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Complex II phosphorylation is triggered by unbalanced redox homeostasis in cells lacking complex III

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Abstract

A marked stimulation of complex II enzymatic activity was detected in cybrids bearing a homoplasmic *MTCYB* microdeletion causing disruption of both the activity and the assembly of complex III, but not in cybrids harbouring another *MTCYB* mutation affecting only the complex III activity. Moreover, complex II stimulation was associated with SDHA subunit tyrosine phosphorylation. Despite the lack of detectable hydrogen peroxide production, up-regulation of the levels of mitochondrial antioxidant defenses revealed a significant redox unbalance. This effect was also supported by the finding that treatment with *N*-acetylcysteine dampened the complex II stimulation, SDHA subunit tyrosine phosphorylation, and levels of antioxidant enzymes. In the absence of complex III, the cellular amount of succinate, but not fumarate, was markedly increased, indicating that enhanced activity of complex II is hampered due to the blockage of respiratory electron flow. Thus, we propose that complex II phosphorylation and stimulation of its activity represent a molecular mechanism triggered by perturbation of mitochondrial redox homeostasis due to severe dysfunction of respiratory complexes. Depending on the site and nature of the damage, complex II stimulation can either bypass the energetic deficit as an efficient compensatory mechanism, or be ineffectual, leaving cells to rely on glycolysis for survival.

Keywords

Mitochondria; Respiratory complex II; Complex III dysfunction; *MTCYB* gene mutation; Cytochrome *b*; Succinate

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1. Introduction

The mitochondrial respiratory chain consists of four large multi-subunit complexes (complexes I–IV) embedded within the inner mitochondrial membrane. The electron transport along complexes I (CI), III (CIII) and IV (CIV) is coupled with the extrusion of protons into the mitochondrial intermembrane space. The electrochemical proton gradient that is built drives ATP synthesis during the oxidative phosphorylation (OXPHOS) process. It is known that under normal conditions, respiratory complexes generate small amounts of reactive oxygen species (ROS) [1,2]. However, under conditions limiting the electron flow through the respiratory complexes, partial reduction of oxygen can lead to production of large excesses of superoxide anions ($O_2^{\cdot -}$), which are rapidly converted into hydrogen peroxide (H_2O_2) either spontaneously or through the superoxide dismutase (SOD)-mediated catalysis. ROS overproduction has been linked to pathological conditions, characterized by perturbation of the redox state of the cell that can lead to damaging effects at the level of cellular proteins, lipids and nucleic acids, depending on the amount and chemical nature of ROS produced. Antioxidant enzymes, such as catalase and peroxiredoxins, as well as the low-molecular-weight antioxidant peptide glutathione, can efficiently scavenge ROS and preserve the integrity of cellular components as much as possible [1,2]. Although ROS are dangerous in large quantities, it is becoming appreciated that low amount of mitochondrial ROS production has advantageous cellular effects, acting as important intracellular signals [3]. Furthermore, there is growing interest on the concept that a small amount of sub-lethal mitochondrial stress can induce an adaptive response as long-lasting metabolic and biochemical changes. This process, termed pre-conditioning or mitohormesis, is not harmful, and seems to render cells less susceptible to subsequent pathological perturbations [4].

Considering the central role of mitochondria in cellular energy production and in metabolic interconnections, it is not surprising that genetic defects in the mitochondrial genome encoding the 13 subunits of the OXPHOS system are often associated with ROS overproduction and redox imbalance. This has been shown to directly impact on the stability of complexes, in particular of CI and CIII [5]. On the other hand, it has been widely demonstrated that organization of these complexes into supra-molecular structures, or supercomplexes, greatly enhances their stability and assembly [5], and also prevents excessive ROS generation [6]. Recent studies showed that the supercomplex association is highly dynamic, and finely tuned by different signaling pathways that are triggered by increased ROS/ H_2O_2 production [7,8], or by mito-energetic dysfunction [9]. This changing organization allows for metabolic compensatory responses that enable mitochondria to promptly sustain the needed levels of cellular ATP.

As a demonstration of this dynamic organization, Acin-Perez et al. [10] recently described a compensatory activation of complex II (CII) in mouse cells with defective CI due to a mutation in the mitochondrial gene encoding the *ND6* subunit. In particular, the tyrosine phosphorylation of the SDHA subunit of CII caused a significant enhancement of its enzymatic activity, which was shown to depend on H_2O_2 -triggered activation of the Src-type tyrosine kinase Fgr, known to be located in the mitochondrial matrix [11]. Remarkably, this CII activation was reported to be specific for CI-deficient cells, because it was not detected

in cells lacking CIII or CIV. Accordingly, CII stimulation was proposed to act as a compensatory mechanism for impaired activity of CI, allowing reorganization of the respiratory chain, switching from NADH to FADH₂ oxidation for the delivery of electrons to the quinone pool and downstream respiratory complexes [10].

In the present study, we investigated CII activation that occurs in cells with defective CIII. We exploited a recently described pathogenic *MTCYB* mutation located on cytochrome *b*, the only CIII subunit encoded by mtDNA. This 18-base pair long, in-frame microdeletion was identified in a patient suffering a severe encephalomyopathy, and leads to the absence of six amino acid (I300-P305) residues in the sixth trans-membrane helix of cytochrome *b*. Cybrids bearing this *MTCYB* micro-deletion in homoplasmic state showed an almost complete loss of CIII activity that was associated with a marked increase of CII activity, which is the only respiratory complex fully encoded by nuclear DNA [12]. We inquired whether the stimulation of CII activity in vitro was strictly dependent on CIII dysfunction, and if so, whether it required a severe loss of CIII activity only or also the disruption of CIII assembly. For comparison, we studied cybrids bearing a *MTCYB* missense mutation (m.15579A > G mutation) in homoplasmic state, causing the amino acid substitution p.278Y > C, previously shown to exert strong inhibition of CIII activity without drastic perturbation of its assembly [13]. In addition, we evaluated the CII activation in vivo by measuring the cellular levels of succinate and fumarate, respectively substrate and product of CII, and determined its effect on cell viability under different metabolic stress conditions.

2. Materials and methods

2.1. Chemicals

2,6-Dichloroindophenol (DCIP), 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (DB), succinate, antimycin A, malonate, rotenone, 3-nitropropionic acid (3-NPA), sulforhodamine B (SRB), digitonin, do-decylmaltoside (DDM), *N*-acetylcysteine (NAC) were purchased from Sigma (Milan, Italy). The antibodies were: NDUFA9 and NDUFS3 (CI), subunit SDHA (CII), core2 (CIII) and subunit IV (CIV) from MitoSciences (Eugene, OR, USA), and actin antibodies were from Santa Cruz Biotechnology SC-1615 (Heidelberg, Germany). Secondary antibodies were from Jackson ImmunoResearch Europe Ltd. (Soham, Cambridgeshire, UK).

2.2. Cell lines and culture conditions

In this study cybrids bearing the m.15649–15,666 microdeletion (I300-P305) in heteroplasmy or homoplasmy (80% or 100% mutation load, respectively) or bearing isogenic wild type (WT) mtDNA [12] and cybrids with the homoplasmic missense mutation m.15579A > G (p.278Y > C) and isogenic WT mtDNA [13] were used. Cells were grown in high glucose (25 mM) Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FBS) (Gibco, Invitrogen, Italy), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 mM sodium-pyruvate, in an incubator with a humidified atmosphere of 5% CO₂ at 37 °C. The growth medium of the homoplasmic cells bearing the I300-P305 microdeletion was supplemented with 50 µM uridine. Where indicated, cells were incubated in low glucose (5 mM) DMEM supplemented with 10%

FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin, or in glutamine-free high glucose DMEM with 100 U/ml penicillin, 100 mg/ml streptomycin and 10% FBS.

2.3. Subcellular fractionation

Crude mitochondria were obtained by harvesting approximately 15×10^6 cells in phosphate buffered saline (PBS). After centrifugation, the pellet was suspended in the isolation buffer containing 200 mM D-Mannitol, 70 mM Sucrose, 1 mM EGTA, 10 mM HEPES, pH 7.5, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and homogenized using a glass teflon homogenizer. This and all the subsequent procedures were carried out at 4 °C. Unbroken cells and nuclei were centrifuged at $600 \times g$ for 10 min, and the supernatant was re-centrifuged at $10,000 \times g$ for 20 min. The mitochondrial pellet was re-suspended with the isolation buffer and stored at -80 °C, after determination of protein content [14].

2.4. Spectrophotometric analysis of the activities of respiratory complexes

Frozen/thawed crude mitochondria were incubated in hypotonic buffer containing 50 mM potassium phosphate, pH 7.8, 0.35% bovine serum albumin and 0.3 mM KCN. Enzymatic activities of redox proteins were measured at 37 °C by using a dual-wavelength spectrophotometer (V550 Jasco Europe, Italy). The CI (NADH:DB:DCIP oxidoreductase) and the CIII (antimycin A-sensitive ubiquinol: cytochrome *c* reductase) activities were measured as described in [13]. The CII activity (succinate:DB:DCIP oxidoreductase) was determined in the presence of 60 μ M DCIP (λ : 600 nm; ϵ_{DCIP} : $19.1 \text{ mM}^{-1} \text{ cm}^{-1}$), 70 μ M decyl benzoquinone (DB). After 10 min incubation with 5 mM succinate, DB was added to start the reaction. The malonate (5 mM)-insensitive activity was subtracted from overall activity.

2.5. SDS-PAGE and Western blotting

Mitochondria were solubilized in radioimmuno-precipitation assay (RIPA) buffer, and protein content was measured [14]. 40 μ g of proteins were separated by 12% SDS-PAGE. Gels were transferred on nitrocellulose membrane (BioRad) for 1 h at 100 V. The nitrocellulose membranes were incubated overnight at 4 °C with the indicated antibody. Primary antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies (1:2000). The chemiluminescence signals were revealed using an ECL Western blotting kit (Amersham Bioscience, Buckinghamshire, UK) and measured with Gel Logic 1500 Imaging System, Kodak.

2.6. Blue native PAGE and 2D blue native/SDS PAGE

For respiratory complexes analysis, approximately 10×10^6 cells were incubated with 50 μ g/ml digitonin and mitoplasts were isolated, as in [15]. Mitoplasts were suspended in a buffer containing 750 mM aminocaproic acid, 50 mM bis-tris, pH 7.0, and solubilized with DDM (20%), corresponding to a DDM/protein ratio of 2.5 (g/g). The suspension was incubated on ice for 10 min and then centrifuged at $13,000 \times g$ for 15 min. Samples were loaded on a 4–12% gradient native gel. After electrophoresis, gels were transferred on nitrocellulose membrane and Western blotting was carried out [13] as described above.

For 2D Blue Native/SDS PAGE, lanes were cut out from the first dimension gel and incubated for 90 min with a dissociating solution (1% SDS and 0.1% β -mercaptoethanol). Electrophoresis was then performed onto 10% polyacrylamide gel by using cathode (100 mM Tris-HCl, 100 mM Tricine, 0.1% SDS, pH 8.2) and anode (200 mM Tris-HCl pH 8.9) buffers [13]. Initial voltage was 30 V, which was shifted to 100 V after ~1 h. Gels were transferred onto nitrocellulose membrane and Western blotting carried out as described above.

2.7. Respiratory supercomplexes assembly

Mitoplasts were suspended (5 mg protein/ml) in 150 mM K-acetate, 30 mM HEPES, pH 7.4, 10% glycerol, 1 mM PMSF, 10 mg/ml digitonin and incubated on ice for 30 min. After centrifugation at $600 \times g$ for 3 min at 4 °C, 100 μ g sample was loaded on native 3–12% gradient gel, after addition of 5% Serva G Blue in 750 mM aminocaproic acid [13]. After electrophoresis, gels were processed for CI in-gel-activity (IGA) [16].

2.8. Determination of glutathione content

The GSH and GSSG content of cybrids was determined enzymatically, exactly as described in [17].

2.9. Dicarboxylic acids determination by LC-MS/MS

Approximately 10×10^6 cells were washed with ice-cold PBS, centrifuged and suspended in 1 ml PBS. Aliquots were kept for protein determination [14]. The subsequent procedures were carried out at 4 °C. After centrifugation at $600 \times g$, the supernatant was removed and 1 ml cold methanol/acetonitrile (1:1) was added. The pellet was carefully mixed, centrifuged at $13,000 \times g$ for 5 min, and the supernatant was solved in 1 ml of 0.1% formic acid in water/methanol 95:5 (v/v), sonicated and filtered with a 0.45 μ m syringe filter. Stock standard solution was prepared by dissolving dicarboxylic acids (malic, succinic and fumaric acid) in water, further dilutions for the calibration curve were prepared daily in 0.1% formic acid in water/methanol (95:5) in the concentration range of 0.025–10 μ g/ml. Standards and samples were injected into the liquid chromatography – mass spectrometry (LC-MS) system.

Chromatographic analysis were performed on a Sunfire C18 (2.1 $\times$ 100 mm, 3.5 μ m; Waters) column, using the following gradient elution: 0.2% formic acid in water (A)/methanol (B) 95:5 for 3 min, to A/B 68:32 in 15 min and to A/B 95:5 in 22 min. The column temperature was set at 40 °C. The injection volume was 1 μ l and the flow rate was 0.6 ml/min. LC-MS/MS analysis was performed on a Series200 modular HPLC (PerkinElmer) interfaced with an API 4000-QTrap triple quadrupole (AB-Sciex Framingham, MA, USA) with an electro-spray ionization (ESI) source. MS parameters were optimized by infusing standard solutions at a concentration of 5 μ g/ml into the TurboIonSpray1 source operating in negative ion mode by a syringe infusion pump. The optimized ionization parameters were: 350 °C probe temperature, 30 psi curtain gas, 50 psi nebulizing gas and heating gas, –4500 kV ion spray voltage. Quantitative analysis was carried out in multiple reaction monitoring (MRM) on transitions 134.1/71 m/z for malic acid, 118.1/73 m/z for succinic acid and 116/71 m/z for fumaric acid; de-clustering potential, entrance potential, collision energy and cell exit potential for quantitative and qualitative transitions were optimized for each acid.

Data processing and quantitation were performed by Analyst software (AB-Sciex). Calibration was done by linear regression with a 1/x weighting. The calibration curves were obtained by plotting fragment ion peak area versus the corresponding concentration, expressed as ng/ml. The linear correlation coefficient (r^2) exceeded 0.998, indicating a linear behavior of the response over two order of concentration. The endogenous amounts of the dicarboxylic acids in each sample were back calculated by interpolation on the regression curve.

2.10. Lactate production

Cybrids were seeded in 6-well plates (3×10^5 /well) in high glucose DMEM. After 24 h, aliquots of the growth medium were collected, mixed with 6% perchloric acid, and then incubated on ice for 1 min. After centrifugation at 13,000 rpm at 4 °C for 2 min, aliquots were incubated for 30 min at 37 °C in a buffer containing 320 mM glycine, 320 mM hydrazine, 2.4 mM NAD^+ and 2 U/ml L-lactic dehydrogenase (LDH, Sigma-Aldrich). Lactate concentration was determined spectro-photometrically by measuring the amount of NADH at $\lambda 340$ nm ($\epsilon_{\text{NADH}} 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$). Data were normalized for the number of cells used.

2.11. Cell viability

Cells were seeded in 24-well plates (2×10^4 /well) and incubated as indicated in the figure legend. Cell viability was determined by using the sulforhodamine B (SRB) assay [18].

2.12. Statistical analysis

The results are presented as means $\pm$ SD of at least three independent experiments, unless otherwise indicated. Significant differences were evaluated with the Student *t*-test. Western blot results are representative of at least three different experiments.

3. Results

3.1. Stimulation of CII activity is associated with the physical absence of CIII

CII couples the Krebs cycle to the respiratory chain by oxidizing succinate to fumarate and then transferring electrons to coenzyme Q, hence is also called succinate: coenzyme Q reductase (SQR) [19]. SQR activity was determined in isolated mitochondria by recording spectrophotometrically the reduction of DCIP in the presence of succinate as an electron donor and DB as an electron acceptor. We have previously reported a significant stimulation of SQR activity in human cybrids bearing the 18-bp *MTCYB* I300-P305 homoplasmic *MTCYB* micro-deletion [12]. We first asked whether this CII activity stimulation was strictly dependent on the lack of CIII, so we utilized another cybrid clone developed in our lab, bearing the very same *MTCYB* I300-P305 microdeletion in heteroplasmic conditions, i.e. 80% mutant *MTCYB* [12] (Fig. 1). Cybrids bearing the homoplasmic *MTCYB* microdeletion (100% mutant mtDNA) exhibited a significant reduction of CI activity, compared with control cybrids bearing 100% wild-type (WT) mtDNA (Fig. 1A), whereas the 80% mutant clone showed increased activity, likely as a compensatory mechanism. The CII activity was markedly increased in homoplasmic *MTCYB* microdeletion cells only (Fig. 1A). The CIII enzymatic activity was almost completely absent in the homoplasmic

MTCYB microdeletion clone, and only a partial activity was present in the 80% heteroplasmic clone (Fig. 1A), in agreement with our previous report [12]. In parallel, we also examined cybrids bearing the pathogenic missense *MTCYB* p.278Y > C substitution, previously shown by us to cause the almost complete loss of CIII activity, without any significant perturbation of its assembly [13]. As also shown in Fig. 1A, no stimulation of CII activity was detected in these cells, despite the almost complete absence of CIII activity like the *MTCYB* microdeletion cells. Evaluation of the assembly state of CIII was carried out in dodecyl maltoside (DDM) solubilized mitochondria by Blue Native (BN)-PAGE, which allows separation and characterization of respiratory complexes under non-denaturing conditions [15]. The data showed that the bands corresponding to CIII₂ and CIII₂ + CIV, identified by the anti-Core-2 subunit antibodies, were almost undetectable in cybrids with the homoplasmic *MTCYB* microdeletion, whereas the band corresponding to CIII₂, although of reduced intensity, was present in cybrids with 80% heteroplasmic mutation. In contrast, in cybrids bearing the p.278Y > C, the bands corresponding to CIII₂ and CIII₂ + CIV were clearly detectable, although with slightly lower intensities than their counterparts in the control cells (WT) (Fig. 1B).

A similar behavior of supramolecular organization of the respiratory complexes is also seen in digitonin-solubilized mitochondria by measuring the CI-associated in-gel activity after BN-PAGE [13]. The bands corresponding to the CI-containing supercomplexes (CI + CIII₂, CI + CIII₂ + IV, CI + III₂ + IV_n) and isolated CI were similar in WT and 80% heteroplasmic cybrids with the *MTCYB* microdeletion, whereas in the homoplasmic clone much weaker bands corresponding to CI and CI + III₂ supercomplex were seen. In cybrids bearing the p. 278Y > C mutation, the respiratory supercomplexes were similar to WT (Fig. 1C), in agreement with previously reported data [13].

To conclude, the data suggested that the stimulation of SQR activity was not associated with the impairment of CIII activity only, but rather a complete disappearance of CIII and most of the CIII containing supercomplexes was required.

3.2. SDHA subunit of CII is tyrosine phosphorylated

The stimulation of CII activity was previously associated with tyrosine (Y604) phosphorylation of its SDHA subunit [10]. Thus, we separated SDHA subunit of mitochondrial fractions by SDS-PAGE and probed it with a phosphotyrosine specific antibody. As reported in Fig. 2A and B, the band corresponding to the SDHA subunit was tyrosine phosphorylated in *MTCYB* microdeletion cybrids only, whereas no positive signal was detected in WT and *MTCYB* p.278Y > C substitution cybrids. As a more specific alternative approach, we also separated DDM-solubilized mitochondria isolated from WT and I300-P305 cybrids by BN-PAGE, cut the BN-PAGE lanes, run them on a SDS-PAGE (2D BN/SDS-PAGE analysis) and performed Western blotting. We observed that the SDHA-positive smear seen in *MTCYB* microdeletion cybrids (Fig. 2C) was also positive to anti-P-tyrosine antibody. These results support a mechanism where the stimulation of SQR activity is associated with tyrosine phosphorylation of its SDHA subunit, as previously reported [10].

3.3. *MTCYB* microdeletion is associated with unbalanced redox homeostasis

Given that SDHA subunit tyrosine phosphorylation and CII stimulation have previously been shown to be triggered by H_2O_2 over-production [10], we measured 2',7'-dichloro dihydrofluorescein diacetate (H_2DCFDA) fluorescence in WT and mutant cybrids. We were unable to detect a significant increase in H_2O_2 production in cybrids bearing either of the two *MTCYB* mutations (not shown). Considering the low sensitivity of this method, we determined the content of glutathione, the main intracellular antioxidant known to act in the detoxification of a number of oxidizing species. The absolute values of reduced (GSH) and oxidized (GSSG) glutathione were different among the different cell clones (Table 1). However, there were not significant differences between mutated cells and their respective isogenic WT clones, both in the GSH and GSSG absolute values as well as in the GSSG/GSH + GSSG ratio, which was slightly decreased in cybrids harbouring the *MTCYB* microdeletion. Although these results confirmed the lack of significant ROS overproduction, analysis by Western blotting indicated a general up-regulation of the mitochondrial antioxidant enzymes in cybrids harbouring the *MTCYB* microdeletion (Fig. 3A). This effect became more visible when the intensity of appropriate bands was normalized to that of actin used as a control (Fig. 3B). In particular, the protein level of mitochondrial superoxide dismutase (SOD2) was significantly increased, and that of peroxiredoxin-3 was strongly enhanced. Conversely, the amounts of ROS combating enzymes were not affected in the p. 278Y > C mutant cybrids, except for a tendency to increase SOD2 level, in agreement with our previous reports showing a weak SOD2-sensitive CIII-specific ROS overproduction in these cells [13].

Thus, it appears that despite the lack of measurable H_2O_2 production and GSH perturbation, a significant unbalance of mitochondrial redox homeostasis occurs in cybrids with the homoplasmic *MTCYB* microdeletion. This finding prompted us to test the effect of *N*-acetylcysteine (NAC), a compound that could act as a ROS scavenger, but is also a precursor of glutathione, thus affecting the redox status of the cell [20]. Three days incubation of the homoplasmic *MTCYB* microdeletion cybrids in the presence of 5 mM NAC completely blunted the stimulation of CII activity, without affecting the CII activity of the control cybrids (WT) (Fig. 4A). Moreover, the band corresponding to SDHA subunit was no longer recognized by P-tyrosine antibody, and the protein level of peroxiredoxin-3 of the homoplasmic microdeletion cybrids became similar to controls (Fig. 4B). Altogether these results suggest that the experimental conditions leading to a more reduced intracellular state were able to attenuate the up-regulation of anti-oxidant defenses and the increased SQR activity seen in the absence of CIII in homoplasmic *MTCYB* microdeletion cybrids.

3.4. Severe metabolic perturbation in *MTCYB* mutant cybrids

In order to assess directly the metabolic consequences of CII stimulation in mutant cells, we evaluated the content of succinate and fumarate, which are the substrate and product, respectively of CII, together with malate in cellular extracts, by optimizing a liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) method. Malate was the most abundant dicarboxylic acid in WT cybrids, with its level being approximately two-fold higher than that of succinate or fumarate. In cybrids harbouring the *MTCYB* microdeletion the level of succinate was more than seven-fold higher than that seen

with WT cybrids, whereas the amounts of malate and fuma-rate were significantly decreased (Fig. 5A). This unpredicted massive accumulation of succinate suggested that CII was not functional in cells lacking CIII as a physical entity. It has been recently shown that the major consequence of the absence of CIII is the blockade of CoQH₂ oxidation [8]. Thus, no oxidized CoQ was available to allow CII activity, even though this enzyme was phosphorylated to enhance its activity. It is noteworthy that during the in vitro CII assay DB is provided as an electron acceptor to circumvent the substrate shortage.

We also measured the dicarboxylic acids content in cybrids with the *MTCYB* p.278Y > C substitution, which showed an increased level of succinate (more than five-fold) compared with isogenic WT cybrids (Fig. 5B). The amount of malate also increased (1,5-fold) whereas that of fumarate was unaffected. It therefore appeared that succinate was accumulated due to the strong reduction of CIII activity in these cybrids, although a significant amount of it could still be oxidized to fumarate subsequently yielding malate.

These findings were further confirmed by measurements of the amount of lactate released to the growth medium during the first 24 h of growth, providing a direct indication of a compensatory activation of glycolytic metabolism to cope with severe OXPHOS dysfunction. Lactate produced by cybrids bearing the p.278Y > C substitution was similar to WT cells. However, it was significantly higher in the homoplasmic cybrids bearing the *MTCYB* microdeletion as compared with the 80% heteroplasmic clone and the WT cells (Fig. 5C). This latter result is in line with previous reports showing that mitochondrial dysfunction becomes apparent when the cytochrome *b* content falls below 10% of normal levels [21].

3.5. Inhibition of CII does not affect the viability of cybrids bearing the *MTCYB* microdeletion

In order to further confirm that CII is inactive in cybrids bearing the *MTCYB* microdeletion, we investigated whether pre-incubation with the CII specific inhibitor 3-nitropropionic acid (3-NPA) [22] affected the viability of these cybrids when they are subjected to different metabolic stresses. WT and mutated cybrids were incubated either in high (25 mM) glucose DMEM or in low (5 mM) glucose DMEM, which is known to force the cells to rely more on oxidative metabolism for ATP production. Furthermore, given that many proliferating cell lines exhibit massive utilization of L-glutamine as a major precursor for anaplerosis as well as for synthesis of nucleic acids, proteins, glutathione and NADPH [23], we utilized a L-glutamine-free DMEM supplemented with 25 mM glucose as a metabolic stress condition. As shown in Fig. 6B and C, control cybrids were able to grow in low glucose at a slower rate than high glucose, and in both cases the presence of 3-NPA significantly reduced the number of viable cells in comparison with untreated cells. In glutamine-free DMEM, control cybrids were still able to grow, although at much lower rate. However, the effect of 3-NPA was dramatic as > 80% of cells died after 12 h, indicating that, under this latter growth condition control, cybrids are more strongly dependent on the respiratory chain function for survival than in the earlier conditions. On the other hand, cybrids bearing the homoplasmic *MTCYB* microdeletion grew at a slower pace than the controls in high glucose DMEM, but they underwent cell death in low glucose, and in L-glutamine-free DMEM. However, with these

cybrids no significant survival difference between the absence or presence of 3-NPA was detected in any growth medium (Fig. 6A–C). This finding suggested that inhibition of CII does not affect oxidative ATP production as if it were already inactive in the *MTCYB* microdeletion cells.

Finally, we addressed whether the redox unbalance caused by *MTCYB* microdeletion-dependent mitochondrial dysfunction could provide a functional advantage against an external oxidative insult, via a pre-conditioning mechanism also referred to as mitohormesis [4]. Cybrids were incubated with increasing concentrations of H₂O₂ for 24 h and cell viability determined. As shown in Fig. 6D, the homoplasmic *MTCYB* microdeletion cybrids were more sensitive to the exogenous oxidative stress than their heteroplasmic counterparts or the control cybrids. Thus, the mitochondrial redox unbalance observed here does not seem to enable oxidatively-stressed cells to be less susceptible to subsequent oxidative insults.

4. Discussion

In this study we used our collection of cybrids with different *MTCYB* mutations and bearing different mutation loads to document that stimulation of SQR activity is strictly dependent on the physical absence of CIII via its disassembly and degradation, whereas loss of CIII activity alone, leaving a physically present but inactive CIII is not sufficient. We show that the enhanced SQR activity is associated with tyrosine phosphorylation of its SDHA subunit in cells bearing the homoplasmic *MTCYB* microdeletion. Unlike the previous findings [10], it thus appears that this stimulation is not strictly associated with H₂O₂ over-production, but it rather reflects a subtle unbalance of redox homeostasis.

The homoplasmic *MTCYB* microdeletion cybrids are completely devoid of CIII, and they cannot oxidize succinate via CII, despite its phosphorylation and activation. Yet, CII is highly active in in vitro based on enzymatic assays. Thus, the second substrate of CII, which is the oxidized CoQ, is lacking or strongly reduced in the complete absence of CIII, consequently, the enzymatically active CII cannot contribute to OXPHOS function. We note that this interpretation is also consistent with the lack of any effect of the CII inhibitor, 3-NPA, on the viability of mutant cybrids exposed to different stressful growth conditions. It therefore appears that in this cell model SDHA tyrosine phosphorylation and stimulation of CII enzymatic activity are molecular mechanisms triggered by mitochondrial redox unbalance, possibly unrelated to the compensatory and adaptive responses, which are unable to improve the energetic efficiency and provide beneficial effects against exogenous oxidative stressors via mitohormesis.

The tyrosine phosphorylation of SDHA subunit occurs in homoplasmic *MTCYB* microdeletion cybrids that produce neither detectable amount of H₂O₂ nor GSH/GSSG change, but that exhibit a significant perturbation of cellular redox balance, as indicated by the increased SOD2 and peroxiredoxin-3 protein levels. H₂O₂, which can be produced by the SOD activity, is enzymatically degraded by a large group of antioxidant enzymes, among them peroxiredoxins possess the highest affinity for H₂O₂. Peroxiredoxin-3 is localized exclusively in the mitochondrial matrix, where it is much more abundant than the other

antioxidant enzymes, and is believed to be the main scavenger of mitochondrial H_2O_2 [24]. The identity of the ROS species and their site(s) of production within mitochondria of *MTCYB* microdeletion cybrids require elaborate experimentation and are beyond the scope of this study. In addition to the respiratory complexes, depending on the substrates available, the 2-oxoglutarate dehydrogenase, branched chain 2-oxoacid dehydrogenase and pyruvate dehydrogenase complexes are also capable of producing considerable amounts of superoxide/ H_2O_2 [25]. We note that the cybrids bearing the p.278Y > C *MTCYB* mutation, previously shown to exhibit enhanced CIII-dependent superoxide production [13], do not present any CII stimulation or up-regulation of antioxidant enzymes.

A strong evidence in support of a redox imbalance in the homoplasmic *MTCYB* microdeletion cybrids stems from the results obtained with NAC pre-incubation, which dampened CII enzymatic activity as well as SDHA subunit tyrosine phosphorylation, and restored in particular the wild type levels of peroxyredoxin-3 protein. The beneficial effects of NAC may not only depend on its free radical scavenging activity, but also on its ability to maintain a reduced intracellular environment [20]. In this regard, an elegant redox proteomic analysis recently identified a number of critical cysteine residues located in the iron-sulfur clusters and in the hydrogenase and NADH dehydrogenase modules of CI that can undergo transient oxidation during re-oxygenation after hypoxia [8]. Specific cysteines oxidation has been proposed as a potential molecular mechanism that destabilizes proteins, possibly triggering their degradation, and in turn, inducing complexes/supercomplexes disassembly [8]. We can only speculate that the experimental conditions that promote a more reduced state of critical cysteines within complexes might prove advantageous.

Our accurate analysis of dicarboxylic acids levels revealing a strong accumulation of succinate in both *MTCYB* mutant cybrids, is also in agreement with a previous report in CIII deficient cells [26]. In the latter study, a detailed metabolomics analysis using stable isotope tracing identified high levels of succinate primarily generated through oxidative glutamine metabolism. It is noteworthy that an undetectable level of cytochrome *b* was reported in the previous biochemical characterization of these cells, as expected for an out-of-frame deletion located 40 bp down from the initiator methionine codon [27]. Consequently, CIII assembly was severely impaired and led to significant H_2O_2 overproduction, but no change in CII activity was reported [27].

It is noteworthy that both our *MTCYB* mutant cybrids exhibited elevated succinate levels, but those harbouring the p.278Y > C substitution showed increased malate and normal fumarate, suggesting that some of succinate can be oxidized to fumarate which in turn produces malate. This is in agreement with previous findings, which showed that the overall energetic ability of these cells was only weakly affected, despite the dramatic decrease of CIII activity caused by the p.278Y > C mutation. The deleterious effects of this mutation are mitigated by CIII assembly within the $CI + III_2 + IV_n$ supercomplexes [13], allowing for significant electron transport and CoQ oxidation, which was also indicated by the lack of compensatory stimulation of glycolysis.

To conclude, in the present study we show that in cell lacking CIII due to the homoplasmic *MTCYB* microdeletion, despite SDHA tyrosine phosphorylation and stimulation of CII

activity, CII is not functioning and cannot contribute to improve the OXPHOS efficiency. Therefore, we can envisage two different scenarios depending on whether the cells have a faulty CI or CIII, as outlined in Fig. 7. On the one hand, in the case of CI dysfunction, H₂O₂ overproduction triggers SDHA tyrosine phosphorylation and stimulation of CII activity, allowing for remodelling of the respiratory complexes organization and optimizing cells capability to utilize FADH₂ derived electrons in the presence of CIII. The engagement of this compensatory mechanism permits to overcome the CI dysfunction and maintain a significant OXPHOS efficiency, as proposed by [10]. On the other hand, in the case of CIII deficit, the mild redox unbalance still involves a tyrosine kinase dependent step, leading to SDHA subunit phosphorylation and CII activity stimulation as a response similar to that described above. However, in the complete absence of CIII, this increased CII activity does not provide any improvement of the energetic efficiency, probably due to the extremely limited availability of oxidized CoQ, and leads to succinate accumulation. Accordingly, the severe mitochondrial dysfunction makes these cells heavily dependent on glycolysis for survival, through a mechanism that might directly involve the inhibition of the activity of prolyl hydroxylases by succinate in the cytosol, leading to the pseudo-hypoxic stabilization of HIF1- α [28]. Further studies are required to assess this attractive possibility.

Altogether, these findings highlight the central role of CII, and in particular its SDHA subunit, as a target for oxidative (ROS) derangement, together with other TCA cycle enzymes such as aconitase and 2-oxoglutarate dehydrogenase that are known to be down-regulated by ROS increase [29,30]. This conclusion is in line with previous reports showing that SDHA subunit is also a binding partner and substrate for SIRT3 deacetylase activity [31,32], thereby bridging energetic metabolism to nutrient stress. It seems therefore that CII represents a common target for different post-translational modifications that allow the fine-tuning of the efficiency of OXPHOS and the adaptation of cellular metabolism.

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Fig. 1. Activities, protein levels and assembly of respiratory complexes

A) The CI (NADH:DB:DCIP), CII (succinate:DB: DCIP) and CIII (DBH₂:cytochrome *c*) enzymatic activities were determined in crude mitochondria, isolated from cybrids bearing the heteroplasmic (80%) or homoplasmic (100%) Δ 300-P305 *MTCYB* microdeletion and isogenic control cybrids (WT), and from cybrids bearing the homoplasmic p.278Y > C mutation and isogenic control cybrids, as described in Materials and Methods. The data, expressed as % of activity of respective isogenic WT cybrids, are mean $\pm$ SD of at least six determinations. Asterisks denote values significantly different from controls (* $p < 0,05$ and ** $p < 0,001$).

B) Analysis of CIII containing complexes in DDM-solubilized mitoplasts from the indicated cybrids, after BN-PAGE and Western blotting, by using antibodies against the CIII representative Core 2 subunit; the SDHA subunit of CII was used for normalization. C) Analysis of supercomplexes assembly in digitonin-solubilized mitoplasts after BN-PAGE, by measuring the CI in-gel-activity. Each blot is representative of at least other three similar.

Fig. 2. Analysis of CII SDHA-subunit tyrosine phosphorylation

Mitochondrial fractions isolated from (A) cybrids with the homoplasmic p.278Y > C mutation and isogenic controls (WT) or (B) cybrids with the homoplasmic (100%) *MTCYB* microdeletion and isogenic controls (WT) were separated by SDS-PAGE, as described in Methods, and Western blot analysis was performed using antibodies against SDHA subunit of CII and P-Tyr. The plots are representative of two similar. (C) DDM-solubilized mitoplasts isolated from cybrids with the *MTCYB* microdeletion and isogenic controls were separated by 2D BN/SDS-PAGE as described in Methods section, and Western blot analysis was carried out using antibodies against SDHA and P-Tyr. The plot is representative of two similar.

Fig. 3. Level of antioxidant enzymes

(A) Western-blot analysis of antioxidant enzymes level was carried out in cellular lysates, by using antibodies against catalase, peroxiredoxin 3 and SOD2. Actin was used as a loading control. A representative blot of two similar is shown. (B) In each cell line, the intensity of bands was normalized over that of actin. Data are mean $\pm$ SEM of three experiments. Asterisks denote values significantly different from WT cells: *p < 0,05 and **p < 0,001.

Fig. 4. Effect of pre-incubation with NAC

WT and homoplasmic *MTCYB* microdeletion cybrids were incubated for three days in the absence or presence of 5 mM NAC. A) The CII enzymatic activity was determined as in Fig. 1B. * denotes values significantly different from untreated cells ($p < 0,05$). B) Determination of P-tyr phosphorylation of SDHA subunit was carried out as in Fig. 2A. Western blotting of peroxiredoxin-3 was carried out as in Fig. 3A. A representative, out of three similar blots, is shown.

Fig. 5. Measurement of cellular dicarboxylic acids levels and lactate production

The cellular content of malate, succinate and fumarate in homoplasmic *MTCYB* microdeletion cybrids and isogenic controls (WT) (A) and in homoplasmic p.278Y > C *MTCYB* mutation cybrids and isogenic controls (B) was determined by LC-ESI-MS/MS, as described in Methods. The data are mean $\pm$ SD of 3 determinations. Asterisks denote values significantly different from WT cells: * $p < 0,01$ and ** $p < 0,001$. C) L-lactate released to the medium during the first 24 h of growth by the heteroplasmic (80% mutant) and homoplasmic (100%) cybrids bearing the *MTCYB* microdeletion and isogenic control (WT) as well as the homoplasmic p.278Y > C *MTCYB* mutation carrying cybrids and their isogenic control (WT). Data are mean $\pm$ SEM) ($n = 3$); asterisk denotes values significantly different from WT cells ($p < 0,05$).

Fig. 6. Effect of 3-NPA on viability of *MTCYB* microdeletion cybrids incubated in different growth media

WT and mutant cybrids were incubated for 48 h with high glucose DMEM (A) or low glucose DMEM (B) or for 12 h with glutamine-free high glucose DMEM (C), in the absence or presence of 0.5 mM 3-nitropropionic acid (3-NPA). Cell viability was determined by the SRB assay. The data are the mean $\pm$ SD values of at least 3 different experiments. Asterisks denote values significantly different from untreated cells: * $p < 0,05$ and ** $p < 0,001$. (D) Heteroplasmic and homoplasmic cybrids bearing the *MTCYB* microdeletion and isogenic controls (WT) were incubated for 24 h in the presence of the indicated amounts of H₂O₂. Cell viability was determined as in panels A–C. Data are mean $\pm$ SD of at least 3 determinations. Asterisk denotes values significantly different from controls (WT): * $p < 0,05$.

Fig. 7. Model of CII stimulation in cells with defective CI or CIII

In the case of CI dysfunction, stimulation of CII activity optimizes the delivery of FADH_2 derived electrons to CIII, thus overcoming the CI dysfunction and allowing a significant OXPHOS efficiency. In the case of CIII deficiency, CII is also tyrosine phosphorylated and activated. However, CII is unable to improve the energetic efficiency due to blockage of electron flow and shortage of oxidized CoQ. In the absence of OXPHOS, cells become heavily dependent on glycolysis for survival.

Table 1

GSH and GSSG content (nmol/mg protein) in control and mutant cybrids.

Cell line	GSH	GSSG	% GSSG/GSH + GSSG
WT	27,4 ± 3,3	0,10 ± 0,02	0,36 ± 0,07
Y278C	21,7 ± 4,6	0,09 ± 0,01	0,40 ± 0,11
WT	14,1 ± 3,2	0,17 ± 0,02	1,10 ± 0,5
I300-P305	13,9 ± 3,2	0,12 ± 0,02	0,80 ± 0,2

The results are means ± SEM from three independent experiments carried out in duplicate.