Functional Analysis of Lymphoblast and Cybrid Mitochondria Containing the 3460, 11778, or 14484 Leber’s Hereditary Optic Neuropathy Mitochondrial DNA Mutation*

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Leber’s hereditary optic neuropathy (LHON) is a form of blindness caused by mitochondrial DNA (mtDNA) mutations in complex I genes. We report an extensive biochemical analysis of the mitochondrial defects in lymphoblasts and transmitochondrial cybrids harboring the three most common LHON mutations: 3460A, 11778A, and 14484C. Respiration studies revealed that the 3460A mutation reduced the maximal respiration rate 20–28%, the 11778A mutation 30–36%, and the 14484C mutation 10–15%. The respiration defects of the 3460A and 11778A mutations transferred in cybrid experiments linking these defects to the mtDNA. Complex I enzymatic assays revealed that the 3460A mutation resulted in a 79% reduction in specific activity and the 11778A mutation resulted in a 20% reduction, while the 14484C mutation did not affect the complex I activity. The enzyme deficit of the 3460A mutation transferred with the mtDNA in cybrids. Overall, these data support the conclusion that the 3460A and 11778A mutants result in complex I defects and that the 14484C mutation causes a much milder biochemical defect. These studies represent the first direct comparison of oxidative phosphorylation defects among all of the primary LHON mtDNA mutations, thus permitting insight into the underlying pathophysiological mechanism of the disease.

Leber’s hereditary optic neuropathy (LHON) is a maternally inherited disease characterized by midlife acute or subacute, progressive and bilateral central visual loss due to optic nerve atrophy. At least 17 different mtDNA sequence variants have been associated with LHON (1). However, only four mutations at (np) 3460A, 11778A, 14484C, and 14459A are sufficiently strong risk factors for blindness to be designated “primary” mutations. The primary mutations have been observed in multiple unrelated LHON families, very rarely co-occur with each other, and have not been detected in a large number of control mtDNAs (1–5). Together, the 3460A, 11778A, 14484C, and 14459A mutations account for greater than 90% of reported LHON cases. With the exception of the 14459A mutation, which is also associated with early onset, generalized dystonia (5, 6), LHON is typically the primary clinical manifestation of these mutations.

All primary LHON mutations alter mtDNA-encoded intrinsic membrane proteins that contribute to NADH:ubiquinone oxidoreductase (EC 1.6.5.3) or complex I. Complex I is a large, multisubunit enzyme comprised of seven mtDNA-encoded (ND1, -2, -3, -4, -4L, -5, and -6) and roughly 35 nuclear-encoded polypeptides (7). In oxidative phosphorylation (OXPHOS), electrons enter the mitochondrial electron transport chain from NADH + H+ via complex I. In complex I, the electrons transverse a FMN, 5–7 iron-sulfur centers, and ultimately reduce ubiquinone to ubiquinol. From ubiquinol, the electrons are transferred to complex III (ubiquinol:cytochrome c oxidoreductase (EC 1.10.2.2)), then to cytochrome c, next to complex IV (cytochrome c oxidoreductase (EC 1.9.3.1)), and finally to atomic oxygen. Electrons can be donated from succinate to complex II, which then reduces coenzyme Q (CoQ10), bypassing complex I. As electrons transverse complexes I, III, and IV, protons are translocated from the matrix to the inner membrane space, creating an electrochemical gradient (ΔΨ). ΔΨ is utilized by complex V (ATP synthase (EC 3.6.1.34)) to condense ADP and inorganic phosphate to ATP. Thus, the addition of ADP to respiring mitochondria increases the respiration rate, as the ADP is phosphorylated (state III), and returns to the basal rate (state IV) when all of the ADP is phosphorylated. The addition of an agent that collapses ΔΨ uncouples electron transport from ATP synthesis, resulting in an accelerated respiration rate.

Although the clinical and genetic aspects of LHON have been well studied, we know little about the pathophysiological mechanism(s) that results in central vision loss. Previous studies have investigated the biochemical defects associated with the various primary LHON mutations (8–15). However, considerable variability exists in the reported results, making it difficult to draw definitive conclusions concerning the underlying biochemical dysfunction. Such variation is apparent in assay methodologies (methods for mitochondrial isolation, preparation of submitochondrial particles, substrates employed), starting material (whole cell versus isolated mitochondria respiration), and mitochondrial buffering/storage procedures. There is also discontinuity in the patient cell type studied (leukocytes, platelets, skeletal muscle, lymphoblasts, and fibroblasts), and rarely is more than one LHON mutation investigated, making comparative analysis of OXPHOS dysfunction difficult. Finally, some reports have examined insufficient numbers of patients and/or controls to permit a definitive statistical analysis of the results.

To further characterize the OXPHOS defects associated with the LHON mutations, we performed respiration and enzymo-
logical analysis on lymphoblast mitochondria isolated from a large number of LHON patients containing the 3460A, 11778A, or 14484C mutations. Transmitochondrial cybrids derived from patient lymphoblasts were also subjected to biochemical analysis. Significant respiratory defects were found associated with site I substrates for the 3460A and 11778A mutations and a striking complex I enzyme defect for the np 3460A mutation, all of which transferred in cybrid studies. No significant defects were found for the np 14484C mutation. Together with our previous study, these data indicate that the biochemical defect is most severe for the np 14459A and np 3460A mutations, intermediate for the np 11778A mutation, and mildest for the np 14484C mutation.

EXPERIMENTAL PROCEDURES

Cell Lines and Cybrid Construction—All patient cell lines reported here were derived from patients who exhibited typical clinical signs of LHON and had an age of onset in young adulthood (16, 17). Patient lymphoblastoid cell lines were constructed from five independent male np 3460A patients, six male and one female np 11778A patients, and five male and two female 14484C patients. All subjects were Caucasian except for one 14484C patient, who was an African-American female. Control lymphoblast cell lines were established from lymphoblasts of six normal men and six normal women. The age range for the control group was 22–45 years. Twenty lymphoblast controls were used for most respiration studies, 12 of whom were males.

Lymphoblast cell lines were established by EBV transformation of leukocytes isolated from whole blood by Ficol-Hypaque gradients. All EBV-transformed cell lines were maintained in RPMI 1640 medium (Bio-Whitaker, Walkersville, MD) supplemented with 15% (v/v) heat-inactivated fetal bovine serum (Life Technologies, Inc., Grand Island, NY). EBV-transformed lymphoblast cell lines had been maintained in culture for 10–20 population doublings at the time of mitochondrial isolation.

The WAL-2A-p0 line lacks mtDNA and is hypoxanthine-guanine phosphoribosyltransferase-deficient. It is grown in RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum, 4 mg/ml glucose, 50 μg/ml uridine, 1 mM pyruvate (GUP medium) containing 1 μM/ml of 6-thioguanine.

Transmitochondrial cybrids were prepared by nucleating EBV-transformed lymphoblastoid cell lines from LHON patients and controls and fusing 2 × 10^6 mitochondria-containing cytoplasts with the 10^7 WAL-2A-p0 cells by electroporation (18). Cybrids were selected in 25 ml of RPMI 1640 medium supplemented with 10% (v/v) dialyzed fetal bovine serum (Life Technologies, Inc.), 2 mg/ml glucose, and 1 μg/ml 6-thioguanine. Rapid growth of the cybrid cultures was observed 20–28 days postfusion, and cybrid lines were passaged 5–10 population doublings prior to mitochondrial isolation.

mtDNA Analysis—Genomic DNA was isolated from approximately 10^6 EBV-transformed lymphoblasts or cybrids using Chelex 100 (Cetus, Emeryville, CA). The 3460A, 11778A, and 14484C LHON mutations were detected by polymerase chain reaction amplification followed by mutation-specific restriction endonuclease digestion (3). All lymphoblastoid cell lines and cybrid constructs were homoplasmic.

Mitochondrial Isolation, Polarography, and OXPHOS Enzymology—Procedures for mitochondrial isolation, polarographic analysis, and OXPHOS enzymology of submitochondrial particles have been described (6, 18). Protein concentrations were determined by the Lowry assay. Lymphoblast and cybrid mitochondria were assayed at concentrations ranging from 3.5 to 13 mg protein/ml. For respiration analysis of mitochondria, two polarographic runs were performed for each substrate (malate plus pyruvate or malate plus glutamate (site I) and succinate (site II)) involving two additions of ADP (125 nmol for site I and 75 nmol of ADP for site II substrates). The experiment was concluded by the addition of the OXPHOS uncoupler 2,4-dinitrophenol. All runs were performed with 250–500 μg of mitochondrial protein. The respiratory control ratio is the mean state III respiration rate divided by the mean state IV respiration rate. All mitochondrial isolates included in this study had a respiratory control ratio greater than 2 to meet our minimum standard of mitochondrial integrity. The state III ratio is the mean of the state III respiration rates using site I substrates divided by the mean of the state III respiration rates using site II substrates.

OXPHOS enzyme activities were assayed on sonicated mitochondria and measured spectrophotometrically with a dual beam UV-visual spectrophotometer (model DW-2000; SLM-Aminco, Urbana, IL). Complex I activities were assayed in triplicate using 30 μg of mitochondrial protein and monitoring the reduction of 10 μM decylubiquinone at 272 nm by 30 μM NADH. Consistently, 90–100% of the total complex I activity was rotenone-sensitive. Complex II plus III activities were assayed in duplicate with 15 μg of mitochondrial protein by the antimycin A-sensitive reduction of cytochrome c by succinate. Complex III activity was assayed in duplicate using 7.5 μg of mitochondrial protein by the antimycin A-sensitive oxidation of reduced decylubiquinone by cytochrome c. Complex III activity was 75–100% antimycin A-sensitive. Complex IV activities were determined in duplicate using 7.5 μg of mitochondrial protein by the cyanide-sensitive oxidation of cytochrome c. Citrate synthase (CS) was assayed in duplicate, using 15 μg of mitochondrial protein.

Statistical Analysis—p values were calculated by the Mann-Whitney, unpaired, two-tailed test (Instat for Macintosh, version 2.03, Graphpad Software, Inc.).

RESULTS

Polarographic Analysis Reveals Significant Respiration Defects in np 3460 and 11778 Mutant Mitochondria—ADP-stimulated (state III) oxygen consumption rates using the site I/complex I substrates (malate + pyruvate or glutamate) were significantly reduced in patient lymphoblasts, relative to controls, for the np 3460A and np 11778A mutants (Table I, top, left column). The np 3640A mutation reduced the state III rate 23–30%, and the np 11778A mutation reduced state III respiration 27–35%, but the np 14484C mutation reduced respiration only 3–12%. The np 11778A mutation also exhibited a significantly slower uncoupled rate (approximately 70% of controls) using complex I substrates. The state III and uncoupled respiration rates for all three mutations were essentially identical to controls using the complex II substrate, succinate. The mutant ADP/O ratios and state IV rates were nearly equivalent to controls in all cases.

These differences were even more apparent when the state III ratios were calculated. The np 3460A mutation resulted in a 20–28% reduction, the np 11778A mutation a 30–36% reduction, and the np 14484C mutation a 10–15% reduction in the state III ratio, with all three showing significant differences relative to the controls. These results are consistent with the LHON mutations imparting a complex I defect.

The complex I-linked state III respiratory defects seen in the np 3460A and 11778A mitochondria were transferred along with the mtDNA into WAL-2A-p0-derived transmitochondrial cybrids. The cybrid 3460A state III rates were reduced 16–25%, and the state III ratio was reduced 18–22%, which were significantly different from controls (Table I, bottom). The state III ratios were significantly reduced for the np 11778A mutation as well. However, the state III respiration rates and state III ratios of the np 14484C cybrids remained within the normal range. The uncoupled site I respiration rate for the np 3460 mutant was also significantly reduced. Again, the complex II substrate respiration rates of the mutant cybrids were the same as controls.

In a comparison of respiration parameters in control cell lines, transmitochondrial cybrids demonstrated slower respiration rates and lower ADP/O ratios than did the EBV-transformed lymphoblasts. In virtually all comparisons of respiration data between the lymphoblasts and the cybrids, the cybrid cells with the WAL-2A nucleus were 15–40% lower (Table II, top). Normalized respiration rates (state III ratios), however, did not differ between lymphoblast and cybrid controls. Identical results were observed when only those lymphoblasts and cybrids that shared the same mtDNA were compared (Table II, bottom), indicating that the reduced respiratory function in the cybrid nuclear background was probably due to the nuclear genetic background difference instead of statistical sampling error.

OXPHOS Enzyme Analysis Reveals Complex I Defect in np 3460A Mutant Mitochondria—Complexes I, II + III, III, and IV
and CS activities were assayed on mitochondria from np 3460A, 11778A, and 14484C patients and control mitochondria. Only mitochondria from the np 3460A patients showed a significant defect in enzyme specific activity, a 79% complex I deficiency relative to controls (p < 0.0001) (Table III, top). This same defect was apparent when normalized to citrate synthase activity, with the np 3460A mitochondria having a 62% reduction in activity (p = 0.0009). The np 11778A mutant mitochondria were also found to have a slight but significant reduction in complex I activity of 25% when normalized to citrate synthase (p = 0.0137). All other enzyme activities were equal to or exceeded the mean normal values.

The complex I defect of the np 3460A mutation was transferred along with the mtDNA in transmitochondrial cybrids. In the cybrids, the 3460A complex I specific activity was reduced 73% (p = 0.0010) relative to controls, and the citrate synthase normalized activity was 70% less (p = 0.0010) than control activity (Table III, bottom). Hence, the np 3460A mtDNA mutation imparts a striking defect in complex I activity, which is not seen for the np 11778A and 14484C mutation.

The effect of nuclear background on respiration rates was also seen for OXPHOS enzyme specific activities. In virtually every comparison between control cell lines, the mitochondrial enzyme specific activities were reduced 20–30% in the WAL-2A-p53 cybrids relative to the lymphoblastoid cell lines (Table IV, top). Surprisingly, the complex III activity was an exception, being higher in the cybrids than in the lymphoblast cell lines. The same alterations were found when only those lymphoblasts and cybrids that shared the same mitochondria were compared (Table IV, bottom). However, as observed in the
respiration data, citrate synthase-normalized complex I activity was essentially equivalent between the two groups.

**DISCUSSION**

The detailed analysis of 19 different patient lymphoblastoid cell lines as well as patient cybrids demonstrates that both the np 3460A and np 11778A mutations impart a moderate complex I respiration defect and that the np 3460A mutation also imparts a severe complex I enzyme defect. The np 3460A complex I defect is similar to that which we previously observed in mitochondria harboring the np 14459A LHON and dystonia mutation. Interestingly, no statistically significant complex I-linked dysfunction could be detected in patients harboring the np 14484C mutation. Combining our analysis of all LHON lymphoblastoid mitochondria thus indicates that the np 14459A and 3460A mutations impart the most severe reduction of complex I function, with the 11778A mutation showing moderate impairment and the 14484C mutation causing little complex I perturbation (Table V). These findings roughly correlate with the clinical features of the LHON mutations. The 14459A mutation causes the most severe symptoms, including LHON and/or early onset, generalized, dystonia due to basal ganglia degeneration, while the 14484C mutation causes only LHON and has the highest spontaneous visual recovery rate among the LHON mutations (19). Moreover, in roughly 80% of European-derived np 14484C LHON cases, the mutation is associated with a specific mtDNA background, European haplogroup J, which is found in only 10% of the general European population. Thus, expression of the np 14484C mutation appears to be influenced by mtDNA background, unlike the more severe np 14459A and 3460A mutations (20, 21).

These data represent the first report on np 14484C respiration and cybrid analysis, and, considering all LHON mutations, our results correspond well with other functional studies conducted on lymphoblasts (9), platelets (22, 23), fibroblasts (13, 24), and cybrids (25, 26). Moreover, because a large number of patient lymphoblast and cybrid cell lines were examined for each mutation, they tend to clarify the somewhat disparate observations in the literature that may result from differences in experimental methods and materials. One important experimental variable that has been illuminated by the current study is the potential effect of the nuclear background on the expression of the mtDNA defect in cybrids. As seen for both respiration and OXPHOS enzyme specific activity parameters, the mitochondrial values are consistently 20–30% lower in the cybrids with the WAL-2A<sup>p</sup>-derived nucleus than in the parental lymphoblast cell lines. This observation also held true for control lymphoblast-cybrid comparisons, even when the same mitochondria were being studied in lymphoblasts and cybrids (Tables II and IV). Thus, the relationships between any mutation group and controls were generally consistent when making comparisons between the same cell type, but there was a consistent slight reduction in the biochemical activity in cybrids compared with lymphoblasts. For example, the 3460A-associated state III rate using pyruvate plus malate as a substrate was 70% of the control rate in lymphoblasts, while it was 75% of the control rate in the cybrid comparison. For normalized (state III or CS ratios) data, patient OXPHOS defects were generally similar between the patient lymphoblasts and the cybrids, and normalized control data also showed no differences (see last column of Tables II and IV). This phenomenon is probably due to the WAL-2A<sup>p</sup>-derived cybrids having lower mitochondrial OXPHOS activity than lymphoblasts, but this effect is minimized when ratios such as the state III ratio or CS normalized enzyme activities are used. Since an overall reduction in OXPHOS levels for all cybrids would reduce the percentage differences between patient mtDNA defects and controls, it would be expected that subtle differences detected in the initial EBV-transformed lymphoblasts would be lost in the cybrids. Consequently, differences in respiration rates between np 14484C lymphoblasts and control lymphoblasts may be real, although they are not preserved at a statistically significant level in the WAL-2A cybrids. Clearly, considerably more study is required for understanding the role of nuclear background on the expression of mtDNA mutations.

**The Pathophysiology of LHON**—Given that it is now confirmed that all of the primary LHON mutations affect complex I, what might we conclude about the pathophysiology of LHON? The most severe LHON mutation is the np 14459A mutation in the ND6 gene, which can cause both LHON and dystonia. This mutation causes a severe complex I enzyme defect in lymphoblasts (6) and in skeletal muscle (27) but only a minimal respiratory defect. Extensive studies on the kinetics of mutant enzyme revealed that it had a reduced V<sub>max</sub> but normal K<sub>m</sub> and that the enzyme activity increased with the concentration of the artificial CoQ electron acceptor until about 5 mM, after which activity markedly declined. The enzyme also showed a strong product inhibition with increasing concentrations of reduced decylubiquinone (6). Thus, the ND6 np 14459A mutation appears to alter the interaction between complex I and CoQ<sub>9</sub>.

The biochemical defect of the np 3460A mutation in the ND1 gene is similar to that of the np 14459A mutations. Both mutations cause a severe complex I enzyme defect. The np 3460A mutation also imparts a mild respiratory defect. Biochemical studies by others have suggested that the np 3460A mutation might also affect the ability of complex I to interact

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**Table III**

| LHON mutation | Number of cell lines | Mean specific activity | Complex I/CS ratio |
|---------------|----------------------|-----------------------|--------------------|
|               |                      | Complex I             | Complex II + III   | Complex III        | Complex IV | CS |
| Lymphoblasts  |                      |                       |                    |                    |            |    |
| 3460A         | 5                    | 11 ± 9<sup>a</sup>    | 308 ± 138          | 539 ± 64           | 668 ± 158  | 486 ± 174 | 0.03 ± 0.02 |
| 11778A        | 7                    | 43 ± 18               | 301 ± 137          | 508 ± 87           | 825 ± 164  | 690 ± 177 | 0.06 ± 0.02 |
| 14484C        | 7                    | 50 ± 24               | 409 ± 207          | 536 ± 58           | 714 ± 261  | 701 ± 327 | 0.07 ± 0.03 |
| Control       | 22                   | 53 ± 20               | 311 ± 91           | 481 ± 101          | 596 ± 250  | 599 ± 177 | 0.08 ± 0.02 |
| Cybrids       |                      |                       |                    |                    |            |    |
| 3460A         | 5                    | 12 ± 6<sup>a</sup>    | 238 ± 100          | 695 ± 125          | 589 ± 141  | 488 ± 146 | 0.03 ± 0.01 |
| 11778A        | 7                    | 46 ± 20               | 398 ± 264          | 681 ± 67           | 574 ± 230  | 565 ± 203 | 0.09 ± 0.04 |
| 14484C        | 7                    | 61 ± 19               | 407 ± 179          | 690 ± 141          | 605 ± 129  | 568 ± 198 | 0.10 ± 0.04 |
| Control       | 9                    | 44 ± 14               | 209 ± 71           | 711 ± 197          | 455 ± 75   | 471 ± 176 | 0.10 ± 0.03 |

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*a Statistical significance (p < 0.05) by the two-tailed, Mann-Whitney U test for unpaired samples. Statistical significance, with the exception of the cybrid complex II + III/CS (for 11778 mutation group only) ratio, was not reached for any mutation group when complex II + III, III, or IV activities were normalized using CS activity.*
with CoQ\textsubscript{10} (11, 12). Indeed, kinetic analysis using ubiquinone analogs suggested that the mutant enzyme was subject to substrate inhibition (11). Hence, it is possible that both the np 14459A and np 3640A mutations might interfere with CoQ\textsubscript{10} binding.

The np 11778A mutation in the ND\textsubscript{4} gene generally results in isolated LHON. Only rarely is it also associated with neurodegenerative disease such as dystonia. While the np 11778A mutation does not impart a strong complex I enzyme defect, it does cause a reproducible respiration defect with complex I-linked substrates. Thus, the combined complex I enzyme defect and respiration defect of the np 3460A mutation forms a bridge between the complex I enzyme defect of the np 14459A mutation and the respiration defect of the np 11778A mutation. The complex I respiration defect and absence of enzyme defect have led one group to propose that the np 11778A mutation impedes the interaction between complex I and NAD-linked matrix dehydrogenases (11). However, other biochemical studies have demonstrated perturbed interactions between ubiquinone (or rotenone) and the mutant complex I, which has led to the proposal that this enzyme interacts inefficiently with CoQ\textsubscript{10}, possibly resulting in the destabilization of ubisemiquinone and increased generation of reactive oxygen species (10, 12). Hence, it is possible that both the np 14459A and np 3640A mutations might interfere with CoQ\textsubscript{10} reduction by complex I.

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Functional Analysis of LHON mtDNA Mutations

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