Phosphorus MR spectroscopy shows a tissue specific in vivo distribution of biochemical expression of the G3460A mutation in Leber’s hereditary optic neuropathy

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Occipital lobe and calf muscle energy metabolism were studied in vivo by magnetic resonance spectroscopy (31P-MRS) in four members of a family harbouring the mitochondrial DNA G3460A mutation causing Leber’s hereditary optic neuropathy (LHON). Three siblings carried 100% mutated mitochondrial DNA (homoplasy), while their mother had coexistence of mutated and wild-type mitochondrial DNA (heteroplasm). Indices of brain energy metabolism on 31P-MRS were abnormal in all subjects examined, but the muscle oxidative phosphorylation rate was normal. These findings indicate a tissue specific distribution of the biochemical expression of the G3460A LHON mutation and suggest that extramitochondrial factors, such as nuclear genes, may influence expression of this mutation in vivo.

Leber’s hereditary optic neuropathy (LHON), the commonest cause of isolated blindness in young men, is a maternally inherited form of bilateral acute or subacute loss of central vision associated with retinal ganglion cells and optic nerve degeneration. A mitochondrial DNA point mutation at one of three nucleotide positions (11778, 14484, or 3460) is found in the majority of LHON pedigrees. All three mutations affect genes coding for different subunits of complex I of the mitochondrial respiratory chain. The G3460A mutation affects the ND1 subunit and is found in 4–19% of all complex I of the mitochondrial respiratory chain. The G3460A mutations affect genes coding for different subunits of complex I and result in a 60–80% reduction in complex I specific activity in a single LHON patient.

Methods

Patients

We studied four individuals (I-2, II-1, II-2, and II-3) from a family harbouring the G3460A mutation previously shown to have a reduced complex I activity in platelets (family 4 in Carelli et al). The proband (II-1) suffered typical LHON at 18 years of age. His visual acuity is now reduced to counting fingers in both eyes, with severe optic atrophy. His twin sisters (II-2, II-3) never complained of any visual disturbance. The proband’s mother never complained of visual loss, nor shows optic atrophy on fundoscopic examination. A proband’s maternal aunt, now deceased, also suffered LHON in her twenties.

Informed consent was obtained from each patient and from normal volunteers (see below), and the studies were carried out with the approval of the local hospital ethics committee.

Molecular genetic studies

Total DNA was extracted from multiple tissues (whole blood, leucocyte or platelet enriched pellets, urinary epithelium, and liver biopsy, as shown in table 1) by a standard phenol-chloroform method. Heteroplasmy of mitochondrial DNA was evaluated by polymerase chain reaction (PCR) amplification of a convenient mitochondrial DNA fragment encompassing the G3460A mutation, followed by restriction fragment

### Table 1  Mutation load and brain phosphorous magnetic resonance (MR) spectroscopy data

<table>
<thead>
<tr>
<th>Case</th>
<th>Age/sex</th>
<th>Whole blood</th>
<th>Platelets</th>
<th>Leucocytes</th>
<th>Liver biopsy</th>
<th>Urinary epithelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-2</td>
<td>56/F</td>
<td>56</td>
<td>24</td>
<td>36</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>I-1</td>
<td>33/M</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>I-2</td>
<td>25/F</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>I-3</td>
<td>25/F</td>
<td>100</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>100</td>
</tr>
<tr>
<td>Controls (n=26)</td>
<td>Mean (SD)</td>
<td>[PCr] (mM)</td>
<td>[Pi] (mM)</td>
<td>[ADP] (µM)</td>
<td>AW (mM²)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.30</td>
<td>1.30</td>
<td>45</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.03</td>
<td>1.30</td>
<td>45</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.78</td>
<td>1.50</td>
<td>35</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.63</td>
<td>1.48</td>
<td>33</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>4.39 (0.25)</td>
<td>1.31 (0.13)</td>
<td>29 (2)</td>
<td>80 (6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>[3.97 to 4.78]</td>
<td>[1.02 to 1.47]</td>
<td>(24 to 33)</td>
<td>(71 to 98)</td>
<td></td>
</tr>
</tbody>
</table>

no, not available; PCr, phosphocreatine; Pi, inorganic phosphate; PP, phosphorylation potential (=ATP/ADP-Pi).
length polymorphism (RFLP) analysis after digestion with the restriction endonuclease BsaHI.

**Phosphorus MR spectroscopy**

"P-MRS was carried out in a whole body GE 1.5 Tesla Signa system using a 7.5 cm diameter surface coil.

Brain "P-MRS was done on the occipital lobes by positioning a surface coil on the occipital region after imaging the brain, as previously described. The DRESS (depth resolved surface coil spectroscopy) localisation technique was used to avoid any contributions to the signal from neck muscles, skin, and other interposed tissues. On the basis of proton sagittal images of the brain, a 2.5 mm thick coronal slice was selected and the profile of the sensitivity volume of the 7.5 cm diameter coil was routinely overlapped to the images to avoid any contributions to the signal from neck muscles, skin, and other interposed tissues (fig 1A). The resulting volume of interest was selected by the DRESS localisation technique for the occipital region and the profile of the sensitivity volume of the 7.5 cm diameter coil was routinely overlapped to the images to avoid any contributions to the signal from neck muscles, skin, and other interposed tissues. On the basis of proton sagittal images of the brain, a 2.5 mm thick coronal slice was selected and the profile of the sensitivity volume of the 7.5 cm diameter coil was routinely overlapped to the images to avoid any contributions to the signal from neck muscles, skin, and other interposed tissues (fig 1A). The resulting volume of interest can be estimated to be about 90 cm$^3$. The repetition time was 5 s. The flip angle in the selected volume was approximately 30°, and it was not necessary to introduce any correction for saturation effects caused by repetition time. To obtain a signal to noise ratio of 9 to 12 for β-ATP 400 free induction decays were accumulated. A computerised curve fitting program was used to quantify the individual peaks of the spectrum.

Phosphocreatine (PCr) and inorganic phosphate (Pi) concentrations were calculated by assuming a cytosolic [ATP] of 3 mM. In order to determine the phosphorylation potential (PP = [ATP] / ([ADP] × [Pi])), a global index of readily available free energy in the cell, free cytosolic ADP concentration was calculated from the creatine kinase equilibrium.

Muscle "P-MRS was performed on the right calf muscle by the pulse and acquire technique (repetition time of five seconds) at rest, during in-magnet aerobic incremental exercise of plantar flexion as previously described. Postexercise spectra were collected for five minutes, with a time resolution of 10 seconds during the first 60 seconds and 30 seconds thereafter. The limits of all the peaks were marked manually on each spectrum after phasing, and areas calculated between the limits. Muscle mitochondrial ATP production was assessed by measuring the rate of entirely oxidative PCR resynthesis during recovery. The rate of PCR resynthesis was calculated from the monoeponential equation best fitting the experimental points, and reported as time constants. Intracellular pH was calculated from the chemical shift of Pi relative to PCr.

Control subjects were healthy volunteers: n 36 (mean (SD) age 37 (17) years) for brain studies, and n 35 (38 (16) years) for skeletal muscle studies. Data are presented as mean (SD). Individual results were taken as abnormal when they fell outside the normal range.

**RESULTS**

All tissues examined were homoplasmic for the G3460A mutation in the index case (II-1) and in his twin sisters (II-2 and II-3), but their mother (I-2) showed a variable degree of heteroplasmacy in the examined tissues (table 1). Brain indices of mitochondrial function such as [PCr], free [ADP], and PP concentration were abnormal in all individuals carrying the G3460A mtDNA mutation (table 1, fig 1B). Occipital lobe [Pi] was within the normal range only in case I-2. We do not know the load of mutated mitochondrial DNA in the brain of case I-2. However, the mild degree of brain bioenergetic impairment found in case I-2 compared with her offspring suggests the presence of some degree of heteroplasmacy in her brain, which in turn would modulate the extent of the energy metabolism deficit (table 1). The rate of PCR postexercise resynthesis was within normal range in all four subjects carrying the G3460A mutation (fig 2).

**DISCUSSION**

Our observations indicate that the biochemical expression of the G3460A mitochondrial DNA mutation in vivo is tissue specific. We found a severe defect of brain bioenergetics in both heteroplasmic and homoplasmic individuals carrying the
G3460A mitochondrial DNA mutation, but we could not demonstrate an impairment of skeletal muscle mitochondrial oxidative metabolism in any subject. All subjects apart from case I-2 were homozygous for the G3460A mitochondrial DNA mutation. Muscle biopsy was not performed in these LHON patients and we do not know the level of mutated mitochondrial DNA in their skeletal muscle. However, at least for the three siblings in the second generation there is consistent evidence that they are homoplasmic mutant in all tissues examined, suggesting that the same probably also applies to skeletal muscle or any other tissue. Thus it seems very unlikely that normal muscle ATP production in these cases could be related to heteroplasmic mitochondrial DNA mutation. The proband’s mother showed heteroplasmic mitochondrial DNA in blood cells and—considering the rarity of skewed segregation among tissues in LHON—it is conceivable that a fairly similar mutant load would be present in her skeletal muscle.

There is a complex relation between mitochondrial DNA mutations, respiratory chain enzyme specific activity, and the rate of mitochondrial ATP synthesis. The role played by nuclear background has recently been underscored by cybrid cell studies. The expression of defective complex I specific activity associated with the G3460A mutation was modulated by changing the nuclear background of the cybrid cell system used in one study. Similarly, the coexpression of respiratory function between LHON lymphoblast cell lines and lymphoblast derived cybrids harbouring each of the three LHON pathogenic mutations showed differences related to the change in nuclear background. These observations also seem to apply to mitochondrial DNA mutations in TRNA genes.

Previous in vivo 31P-MRS studies have shown that the rate of mitochondrial ATP production is severely impaired in the skeletal muscle of subjects carrying the G11778A LHON mitochondrial DNA mutation. The proband’s mother showed heteroplasmic mitochondrial DNA in their skeletal muscle. However, at least for the three siblings in the second generation there is skewing segregation among tissues in LHON—i.e. it is conceivable that a fairly similar mutant load would be present in her skeletal muscle.

The relation between primary LHON mutations and complex I dysfunction is still debated, and various factors of both mitochondrial and nuclear origin, may play a role in their biochemical expression in vivo.

We provided in vivo evidence of a tissue specific distribution of the biochemical expression of the G3460A LHON mutation which is associated with mitochondrial dysfunction in the occipital lobes, but normal oxidative metabolism in an extraneural tissue (skeletal muscle). This finding—and those of another recent in vivo study investigating the A3243G mitochondrial DNA mutation—supports the role of the nuclear genetic environment in the biochemical expression of mitochondrial DNA mutations in vivo. Our observations may be important in understanding the molecular mechanisms responsible for the variable penetrance of LHON, and for the fact that its clinical expression is highly restricted to retinal ganglion cells and the optic nerve; it appears that these tissues may express the oxidative phosphorylation deficit produced by specific mitochondrial DNA mutations more severely than other tissues.

ACKNOWLEDGEMENTS
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