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Biochemical phenotypes associated with the mitochondrial ATP6 gene mutations at nt8993

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Abstract

Two point mutations (T>G and T>C) at the same 8993 nucleotide of mitochondrial DNA (at comparable mutant load), affecting the ATPase 6 subunit of the F_1F_0-ATPase, result in neurological phenotypes of variable severity in humans. We have investigated mitochondrial function in lymphocytes from individuals carrying the 8993T>C mutation: the results were compared with data from five 8993T>G NARP (Neuropathy, Ataxia and Retinitis Pigmentosa) patients. Both 8993T>G and 8993T>C mutations led to energy deprivation and ROS overproduction. However, the relative contribution of the two pathogenic components is different depending on the mutation considered. The 8993T>G change mainly induces an energy deficiency, whereas the 8993T>C favours an increased ROS production. These results possibly highlight the different pathogenic mechanism generated by the two mutations at position 8993 and provide useful information to better characterize the biochemical role of the highly conserved Leu-156 in ATPase 6 subunit of the mitochondrial ATP synthase complex.

Keywords: mtDNA; T8993C; ATP synthase; Membrane potential; ROS; Mitochondria

1. Introduction

NARP (*N*europathy, *A*taxia, and *R*etinitis *P*igmentosa) and MILS (*M*aternally *I*nherited *L*eigh's *S*yndrome) are progressive neurodegenerative disorders of infancy and early childhood [1]. Clinical features of NARP may variably include peripheral neuropathy, retinitis pigmentosa, cerebellar atrophy, mental retardation, and less frequently epileptic seizures, cardiomyopathy, deafness and optic atrophy. MILS is dramatically characterized by bilateral subacute lesions of the basal ganglia, which extending to the brainstem, lead to death of the patient.

Point mutations (T>G and T>C) at nucleotide 8993 of mtDNA, affecting the ATPase 6 subunit of the F_1F_0-ATPase (ATP synthase), have been associated with both syndromes, and are always found as heteroplasmic mutations (wild-type and mutant mtDNA coexist within the same cell or tissue). Recent studies indicate a tight correlation between clinical, genetic and biochemical features in patients carrying variable loads of the mtDNA 8993T>G transversion [2,3]), and suggest that reduction in the rate of ATP synthesis is a major contributor to the pathomechanism of this mutation [4].

Abbreviations: F_0, membrane sector of the F_1F_0-ATPase complex; F_1, catalytic sector of the F_1F_0-ATPase complex; CS, citrate synthase; SODs, Mn- and CuZn-superoxide dismutases; ROS, reactive oxygen species; RH-123, Rhodamine-123; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′tetraethylbenzimidazolo-carbocyanine iodide; FCCP, carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone; CM-DCFDA, 5-(and -6) chloromethyl-2,7-dichlorofluorescein diacetate; $\Delta\Psi_m$, mitochondrial electrical membrane potential; PBMCs, Peripheral Blood Mononuclear Cells; FQR, fluorescence quenching rate; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; NARP, Neuropathy, Ataxia and Retinitis Pigmentosa; MILS, Maternally Inherited Leigh Syndrome

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A second mutation at the same nucleotide, the 8993T>C transition leads to similar NARP/MILS phenotypes, although in a milder form [5]. Neither the pathogenic mechanism, nor the biochemical effect of the 8993T>C mutation are fully understood. Some reports indicate that the maximal rate of ATP production is only slightly decreased in cells from patients and in transmitochondrial cytoplasmic hybrids (cybrids) carrying the 8993T>C mutation [6,7]. This suggests that the pathomechanism for the latter mutation may not be exclusively linked to a decrease in ATP production.

Mutations in mtDNA or nDNA that affect oxidative phosphorylation (OXPHOS) may induce overproduction of free radicals (reactive oxygen species, ROS) [8,9]. Oxidative stress, resulting from the imbalance between ROS formation and antioxidant defences, plays a major role in the pathogenesis of neurological diseases related to mitochondrial dysfunction (for recent reviews, see [10,11]). Intriguingly, both cells of patients and cybrids carrying the 8993T>G mutation displayed an abnormal production of ROS, irrespective of effects in mitochondrial membrane potential [12,13]. To better understand the pathogenic mechanism of the 8993T>C mutation we compared mitochondrial function of lymphocytes derived from two variably affected individuals harbouring different mutant loads with that of five NARP or NARP/Leigh patients carrying the 8993T>G heteroplasmic mutation. We report here the measurement of ATP synthesis rate in permeabilized lymphocytes, the fluorometric evaluation of mitochondrial electrical membrane potential ($\Delta\Psi_m$) by both flow cytometry and fluorescence quenching analysis, and we relate the bioenergetics of 8993T>C mutant cells to ROS production and pattern of ROS-detoxifying enzymes. Based on the current results we suggest that increased oxidative stress may play a major contribution to the pathomechanism associated with the 8993T>C mutation. Furthermore, the present results provide useful information on the importance of the Leu-156 residue within the ATPase 6 subunit of the mitochondrial ATP synthase.

2. Materials and methods

2.1. Patients

All patients were previously reported. The five 8993T>G NARP patients are the same as in Carelli et al. [3] and Sgarbi et al. [4]. The 8993T>C family has been recently reported by Sciacco et al. [14]. This latter family is composed by a female NARP proband (subject II-1 in Fig. 1) and her mother (I-1 in Fig. 1). The proband is a 30-year-old woman, currently suffering of visual loss due to retinitis pigmentosa, sensorineural deafness, cerebellar ataxia, muscle weakness, and mild mental retardation. Her mother, a 53-year-old woman, is short and suffering a mild form of peripheral neuropathy, confirmed by neurophysiological investigations. Finally, a proband's sister died at 3 years of age with encephalopathic features compatible with the diagnosis of Leigh syndrome.

2.2. Cell culture

PBMCs (Peripheral Blood Mononuclear Cells) were obtained by Ficoll-Hypaque Plus centrifugation of EDTA-treated blood, washed twice in PBS (Phosphate Buffered Saline) and resuspended in RPMI 1640 medium supplemented with 10% heat inactivated FBS (Fetal Bovine Serum), 50 IU/ml penicillin and 50 mg/ml streptomycin. PBMCs (10^6 cells/ml) were cultured in T-75 flasks for 3 h at 37 °C in a humidified incubator. Lymphocytes (non adherent cells) obtained as previously reported [4]) were cultured for 48 h. Viability was usually around 90% in both control and patient cells and cell population homogeneity (95% lymphocytes) was assessed by flow cytometry.

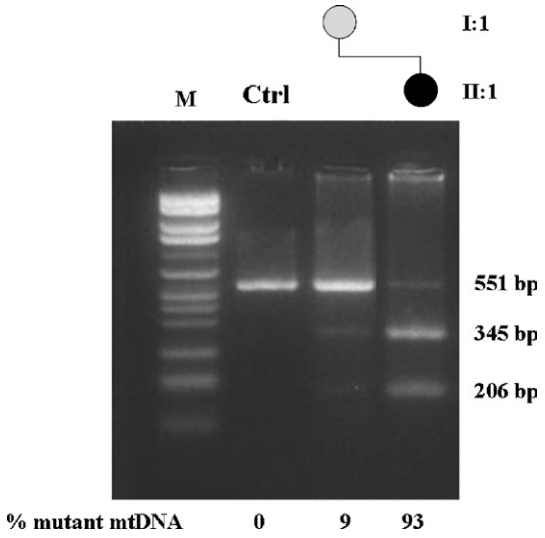

Fig. 1. Mitochondrial DNA mutant load in lymphocytes of 8993T>C patients. Electrophoretic gel showing the *Hpa*II digestion of PCR products. mtDNA heteroplasmy was evaluated by relative densitometric quantification of the *Hpa*II digested fragments. *TOP* Pedigrees of the Italian family investigated: the grey symbol indicates the carrier of the mutation with non-specific NARP symptoms; the dark grey symbol, the NARP patient. Note that the mutant loads after correction for the heteroduplex artefact were estimated up to 30% and 94% for the proband's mother (I-1) and the proband (II-1).

2.3. Mitochondrial DNA analysis

Total DNA was extracted from a pellet of the same lymphocytes used for biochemical investigations by means of the standard phenol-chloroform method. The point mutations at nt8993 were detected by restriction fragment length polymorphism (RFLP) analysis using the restriction endonucleases *Ava*I for the 8993T>G mutation and *Hpa*II for the 8993T>C mutation followed by electrophoresis, as previously reported [3,15]. Quantitative evaluation of the mutant loads in patients with 8993T>G mutation has been previously reported by Carelli et al. [3]. In the case of the two subjects carrying the 8993T>C mutation we evaluated the mutant loads by densitometry on the agarose gels stained with ethidium bromide as reported by Baracca et al. [15], followed by a mathematical correction for the heteroduplex PCR artefact as suggested by Shoffner et al. [16].

2.4. ATP synthesis and citrate synthase assay

Mitochondrial ATP synthesis rate in digitonin-permeabilized lymphocytes was assayed as recently detailed [4]. Briefly, lymphocytes (20×10^6 cells/ml) were incubated for 20 min at room temperature with 60 μg/ml digitonin, 2 mM iodoacetamide, and the adenylate kinase inhibitor, P^1,P^5-Di(adenosine-5′) pentaphosphate pentasodium salt (25 μM)), in 10 mM Tris/Cl (pH 7.4), 100 mM KCl, 5 mM KH_2PO_4, 1 mM EGTA, 3 mM EDTA, 2 mM $MgCl_2$. Complex II driven ATP synthesis was induced adding 20 mM succinate, 4 μM rotenone, and 0.5 mM ADP to the sample. The reaction was carried out at 30 °C and after 5 min it was stopped by addition of 80% dimethylsulfoxide. ATP synthesized was extracted from the cell suspension and assayed by the luminometric method as described by Stanley and Williams [17]: as a blank a sample not energized with succinate, but containing both 18 μM antimycin A and 2 μM oligomycin was used. Digitonin concentration was chosen as a result of a careful analysis to minimize any possible side effect.

Citrate synthase activity was quantified essentially according to Trounce et al. [18].

2.5. Mitochondrial membrane potential in human lymphocytes

Cytofluorometric analysis of mitochondrial membrane potential was performed in intact lymphocytes with 5,5′,6,6′-tetrachloro-1,1′,3,3′tetraethylbenzimidazolocarbocyanine iodide (JC-1) (Molecular Probes, Eugene, OR) on the Epics Elite (Beckman-Coulter) flow cytometer equipped with a 15 mW argon laser. Cells washed twice in PBS and re-suspended at 5×10^5 cells/ml in RPMI 1640 medium were incubated with 2 μM JC-1 for 30 min at 37 °C. The dye is electrophoretically accumulated in mitochondria, and after excitation at 488 nm the fluorescence emission was detected at both 525 nm (JC-1 monomeric form) and 575 nm (aggregate). A minimum of 10.000 events were acquired in linear scale for each measurement and the data were analysed with the cytometer software. When used, 2 μM oligomycin was pre-incubated with cells at 37 °C for 5 min.

The steady state mitochondrial electrical potential of permeabilized lymphocytes was assessed by means of the fluorescent cationic dye Rhodamine-123 (RH-123), which distributes electrophoretically into the mitochondrial matrix in response to the electrical potential across the inner mitochondrial membrane [19]. Briefly, lymphocytes (4×10^6/ml) suspended in a respiratory buffer (250 mM sucrose, 10 mM HEPES, 0.1 mM K-EGTA, 2 mM $MgCl_2$, 4 mM KH_2PO_4, pH 7.4) containing an ADP regenerating system (5 U/ml hexokinase and 10 mM glucose) and 50 nM Rhodamine-123 were incubated with 33 nM cyclosporine A, 2.5 μM rotenone, 200 μM ADP and then permeabilized adding 15 μg/ml digitonin. RH-123 fluorescence decay kinetics ($\lambda_{exc}=503$ nm; $\lambda_{em}=527$ nm) were induced by the addition of 20 mM succinate to the sample either pre-incubated (state 4 respiratory condition) or not (state 3 respiratory condition) with 2 μM oligomycin. Further details on reliability and suitability of the method for $\Delta\Psi_m$ measurements are reported in Baracca et al. [20].

2.6. Mn-SOD, Cu/Zn-SOD and catalase activity assays

Lymphocytes ($10–15\times10^6$ cells/ml) were suspended in phosphate buffer (50 mM KH_2PO_4, pH 7.8) containing 0.1% bovine serum albumin and the protease inhibitor cocktail (Sigma), and exposed to sonic oscillation at maximum output of a Brown apparatus for 45 s, in 15 s burst, at 4 °C. After centrifugation at 100,000×*g* for 30 min, the activity of both Mn-SOD and Cu/Zn-SOD was measured in the supernatant according to Oberley and Spitz [21]. Since the Mn-SOD is a mitochondrial enzyme, comparison of its activity in control and in patient cells was done following normalisation with respect to the activity of citrate synthase, which is a general marker for mitochondrial volume in cells. The catalase activity was measured spectrophotometrically at 240 nm following the hydrogen peroxide decomposition according to Aebi et al. [22] and the cyanide-sensitivity of the enzyme was assessed in the presence of 0.2–0.4 mM KCN, as suggested by Heck et al. [23].

2.7. Protein determination

Protein concentration of samples was assessed by the method of Lowry et al. [24] in presence of 0.3% (w/v) sodium deoxycholate. Bovine serum albumin was used as standard.

2.8. ROS detection

Lymphocytes were incubated for 24 h in Dulbecco's modified Eagle medium (D-MEM) (devoid of both pyruvate and glucose) and 5% heat inactivated and dialyzed FBS containing either 10 mM glucose or 5 mM D-galactose and 110 mg/l pyruvate. The cells were then pelleted and re-suspended at $0.5–1\times10^6$ cells/ml in 10 mM HEPES, 142 mM NaCl, 2 mM KCl, 1.2 mM KH_2PO_4, 1 mM $MgSO_4$, 1.3 mM $CaCl_2$, pH 7.4 and either of the two above substrates, and finally cells were incubated with 5 μM 5-(and -6) chloromethyl-2,7-dichlorofluorescein diacetate (CM-DCFDA) for 30 min at 37 °C. Reactive species production was detected by measuring the fluorescence emission at 535 nm in a Wallac Victor2 1420 multilabel counter (Perkin-Elmer), according to a slightly modified method of Degli Esposti [25]. ROS detection was occasionally carried out by incubating the cells for 2 h in the presence of 2 μM carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP).

2.9. Statistical analysis

All results are expressed as mean ± SD. Statistical significance of differences between sample populations was evaluated using one-way ANOVA followed by the Bonferroni post-test using a statistical software program (OriginPro 7.5, OriginLab Corporation, MA, USA) for multiple comparison tests. Statistical differences of $p\leq0.05$ were considered to be significant.

3. Results

3.1. Mitochondrial DNA analysis of cells

Molecular analysis of lymphocytes mtDNA from the two subjects carrying the 8993T>C mutation is shown in Fig. 1, whereas for all the other patients we referred to the previously reported evaluations [3,4]. Overall, the 8993T>G patients ranged between 79% and 94% mutant loads. Concerning the densitometric evaluation of the two 8993T>C subjects, the mutant loads were estimated as 9% and 93%, which corrected for the heteroduplex PCR artifact were about 30% and 94% for the proband's mother (I-1 in Fig. 1) and the proband (II-1 in Fig. 1), respectively [16].

3.2. Rate of ATP synthesis in digitonin-permeabilized 8993T>C heteroplasmic cells

The main function of mitochondria is to synthesize ATP. The process is catalyzed by the ATP synthase and it is thermodynamically driven by the electrochemical proton gradient across the inner membrane. In normally functioning mitochondria, the $\Delta\Psi_m$ is generated by the proton translocation from the matrix to the intermembrane space coupled with the electron transfer from reduced nicotinamide and flavin mononucleotides to oxygen, which is carried out by the respiratory chain complexes. To correlate ATP synthesis with mutant load, we measured mitochondrial ATP synthesis rate in digitonin-permeabilized cells energized with succinate. Fig. 2 shows that ATP synthesis decreases only 20% in lymphocytes carrying the 8993T>C mutation from the patient with 94% heteroplasmy. The patient's mother, carrying 30% mutant load, was not affected. By contrast, the rate of ATP synthesis in lymphocytes from five 8993T>G NARP patients (79–94% mutant load) decreases 65% or more compared with controls.

3.3. Membrane potential of mitochondria carrying the 8993T>C mutation

The synthesis of ATP by the mitochondrial ATP synthase slightly decreases $\Delta\Psi_m$ by utilizing the proton current, therefore it follows that inhibition of ATP synthase will hyperpolarize the mitochondria; addition of the specific ATP synthase inhibitor oligomycin acutely hyperpolarizes the mitochondria. Conversely, mitochondria that have inhibited respiratory chains or that have leaky inner membranes can reverse the ATP synthase to maintain a suboptimal $\Delta\Psi_m$ by hydrolyzing glycolytic ATP. By monitoring the initial rate of Rhodamine 123 (RH-123) fluorescence quenching induced by respiration, a quantitative estimation of the mitochondrial mem-

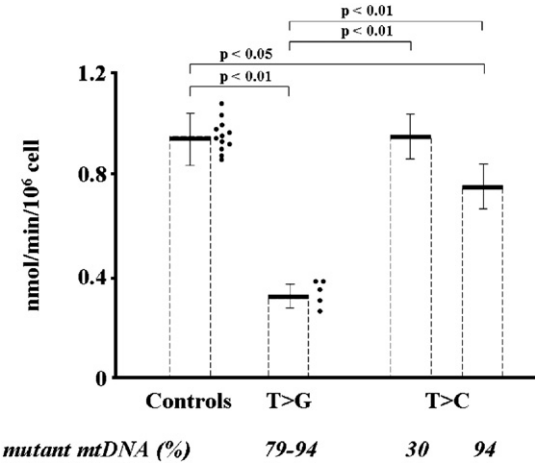

Fig. 2. Rate of ATP synthesis by permeabilized 8993T>C/G lymphocytes energized with succinate. Data reported for individuals harbouring the 8993T>C mutation are presented as mean±SD of three determinations on each lymphocyte preparation, whereas mean±SD of two to four assays are reported in single dots for each control and 8993T>G patient. Dots represent mean values of each individual.

brane potential can be achieved [20]. Protons are extruded from the mitochondrial matrix by the respiratory complexes into the intermembrane space, and easily diffuse back in through F_0. However, a significant fluorescence quenching is maintained at steady state as a balance between activities of respiration and proton flow through F_0. The initial rate of respiration-induced RH-123 fluorescence quenching increases with increasing $\Delta\Psi_m$ [20].

The membrane potential generated by proton movements across the inner mitochondrial membrane in digitonin-permeabilized lymphocytes of two individuals carrying the 8993T>C mutation was evaluated and compared with controls and patients harbouring the 8993T>G mutation. Fig. 3 shows the rate of RH-123 fluorescence decay upon energization with succinate under conditions of maximal ADP phosphorylation (state 3 respiration) for mitochondria from normal or mutant permeabilized lymphocytes. In the mutant cells, a slightly higher level of mitochondrial $\Delta\Psi_m$ was observed during ADP phosphorylation. In T>C mutant lymphocytes, the increase reached statistical significance only when heteroplasmy was 94%. A significant increase was also observed in the T>G mutant lymphocytes. Clearly, oligomycin enhanced mitochondrial $\Delta\Psi_m$ to a much higher extent than the mutations, indicating an impairment of proton translocation through the mutant F_0, which however is far from the block of F_0 proton permeability as induced by the specific ATP synthase inhibitor oligomycin.

The mitochondrial $\Delta\Psi_m$ of intact cells kept in glucose medium was analysed by cytofluorometry using the membrane potential-sensitive cationic probe JC-1. The $\Delta\Psi_m$ of 8993T>C mutant cells was similar to controls (Fig. 4a and c, top, and Fig. 4, bottom), whereas $\Delta\Psi_m$ of cells with the 8993T>G mutation was increased compared to controls (Fig. 4a and b, top, and Fig. 4, bottom), suggesting that under condition of low energy cell demand, the T>G mutant cells, having ATP synthesis by OXPHOS severely reduced, possibly produce ATP only

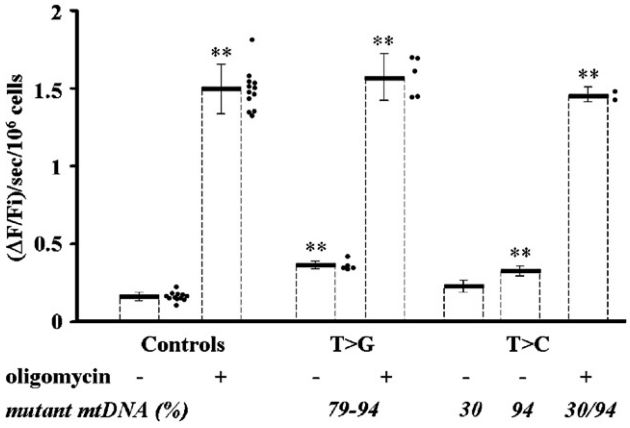

Fig. 3. Mitochondrial membrane potential in digitonin-permeabilized lymphocytes of individuals harbouring the mtDNA 8993T>C/G mutation. (ΔF/Fi/sec) is an expression of the decay rate of RH-123 fluorescence, strictly related with $\Delta\Psi_m$ [20]. Mean±SD of three measurements on each sample preparation of T>C patients is reported in presence or absence of oligomycin, whereas mean±SD of two to four assays are reported in single dots for each control (n=12) and T>G patient (n=5) both exposed or not to oligomycin. Asterisks indicate statistically significant differences (p<0.01) between patient and control lymphocytes or between oligomycin-treated and untreated lymphocytes. Dots represent mean values of each individual.

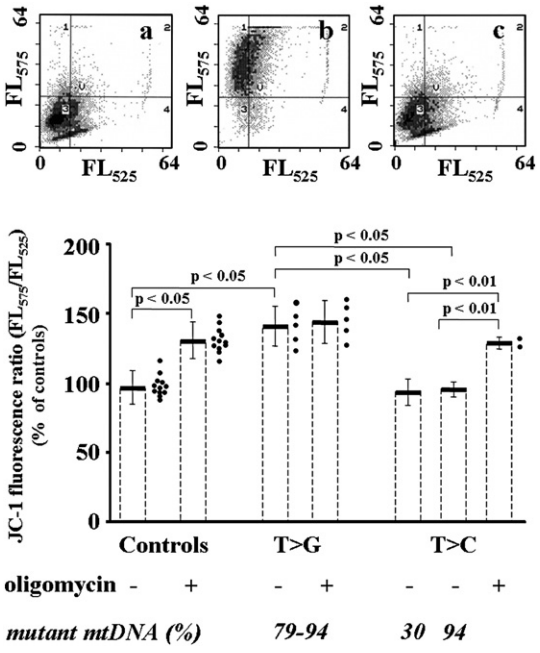

Fig. 4. Mitochondrial membrane potential of intact lymphocytes carrying the 8993T>G and T>C mutation. Cytofluorometric analysis of $\Delta\Psi_m$ was performed in glucose-grown intact lymphocytes previously loaded with 2 μM JC-1. *TOP* Left panel, a, typical dot plot of controls; centre panel, b, typical dot plot of 8993T>G mutant cells; right panel, c, typical dot plot of 8993T>C mutant cells. Dots in region 3 indicate cells expressing low red fluorescence (corresponding to J-aggregates), which increases when mitochondrial membrane becomes hyperpolarized, as in b. *BOTTOM* Quantification of cytofluorometric data is shown. Mean values±SD of JC-1 fluorescence ratio (F_{575}/F_{525}) of lymphocytes examined in presence and absence of 2 μM oligomycin are reported. Dots represent mean values of each individual.

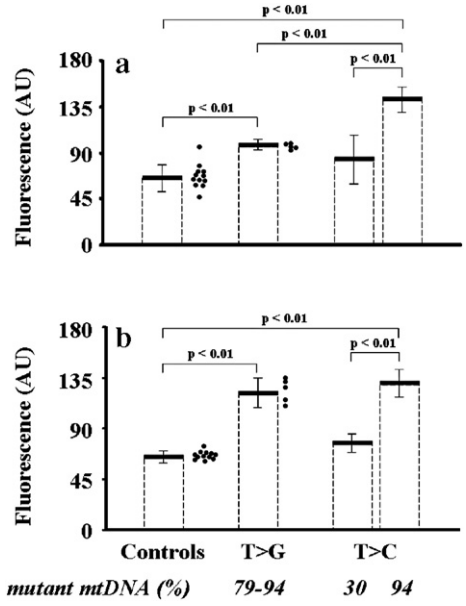

Fig. 5. ROS production in intact lymphocytes of 8993T>G/C patients. Reactive species were evaluated by measuring CM-DCFDA fluorescence in cells maintained with media containing glucose/pyruvate (a), or galactose/pyruvate (b) substrates. Bars represent means±SD of triplicates carried out on 12 controls, the five NARP patients carrying the 8993T>G mutation, and the 8993T>C patients. Dots represent mean values of each individual.

through the anaerobic glycolytic pathway. At variance, T>C mutant cells, similarly to controls, can satisfy the energy demand by predominantly using the oxidative phosphorylation process. This interpretation finds support on the observation that oligomycin pre-treatment of cells results in a JC-1 fluorescence ratio increase in both controls and T>C mutant cells reaching a value similar to the fluorescence ratio of untreated T>G mutant cells (Fig. 4, bottom).

3.4. ROS overproduction and enhanced activity of detoxifying enzymes in lymphocytes from NARP patients

ROS levels are affected by rates of ROS production (mostly via respiratory chain complexes) and ROS destruction by detoxifying enzymes, including superoxide dismutases. Impaired energy conservation, and increased $\Delta\Psi_m$ values in mitochondria, are often accompanied by increased ROS production as the electron transfer compounds become over reduced. ROS levels were measured in the mutant cells using the fluorescent probe CM-DCFDA [25]: the fluorescence of the probe incubated for 30 min with lymphocytes of controls and patients carrying the two mutations is reported in Fig. 5. T>G NARP cells grown in glucose medium (Fig. 5a) produced about 50% more ROS than controls, whereas T>C NARP cells showed double ROS production. Under conditions in which ATP synthesis depends primarily on oxidative metabolism, that can be induced by growing cells in the absence of glucose, NARP cells with either mutation showed a similar 2-fold enhancement compared to controls (Fig. 5b).

In NARP T>G mutated cells the activity of both CuZn-SOD and mitochondria-compartmentalized Mn-SOD, the latter expressed as the ratio between Mn-SOD and citrate synthase (CS) activity, increased compared to control cells ($p<0.05$) (Fig. 6a, b). In NARP T>C mutated cells both SOD activities showed a mild tendency to increase, but the enhancement was not statistically significant (Fig. 6a, b). Moreover, catalase activity did not change significantly in any of the NARP samples (Fig. 6c).

4. Discussion

We here report on the biochemical phenotype associated with the 8993T>C mutation in circulating lymphocytes, and the results are compared to those of the better characterized 8993T>G mutation.

Predictably, ATP synthesis rate was only mildly reduced by the 8993T>C mutation at 94% heteroplasmy, thus not fully explaining the severity of NARP and MILS phenotypes seen in patients. Similarly, only 22% reduction of ATP synthesis rate has been previously reported in homoplasmic 8993T>C cybrids [7]. Thus, a bioenergetic defect is unlikely to be the main reason for the disease pathogenesis. In account for pathogenesis, additional mechanisms must be considered.

In intact cells of 8993T>G NARP patients, the $\Delta\Psi_m$ was significantly increased compared to controls (Fig. 4), whereas $\Delta\Psi_m$ of cells carrying the 8993T>C mutation was close to normal, in agreement with Vilarinho et al. [26]. Nonetheless, ROS levels sharply increased in glucose-grown lymphocytes

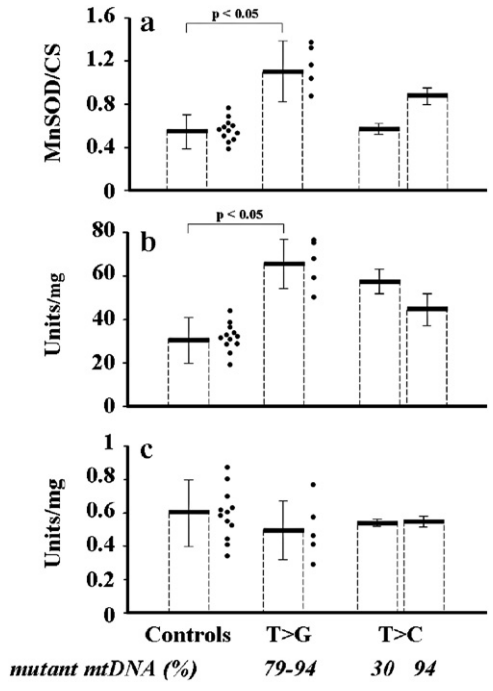

Fig. 6. Antioxidant enzyme activities in lymphocytes of 8993T>G/C patients. Activities were measured as detailed in Materials and methods. (a) The mitochondrial Mn-SOD was normalised by citrate synthase to take account of the mitochondrial mass in different cells, (b) CuZn-SOD and (c) catalase activity. Values are mean±SD obtained from duplicate measurements on 12 controls, and 7 individuals carrying 8993T>G ($n=5$) and 8993T>C ($n=2$) mutations. Dots represent mean values of each individual.

harbouring the 8993T>C mutation (+120% at 94% mutant load (m.l.) and +30% at 30% m.l. compared to controls) but only moderately with the 8993T>G mutation (+50% at m.l. above 79%). The strong increase of ROS in cells carrying either mutation occurred concurrently with a tendency to increased SOD activities. Our results on both ROS cellular level and SOD activities in 8993T>G and 8993T>C mutant cells are only apparently in contrast since increased SOD activities could enhance the hydrogen peroxide level without significant effects on DCFDA fluorescence due to different sensitivity of the probe towards the biological reactive species: in particular it promptly detects reactive species as hydroxyl radical and peroxynitrite [27].

The prevailing hypothesis to explain ROS overproduction in mutant mitochondria is an accumulation of reduced species within redox centres of complexes I and III of the respiratory chain associated with increased $\Delta\Psi_m$ [8]. Accordingly, the results here presented show an enhanced endogenous $\Delta\Psi_m$ under condition of maximal ATP synthesis rate (i.e. state 3 respiration) in mutant cells (Fig. 3), and concurrently ROS levels are higher compared to controls (Fig. 5b).

In glucose-grown intact cells, ROS were observed at higher concentration in 8993T>C than in NARP 8993T>G mutant cells (Fig. 5a), whereas $\Delta\Psi_m$ was higher than controls only in 8993T>G mutant cells (Fig. 4). This might be the consequence of the different metabolic pathway used by 8993T>G mutant cells with respect to 8993T>C and control cells under condition of both low energy requirement and glucose availability as metabolic substrate. In fact in 8993T>G mutant cells most ATP is produced by anaerobic glycolysis since oxidative phosphorylation rate is highly decreased. Therefore the electron flow through the respiratory chain is severely reduced, consequently the uncontrolled electron leak may be minimized. This is consistent with the observation that in digitonin-permeabilized 8993T>G mutant cells the respiration rate under phosphorylating condition is over 40% reduced [7,13,28], whereas in 8993T>C mutant lymphocytes from the NARP patient, the respiration rate is only 16% lower than controls [unpublished results], in agreement with data we previously reported for cybrids [7]. The occurrence of reduced electron flow through the respiratory chain (induced by the ATP synthase impairment) together with the mainly aerobic metabolism of the 8993T>C mutant cells might give reason for the overproduction of ROS with respect to both 8993T>G and control lymphocytes as grown in glucose medium. This interpretation is consistent with the idea that mitochondria highly contribute to ROS production in 8993T>C NARP cells. To support this hypothesis, we analysed the uncoupler (FCCP) effect on the DCFDA fluorescence of 8993T>C lymphocytes grown in glucose medium. We found that the DCFDA fluorescence of FCCP-treated cells was 39% and 33% reduced in 8993T>C and control lymphocytes, respectively. Therefore, FCCP similarly prevented $\Delta\Psi_m$-dependent ROS production in both 8993T>C and control lymphocytes. To account for the overall ROS production in 8993T>C mutant cells, we suggest that other mechanisms are involved, including loss of electrons due to impaired respiratory chain complexes, as observed by Mattiazzi et al. [13] in cells carrying the 8993T>G mutations, and dysfunction of the alfa-ketoglutarate dehydrogenase complex under conditions of altered NAD(P)H/NAD(P)$^+$ ratios [29], that likely occurs in cells harbouring mtDNA mutations.

Based on the present results, we suggest that ROS overproduction is a major pathogenic mechanism contributing to the occurrences of NARP and MILS phenotypes with the T>C mutation at position 8993 of the ATPase 6 subunit gene. Although overproduction of ROS is also reported in 8993T>G NARP cells [13], the dramatic bioenergetic impairment shown by these cells [4,15] suggest the energy deficiency as the prevalent component of the pathomechanism. The difference in biochemical behaviour of the two distinct nucleotide changes at the same 8993 mtDNA position may justify the less severe phenotype and possibly a later onset of symptoms in the presence of similar mutant loads associated with the 8993T>C mutation. A further element that may induce a slightly different clinical expression of the two mutations, and possibly a different severity in the tissues involved, may be the specific capability of different cell types to cope with increased ROS, deficient ATP synthesis or various combinations of both pathogenic components.

In summary, a battery of biochemical experiments performed in the present study demonstrated that different pathogenic mechanisms may be generated by two different mutations at the same position of the ATP 6 gene of mtDNA, which might lead to different pharmacological avenues to the treatment of the associated mitochondrial disease. In addition, the present results might help to characterize the structural and/or the functional role of Leu-156 of the ATPase 6 subunit of the human mitochondrial ATP synthase.

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