, and has to be differentiated by measurement of complex II from an isolated reduction of mtDNA as found in patients with mitochondriocytopathies and the so-called mtDNA depletion syndrome.

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Introduction

Childhood-onset spinal muscular atrophy (SMA) is a severe neuromuscular disorder, characterized by loss of lower motor neurons in the spinal cord, causing progressive weakness and muscle atrophy. This autosomal recessive disorder is the most common genetic cause of childhood morbidity affecting 1:6,000 to 1:10,000 live births [28]. SMA is classically subdivided into three clinical groups based on the age of onset and clinical course [37].

Molecular genetic studies have shown that all three forms of childhood SMA map to a single genetic locus on chromosome 5q11.2-q13.3 [4, 8, 19]. This complex region contains a large inverted duplication, consisting of at least four genes, which are each present in a telomeric and a centromeric copy. However, it is now well established that the survival motor neuron (SMN-1) gene is the SMA-determining gene [13]. The telomeric copy of the SMN gene is reported to be deleted in approximately 95% of cases and mutated in the remaining SMA patients, regardless of SMA type [13, 42].

Functional investigation of SMA muscle tissue revealed diminished respiration rates and decreased enzyme activities of the respiratory chain including all respiratory chain enzyme complexes I–IV [35]. Since these enzyme investigations included only a combined determination of complex II and III, the question remained open if these findings could be explained by a mitochondrial DNA (mtDNA) depletion.

Quantitative defects of the mtDNA content were first described in patients with fatal mitochondrial disease of early infancy and mitochondrial myopathy of childhood [22, 39] and have been termed mtDNA depletion syndromes. A number of patients with a wide range of clinical manifestations have been reported to be associated with isolated mtDNA depletion [1, 15, 16, 18, 23, 25, 26].

Biochemically, a selective reduction of the activities of complexes I, III and IV of the respiratory chain in the target tissue can be found, sparing complex II, which is entirely encoded by nuclear genes [9].

To determine if the diminished energy supply in SMA muscle tissue might also be due to mtDNA depletion, we investigated the proportion of mtDNA to nuclear DNA as a measure of the mtDNA content, and included complex II measurements to differentiate between nuclear and mtDNA involvement. Since various muscular disorders with a certain degree of reduction of mtDNA and diminished respiratory chain activities have been described [31], these additional investigations were necessary to distinguish between a primary and secondary respiratory chain defect. This is of high clinical relevance because patients with severe muscular atrophy could be falsely classified as having a primary mitochondriopathy.

Materials and methods

Patients

Muscle biopsy specimens of 20 unrelated SMA patients (age 2 months–16 years; mean 24 months) were selected on the basis of clinical, electrophysiological and histological criteria during 1988 and 1997. They all fulfilled the diagnostic criteria for SMA as defined by the International SMA Consortium [24]. Clinically, 13 patients were classified as SMA type I, 6 as SMA type II and 1 as SMA type III.

Control muscle specimens were obtained from patients undergoing orthopedic hip replacement surgery and patients referred for exclusion of malignant hyperthermia (n=27; age 12–80 years; mean 59 years). Muscle biopsy samples of age-matched controls were obtained from four children (ages 3, 5, 9 and 20 months; mean 9.3 months) without clinical or histological evidence of SMA and mitochondrial myopathy. In addition, two muscle biopsy specimens from patients with severe neurogenic muscular atrophy, one due to diabetic neuropathy (age 55 years) and one due to polyradiculitis (age 56 years) were investigated.

Quantitative Southern blot analysis was applied to all tissue specimens. The selection of biopsy tissue for immunofluorescence, enzyme measurements and Western blot analysis was based on tissue availability.

PCR analysis of deletions in SMN

Deletions in SMN-1 exons 7 (primers R111 and X7-Dra) and 8 (primers 541C960 and 541C1120) were detected by polymerase chain reaction (PCR) amplification as described elsewhere [13, 41]. Positive and negative controls were included in each PCR run.

Quantitative Southern blot analysis

Total cellular DNA was isolated from frozen muscle biopsies according to standard procedures. Southern blot analysis was carried out as previously described [3]. The same mixture of mitochondrial and nuclear probe was used in each experiment. To determine the intensity of the signals, densitometry on autoradiographs was performed. The relative amount of mtDNA was expressed as the ratio of the signal of the mitochondrial probe to that of the nuclear probe (m/r ratio).

Immunofluorescence

Immunofluorescence with a monoclonal antibody directed against a 65-kDa human mitochondrial protein (Chemicon International Inc., Temecula, Calif.) and COX IV subunit I (A-6403; Molecular Probes, Ore.) was performed according to the manufacturer's instructions. Briefly, 5-µm-thick sections from frozen muscle were fixed in 4% formaldehyde in 100 mM $CaCl_2$ pH 7.4 for 30 min, washed and dehydrated in serial alcohol dilutions. After rehydration in 1× phosphate-buffered saline (PBS; 137 mM NaCl, 2.88 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4), the slides were incubated with the primary antibody (65-kDa mitochondrial protein: diluted 1:40 in PBS; COX IV subunit I: diluted 1:30 in PBS). After 2 h, the sections were washed and incubated for 30 min with the appropriate biotin-labeled secondary antibodies (Amersham, Pharmacia Biotech, Buckinghamshire, UK) diluted 1:100 in PBS. The sections were washed, incubated for 30 min with streptavidin-Texas Red (Amersham, Pharmacia Biotech) diluted 1:100 in PBS, washed again with PBS and mounted in 50% glycerol in PBS.

Sample preparation for enzyme measurements and Western blot analysis

Muscle specimens (15–25 mg) were minced with a pair of scissors followed by homogenization with a tissue disintegrator (Ultraturrax, IKA, Staufen, Germany) in extraction buffer (20 mM TRIS/HCl pH 7.6, 250 mM sucrose, 40 mM KCl, 2 mM EGTA) and final homogenization with a motor-driven Teflon-glass homogenizer (Potter S, Braun, Melsungen, Germany). The homogenate was centrifuged at 600 g for 10 min at 4°C. The supernatant was taken for measuring the mitochondrial enzymes and for Western blot analysis.

Enzyme measurements

Citrate synthase was determined according to Srere [36] with modifications. Briefly, the reaction mixture contained 50 mM TRIS/HCl pH 8.1, 0.1% bovine serum albumin (BSA), 0.1% Triton X-100, 0.2 mM 5,5'-dithio-bis(2-nitrobenzoic acid), 0.15 mM acetyl-CoA and the extract (final protein concentration 10–50 µg/ml). The reaction was started by addition of 0.5 mM oxaloacetate and followed at 412 nm for 8 min at 37°C.

Cytochrome c oxidase was measured according to Trounce at al. [40] with some modifications. The reaction mixture contained 50 mM potassium phosphate pH 7.0, 0.2% Tween-20, 0.1% BSA and the extract (final protein concentration 10–50 µg/ml). The reaction was started by addition of 60 µM reduced cytochrome c and followed at 550 nm for 6 min at 37°C. To reach the end point of the reaction, 15 µg bovine heart mitochondria were added and the final absorbance was determined after 10 min. The logarithmic reaction constant was calculated and used to determine the initial activity.

Complex II was measured according to Rustin et al. [33] with the following modifications. The reaction mixture contained 50 mM potassium phosphate pH 7.8, 2 mM EDTA, 0.1% BSA, 3 µM rotenone, 80 µM 2,6-dichlorophenolindophenol, 50 µM decylubiquinone, 1 µM antimycin A, 0.2 mM ATP, 0.3 mM KCN and the sample (final protein concentration 10–50 µg/ml). The mixture was preincubated for 10 min at 37°C, started by addition of 10 mM succinate, and followed for 6 min at 600 nm at 37°C.

Western blot analysis

After separation of the 600 g homogenate (4 µg protein) on 5–20% polyacrylamide gels, proteins were transferred to PVDF membranes at constant voltage (200 mA, 100 min) in 48 mM TRIS, 39 mM glycine, 20% (v/v) methanol, 0.05% (w/v) SDS. Immunological detection was carried out with the Western-Star Protein Detection Kit (Tropix, Bedford, Mass.) according to the manufacturer's protocol. The following antibodies were used: mouse monoclonal antibodies against COX IV subunit I (A-6403; Molecular Probes; 1:250); COX IV subunit IV (A-6431; Molecular Probes; 1:1,500) and alkaline phosphatase-conjugated rabbit anti mouse

immunoglobulins (Dako, Glostrup, Denmark; 1:5,000). Images were analyzed by densitometry with image analysis software (Molecular Analyzer; Bio-Rad).

Statistical analysis

Significance of the differences among the patient and control groups was examined by the Mann Whitney test using Prism 2.0 software (GraphPad Software Inc., San Diego, Calif.). A P value of less than 0.05 was considered to be statistically significant.

Results

Genetic analysis of SMA patients

All 20 SMA patients showed deletions in exons 7 and/or 8 of the SMN-1 gene.

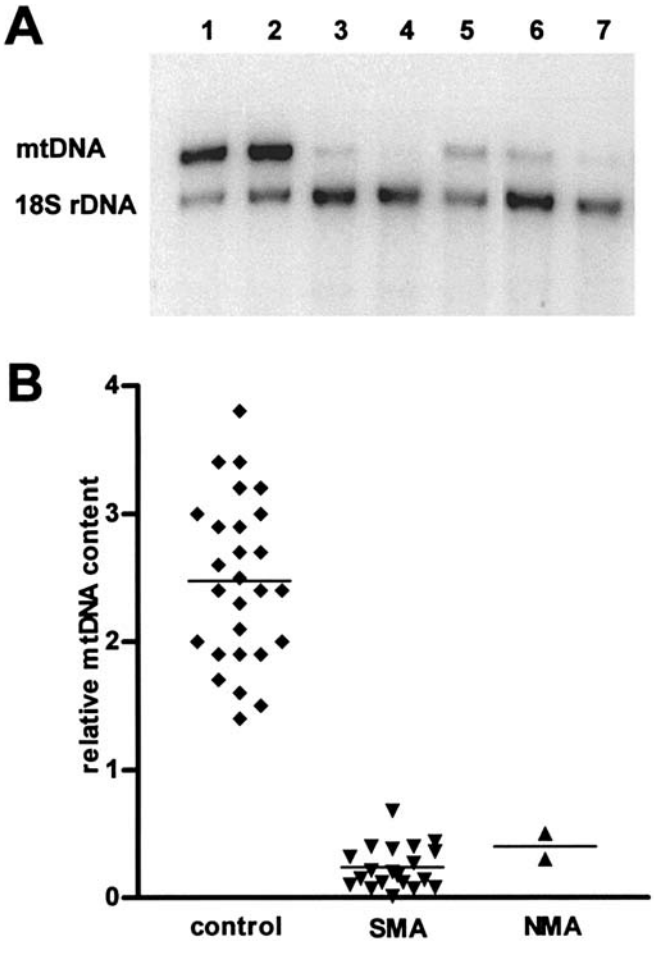

Fig. 1 **A** Southern blot analysis of total DNA extracted from skeletal muscle of two controls (*lanes 1* and *2*) and five SMA patients (*lanes 3–7*) hybridized simultaneously with a ^{32}P-labeled mixture of mtDNA and 18S rDNA probes. **B** Relative mtDNA content (m/r ratio: mtDNA/nDNA) of SMA patients and controls; *bars* represent mean relative mtDNA content (mean ± SD, SMA 0.24±0.20, controls 2.41±0.61); muscle biopsy samples from two patients with NMA showed an m/r ratio of 0.3 and 0.5, respectively (*SMA* spinal muscular atrophy, *mt/r/nDNA* mitochondrial/ribosomal/nuclear DNA, *NMA* neurogenic muscle atrophy)

mtDNA depletion analysis

Southern blot analysis revealed a severe homogeneous mtDNA reduction ranging from 65.6% to 99.6% (mean ± SD, 90.3±7.8%) in skeletal muscle of 20 SMA patients compared to mtDNA levels of controls (Fig. 1A). The average relative ratio of mtDNA to 18S rDNA (m/r ratio) of the 27 controls was 2.46±0.63. The four age-matched controls showed an average m/r ratio of 2.08±0.33. The average m/r ratio of all SMA patients (0.24±0.20) lies significantly under the average m/r ratio of all 31 controls (2.41±0.61; P<0.0001) (Fig. 1B). Two muscle biopsy samples from patients with severe neurogenic atrophy also showed a severe reduction of mtDNA (m/r ratio 0.3 and 0.5).

The inter-assay variability of the quantitative Southern blot analysis ranged from 5% to 15%.

Analysis of the content of mitochondria in SMA muscle tissue

To exclude that the observed low mtDNA content in SMA muscle tissue is due to a reduction of the amount of mito-

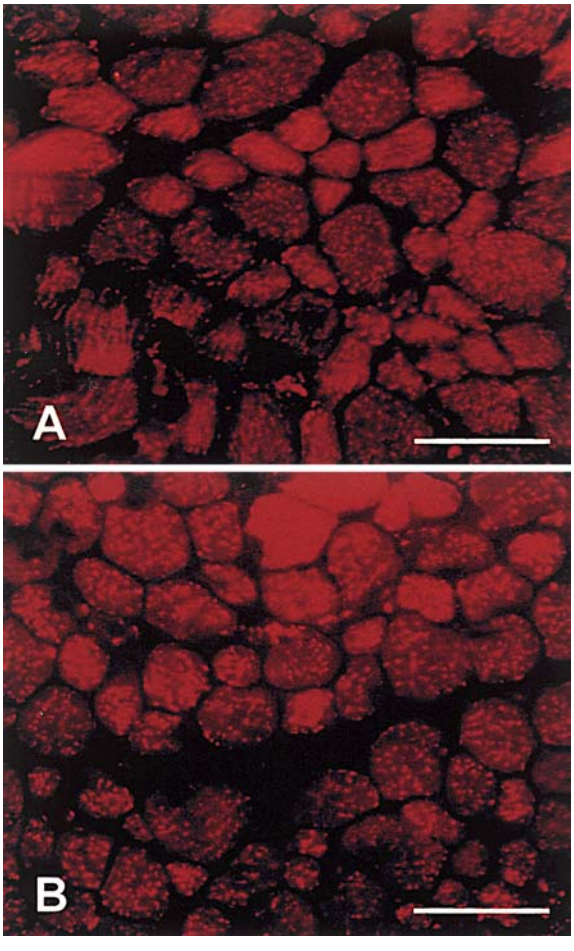

Fig. 2A, B Immunofluorescence performed with an antibody against mitochondrial protein on skeletal muscle sections. **A** Control group subject, **B** SMA I patient. *Bars* 25 µm

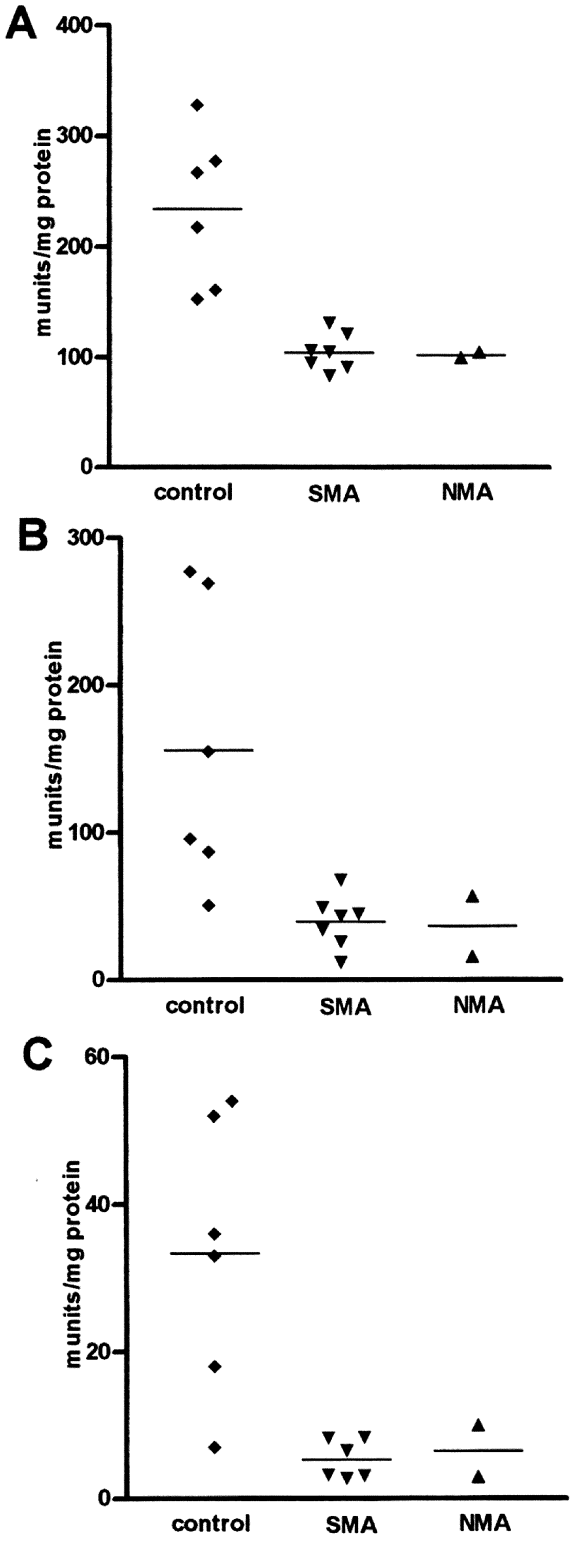

Fig. 3A–C Enzymes of the mitochondrial energy metabolism in SMA patients, controls and two patients with NMA. **A** Citrate synthase activity; **B** COX activity; **C** complex II activity. Enzyme activities are expressed in mU/mg protein, *bars* represent mean enzyme activity

chondria, tissue sections were analyzed by immunofluorescence with an antibody directed against mitochondria. The frozen sections from skeletal muscle of the controls showed a typical mitochondrial staining pattern in the sarcoplasm (Fig. 2A). Staining of SMA muscle tissue with antibodies against mitochondria revealed the same staining pattern in the sarcoplasm as observed in controls. No reduction of the content of mitochondria in SMA muscle tissue was detected (Fig. 2B). Immunostaining with the antibody against subunit I of COX also revealed the same mitochondrial staining pattern in controls and SMA. However, the intensity of the staining was reduced, whereas the number of mitochondria remained the same (data not shown).

Enzyme activities in SMA

Citrate synthase activity in muscle tissue of SMA patients (n=7) was found to be significantly reduced in SMA muscle (58%; P<0.005; mean ± SD, 104±17 mU/mg protein) as compared to controls [234±69 mU/mg protein (n=6); Fig. 3].

The activity of COX was 39±17 mU/mg protein (n=7) for SMA muscle tissue and 156±96 mU/mg protein for controls (n=6), showing a significant reduction of COX activities in SMA muscle (75%, P<0.005; Fig. 3).

Complex II in muscle of SMA patients was 5.3± 2.5 mU/mg of protein (n=7) and in controls 33±7.5 mU/mg protein (n=6). The highest reduction of enzyme activity was therefore detected for complex II (85%; P<0.01) in SMA patients compared to controls (Fig. 3). The correlation of citrate synthase as well as complex II to COX did not reveal different ratios between the SMA and the control group.

Enzyme activities of citrate synthase (100 and 105 mU/mg protein), COX (57 and 16 mU/mg protein) and complex II (10 and 3 mU/mg protein) in the muscle tissue of two patients with severe neurogenic muscular atrophy was reduced to a similar extent to that seen in SMA muscle (Fig. 3).

Western blot analysis

The effect of the mtDNA reduction on the expression of mitochondrial- and nuclear-encoded COX subunits was

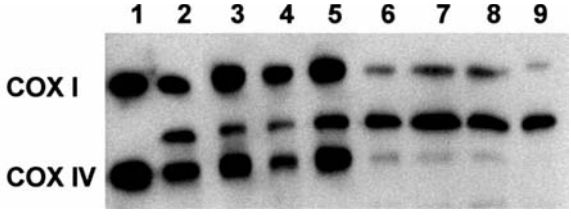

Fig. 4 Western blot analysis of COX subunits in muscle extracts of SMA patients and controls. *Lane 1* Isolated mitochondria from control tissue (4 µg protein); *lanes 2–5* control muscle tissue; *lanes 6–9* muscle tissue of SMA patients; COX I: 57 kDa; COX IV: 19.6 kDa

investigated by Western blot analysis. In SMA muscle tissue a significant reduction of nuclear- and mitochondrial-encoded subunits was found compared to controls (Fig. 4). However, the average reduction of the mitochondrial-encoded subunit I of COX (79%) was significantly higher than the reduction of the nuclear-encoded subunit IV (45%; $P<0.001$).

Discussion

Former enzymatic and polarographic measurements in muscle tissue of SMA patients showed a clear reduction of respiratory chain enzymes as well as oxidation rates [35]. These findings might be explained by either a mtDNA depletion and/or a reduction of mitochondria in the affected muscle tissue. Quantitative Southern blot analysis revealed a homogeneously and severely reduced mtDNA content in SMA muscle tissue as compared to controls, whereas immunohistochemical investigation with an antibody against mitochondrial protein revealed no differences in the density of mitochondria. Therefore, in the present study we show for the first time that the mtDNA content is severely reduced in skeletal muscle of patients with genetically proven SMA, indicating a mtDNA depletion. This mtDNA depletion has to be differentiated from the so-called mtDNA depletion syndromes, which have been described among the mitochondriocytopathies with an isolated mtDNA reduction in muscle tissue [9, 14, 17, 27, 29, 30, 31, 44].

To further differentiate the finding of a mtDNA depletion in SMA muscle, we measured complex II (succinate dehydrogenase), which is entirely encoded by nuclear DNA. In our study we found reduced complex II, as well as reduced nuclear-encoded citrate synthase activities. These results indicate that both nuclear- and mitochondrial-encoded subunits of the respiratory chain are down-regulated in SMA muscle tissue. Western blot analysis of COX subunits confirmed this observation by showing that both mitochondrial-encoded subunit I and nuclear-encoded subunit IV were significantly reduced compared to controls. In addition, immunostaining using subunit I of COX revealed a reduced intensity of the signal in sections of SMA muscle tissue.

Taken together, our findings show that the mtDNA depletion in SMA seems to be clearly distinct from the mtDNA depletion syndrome. The mtDNA depletion found in SMA muscle might be a relatively nonspecific response of muscle tissue to neurogenic atrophy. Accordingly, in muscle tissue of two patients with severe peripheral neuropathy, one with diabetic neuropathy and one with polyradiculitis, mtDNA depletion was found to a similar extent as in our SMA patients.

Poulton et al. [31] investigated patients with mitochondrial myopathies and other forms of muscle disease and showed that mtDNA depletion to various degrees seems to be a rather common feature in patients with muscle disorders. However, there are only a few reports of patients with lower motor neuron disease and mitochondrial disturbances. Pons et al. [30] reported a patient with a severe progressive neuromuscular disorder resembling spinal muscular atrophy who did not show deletions in exons 7 and 8 of the SMN-1 gene. Immunohistochemistry using anti-DNA antibodies revealed only nuclear staining in skeletal muscle of this patient, suggesting a mtDNA depletion. Enns et al. [5] also presented a patient with mtDNA depletion and features of SMA but no deletions in the SMN gene. Biochemical analysis showed normal complex I–IV activities, when referred to low citrate synthase. These features are similar to those of our patients, which might indicate that the described patient belongs to the 5% of SMA patients who carry mutations but no deletions in exons 7 and/or 8 of the SMN gene [13, 42]. Therefore, mutation analysis would be necessary to further clarify this case. Rubio-Gozalbo et al. [32] observed a COX deficiency in muscle tissue of a patient with typical clinical and histological findings of SMA. Kirches et al. [12] found a mtDNA depletion in a 3-year-old girl with a multisystem disorder and an unspecific severe myopathy. No respiratory chain enzyme measurements have been performed, so that the finding of depletion alone does not allow speculation on a primary mitochondrial disease. These and other reports [6, 7, 43] illustrate that it is not easy to distinguish between primary and secondary mitochondrial alterations in neuromuscular disorders. At least mtDNA depletion alone is no conclusive evidence of a primary mitochondrial defect, because muscle atrophy could also lead to a down-regulation of oxidative phosphorylation in muscle tissue, as shown in our study. Therefore, some caution is necessary in interpreting mtDNA levels alone. Our findings of low mtDNA and diminished respiratory chain enzyme activities fit very well to the severe muscle weakness of SMA as a consequence of the lower motor neuron affection.

In contrast, in patients with mtDNA depletion syndromes, nuclear-encoded enzymes are usually found at high amounts, whereas all other respiratory chain enzymes are reduced [9, 17, 27, 29, 39, 44]. From these data we conclude that, in the absence of deletions of the SMN-1 gene in patients with clinical features of SMA, enzymatic measurement of OXPHOS enzymes, especially of succinate dehydrogenase, is necessary to distinguish between a mtDNA depletion syndrome and a nonspecific response of muscle to atrophy.

Low amounts of mtDNA in muscle of children could also be an age-related phenomenon rather than a pathological mtDNA reduction. It has been suggested by some authors that muscle tissue of younger controls contains a two- to threefold lower proportion of mtDNA to nuclear DNA (nDNA) than those of older controls, i.e., the ratio of mtDNA to nDNA is very low in fetal muscle and low in neonatal muscle of about 3 months of age compared to levels in older children and adults [2, 31]. This is consistent with the known increases in mitochondrial enzyme activities with age [20, 34]. However, age dependency cannot explain the homogeneous finding of a severe mtDNA reduction in our study since all three types of childhood-onset SMA were included and most patients were biop-

sied at an age of more than 3 months. We could also demonstrate that muscle from age-matched controls contains similar amounts of mtDNA as muscle from older controls.

The mechanisms occurring after mtDNA depletion in muscle atrophy are at the moment only speculative. Progressive atrophy seems to result in a coordinated down-regulation in the mitochondrial energy metabolism. During this process nuclear- and mitochondrial-encoded proteins decrease at a similar extent. The series of factors involved in this development is not known so far but studies in the recently developed mouse model of SMA could bring more insight in this probably common process [10, 11, 21]. Also, an active disintegrative mechanism like apoptosis [38] should be considered.

In conclusion, the results of our study on SMA muscle tissue demonstrate that mtDNA depletion can be a consequence of severe atrophy and has nothing to do with the isolated reduction of mtDNA that is found in patients with so-called mtDNA depletion syndromes. Measurements of respiratory chain enzymes, especially of the nuclear-encoded complex II, are a prerequisite for differentiation of mtDNA depletion in patients with muscular atrophy. A mtDNA depletion alone does not necessarily indicate a primary mitochondrial defect since muscle atrophy can also lead to a down-regulation of both nuclear- and mitochondrial-encoded respiratory chain complexes.

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