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Free radicals-mediated damage in transmitochondrial cells harboring the T14487C mutation in the ND6 gene of mtDNA

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Abstract **We have studied the production of reactive oxygen species (ROS) in transmitochondrial cells, harboring homoplasmic levels of the T14487C mtDNA mutation in the *ND6* gene of mitochondrial DNA (mtDNA). Previous work has shown that this mutation causes complex I deficiency. Here, we show that this mutation causes an overproduction of ROS leading to an increase in the oxidation of lipids and mtDNA without modification of antioxidant enzyme activities. We suggest that mutations in mtDNA affecting complex I activity may result in oxidative cellular damage, and reinforce the possible role of ROS-mediated mechanisms participating in some mtDNA-related disorders.**

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Keywords: Oxidative damage; ROS; mtDNA mutations; Complex I; ND6

1. Introduction

Mitochondrial DNA (mtDNA) point mutations have been associated with a wide range of clinical presentations ranging from pure myopathies to multisystem disorders [1]. Despite the great efforts made in recent years to understand the genotype–phenotype relationship in mitochondrial disorders, the precise mechanisms that result in a particular clinical phenotype remain far from being understood. Although it is believed that impairment of ATP synthesis plays a key role in the clinical presentation of mitochondrial disorders, there is growing evidence that other biochemical pathways may be involved. In recent years, a substantial amount of work has suggested that reactive oxygen species (ROS)-mediated damage plays an important role in the pathogenesis of cell damage caused by mtDNA mutations [2]. However, very few systematic studies in controlled experimental models have been performed to assess this hypothesis.

We present biochemical data on a cell model of the T14487C mutation in the ND6 gene of mtDNA. This mutation has been recently identified independently by three groups in patients with a Leigh-like or bilateral striatal necrosis syndrome [3–5]. Here we report that transmitochondrial cybrids harboring homoplasmic levels of the mutation showed an increase in ROS production which results in lipid and mtDNA oxidative damage. Interestingly, in this model, the over-production of ROS did not result in an increase in antioxidant enzyme defenses.

These results suggest that mutations in complex I (nuclear or mtDNA) genes may lead to a significant increase in ROS production and a subsequent increase in oxidative damage in cell lipids and mtDNA.

2. Materials and methods

Biochemical studies were carried out on a transmitochondrial cell line harboring homoplasmic levels of the T14487C mutation (ND6 100%), and wild-type control cybrids (WT) whose biochemical phenotype has been previously characterized [3].

2.1. ROS production

Cells (2.5×10^5) were collected by trypsinization and resuspended in PBS. Twenty-four microliters of the 1.25 mM cell-permeate probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) solution was added to each cell suspension and incubated for 15, 30, and 45 min at 37 °C. Fluorescence was analyzed in a fluorimeter (SFM 25, Kontron Instruments. 488 nm excitation and 525 nm emission). Results were referred to a standard curve of dichlorofluorescein (DCF). DCF fluorescence has been previously described that is linear in the nanomolar range with hydrogen peroxide [6], thus the results can be expressed as fmol of hydrogen peroxide.

2.2. Antioxidant enzyme activities

Cells were grown to confluence, and 60–70 million cells were harvested for each sample with trypsin–EDTA, washed twice, suspended in PBS and frozen at −80 °C until analysis. Samples were homogenized in PBS, sonicated for 5 min and centrifuged to isolate the supernatant. Total superoxide dismutase (SOD) activity was determined according to McCord and Fridovich [7], measuring the inhibition of the cytochrome *c* reduction by superoxide radical. Mn-SOD activity was measured in the presence of 10 mM KCN. Glutathion peroxidase (GSH-Px) was measured using the method of Lawrence and Burk [8] in which GSH-Px activity is coupled to the oxidation of NADPH by glutathione reductase monitored at 340 nm. Catalase (CAT) assay was performed spectrophotometrically according to Aebi's method by monitoring the degradation of H_2O_2 at 240 nm [9].

2.3. Lipid peroxidation

Lipid peroxidation was assessed by measuring the levels of the malondialdehyde–thiobarbituric acid (MDA–TBA) adduct in cell extracts as previously described [10]. Briefly, cells (6.0×10^6) were collected and

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homogenized in 0.44 M orthophosphoric acid and butylated-hydroxitoluene (3 mM) was added to avoid de novo peroxidation. Samples were incubated in the presence TBA (42 mM). After incubation, 0.1 ml of 70% perchloric acid was added and the sample was centrifuged. Twenty microliters of the supernatant was injected into the HPLC system (Alliance 2695 system, Waters Corporation, USA, Symmetry C_{18} 5 μm column). The mobile phase was composed of 60% potassium phosphate 50 mM, pH 6.8, and 40% methanol. The eluate was monitored fluorometrically, and the adduct MDA–TBA quantified based on comparison with a calibration curve of 1,1,3,3-tetraethoxypropane.

2.4. Measurement of mtDNA oxidation (QPCR)

The basis of the QPCR assay is that lesions present in the DNA block the progression of any thermostable polymerase on the template, resulting in the decrease of DNA amplification in the damaged template [11,12]. Briefly, total DNA was extracted using QIamp DNA Mini Kit (QiAgen, GmbH, Germany) and quantitated spectrophotometrically. Aliquots of DNA (50–100 ng) were then subjected to QPCR. Two fragments of mtDNA were amplified (mtDNA primers for fragment of 8.9 kb; sense 5′-TTTCATCATGCGGAGATGTTGGATGG-3′ and antisense 5′-TCTAAGCCTCCTTATTCGAGCCGA-3′; fragment of 207 bp; sense 5′-ACTTCCTACCACTCACCCTA-3′ and antisense 5′-TGATAGGTGGCACGGACAAT-3′). QPCRs were performed with the GeneAmp XL PCR kit (Perkin–Elmer) as previously described [12]. An aliquot of each PCR product was resolved on a 0.8% agarose gel and electrophoresed in TBE at 90 V for 45 min. The gels were stained with EtBr and the density of the bands were obtained and processed with the Quantity One® software (Biorad, Hercules, CA).

2.5. Protein oxidation

Western blot analysis of carbonyl groups content in proteins from cell extracts was used to assess the levels of protein oxidation [13]. Briefly, cells were homogenized and proteins were derivatized with 2,4-dinitrophenylhydrazine and separated (10 μg protein per lane) by SDS–PAGE (12%). After electrophoresis, proteins were transferred to an Immobilon-P PVDF membrane (Millipore Corporation, Bedford, MA). Primary antibody was anti-2,4-dinitrophenol (DNP) developed in rabbit (DAKO A/S Denmark) at a 1:100 000 dilution. A goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (DAKO A/S Denmark) (1:5000) was used to estimate the relative amount of protein oxidation, using ECL + plus Western blotting detection system (Amersham Biosciences, England). Densitometric analyses of the immunoblots were performed and intensities normalized by protein content (Coomassie blue staining).

2.6. Protein determination

Protein content was determined using the Coomassie plus protein assay reagent (Pierce®, Rockford, IL). Antioxidant enzyme activities and MDA were expressed per mg of protein.

3. Results

3.1. ROS production

ROS production was studied in T14487C/ND6 mutant cybrids using the cell-permeate probe DCFH-DA. ROS production was studied in T14487C/ND6 mutant cybrids using the cell-permeate probe DCFH-DA. DCFH reacts very fast with endogenous reactive intermediates, including superoxide anions, hydrogen peroxide and hydroxyl radicals in the moment that the assay is performed. DCFH reacts very fast with endogenous reactive intermediates, including superoxide anions, hydrogen peroxide and hydroxyl radicals in the moment that the assay is performed. Fig. 1 shows that ROS production significantly increased in the transmitochondrial cell line harboring homoplasmic levels of the mutation ($P < 0.001$). A 1.32-fold increase in hydrogen peroxide ROS production was observed when compared to controls (1.28 vs. 1.69 fmols H_2O_2 produced/min cell).

3.2. Antioxidant enzyme activities

Fig. 2 shows the results of the antioxidant enzyme activities. Panel A shows total SOD and mitochondrial SOD (Mn-SOD) activities, panel B CAT activity and panel C total GSH-Px and Se-GSH-Px form. There were no significant differences in any of the antioxidant enzymes analyzed ($P > 0.05$).

3.3. Lipid, mtDNA and protein oxidation

In this cell model, oxidative damage to lipid, mtDNA and protein was studied. Fig. 3 shows a significant increase in both lipid peroxidation (assessed by the MDA–TBA assay, panel A; control cell line 0.29 ± 0.071 vs. mutant cell line 0.39 ± 0.13 nmolMDA/mg prot), and mtDNA oxidation (measured by QPCR, panel B; control cell line 100 ± 19.1 vs. mutant cell line 74.4 ± 24.6, measured as relative amplification%). However protein oxidation, estimated as the levels of carbonyl group contents in proteins of cell homogenates, was not significantly increased in T14487C/ND6 mutant cybrids (panel C; control cell line 100 ± 22 vs. mutant cell line 127 ± 91, measured as protein oxidation increase%).

4. Discussion

Using a transmitochondrial cell line harboring homoplasmic levels of the T14487C mtDNA mutation, we aimed to study the production of ROS as well as the putative damage produced by these species on cell biomolecules. We previously demonstrated [3] that this mutation causes isolated complex I deficiency in muscle and a virtual abolition of oxygen consumption in the same transmitochondrial cell line. The same mutation has been identified by Ugalde et al. and Lebon et al. in patients with Leigh-like syndrome [4,5] showing that the mutation causes reduction of complex I activity in both patient's fibroblasts and cybrids [4]. Ugalde et al. [4] showed an alteration in levels of fully assembly complex I (measured by blue native electrophoresis) in the patient's fibroblasts and cybrids, suggesting that this mutation may impair complex I assembly and mobility. Here, we show that the production of hydrogen peroxide increases in the transmitochondrial cell line harboring homoplasmic levels of the mutation (1.28 vs. 1.69 fmols H_2O_2 produced/min cell) This is not surprising, as complex I is, with complex III, the major source of ROS in cells [14,15]. Mutations in subunits of complex I or III may lead to a similar situation. The rate of $O_2^{-\bullet}$ formation by the respiratory chain is controlled by mass action, and increases when the electron flow slows down [16]. Defectively assembled complexes of the electronic respiratory chain may lead to this situation.

A study performed in patients harboring mutations in nuclear-encoded complex I subunits showed a significant decrease in the levels of intact complex I, indicating that complex I assembly and/or stability is compromised [17]. Furthermore, in the same study the authors suggest that mutations in complex I can also affect the stability of other respiratory chain complexes; therefore, we cannot rule out the possibility that the T14487C mutation affects not only complex I, but also complex III, increasing the production of ROS, as complexes I and III are the main sources of ROS. This hypothesis has been recently reinforced by the demonstration that complex I assembly is dependent on the assembly of complex III [18].

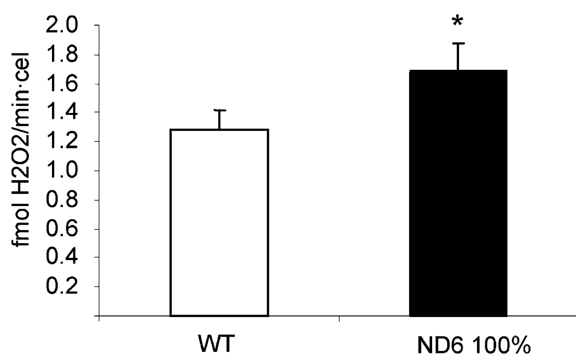

Fig. 1. ROS production in wild type (WT) and mutant cybrid cells lines. Mutant cells show a 1.3-fold increase when compared to the WT cells. Results of three independent assays are depicted. All samples were analysed in duplicates. Mean and standard deviation are represented ($^{*}P < 0.001$; Mann–Whitney test).

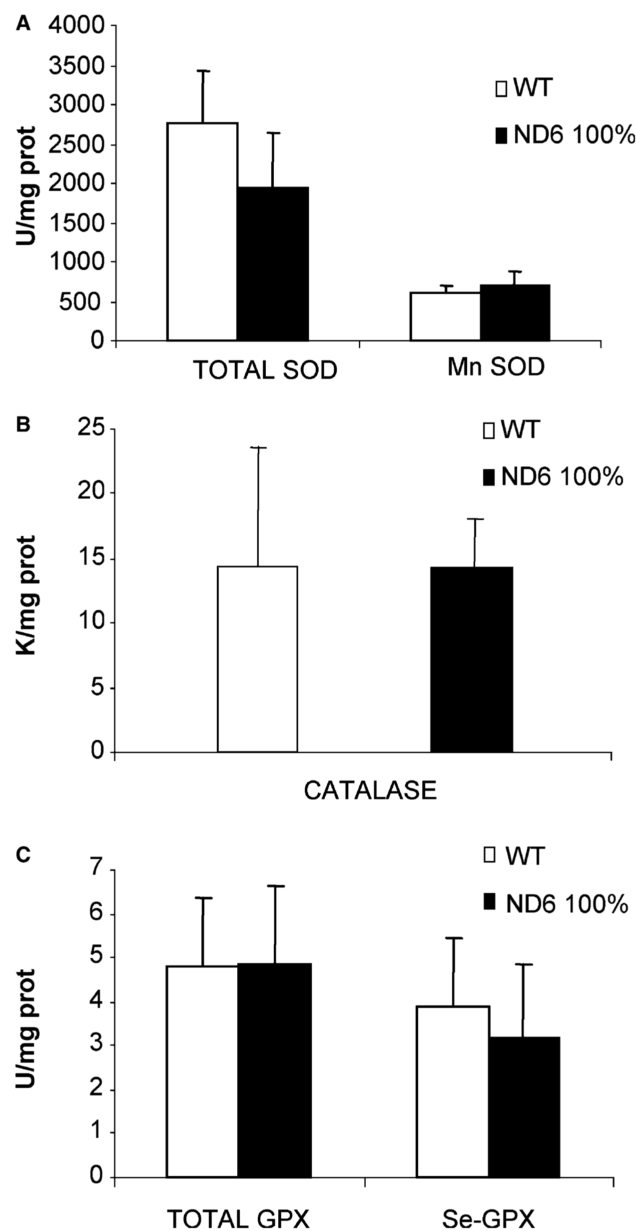

Fig. 2. Antioxidant enzyme activities. Panel A: Activities of total-SOD and Mn-SOD. Panel B: CAT activity. Panel C: Activities of total (GSH-Px) and selenium dependent glutathion peroxidase (SeGSH-Px). No differences were observed in any of the activities of these enzymes between mutated and WT cells ($P > 0.05$). All samples were analysed in duplicates and results of six independent assays are depicted. Mean and standard deviation are represented (Mann–Whitney test).

Direct or indirect evidence of an increase in ROS production due to mtDNA mutations has been extensively studied with the use of electronic transport chain inhibitors [19], however there are only a few works reporting ROS production associated with mutations in the mtDNA using the model of xenomitochondrial cybrids [20,21]. In one of these studies, Rana et al. [21] showed an increase in hydrogen peroxide production in transmitochondrial cybrids harboring a microdeletion in the mitochondrial cytochrome *b* gene. This was the first solid evidence of increase in ROS production due to a mtDNA mutation in a cybrid cell system and the authors suggested that the neurodegeneration features observed in the patient could be related to the damage produced by ROS. More recently, it has been shown that cybrids harboring Leber's disease-related mtDNA mutations have an increase in ROS production [20,22].

We showed in a study on cybrids harbouring mtDNA tRNA mutations (A3243G, A8344G) [23], that an increase in antioxidant enzyme activities is produced in response to an overproduction of ROS in MELAS and MERRF cybrids, preventing oxidative damage. Oxidative damage results from an imbalance between free radical generation and detoxification by antioxidant defences. Here, cybrid cells harboring the 14487/ND6 mutation show overproduction of ROS leading to lipid and mtDNA oxidative damage. This observation, suggests that this damage could be produced by the failure of upregulate the activities of antioxidant enzymes. The reason why ROS generated by complex I deficiency fail to induce upregulation of antioxidant enzymes is not clear. A possible explanation could be the specific site of production of ROS in the electronic transport chain. Therefore, ROS generated only in complex I (as in the 14487/ND6 mutation) are released to the mitochondrial matrix, while ROS generated from complexes I + III (as in the tRNA mutations) are released to both mitochondrial matrix and intermembrane space [14,15].

Floreani et al. [24] showed in cybrids harbouring the 14484/ND6 mutation, no variations in the activity of catalase and GSH-Px. Moreover, Pitkänen et al. [25], using fibroblast cultures with complex I deficiency, demonstrated low levels of MnSOD in cells with high ROS production, and Esposito et al. [26], did not find MnSOD increase in heart mitochondria of *Ant1*-deficient mice. Some hypotheses have been posed to explain this phenomenon, postulating a different tissue regulation in the expression of antioxidant enzymes [26], influence of redox state of the cell in enzyme activation [25] or decrease in antioxidant defenses due to mutations which produces high levels of ROS [24]. Further studies are needed to clarify the antioxidant enzymes regulation in these situations.

A pathogenic role of ROS in mitochondrial disorders has been hypothesized, on the basis of the ability of mitochondria to produce abnormally high levels of these toxic species when the respiratory chain is impaired [26], and also because of the ROS-related clinical phenotypes of some mitochondrial encephalomyopathies [27]. Interestingly, in this study, the

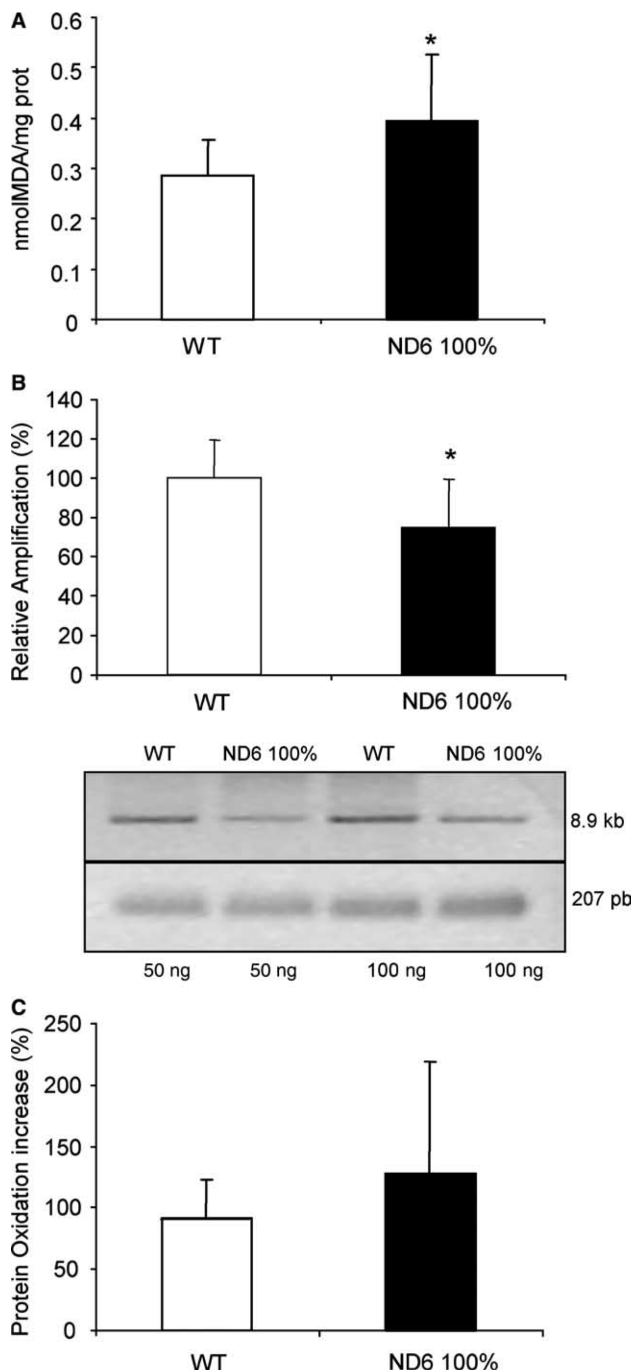

Fig. 3. Oxidative damage to lipids, mtDNA and proteins. Panel A: Lipid peroxidation assessed by the MDA–TBA assay. Mutated cells show higher values of MDA than control cells ($^*P < 0.001$, Mann–Whitney test). Panel B: mtDNA oxidative damage. Mutated cells show higher oxidative damage than control cells ($^*P < 0.05$, Willcoxon test). Upper panel depicts the different amplification efficiency of mutated cell line vs. control cell line. Lower panel shows a representative experiment of a QPCR amplification product of the 8.9 kb and 207 pb fragments. Panel C: Protein oxidative damage assessed by the carbonyl assay. Mutant cells show no significant increase in carbonyl groups.

T14487C mutated transmitochondrial cell line shows not only increases in ROS production but also clear evidence of oxidative damage to cell biomolecules, suggesting a possible contribution of oxidative stress to the pathogenesis of the ND6 mutation.

These results indicate that mutations in mtDNA leading to complex I deficiency may result in an increase in ROS. Therefore, in vivo studies are needed to confirm a putative role of ROS in the clinical presentation of mtDNA-related disorders.

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