The mtDNA T8993G (NARP) mutation results in an impairment of oxidative phosphorylation that can be improved by antioxidants

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A T8993G point mutation in the mtDNA results in a Leu156Arg substitution in the MTATP6 subunit of the mitochondrial F1F0-ATPase. The T8993G mutation causes impaired oxidative phosphorylation (OXPHOS) in two mitochondrial disorders, NARP (neuropathy, ataxia and retinitis pigmentosa) and MILS (maternally inherited Leigh’s syndrome). It has been reported, in some studies, that the T8993G mutation results in loss of assembled F1F0-ATPase. Others reported that the mutation causes impairment of proton flow through F0. In addition, it was shown that fibroblasts from NARP subjects have a tendency to undergo apoptotic cell death, perhaps as a result of increased free radical production. Here, we show that the T8993G mutation inhibits oxidative phosphorylation and results in enhanced free radical production. We suggest that free radical-mediated inhibition of OXPHOS contributes to the loss of ATP synthesis. Importantly, we show that antioxidants restore respiration and partially rescue ATP synthesis in cells harboring the T8993G mutation. Our results indicate that free radicals might play an important role in the pathogenesis of NARP/MILS and that this can be prevented by antioxidants. The effectiveness of antioxidant agents in cultured NARP/MILS cells suggests that they might have a potential beneficial role in the treatment of patients with NARP.

INTRODUCTION

The F1F0-ATPase (mitochondrial ATP synthase) couples the synthesis of ATP from ADP and inorganic phosphate to the passage of protons from the intermembrane space to the matrix (1,2). Protons are translocated from the mitochondrial matrix to the intermembrane space at specific sites of the respiratory chain and thus, because of the low permeability to protons of the inner membrane that limits simple diffusion, a proton gradient is formed across the inner mitochondrial membrane. Protons re-enter the matrix through a channel formed by the mitochondrial DNA (mtDNA)-encoded MTATP6. The proton flow is coupled to rotation of the c-ring (13) that is transmitted to the F1 portion and allows for ADP phosphorylation. NARP (neuropathy, ataxia and retinitis pigmentosa) and MILS (maternally inherited Leigh’s syndrome) are mitochondrial disorders associated with mutations in MTATP6 (accession code NP_776050). The most common and studied NARP/MILS mutation is a T!G transversion at mtDNA nucleotide 8993 (T8993G) converting a highly conserved leucine into arginine (4,5). The clinical phenotype associated with the T8993G mutation is determined by the proportion of mutant mtDNAs (6). Typically, when the mutation load is between 70 and 90%, patients present with the NARP phenotype. A higher mutational burden may cause the fatal infantile encephalopathy MILS. The T8993G mutation is thought to impair the function of the F0 portion of ATPase causing ATP synthesis defects (7). In cells harboring homoplasmic (i.e. 100%) T8993G mutation, mitochondrial ATP synthesis is reduced by 50–70%, depending on the cell type and the assay used (8–13). In addition, it was proposed that the T8993G mutation enhances the production of toxic reactive oxygen species (ROS), because in fibroblasts
from patients with the T8993G mutation there was an upregulation of the activity of ROS scavenging enzymes associated with apoptotic cell death (14). Increased ROS production was also found in other disorders associated with mtDNA mutations (15). For example, a complex I (NADH dehydrogenase-coenzyme Q reductase) defect caused increased ROS production in human cells (16,17) as well as in xenomitochondrial cybrids (18). Although it is not clear to what extent mitochondrial dysfunction is exacerbated by free radical damage, numerous indications suggest that ROS might contribute to the pathogenesis of these diseases.

To better understand the relationship between the burden of T8993G mutant genomes and the biochemical phenotype in NARP cells, we established transmitochondrial cybrid cell lines (cybrids) harboring various proportions of the T8993G mutation. We found that ATP synthesis decreased with increasing mutation loads. Surprisingly, the biochemical impairment was not confined to the F1F0-ATPase, but it also involved other components of the OXPHOS machinery that were not affected by the genetic defect. We found increased ROS production, free radical damage to lipids, and mitochondrial superoxide dismutase activity in mutant cells, which were associated with a decline in mitochondrial respiration. These findings prompted us to treat mutant cells with antioxidants, which improved mitochondrial respiration and ATP synthesis.

RESULTS

Correlation between T8993G mutation load and ATP synthesis in cybrid cells

The first type of cells utilized in this study was obtained by the fusion of platelets containing heteroplasmic levels of the T8993G mtDNA mutation with human osteosarcoma cells devoid of mtDNA (143Bp0 cells). Since platelets do not contain nuclei, this technique allows the generation of cytoplasmic hybrids (also known as transmitochondrial cybrids or cybrids), which have the patient's mtDNA and the nuclear DNA from the host osteosarcoma cells. These cells can be used to study the effects of mtDNA mutations in a neutral nuclear background (19). To obtain cybrid cell lines containing various proportions of T8993G mutant mtDNA, a previously characterized cybrid clone with 84% mutant mtDNAs (10) was subjected to treatment with ethidium bromide (EB) for 10 days. EB inhibits mtDNA replication, thus reducing the average number of mtDNAs per cell and increasing the likelihood of skewed mitotic segregation of wild-type and mutant molecules upon EB removal and ensuing mtDNA repopulation. EB-treated cybrids were cloned, and clones harboring variable proportions of mutant mtDNA, ranging from 0 to 100%, were identified.

We noticed that, in several of the heteroplasmic (i.e. containing a mixture of T8993G mutant and wild-type mtDNA) cybrid clones, the proportion of mutant mtDNA was not constant over time during continuous culture. Thus, in order to obtain an accurate correlation between the proportion of mutated mtDNA and the various biochemical parameters, we performed PCR-RFLP measurement of the proportion of mutated mtDNAs on each clone at each time point when experiments were performed. ATP synthesis was measured either with succinate (Fig. 1A) or with malate plus pyruvate (Fig. 1B) as substrates in clones harboring mutant mtDNA. With both substrates, we found a statistically significant negative correlation of ATP synthesis with proportion of mutated mtDNA ($P < 0.0001$). We tested the ability of cybrids to grow in medium lacking glucose and containing pyruvate and galactose, where ATP synthesis depends primarily on oxidative metabolism (20). After one day in galactose, the growth rate of mutant cybrid clones harboring mutated genomes was decreased as compared with wild-type clones. After 2 days, mutant cybrids appeared unable to replicate any longer and started to die, whereas wild-type cells maintained a steady growth rate and became confluent by day 3 (Fig. 1C). A statistically significant difference ($P < 0.005$) in the average number of cells attached to the plate between wild-type clones and mutant ones was observed as early as day 3 in a clone harboring 30% mutant mtDNA. Clones with higher mutation loads showed a statistically significant decrease of viable cells ($P < 0.001$) as early as day 2 (Fig. 1C).

Correlation between T8993G mutation load and mitochondrial respiration

A statistically significant negative correlation of intact cell respiration with the proportion of mutated mtDNA was found in mutant clones (Fig. 2A). In an independent set of experiments, mitochondria were uncoupled with the protonophore trifluromethoxyphenyl-hydrazone (FCCP). Because of the loss of respiratory control by the F1F0-ATPase (21), FCCP increased oxygen consumption in all cybrid lines (Fig. 2B). As indicated by the almost identical slopes in Fig. 2A and B, the increase in oxygen consumption after treatment with FCCP was proportionally similar in mutant and wild-type cybrids, suggesting that, prior to the addition of FCCP, mutant mitochondria were not more uncoupled than wild-type ones. Thus, after FCCP addition, oxygen consumption was still negatively correlated with the proportion of mutant mtDNA (Fig. 2B). This failure to restore a normal uncoupled respiration by FCCP suggested that the loss of mitochondrial respiration in mutant cybrids was not simply due to enhanced respiratory control resulting from the T8993G mutation.

Oligomycin, a specific inhibitor of the F1F0-ATPase, decreased mitochondrial respiration more markedly in wild-type cells and in cells with lower mutation loads than in cells harboring higher levels of mutant mtDNA ($P < 0.001$; Fig. 2C and D). This suggested that the mutation rendered mutant cybrids less sensitive to oligomycin inhibition.

Oxygen consumption was also measured in digitonin permeabilized cells stimulated with ADP using succinate as substrate plus the complex I inhibitor rotenone, with and without the addition of FCCP. There was a reduction of state 3 respiration (i.e. stimulated by ADP) in mutant cybrids (Fig. 2E and F), which paralleled the one found in intact cells. These data suggested that, in addition to a defective F1F0-ATPase, mutant cybrids had an inhibition or down-regulation of other enzymes involved in cell respiration. In agreement with this hypothesis we found reduced cytochrome c oxidase (COX) activity in mutant compared with wild-type cybrids. There was a statistically significant negative correlation between COX activity and the proportion of mutant mtDNA (Fig. 3A). The activities of respiratory chain complexes I and II (succinate-coenzyme Q-reductase) were also significantly...
decreased in cybrids harboring 60% or more mutant mtDNA compared with wild-type clones (Fig. 3B and C). The activity of aconitase, an enzyme of the citric acid cycle that contains iron–sulfur groups, which renders it particularly sensitive to inactivation by ROS, was significantly decreased in cells harboring high levels (84 and 100%) of T8993G mutant mtDNA as compared with wild-type cells ($P < 0.04$; Fig. 4A), further suggesting that the OXPHOS machinery was impaired or inhibited in T8993G mutant cells. Conversely, the activities of complex II measured in the presence of inhibitors of the electron transfer to coenzyme Q (using instead an external acceptor, 2,6-dichloro-indophenol), as well as the mitochondrial matrix enzyme citrate synthase were unchanged in mutant and wild-type cybrids (not shown). Finally, as previously reported in NARP fibroblasts (14) there was a statistically significant increase in the activity of the mitochondrial ROS scavenging enzyme MnSOD in cybrids harboring 50 and 100% mutant mtDNA compared with wild-type cybrids ($P < 0.05$ and 0.0003 respectively; Fig. 4B), presumably in response to increased mitochondrial ROS production.

To exclude the possibility that the respiratory chain defects were due to loss of mtDNA-encoded polypeptides resulting, for example, from a low mtDNA copy number, we assessed the expression levels of the mtDNA-encoded COX subunits I and II by western blot analyses and found that mutant cells contained normal amounts of COX subunits (not shown).

Mitochondrial $\Delta \psi$ and matrix pH in T8993G mutant cybrids

We measured mitochondrial membrane potential ($\Delta \psi$) with the fluorescent potentiometric dye tetramethylrhodamine methyl ester (TMRM) in two independent 100% mutant cybrid clones, in a clone harboring 50% mutant mtDNA, and in a wild-type clone. $\Delta \psi$ was first measured in medium containing glucose, followed by the addition of oligomycin to hyperpolarize mitochondria, and, finally, of FCCP to collapse $\Delta \psi$. Surprisingly, we found that, despite the decrease of respiratory chain activities, mutant cybrids clones did not appear to have reduced $\Delta \psi$. Rather, there was a very small but statistically significant increase of TMRM fluorescence in both 50% ($P < 0.05$) and 100% ($P < 0.003$) mutant cybrids as compared to wild-type cybrids (Fig. 5A). As expected, oligomycin increased $\Delta \psi$ in all cybrid clones without statistically significant differences between mutant and wild-type cybrids, and FCCP equally collapsed $\Delta \psi$ in all clones. These results suggested that, in mutant clones, the loss of respiratory chain function did not result in a loss of mitochondrial $\Delta \psi$.

To investigate the effect of the T8993G mutation on the mitochondrial matrix pH, we used a ratiometric pH-sensitive green fluorescent protein (pHluorin) (22,23) targeted to the mitochondrial matrix (24). Wild-type and 100% mutant cells were transfected with mito-pHluorin (Fig. 5B) and fluorescence was measured 24 h after transfection. The ratio between the fluorescence emitted by mito-pHluorin excited at 410 and 470 nm was proportional to pH, as determined by a pH calibration curve obtained in permeabilized cells (Fig. 5C). We found that the matrix pH in 100% mutant cybrids was significantly higher (8.24 ± 0.51) than in wild-type cybrids (7.68 ± 0.34; $P < 0.004$; $n = 17$; Fig. 5D).
Figure 2. Mitochondrial respiration in NARP cybrids. (A) Measurements of oxygen consumption in intact cybrids. (B) Uncoupled respiration in intact cells after the addition of 1 μM FCCP. (C) Oligomycin inhibition of mitochondrial respiration. In each clone, mitochondrial respiration in intact cells was measured before (light gray bars) and after the addition of 10 μg/ml of the ATPase inhibitor oligomycin. (D) Statistical analysis of oligomycin inhibition of mitochondrial respiration relative to uninhibited respiration. For each clone, the values indicate the percentage by which respiration in intact cells (light gray bars in C) was reduced after the addition of oligomycin (dark gray bars in C). (E) Oxygen consumption in digitonin permeabilized cybrids stimulated with ADP and succinate with the addition of rotenone. (F) Oxygen consumption in digitonin permeabilized cybrids stimulated with ADP and succinate with the addition of rotenone and uncoupled with 1 μM FCCP. In (A, B, D, E and F), each open circle represents an independent measurement. The proportion of mutated genomes was determined for each clone at the time when each measurement was performed. In (A, B, E and F), there was a statistically significant negative correlation of oxygen consumption with increasing mutation loads. In (D) there was a statistically significant negative correlation of the percentage of oligomycin inhibition with increasing loads of mutant mtDNA. The $r^2$ and $p$-values of the slopes are indicated. The dashed lines indicate the 95% confidence interval on slope.
**ROS production and lipid peroxidation**

We measured the production of ROS by 5-(and-6) carboxy-2′,7′-dichlorofluorescein diacetate (carboxy-H2DCFDA) fluorescence in wild-type and mutant cybrids clones. There was a statistically significant increase in ROS production in cybrids harboring 50 and 100% mutant mtDNA as compared with wild-type cybrids \((P < 0.005 \text{ and } 0.001, \text{respectively}; \text{Fig. 6A})\). Furthermore, relative to wild-type cells, we found a 3- and a 2-fold increase in the lipid hydroperoxides malondialdehyde and 4-hydroxyalkenals in cybrids harboring 50 and 100% mutant mtDNA, respectively (Fig. 6B and C). These results correlated with the increase in the activity of the mitochondrial ROS scavenging enzyme MnSOD (Fig. 4B). Conversely, we found no change in the predominantly cytosolic form of superoxide dismutase, CuZnSOD (not shown).

We hypothesized that the inhibition of F1F0 ATPase caused by the mutation may be involved in the increased ROS production. To test this hypothesis we attempted to mimic the defect of mutant F1F0 ATPase in wild-type cells using the F1F0 ATPase inhibitor oligomycin. One-hundred per cent wild-type cybrids were incubated for 16 h in medium containing the following oligomycin concentrations: 0, 0.1, 1.0 and 5.0 \(\mu\)M. F1F0 ATPase inhibition resulted in a statistically significant increase in hydroperoxides production as shown by H2DCFDA fluorescence. There was statistically significant increase in H2DCFDA fluorescence with 0.1 \(\mu\)M oligomycin \((P < 1 \times 10^{-7}; \text{n} = 18; \text{Fig. 7})\). No further significant increases in ROS production were observed with the higher oligomycin concentrations. As expected, the addition of an antioxidant, N-acetylcysteine (NAC), significantly prevented the oligomycin-induced ROS production.

**Antioxidants improve respiratory chain functions and ATP synthesis both in mutant cybrids and in patient’s fibroblasts**

We hypothesized that increased ROS production could participate in causing respiratory chain dysfunction in T8993G mutant cells. Thus, in an attempt to reverse the effects of enhanced ROS production, we treated cybrids with...
antioxidant agents. We used 2.5 mM NAC and 10 μM dihydrolipoic acid (DHLPA), which have been shown to protect cells from ROS damage induced by complex I inhibitors (25), and 1 μg/ml coenzyme Q10 (CoQ10), which has been shown to protect platelets from oxidative stress (26). After 48 h of culture (corresponding to ~2.6 cell divisions) with 2.5 mM NAC, ROS were decreased in mutant cells to levels comparable to those of wild-type cells (Fig. 6A). Consistent with the decrease in ROS production, lipid hydroperoxydes in mutant cells were also markedly decreased by NAC (Fig. 6B and C). The decreases in ROS production and lipid peroxidation in response to the antioxidants were statistically significant. Antioxidants improved the activity of the respiratory chain enzyme COX compared with untreated mutant cybrids (Fig. 6D). Presumably, because of the increase in the activities of OXPHOS enzymes, mitochondrial respiration both without (Fig. 6E) and with FCCP (not shown) as well as ATP synthesis (Fig. 6F) were improved in cybrids treated with NAC. Similar results were obtained after 48 h of treatment with DHLPA (10 μM) or CoQ10 (1 μg/ml), both of which resulted in increased oxygen consumption and ATP synthesis in mutant cybrids (not shown). Furthermore, the viability in galactose medium was partially restored in mutant cybrids after NAC treatment (Fig. 6G). Although the growth rate of mutant cybrids was slower than that of wild-type ones, the total number of mutant cells remained stable over 4 days, suggesting that the death rate of NAC-treated mutant cells in galactose medium was less than that of untreated cells (Fig. 1C). We have not measured apoptotic cell death in our cybrids, but based on the results of Geromel and colleagues in NARP fibroblasts (14) it is likely that, in addition to improving OXPHOS activities, antioxidants contributed to reduce ROS-mediated apoptosis. Finally, treatment with NAC restored a normal matrix pH in mutant cybrids. Matrix pH measured after 48 h of NAC treatment was 7.8 ± 0.38 and 7.70 ± 0.14 (n = 14), respectively, in wild-type and 100% mutant cybrids and were not significantly different. This was presumably due to the improvement of state 3 (phosphorylating) cell respiration resulting in enhanced exchange of ions across the mitochondrial inner membrane.

To ensure that the beneficial effect of antioxidants was not a phenomenon confined to osteosarcoma-derived cybrids, we tested the effect of antioxidants in primary fibroblasts harboring 97% T8993G mutant mtDNA obtained from a patient with NARP. Antioxidants significantly improved mitochondrial

**Figure 5. Mitochondrial membrane potential and matrix pH.** (A) For mitochondrial membrane potential measurements cybrids were loaded with TMRM and fluorescence was measured after 30 min at baseline (i.e. in medium containing glucose and pyruvate), after the addition of 1 μg/ml oligomycin, and after the addition of 1 μM FCCP. Values are averages ± SD of the relative fluorescence measured in 300 individual cells from 0% mutant cybrids, 200 individual cells from 100% mutant cybrids and 100 individual cells from 50% mutant cybrids. In brackets are indicated the numbers of clones analyzed and averaged for each group. The P-values of statistically significant differences are shown. (B) An example of microscopically visualized wild-type cybrids transfected with mito-pHluorin to measure matrix pH. (C) pH calibration curves of 0 and 100% mutant cybrids. Mito-pHluorin transfected cells were permeabilized with nigericin and fluorescence ratios (410/470 nm excitation) were measured in calibration buffers with various pH ranging from 5.0 to 9.5. (D) Average fluorescence ratios and corresponding pH values ± SD measured in 0 and 100% cybrids (n = 17 cells for each group). The P-value of the difference between wild-type and 100% mutant cells is shown.
respiration and ATP synthesis also in these fibroblasts (Fig. 8A and B). These results suggested that the protective effects of antioxidants on NARP cells were not limited to the cybrid model but could also be seen in patient derived cells.

**DISCUSSION**

The pathogenic mechanisms leading to decreased ATP synthesis in cells harboring the T8993G mutation are controversial. It was proposed that the mutation destabilizes the F0 complex resulting in the loss of assembled F1F0 ATPase (13, 27). In contrast, the F1F0-ATPase was normally assembled in fibroblasts harboring high proportions of T8993G mutation (11). A second model based on studies that modeled the T8993G mutation in *E. coli* predicts that the T8993G mutation impairs proton translocation through the F0 (28–30). However, studies performed on submitochondrial particles from platelets of NARP patients did not confirm those findings (12).

The ATP synthesis defect in our homoplasmic mutant cybrids was comparable to those previously reported by us and by others (9, 10, 13). We studied cybrid clones harboring various proportions of mutant T8993G mtDNA, and found that there was a significant negative correlation between ATP synthesis and the mutation load. However, this correlation was not perfectly linear due in part to the variability among clones with similar mutation loads. This interclonal variability emphasizes the importance of analyzing numerous clones in this type of cybrid study in order to achieve meaningful results. Similar findings have been previously reported in platelets from asymptomatic family members of NARP patients, which harbored ~35% of T8993G mutant mtDNA resulting in approximately a 65% decrease in ATP synthesis (31).

There is conflicting evidence in the literature on the existence of a mitochondrial respiratory chain defect in NARP cells. Although some studies reported an oxygen consumption defect in isolated mitochondria from cells with high levels of T8993G mutation (32), other studies in intact cybrids failed to confirm these findings (13). These discrepancies may be related to the inherent differences in measuring respiration in isolated mitochondria versus whole cells, or to the different sensitivities and specificities of the assays.
In this study, we found reduced oxygen consumption in both mutant cybrids and in fibroblasts from a patient with the T8993G mutation. At first, we thought that the slower respiration rate was caused by the loss of ADP phosphorylation leading to an inhibitory control on the respiratory chain (32). However, the failure to restore oxygen consumption by FCCP, which should have resolved the respiratory block caused by an impaired F_{0}, suggested that, in addition to an increased proton flux is sufficient to build a proton motive force (see 33 for a definition) which is higher as compared with normally respiring wild-type cells. In other words, the L156R substitution might reduce the electrogenic protons entering the matrix through ATPase and lower the proton motive force, simultaneously decreasing state 3 respiration and impaired proton flux is sufficient to build a proton motive force (see 33 for a definition) which is higher as compared with normally respiring wild-type cells. In addition, Nicholls and colleagues (35) showed that, in cultured fibroblasts from NARP subjects (14). In their report, Geromel and colleagues (14) suggested that free radical scavenging enzymes were elevated in response to ROS production, which, presumably, rendered mutant cells more susceptible to apoptotic cell death. Since, unlike fibroblasts, our cybrid clones contained a homogenous and neutral nuclear background, our findings confirmed that increased ROS production was indeed a result of the T8993G mutation.

Our results were in agreement with previous ones showing increased levels of free radical scavenging enzymes in fibroblasts from NARP subjects (14). In their report, Geromel and colleagues (14) suggested that free radical scavenging enzymes were elevated in response to ROS production, which, presumably, rendered mutant cells more susceptible to apoptotic cell death. Since, unlike fibroblasts, our cybrid clones contained a homogenous and neutral nuclear background, our findings confirmed that increased ROS production was indeed a result of the T8993G mutation.

Furthermore, we have investigated the biochemical mechanisms leading to increased ROS production in NARP cells. As explained above, we believe that the combination of reduced state 3 respiration and the persistence of a high proton motive force might be responsible for increased ROS production in mutant cells.

We used oligomycin to try to mimic the effect of the L156R substitution in wild-type cells. Oligomycin was previously shown to increase ROS in cultured preadipocytes (36) and to induce MnSOD expression in wild-type fibroblasts (37). Using a similar experimental paradigm, we found that the inhibition of the F_{1}F_{0}-ATPase by oligomycin resulted in increased ROS production. However, we note that the increase in ROS production in wild-type cells treated with oligomycin was not as pronounced as that spontaneously occurring in T8993G mutant cybrids. This suggests that either the inhibition of the F_{1}F_{0}-ATPase is not the only cause of increased ROS production in mutant cells or that the effects of the T8993G mutation and oligomycin may be similar, but not identical. It is also possible that the endogenous cellular antioxidant defenses become more depleted in mutant cybrids that are chronically exposed to increased ROS production than in wild type cells acutely exposed to oligomycin.

We reasoned that ROS caused inhibition of OXPHOS enzymes, which, in turn, could accentuate the ATP synthesis defect. Therefore, we attempted to brake this ‘vicious’ cycle by treating cells with various antioxidant agents that promote ROS scavenging, such as NAC, DHLPA (25) and CoQ_{10} (26). We showed that antioxidants reduced ROS and, most importantly, restored cell respiration and partially improved ATP synthesis both in mutant cybrids and in fibroblasts from a patient with NARP, which harbored high levels of the T8993G mutation. Clearly, it is possible that antioxidants might exert their effects on OXPHOS via some mechanisms other than ROS inactivation. For example, antioxidants might directly introduce reducing equivalents into the mitochondrial matrix, thereby reducing its pH. Modification of the matrix pH might have important consequences on the regulation of mitochondrial respiratory chain enzymes. Further studies are needed to address these potential additional mechanisms.

The therapeutic tools currently available for the treatment of mitochondrial diseases due to mtDNA mutations are few and their efficacy is not yet well established. We and others have recently demonstrated that one potentially viable approach to...
treating mitochondrial disorders due to mutations in protein coding genes, such as NARP/MLS and Leber’s hereditary optic neuropathy, is the expression of mtDNA-encoded proteins allotopically from the nucleus (38–40).

Antioxidant agents such as NAC, and CoQ_{10} are routinely administered to patients with a variety of disorders including mitochondrial disorders, albeit with conflicting results in the latter (41). Because of the mitochondrial hyperpolarization, ROS production in NARP may be more pronounced than in other mtDNA-related disorders. However, to our knowledge, antioxidants have not been systematically administered to NARP patients. Therefore, we suggest that, based on our present results and on their ease of administration, antioxidants may be considered as a potentially useful tool, perhaps in conjunction with other therapeutic approaches, in the treatment of NARP and of other mitochondrial disorders in which ROS play an important pathogenic role.

**MATERIALS AND METHODS**

**Cell culture**

Two types of cells were used for this study. The first type was obtained by fusion of platelets containing heteroplasmic levels of the T8993G mtDNA mutation with human osteosarcoma cell devoid of mtDNA (143Bp0 cells) (19). Ethidium bromide was used to manipulate the relative proportions of mutant and wild-type mtDNA as described (42). In brief, a heteroplasmic cybrid cell line containing 84% T8993G mtDNA mutation and 16% wild type mtDNA was treated for 10 days with EB (50 ng/ml) in Dulbecco modified Eagle’s medium (DMEM) containing 4.5 g/l glucose and 100 mM pyruvate, supplemented with 10% fetal bovine serum and 50 μg/ml uridine. After 10 days, EB was removed from the medium and mtDNA was allowed to repopulate the cybrids. At this stage, uridine was removed from the medium in order to eliminate potential p0 cells resulting from the treatment (19). Clones were isolated by the cloning cylinder method and the proportions of mutated mtDNA were assessed in each clone by restriction fragment length polymorphism analysis of PCR fragments of mtDNA encompassing the T8993G mutation as described previously (10).

The second type of cells were skin fibroblasts harboring 97% T8993G mutation which were directly obtained from a patient with NARP with informed consent and cultivated in DMEM supplemented with 15% FBS.

Growth curves of cybrids in strict oxidative conditions (i.e. galactose medium) were obtained by seeding 35 mm² plastic Petri dishes with 5 × 10⁴ cells in 3 ml of DMEM without glucose, supplemented with 5% dialyzed FBS, 110 mg/l sodium pyruvate, 5 mM galactose. For each clone, equal numbers of cells were seeded in 12 dishes, so that cell counts could be obtained in triplicate each day for 4 days.

For antioxidant treatments, both cybrids and fibroblasts were grown for 48 h in DMEM medium (as above) containing one of the following antioxidants: NAC (2.5 mM final concentration), DHLPA (10 μM final concentration) and CoQ_{10} incorporated in liposomes as described (43) and used at a final concentration of 1.0 μg/ml.

To determine the effects of mitochondrial F_{1}F_{0} ATPase inhibition by oligomycin on ROS production in wild-type cells, cybrids containing 100% wild-type mtDNA were grown in DMEM containing 4.5 g/l glucose and 100 mM pyruvate, supplemented with 10% fetal bovine serum with the addition of different concentrations of oligomycin, ranging from 0 to 5 μM. Oligomycin (Sigma) was serially diluted in ethanol in order to maintain the ethanol concentration constant at 1 : 600 (vol : vol) in all experiments. Aliquots of 7.5 × 10⁴ cells were plated in 48-well dishes 24 h prior to the experiments. Cells were incubated with oligomycin for 16 h. Each experiment was performed in six replicates and, in one set of experiments, NAC (2.5 mM) and 5 μM oligomycin were added to the medium in order to test its antioxidant effect on ROS production induced by oligomycin.

**Biochemistry and immunochemistry**

ATP synthesis in cybrid clone and patient’s fibroblasts was measured with malate plus pyruvate or with succinate (in the presence of 1 μM rotenone) as substrates using a luciferin luciferase method as described (44).

Oxygen consumption in intact cells was measured with a Clark-type electrode (Hansatech) in an oxygen chamber as described (42). Oligomycin (10 μg/ml) was added to inhibit mitochondrial ATPase. FCCP, 1 μM, was added to uncouple mitochondrial respiration. Oxygen consumption was also measured with and without the addition of 1 μM FCCP in cells permeabilized with digitonin (50 μg/ml) in a respiration buffer containing 0.25 mM sucrose, 50 mM HEPES, 2 mM MgCl_2, 1 mM EGTA, 10 mM KH_2PO_4, pH 7.4. 20 mM succinate (plus 1 μM rotenone) and 300 mM ADP were used to stimulate state 3 respiration.

COX and citrate synthase were measured on cell lysates as described (32). Since the activities of respiratory chain complexes I, II (succinate–CoQ oxidoreductase), and SDH (succinate dehydrogenase) are difficult to measure accurately in cell lysates, they were measured on isolated mitochondria as described (32). Complex II activity was measured in two ways. One (succinate-CoQ oxidoreductase) using coenzyme Q1 as electron acceptor, the other (succinate dehydrogenase) in the presence of phenazine methosulfate (4 mM) and 1 mM TTFA [4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione]. In the second reaction, electrons are shunted from complex II to an acceptor, 2,6-dichloro-indophenol, without oxidizing coenzyme Q.

Aconitase activity was measured spectrophotometrically in whole cell lysates following the reduction of β-nicotinamide adenine dinucleotide phosphate (β-NADP) in the presence of citrate, isocitric dehydrogenase, Fe(NH_4)_2(SO_4)_2, MnSO_4, and L-cysteine, as previously described (45).

Immunoblot analyses of equal amounts of total cell proteins (30 μg) were performed as described (46) using mouse monoclonal antibodies against COX subunits I and II (Molecular Probes). Bands detected by anti-β-tubulin antibodies were used as gel loading controls.

**Mitochondrial membrane potential and matrix pH**

Mitochondrial membrane potential was measured with the potentiometric fluorescent dye TMRM on attached cells as described (47). Cell fluorescence was captured from 100 cells...
from each clone with an inverted microscope equipped with a CCD camera. Quantification of fluorescence was performed with the image analysis software Openlab™ (Improvision).

Mitochondrial matrix pH was measured with the ratiometric pHluorin construct targeted to mitochondrial matrix (mito-pHluorin) by the addition of the first 12 amino acids of the mitochondrial targeting sequence of subunit IV of COX through an Arg-Ser-Gly-Ile linker (24). Cybrids were transfected with mito-pHluorin using a DMRIE-C transfection reagent (Invitrogen). Twenty-four hours after transfection, cells were imaged as previously described (23). Fluorescence quantification was performed using MetaFluor 3.0 software (Universal Imaging). A Calibration of the system was performed on wild type and mutant cybrids permeabilized with 10 μM nigericin (Sigma) in calibration buffers (30 mM HEPES, 125 mM KCl, 20 mM NaCl, 0.5 mM CaCl₂, 0.5 mM MgCl₂) ranging in pH from 5 to 10. The imaging buffer consisted of 30 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM Kcl, 1 mM MgCl₂, 2 mM CaCl₂ and 25 mM glucose.

ROS, lipid hydroperoxides and ROS scavenging enzymes

ROS were measured as described (48) in cells loaded with 50 μM 5-(and-6)-carboxy-2’,7’-dichlorofluorescein diacetate (carboxy-H2DCFDA, Molecular Probes). After 30 min incubation at 37°C in the dark in Optimem™ medium (Invitrogen) without phenol red, cells were lysed and cell fluorescence was measured in a HITS 7000 plus plate reader (Perkin Elmer). Lipid hydroperoxides (malondialdehyde and 4-hydroxyalkenals) were measured in cell lysates with a lipid peroxidation assay kit (Calbiochem) according to the manufacturer’s guidelines. The activities of manganese superoxide dismutase (MnSOD) and copper, zinc superoxide dismutase (Cu, ZnSOD) were measured in cell lysates as described (49).

Statistical analyses

For the analyses of the correlations between the proportion of mutated mtDNA and biochemical parameters such as ATP synthase, cell respiration, enzymatic activities, to determine whether the two variables were positively or negatively correlated, a line was fit to the data by the least square method. The 95% confidence interval on the slope was calculated. Correlations were considered statistically significant if the confidence intervals (95%) did not include the zero-slope. Calculations were performed with Statview software.

For statistical comparison between two groups of data such as those from the TMRM, the mito-pHluorin, the oligomycin-induced ROS and the antioxidant treatments, since F-test analyses revealed an homogeneity of variances between groups, P-values of the differences between wild-type and mutant cells were calculated by unpaired two-tailed Student’s t-test. A level of confidence of P < 0.05 was adopted.

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