Respiratory Complex I Dysfunction Due to Mitochondrial DNA Mutations Shifts the Voltage Threshold for Opening of the Permeability Transition Pore toward Resting Levels*

Received for publication, September 22, 2008 Published, JBC Papers in Press, December 1, 2008 DOI 10.1074/jbc.M807321200

Anna Maria Porcelli§, Alessia Angelin§, Anna Gelli§, Elisa Mariani§, Andrea Martinuzzi†, Valerio Carelli§, Valeria Petronilli§, Paolo Bernardi§*, and Michela Rugolo§

From the Departments of 4 Evolutionary and Experimental Biology and 1 Neurological Sciences, University of Bologna, 40126 Bologna, the *Istituto di Ricovero e Cura a Carattere Scientifico “E. Medea”, Conegliano Veneto 31015, and the †Department of Biomedical Sciences and Consiglio Nazionale delle Ricerche, University of Padova, 35121 Padova, Italy

We have studied mitochondrial bioenergetics in HL180 cells (a cybrid line harboring the T14484C/ND6 and G14279A/ND6 mtDNA mutations of Leber hereditary optic neuropathy, leading to an ~50% decrease of ATP synthesis) and XTC.UC1 cells (derived from a thyroid oncocytoma bearing a disruptive frame-shift mutation in MT-ND1, which impairs complex I assembly). The addition of rotenone to HL180 cells and of antimycin A to XTC.UC1 cells caused fast mitochondrial membrane depolarization that was prevented by treatment with cyclosporin A, intracellular Ca2+ chelators, and antioxidant. Both cell lines also displayed an anomalous response to oligomycin, with rapid onset of depolarization that was prevented by cyclosporin A and by overexpression of Bcl-2. These findings indicate that depolarization by respiratory chain inhibitors and oligomycin was due to opening of the mitochondrial permeability transition pore (PTP). A shift of the threshold voltage for PTP opening close to the resting potential may therefore be the underlying cause facilitating cell death in diseases affecting complex I activity. This study provides a unifying reading frame for previous observations on mitochondrial dysfunction, bioenergetic defects, and Ca2+ deregulation in mitochondrial diseases. Therapeutic strategies aimed at normalizing the PTP voltage threshold may be instrumental in ameliorating the course of complex I-dependent mitochondrial diseases.

NADH:ubiquinone oxidoreductase (respiratory complex I EC 1.6.5.3) is the first multiprotein complex of the oxidative phosphorylation system. Complex I contributes to the formation of the proton electrochemical gradient across the inner mitochondrial membrane by coupling proton translocation to electron transfer from NADH to ubiquinone. The proton gradient provides the driving force for ATP synthesis, ion transport, and maintenance of antioxidant defenses (1). Complex I is the largest respiratory chain complex with an estimated molecular mass of 900,000 Da; it is made up of seven subunits encoded by mtDNA (ND1–6 and ND4L) and 35 or more subunits encoded by nuclear genes (2, 3). Mutations in both the mitochondrial-encoded and the nuclear-encoded human genes are known to cause complex I dysfunction, which is associated to a wide array of neurodegenerative diseases (3, 4) and to some types of tumor (5, 6). Pathogenic mutations have been identified in each of the seven NADH dehydrogenase (ND)3 genes encoded by mtDNA. The clinical symptoms range from single organ or tissue diseases like Leber hereditary optic neuropathy (LHON) (7) to multisystemic disorders like mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) and Leigh syndrome (3, 4).

It is established that mitochondrial dysfunction is strictly associated to activation of the intrinsic apoptotic pathway activated by the release of cytochrome c from mitochondria (8). In particular, complex I deficiency may sensitize cells to the action of death agonists that permeabilize the outer membrane, such as Bax, through mitochondrial oxidative damage. A fraction of cytochrome c is bound to cardiolipin (9), and complex I-dependent oxidative damage to cardiolipin could increase the pool of free cytochrome c in the intermembrane space and therefore the fraction of released cytochrome c, making cells more prone to apoptosis (10). Cell death may also occur following opening of the inner membrane permeability transition pore (PTP), a high conductance channel that causes depolarization and membrane permeabilization to solutes up to about 1,500 Da (11). Prolonged PTP openings are indeed accompanied by swelling and cytochrome c release in situ (12, 13). Outer membrane permeabilization by Bax and PTP opening are synergistic rather than mutually exclusive mechanisms (14). Indeed, cristae remodeling by PTP opening increases the fraction of released cytochrome c that is available for release (15, 16), and full outer membrane permeabilization appears to be
Complex I Dysfunction Shifts the PTP Threshold

essentially for the release of intermembrane apoptotic factors that are too big for being released through Bax/Bak channels (14, 17,18). An interesting link between the two pathways is suggested by the effects of antiapoptotic Bcl-2, which decreases the probability of opening of the PTP, thus maintaining the reduced pyridine nucleotide pool and inhibiting the mitochondrial release of apoptotic factors (19, 20).

In the present study, we have investigated mitochondrial bioenergetics in two cell models bearing mtDNA mutations in complex I. The first is a hybrid cell line (HL180) harboring two missense mutations at nucleotide positions T14484C/ND6 and G14279A/ND6 of mtDNA, which are both associated with LHON (21, 22). The second cell line (XTC.UC1) is derived from a thyroid oncocyte (23) and bears a disruptive frameshift mutation in MT-ND1, causing the lack of ND1 and other complex I subunits and a dramatic reduction of its enzymatic activity (24).

We found that HL180 and XTC.UC1 cells displayed an anomalous depolarizing response to respiratory inhibitors and to oligomycin that was prevented by decreasing the probability of PTP opening with cyclosporin (Cs) A, which also protected from depolarization after switching the cultures from glucose to galactose. These findings suggest that, like in muscular dystrophy due to collagen VI deficiency (25), the voltage threshold for PTP opening is altered in HL180 and XTC.UC1 cells, and that PTP opening may represent the final common pathway through which complex I defects cause cell death.

EXPERIMENTAL PROCEDURES

Materials—Oligomycin, rotenone, antimycin A, carbonylcyanide-p-trifluoromethoxyphenyl hydrazone (FCCP), 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ATP monitoring kit, Lipofectamine 2000, lauryl maltoside, protease inhibitors mixture, Coomassie Blue G, and G418 were from Sigma (Milan, Italy). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 1,2-bis(o-aminophenoxy)ethane-N,N',N''-tetraacetic acid (Trolox) and 1,2-bis(o-aminophenoxy)ethane-N,N',N''-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM) were from Invitrogen (Milan, Italy). 5-(6-Chloro-2,4-dioxo-1,3,4,10-tetrahydro-2-ethyl ester (BAPTA-AM) were from Invitrogen (Milan, Italy).

Cell Culture and Growth Conditions—HPS11 and HL180 cells are previously characterized cybrids (26, 27) generated by fusion of enucleated fibroblasts derived from one control (HPS11) and one LHON patient (HL180) into osteosarcoma 143B:1K− cells deprived of their own mtDNA. Complete mtDNA sequencing revealed that HPS11 belongs to haplogroup T2c (data not shown), whereas HL180 harbors the 14484/ND6 and 14279/ND6 LHON mutations causing M64V and S132L amino acid substitutions in the MT-ND6 subunit, respectively (28). Although the T14484C/ND6 mutation was previously shown not to affect complex I activity (29, 30), it was reported to sharply reduce mitochondrial ATP synthesis driven by complex I substrates (31). XTC.UC1 cells, derived from a human thyroid carcinoma (23), bear a C insertion at bp3571 in ND1, generating a premature stop codon at amino acid 101 of ND1 subunit (24). Despite the different severity of complex I dysfunction, both cell lines died when switched from glucose to galactose as the energy source (24, 26), a condition that forces ATP production through oxidative phosphorylation only (32). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cultures were grown in a humidified incubator at 37 °C with 5% CO2. In some experiments, cells were incubated in DMEM or glucose-free DMEM supplemented with 5 mM galactose and 5 mM pyruvate (DMEM-galactose).

Blue Native Electrophoresis and Complex I In-gel Activity—Isolated mitochondria (4 mg/ml) were solubilized using 0.4% lauryl maltoside, and ~80 μg of protein was loaded on each of two 5–12% gradient polyacrylamide gels. One gel was stained with Coomassie Blue G (33); in the other, the complex I in-gel activity was determined by incubating with 2 mM Tris-Cl (pH 7.4), 0.15 mM NADH, and 2.5 mg/ml MTT at room temperature for 1 h as described in Ref. 34. The reaction was stopped with 50% methanol and 10% acetic acid, and the gel was analyzed with a Fluor-2 MAX multimager system (Bio-Rad).

Mitochondrial Membrane Potential (Δψm)—Cells were seeded onto 24-mm-diameter round glass coverslips and grown for 2 days in DMEM. Δψm was measured based on the accumulation of TMRM in the presence of 1.6 μM CsH, which inhibits the multidrug resistance pump, but not the PTP (17, 35). Cells were incubated in bicarbonate- and phenol red-free Hank’s balanced salt solution supplemented with 10 mM Hepes and 1.6 μM CsH and loaded with 20 nM TMRM for 30 min. At the end of each experiment, mitochondria were fully depolarized by the addition of 4 μM of the protonophore FCCP. Cellular fluorescence images were acquired with an Olympus IX71/IX51 inverted microscope, equipped with a xenon light source (150 watts) for epifluorescence illumination, and with a digital camera. For detection of fluorescence, 568 ± 25-nm bandpass excitation and 585-nm longpass emission filter settings were used. Images were collected with an exposure time of 100 ms (6% illumination intensity) using a ×40, 1.3 NA oil immersion objective (Olympus). Data were acquired and analyzed using Cell R software (Olympus). Clusters of several mitochondria (10–30) were identified as regions of interest, and fields not containing cells were taken as the background. Sequential digital images were acquired every minute, and the average fluorescence intensity of all relevant regions was recorded and stored for subsequent analysis.

Measurements of Oxygen Consumption, Cellular ATP Content, and Mitochondrial ATP Synthesis—The rate of oxygen consumption driven by substrates of complex I (malate and pyruvate) or complex II (succinate plus rotenone) in the presence of 1 mM ADP (state 3) was measured in digitonin-permeabilized cells at 30 °C using a Clark type oxygen electrode as described previously (36). ATP content was measured by using the luciferin/luciferase assay (37). The rate of mitochondrial ATP synthesis supported by complex I and complex II substrates was determined in digitonin-permeabilized cells exactly as reported previously (24). Data were normalized for citrate synthase activity (38).
Bcl-2 Transfection and Clone Generation—Cells were transfected with the pcDNA3 plasmid containing the full-length bcl-2 cDNA (a gift from G. Manfredi, Weill Medical College of Cornell University, New York, NY) or pcDNA3 empty vector. Transfection was performed using Lipofectamine 2000 as described by the manufacturer. Forty-eight hours after transfection, clones were selected in the presence of 800 μg/ml G418. Resistant cells were pooled and maintained in culture with 200 μg/ml G418. No functional differences were observed between naive and mock-transfected cells, so the former were routinely used as controls in the TMRM fluorescence measurements.

Cell Lysis and Western Blotting—Cells (4 x 10⁶) were resuspended in 0.1 ml of buffer containing 150 mM NaCl, 50 mM Tris-Cl, pH 7.6, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, and 100 μM/ml of protease inhibitors mixture, sonicated, and centrifuged at 10,000 x g. Protein content of the supernatant (cell lysate) was then determined (39). Lysates (50 μg) were separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad, Hertfordshire, UK). Primary antibodies (dilution 1:1000 for Bcl-2 and 1:500 for actin) were incubated at 4 °C for 2 h. Primary antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies. Signals were detected using ECL (Amer sham Biosciences), and densitometry was performed with a Fluor-2 MAX Multimager system (Bio-Rad).

Statistical Analysis—All the experiments were repeated at least three times, and the results are presented as mean ± S.D. Statistical analysis was performed using the Student’s t test, with p < 0.05 as the level of significance.

RESULTS

We measured the amount of respiratory complex I assembled in mitochondria by blue native gel electrophoresis (Fig. 1A, top portion). The ~900-kDa band corresponding to native complex I (CI) was apparent both in HPS11 cells bearing wild-type mtDNA (lane 1) and in HL180 cybrids (lane 2), whereas it was not detectable in XTC.UC1 cells (lane 3). On the other hand, no major alteration in the pattern of the other mitochondrial respiratory (CII-CIV) and ATP synthase complexes (CV) was observed (Fig. 1A, top). In-gel enzymatic activity revealed that complex I activity could be detected in HPS11 and HL180, but not XTC.UC1 cells (Fig. 1A, bottom portion, compare lanes 3 with lanes 1 and 2). Taken together, these findings indicate that the frameshift mutation in the ND1 subunit is associated with the lack of complex I assembly in XTC.UC1 cells, resulting in a more severe dysfunction than caused in HL180 cells by the missense point mutations in the ND6 subunit.

To explore the bioenergetic competence of these cell models, we measured the rate of respiration and of mitochondrial ATP synthesis in digitonin-permeabilized cells in the presence of different substrates. In agreement with previous reports (31), the respiratory rates of HL180 cells in the presence of substrates of complex I (pyruvate and malate) or complex II (succinate) were not significantly different from those of control HPS11 cells. On the other hand, in XTC.UC1 cells oxygen consumption was selectively decreased with complex I substrates (Fig. 1B). The rate of ATP synthesis driven by complex I substrates was decreased by 50% in HL180 cells and by nearly 90% in XTC.UC1 cells, whereas no significant differences emerged when succinate was the oxidizable substrate (Fig. 1C).

We next monitored the mitochondrial membrane potential of these cell lines based on the mitochondrial accumulation of TMRM, a fluorescent cationic probe that accumulates in polarized mitochondria and is released when the mitochondrial depolarization (ΔΨm) decreases. As expected (25), accumulation of TMRM was marginally affected by the addition of the specific complex I inhibitor, rotenone (Fig. 2A, open symbols), or of the specific F1FO ATPase inhibitor, oligomycin (Fig. 2B, open symbols), whereas rapid release of the probe (i.e. mitochondrial depolarization) readily followed the addition of both inhibitors (Fig. 2, A and B, closed symbols). Similar levels of TMRM accumulation were observed in HL180 and XTC.UC1 cells, suggesting that these lines maintain a transmembrane proton electrochemical gradient irrespective of the overall activity of complex I (Fig. 3, A and A’). However, the addition of rotenone caused a rapid and complete mitochondrial
Complex I Dysfunction Shifts the PTP Threshold

![Fluorescence changes induced by rotenone or antimycin A.](Image)

**FIGURE 3.** Effect of CsA, BAPTA-AM, and Trolox on mitochondrial TMRM fluorescence changes induced by rotenone or antimycin A. HL180 (A–C) and XTC.UC1 cells (A’–C’) were loaded with TMRM as described under "Experimental Procedures" in the absence of further treatments (A and A’) or in the presence of 5 μM BAPTA-AM (B and B’) or of 0.5 μM Trolox (C and C’). In panels A–C, where indicated, 4 μM rotenone (R) and 4 μM FCCP (F) were added in the absence (closed symbols) or presence (open symbols) of 0.8 μM CsA. In panels A’–C’, where indicated, 4 μM antimycin A (AA) and 4 μM FCCP (F) were added in the absence (closed symbols, line a) or presence (open symbols, line b) of 0.8 μM CsA. In A’, only closed triangles, line a’ document the effect of the addition of 4 μM rotenone (R). The results shown are mean ± S.D. of at least five repeats. To facilitate comparison of the various treatments to the responses to rotenone or antimycin A alone, the latter are outlined in the dashed traces of panels B, C, B’, and C’, respectively. Fluorescence readings following the addition of rotenone and preceding that of FCCP revealed a statistically significant difference (p at least <0.05) for all time points and treatments (panels A–C). Fluorescence readings following the addition of antimycin A and preceding that of FCCP revealed a statistically significant difference (p at least <0.05) for the indicated treatments up to 16 min (panel A’), 32 min (panel B’), and 19 min (panel C’).

Depolarization in HL180 cells (Fig. 3A, closed symbols, line a). Remarkably, depolarization was delayed by the PTP desensitizer CsA (Fig. 3A, open symbols, line b), by the cell-permeant divalent metal chelator BAPTA-AM, and by the vitamin E-derived antioxidant Trolox (Fig. 3, B and C, closed symbols, lines a, respectively), with a small but measurable additive effect of the latter compounds with CsA (Fig. 3, B and C, open symbols, lines b, respectively). In keeping with the finding that complex I is not assembled in XTC.UC1 cells (Fig. 1A), rotenone did not affect their mitochondrial membrane potential (Fig. 3A’, closed triangles, line a’). Of note, on the other hand, depolarization was promptly elicited by the addition of the complex III inhibitor antimycin A (Fig. 3A’, closed squares, line a). Depolarization was considerably delayed by CsA (Fig. 3A’, open symbols, line b), BAPTA-AM, and Trolox (Fig. 3, B’ and C’, closed symbols, lines a, respectively), again with a measurable additive effect with CsA at the later time points (Fig. 2, B’ and C’, open symbols, lines b, respectively).

We then tested the effect of the F1F0 ATPase inhibitor oligomycin and also detected a rapid decrease of mitochondrial TMRM fluorescence that was particularly marked for the XTC.UC1 cell line (Fig. 4, A and A’, closed symbols). Treatment with CsA normalized the Δψm response to oligomycin in both HL180 and XTC.UC1 cells (Fig. 4, A and A’, open symbols) suggesting that PTP opening is responsible for oligomycin-induced depolarization. Preincubation with BAPTA-AM (Fig. 4, B and B’, closed symbols) or with the vitamin E derivative Trolox (Fig. 4, C and C’, closed symbols) did not significantly change oligomycin-dependent depolarization, but Trolox, which mitigates the effect of complex I deficiency in human fibroblasts (40), somewhat enhanced the protective effects of CsA (Fig. 4C’, open symbols).

Bcl-2 both desensitizes the PTP (19, 41) and improves mitochondrial function in cells with pathogenic mutations of mitochondrial tRNA genes (42). We therefore tested whether Bcl-2 overexpression affects the mitochondrial response to oligomycin of HL180 and XTC.UC1 cells (Fig. 5). As reported in Fig. 5, A and A’, the level of Bcl-2 protein was markedly increased in HL180 and measurably increased in XTC.UC1 cells. TMRM accumulation showed no significant difference after Bcl-2 transfection (result not shown), suggesting that the Δψm levels were the same. Oligomycin caused negligible depolarization (Fig. 5, B and B’, closed triangles, lines a), suggesting that the latent mitochondrial dysfunction was prevented by Bcl-2. The specificity of the protective effect of Bcl-2 overexpression on mitochondrial function was tested by measuring the effect of EM20–25, a small organic molecule that binds to the BH3 domain of Bcl-2 and counteracts its inhibitory effects on the PTP (41). Preincubation with EM20–25 restored the depolarizing response to oligomycin fully in HL180 and partially in XTC.UC1 cells (Fig. 5, B and B’, closed squares, lines b,
respectively, compare with Fig. 4, A and A’), and CsA prevented the sensitizing effects of EM20–25 (Fig. 5, B and B’, open squares, lines c). These results clearly indicate that the protective effect of Bcl-2 on mitochondrial function specifically involves PTP regulation.

Bcl-2 overexpression failed to increase either the rate of mitochondrial ATP synthesis driven by complexes I and II in both cell lines or the steady-state levels of respiratory chain complex I–IV subunits (results not shown), suggesting that Bcl-2 did not exert its effect by increasing the activity/level of mitochondrial enzymes. On the other hand, Bcl-2 markedly increased the ATP content of HL180 but not XTC.UC1 cells during incubation in galactose medium (Fig. 6A, compare open and closed symbols). Consistent with the results described above for oligomycin, treatment with EM20–25 completely prevented the increase in ATP content of HL180 Bcl-2 cells (Fig. 6B).

We finally assessed the mitochondrial membrane potential after switching the energy source from glucose to galactose, a treatment that causes cell death in these cell lines (24, 26). A marked depolarization was observed both in HL180 and in XTC.UC1 cells (Fig. 7, A and C, respectively). In keeping with the results obtained with rotenone and oligomycin, in HL180 cells galactose-dependent depolarization was fully prevented by both CsA and Bcl-2 overexpression (Fig. 7, A and B), although depolarization was delayed only by CsA at 2 h of incubation in the case of XTC.UC1 cells (Fig. 7, C and D).

DISCUSSION

The present study sheds new light on the basis for mitochondrial impairment due to defective complex I. We have demonstrated that HL180 cells (harboring the T14484C/ND6 and G14279A/ND6 mtDNA mutations of LHON causing an ~50% decrease of complex I-dependent ATP synthesis) and XTC.UC1 cells (bearing a disruptive frameshift mutation in MT-ND1 that prevents complex I assembly) maintain a normal mitochondrial membrane potential under resting conditions yet are very prone to undergo PTP opening upon inhibition of respiration or of the F1F0 ATP synthase or when switched from glucose to galactose as the energy source. It is remarkable that...
Complex I Dysfunction Shifts the PTP Threshold

the addition of rotenone caused rapid depolarization of HL180 cells, whereas XTC.UC1 cells were totally resistant to rotenone and depolarized only after the addition of antimycin A. This finding indicates that complex I deficiency can be compensated through the oxidation of complex II substrates, in agreement with the results of respiration and ATP synthesis obtained in the presence of succinate plus rotenone, which were not significantly different from control cells.

Mechanism of Depolarization Induced by Respiratory Inhibitors and Oligomycin—The PTP-dependent depolarization caused by respiratory inhibitors in HL180 and XTC.UC1 cells can be easily explained within the framework of the PTP voltage dependence (43). Pore opening can occur either because depolarization is large enough to reach the voltage threshold for opening (44) or because the threshold is shifted toward the resting potential by pathophysiological effectors like Ca\(^{2+}\), Pi, and reactive oxygen species (45), two mechanisms that can synergize. In healthy cells, the initial decrease of mitochondrial membrane potential caused by the addition of rotenone is readily compensated by reversal of the ATP synthesize. Because the threshold voltage for PTP opening is not reached, the inner membrane permeability remains low, allowing the maintenance of the membrane potential at the expense of ATP hydrolysis. In HL180 and XTC.UC1 cells, the PTP threshold would instead be close to the resting potential, allowing PTP opening even for small depolarizations. Once this occurs, repolarization cannot take place despite ATP hydrolysis. Based on the protective effect of the intracellular Ca\(^{2+}\) chelator BAPTA-AM and of the antioxidant Trolox, we think that defective complex I sensitizes the PTP through Ca\(^{2+}\) overload and increased production of reactive oxygen species (46), which have indeed been described in several mtDNA disease models (47–57).

The sarco-endoplasmic reticulum is crucial in Ca\(^{2+}\) homeostasis in close collaboration with the mitochondria (58, 59), which provide the ATP required for Ca\(^{2+}\) uptake. This is critical for muscle relaxation in excitation-contraction coupling of skeletal muscle fibers (60, 61), which are almost invariably affected in mtDNA diseases (62). In this context, it is worth noting that fibroblasts derived from patients with complex I deficiencies due to mutations in the nuclear-encoded subunits exhibited reduced Ca\(^{2+}\) content of the endoplasmic reticulum and decreased hormone-stimulated mitochondrial ATP production (57). It appears likely that a similar alteration is present in cells with complex I deficiency due to mutations of mtDNA. The decreased ATP supply to the Ca\(^{2+}\) pumps would then reduce Ca\(^{2+}\) uptake in intracellular stores and cause increased cytosolic free [Ca\(^{2+}\)] followed by mitochondrial Ca\(^{2+}\) and Pi uptake, resulting in a shift of the PTP voltage threshold. The depolarizing effect of oligomycin, which was also observed in cybrids from a MELAS patient harboring a A13528G/ND5 mtDNA mutation (55) and in patients affected by Ullrich congenital muscular dystrophy (63, 64), can therefore be explained on the basis of decreased ATP levels, eventually resulting in a shift of the PTP threshold above the resting membrane potential (25).

Mitochondrial depolarization by oligomycin was also markedly prevented by Bcl-2 overexpression. Bcl-2 has been shown to decrease the probability of PTP opening and to prevent cell death through inhibition of cytochrome c release (19, 20). Although the effects of Bcl-2 are complex and its apoptotic actions are not necessarily linked to mitochondria, our results are consistent with a PTP-dependent effect. Indeed, treatment with EM20–25, which displaces proapoptotic Bax from Bcl-2 and sensitizes the PTP to opening (41), reverted the protective effects of Bcl-2 overexpression and restored oligomycin-dependent, CsA-sensitive depolarization of HL180 and XTC.UC1 cells.

Recovery of the Energetic Competence in Galactose Medium—Although both HL180 and XTC.UC1 cells are unable to survive when switched from glucose to galactose as the energy source (24, 26), they displayed a differential response to Bcl-2 overexpression in that ATP levels and Δψ\(_{\text{m}}\) were preserved by Bcl-2 in HL180 but not in XTC.UC1 cells. Given that Bcl-2 similarly delayed mitochondrial depolarization after the addition of oligomycin in both cell lines, it is apparent that in XTC.UC1 cells, activity of the respiratory chain downstream of complex I has little or no reserve capacity under stress conditions. Conversely, the residual complex I activity of HL180 cells may synergize with Bcl-2 and maintain the energetic competence for several hours. This may be part of the protective strategies of the cells as Bcl-2 has been reported to improve mitochondrial energetic function in cells harboring pathogenic mutations in mitochondrial tRNA genes (42) and to increase resistance to Ca\(^{2+}\) of mitochondria isolated from the heart of mice with a high load of mtDNA mutations due to expression of an error-prone DNA polymerase γ (65). It should be noted that even in the latter model, PTP opening plays a key pathogenic role in vivo, as shown by the efficacy of CsA at preventing onset of the otherwise lethal dilated cardiomyopathy (66).

The PTP in the Pathogenesis of mtDNA Diseases—Earlier evidence that the PTP may be involved in mtDNA diseases was obtained by Wong and Cortopassi (67), who observed that cybrids harboring the MELAS, LHON, or myoclonic epilepsy with ragged red fiber mutations were sensitized to the toxic effects of added hydrogen peroxide in a CsA-sensitive manner. Similar protection by CsA and minocycline was also reported in LHON cybrids following Ca\(^{2+}\) deregulation with thapsigargin (68). Although this issue was not addressed directly, a large number of additional studies of mtDNA diseases, which are not limited to isolated defects of complex I, have revealed features that are consistent with PTP deregulation. These include alterations of Ca\(^{2+}\) homeostasis (47, 48, 52, 53, 57), increased reactive oxygen species production (50, 51), early alterations of respiration and/or membrane potential (69–72), and sensitization to Fas-induced apoptosis (73). Assessing whether the altered threshold for PTP opening identified here is a general feature of mtDNA diseases may not only add to our understanding of their pathogenesis but also provide a strategy for therapeutic intervention.

Acknowledgments—We thank Dr. Giovanni Manfredi, Cornell University, New York, NY, for the generous gift of the pcDNA3-Bcl-2 vector, and Dr. Arcangela Iuso, University of Bari, for help with the blue native gel electrophoresis experiment.
Complex I Dysfunction Shifts the PTP Threshold

62. DiMauro, S., and Davidzon, G. (2005) *Ann. Med.* 37, 222–232
63. Angelin, A., Tiepolo, T., Sabatelli, P., Grumati, P., Bergamin, N., Golfieri, C., Mattioli, E., Gualandi, F., Ferlini, A., Merlini, L., Maraldi, N. M., Bonaldo, P., and Bernardi, P. (2007) *Proc. Natl. Acad. Sci. U. S. A.* 104, 991–996
64. Merlini, L., Angelin, A., Tiepolo, T., Braghetta, P., Sabatelli, P., Zamparelli, A., Ferlini, A., Maraldi, N. M., Bonaldo, P., and Bernardi, P. (2008) *Proc. Natl. Acad. Sci. U. S. A.* 105, 5225–5229
65. Mott, J. L., Zhang, D., Chang, S. W., and Zassenhaus, H. P. (2006) *Biochim. Biophys. Acta* 1757, 596–603
66. Mott, J. L., Zhang, D., Freeman, J. C., Mikolajczak, P., Chang, S. W., and Zassenhaus, H. P. (2004) *Biochem. Biophys. Res. Commun.* 319, 1210–1215
67. Wong, A., and Cortopassi, G. (1997) *Biochem. Biophys. Res. Commun.* 239, 139–145
68. Haroon, M. F., Fatima, A., Scholer, S., Gieseler, A., Horn, T. F., Kirches, E., Wolf, G., and Kreutzmann, P. (2007) *Neurobiol. Dis.* 28, 237–250
69. Byrne, E., Trounce, I., Marzuki, S., Dennett, X., Berkovic, S. F., Davis, S., Tanaka, M., and Ozawa, T. (1991) *Acta Neuropathol.* 81, 318–323
70. Hofhaus, G., Johns, D. R., Hurko, O., Attardi, G., and Chomyn, A. (1996) *J. Biol. Chem.* 271, 13155–13161
71. James, A. M., Wei, Y. H., Pang, C. Y., and Murphy, M. P. (1996) *Biochem. J.* 318, 401–407
72. Jun, A. S., Trounce, I. A., Brown, M. D., Shoffner, J. M., and Wallace, D. C. (1996) *Mol. Cell. Biol.* 16, 771–777
73. Danielson, S. R., Wong, A., Carelli, V., Martinuzzi, A., Schapira, A. H., and Cortopassi, G. A. (2002) *J. Biol. Chem.* 277, 5810–5815