Severe Impairment of Complex I–Driven Adenosine Triphosphate Synthesis in Leber Hereditary Optic Neuropathy Cybrids

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Background: Leber hereditary optic neuropathy (LHON) is a maternally inherited form of central vision loss associated with mitochondrial DNA point mutations that affect the ND subunits of complex I.

Objective: To elucidate the bioenergetic consequences of complex I dysfunction in LHON.

Design: The biochemical phenotypes of LHON mutations have been investigated using the transmitochondrial cytoplasmic hybrid (cybrid) cell model derived from the osteocarcoma parental cell line 143B.TK−.

Setting: Research laboratories at neuroscience and biochemistry departments at the University of Bologna, Scientific Institute “E. Medea,” and University of College Medical School.

Participants: Fibroblast cell lines were obtained from patients affected with LHON, as defined by the presence of 1 pathogenic mutation, and from healthy volunteers as controls to construct cybrid cell lines.

Main Outcome Measures: Complex I (glutamate-malate)– and complex II (succinate)–dependent adenosine triphosphate (ATP) synthesis, their respective respiratory rates, and total cellular ATP content were investigated using digitonin permeabilized cybrid cells. Multiple cybrid cell lines were constructed, introducing into osteosarcoma-derived rho0 cells either wild-type or LHON mutant mitochondria carrying each of the 3 common mutations at positions 11778/ND4, 3460/ND1, and 14484/ND6.

Results: All 3 LHON mutations impaired ATP synthesis and the respiratory control ratio driven by complex I substrates. In contrast, succinate-driven ATP synthesis, respiration rates, and respiratory control ratios were not affected. However, the defective ATP synthesis with complex I substrates did not result in reduced ATP cellular content, indicating a compensatory mechanism.

Conclusions: The LHON pathogenic mutations profoundly impair complex I–dependent synthesis of ATP, providing a common biochemical feature that may play a major role in LHON pathogenesis. Stratification of the results by mutation suggests that the 11778/ND4 mutation may induce an uncoupling of cybrid respiration, whereas the other 2 mutations impair the oxygen consumption rate.

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eral, no differences were detectable between cells derived from patients with LHON and cells from unaffected carriers of LHON mutations.6,8,9,11,13

Polarographic assessment of complex I–dependent (pyruvate or glutamate-malate) respiration in isolated mitochondria from muscle,11 lymphoblasts,7,17,18 and LHON cybrids13,17 showed variable impairment with the 11778/ND4 and 3460/ND1 mutations. In the most extended study,17 this impairment was quantified as a 30% to 36% decrease for the 11778/ND4 mutation, as a 20% to 28% decrease for the 3460/ND1 mutation, and as a 10% to 15% decrease for the milder 14484/ND6 mutation. Thus, an apparent discrepancy is observed between spectrophotometric evaluation of complex I–specific activity and complex I–dependent respiration.9,13,17 Moreover, no common feature seems to characterize the 3 most common LHON pathogenic mutations.6

Further investigations9,13,18,19 that assessed the sensitivity of complex I to different specific inhibitors consistently suggested that all 3 LHON mutations interfere with the interaction of complex I with the ubiquinone substrate (coenzyme Q10). These results have been interpreted as an indication that ubiquinone binding may be affected by the LHON mutations and hence that ubiquinonemobile intermediates may have reduced stability.20 At least 3 major downstream consequences may derive from complex I dysfunction in LHON: (1) respiratory function may be disturbed at the level of quinol product release because of impaired electron flow; (2) proton pumping through complex I may be defective and affect energy conservation; and (3) unstable ubisemiquinone radicals may rapidly dismutate, reacting with oxygen to increase reactive oxygen species production.

The first 2 hypothesized mechanisms would possibly result in a decrease in net adenosine triphosphate (ATP) synthesis driven by complex I. This issue has been poorly investigated so far. Direct measurements of ATP synthesis in fibroblasts that carry the 3460/ND1 mutation were reported as normal in 1 study,10 whereas 2 recent studies21,22 documented that osteosarcoma-derived cybrids that carry the 11778/ND4 mutation had an approximately 60% reduction in ATP synthesis with complex I–dependent substrates. Another study23 of the ATP cellular content after extraction with 80% (vol/vol) dimethylsulfoxide reported as normal in 1 study,10 whereas 2 recent studies24,25 showed that adenylate kinase activity may be disturbed in LHON disease conditions.26

In the present study, we investigated ATP synthesis and ATP cellular content in multiple osteosarcoma-derived cybrids obtained from unrelated probands with LHON carrying each of the 3 common LHON pathogenic mutations.

METHODS

CYBRID CELL LINES AND CULTURE CONDITIONS

Cybrid cell lines were constructed using enucleated fibroblasts from 3 control individuals and 6 unrelated probands with LHON as mitochondria donors and the osteosarcoma (143B.TK–)–derived 206 cell line as an acceptor rho0 cell line (the 143B.TK– and rho0 206 cell lines were provided by Giuseppe Attardi, MD, and Michael King, MD). All fibroblast cell lines were established from skin biopsy samples or from umbilical cord specimens after having obtained the informed consent of patients with LHON and controls. Cell fusions of fibroblast-derived cytoplasts (enucleated fibroblasts) with the rho0 206 cells were performed as previously reported elsewhere.24 Definition of the mtDNA haplogroup and identification of the LHON pathogenic mutations were performed using the polymerase chain reaction/restriction fragment length polymorphism method carried out as previously reported elsewhere.24,25 Parental and cybrid cell lines were grown in Dulbecco Modified Eagle Medium supplemented with 10% fetal bovine serum, 2mM levoglucosamine, penicillin G sodium (100 U/mL), streptomycin sulfate (100 µg/mL), and bromodeoxyuridine (0.1 mg/mL).

ATP AND CITRATE SYNTHASE ASSAYS

The ATP synthesis rate was assayed by incubating cells (5 × 106/mL) permeabilized by digitonin exposure according to the method described by Ouhabi et al.27 The reaction was started by adding 20mM succinate (plus 4µM rotenone) or 10mM glutamate–10mM malate (plus 0.6mM malonate) and 0.5mM adenosine diphosphate (ADP). Incubation was conducted for 5 minutes at 30°C, and the reaction was stopped by the addition of 80% (vol/vol) dimethylsulfoxide. The ATP content of the vial was measured using the luciferin-luciferase chemiluminescent method.28 The same method was used to assay cellular ATP content after extraction with 80% (vol/vol) dimethylsulfoxide added to cell samples (0.5–1.0 × 106/mL). Citrate synthase activity was assayed essentially according to the method of Trounce et al29 by incubating cell samples with 0.02% (vol/vol) Triton X-100 and following the reaction spectrophotometrically by measuring the rate of free coenzyme A release.

PROTEIN DETERMINATION AND RESPIRATION MEASUREMENTS

The protein concentration of cybrid samples was assessed using the Lowry colorimetric method in the presence of 0.3% (wt/vol) sodium deoxycholate.30 Bovine serum albumin was used as the standard.

Respiratory rates of digitonin (40 µg/mL)–permeabilized cell samples were measured at 30°C using a Clark-type oxygen electrode as previously reported by Aicardi and Solaini.31 The respiratory control ratio (RCR) (respiration rate in state 3–respiration rate in state 4, where state 3 refers to maximal respiration, ADP induced) was evaluated using either glutamate-malate or succinate as substrate.

STATISTICAL ANALYSIS

The data are presented as mean ± SD. Differences in measurements were evaluated using 1-way analysis of variance followed by the Bonferroni posttest using a statistical software program (SigmaStat; Systat Software Inc, Point Richmond, Calif). A value of P < .05 was considered statistically significant.

RESULTS

CYBRID CELL LINES INVESTIGATED

We established cybrid cell lines from probands with LHON belonging to unrelated families carrying each of the most common pathogenic mutations at positions 11778/ND4, 3460/ND1, and 14484/ND6 (Table 1). Two
cell lines for each mutation were available for experiments, and the cybrid clones used herein are the same as those used in previous studies.14,21,25,26,32 Each cybrid cell line was stably homoplasmic for the LHON pathogenic mutation, as verified by regular checks of the mutation-specific polymerase chain reaction/restriction fragment length polymorphism pattern. Furthermore, LHON and control cybrid cell lines were defined for their mtDNA haplogroup (Table 1).

### ATP SYNTHESIS

We tested the efficiency of oxidative phosphorylation in LHON and control cybrids by assaying the ATP synthase activity driven by complex I (glutamate-malate) and complex II (succinate) substrates. The rate of ATP synthesis was sharply reduced (<10% residue) in cells carrying the 3460/ND1 and 14484/ND6 mutations with nicotinamide adenine dinucleotide–dependent substrates, which implies electron transfer through complex I, whereas a milder reduction (35% residue) was observed with the 11778/ND4 mutation (Figure 1A). When the substrate was succinate, implying electron transfer through complex II, the ATP synthesis rate was not affected in LHON cybrids even if a nonsignificant tendency toward a reduction was observed with the 11778/ND4 and 14484/ND6 mutations (Figure 1A).

The assay of citrate synthase activity in LHON cybrids, used as a well-known marker of mitochondrial mass content, indicated a significant decrease (~21%) for the 11778/ND4 mutation compared with control cybrids (Figure 1B). This finding was somewhat surprising given that slight mitochondrial proliferation has previously been reported in patient-derived tissues, such as platelets,6,8 lymphoblasts,7,18 fibroblasts,10 and skeletal muscle.11 We then corrected the ATP synthesis rate for citrate synthase activity (ATP synthase–citrate synthase activity ratio), confirming the result of dramatic reductions with the glutamate-malate–driven oxidative phosphorylation for all LHON mutations (Figure 1C). The 11778/ND4 mutation remained the least affected in ATP synthesis (~60%). Correcting for citrate synthase activity, the succinate-driven oxidative phosphorylation did not substantially change the previous results (Figure 1C).

### TOTAL CELLULAR ATP CONTENT

To assess the bioenergetics of LHON cybrids, we also determined the cellular ATP content. Figure 2 shows a normal level of ATP in the 11778/ND4 cybrid, whereas the 14484/ND6 and 3460/ND1 cybrids were slightly reduced compared with control cybrids. These reductions did not reach statistical significance (P=.36 for 3460/ND1; P>.99 for 14484/ND6, with respect to controls).

### CYBRID OXYGEN CONSUMPTION RATE

Our results on ATP synthesis driven by complex I–dependent substrates showed a profound defect in LHON cybrids, with the 11778/ND4 mutation being the least affected. This functional defect in oxidative phosphorylation may imply that the enzyme is incapable of either transporting electrons and/or translocating protons through the inner mitochondrial membrane.30 To clarify which of the 2 proposed mechanisms was effective, the
respiration rates of cybrids were measured and then were normalized for citrate synthase activity.

The rate of glutamate–malate–driven respiration in the controlled state (state 4) (Figure 3A) was essentially normal in 3460/ND1 and 14484/ND6 cybrids, whereas in the presence of saturating ADP (state 3) (Figure 3B), respiration rates tended to decrease (–42% and –53% for 3460/ND1 and 14484/ND6 mutations, respectively) (Table 2). In contrast, the 11778/ND4 cybrids showed an opposite trend toward increased glutamate–malate–driven respiration rates (+51%) (Figure 3A) and normal ADP-stimulated respiration rates compared with controls (Figure 3B). However, none of the reported variations reached statistical significance (state 4 respiration rate: P = .99 for 3460/ND1, P = .10 for 11778/ND4, and P = .99 for 14484/ND6; state 3 respiration rate: P = .46 for 3460/ND1, P = .99 for 11778/ND4, and P = .22 for 14484/ND6, with respect to the corresponding controls). The RCR values of the 3 LHON mutant cybrids with glutamate-malate as substrates were all statistically significantly reduced compared with the control cybrids (Figure 3C and Table 2). The RCR faithfully expresses mitochondrial function and hence cellular ATP synthesis capability through oxidative phosphorylation. In our hands, the RCR values reflected the results of ATP synthesis driven by complex I–dependent substrates, with 3460/ND1 and 14484/ND6 cybrids being similarly reduced and the 11778/ND4 cybrid being the least affected.

When the respiratory substrate was succinate, the oxygen consumption rate was barely affected in either state 3 or state 4 respiration (Figure 4A and B and Table 3), and the RCR values seem to be affected only to a minor extent, with no statistical significance (state 4 respiration rate: P > .99 for 3460/ND1, P = .26 for 11778/ND4, and P > .99 for 14484/ND6; state 3 respiration rate: P > .99 for 3460/ND1, P > .99 for 11778/ND4, and P > .99 for 14484/ND6; RCR: P > .99 for 3460/ND1, P = .42 for 11778/ND4, and P = .89 for 14484/ND6, with respect to the corresponding controls (Figure 4C).

**COMMENT**

Expanding on previous results limited to the 11778/ND4 mutation, this study shows that all the common LHON pathogenic mutations severely impair complex I–driven ATP synthesis compared with control cybrids. The reduction was less pronounced with the 11778/ND4 mutation. This impairment was not compensated efficiently by the parallel succinate pathway, as frequently seen in patient-derived tissues or cell lines. In fact, complex II–driven ATP synthesis was essentially unaffected by all 3 LHON mutations. However, total cellular ATP content of LHON cybrids was not significantly decreased. Thus, despite a potentially harmful defective ATP synthesis through oxidative phosphorylation, a metabolic balance seems to be maintained by LHON cybrids in glucose medium, mostly by glycolytic ATP production. This was also confirmed by the lack of mitochondrial proliferation in LHON cybrids, as indicated by normal citrate synthase activity. Variable levels of mitochondrial proliferation are normally seen in patient-derived tissues.
respiration was 20mM succinate, and 4µM rotenone was present to inhibit synthase activity of the corresponding cell samples. The substrate for after normalization of the polarographic oxygen consumption rates to citrate II–dependent substrate. Respiration rates of state 4 and state 3 are reported

Table 2. Glutamate-Malate–Sustained Respiration in Digitonin-Permeabilized Cybrids

<table>
<thead>
<tr>
<th>Respiratory Rate Changes, %</th>
<th>Control, %</th>
<th>3460/ND1</th>
<th>11778/ND4</th>
<th>14484/ND6</th>
</tr>
</thead>
<tbody>
<tr>
<td>State 4</td>
<td>100</td>
<td>+2</td>
<td>+51</td>
<td>+13</td>
</tr>
<tr>
<td>State 3</td>
<td>100</td>
<td>−42</td>
<td>−2</td>
<td>−53</td>
</tr>
<tr>
<td>Respiratory control rate</td>
<td>100</td>
<td>−54*</td>
<td>−42†</td>
<td>−57*</td>
</tr>
</tbody>
</table>

*P<.01.
†P<.05.

To better clarify how the ATP synthesis defect is generated by complex I dysfunction, we also studied respiratory rates using complex I– and complex II–dependent substrates. The respiratory behavior of the 11778/ND4 mutation seems to differ from the other 2 mutations, suggesting a possible uncoupling effect on complex I function. This interpretation is based on the observation that ADP-stimulated respiration (state 3) with the 11778/ND4 mutation is normal, whereas state 4 respiration is significantly enhanced. On the contrary, state 3 respiration was reduced with 3460/ND1 and 14484/ND6 mutations, and state 4 respiration was similar to that of controls. Our results are consistent with those previously reported in a different WAL-2A–derived cybrid cell system. Overall, the best fit between our polaro graphic respiration experiments and the luminometric ATP synthesis rate was observed considering the RCR with complex I–dependent substrates. In fact, all 3 LHON mutant cybrids were significantly reduced compared with controls, with the 11778/ND4 mutation being the least affected, paralleling the ATP synthesis results.

These results obtained in LHON cybrids do not match the severity of the disease in patients and the results of other biochemical measurements. Considering the rate of spontaneous visual recovery in patients with LHON, the phenotypic severity would decrease from the 11778/ND4 > 3460/ND4 > 14484/ND6 mutation. However, considering the impairment of complex I activity, as measured spectrophotometrically in patient-derived tissues, cell lines, or cybrids, the severity would decrease from the 3460/ND1 > 11778/ND4 > 14484/ND6 mutation. Based on the present results, the severity of the biochemical phenotype would decrease from the 14484/ND6 > 3460/ND1 > 11778/ND4 mutation. These latter data match our results on the rate of apoptotic cell death of LHON cybrids when glucose is replaced by galactose in the medium, with the 11778/ND4 mutation being the least severe. In line with these findings, in vivo assessment by phosphorus 31 magnetic resonance spectroscopy of the maximum rate of ATP production in skeletal muscle also revealed a 53% residual rate with the 14484/ND6 mutation compared with controls. In contrast, the 3460/ND1 mutation had a normal rate, whereas the 11778/ND4 mutation was more severely reduced to 27% of controls.

The biochemical effect of LHON pathogenic mutations has been studied for more than a decade and still remains controversial. This study clarifies that the ATP synthesis defect that depends on complex I substrates is severe with all 3 common LHON mutations. However, there is also evidence that the cells may effectively compensate for this impairment in most human tissues, as also happens in our cell system. This leads to the absence of any evident pathologic features in most individuals with the homoplasmic LHON mutation. However, the compensation is probably an unstable equilibrium that may be upset by various factors, such as the frequently suggested nuclear modifying genes and environmental triggers. On the other hand, the rare reports of LHON cases complicated by a syndromic neurologic involvement, such as Leigh disease, multiple sclerosis–like features, cerebellar atrophy, or dystonia associated with basal ganglia lesions, all support the possibility that the energetic failure may spread.
There is increasing evidence that the nuclear gene expression may profoundly modify the pathogenic expression of the LHON mutations. This has been shown in cellular studies\(^6\) and with phosphorus 31 magnetic resonance spectroscopy investigation of different tissues in the same patients carrying the 3460/ND1 mutation.\(^6\) Thus, incongruent results among different studies performed in different cell types or patient-derived tissues may depend on nuclear gene expression. We also noted this feature having assayed the complex I–dependent ATP synthesis of lymphocyte mitochondria from the same patient homoplasmic for the 3460/ND1 mutation used to generate 1 of the cybrid cell lines reported herein (HMM in Table 1; also, patient II-1 in the study by Lodi et al\(^36\)). The reduction amounted to approximately 30% compared with controls, which was a much milder defect than that observed in its cybrids (A.B., G. Solaini, G. Sgarbi, and V.C., unpublished data, 2003).

The present results are not in contrast with the currently most widely accepted hypothesis that besides an ATP defect, overproduction of reactive oxygen species may represent a major element in the pathogenesis of LHON.\(^5,6\) In the long term, bioenergetically compensated cells may experience chronic reactive oxygen species overproduction, and the compromised oxidative phosphorylation in LHON may play a major role once the threshold for cell death is crossed. The present model of apoptotic cell death of LHON cybrids grown in galactose\(^32\) shows a rapid course of cellular ATP depletion, which precipitates a caspase-independent mode of apoptosis.\(^41\) The exact understanding of the reciprocal role of ATP defective synthesis and reactive oxygen species production will be crucial to design appropriate therapeutic approaches, and the current evidence of some differences in the pathophysiological mechanism of LHON mutations may prove to be important for the administration of agents such as coenzyme Q10 or the quinone analog idebenone.

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Table 3. Succinate-Sustained Respiration in DCCP-Permeabilized Cybrids

| State 4 | 100 | +5 | +40 | +2 |
| State 3 | 100 | −5 | +5 | −23 |
| Respiratory control ratio | 100 | −15 | −27 | −19 |

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