α -Tocopherol/lipid ratio in blood is decreased in patients with Leber's hereditary optic neuropathy and asymptomatic carriers of the 11778 mtDNA mutation

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Abstract

Objectives—Leber's hereditary optic neuropathy (LHON) is a maternally inherited disease characterised by acute or subacute bilateral visual loss in young patients. The primary aetiological event is a mutation in the mitochondrial genome (mtDNA) affecting in most cases mtDNA-encoded subunits of the respiratory chain NADH: coenzyme Q oxidoreductase (complex I). The impaired function of complex I leads to a decline in mitochondrial energy production and enhances free radical generation.

Methods—The concentrations of some non-enzymatic antioxidants (α -tocopherol, β -carotene, lycopene, glutathione, free sulphhydryl groups) and the lipid peroxides in the blood of patients with LHON, carriers with homoplasmic DNA mutation at 11778, and controls were investigated using high performance liquid chromatography and spectrophotometric methods to assess the function of their antioxidant defence systems.

Results—The α -tocopherol/cholesterol+triglyceride ratio was significantly reduced ($p < 0.05$) both in the patients and asymptomatic carriers. The concentrations of the other antioxidants and the lipid peroxides were not different from those of control subjects.

Conclusion—The low concentration of plasma α -tocopherol most probably reflects the consumption of the antioxidant by the affected tissues. Furthermore, it suggests that α -tocopherol may be the primary scavenger molecule against the free radicals induced by complex I deficiency.

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the respiratory chain NADH:coenzyme Q oxidoreductase (complex I).² In more than 95% of the genetically defined families LHON is associated with one of three pathogenic mtDNA mutations, at bp 11778, 3460, or 14484.² The mtDNA mutation is necessary but not sufficient for the manifestation of the disease—for example, only about 40%–50% of males and 5%–15% of females carrying the 11778 mutation actually have visual loss.³

The low penetrance of the disease has pointed to the possible role of secondary factors including X chromosome-linked visual loss susceptibility locus, the influence of nuclear background, mtDNA heteroplasmy, environmental factors, and autoimmunity.³

Complex I is the first member of five multimeric complexes embedded in the mitochondrial inner membrane mediating electron transport and oxidative phosphorylation. It catalyzes electron transport from NADH to ubiquinone (coenzyme Q), a hydrophobic, redox active quinone derivative. All three pathogenic LHON mutations involve complex I subunits; therefore it is assumed that they affect some functional properties of this enzyme complex. Complex I defect, however, can be subtle and difficult to detect using only the NADH-ubiquinone (Q) reductase activity of the enzyme. Reduced complex I activity has been found in patients with LHON with the A3460G and T14484C mutations, and polarography has shown reduced oxygen consumption with complex I-linked substrates in A11778G positive patients. However, the relation between complex I dysfunction and disease pathogenesis is unclear, as similar defects have been seen in all the tissues studied including platelets, lymphocytes and muscle in both clinically affected and unaffected people.⁴ The various mtDNA mutants are associated with reductions up to 60% in the catalytic activity of complex I in different tissues (muscle, fibroblasts, platelets, leucocytes) in patients with LHON.^{5–8} In vivo phosphorus 31 magnetic resonance spectroscopy showed defective bioenergetics in the brain or muscle both in patients and asymptomatic carriers.^{9–10}

Impaired complex I activity not only leads to a decline in mitochondrial energy production but also enhances the generation of free radicals and initiates lipid peroxidation. Enhanced formation of reactive oxygen species in genetically or chemically induced complex I deficiency has been shown in submitochondrial particles and in different cell systems.^{11–13} Lipid

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Leber's hereditary optic neuropathy (LHON) is a disease which may cause acute or subacute bilateral visual impairment, predominantly in young men. A characteristic clinical sign is the loss of central vision due to degeneration of the retinal ganglion cells and optic nerve axons, but additional neurological or cardiological symptoms might also be present.¹

The primary aetiological event is a mutation in the mitochondrial genome (mtDNA) affecting in most cases mtDNA encoded subunits of

peroxidation was reported to increase linearly until about 75% complex I inhibition and to result in damage to the mitochondrial membranes.¹³ The importance of free radicals is underlined by neuroprotective effects of spin traps *in vivo* against injuries induced by complex I inhibitor.¹⁴

The cells have their endogenous defence systems to neutralise reactive oxygen species or the damage elicited by these toxic species. The main hydrophilic scavenger found in cytosolic, mitochondrial, and nuclear compartments is reduced glutathione (GSH), whereas its hydrophobic equivalent localised in membranes is α -tocopherol. Both GSH and α -tocopherol are potent inhibitors of lipid peroxidation; whereas the role of GSH in this process is primarily, if not solely, to prevent the initiation of radical formation; α -tocopherol inhibits the propagation of the chain reaction.¹⁵ We consider that these two factors provide a very important defence system against oxidative stress.

It was previously reported that cells carrying the pathogenic LHON mutation at 11778 are significantly more sensitive to death induced by oxidative stress and this cell death is Ca^{2+} dependent.¹⁶

The objective of the present study was to determine whether patients with LHON, and carriers, display any alterations in these main antioxidant systems pointing to enhanced free radical reactions. The concentration of GSH, α -tocopherol, and some other antioxidants (free SH groups, β -carotene, lycopene) were determined in the peripheral blood of controls, carriers, and patients with LHON.

Patients and methods

Blood samples were obtained from nine patients (six male and three female, mean age 36.6 (SEM 3.6) years.), seven carrier maternal relatives (two male and five female, mean age 34.8 (SEM 6.7) years), and 15 control subjects (six male and nine female, mean age 33.4 (SEM) 3.5) years) while fasting during the morning hours. Molecular analysis confirmed that all of the patients with LHON and the carriers had the homoplasmic 11778 point mutation in the mtDNA of their blood cells. They received no steroid therapy or vitamin supplementation during the 3 months before the investigation and all of them were non-smokers. In the symptomatic group patients had severe visual impairment and pathological visually evoked potentials (VEPs), whereas in the carrier and control groups subjects had no visual problems and had normal VEPs.

The study was approved by the human investigation review board of the University, and informed consent was obtained from each patient participating in the study.

CHEMICALS

Glutathione reductase (GR), 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), N-ethylmaleimide (NEM), reduced nicotinamide adenine dinucleotide phosphate (NADPH), 2-thiobarbituric acid (TBA), lycopene, β -carotene, α -tocopherol, and α -tocopherol acetate were purchased from Sigma Chemical Co (St Louis, USA).

Malonaldehyde-bis-(diethyl acetal) was from Schuchardt (Munich, Germany). Sephadex G 10 was obtained from Pharmacia Biotech (Uppsala, Sweden). All other chemicals were of reagent grade.

MEASUREMENTS

Plasma α -tocopherol, lycopene, and β -carotene

Heparinised plasma samples stored at -70°C were used for the analysis. α -Tocopherol, lycopene, and β -carotene were determined by high performance liquid chromatography (HPLC) plus UV detection according to the method of Hess *et al.*¹⁵ The concentration of α -tocopherol was expressed with reference to plasma cholesterol+triglyceride, as accepted in the literature.¹⁷

Lipid peroxides

Venous blood anticoagulated with heparin was immediately separated by centrifugation (4°C , 1500 g, 10 minutes). EDTA and GSH were added to the plasma samples in final concentrations of 1.34 and 0.65 mM and samples were stored at -70°C until measurement. Lipid peroxides were determined as malondialdehyde-thiobarbituric acid adducts by HPLC and spectrophotometric detection.¹⁸

Reduced and oxidised glutathione

The concentrations of total and oxidised glutathione in whole blood haemolysate were measured by combining previously accepted standard methods.¹⁹ The action of DTNB and NADPH in the presence of glutathione reductase results in a reaction cycle, the rate of which depends on the total concentration of glutathione recorded spectrophotometrically at 412 nm during the first 6 minutes. As the assay responds to both GSH and GSSG, GSSG must be determined separately after alkylation of GSH with N-ethylmaleimide (NEM). Separation of GSSG and NEM was achieved by gel filtration with Sephadex G-10. The concentrations of the thiols were expressed with reference to haemoglobin (hgb) determined by the cyanmethaemoglobin method.

Plasma free SH groups

The concentration of SH groups was determined spectrophotometrically at 412 nm.²⁰ The protein content of plasma samples was measured using the method of Lowry *et al.*²¹

STATISTICAL ANALYSIS

All data were expressed as mean (SEM). One way analysis of variance (ANOVA) was followed by the LSD (least significant difference) test to determine significant differences between groups. A p value <0.05 was considered statistically significant.

Results

There were no significant changes in the concentrations of β -carotene, lycopene, GSH, GSSG, malondialdehyde (MDA), protein, cholesterol, and triglyceride, or the GSSG/GSH ratio and free-SH groups/protein ratio, as shown in the table. The α -tocopherol/lipid ratio

Concentrations (mean (SEM)) of various antioxidants in the three groups

	Controls (n=15)	Carriers (n=7)	LHON patients (n=9)
GSH (μmol/g hgb)	6.81 (0.14)	6.55 (0.22)	6.86 (0.36)
GSSG (nmol/g hgb)	9.25 (0.73)	9.66 (1.54)	8.78 (0.71)
GSSG/GSH	0.14 (0.01)	0.15 (0.03)	0.13 (0.01)
α-Tocopherol (μmol/l)	24.57 (1.02)	24.17 (1.72)	23.41 (1.52)
Cholesterol (mmol/l)	4.91 (0.2)	5.71 (0.37)	5.89 (0.23)
Triglyceride (mmol/l)	1.07 (0.28)	1.27 (0.24)	1.16 (0.13)
β-Carotene (μmol/l)	0.43 (0.06)	0.74 (0.24)	0.45 (0.08)
Lycopene (μmol/l)	0.56 (0.12)	0.38 (0.05)	0.55 (0.16)
SH Groups/protein (μmol/g)	2.33 (0.05)	2.51 (0.10)	2.57 (0.14)
Protein (g/l)	78.21 (1.01)	81.83 (1.51)	77.29 (1.48)
MDA (μmol/l)	0.41 (0.03)	0.49 (0.06)	0.42 (0.05)

hgb=Haemoglobin.

Ratio of α-tocopherol to cholesterol plus triglyceride in controls, asymptomatic carriers, and patients with LHON (mean (SEM); * $p < 0.05$).

was significantly reduced ($p < 0.05$) in patients with LHON and carriers compared with control subjects (figure).

Discussion

In the present study we found marked reduction in the plasma α-tocopherol/lipid ratio both in the patients and carriers homo-plasmic for the 11778 LHON mtDNA mutation.

Mutation at position 11778 affects the subunits of the respiratory chain enzyme complex I and results in a marked reduction in the mitochondrial respiration rate in intact mitochondria.⁴ In genetic and drug induced models, complex I impairment was found to be correlated with cell respiration, cell growth, free radical production, lipid peroxidation, mitochondrial membrane potential, and apoptosis.^{8, 22} Furthermore, cell death was quantitatively associated with free radical production rather than with a decrease in respiratory chain function.

Mitochondria are the major source of cellular reactive oxygen species; under physiological conditions up to 5% of the oxygen consumed by mitochondria is converted to oxygen radicals.^{23, 24} It has been shown that superoxide anion is generated at the level of the three enzymatic complexes interacting with ubiquinone.²⁵ Inhibition of electron transfer through complexes I-III enhances production of reactive oxygen species; increased free radical generation under conditions of impaired complex I activity is supported by several studies.^{8, 11-13, 22, 26} Furthermore, the increased concentration of reactive oxygen species has been found to induce lipid peroxidation, thereby leading to membrane damage in different models of complex I inhibition.^{8, 13, 22, 27}

Mitochondria possess powerful antioxidants—namely glutathione in the matrix and

α-tocopherol in the membrane. Moreover, mitochondria have the highest concentration of α-tocopherol and most of it is in the inner membrane²⁷. As complex I and ubiquinone are also located there, reactive oxygen species production and initiation of lipid peroxidation occur just at the site where the scavenger molecule is the most abundant. Thus α-tocopherol may effectively break the chain reaction while turning into tocopheroxyl radical. We suggest that the low concentration of α-tocopherol in the plasma of patients with LHON and carriers most probably reflects the high consumption of the scavenger molecule by affected tissues. Although LHON represents a focal type of neurodegenerative damage predominantly involving the retinal ganglion cells and optic nerve axons, impairment of complex I activity is present in several tissues including the brain, muscle, fibroblasts, platelets, and leucocytes.⁵⁻⁹ Furthermore, the functional consequences of the mtDNA mutations have also been demonstrated in the above mentioned tissues of asymptomatic carriers.¹⁰ These data may explain why the proposed consumption of α-tocopherol could be detected in plasma in the unaffected carriers as well as in the patients.

The importance of α-tocopherol as an antioxidant in the mitochondrial membrane is supported by the finding that damage to this membrane is one of the earliest pathological events in the skeletal muscle of animals deficient in α-tocopherol.²⁸ Furthermore, α-tocopherol was reported to regulate mitochondrial hydrogen peroxide production in a dose dependent manner.²⁹ The reduced form of ubiquinone, ubiquinol, has also been recognised as an important antioxidant in the inner mitochondrial membrane.^{30, 31} Although ubiquinol is able to scavenge radicals directly, it was suggested that it acts mainly indirectly as an antioxidant, regenerating α-tocopherol from its phenoxyl radical.^{32, 33} Ubiquinone intake has been shown to exert a sparing effect on α-tocopherol in mitochondria *in vivo*.³⁴ The mitochondrial membrane of different LHON mtDNA mutant lymphoblasts has an increased concentration of ubiquinone-10.³⁵ This finding may accord with a recent finding that vitamin E and selenium deficiency induces expression of the ubiquinone-dependent antioxidant system at the plasma membrane.³⁶

Blood GSSG and GSH concentrations are a sensitive index of whole body oxidative stress.³⁷⁻³⁹ Yet neither the glutathione system, nor the lipophilic antioxidants lycopene and β-carotene exhibited any differences between the patients with LHON, carriers, and controls. Neither did we find changes in the free SH groups/protein ratio. This suggests that the primary antioxidant against free radicals elicited by complex I impairment is α-tocopherol. However, changes in the glutathione system limited to the mitochondrial matrix cannot be excluded. Whereas complex I deficiency has been shown in several tissues in patients with LHON, tissue injury is limited to the retinal ganglion cells and optic nerve. Our failure to detect enhanced lipid peroxidation is most probably related to these localised types of tis-

sue injury. The specific sensitivity of the different tissues to complex I deficiencies is an interesting problem which has also emerged in Parkinson's disease. As an explanation for the phenomenon a threshold theory was suggested—namely a limit of complex I inhibition that still allows cells to grow and that may change with the cell type.¹³

In conclusion, patients with LHON and asymptomatic carriers display a reduced α -tocopherol/lipid ratio in their plasma which most probably reflects the increased free radical generation and α -tocopherol consumption in the affected tissues. The finding may have therapeutic implications, although it is necessary to be cautious about the expectations. Treatment of patients with Parkinson's disease with α -tocopherol, another neurodegenerative disorder with complex I impairment, was no help,⁴⁰ although it has been suggested that high intake of dietary tocopherol protects against the occurrence of the disease.⁴¹

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