Human NADH:ubiquinone oxidoreductase deficiency: radical changes in mitochondrial morphology?

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¹Department of Membrane Biochemistry, Nijmegen Centre for Molecular Life Sciences, and ²Department of Paediatrics, Nijmegen Centre for Mitochondrial Disorders, and ³Microscopical Imaging Centre of the Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

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KOOPMAN WJ, VERKAART S, VISCH HJ, VAN EMST-DE VRIES S, NIJTMANS LG, SMEITINK JA, WILLEMS PH. Human NADH:ubiquinone oxidoreductase deficiency: radical changes in mitochondrial morphology? Am J Physiol Cell Physiol 293: C22–C29, 2007. First published April 11, 2007; doi:10.1152/ajpcell.00194.2006.—Malfunction of NADH:ubiquinone oxidoreductase or complex I (CI), the first and largest complex of the mitochondrial oxidative phosphorylation system, has been implicated in a wide variety of human disorders. To demonstrate a quantitative relationship between CI amount and activity and mitochondrial shape and cellular reactive oxygen species (ROS) levels, we recently combined native electrophoresis and confocal and video microscopy of dermal fibroblasts of healthy control subjects and children with isolated CI deficiency. Individual mitochondria appeared fragmented and/or less branched in patient fibroblasts with a severely reduced CI amount and activity (class I), whereas patient cells in which these latter parameters were only moderately reduced displayed a normal mitochondrial morphology (class II). Moreover, cellular ROS levels were significantly more increased in class I compared with class II cells. We propose a mechanism in which a mutation-induced decrease in the cellular amount and activity of CI leads to enhanced ROS levels, which, in turn, induce mitochondrial fragmentation when not appropriately counterbalanced by the cell’s antioxidant defense systems.

MITOCHONDRIAL FUNCTION AND COMPLEX I DEFICIENCY

Mitochondria produce the large majority of cellular ATP during aerobic respiration; harbor essential parts of the urea cycle; and are crucial for the breakdown of fatty acids and generation of heat and biosynthesis of heme, pyrimidines, amino acids, phospholipids, and nucleotides (19, 36). Additionally, mitochondria are key players in apoptosis (1, 15, 57), innate immune defense (51), generation of reactive nitrogen species, biosynthesis of heme, pyrimidines, and NADH dehydrogenase (ND) subunits (NDUFS1, NDUFS2, NDUFS3, NDUFS4, NDUFS5, NDUFS6, NDUFS7, and NDUFS8), and seven hydrophobic protein subunits (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6). All NADH dehydrogenase (ND) subunits are mitochondrial DNA encoded, whereas the remainder are encoded by the nuclear genome (53). Proper assembly and function of the OXPHOS system further requires at least 40 ancillary nuclear-encoded proteins (17).

CI consists of 45 different subunits, together having a molecular mass of close to 1 MDa (10). The catalytic core of CI consists of 14 evolutionary conserved proteins (8). These core subunits have been classified as part of a flavoprotein, iron-sulfur protein, or hydrophobic protein fraction. The CI core consists of two flavoprotein subunits (encoded by the \( \text{NDUFV1} \) and \( \text{NDUFV2} \) genes in humans), five iron-sulfur protein subunits (\( \text{NDUFS1} \), \( \text{NDUFS2} \), \( \text{NDUFS3} \), \( \text{NDUFS7} \), and \( \text{NDUFS8} \)), and seven hydrophobic protein subunits (\( \text{ND1} \) to \( \text{ND6} \) and \( \text{ND4L} \)). All NADH dehydrogenase (ND) subunits are mitochondrial DNA encoded, whereas the remainder are encoded by the nuclear genome. Assembly and maintenance of this large multiprotein complex requires assistance of specific factors, such as the recently discovered \( \text{NDUFAF1} \) (65), \( \text{B17.2L} \) (40), and \( \text{Ecsit} \) (66). In most cases, CI deficiency is caused by autosomal recessive mutations involving subunits (\( \text{ND1} \), \( \text{ND2} \), \( \text{ND3} \), \( \text{ND4} \), \( \text{ND4L} \), \( \text{ND5} \), and \( \text{ND6} \)), resulting in a complex multisystem syndromes (17, 53, 56, 72). Inherited disorders of the OXPHOS system are observed once every 10,000 live births and usually occur within the first 2 yr of life. In 40% of these cases, the decrease in OXPHOS activity is associated with an isolated (25% of the cases) or combined (15%) deficiency (OMIM 252010) of CI (NADH:ubiquinone oxidoreductase; EC 1.6.5.3), the first and largest complex of the OXPHOS system (55). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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function at the molecular level. Rather than using overexpression and/or downregulation of relevant proteins, we use cells derived from healthy subjects and patients with inherited diseases of the OXPHOS system, with particular emphasis on nuclear inherited CI deficiency.

DOES CI DEFICIENCY ALTER MITOCHONDRIAL SHAPE AND/OR NUMBER?

Mitochondrial shape and size are significantly influenced by the cell’s developmental and differentiation stage, cell cycle phase, mitochondrial DNA content, and metabolic state (11, 19–21, 26, 49, 69). The first question we asked ourselves was whether the reduction in cellular CI activity was accompanied by a change in mitochondrial shape and/or number. To this end, we developed an automated protocol for the quantitative analysis of mitochondrial morphology in living cells by video rate confocal imaging of cells stained with the fluorescent cation rhodamine 123 (29, 31). This approach revealed marked differences in mitochondrial form factor \( F \) (a combined measure of mitochondrial length and degree of branching) and the number of mitochondria per cell \( N_c \) between patient fibroblasts (Table 1; Ref. 30). In sharp contrast, no significant differences were observed between control cells. The fact that some patient cells displayed an increased \( N_c \) and normal \( F \) (i.e., nos. 4608, 5175, and 5171) suggests that individual mitochondria are more elongated. To demonstrate the latter, patient cells were transduced with mitochondria-targeted enhanced yellow fluorescence protein (mito-EYFP) using a baculoviral vector and subsequently subjected to FRAP (fluorescence recovery after photobleaching) analysis. This revealed that mito-EYFP fluorescence rapidly reappeared in the area of the organelle that received the bleach pulse, whereas, at the same time, fluorescence decreased in the remainder of the mitochondrion (not shown). Therefore, the observed mitochondrial elongation is genuine and does not merely reflect juxtaposition of individual organelles. Relatively large reductions in CI activity occurred in association with a decrease in \( F \) and/or an increase in \( N_c \) (Table 1), suggesting a decrease in mitochondrial mass and/or enhanced fission. On the other hand, moderate reductions in CI activity were found to be associated with an increase in \( F \) and never with a decrease in \( N_c \), clearly pointing to an increase in mitochondrial mass rather than enhanced fusion. Importantly, the data obtained with rhodamine 123 were quantitatively identical to those obtained with mito-EYFP, independent of the affected subunit, not due to alterations in cell cycle phase, and restored upon complementation of the genetic defect by somatic fusion (30).

To describe mitochondrial morphology, we introduced the operational parameter “mitochondrial complexity” (30),

Table 1. Mutations, CI activity, mitochondrial morphology, and ROS levels in control and patient fibroblasts

<table>
<thead>
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<th>Cell Line No.</th>
<th>Mutation</th>
<th>CI Activityb</th>
<th>Mitochondrial Morphology</th>
<th>ROS Levels</th>
<th>Ref. No.</th>
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<td></td>
<td></td>
<td>F</td>
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</tr>
<tr>
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<tr>
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<td>103</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>6173</td>
<td>S1-R557X/D816N</td>
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<td>107±5</td>
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Morphological and reactive oxygen species (ROS) data are given as average ± SE and expressed as percentage of control no. 5120 measured on the same day. Values in parentheses represent the number of individual cells analyzed on at least 2 days. Cell line numbers indicate the designation of the cell lines within the Nijmegen Centre for Mitochondrial Disorders (NCMD). Human subunit designation corresponds to NDUSF1 (S1), NDUSF2 (S2), NDUSF4 (S4), NDUSF7 (S7), NDUSF8 (S8), and NDUSFV1 (V1). Mutations are given at the protein level (aVPEEHI67/VEKSIstop). bEnzymatic activities were measured in mitochondrial-enriched fractions and expressed as percentage of the lowest control value (110–260 mU/μl complex IV; values in bold are below the lowest control value). \( F \) is the degree of mitochondrial branching; \( N_c \) represents the number of mitochondria per cell (29, 31). The ratio between these parameters (\( F/N_c \)) is a measure of mitochondrial complexity (30). Ethidium (Et) fluorescence and the rate of CM-DCF formation represent the level of cellular superoxide and its downstream products, respectively, and are determined in a mitochondrial region (Et) and cytosol (CM-DCF), as described previously (29, 32, 62). Significantly different from ‘control no. 5120 (\( F, N_c \)), significantly lower than ‘control no. 4979 (\( F/N_c \)), or significantly higher than ‘control no. 5119 (Et) and ‘control no. 5118 (CM-DCF): \( P < 0.05 \). CI, complex I; IP, manuscript in preparation; ND, not determined.

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which is given by the ratio of $F$ to $N_c$ ($F/N_c$). Based on this parameter, patient fibroblasts could be divided into two groups (Fig. 1, A and B): one in which the value of $F/N_c$ was significantly lower than that of the lowest control (“class I cells”; shaded bars), and another one in which this value was indistinguishable from control (“class II cells”; open bars). Unsupervised cluster analysis of mitochondrial complexity and biochemical CI activity revealed the same classes (Fig. 1, C and D). In agreement with previous findings, obtained with a subset of patients (30), mitochondrial complexity and CI activity were linearly correlated (Fig. 1D; $R = 0.64; P = 0.003$). On average, class I cells had a lower mitochondrial complexity than class II cells (Fig. 2A), which was due to a significant decrease in $F$ and increase in $N_c$ (Fig. 2, B and C). Moreover, the average amount of fully assembled CI, as determined by blue native gel electrophoresis, and its residual activity were significantly more reduced in class I cells than in class II cells (Fig. 2, D and E). Taken together, these findings suggest that

![Fig. 1. Mitochondrial morphology, complex I (CI) activity/expression, and reactive oxygen species (ROS) handling in control, patient, and rotenone-treated control fibroblasts.](image-url)
the degree of mitochondrial complexity and CI deficiency are mutually dependent.

At the cellular level, mitochondrial complexity is determined by the balance between organelle fusion and fission. In mammals, fusion is mediated by three dynamin-related GTPases: mitofusin 1, mitofusin 2, and the optic atrophy 1 protein (46), whereas fission requires recruitment of another dynamin-related GTPase (dynamin-related protein 1 or Drp1) to the mitochondrial outer membrane. The latter process involves the direct or indirect interaction of Drp1 with the outer mitochondrial membrane protein hFis1. It is tempting to speculate that the CI-deficient condition alters mitochondrial complexity by affecting the expression and/or recruitment of these fission/fusion proteins. Presently, studies investigating this possibility are underway in our laboratory.

**ARE CHANGES IN MITOCHONDRIAL MORPHOLOGY DURING CI DEFICIENCY RELATED TO CELLULAR ROS?**

Previous evidence revealed that NADH-stimulated mitochondrial superoxide production, hydroxyl radicals levels, and aldehydic lipid peroxidation were increased in mitochondrial membranes isolated from skin fibroblasts of patients with CI deficiency (35, 45). Interestingly, chronic exogenous application of hydrogen peroxide increased mitochondrial mass in human lung fibroblasts (33), whereas in cybrid cells containing predominantly mutant mitochondrial DNA [tRNA^{leu(UUR)}], reduced CI activity was accompanied by mitochondria with a less elongated or even dotted appearance (61).

To obtain a quantitative understanding of the relationship between CI deficiency, mitochondrial morphology, and cellular ROS levels, we compared these parameters between control and patient fibroblasts (Table 1; Ref. 62). Superoxide levels were assessed using hydroethidine (HEt), a membrane-permeable derivative of ethidium bromide that is specifically converted by superoxide into 2-hydroxyethidium and ethidium (29). The fluorescent products formed during HEt oxidation accumulated predominantly in the nucleus and a widespread network of tubular structures located within the cytosol (62). Dissipation of the mitochondrial membrane potential by application of the protonophore FCCP [carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone] lead to a rapid decrease in tubular fluorescence, demonstrating that the positively charged HEt oxidation products are retained in a Δψ-dependent manner. The FCCP-induced decrease in tubular fluorescence was mirrored by a concomitant increase in nuclear fluorescence, indicating the translocation of HEt oxidation products from the mitochondria to the nucleus (62). The results obtained with FCCP demonstrate that HEt oxidation products can easily pass mitochondrial membranes. Therefore, it is not possible to make a statement concerning the exact cellular site(s) of HEt oxidation. Digital imaging microscopy revealed that cellular superoxide levels were significantly, but to a variable degree, in-
creased in all but two patient cell lines (Table 1) and inversely related to CI activity ($R = -0.81$, $P < 0.0001$; Ref. 62). The results obtained with HEt were confirmed using the mitochondrial superoxide indicator MitoSOX Red.

Downstream products of superoxide were quantified by monitoring the oxidative conversion of 5- (and -6)-chloromethyl-2',7'-dichlorodihydrofluorescein (CM-H$_2$DCF) into fluorescent 5-(and -6)-chloromethyl-2',7'-dichlorofluorescein (CM-DCF) by video-rate confocal microscopy. We argued previously that formation of CM-DCF in cellular systems can best be considered as a marker of oxidant levels rather than as a direct reporter of a specific ROS/RNS species (32). CM-DCF fluorescence increased linearly in time and displayed zero-order kinetics, indicating that [CM-H$_2$DCF] was not rate-limiting and that the rate of CM-DCF formation was a function of the level of cellular oxidants (32). These levels were increased in all except one patient cell line (Table 1), positively correlated to superoxide levels ($R = 0.74$, $P = 0.002$), and inversely related to CI activity ($R = -0.86$, $P < 0.0001$).

It has been proposed that the increased mitochondrial mass in muscle evoked by chronic exercise lowers the rate of respiration per mitochondrion for any given workload, thus reducing the level of potentially damaging ROS (22). In another study, it was suggested that mitochondrial function directly benefits from a nonfragmented mitochondrial phenotype because this facilitates sharing of intramitochondrial antioxidants, matrix solutes, and ROS-damaged mitochondrial constituents (38). When not appropriately counterbalanced by the cell’s endogenous antioxidant systems (68), ROS can damage proteins like CI, lipids, and mitochondrial DNA, thereby further compromising mitochondrial function (12, 13, 18). Interestingly, fragmented mitochondria were also functionally impaired (44).

Using rat myoblasts and HeLa cells, it was recently demonstrated that, during high- and low-glucose conditions, ROS production reversibly increased and decreased, respectively (71). Interestingly, the increase in ROS production stringently required fragmentation of the mitochondrial network, and it was concluded that mitochondrial fragmentation leads to increased respiration, reflected by increased ROS generation. Using an inducible overexpression system (T-Rex HeLa Drp1 cells), we observed that Drp1-induced mitochondrial fragmentation ($F/Nc$ reduced by 25%) was not accompanied by a change in CM-DCF fluorescence. This result indicates that mitochondrial fragmentation per se does not lead to increased ROS levels. A recent study using an immortalized normal

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**Fig. 3.** Expression of key mitochondrial proteins in control, patient, and rotenone-treated control cells (CT+ROT). **A:** expression analysis of mitochondrial proteins as determined by Western blotting of whole cell homogenates in a representative set of cell lines. CI-39, CII-70, CIII-core 2, CIV-II, and CV- represent oxidative phosphorylation subunits; porin is a mitochondrial outer membrane protein (a.k.a., voltage-dependent anion channel); and mtHSP70 represents mitochondrial heat shock protein 70 present in the mitochondrial matrix. The typical blot depicted in this panel was obtained using a control cell line (no. 5120; marked by a box) and four patient cell lines (nos. 5170, 5067, 4608, 5175). **B:** average protein expression levels (see also Table 2) in control cells (black bars), class I patients (light gray bars), class II patients (open bars), and control cells cultured in the presence of rotenone (100 nM, 72 h; shaded bars). Average values were expressed as percentage of those obtained in (vehicle-treated) control cells (no. 5120). Significant differences (*$P < 0.05$) with *control cells, *class I cells, and *class II cells. **C:** effect of chronic rotenone treatment (100 nM, 72 h; marked by + signs) on the expression of mitochondrial proteins in control cells (no. 5120). For the CII-70 antibody, the top band represents the specific signal. The blots in **A** and **C** were contrast optimized for visualization purposes.

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Perspectives In Cell Physiology

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hepatocyte cell line showed that chronic (12-h) application of a high concentration of hydrogen peroxide (1 mM) induced mitochondrial fragmentation, whereas lower doses (100–200 μM) resulted in the formation of elongated "giant" mitochondria (70). In agreement with these findings, we found that ROS levels were significantly higher in cells with fragmented mitochondria (class I; Fig. 2, F and G) than in cells with normal mitochondria (class II). At present, it is unclear whether this difference in ROS level is due to enhanced production, decreased detoxification, or a combination of both.

ARE CHANGES IN MITOCHONDRIAL MORPHOLOGY RELATED TO MITOCHONDRIAL PROTEIN EXPRESSION?

Changes in mitochondrial metabolism, structure, and cellular ROS levels are often paralleled by alterations in mitochondrial protein expression (19, 27, 43, 49). To determine how CI deficiency affects the expression of key mitochondrial proteins, we quantified this parameter in 10 representative patient cell lines using quantitative Western blotting of whole cell homogenates (Fig. 3A and Table 2). In agreement with the CI assembly data depicted in Fig. 2E, it was found that the total cellular amount of the CI 39-kDa subunit was significantly more reduced in class I than in class II cells (Fig. 3B). Although no significant difference between class I and II was observed for other proteins [CII-70, CIII-core2, CIV-II, CV-α, porin, and mitochondrial heat shock protein 70 (mtHSP70)], their expression tended to be higher in class II fibroblasts. These data are compatible with the observed increase in mitochondrial complexity in these cells.

CHRONIC ROTENONE TREATMENT: A VALID MODEL FOR CI DEFICIENCY?

In living cells, specific (submaximal) inhibition of CI by rotenone increased radical production, which was paralleled by increased lipid peroxidation, depolarization of ΔΨm, and decreased ATP production (3, 16, 29). To assess to which extent the changes in CI amount, ROS levels, mitochondrial complexity, and mitochondrial protein expression were related to the pathological reduction in CI activity, we chronically treated fibroblasts of a healthy control subject (no. 5120) with rotenone (100 nM, 72 h). It was found that this treatment decreased the residual activity of CI by 80% (Fig. 2D; Ref. 29). The latter was paralleled by an increase in mitochondrial branching (Fig. 2B), complexity (Fig. 2A), and superoxide levels (Fig. 2F; Refs. 29, 62). In contrast, the number of mitochondria per cell (Fig. 2C) and cellular ROS levels (Fig. 2G) were not affected by this treatment. Blue native analysis revealed that chronic treatment with rotenone significantly increased the amount of fully assembled CI (Fig. 2E). In agreement with this result, Western blot analysis of whole cell homogenates showed that the expression of CI-39 was significantly increased (Fig. 3, B and C). On the other hand, rotenone did not alter the expression of CII-70, CIII-core 2, and CIV-II, whereas it increased the amount of CV-α, porin, and mtHSP70 (Fig. 3B). The finding that rotenone increased rather than decreased the amount of fully assembled CI in control cells suggests that the elevated superoxide levels observed in patient fibroblasts primarily result from the reduction in cellular CI activity and are not due to increased electron leakage from mutationally malformed complexes (62).

Importantly, mitoquinone (24), a mitochondria-targeted derivative of coenzyme Q10, prevented the rotenone-induced increase in lipid peroxidation and mitochondrial branching (29). Because mitoquinone has been demonstrated to react mainly with lipid peroxidation products (24, 52), this result suggests that rotenone acts through these products to increase mitochondrial network complexity. In agreement with this idea, it has been reported that CI inhibition induces peroxidation of cardiolipin (42) and that depletion of this mitochondria-specific lipid results in a further decrease in CI activity (41) and increase in ROS production (14).

In conclusion, chronic rotenone treatment of healthy fibroblasts does not fully mimic the alterations observed in fibroblasts of patients with a mutation in a nuclear DNA-encoded CI gene. The important differences are that rotenone does not induce an increase in superoxide-derived ROS and increases rather than decreases the amount of fully assembled CI. However, the rotenone model revealed an adaptive mechanism.

Table 2. Protein expression in (rotenone-treated) control and patient fibroblasts

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<th>Cell Line No.</th>
<th>CI-39</th>
<th>CII-70</th>
<th>CIII-Core 2</th>
<th>CIV-II</th>
<th>CV-α</th>
<th>Porin</th>
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<td>100±15 (9)</td>
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<td>98±11 (7)</td>
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<td>124±20 (3)</td>
<td>126±18 (3)</td>
<td>90±7 (3)</td>
<td>174±59 (3)</td>
<td>93±8 (3)</td>
<td>107±12 (3)</td>
</tr>
<tr>
<td>5077</td>
<td>46±1* (3)</td>
<td>115±18 (3)</td>
<td>106±12 (3)</td>
<td>92±8 (3)</td>
<td>119±28 (3)</td>
<td>98±1 (3)</td>
<td>89±43 (3)</td>
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<tr>
<td>5175</td>
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<td>84±9 (4)</td>
<td>98±11 (4)</td>
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<td>139±28 (4)</td>
<td>216±38* (4)</td>
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<tr>
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<td>130±16 (3)</td>
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<tr>
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<td>81±16 (5)</td>
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<td>96±12 (5)</td>
<td>125±37 (4)</td>
<td>129±16 (5)</td>
<td>205±45* (5)</td>
</tr>
</tbody>
</table>

Cell line numbers indicate the designation of the cell lines within the NCMD (see also Table 1). Pooled CT is the average value obtained from three controls (nos. 4996, 5118, and 5120) expressed as percentage of CT no. 5120. Expression levels were determined by Western blotting of whole cell homogenates and successive integrated optical density analysis. Integrated optical density values were expressed as percentage of CT no. 5120. Numbers in parentheses are number of independent Western blots. Antibodies were obtained from Invitrogen (Breda, The Netherlands) and directed against the 39-kDa subunit of CI (CI-39), the 70-kDa subunit of complex II (CII-70), the core 2 subunit of complex III (CIII-core2), subunit II of complex IV (CIV-II), the α-subunit of complex V (CV-α), mitochondrial porin (or voltage-dependent anion channel), and mitochondrial heat shock protein 70 (mtHSP70). ROT, rotenone. Statistics: *significant differences (P < 0.05) with the pooled control value.
involving an increase in mitochondrial complexity and a selective increase in certain OXPHOS complexes (CI, CV) and other mitochondrial proteins (porin, mtHSP70). Our observation that mitochondrial complexity was normal in class II cells and expression of CV and mtHSP70 was significantly increased might indicate that this adaptive mechanism is also operational in these patient cells.

CONCLUSIONS

In this brief overview, we provide evidence for the existence of two classes of CI-deficient patient fibroblasts, in which the mitochondria have a fragmented (class I) and normal appearance (class II), respectively. Because ROS levels are significantly higher in class I cells than in class II cells, we propose that ROS is an important determinant of mitochondrial shape. The latter may be due to differences in ROS production and/or antioxidant capacity. Presently, we are investigating whether exogenous antioxidants can restore mitochondrial shape and/or function in CI-deficient patient fibroblasts.

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