Dysfunctions of Cellular Oxidative Metabolism in Patients with Mutations in the NDUFS1 and NDUFS4 Genes of Complex I

The pathogenic mechanism of a G44A nonsense mutation in the NDUFS4 gene and a C1564A mutation in the NDUFS1 gene of respiratory chain complex I was investigated in fibroblasts from human patients. As previously observed the NDUFS4 mutation prevented complete assembly of the complex and caused full suppression of the activity. The mutation (Q522K replacement) in NDUFS1 gene, coding for the 75-kDa Fe-S subunit of the complex, was associated with (a) reduced level of the mature complex, (b) marked, albeit not complete, inhibition of the activity, (c) accumulation of H$_2$O$_2$ and O$_2^.$ in mitochondria, (d) decreased cellular content of glutathione, (e) enhanced expression and activity of glutathione peroxidase, and (f) decrease of the mitochondrial potential and enhanced mitochondrial susceptibility to reactive oxygen species (ROS) damage. No ROS increase was observed in the NDUFS4 mutation. Exposure of the NDUFS1 mutant fibroblasts to dibutyryl-cAMP stimulated the residual NADH-ubiquinone oxidoreductase activity, induced disappearance of ROS, and restored the mitochondrial potential. These are relevant observations for a possible therapeutic strategy in NDUFS1 mutant patients.

Deficiency of complex I (NADH ubiquinone oxidoreductase, EC 1.6.5.3) of the respiratory chain is a major cause of inborn mitochondrial disease (1–6). Leigh syndrome (early onset fatal neurodegenerative disorder) and Leigh syndrome-like disease are the most common clinical phenotypes associated with complex I deficiency. Impairment of complex I has also been reported in Parkinson (7), Alzheimer (8, 9), and Huntington (10) diseases.

Complex I is the largest of the respiratory chain enzymes, being composed of seven mitochondrial DNA and at least 39 nuclear DNA-encoded subunits (11). Mutations in structural subunits have been found in ~40% of the patients with inborn deficiency of complex I. Reported mutations include all of the mitochondrial DNA-encoded subunits (12) and twelve nuclear-encoded subunits (3, 6, 13–15).

The nuclear NDUFS4 gene codes for an 18-kDa subunit of the complex (11), which in high eukaryotes contains potential phosphorylation sites for cAMP-dependent protein kinase in both the precursor and the carboxyl-terminal region (EMBL Data Bank). In mammalian (16–18) and human (19) cell cultures, cAMP promotes the phosphorylation of the NDUFS4 protein and enhances the functional capacity of complex I. Three recessive mutations in the nuclear NDUFS4 gene have been identified in three unrelated affected children by Leigh syndrome-like syndrome with deficiency of complex I, including an AAGTC duplication at position 466–470 in exon 5 (20), a single base deletion at position 289/290 in exon 3 (21), and a G44A nonsense mutation in the first exon of the gene, introducing a premature termination codon in the sequence coding for the mitochondrial leader peptide (13). All three mutations resulted in the disappearance of the 18-kDa subunit and defect in both the activity and assembly of the complex (22). In the 289/290 deletion in exon 3, which predicts the synthesis of an aberrant and prematurely truncated protein, we found almost complete absence of the NDUFS4 transcript (22), apparently degraded by Nonsense Medi- ated Decay (23). The G44A nonsense mutation in the first exon resulted in stabilization of three alternatively spliced transcript variants of the NDUFS4 gene (24). Recently, large-scale deletion and point mutations in the NDUFS1 gene coding for the 75-kDa Fe-S subunit of complex I have been found (6, 25) in children with mitochondrial encephalopathy.

In this report a detailed study is presented on the functional consequences of the G44A mutation in NDUFS4 and of a C1564A mutation in the NDUFS1 gene (Q522K replacement) (6). The assembly and catalytic activity of complex I, mitochondrial energy-transfer, and oxygen-free radical balance were investigated in primary fibroblast cultures of two patients.

EXPERIMENTAL PROCEDURES

Reagents and Cell Lines—DMEM; 2 PBS, trypsin (0.05%), EDTA (0.02%), penicillin, streptomycin, calf serum, and fetal bovine serum were from EuroClone. Cholera toxin, dibutyryl cyclic AMP, diphenylethioiodon, glutathione reductase, reduced glutathione, and rotenone were from Sigma. NADH was from Boehringer, dicyclofubin and 3-isobutyl-1-methylxanthine (IBMX) from Calbiochem, and 2',7'-dichlorofluorescein diacetate, MitoCapture, from Biovision. Mito Tracker Red and MitoSOX was from Molecular Probes; TRIZol, Super-

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1 To whom correspondence should be addressed: Dept. of Medical Biochemistry, Biology, and Physics, University of Bari, 70124 Bari, Italy.

2 The abbreviations used are: DMEM, Dulbecco’s modified Eagle’s medium; DCF-DA, dichloro-7-amino-2,7-fluorescein diacetate; SOD, superoxide dismutase; PBS, phosphate-buffered saline; LSCM, laser scanning confocal microscopy; ROS, reactive oxygen species.
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FIGURE 1. Immunoblots of two-dimensional electrophoretic gels of mitoplasts from control and patient's fibroblasts with mutations in the NDUFS4 (A) and NDUFS1 (B1 and B2) genes of complex I. Respiratory complexes (I, III, and IV) and ATP synthase complex (V) were separated by blue native electrophoresis and resolved in their subunits in a second dimension by SDS-PAGE. Blotted onto nitrocellulose, and tested with different antibodies. The proteins of the complexes were detected by specific antibodies against the 18- and 12-kDa subunits of complex I (CI), core II subunit of complex III (CIII), and β subunit of ATP synthase (CV) (panel A). NDUFS1, 39, 20, and 17 kDa of complex I (CI) (panel B1), 39 and 18 kDa of complex I (CI) (panel B2), core II subunit of complex III (CIII), subunit IV of complex IV (Cox), and β subunit of ATP synthase (CV) (panels B1 and B2). It can be noted that core II was immunodetected in both monomeric and dimeric complex III. For details see “Experimental Procedures.”

TABLE 1
NADH ubiquinone oxidoreductase, NADH ferricyanide oxidoreductase, and cytochrome c oxidase activities in mitoplasts from control and patient's fibroblasts

Activities are expressed as nmoles/min/mg proteins. Mitoplasts were exposed to ultrasound energy for 15 s at 0 °C. The activity of complex I was measured using 50 μg of proteins in 700 μl of 50 mM potassium phosphate buffer, MgCl2 5 mM, in the presence of 0.2 mM decylubiquinone and 3 mM KCN, pH 7.4, 37 °C. The reaction was started with different concentrations of NADH (2.5–15 μM). The activity was corrected for the reaction in the presence of rotenone (1 μM/mL). From the activity measurements Lineweaver-Burk plots were drawn to calculate Vmax and Km [NADH] of NADH-UQ oxidoreductase. The rate of NADH-ferricyanide oxidoreductase and the oxidation rate of 10 μM ferrocyanochrome c were determined at 37 °C as described under “Experimental Procedures.” n, number of determinations; M ± S.E., mean values of measurements ± standard error. Aver., range of measured values. p, Student’s t-test analysis of patients vs. controls. ND, not detectable. NS, not significant.

| Enzymatic activities   | NHDF                        | NDUFS4 (G44A null mutation) | NDUFS1 (C1564AQ522K substitution) |
|------------------------|-----------------------------|-----------------------------|-----------------------------------|
|                        | n   | Mean ± S.E. | Aver. | n   | Mean ± S.E. | Aver. | p   | n   | Mean ± S.E. | Aver. | p   |
| NADH-UQ oxidoreductase |     |             |       |     |             |       |     |     |             |       |     |
| Vmax                   | (8) | 24.42 ± 1.3 | (28–19) | (2) | ~0.00       |       |     | (4) | 5.17 ± 1.4 |       | <0.001 |
| Ks                     | (8) | 16.72 ± 2.3 | (23–12) | ND |             |       |     | (4) | 6.38 ± 2.2 | (10–2.0) | <0.01 |
| NADH-FeCN oxidoreductase| (6) | 952 ± 70  | (1200–759) | (3) | 1353 ± 53 | (1448–1263) | <0.01 | (4) | 1512 ± 139 | (1874–1240) | <0.01 |
| Cytochrome c oxidase   | (8) | 14.87 ± 2.0 | (25–9) | (5) | 11.12 ± 1.7 | (14–7.0) | NS | (5) | 13.41 ± 1.5 | (18–10) | NS |

Script reverse transcriptase, and primers from Invitrogen; ATP assay from Promega, and neonatal human dermal fibroblasts (NHDF-neo CC-2509) from Cambrex.

Case Report—The patient with the homozygous G44A nonsense mutation in the NDUFS4 gene was a baby diagnosed as a case of Leigh syndrome. The patient with severe lactic acidosis died at the age of 7 months. The homozygous C1564A mutation in the NDUFS1 gene was found in two brothers diagnosed for leukodystrophy; at six months a lactic acidosis appeared in both children, who had a progressive disease course.

Cell Culture and Mitoplast Preparation—Control fibroblasts (neonatal human dermal fibroblasts-neo) and patient’s fibroblasts were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml of penicillin, and 100 IU/ml of streptomycin. The growth of the fibroblasts from the NDUFS4 mutant was depressed (doubling time >72 h) as compared with the fibroblasts from the NDUFS1 mutant, whose growth was comparable with that of controls (doubling time ~24 h).

For mitoplast preparation fibroblasts were harvested with 0.05% trypsin and 0.02% EDTA and washed in phosphate-buffered saline, pH 7.4, with 5% calf serum. Cells in PBS were exposed 10 min on ice to 0.2 mg of digitonin/mg cellular protein. Mitoplasts were pelleted at 14,000 × g and resuspended in PBS.

Rotenone Titration of Cellular Respiration—Respiration was monitored by oxygen polarography.

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Enzymatic Spectrophotometric Assay—NADH-UQ oxidoreductase activity: mitoplasts were exposed to ultrasound energy for 15 s at 0 °C, and $V_{\text{max}}$ and $K_m$ were determined as in Ref. 22. NADH ferricyanide oxidoreductase activity: mitoplasts were added to 700 μl of 50 mM potassium phosphate buffer, MgCl$_2$ 5 mM, pH 7.4, in the presence of 1 μg/ml of Antimycin A and 3 mM KCN, and the reduction of 250 μM ferricyanide was measured spectrophotometrically (26). Cytochrome c oxidase activity was determined on mitoplasts in 700 μl of 10 mM phosphate buffer, pH 7.4, following the oxidation of 10 μM ferrocytochrome c (27). Glutathione peroxidase activity was determined as described in Ref. 28. Cells were suspended in PBS and disrupted by exposure to ultrasound.

Electrophoretic Procedures and Antibodies—Two-dimensional gel analysis (blue native PAGE/SDS-PAGE) of the mitoplasts and immunoblotting were carried out as in Ref. 22. Rabbit antiserum against the 20 residues of the carboxyl terminus with phosphorylated Ser-131 of the NDUFS4 protein (18 kDa) was produced by Neosystem, Strasbourg, France. Monoclonal antibodies against other subunits of complex I (39, 20, 17, 12 kDa), the core II subunit of complex III, the subunit IV of complex IV, and the β subunit of complex V were from Molecular Probes (Eugene, Oregon).

Laser Scanning Confocal Microscopy Analysis (LSCM)—Cells were seeded onto fibronectin-coated 35-mm glass-bottom dishes. Cells were incubated 20 min at 37 °C with MitoCapture (1/1000 dilution) or 0.5 μM Mito Tracker Red to monitor mitochondrial membrane potential and 10 μM dichlorofluorescein-diacetate (DCF-DA) or 3 μM MitoSOX for detection of H$_2$O$_2$ and O$_2^{-}$, respectively. Stained cells were examined by a Nikon TE 2000 microscope (images collected using a ×60 objective 1.4 NA) coupled to a Radiance 2100 dual laser (four-lines Argon-Krypton, single-line Helium-Neon) scanning confocal microscope (Bio-Rad). The fluorescent signal of the MitoCapture double-emitter probe was examined sequentially, exciting first with the Ar-Kr laser beam (λex 543 nm) and then with the He-Ne laser beam (λex 633 nm) (29). The fluorescence signal of the cationic membrane permeant probe Mito Tracker Red was monitored exciting with the He-Ne laser beam (λex 545 nm). DCF-DA is hydrolyzed by intracellular esterases and converted to the H$_2$O$_2$-reacting product DCF (30). The green fluorescence of oxidized DCF was analyzed by exciting the sample with the Ar-Kr laser beam (λem 488 nm).

FIGURE 2. Rotenone titration of endogenous cellular respiration in control and patient's fibroblasts. The respiratory rate was measured at 37 °C on 2·10^6 cells suspended in 500 μl of a reaction medium containing 75 mM sucrose, 30 mM Tris-HCl, 50 mM KCl, 0.5 mM K-EDTA, 0.5 mM MgCl$_2$, and 2 mM potassium phosphate buffer (pH 7.4) in the presence of 50 mM meta-chlorobenzylicyanide phenylhydrazone (CCCP) uncoupler. Experimental points were fitted by sigmoidal curve of SigmaPlot program. Filled circles, control fibroblasts. Empty circles, fibroblasts from the NDUFS1 mutant patient. Open squares, fibroblasts from the NDUFS4 mutant patient. For details see “Experimental Procedures.”

ATP Content Measurements—Cellular ATP was extracted by boiling water (31) and assayed by bioluminescence using a luciferin-luciferase system (Promega).

Measurement of Glutathione—Cells harvested from Petri dishes with trypsin-EDTA were washed in PBS supplemented with 5% serum, suspended in PBS, and homogenized. After protein precipitation with 2% sulfosalicylic acid, glutathione was determined as described in Ref. 32. For measurement of reduced glutathione, proteins were precipitated with 10% perchloric acid and the supernatant analyzed by high-performance liquid chromatography. The samples were loaded onto a Synergi 4μ Hydro-RP column (150 × 4.6 mm) and eluted with an isocratic mobile phase of 220 mM potassium phosphate, pH 2.7/acetonitrile (99:1).

Reverse Transcription Polymerase Chain Reaction—2 μg of total cellular RNA isolated by TRIzol reagent was reverse transcribed to cDNA with specific antisense primers (50 pmol each) by SuperScript reverse transcriptase. Primers used were cytosolic CuZn superoxide dismutase (SOD1), 5′-TGAAGAGAGGCATGTGAG-3′, 5′-TCTTCTATTTCCACCTTGGCC-3′ (Tm 55 °C); mitochondrial Mn superoxide dismutase (SOD2), 5′-GACAACCCCTAGGCTT-3′, 5′-TGCTCCCAACATCATTT-3′ (Tm 57 °C); cytosolic glutathione peroxidase, 5′-AAATTCCTCAAGTACGTCCG-3′, 5′-GACGACATGGGAA-3′, 5′-ACCAACTGGGAGCAGACATGGGAG-3′, 5′-GACGACATGGGAG-3′. 

FIGURE 4. Cellular ATP content in control and patient’s fibroblasts. Fibroblasts were grown in the presence of 10% fetal bovine serum in DMEM (white bars) or 0.5% fetal bovine serum in DMEM (gray bars). Each bar represents the average of six independent measurements ± S.E. The statistical difference in ATP content between controls and NDUFS4 mutant fibroblasts was evaluated by two-tail Student’s t-test analysis. a, control fibroblasts. b, fibroblasts from the NDUFS1 mutant patient. c, fibroblasts from the NDUFS4 mutant patient. For details see “Experimental Procedures.”

FIGURE 3. LSCM images of mitochondria accumulating the membrane potential probe in control and patient’s fibroblasts. Fibroblasts seeded on fibronectin-coated glass-bottom dishes were stained at 37 °C with the ΔΨ probe Mitocapture. a, control fibroblasts. b, fibroblasts from the NDUFS1 mutant patient. c, fibroblasts from the NDUFS4 mutant patient. Scale bars, 20 μm. For details see “Experimental Procedures.”

FIGURE 3.
The inhibition of respiratory rate decreased from 2.4 to 1.5 attomol/cell). (Fig. 2) showed in the normal range. This effect was exacerbated by the NDUFS1 mutation (Fig. 3). No significant decrease in cellular ATP was found for either mutation when fibroblasts were grown in the presence of 10% fetal bovine serum (Fig. 4). Under conditions of serum limitation a higher content of cellular ATP was found in both mutant fibroblasts, in particular in the NDUFS4 mutant, as compared with control fibroblasts.

**Cellular Reactive Oxygen Species and Scavenger Systems**—In the NDUFS1 mutant fibroblasts we found markedly high levels of H$_2$O$_2$ detected by the green fluorescence of 2′,7′-dichlorofluorescein. However, we found no difference in the H$_2$O$_2$ content of the NDUFS4 mutant fibroblasts compared with control cells (Fig. 5). In the NDUFS1 mutant fibroblasts the H$_2$O$_2$DCF green fluorescence merged with the red fluorescence of the mitochondrial probe, MitoTracker Red, giving an orange/yellow fluorescence (Fig. 5). The MitoSOX probe, a fluorochrome specific to oxygen superoxide (O$_2^-$) produced in the inner mitochondrial compartment, showed higher staining in the NDUFS1 fibroblasts as compared with both control and NDUFS4 mutant fibroblasts (Fig. 5).

In contrast with the NDUFS4 mutation, which caused a marked depression, the NDUFS1 mutation did not affect the fibroblast growth (see "Experimental Procedures"). Marked cell shrinkage was, however, detected in NDUFS1 mutant fibroblasts when exposed to H$_2$O$_2$. No such effect was observed in the NDUFS4 mutant fibroblasts (Fig. 6).
Reverse transcription PCR analysis showed that the transcript levels of both cytosolic CuZn-superoxide dismutase (SOD1), Mn-superoxide dismutase (SOD2), and glutathione peroxidase (GPX1) activity and glutathione levels in control fibroblasts (black bars, a), fibroblasts from the NDUFS1 mutant patient (gray bars, b), and fibroblasts from the NDUFS4 mutant patient (empty bars, c). Transcript levels (A) were determined by reverse transcription PCR on extracts of total cellular RNA. Glutathione peroxidase activity (B) and levels of total glutathione and reduced glutathione (C) were determined on cellular extracts as described under “Experimental Procedures.” Student’s t-test analysis b versus a: *, p < 0.05; **, p < 0.01.

Effect of Dibutyryl-cAMP on Functional Parameters of NDUFS1 Mutant Fibroblasts—Exposure of the NDUFS1 mutant fibroblasts to dibutyryl-cAMP resulted in stimulation of the residual forward electron transport (Fig. 8). Under these conditions, the NADH-ubiquinone oxidation was increased (A). MitoCapture for detection of low (green fluorescence) and high (red fluorescence) mitochondrial membrane potential (B). DCF-DA detection of H2O2 (green fluorescence) (C). Bars, 20 μm. Semiquantitative LSCM analysis of the fluorescent signals. Confocal planes of 0.2-μm thickness were examined along the z-axes. Acquisition, storage, and analysis of data were made by using LaserSharp and LaserPix software from Bio-Rad. Quantification of the emitted fluorescent signal was achieved by producing an xz intensity profile of the average value of the pixels within intracellular areas (circles of 5–10 μm) as a function of each focal plane. The integrated value of the xz profile was taken as a measure of the fluorescence intensity and quantified in arbitrary units. For details see the legends to Table 1 and Figs. 3 and 5 and “Experimental Procedures.”

Effect of Dibutyryl-cAMP on Functional Parameters of NDUFS1 Mutant Fibroblasts—Exposure of the NDUFS1 mutant fibroblasts to dibutyryl-cAMP resulted in stimulation of the residual forward electron transport (Fig. 8). Under these conditions, the NADH-ubiquinone oxidation was increased (A). MitoCapture for detection of low (green fluorescence) and high (red fluorescence) mitochondrial membrane potential (B). DCF-DA detection of H2O2 (green fluorescence) (C). Bars, 20 μm. Semiquantitative LSCM analysis of the fluorescent signals. Confocal planes of 0.2-μm thickness were examined along the z-axes. Acquisition, storage, and analysis of data were made by using LaserSharp and LaserPix software from Bio-Rad. Quantification of the emitted fluorescent signal was achieved by producing an xz intensity profile of the average value of the pixels within intracellular areas (circles of 5–10 μm) as a function of each focal plane. The integrated value of the xz profile was taken as a measure of the fluorescence intensity and quantified in arbitrary units. For details see the legends to Table 1 and Figs. 3 and 5 and “Experimental Procedures.”

The same cells, the total content of glutathione was reduced, especially the reduced fraction. No such changes were observed in the NDUFS4 mutant fibroblasts (Fig. 7).
NADH-ubiquinone oxidoreductase activity of complex I and restored the mitochondrial potential, whereas the H$_2$O$_2$ virtually disappeared (Fig. 8).

**DISCUSSION**

The homozygous *NDUFS1* and *NDUFS4* mutations are associated with remarkably different patterns of biochemical dysfunction. These differences can explain the different clinical features in the two patients and may possibly suggest rational therapeutic strategies. The G44A nonsense *NDUFS4* mutation was associated with earlier onset and more severe disease course. Similar to other mutations of this gene (22), the mutation caused the disappearance of the corresponding protein (18-kDa subunit), the incomplete assembly of complex I, the complete abolition of the rotenone-sensitive NADH-ubiquinone oxidoreductase activity of the complex, and the marked depression of both respiration and cell growth. The C1564A *NDUFS1* mutation, which determines a Q522K replacement in the 75-kDa Fe-S protein of complex I, was associated with a later onset, more progressive disease course. The mutation did not abolish the rotenone-sensitive NADH-ubiquinone oxidoreductase completely, caused little decrease of fibroblast respiration, and did not affect their growth rate. However, the *NDUFS1* mutation determined a decrease of the mitochondrial membrane potential, which was not observed in the *NDUFS4* mutant fibroblasts. In the latter, complete abolition of complex I activity and marked depression of respiration failed to produce a decrease in the cellular ATP content (Fig. 4), possibly because in cell culture ATP is produced in high amount by both glycolysis and the mitochondrial oxidation of glycolytic NADH mediated by the glycerophosphate shuttle, which bypasses complex I (34). Mitochondrial hydrolysis of glycolytic ATP and/or electron flow through the second and third sites of the respiratory chain can contribute to maintaining the mitochondrial potential. Consistent with this possibility is the observation that under serum limitation growth conditions the cellular ATP content increased, particularly in the *NDUFS4* mutant fibroblasts. This compensatory mechanism is clearly insufficient to prevent the progression of the disease in the whole organism and can indeed explain the severe lactic acidosis documented in the patient.

Direct monitoring of ROS level and glutathione showed ROS overproduction in the *NDUFS1* mutant fibroblasts. The mutation was associated with a large increase in the level of H$_2$O$_2$ in/around mitochondria, accumulation of O$_2^-$ in the inner mitochondrial compartment, increased expression of glutathione peroxidase, and a decrease in the cellular reserve of glutathione and in its reduction level. No such changes were observed in the fibroblasts from the *NDUFS4* patient.

These observations substantiate the view that complex I is the major source of O$_2^-$ and ROS derivatives in human fibroblasts (1, 35, 36). The complete abolition of the normal rotenone-sensitive NADH-ubiquinone oxidoreductase caused by the deletion of the *NDUFS4*-encoded 18-kDa subunit is likely to result from inhibition of a redox step that is also involved in the direct reduction of O$_2$ to O$_2^-$ (37). This step, which remains to be identified, might also be controlled by cAMP-dependent phosphorylation of the *NDUFS4* 18-kDa subunit of complex I (16, 19). In the case of the *NDUFS1* mutation, the inhibition of the NADH-ubiquinone oxidoreductase can be attributed to altered function of the 75-kDa Fe-S protein encoded by this gene. The Q522K substitution can promote direct oxidation by molecular oxygen of the *NDUFS1* Fe-S center once it is reduced by NADH. Both mutations are likely to involve redox step(s) below the site where ferricyanide accepts electrons from the complex, because both were associated with increased rotenone-insensitive NADH-ferricyanide oxidoreduction activity.

In the *NDUFS1* mutant fibroblasts, dibutyryl-cAMP stimulated the residual rotenone-sensitive NADH-ubiquinone oxidoreductase activity, determined the disappearance of ROS, and restored the mitochondrial potential. The pattern of complex I assembly in *NDUFS4* and *NDUFS1* mutant fibroblasts provides clues to explain our biochemical results (cf. Ref. 38). The absence of the 18-kDa subunit blocked a late step in the assembly of a mature functional complex, determining the formation of an inactive subcomplex whose molecular mass was ~100 kDa lower than normal (see also Ref. 22). The Q522K substitution in the 75-kDa Fe-S subunit of complex I was associated with reduced level of the fully assembled complex and the presence of a subcomplex of similar molecular mass to the subcomplex observed in the *NDUFS4* mutant. The *NDUFS4* 18-kDa protein detected in the residual amount of the mature complex was absent in the subcomplex. The mutation of the 75-kDa Fe-S protein could have impaired the last step in the assembly of the complex and/or induced oxidative degradation of the complex as a consequence of enhanced ROS production (cf. Ref. 39).

In conclusion, the lack of a completely assembled, functional NADH-ubiquinone oxidoreductase complex I and the consequent severe acidosis due to accumulation of pyruvate/lactate and other NAD-linked substrates could explain the early onset, fatal course of the disease in the *NDUFS4* mutant patient. In the *NDUFS1* mutant patient, the partial depression of the NADH-ubiquinone oxidoreductase activity of complex I could explain the less severe clinical course. In this mutation an additional adverse event, however, results from the enhanced production of ROS, which could in turn trigger oxidative stress in a “vicious circle” leading to amplification of biochemical damage and disease progression. Selected antioxidants and β-agonists could offer a rational therapeutic strategy in patients carrying mutations in *NDUFS1* or in other complex I genes that result in biochemical dysfunction centered on excess of ROS production and oxidative stress.

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