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Review

Bioenergetics of mitochondrial diseases associated with mtDNA mutations

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Abstract

This mini-review summarizes our present view of the biochemical alterations associated with mitochondrial DNA (mtDNA) point mutations. Mitochondrial cytopathies caused by mutations of mtDNA are well-known genetic and clinical entities, but the biochemical pathogenic mechanisms are often obscure.

Leber's hereditary optic neuropathy (LHON) is due to three main mutations in genes for complex I subunits. Even if the catalytic activity of complex I is maintained except in cells carrying the 3460/ND1 mutation, in all cases there is a change in sensitivity to complex I inhibitors and an impairment of mitochondrial respiration, eliciting the possibility of generation of reactive oxygen species (ROS) by the complex.

Neurogenic muscle weakness, Ataxia and Retinitis Pigmentosa (NARP), is due to a mutation in the ATPase-6 gene. In NARP patients ATP synthesis is strongly depressed to an extent proportional to the mutation load; nevertheless, ATP hydrolysis and ATP-driven proton translocation are not affected. It is suggested that the NARP mutation affects the ability of the enzyme to couple proton transport to ATP synthesis.

A point mutation in subunit III of cytochrome *c* oxidase is accompanied by a syndrome resembling MELAS: however, no major biochemical defect is found, if we except an enhanced production of ROS. The mechanism of such enhancement is at present unknown.

In this review, we draw attention to a few examples in which the overproduction of ROS might represent a common step in the induction of clinical phenotypes and/or in the progression of several human pathologies associated with mtDNA point mutations.

Keywords: Mitochondrial cytopathy; LHON; NARP; Complex I; ATP synthase; Cytochrome oxidase subunit III

1. Introduction

Mitochondrial diseases (mitochondrial cytopathies) are a heterogeneous group of disorders characterized by dysfunction of mitochondrial oxidative phosphorylation (OXPHOS) and caused by mutations of either nuclear DNA or mitochondrial DNA (mtDNA) [1]. Point mutations in mitochondrial genes encoding for either ribosomal RNA, transfer RNA, or polypeptides are maternally inherited, while large-scale rearrangements of mtDNA (deletions and/or duplications) are usually sporadic. Mutations in the mitochondrial genome have been described in a heterogeneous group of disorders in which the nervous system and the skeletal muscle are predominantly affected.

Diseases due to mtDNA mutations are well-defined clinical syndromes and their genetics is known; however, the pathogenesis and the relation between molecular target of the mutation and clinical phenotype are often obscure [2]. The main biochemical consequence of deletions and pathogenic mtDNA mutations affecting tRNAs is impairment in respiratory capacity of the cell and ATP synthesis, possibly due to defects in the mitochondrial translation mechanism [1]. A bioenergetic defect is generally apparent only above a threshold of mutation load, indicating that the normally synthesized complexes may take over OXPHOS to a sufficient extent for the cellular needs.

In the case of point mutations affecting structural genes, a simple direct biochemical consequence of the protein alteration due to the mutation cannot be usually found. The association of different syndromes with the same mutation, and of different mutations with the same syndrome, has prompted speculation that additional mech-

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anisms such as genetic background, immunological or environmental factors, or even the aging process itself, may be important contributory determinants of phenotypic expression [2]. In addition, there is insufficient knowledge on some basic aspects of mitochondrial structure and function in different tissues, such as the rate-limiting steps and the organization of the OXPHOS system as individual complexes or supercomplexes [3], the role of the respiratory chain in production of reactive oxygen species (ROS) [4], and the role of mitochondrial lesions within the cell environment, in particular the cross-talk between altered mitochondria and nucleus [5]. All of these aspects may be critical in the pathogenesis of the mitochondrial cytopathies and their expression in selected tissues.

Diagnosis of mitochondrial diseases is based on biochemical, morphological and molecular genetic analyses of blood, muscle or skin biopsies. Because it is often difficult to obtain enough material to study pathogenetic mechanisms, patient-derived cell culture models have been used to establish bioenergetic deficits and to analyze the biochemical phenotypes. A cell culture model most extensively used for the study of mitochondrial disorders is the cybrids (cytoplasmic hybrids) system, first described by King and Attardi [6]. Cybrids are generated by fusing enucleated cytoplasts from patients' cells harboring mtDNA mutations with mtDNA-less (ρ°) cells, grown under selection, and subcloned. This technique permits the analysis of cybrids that are isogenic for mtDNA and have a neutral nuclear background. Cybrids may contain not only homoplasmic wild-type and homoplasmic mutant mtDNA, but also different percentages of mutant molecules from the same patient. Several mtDNA mutations have been studied and characterized using this system.

2. Biochemical impairments in Leber's hereditary optic neuropathy (LHON)

Since mtDNA mutations show a wide spectrum of clinical, biochemical, and tissue-specific phenotypes, it is helpful to examine multiple aspects of bioenergetics in culture models to understand their underlying pathophysiology. This involves analysis of check points in energy metabolism, such as (a) the end point of glycolysis at the level of lactate and pyruvate, (b) the terminal step of oxidative phosphorylation, resulting in ATP synthesis, and (c) overall respiratory chain function, as reflected by rates of oxygen consumption. In this study we have analyzed some key biochemical parameters that reflect the global bioenergetic status of LHON cybrid cell lines. This is a maternally inherited form of degeneration of the retinal ganglion cells, leading to loss of central vision [7]. Three frequent, and a few other rare mutations affecting ND subunits of complex I [7,8] are established as pathogenic. The three most frequent mutations are those found at positions G11778A/ND4, G3460A/ND1, and T14484C/ND6.

Biochemical investigations of the three most frequent mutations revealed some biochemical changes in measurable aspects of complex I function that are usually subtle and of modest entity [9]. Only the 3460/ND1 mutation showed a consistent reduction in complex I electron transfer activity (60–80%) [10–12], while both 11778/ND4 and 14484/ND6 mutations had normal or slightly reduced activities [12–14]. Moreover, no differences were usually detected between cells derived from LHON patients and cells from unaffected carriers of LHON mutations [12,13].

Further studies on the sensitivity of the complex to different specific inhibitors reported a reduced sensitivity to rotenone and an enhanced sensitivity to quinol product inhibition, while sensitivity to other complex I inhibitors not interfering with the CoQ binding site, such as rolliniastatin-2 or amytal, was unchanged (Table 1). These results consistently suggest that all three LHON mutations interfere with the interaction of complex I with the ubiquinone substrate (CoQ) [12,13,15,16], indicating that the ubiquinone binding site may be affected by the LHON mutations and/or that ubisemiquinone intermediates may have a reduced stability [17].

Three major consequences may derive from the complex I dysfunction reported in LHON: first, respiratory function may be disturbed at the level of quinol product release because of the impaired electron flow, leading to decreased total respiratory activity; in addition, due to alteration of the hydrophobic quinone binding site(s), proton pumping through complex I may be defective thereby affecting energy conservation; finally, an increase of ROS generation may occur as a consequence of altered electron flow, as reported in the case of nuclear complex I mutations [18], or because unstable ubisemiquinone radicals may rapidly dismutate after reacting with oxygen [17]. Studies are in due course in our laboratory using osteosarcoma-derived cybrids carrying each of the LHON mutations to evaluate NAD- and succinate-dependent respiration and ATP synthesis. Preliminary data seem to indicate that complex I-dependent ATP synthesis is affected by all three mutations, as reflected by the slight reduction of total ATP

Table 1
Complex I redox activity, complex I-dependent respiration and inhibitor sensitivity in LHON patients (see Refs. [12,13,15–17])

	3460/ND1	11778/ND4	14484/ND6
Complex I redox activity	strongly reduced	usually normal	usually normal
K_m for CoQ_2	–	increased	–
NAD-dependent respiration	slight decrease	slight decrease	slight decrease
Inhibitor sensitivity			
Rotenone	decreased	decreased	decreased
Rolliniastatin-2	normal	normal	normal
Amytal	normal	normal	normal
Myxothiazol	–	increased	increased
$NBQH_2$	–	increased	increased

Table 2
Respiratory activities in digitonin-permeabilized cybrids carrying the 11778/ND4 mutation

	Wild-type cybrids		11778/ND4 cybrids	
	Glutamate/Malate	Succinate	Glutamate/Malate	Succinate
	(nmol O_2/min/mg)		(nmol O_2/min/mg)	
State 4	0.030 ± 0.006	0.057 ± 0.015	0.046 ± 0.003	0.080 ± 0.004
State 3	0.111 ± 0.029	0.172 ± 0.029	0.107 ± 0.040	0.180 ± 0.043
RCR	3.7 ± 0.5	3.1 ± 0.5	2.1 ± 0.9*	2.3 ± 0.6

$P < 0.05$.

cellular content observed. Complex II-dependent ATP synthesis does not appear significantly affected.

Analysis of State 4 and State 3 respiration in cybrids carrying the 11778/ND4 mutation reveals that the decrease of respiratory control ratio is due to real uncoupling, with increase of State 4 activity; such uncoupling occurs both with glutamate plus malate and with succinate as substrates, and therefore appears to be a secondary effect (Table 2).

The present results are not in contrast with the currently favored hypothesis that besides an energy defect, overproduction of ROS may represent a major element in LHON pathophysiology [19]. Actually, the uncoupling effect found in the 11778/ND4 mutation with succinate may suggest that the effect is the result of ROS overproduction. Inhibition of complex I with rotenone and other CoQ antagonists induces an enhancement of superoxide radical production by the complex [20]; the site of ROS production is identified in a region upstream of bound ubiquinone, such as FMN [21], or iron sulfur clusters N2 [20] or N1a [22], thus, an enhanced ROS production is likely to occur under conditions when complex I activity is impaired, as in cells carrying the G3460A/ND1 mutation. This hypothesis is supported by the increased ROS generation after partial complex I inhibition [23]. However, the hypothesis is more difficult to reconcile with the 11778 mutation where electron flow is normal. Although ubiquinone does not normally represent a source of ROS in complex I, it was suggested that the destabilization of bound ubiquinone [17] in the complex may enhance the production of ROS by the complex; the apoptotic cell death occurring in LHON cybrids carrying the 11778 mutation, as well as others, incubated in galactose medium [24] may be the result of increased ROS generation. On the long term, bioenergetically compensated cells may suffer from chronic ROS overproduction and the compromised oxidative phosphorylation in LHON may play a major role once the threshold for cell death is crossed [24].

3. Impairment of ATP synthase in NARP syndrome

The Neurogenic muscle weakness, Ataxia and Retinitis Pigmentosa (NARP) syndrome, and the maternally inherited Leigh syndrome (MILS), among others, are both caused by mutations at the 8993 bp in mtDNA [25,26]. The T → G mutation changes Leu-156 with Arg in the ATPase-6 subunit gene (subunit a) of the mitochondrial ATP synthase complex. The variable degree of heteroplasmy is associated with different clinical expressions [25–27].

Coupled submitochondrial particles prepared from platelets of NARP patients showed a dramatic decrease of succinate-driven ATP synthesis rate, but both ATP hydrolysis rate and ATP-driven proton translocation were minimally affected [28]. This implies a different effect of the mutation on the F_0F_1 function and/or an unidirectional impairment of proton flow, only when the enzyme works physiologically (i.e. cytosol to matrix proton flow). There was a close relationship between tissue heteroplasmy, expression of the biochemical defect of ATP synthesis in platelets, and clinical involvement [29]. Furthermore, little variation in heteroplasmy among different tissues in each patient was reported. Fig. 1 shows that the biochemical defect is strictly correlated with the degree of heteroplasmy without any evidence of a biochemical threshold. However, 60% to 75% mutant mtDNA is required for clinical expression of typical central nervous systems symptoms [30,31].

In order to unravel the biochemical mechanism underlying decreased ATP synthesis, we are measuring the proton transport from cytosol to matrix during ATP synthesis in digitonin-permeabilized lymphocytes of NARP patients. The method involves exploitation of the kinetics of Rhodamine-123 fluorescence quenching in presence of succinate and ADP, when protons are both pumped out of the matrix by the respiratory chain complexes and allowed to diffuse back in the matrix through ATP synthase during ATP synthesis; the overall quenching rate is proportional to the steady-state membrane potential under State 3 conditions. The initial rate of quenching is significantly enhanced by oligomycin, which abolishes the ADP-driven potential decrease due to backflow of protons during ATP synthesis [32].

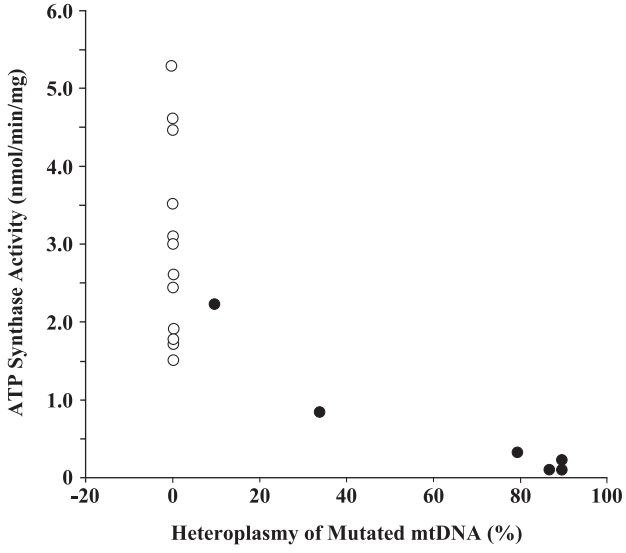

Fig. 1. Correlation of the rate of ATP synthesis with the extent of heteroplasmy in platelets from NARP subjects (cf. Ref. [29]).

Preliminary experiments in lymphocytes from a NARP patient show that the mitochondrial membrane potential, studied as above, is slightly enhanced with respect to controls, suggesting that mutation seems to hardly affect the F_0 permeability to protons. The results are also in line with the previously found normal ATP-driven proton translocation [28]. Furthermore, the reduced backflow of protons might induce substantial overproduction of ROS [33] as an additional defect superimposed to the defect of extremely reduced ATP synthesis, especially when nearly homoplasmic levels of mutant mtDNA are reached: this may account for the shift from the rather mild NARP to the devastating Leigh disease [29].

4. Enhanced ROS generation in cybrids harboring a COX III mtDNA mutation (T9957C) associated with MELAS

A missense mutation in the gene encoding COX III at position 9957 of mtDNA which causes a F251L amino acid substitution, a highly conserved residue among different species, has been reported in a patient affected by mitochondrial encephalomyopathy clinically resembling MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes) [34]. The COX III residue Phe-251 in humans is located in the hydrophobic core of the last transmembrane helix of the subunit [35], and is homologous to Phe-263 in *Paracoccus denitrficans* [34]. When this residue was converted to Leu, there was a reduced growth of the mutant strain, reduction of proton pumping stoichiometry of COX, and reduction of membrane potential [36]. Therefore, an intrinsic uncoupling of COX (reduced efficiency of $\Delta\mu_{H+}$ generation) may be the cause of the mitochondrial dysfunction in the patient harboring the 9957 mtDNA mutation. A role of Subunit III in proton translocation through the D proton pathway has been suggested [37,38]. Alternatively, a defect of COX III might result in altered subunit assembly [39]: there is increasing evidence for a role of subunit III in COX assembly [40–43].

Cybrids produced from the patient's fibroblasts and an osteosarcoma-derived cell line have been kindly provided by Dr. G. Manfredi and investigated in order to enlighten the biochemical defect responsible for the disease.

We found no major decrease of either cytochrome oxidase activity or total respiration nor any change of mitochondrial membrane potential in the cybrids in comparison with the parent osteosarcoma cell line; on the other hand, we did find a significant increase of ROS production by incubation of the cells with dichlorofluorescin diacetate and analyzing the fluorescence of the oxidized product dichlorofluorescein (Fig. 2).

The mechanism of enhanced ROS production in the mutant cells is not clear: in general, ROS are produced by complexes I and III when the electron flow is lowered and the electron carriers are mostly in the reduced state [33].

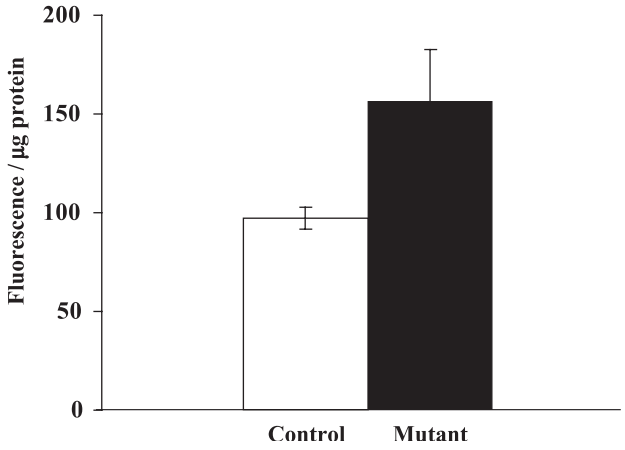

Fig. 2. ROS generation by cybrids carrying the 9957 COX III mutation in comparison with the parent osteosarcoma cell line. ROS were evaluated using the fluorescent probe DCFA [44].

Since no change in oxidation rate of the chain is apparent, this mechanism has little support. On the other hand, no case has been described so far of ROS generation directly at the level of cytochrome oxidase, where the electron transfer to oxygen does not lead to release of partially reduced oxygen intermediates.

5. Conclusions

The elusive nature of the biochemical defects responsible for the clinical phenotype of mtDNA genetic disorders is largely due to technical difficulties in the biochemical characterization of the dysfunctions. The availability of transmitochondrial cell lines (cybrids) greatly facilitates the efforts to unveil the biochemical mechanisms of mitochondrial diseases. Even when no clear alterations are apparent in the simple enzymology of the affected complexes, in-depth analysis may reveal molecular defects responsible for decreased ATP synthesis or enhanced production of ROS. Both such defects may coexist in the same cells, but to different extents in different tissues, thus complicating the understanding of the pathogenic mechanisms of the diseases. Nevertheless, the results discussed in the present mini-review based on data from our and other laboratories are relevant in order to better understand the biochemical mechanisms altered by the mtDNA point mutations described, and might be important to design appropriate therapeutic strategies to at least slow the progression of the mitochondrial diseases.

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