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Review

Bioenergetics shapes cellular death pathways in Leber's hereditary optic neuropathy: a model of mitochondrial neurodegeneration

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Abstract

Leber's hereditary optic neuropathy (LHON) was the first maternally inherited disease to be associated with point mutations in mitochondrial DNA and is now considered the most prevalent mitochondrial disorder. The pathology is characterized by selective loss of ganglion cells in the retina leading to central vision loss and optic atrophy, prevalently in young males. The pathogenic mtDNA point mutations for LHON affect complex I with the double effect of lowering the ATP synthesis driven by complex I substrates and increasing oxidative stress chronically.

In this review, we first consider the biochemical changes associated with the proton-translocating NADH-quinone oxidoreductase of mitochondria in cybrid cells carrying the most common LHON mutations. However, the LHON cybrid bioenergetic dysfunction is essentially compensated under normal conditions, i.e. in glucose medium, but is unrevealed by stressful conditions such as growing cybrids in glucose free/galactose medium, which forces cells to rely only on respiratory chain for ATP synthesis. In fact, the second part of this review deals with the investigation of LHON cybrid death pathway in galactose medium. The parallel marked changes in antioxidant enzymes, during the time-course of galactose experiments, also reveal a relevant role played by oxidative stress.

The LHON cybrid model sheds light on the complex interplay amongst the different levels of biochemical consequences deriving from complex I mutations in determining neurodegeneration in LHON, and suggests an unsuspected role of bioenergetics in shaping cell death pathways.
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Keywords: LHON; Mitochondria; Complex I; ATP synthesis; Apoptosis; ROS

1. Leber's hereditary optic neuropathy (LHON): a complex I disease

LHON is an inherited form of acute or subacute loss of central vision affecting predominantly young males [1]. The rapid loss of central vision in one eye is usually painless and followed by similar involvement of the other eye. Visual acuity reaches stable residual values at or below 20/200 within a few months and visual field defect is characterized by a large centro-cecal absolute scotoma. Optic atrophy with permanent severe loss of central vision is the usual endpoint of the disease. This is reflected at the histopathological level by a striking loss of retinal ganglion cells (RGCs) and atrophy of the optic nerve with severe diffuse demyelination. A complete loss of central fibers with various degrees of axonal sparing in the periphery is observed by light microscopy. Larger axon profiles are selectively spared, immersed in the reactive gliotic tissue. Wide variability in myelin thickness characterizes the spared fibers at the ultrastructural level, some axons being almost denuded of myelin sheath. Most of the demyelinated fibers showed mitochondrial accumulation, sometimes completely filling the axonal profile.

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The maternal pattern of inheritance of LHON led to the identification of pathogenic mtDNA point mutations, the three at positions 11778/ND4, 3460/ND1, and 14484/ND6 being the most common worldwide [2–6]. More recently, an array of further rare but truly pathogenic mtDNA point mutations has been described (see Ref. [1] for an updated list), and they all affect different subunits of complex I, the ND6 being a well-recognized hot spot. In most LHON families the mtDNA pathogenic mutation affects all the individuals on the maternal line in a homoplasmic fashion (100% of the mtDNA molecules are mutant); however, not all maternally related individuals develop LHON. Thus, the mtDNA mutation is a necessary but not sufficient condition to determine the pathology and the existence of further genetic determinants, such as nuclear modifying genes, has been largely hypothesized [7] and debated [8]. The variability in penetrance observed in LHON families may also be due to environmental factors, which may act as triggers of the pathology, and tobacco smoking and alcohol consumption are the most likely risk factors [9].

2. Biochemical features of complex I dysfunction and bioenergetic consequences of LHON mutations

The proton-pumping NADH:ubiquinone oxidoreductase, also known as complex I, is the entry point for electrons into the respiratory chains of many bacteria and mitochondria of most eukaryotes [10,11]. It couples electron transfer with the translocation of protons across the membrane, thus contributing to the proton motive force essential for energy-consuming processes. The current knowledge about human complex I indicates that it is built up by the co-ordinated expression and assemblage of 46 different subunit proteins [12], 39 being encoded by the nuclear DNA and 7 by mtDNA. The available three-dimensional structure of the enzyme reveals a characteristic L shape [10], with the short arm embedded in the inner mitochondrial membrane and the long arm projecting out of the membrane into the mitochondrial matrix (Fig. 1). The seven mtDNA-encoded subunits of complex I (ND1 to ND6, and ND4L) belong all to the membrane embedded domain, the short arm of the L. The amino acid changes induced by the three most common LHON mutations are listed in Table 1.

The biochemical effect of LHON pathogenic mutations has been studied for over a decade and still remains quite controversial (see for discussion the reviews by Brown [13], Howell [14], and Carelli et al. [1]) With the exception of the 3460/ND1 mutation, which consistently decreases the electron transport activity of complex I, the other LHON mutations induce only modest or subtle changes in measurable aspects of complex I function (Table 1). However, all LHON mutations clearly induce a complex I-dependent impairment of mitochondrial respiration.

The initial series of our biochemical investigations on LHON-associated complex I mutations [15–18] failed to show a significant reduction of complex I activity with the 11778/ND4 and 14484/ND6 mutations, although this reduction was evident with the 3460/ND1 mutation. These findings were similar to those reported by others [1,13,14].

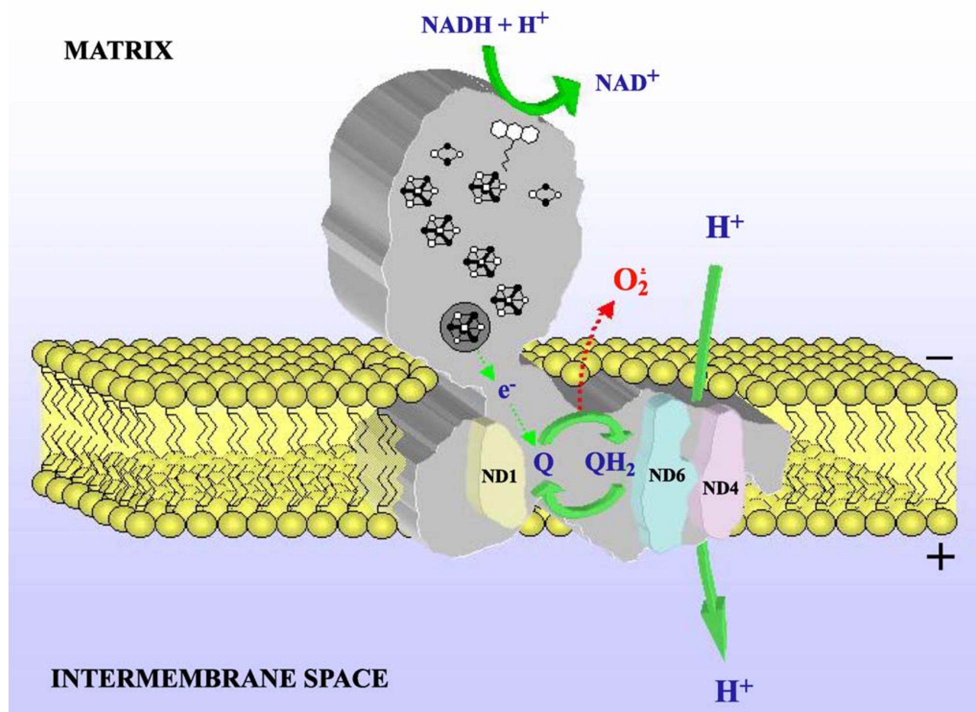

Fig. 1. Complex I model (adapted from Refs. [10,11]).

Table 1
Biochemical features of the most common pathogenic mutations in LHON

nt position	mtDNA gene	Amino acid	Protein domain	Complex I electron transport activity
G3460A	ND1	A 52 T (moderately conserved)	extramembrane loop on the negative side of the membrane	reduced (60–80%)
G11778A	ND4	R 340 H (highly conserved)	transmembrane helix close to the negative side of the membrane	normal or slightly reduced (0–25%)
T14484C	ND6	M 64 V (low conserved)	central part of a transmembrane helix	normal

However, our studies consistently found that mitochondria carrying the 11778/ND4 and 3460/ND1 mutations, and to a lesser extent the 14484/ND6 mutation, had a decreased sensitivity to rotenone, a powerful complex I inhibitor that acts as an antagonist of Q intermediates [15–18]. Rotenone affects the binding of semiquinone intermediates and of the quinol product in complex I. We interpreted these changes in rotenone sensitivity as suggestive of an altered stability of the semiquinone intermediates formed during the catalytic cycle of complex I. The latter terminates with the release of a quinol product derived from controlled dismutation of the semiquinones.

We also evaluated the sensitivity of complex I activity to product inhibitors such as quinols and myxothiazol and found that both the 14484/ND6 and 11778/ND4 LHON mutations induced an increased sensitivity of complex I to myxothiazol and nonyl-benzoquinol [1,18], by analogy with what previously reported in cell lines carrying the rare LHON/dystonia/Leigh 14459/ND6 mutation [19]. A product inhibition was also reported for the 3460/ND1 mutation [20]. Thus, alterations in the affinity for quinol products, as well as resistance to the inhibitory effect of rotenone, consistently suggested that LHON mutations affect the interaction of complex I with the Q substrate [1,13–18].

Based on our results, we originally proposed that both a partial decrease of net energy production and a slight chronic increase of oxidative stress may result from complex I dysfunction due to the mtDNA point mutation pathogenetically associated with LHON [15]. Subsequent studies were undertaken in ours and in other laboratories to evaluate the bioenergetic consequences of the LHON mutations.

Considering the metabolic flux control theory [21], which defines the control that every single step in a pathway has over the global flux of that pathway, the impairment of complex I might result in a variably impaired respiration rate and ultimately in different efficiencies of ATP synthesis [22]. The most extensive study on the respiration of lymphoblast and cybrid mitochondria carrying one of three LHON common mutations reached the conclusion that the 3460/ND1 and 11778/ND4 mutations consistently decreased complex I-driven respiration of 20–28% and 30–36%, respectively, whereas the 14484/ND6 mutation induced a much milder defect, quantified in a 10–15% decreased respiration [23]. However, the authors of this study did not assess the ATP synthesis rate with direct measurements to definitively state whether bioenergetics per se is defective and whether ATP availability to the cell is the primary event causing LHON. Another study, limited to the 11778/ND4 mutation, reported that the energy charge, an index of energy availability in cells, was not decreased in lymphocytes from LHON patients [24].

The issue of ATP synthesis and total ATP cellular content in LHON has been partially addressed in a few studies [20,25,26], and it has now been systematically investigated in our laboratory using the cybrid cell model, expanding to all the common LHON mutations and considering the possible compensatory mechanisms operated by complex I-independent substrates (Baracca et al., 2004, submitted for publication). Combining our results and others', we can now state that the complex I-driven ATP synthesis is severely affected with all three common LHON mutations. However, there is also evidence that cells may effectively compensate, mainly through ATP synthesis sustained by alternative pathways (through glycolysis and complex II/glycerol 3-phosphate dehydrogenase), in most human tissues. This set of results on cells, either primary cultures from patients or constructed cybrids, fits with the in vivo results obtained using the ^{31}P magnetic resonance spectroscopy (MRS) in LHON patients, indicating a defective ATP synthesis in skeletal muscle and/or brain [27,28].

Taken all into consideration, it is likely that besides the bioenergetic deficit, other molecular mechanisms contribute to the clinical onset of LHON. Impaired re-oxidation of NADH which may occur in LHON cells would result in increased [NADH]/[NAD^+] ratio, which induces per se decreased pH and increases synthesis of lactate, further lowering the pH. This scenario may exert possible secondary effects on metabolism, particularly when a high-energy demand occurs, and glycolysis rate is increased in highly energy demanding cells. Decreased pH might also synergistically affect the redox centres of the respiratory chain, further sustaining reactive oxygen species (ROS) overproduction, as initially proposed [15], and recently shown to occur in neuronal LHON cybrids [29].

3. Cell death in LHON

It is well established that cells with defects of mitochondrial respiration are able to keep up the energy charge and maintain normal growth rate in glucose containing media. However, severe growth impairment and increased rate of cell death occur when glucose in the growth medium is replaced by galactose [30]. Under this condition, the slow

metabolism of galactose to glucose-1-phosphate is not sufficient for the cells to synthesize the bulk of ATP requirement by glycolysis, and cells are forced to rely on respiratory chain for ATP synthesis. Impairment of cell growth in galactose medium has also been reported in cybrids bearing LHON mutations [31]. Furthermore, we have recently reported that incubation of LHON cybrids, but not of 143B.TK$^-$ parental cell line or of control cybrids, in galactose medium caused cell death characterized by the typical hallmarks of apoptosis, such as changes in nuclear morphology, chromatin condensation and fragmentation of chromosomal DNA [32]. The occurrence of a significant release of cytochrome *c* into the cytosol clearly indicated a mitochondria-dependent cell death pathway [32]. Increased sensitivity to cell death has also been reported in the same LHON cybrid clones after treatment with Fas, a well-known activator of the Fas ligand death receptor pathway [33]. Given that the only relevant difference between LHON cybrids and control cybrids is the mtDNA carrying the LHON pathogenic mutations, it is reasonable to suggest that alteration in complex I structure or function may result in the increased sensitivity to apoptotic cell death.

The hypothesis that a bioenergetic defect plays a critical role in the pathophysiology of LHON has been strengthened by our recent findings showing that the total cellular ATP content of LHON cybrids, which is not significantly different from controls in glucose medium, dramatically decreased during the early times of incubation in galactose medium. Indeed, ATP levels were reduced in all LHON cybrid clones to approximately 30–50% after 3-h incubation and to less than 10% after 24 h, whereas the ATP content of the parental 143B cell line was not affected [34].

The inability of LHON cybrids to maintain their ATP content during incubation in galactose medium is likely to shape the pathway of cell death. In fact, it is well known that different types of programmed cell death can be triggered by mitochondria. In general, the efflux of cytochrome *c* from these organelles is a pivotal event in the classical apoptotic death, since it drives the assembly in the cytoplasm of the apoptosome, a high molecular weight caspase-activating complex. In fact, binding of cytochrome *c* to Apaf-1 promotes its oligomerization, and recruitment of caspase-9 into a multimeric Apaf-1–caspase-9 complex, which results in caspase-9 activation. Caspase-9, in turn, directly processes and activates the effector caspase-3 and -7 [35,36]. Noticeably, cleavage of inhibitor of caspase-activated deoxyribonuclease (I-CAD) by caspase-3 causes CAD release, which enters the nucleus to trigger both chromosomal DNA fragmentation and chromatin condensation [37,38]. The scheme shown in Fig. 2 summarizes the pathway of caspase-mediated apoptotic cell death.

Despite we reported a significant release of cytochrome *c* from mitochondria in LHON cybrids [32], we have found

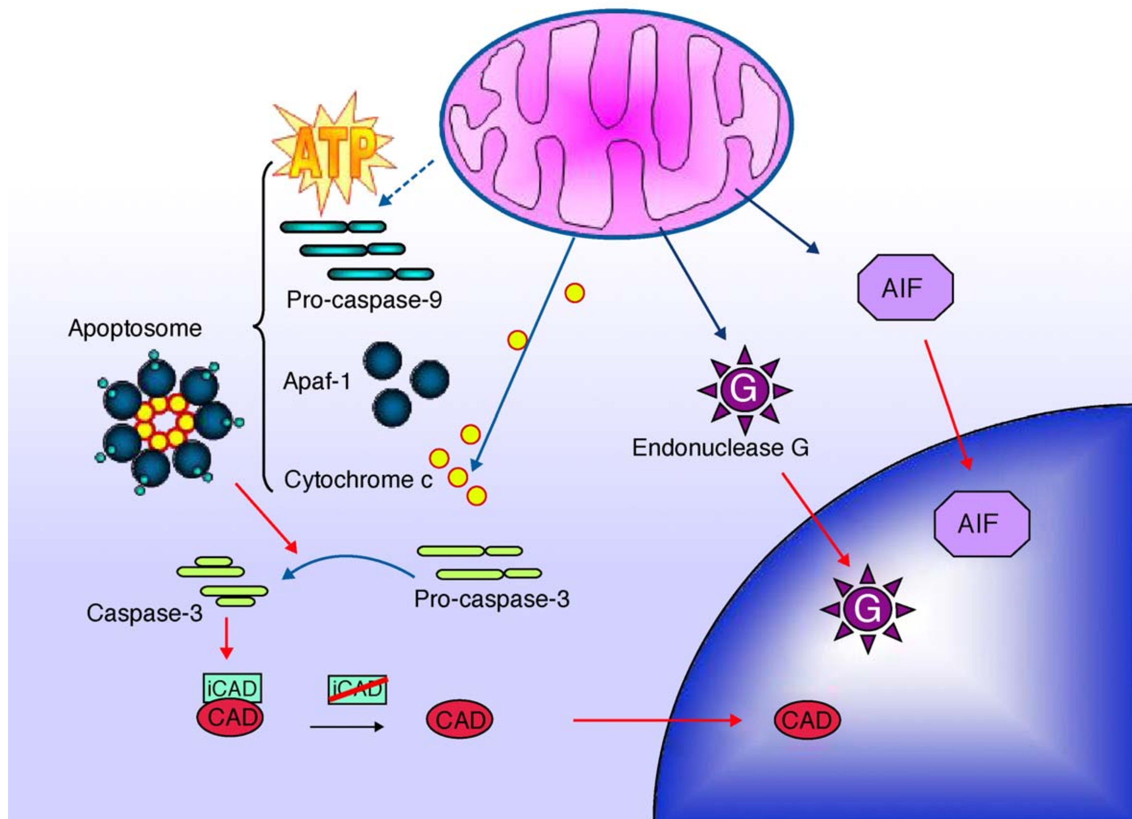

Fig. 2. Apoptotic pathways driven by mitochondria.

that the galactose-induced death process was caspase-independent. In fact, no activation of DEVDase activity or cleavage of procaspase-3 by Western blot could be determined [34]. Furthermore, pretreatment with the pan-caspase-inhibitor z-VAD.fmk (100 μM) was unable to increase the viability of cells incubated in galactose medium, whereas it completely abolished the caspase-3 mediated cell death triggered by staurosporine (Zanna et al., 2004 submitted for publication). Given that both cytochrome *c* and ATP are required for the assembly of apoptosome and subsequent activation of caspase-9 and -3, it is likely that the drastic reduction of ATP levels would impair the formation of the oligomeric complex and consequently the activation of the caspases cascade.

The presence of nuclear fragmentation in the absence of caspase activation suggests the possibility of alternative pathways of cell death [39]. One of these pathways is that mediated by apoptosis inducing factor (AIF) and endonuclease G (EndoG), two apoptogenic factors present in the mitochondrial intermembrane space that translocate to the nucleus upon induction of apoptosis (see also Fig. 2). Both EndoG and AIF have been reported to cause nuclear DNA cleavage and fragmentation [40,41]. Studies are in progress to assess whether these apoptogenic factors are also released from mitochondria during the metabolic stress-induced death of LHON cybrids.

The galactose model developed with LHON cybrids may be extremely helpful, in the absence of a faithful and reliable animal model for this disease, to simulate the pathogenic cascade which may occur in vivo during the acute phase of the pathological process when RGCs rapidly undergo a massive wave of cell death. This model may be instrumental to test rescue strategies that may form the basis for pharmacological neuroprotection.

4. LHON mutations and ROS

One important mechanism by which a defective mitochondrial respiratory chain may lead to cell damage and to final commitment to apoptotic death is the overproduction of ROS, which are common byproducts of oxidative reactions occurring within the mitochondria. A major site of ROS production (superoxide) at the respiratory chain level is complex I [42]. Superoxide is rapidly converted to H_2O_2 by superoxide dismutase (SOD), and H_2O_2 in the presence of reduced transition metals, such as Fe^{2+}, may either undergo further reduction to a very reactive and toxic hydroxyl radical through Fenton reaction, or be detoxified by several lines of antioxidant defenses: catalase, glutathione and glutathione cycling enzymes peroxydase (GPx) and reductase (GRH), scavenging molecules like peroxiredoxins, vitamins A, C, and E or even the ambivalent coenzyme Q, itself a possible culprit of ROS production.

ROS overproduction has been frequently reported in cells harboring mtDNA pathogenic mutations [43,44], particularly when complex I dysfunction is implicated [45,46] like in the recently established model of xenomitochondrial cybrids [47,48]. A role for ROS in the pathogenesis of LHON has been initially proposed [15], and mounting evidence of their involvement is now being accumulated [29,49,50].

We also investigated ROS production in our collection of LHON cybrids. Basal ROS production in cybrids harboring the three most common LHON pathogenic mutations was modestly increased (Ref. [51]), or even undistinguishable from controls [29]. There are indications that the antioxidant defenses are activated, but still sufficient to balance the subtle mitochondrial dysfunction. This was demonstrated by the slight decrease in GSH content and by the changes in SOD expression [51], compatible with normal cell viability in nonselective conditions [52]. A drastic change occurs when cells are stressed or under increased metabolic demand. Under such conditions, ROS production sensibly increases, antioxidant defenses collapse, growth capacity declines, and cell death with the hallmarks of apoptosis takes place (Refs. [32,53], Floreani et al., submitted for publication).

The direct measurement of ROS may be tricky, and the methods currently available all have some drawback. Keeping this caveat in mind, a significant increase in ROS generation has been indeed observed in NT2 neuronal differentiated LHON cybrids carrying the 11778/ND4 and 3460/ND1 mutations [29]. This finding was confirmed using a different detection method (dichlorofluoroscein) in osteosarcoma cybrids carrying the 11778/ND4, 3460/ND1, or the 14484 LHON mutations (Ref. [53]).

We are currently investigating soluble and enzymatic antioxidant defenses in LHON cybrids grown either in glucose rich medium, a condition in which cybrids rely mostly on anaerobic metabolism for energy production [54], or in glucose-free/galactose supplemented medium, where cybrids are forced to rely upon the respiratory chain for ATP synthesis. In glucose medium, the soluble antioxidant defenses of cybrids are not different between LHON and controls, whereas GRH and GPx activities were lower in LHON cybrids carrying the 3460 and the 11778 mutations. Since cybrids with LHON mutations and controls all share an identical nuclear genome, the observed changes are epigenetic, and most likely are the result of a posttranslational inactivation caused by increased chronic ROS production. These features become rapidly significant in LHON cybrids during the time course experiments in galactose medium (Floreani et al., submitted for publication). Thus, we have evidence that drastic changes in the mitochondrial antioxidant enzymatic machinery occur under the stressful conditions imposed by the galactose model, suggesting a possible burst of oxidative stress, which may be implicated in the apoptotic cell death observed in LHON cybrids under these conditions [29].

We need to mention a further interesting implication of the chronic ROS overproduction associated with the LHON

pathogenic mutations. The 143B.TK-osteosarcoma parental cell line, from which cybrids are derived, was recently found to express the EAAT1/GLAST glutamate transporter and the glutamate uptake was significantly reduced in all LHON cybrids compared with control cybrid [54]. This reduction strongly correlated, in a mutation-specific fashion, with the degree of enhanced mitochondrial production of ROS [55]. This observation may become relevant in the context of the inner retina where the Muller cells, which prevent RGCs from excitotoxicity, carry the EAAT1 as the major source of glutamate removal.

5. Conclusions and perspectives

The biochemical effect of LHON mutations revealed to be complex and not univocally interpreted [1,13,14]. The interplay of bioenergetics and oxidative stress with anatomo-physiological tissue-specific features may determine unusual consequences on cell viability, and influence the specific pathways of neuronal cell death. RGCs, the target tissue in LHON, are functionally skewed with high-energy demand in the initial unmyelinated portion of the axons, while the retrobulbar portion of the nerve, which is myelinated and action potentials are saltatory, has a lower energy requirement [1]. Therefore, high numbers of mitochondrial population characterize the first portion, whereas only a few docked mitochondria under nodes of Ranvier are present in the retrobulbar part of the nerve [56]. The axonal transport of mitochondria, a process fueled by mitochondrial ATP, and mitochondrial distribution in axons seem a crucial step for the correct function of the system [57]. Thus, any bioenergetic defect may potentially be harmful either on action potential conduction and/or mitochondrial transport and cell viability [1].

On the other hand, excess of oxidative stress may result damaging on different cell types, like oligodendrocytes providing the myelination of RGCs axons [1]. A chronic impairment of myelin turnover may worsen axonal function and metabolism. The acute loss of visual function in LHON patients suggests that once a threshold is crossed a rapid wave of cell death occurs, possibly due to the triggering of apoptotic pathways, which selectively affect the RGCs viability. Our cellular model in galactose may resemble this last series of molecular events, and we showed how a rapid loss of the bioenergetic cell balance under these circumstances may become relevant in directing the cell death modality, with the possible contribution of an oxidative stress burst.

The LHON cybrid cell model in galactose will be instrumental to rescue experiments, testing each of the different aspects of the pathophysiology, i.e. correcting the complex I-driven defect of ATP synthesis and/or buffering ROS production and/or interfering with the apoptotic pathway. Preliminary results in our laboratory are encouraging towards the design of appropriate therapies rescuing cell death in our cell model.

Besides, the results obtained from such studies on cells from LHON patients will provide valuable information on fundamental aspects of bioenergetics in eukaryotes. Detailed biochemical investigations of the altered complex I carrying the amino acid replacements induced by the LHON mtDNA mutations will certainly shed further light on the role of ND subunits, of the specific amino acid positions and of their chemico-physical characteristics, all relevant information for the enzyme structure and function. At present, this achievement cannot be reached in other ways, and it allows also a comparative analysis of the eukaryotic amino acid changes with those in bacteria, easily obtainable by means of site-directed mutagenesis.

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