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A novel deficiency of mitochondrial ATPase of nuclear origin

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Received May 10, 1999; Revised and Accepted July 5, 1999

We report a new type of fatal mitochondrial disorder caused by selective deficiency of mitochondrial ATP synthase (ATPase). A hypotrophic newborn from a consanguineous marriage presented severe lactic acidosis, cardiomegaly and hepatomegaly and died from heart failure after 2 days. The activity of oligomycin-sensitive ATPase was only 31–34% of the control, both in muscle and heart, but the activities of cytochrome c oxidase, citrate synthase and pyruvate dehydrogenase were normal. Electrophoretic and western blot analysis revealed selective reduction of ATPase complex but normal levels of the respiratory chain complexes I, III and IV. The same selective deficiency of ATPase was found in cultured skin fibroblasts which showed similar decreases in ATPase content, ATPase hydrolytic activity and level of substrate-dependent ATP synthesis (20–25, 18 and 29–33% of the control, respectively). Pulse–chase labelling of patient fibroblasts revealed low incorporation of [35S]methionine into assembled ATPase complexes, but increased incorporation into immunoprecipitated ATPase subunit β, which had a very short half-life. In contrast, no difference was found in the size and subunit composition of the assembled and newly produced ATPase complex. Transmitochondrial cybrids prepared from enucleated fibroblasts of the patient and ρ° cells derived from 143B.TK– human osteosarcoma cells fully restored the ATPase activity, ATP synthesis and ATPase content, when compared with control cybrids. Likewise, the pattern of [35S]methionine labelling of ATPase was found to be normal in patient cybrids. We conclude that the generalized deficiency of mitochondrial ATPase described is of nuclear origin and is caused by altered biosynthesis of the enzyme.

INTRODUCTION

The mitochondrial disorders caused by impairment of mitochondrial oxidative phosphorylation (OXPHOS) predominantly affect muscle, brain and other tissues with high energetic demands. As both nuclear DNA and mitochondrial DNA (mtDNA) encode OXPHOS proteins, mitochondrial diseases can be caused by mutations in both genomes. Many OXPHOS disorders with defects of the respiratory chain enzymes, in particular in complexes I and IV, have been described, whereas the defects of mitochondrial ATPase, the key enzyme of ATP production in mammalian cells, appear to be less frequent.

Mitochondrial ATPase consists of 16 different polypeptides (1), six of which comprise the globular, membrane-extrinsic, F₁-catalytic part, and ten which form the H⁺-translocating, membrane spanning F₀ part. Four of the F₀ subunits form a ‘stalk’ which connects the F₁ and F₀ moieties. Only two of the F₀ subunits—subunit a and subunit A6L (subunits 6 and 8)—are the products of mitochondrial genes (2); the other ATPase subunits are nuclear encoded.

To date, all the known ATPase defects have been caused by maternally inherited mtDNA point mutations in subunit a. The most frequent is a heteroplasmic T8993G mutation (3–7), or T8993C mutation (8), which causes replacement of a Leu156 with Arg or Pro in subunit a and clinically manifests as NARP syndrome (neurogenic muscle weakness, ataxia, and retinitis pigmentosa) (4,5) or Leigh’s syndrome (3,6,7). The 8993 mutation disturbs the function of the F₀ proton channel resulting in a decrease of mitochondrial ATP production (3,9,10). This has been confirmed by site-directed mutagenesis of the ATPase subunit a in the bacterial enzyme (11). Three other missense mtDNA mutations of subunit a have been described recently: a nearly homoplasmic T8851C mutation (9) and two T8993C mutations (10,11). These mutations result in a decrease of ATPase activity (12). The T8851C mutation in subunit a in the human liver mitochondrial ATPase results in a decrease of ATPase activity (13).

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which changes highly conserved Trp to Arg and manifests as bilateral striatal lesion; a homoplasmic or heteroplasmic T9176C mutation which changes highly conserved Leu to Pro and associates with Leigh’s syndrome or sudden death syndrome; and a T9101C mutation which has been found in an LHON patient. Besides that, some of the mtDNA deletions, which include ATPase genes, may also result in defects of ATPase.

In this paper we describe a new selective defect of mitochondrial ATPase caused by altered biosynthesis of the enzyme, which is of generalized character and manifests as fatal neonatal lactic acidaemia with cardiomegaly and hepatomegaly. Analysis of mtDNA and studies of fibroblasts of the patient and derived transmitochondrial cybrids revealed the nuclear origin of the defect.

RESULTS

Activity and content of ATPase in patient tissues

Measurements of mitochondrial enzyme activities in 600 g post-nuclear supernatant prepared from the patient’s muscle showed low activity of oligomycin-sensitive ATPase, which was decreased to 34% of the control (Table [1]), whereas activities of cytochrome c oxidase (COX), citrate synthase (CS) and pyruvate dehydrogenase (PDH) were normal. Similarly, decreased ATPase activity (31% of the control) but normal COX activity were found in heart (Table [1]).

Electrophoretic analysis of heart mitochondrial OXPHOS complexes by Blue Native (BN)–PAGE in the first dimension and SDS–PAGE in the second dimension showed normal size, levels and composition of NADH dehydrogenase (I), ATPase, bc1 complex (III), COX (IV) and of ATPase subunits α and β (data not shown). Quantification of the ATPase from the content of the F1 subunits α and β detected by immunoblotting or staining revealed in patient tissues only 19–32% of the enzyme content detected in the control. This indicated that low activity of mitochondrial ATPase in patient tissues was caused by decreased levels of the enzyme.

Decreased function and levels of ATPase in fibroblasts and restoration of the ATPase defect in derived transmitochondrial cybrids

As shown in Table [1] the ATPase defect was present in patient fibroblasts, in which the ATPase activity decreased to 18% of the control, while the activities of the respiratory chain enzyme complexes were normal. ATP synthesis in digitoninized fibroblasts also showed very low levels of substrate-stimulated ATP production (Table [2]). ATP synthesis in patient cells was 33 and 29% of the control using NADH-dependent substrates or succinate, respectively, in accordance with the low level of oligomycin-sensitive ATP hydrolysis.

Table 1. Low ATPase activities in patient heart, muscle and fibroblasts

<table>
<thead>
<tr>
<th>Activity (nmol/min/mg protein)</th>
<th>ATPase</th>
<th>COX</th>
<th>CS</th>
<th>SCC</th>
<th>PDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle</td>
<td>10.1 (30 ± 4)</td>
<td>44.1 (58.5 ± 33.3)</td>
<td>93.9 (99.7 ± 49.8)</td>
<td>ND (1.5 ± 0.8)</td>
<td>0.8</td>
</tr>
<tr>
<td>Heart</td>
<td>14.4 (46 ± 9)</td>
<td>451 (371 ± 30)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>1.5 (8.5 ± 2.2)</td>
<td>30.5 (29.7 ± 14.8)</td>
<td>70.6 (53.5 ± 32.1)</td>
<td>8.0 (6.7 ± 1.9)</td>
<td>ND</td>
</tr>
<tr>
<td>Cybrids</td>
<td>9.5 (10.5 ± 3.2)</td>
<td>41.3 (36.5 ± 22.5)</td>
<td>80.9 (74.5 ± 17.1)</td>
<td>15.6 (20.2 ± 8)</td>
<td>ND</td>
</tr>
</tbody>
</table>

Mitochondrial enzyme activities were measured in post-nuclear supernatants of heart and muscle and in cultured fibroblasts and cybrids. Control activities (means ± SD, n = 5–18) are shown in parentheses. ATPase, oligomycin-sensitive ATPase; COX, cytochrome c oxidase; CS, citrate synthase; SCC, succinate cytochrome c reductase; PDH, pyruvate dehydrogenase; ND, not determined.

Figure 1. Decreased content of ATPase in patient heart mitochondria revealed by two-dimensional electrophoresis. Protein aliquots (10 µg) of patient and control heart mitochondria were separated in the first dimension by BN–PAGE and in the second dimension by SDS–PAGE. Gels were stained with Coomassie brilliant blue. The positions of NADH dehydrogenase (I), ATPase, bc1 complex (III), COX (IV) and of ATPase subunits α and β are indicated.
Electrophoretic analysis of the patient fibroblasts showed also that the level of ATPase enzyme was decreased to 20–25% of the control (Fig. 2), while the level of COX was normal. Apparently, the ATPase defect was fully manifested in patient fibroblasts.

When the mitochondrial membrane potential (ΔΨ) was measured in digitoninized fibroblasts with tetramethylrhodamine methylester (TMRM) using the FACS approach (Fig. 3), a 17% higher level of TMRM fluorescence was found in the cells of the patient in state 4 (using succinate as the substrate) compared with the controls (fluorescence/5000 cells in three independent experiments, mean ± SD: patient, 1470 ± 136; controls (n = 5), 1254 ± 351; controls with 1 µM FCCP, 194 ± 41; patient with 1 µM FCCP, 210 ± 30). The decrease in the content and activity of mitochondrial ATPase thus seems to be connected with decreased proton leak of the inner mitochondrial membrane, which results in an increased value of ΔΨ.

Analysis of DNA from muscle and fibroblasts of the patient did not show any deletions or rearrangements of mtDNA. Also the screening of the most frequent tRNA mutations and previously described missense mtDNA mutations in ATPase subunit gene was negative. Therefore, we tested the possibility that the ATPase defect is of nuclear origin, and we prepared transmitochondrial cybrids by fusing enucleated fibroblasts of the patient and mtDNA-less ρ° cells. Cybrid cells derived from patient fibroblasts fully restored oligomycin-sensitive ATPase activity (Table 1) and similarly restored the activity of ATP synthesis (Fig. 4). Also, the level of ATPase detected by western blotting was found to be normal in patient cybrids (Fig. 2). Immortalization of the patient fibroblasts did not affect the ATPase defect and the cell line derived from patient fibroblasts retained the low level of ATPase, while the level of COX was normal (data not shown). The restoration of the ATPase defect by control nucleus from the F1-ATPase subcomplex was similarly decreased (Fig. 4), which represents an assembly intermediate of the enzyme (17).

Bioavailability of ATPase in ATPase-deficient cells

In order to get more information about the mechanism responsible for low levels of ATPase in patient cells we performed [35S]methionine pulse–chase labelling experiments. Cells were labelled in culture for 3 and 6 h, mitoplasts were prepared by digitonin treatment and labelled mitochondrial proteins were resolved by two-dimensional electrophoresis. The observed pattern of the labelling showed significantly lower incorporation of [35S]methionine into subunits of the full-sized, assembled ATPase complex (Fig. 4) in fibroblasts of the patient compared with control fibroblasts. This can be best seen with the labelling of the α and β subunits which are the most intensely labelled subunits of the ATPase complex in the control fibroblasts. The labelling of the α and β subunits of the F1-ATPase subcomplex was similarly decreased (Fig. 4), which represents an assembly intermediate of the enzyme (17).

Table 2. Production of ATP by patient fibroblasts and derived cybrids

<table>
<thead>
<tr>
<th>ATP production (nmol ATP/30min/mg protein)</th>
<th>Fibroblasts</th>
<th>Cybrids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>32 ± 13</td>
<td>13 ± 7</td>
</tr>
<tr>
<td>Patient</td>
<td>13 ± 7</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>Ketoglutarate + malate</td>
<td>781 ± 99</td>
<td>255 ± 80</td>
</tr>
<tr>
<td>Succinate + rotenone</td>
<td>640 ± 55</td>
<td>183 ± 13</td>
</tr>
</tbody>
</table>

ATP synthesis by digitoninized cultured skin fibroblasts was measured fluorometrically after 30 min incubation at 37°C in the presence or absence of 10 mM ketoglutarate + 10 mM malate or 10 mM succinate + 5 µM rotenone. Data are the means ± SD of four to six independent measurements.

Figure 2. Restored ATPase content in transmitochondrial cybrids prepared from fibroblasts of the patient. Patient and control fibroblasts were enucleated and fused with ρ° cells to produce transmitochondrial cybrids. Protein aliquots (5 µg) of mitoplasts prepared from fibroblasts and cybrids were separated by BN–PAGE and the gel was analysed by western blotting with anti-F1-ATPase antibody (Anti F1, 1:10 000, upper panel) and anti-COX antibody (Anti COX, 1:5000, lower panel).
of the newly synthesized ATPase, the cells pulsed for 6 h were chased with excess of cold methionine (100 mM) and cultured for another 48 h. As shown in Figure 5, the labelling of ATPase decreased somewhat faster in patient fibroblasts than in control fibroblasts. At 48 h of chase, the labelling of ATPase in patient and control fibroblasts decreased to 44 and 72%, respectively, of the maximum labelling reached. No difference in the decay of ATPase labelling following the 48 h chase was found between the patient and control cybrids, which both exerted a labelling pattern similar to the control fibroblasts.

These data suggest that observed ATPase deficiency is associated with decreased ability of patient cells to form assembled ATPase complexes and that the half-life of the newly produced enzyme could be shorter than that in controls. The total synthesis of mitochondrial proteins, however, was not significantly affected in patient fibroblasts, which supports the view that we investigated a selective defect of only one OXPHOS complex.

**Biosynthesis of the \( \beta \) subunit of ATPase**

Radioactivity recovered in OXPHOS complexes resolved by two-dimensional electrophoresis of mitoplasts prepared by digitonin treatment (Figs 4 and 5) represents the labelling of the membrane-bound proteins, whereas the soluble, intra-mitochondrially located proteins are lost at the digitonin concentration used (18). In order to analyse the labelling of non-assembled subunits, we followed by means of immunoprecipitation the incorporation of \[^{35}S\]methionine into the \( \beta \) subunit of ATPase, which is the main catalytic subunit of ATPase. We solubilized labelled cells with Triton X-100 and the \( \beta \) subunit was immunoprecipitated from the whole cell sample with anti-F1 antibody, which preferentially precipitates the \( \beta \) subunit. Both in control and patient fibroblasts we observed a single immunoprecipitated protein which according to the size (51 kDa) corresponds with the mature form of the \( \beta \) subunit. The labelling of the immunoprecipitated \( \beta \) subunit (Fig. 6) was much higher in patient cells after 3 h and about the same labelling was observed in both types of cell after 6 h. When the cells were chased with cold methionine, a much faster decrease of the \( \beta \) subunit labelling was observed in patient cells. In fact, 3 h after the chase with 100 mM cold methionine the label in immunoprecipitated \( \beta \) subunit from patient fibroblasts decreased to <10%, whereas in the control cells the labelling of the \( \beta \) subunit remained the same or even slightly increased in comparison with the labelling after 6 h pulse. Therefore it appears that in patient cells the \( \beta \) subunit is synthesized, transported to mitochondria and processed to its mature form with normal or increased intensity, but the newly formed subunits are very labile and apparently become quickly degraded. It is likely that this results from decreased assembly of the \( \beta \) subunit into ATPase complexes, as shown in Figure 5.

**DISCUSSION**

Described deficiency of mitochondrial ATPase differs from the other ATPase disorders reported so far. As shown by enzyme activity measurements, electrophoretic and immunochemical analyses, the ATPase defect is selective with respect to other OXPHOS proteins and it has generalized character. The reduction of ATPase levels in the tissues investigated is not accompanied by any detectable changes in the size and composition of the enzyme (within the resolution of the method used) and the defect does not lead to the
occurrence or accumulation of assembly intermediates or aberrant forms of ATPase in the mitochondrial membrane. In cultured fibroblasts the defect manifests functionally as a pronounced decrease in ATPase hydrolytic and synthetic activities, in accordance with the low level of the enzyme. ATPase deficiency presented clinically as fatal neonatal lactic acidosis with cardiomegaly causing death shortly after birth. The main cause must be severely insufficient energy provision, which affects predominantly the heart—the tissue with high energy demands. In accordance with the low oligomycin-sensitive ATPase hydrolytic activity (18% of the control), the measurements in digitoninized fibroblasts showed very low stimulation of ATP synthesis with succinate or ketoglutarate and malate, indicating that in patient tissues glycolysis is the main source of ATP. Interestingly, the mitochondrial production of ATP was even lower than that in the fibroblasts from very severe and fatal cases with a nearly homoplasmic T8993G mtDNA mutation where ∼50% of residual mitochondrial ATP production has been always found (9,10,19).

$F_0$ proton channel of the ATPase contributes significantly to proton conductivity of the inner mitochondrial membrane at steady-state conditions (20,21). In patient fibroblasts the mitochondrial membrane potential ($\Delta\Psi$) values measured with TMRM at state 4 were increased, indicating that ATPase deficiency decreases the proton leak of the inner mitochondrial membrane. Apparently, the observed ATPase defect is a whole enzyme deficiency due to the low number of the assembled ATPase complexes, which do not show any increase in conductivity of the gated $F_0$ proton channels.

Screening of the known mtDNA mutations in the ATPase subunit gene was negative and experiments with cybrids derived from the patient fibroblasts showed full complementation of the content and activities of mitochondrial ATPase by the nuclei from $\rho^0$ cells, thus demonstrating that the defect is of nuclear origin. Further experiments will be necessary to locate the affected gene; however, the selectivity of the defect indicates that some assembly factor specific for ATPase biosynthesis could be missing. Several assembly proteins specific for ATPase have been found in yeast (22–24). Their analogues have to be expected in human cells, as the human analogue of the OXA1 protein has been found, which is needed for COX and ATPase assembly (25). The ATPase defect could also be caused by changes in expression of some of the nuclear-encoded ATPase subunits at the transcriptional or post-transcriptional levels. Both types of event can affect the content of mammalian ATPase as has been shown in brown adipose tissue (26–29) or in liver (30–33).

In vitro pulse–chase labelling experiments with patient fibroblasts showed normal or increased pulse labelling of the immunoprecipitated $\beta$ subunits of ATPase, indicating that the biosynthesis is normal, but the decay of the $\beta$ subunit labelling after the chase was accelerated. It appears that the de novo synthesized $\beta$ subunits cannot be efficiently assembled into functional ATPase complexes and thus become degraded. The question arises of at which stage is the assembly process impaired. It has been shown that mtDNA-encoded subunits assemble at a rather late stage (10,17) and inhibition of intramitochondrial translation leads to transient occurrence of two ATPase subcomplexes: $F_1$-ATPase and the 390 kDa ATPase subcomplex (17). In our patient, however, we did not find any accumulation of the $F_1$-ATPase subcomplex and we were unable to detect any other type of the short-lived and membrane-bound subcomplex containing ATPase subunits. Therefore, it is reasonable to
conclude that the assembly process must be terminated at an earlier stage than that at which the F_{1}-ATPase is formed. The situation in the patient may resemble the situation in mammalian thermogenic organ, brown adipose tissue, where an up to 10-fold selective decrease in the ATPase level is caused by limited availability of ATPase subunit c due to selectively low expression of subunit c genes (28,29).

**MATERIALS AND METHODS**

**Patient**

The parents are first degree cousins and they are healthy. The mother has been pregnant eight times with one miscarriage. Three boys are healthy, but two girls died in the first week of life probably due to severe metabolic acidosis. One boy with a congenital heart defect died at the age of 3 years due to failure to thrive and progressive psychomotor retardation. Three children of the mother’s sister died within the first week of life without any metabolic investigations.

The boy was born in term after the eighth pregnancy with oligohydramnion, birth weight 1950 g (<5 percentiles) and length 45 cm. Small placenta and craniofacial dysmorphia was present including frontal bossing, small mandible, and hypoplasia. Early postnatal adaptation was complicated with progressive hypotonia, hepatomegaly and metabolic acidosis. Sonography revealed cardiomegaly with hypertrophy of the interventricular septum. The boy died in the second day of life due to fatal lactic acidosis and heart failure. Laboratory investigations revealed hypoglycemia (0.2 mmol/l), increased aminotransferases (ALT 1.1 units/l, AST 2.6 units/l), hyperammonemia (297 mmol/l, controls <100 mmol/l), severe lactic acidosis (blood lactate, 30–36 mmol/l, controls <2 mmol/l), CSF lactate, 17 mmol/l, controls <2 mmol/l) and high lactate to pyruvate ratio (160, controls 10–20).

**Ethics**

This study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Committees of Medical Ethics at all collaborating institutions. Informed consent was obtained from the parents.

**Tissues**

Samples of muscle and heart obtained during autopsy 2 h after the death were frozen at −70°C. Heart homogenate (5%) was prepared in 250 mM sucrose, 10 mM Tris–HCl, 1 mM EDTA, pH 7.4 (STE medium), using a glass–Teflon homogenizer and muscle homogenate (5%) in 150 mM KCl, 50 mM Tris–HCl, 2 mM EDTA, pH 7.4 using a hand-driven glass–glass Dounce homogenizer. Homogenates were filtered through a 250-µm nylon screen and centrifuged 10 min at 600 g to obtain post-nuclear supernatants.

**Cell cultures, immortalized cells and preparation of cybrids**

Skin fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum (FCS) in 5% CO_{2} in air. Fibroblasts were immortalized with pRNS-1 plasmid (34) by a lipofectin transfection (FuGene6; Boehringer Mannheim, Mannheim, Germany). Trans mitochondrial cybrids were prepared according to the method of Tiranti et al. (35). Fibroblasts from the ATPase-deficient patient and from controls were enzymatically treated in DMEM containing 10 µg/ml cytochalasin B and then fused with mtDNA-less (p14) tumoral cells (derived from 143B.TK− osteosarcoma cells). Selection was performed by cultivation of cells for 3 weeks in DMEM containing 5% FCS, 100 µg/ml 5-bromodeoxyuridine and lacking uridine. The cells were grown to ∼90% confluence and harvested using 0.05% trypsin and 0.02% EDTA. Enzyme activity measurements cells were resuspended in STE medium and homogenized using a glass–Teflon homogenizer. For electrophoretic analysis mitoplasts were prepared by digitonin treatment (18) using 0.8 mg digitonin/mg protein.

**[35S]methionine labelling**

Fibroblasts or cybrids grown in 25 cm^2 flasks to 70–80% confluence were washed twice with 4 ml phosphate-buffered saline (PBS) and incubated for 3 h in 3 ml methionine-free DMEM containing 10% FCS that was dialysed against PBS prior to use. Medium was then removed and to each flask 2 ml of methionine-free DMEM with dialyzed FCS and 20 µCi [35S]methionine were added. Cells were labelled for indicated time intervals. To chase the cell labelling, the labelling medium was removed, cells were washed twice with 4 ml of DMEM with 100 mM methionine and 10% FCS and then 4 ml of DMEM with 10% FCS and 10 mM methionine were added. Incubations were terminated by addition of 20 µl of 0.5 M chloramphenicol and 20 µl of 0.05 M cyclorrhaphic and cells were immediately harvested by trypsination, washed twice with PBS and resuspended to 5 mg protein/ml. Total incorporation was determined in aliquots spotted onto Whatman 3MM filter paper and precipitated by trichloroacetic acid. For electrophoretic analysis mitoplasts were prepared by digitonin treatment (18) using 0.8 mg digitonin/mg protein.

**Immunoprecipitation of F_{1}-ATPase**

For each point the labelled cells harvested from two 25 cm^2 flasks were solubilized in 0.8 ml of TNET (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) with 2 µg/ml of pepstatin and 0.2 mM PMSF. Insoluble material was sedimented by 30 min centrifugation at 30 000 g. Samples were incubated on a tube rotator at 4°C with 10 µl of rabbit-anti bovine F_{1}-ATPase serum for 16 h, then 20 µl of protein A–Sepharose was added and samples were incubated for 1 h. Protein A–Sepharose beads were washed 3 times with 2 ml TNET, twice with 2 ml TNE and once with 2 ml TE. Immunoprecipitated proteins were solubilized from Protein A–Sepharose with 30 µl of sample loading buffer (2% mercaptoethanol, 4% SDS, 50 mM Tris–HCl, pH 7.0, 10% glycerol) for 5 min at 100°C.

**Electrophoretic methods**

BN–PAGE (36) was used for separation of samples in the first dimension on a 6–15% polyacrylamide gradient as previously described (18). Mitoplasts or mitochondria (isolated from post-nuclear supernatant by 10 min centrifugation at 10 000 g) were solubilized with 1% lauryl maltoside at a protein concentration 1 mg/ml. For two-dimensional analysis, strips of the first dimension gel were incubated for 45 min with 1% SDS and 1%...
β-mercaptoethanol and then subjected to 10% polyacrylamide SDS–PAGE for separation in the second dimension (37). The gels were stained with Coomassie brilliant blue R and then by silver (38). COX and F1-ATPase were detected by western blotting using semi-dry transfer of proteins onto Hybond C-extra membrane (Amersham, Little Chalfont, UK). Specific antisera to F1-ATPase, to ATPase F0 subunits b and c and to COX (28,39) were used at titres of 1:2000–1:10000. Immunocomplexes were detected by the peroxidase-conjugated secondary antibody (FAB fragment 1:3000; Bio-Rad, Hercules, CA) and enhanced chemiluminescence (ECL; Amersham). Dried gels and luminograms exposed on MEDIX-MA X-ray films (FOMA, Hradec Kralove, Czech Republic) were quantified on a Molecular Dynamics Computing Densitometer using Image Quant 3.3 software (Molecular Dynamics, Sunnyvale, CA).

**ATP synthesis**

The rate of the ATP synthesis by digitoninized cultured cells was measured as described by Wanders et al. (40). Cells resuspended at a protein concentration of 0.16 mg/ml were subjected to 40 μg digitonin/ml and incubated with different respiratory substrates (10 mM) for 30 min at 37°C. The ATP production was expressed in nmol/30min/mg protein.

**Measurements of mitochondrial membrane potential**

Cytofluorometric analysis of mitochondrial membrane potential was performed in digitonin-treated fibroblasts with TMRM (Molecular Probes, Eugene, OR) on the FACSsort flow cytometer (Becton Dickinson, San Jose, CA) equipped with an argon laser 488 nm as previously described (41). Cells resuspended in KCl medium were permeabilized with 0.03 mg digitonin/ml and incubated with different mitochondrial membrane potential probes covering the region 1–12641 and 14956–16569 were selected and cycle sequenced using Cy5 labeled malE and M13 universal vector primers. Sequences were read on ALFExpress (Pharmacia, Uppsala, Sweden) and analyzed using the GeneSkipper software (EMBL, Heidelberg, Germany). Origin of mtDNA and nuclear DNA in prepared cybrids was verified by analysis of the mtDNA 500–16130 region of the D-loop and of the highly polymorphic repetitive sequence at the D11S53 locus on chromosome 11q, respectively (35). Radioactivity was detected on a PhosphorImager (Molecular Dynamics).

**Other assays**

The activities of COX (46), CS (47), PDH (48) and oligomyxin-sensitive ATPase (49) were measured according to standard procedures. The protein concentration was measured in lauryl maltsose-solubilized proteins according to Bradford (50), in other samples according to Lowry et al. (51), using bovine serum albumin as a standard.

**ABBREVIATIONS**

ATPase, mitochondrial ATP synthase; mtDNA, mitochondrial DNA; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BN–PAGE, Blue Native–polyacrylamide gel electrophoresis; COX, cytochrome c oxidase; CS, citrate synthase; OXPHOS, oxidative phosphorylation; PDH, pyruvate dehydrogenase; TMRM, tetramethylrhodamine methyl ester.

**ACKNOWLEDGEMENTS**

The expert technical assistance of V. Fialová and Z. Stárková is gratefully acknowledged. This work was supported by grant 303/97/0554 from the Grant Agency of the Czech Republic and by grant 4035–3 from the Grant Agency of Ministry of Health of the Czech Republic.

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