‘Secondary’ 4216/ND1 and 13708/ND5 Leber’s hereditary optic neuropathy mitochondrial DNA mutations do not further impair in vivo mitochondrial oxidative metabolism when associated with the 11778/ND4 mitochondrial DNA mutation

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Summary
The pathogenic role of ‘secondary’ mitochondrial DNA (mtDNA) point mutations, when occurring in patients with Leber’s hereditary optic neuropathy (LHON) in association with ‘primary’ mutations, is still controversial. We used phosphorus magnetic resonance spectroscopy to establish whether two of these ‘secondary’ LHON mtDNA mutations, 4216/ND1 and 13708/ND5 (haplogroup J), further affect in vivo mitochondrial oxidative metabolism in subjects with the ‘primary’ 11778/ND4 mtDNA mutation. Brain and skeletal muscle energy metabolism was assessed in 10 subjects homoplasmic for the 11778/ND4 mtDNA mutation and 10 subjects homoplasmic for the same mutation occurring on the haplogroup J mtDNA background. Brain phosphocreatine concentration and phosphorylation potential were significantly reduced and brain inorganic phosphate concentration was significantly increased compared with controls in both groups of 11778/ND4-positive subjects. The degree of reduction in the phosphocreatine concentration and phosphorylation potential and of increase in the inorganic phosphate concentration was, however, similar in the two groups with the 11778/ND4 mtDNA mutation with or without the haplogroup J. Similarly, the rate of muscle phosphocreatine resynthesis after exercise, a sensitive index of the rate of mitochondrial ATP production, was reduced by the same extent in both groups of LHON subjects. This in vivo study does not support synergism of the 4216/ND1 and 13708/ND5 ‘secondary’ mutations with the 11778/ND4 ‘primary’ mutation in determining the deficit of energy metabolism in LHON.

Keywords: LHON; MR spectroscopy; in vivo metabolism; brain and muscle bioenergetics

Abbreviations: FID = free induction decay; LHON = Leber’s hereditary optic neuropathy; PCr = phosphocreatine; Pi = inorganic phosphate; 31P-MRS = 31P magnetic resonance spectroscopy

Introduction
Leber’s hereditary optic neuropathy (LHON) is a maternally inherited disease characterized by acute or subacute visual loss, with variable recovery, and associated with the degeneration of the retinal ganglion cell layer and optic nerve. It most commonly affects young adult males and is associated with a number of point mutations of mtDNA (mitochondrial DNA) (Riordan-Eva and Harding, 1995). A pathogenic role has been established for the so-called primary point mutations, which are found in the majority of LHON pedigrees (Harding et al., 1995; Riordan-Eva et al., 1995) and affect one of the three nucleotide positions 11778 (Wallace et al., 1988), 14484 (Mackey and Howell, 1992; Johns et al., 1993) and 3460 (Howell et al., 1991; Huoponen et al., 1991) in genes coding for the ND4, ND6 and ND1 genes. A secondary role has been suggested for some other mutations, such as 3460/ND4, 5062/ND1 and 5178/ND2. The pathogenic role of the so-called secondary mutations is still controversial. The ‘primary’ mutations are associated with a number of secondary mutations, which are often found in patients with LHON. The effects of these secondary mutations on mitochondrial function are still unclear. The aim of this study was to investigate the in vivo effects of two secondary mutations, 4216/ND1 and 13708/ND5 (haplogroup J), on mitochondrial energy metabolism in subjects with the ‘primary’ 11778/ND4 mtDNA mutation.

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The identification of heteroplasmic individuals, had a high penetrance (seven affected and six unaffected). Both of these families with the 11778/ND4 mutation took part in the haplotype and phylogenetic study of LHON families in Italy (Torrioni et al., 1997): Family 1 was found to carry the haplogroup U and Family 2 was classified as haplogroup K. We also studied 10 subjects from a third family (three males, age 36 ± 11 years, one affected male) carrying the 11778/ND4 plus the two ‘secondary’ 4216/ND1 and 13708/ND5 mtDNA mutations. This family (Family 3) had five affected subjects (four males and one female) and 11 maternally related, unaffected individuals in the last three generations (Vergani et al., 1995). All the affected individuals were in the high-penetrance, older generation (five affected and two unaffected). Unfortunately, we were able to study only one clinically affected member of this pedigree by 31P-MRS because the other patients had magnetic clips in their brains after surgery for opticochiasmatic arachnoiditis at the time of their visual loss. All the subjects from this pedigree were found to be homoplasmic and to carry the haplogroup J (Torrioni et al., 1997).

All 20 subjects (patients and unaffected individuals) investigated in this study were selected because they were homoplasmic mutant in both leucocytes and platelets and were negative for the other LHON mutations that have been described (3460/ND1, 14484/ND6, 4160/ND1, 5244/ND2, 7444/COI, 3394/ND1, 4917/ND2, 15257/Cytb, 15812/Cytb). Clinically, the six affected patients investigated in this study showed similar reduced visual acuity as a consequence of acute or subacute visual loss. The age at onset was 24–41 years for the five patients carrying only the 11778/ND4 mtDNA mutation and 33 years for the one patient who also carried the two ‘secondary’ mutations, 4216/ND1 and 13708/ND5.

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

Methods

Patients

We studied 10 subjects (including four males and five affected subjects) from two unrelated families carrying only the 11778/ND4 mtDNA mutation (age 44 ± 14 years, mean ± standard deviation). Seven of them (four males, three affected males) were from a first family (Family 1) (Cortelli et al., 1991). This family had three affected males and 18 maternally related unaffected individuals in the last four generations. The low penetrance in this family may be influenced partially by the presence of heteroplasmic individuals in the maternal line (Barboni et al., 1992). The remaining three subjects (three females, two of them affected) were from a second family (Family 2) (Family 3 in Carelli et al., 1997). This family had eight affected subjects (four males and four females) and 13 maternally related, unaffected individuals in the last two generations. All these subjects are distributed in two branches of the genealogical tree, one of which, despite the other interposed tissues from contributing to the signal. The repetition time was 5 s. The flip angle in the selected volume was ~30°, and it was not necessary to introduce any correction for saturation effects due to repetition time. Four hundred free-induction decays (FIDs) were accumulated so as to have...
a signal-to-noise ratio of 9–12 for β-ATP. A computerized curve-fitting program was used to quantify the individual peaks of the spectrum. By assuming a cytosolic ATP concentration of 3 mM (Bottomly and Hardy, 1989), we calculated the concentrations of phosphocreatine (PCr) and inorganic phosphate (Pi). In order to determine the phosphorylation potential ([ATP]/([ADP] × [Pi]), a global index of readily available free energy in the cell (Veech et al., 1979), free cytosolic ADP concentration was calculated from the creatine kinase reaction (Degani et al., 1987).

Muscle 31P-MRS was performed on the right calf muscle by the pulse-and-acquire technique (repetition time 5 s) at rest, during in-magnet aerobic isokinetic exercise (Iotti et al., 1993) and during recovery from exercise. Sixty FIDs were accumulated during rest (5 min); then exercise was begun and data were collected for 1 minute (12 FIDs) for each level of work. As soon as the last minute of work had been completed and the corresponding 12 FIDs had been recorded, one 2-FIDs data block (10 s) was also recorded and was considered zero time, and the exercise was stopped immediately afterwards. During post-exercise recovery, 10 s data blocks (2 FIDs) were recorded during the first 60 s, and 30 s data blocks were recorded thereafter for another 4 minutes. The limits of all the peaks were marked manually on each spectrum after phasing, and areas between the limits were calculated (Iotti et al., 1993).

The rate of mitochondrial ATP production by muscle was assessed by measuring the rate of PCr resynthesis during recovery, which is entirely oxidative (Taylor et al., 1983; Arnold et al., 1984). The rate of PCr resynthesis was calculated from the monoexponential equation best fitting the experimental points of recovery, and was reported as time constants. In view of the influence of the minimum cytosolic pH, reached during recovery, on the PCr recovery rate, all time constants were normalized to a cytosolic pH of 7.00 (Iotti et al., 1993).

![Fig. 1 31P-MR spectra (400 FIDs) from occipital lobes of a healthy volunteer (left), a symptom-free subject carrying the 11778/ND4 mtDNA mutation (centre) and a symptom-free subject carrying the 11778/ND4 + 4216/ND1 and 13708/ND5 mtDNA mutations (right). The phosphomonoester peak is located to the left of the Pi peak; the phosphodiester peak is located between the Pi and PCr peaks. The abscissa shows the chemical shift in parts per million (p.p.m.) and the ordinate the relative intensity.](image)

### Table 1 31P-MRS data from occipital lobes of subjects carrying LHON mtDNA mutations and control subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>[PCr] (mM)</th>
<th>[Pi] (mM)</th>
<th>pH</th>
<th>PP (mM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) 11778/ND4 mutation (n = 10)</td>
<td>3.43 ± 0.37**</td>
<td>1.55 ± 0.14**</td>
<td>7.03 ± 0.01</td>
<td>46 ± 10**</td>
</tr>
<tr>
<td>(B) 11778/ND4 + 4216/ND1 and 13708/ND5 mutations (n = 10)</td>
<td>3.32 ± 0.45**</td>
<td>1.46 ± 0.13*</td>
<td>7.03 ± 0.02</td>
<td>47 ± 12**</td>
</tr>
<tr>
<td>Controls (n = 36)</td>
<td>4.39 ± 0.25</td>
<td>1.31 ± 0.13</td>
<td>7.02 ± 0.02</td>
<td>80 ± 6</td>
</tr>
<tr>
<td>P value, group (A) versus (B)</td>
<td>0.5</td>
<td>0.1</td>
<td>0.9</td>
<td>0.7</td>
</tr>
</tbody>
</table>

PP = phosphorylation potential. Data are expressed as mean ± standard deviation. *P < 0.01; **P < 0.001 versus controls.
Intracellular pH was calculated from the chemical shift of Pi relative to PCr (Petroff et al., 1985). The chemical shift was determined carefully from the centroid of the PCr peak to the centroid of the Pi peak.

**Control data**

Control subjects were healthy volunteers: there were 36 (age 37 ± 17 years) for brain studies and 35 (age 38 ± 16 years) for skeletal muscle studies.

**Statistics**

Data are presented as mean ± standard deviation. Statistical analysis was performed with Student’s t-test for unpaired data, and P < 0.05 was taken to be significant.

**Results**

Figure 1 shows typical spectra of occipital lobes from two symptom-free subjects, one of whom was carrying the ‘primary’ 11778/ND4 mtDNA mutation and the other the 11778/ND4 mutation in association with the ‘secondary’ 4216/ND1 and 13708/ND5 mtDNA mutations (haplogroup J), and from a sex- and age-matched control. In the two unaffected subjects carrying the LHON mutations, the phosphocreatine (PCr) peak was decreased to the same extent. Brain 31P-MRS data are reported in Table 1 and Fig. 2. The PCr concentration was significantly lower than in controls in both groups of 11778/ND4-positive LHON subjects, and it did not differ significantly between the two groups with the 11778/ND4 mtDNA mutation with or without haplogroup J. Similarly, the Pi concentration, compared with controls, showed a similar and significant increase in both groups of J-negative and J-associated 11778/ND4-positive subjects. Mean brain phosphorylation potential was reduced to the same extent in the J-negative and J-positive subjects with the 11778/ND4 mutation. Cytosolic pH was similar in both groups of 11778/ND4-positive subjects and did not differ from the value in controls.

The concentrations of PCr and Pi in skeletal muscle at rest in the two LHON groups were similar to control values, and there was no difference between the two patient groups (data not shown). The rate of ATP production in skeletal muscle mitochondria was assessed from the post-exercise PCr recovery rate after in-magnet exercise. The time constant of post-exercise recovery of PCr in the J-negative and J-associated LHON groups (34.5 ± 11.9 and 33.7 ± 10.7 s, respectively) was significantly higher than in the controls (17.4 ± 4.0 s; P < 0.001 for both groups), but no difference was found between the two LHON groups (P = 1) (Fig. 2D).

Values in the brains of five asymptomatic J-negative 11778/ND4 individuals for PCr (3.59 ± 0.20 mM) and Pi (1.55 ± 0.18 mM) concentrations and phosphorylation potential (47 ± 11 mM−1) were not significantly different from those found in the nine asymptomatic 11778/ND4 individuals with a J haplotype background (3.35 ± 0.47, P = 0.3; 1.47 ± 0.13, P = 0.3; 47 ± 13, P = 0.9; respectively). Similarly, the only 17788/ND4 J-positive LHON patient showed brain PCr (3.06 mM) and Pi (1.40 mM) concentrations and phosphorylation potential (51 mM−1) within the range of those found in the five J-negative 11778/ND4 LHON patients (2.87–3.87 mM, 1.37–1.65 mM and 34–56 mM−1, respectively). The recovery rate of muscle phosphocreatine after exercise in asymptomatic and symptomatic subjects with the J-associated 11778/ND4...
mutation was not significantly different from that found in J-negative 11778/ND4 asymptomatic and symptomatic subjects, respectively (data not shown). This clearly indicates that the similarity in brain and muscle energy metabolism found between J-positive and J-negative subjects is not due to the different number of symptomatic or asymptomatic subjects in the two groups.

No age-dependence was found for any 31P-MRS variable in 11778/ND4-positive individuals (data not shown).

Discussion

Impairment of mitochondrial energy production is an effect common to the three 'primary' mtDNA mutations, 11778/ND4, 14484/ND6 and 3460/ND1, that are pathogenically associated with LHON (Howell et al., 1991; Majander et al., 1991; Barbierioli et al., 1995; Lodi et al., 1997; Carelli et al., 1999). Our results show that in the individuals carrying the 11778/ND4 mtDNA mutation, the 'secondary' 4216/ND1 and 13708/ND5 mtDNA mutations (haplogroup J) do not further impair brain and muscle mitochondrial oxidative metabolism, as assessed in vivo by 31P-MRS.

The 4216/ND1 and 13708/ND5 mtDNA base-pair substitutions have been implicated in the expression of LHON on the basis of the much more frequent occurrence of this haplogroup in subjects carrying the 14484/ND6 and 11778/ND4 mutations (Brown et al., 1997; Torroni et al., 1997). However, haplogroup J does not alter the clinical severity of the disease when it occurs in association with a 'primary' LHON mutation (Oostra et al., 1994; Nikoskelainen et al., 1996), and the role of haplogroup J in increasing penetrance and the risk of disease expression for the 'primary' mutation is still controversial (Nikoskelainen et al., 1996; Howell, 1998). Indeed, the presence of haplogroup J did not influence the expression of the 11778/ND4 mutation in the pedigree we studied compared with the other two pedigrees, which had only the 11778/ND4 mutation. Cybrid cell lines carrying the 11778/ND4 mutation and haplogroup J showed lower oxygen consumption and a longer doubling time than cell lines carrying the 11778/ND4 mutation alone (Vergani et al., 1995). However, these preliminary results were obtained from a limited number of clones carrying the 11778/ND4 mutation plus haplogroup J from a single patient, and more extensive investigations are in progress to confirm these findings.

Typically, in vivo 31P-MRS shows abnormalities in the concentration of high-energy phosphates in the brain of patients carrying mtDNA mutations and, in particular, the concentration of PCr is reduced and that of Pi tends to be increased (Barbiroli et al., 1993; Lodi et al., 1994). The cytosolic PCr concentration is an expression of the readily available free energy in the cell, and is thus a very sensitive index of mitochondrial function in steady-state conditions. The lower the concentration of PCr, the smaller the energy reserve available in the cell. The concentration of Pi is incorporated in the calculation of the phosphorylation potential, which is a global index of the bioenergetic state of the cell. No significantly lower PCr or higher Pi concentrations in the brain or lower phosphorylation potential was found in the presence of the two 'secondary' mtDNA mutations, 4216/ND1 and 13708/ND5, indicating that these had no additive effect on oxidative phosphorylation (Table 1).

The absence of any additive effect of haplogroup J on in vivo oxidative metabolism was confirmed in an extraneuronal tissue, skeletal muscle. The rate of muscle mitochondrial ATP production was estimated from the rate of PCr resynthesis after exercise, a very sensitive index of mitochondrial function (Barbiroli et al., 1995; Lodi et al., 1997; Chinnery et al., 2000). The recovery rate of PCr was significantly slower in both groups of J-positive and J-negative 11778/ND4 subjects than in controls. However, haplotype J did not further impair the rate of recovery of PCr as the groups of J-negative and J-positive subjects showed virtually the same mean PCr recovery rate (Fig. 2).

We do not know if any of our control subjects were carrying haplogroup J, or if such haplogroup had an influence on in vivo mitochondrial oxidative metabolism in our healthy subjects. However, studies evaluating mitochondrial respiratory function in cybrid cell lines obtained from normal subjects with and without haplogroup J did not display any difference in respiratory capacity between the cybrid cell lines investigated (Martinuzzi et al., 1999). Moreover, complex I activity assessed in platelets from healthy subjects carrying haplogroup J did not differ from that found in healthy subjects without haplogroup J (Carelli et al., 1999). These findings and the results of the present study suggest that the 4216/ND1 and 13708/ND5 mtDNA base-pair substitutions do not exert any effect on mitochondrial phosphorylation, either in the presence of a normal mtDNA background or in association with the 11778/ND4 mutation, at least under the experimental conditions described here.

In conclusion, our study does not support a synergistic effect of haplogroup J and the 11778/ND4 mutation on energy metabolism deficiency in LHON. However, this finding does not rule out a role of haplogroup J in LHON, and further studies are needed to investigate the interaction of haplogroup J with nuclear genes and environmental factors.

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