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mtDNA Mutations Confer Cellular Sensitivity to Oxidant Stress That Is Partially Rescued by Calcium Depletion and Cyclosporin A

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The complete mechanism by which pathogenic mtDNA mutations cause cellular pathophysiology and in some cases cell death is unclear. Oxidant stress is especially toxic to excitable nerve and muscle cells, cells that are often affected in mitochondrial disease. The sensitivity of cells bearing the LHON, MELAS, and MERRF mutations to oxidant stress was determined. All were significantly more sensitive to H_2O_2 exposure than their nonmutant cybrid controls, the order of sensitivity was MELAS > LHON > MERRF > controls. Depletion of Ca^{2+} from the medium protected all cell lines from oxidant stress, consistent with the hypothesis that death induced by oxidant stress is Ca^{2+}-dependent. A potential downstream target of Ca^{2+} is the mitochondrial permeability transition, MPT, which is inhibited by cyclosporin A. Treatment of MELAS, LHON, and MERRF cells with cyclosporin A caused significant rescue from oxidant exposure, and in each case significantly greater rescue of mutant than control cells. The pronounced oxidant-sensitivity of mutant cells, and their protection by Ca^{2+} depletion and CsA, has potential implications for both the pathophysiological mechanism and therapy of these mitochondrial genetic diseases.

Pathogenic mutations of the mitochondrial DNA (mtDNA) have been demonstrated to cause several encephalomyopathies (1-3). In many cases, one or more biochemical defects has been identified.

For example, in MELAS (Myoclonic Epilepsy with Lactic Acidosis and Stroke) that results from a mutation in the mitochondrial $tRNA^{Leu}$ gene, defects have been described in protein synthesis (4, 5); electron transport (6, 7); mitochondrial membrane potential (8, 9); and calcium sequestration (9).

In MERRF (Myoclonic Epilepsy with Ragged Red Fibers) that results from mutations at the mitochondrial $tRNA^{Lys}$ gene, defects occur in protein synthesis (10, 11), in respiration (12-14), and in membrane potential (8).

In cells from individuals with LHON (Leber's Hereditary Optic Neuropathy) a deficiency in oxygen consumpation and in complex I activity have been described (15-17).

However, exactly why these mutations preferentially affect excitable tissue, and the complete mechanism of cellular pathophysiology, and in some cases cell death caused by the mutation, has yet to be determined.

Disruption of Ca^{2+} homeostasis is thought to be a major mechanism of toxicity in excitable nerve and muscle tissue (18-24). Several neurotoxic compounds (e.g. glutamate, pro-oxidants, carbon tetrachloride, lead, and mercury) induce a rise in intracellular Ca^{2+}in the course of inducing cell death (24, 25).

Reactive Oxygen Species (ROS) are potent inducers cell death, which is usually preceded by a rise in intracellular Ca^{2+}(24). ROS have been implicated in the pathophysiology of several neurological diseases, including ALS, Parkinson's disease, and Alzheimer's disease (19-21, 26-29).

Given the implication of oxidants and Ca^{2+} in the pathophysiology of other neurological diseases, we wondered if the mutations responsible for the mitochondrial diseases MELAS, LHON, and MERRF would confer sensitivity to oxidant stress, and if so, how modulation of that stress might protect cells from death. The experimental model we have chosen is cellular transmitochondrial cell lines, which allow the comparison of mitochondrial genetic contribution to cellular phenotype in the absence of nuclear genetic variation (30).

METHODS AND MATERIALS

Cell lines and media. Cell cybrid pairs, which contained 100% mutant and 100% normal mtDNA, produced from cells with identical nuclear DNA, were used in all studies, produced as in King and Attardi, 1989 (30). The MERRF (pT1) and LHON (Le1.3.1) cell lines and their parental cell line, 143B, were generous gifts from Drs.

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Giuseppe Attardi and Anne Chomyn (Caltech, Pasadena, CA), who produced and characterized (31) the MERRF and LHON transmitochondrial cell lines. The MELAS (Da12B) cell line and its parental cell line, Da1B, were kindly provided by Dr. Eric Shoubridge (McGill University, Canada). The MERRF cell line carries the mtDNA mutation at nucleotide (nt) 8344, and the LHON cell line possess the mtDNA mutation at nt 11778. The MELAS cell line has the mtDNA mutation at nt 3243. Production of these cell lines are described elsewhere; MERRF (31), LHON (15); and MELAS (32). The cells were grown in Dulbecco modified Eagle medium (DMEM) (Life Technologies, Gaithersberg, MD), supplemented with 10% fetal bovine serum (FBS), 50 mg/ml uridine and 1mM sodium pyruvate.

DNA mutational analysis. The presence of the mtDNA mutations was confirmed by the polymerase chain reaction (PCR) and sequencing of the PCR products. Proteinase K digestion was used to isolate DNA from 1×10^6 cells. To amplify the MERRF mutation, primers were designed at nt 8220-8247 and nt 8592-8616. Thirty cycles were used to amplify the 396 bp product using the following conditions: denaturation 95°C, annealing 60°C and extension 72°C for one minute each. The LHON mutation was amplified using primers at nt 11429-11448 and nt 11910-11929. Primers designed at nt 3072-3095 and nt 3516-3536 were used to amplify the MELAS mutation. The PCR conditions to amplfy the MELAS and LHON mutations were the same as described above, except annealing was performed at 52°C. The PCR products were sequenced on an Applied Biosystems automated DNA sequencer and the mutations were confirmed by reading the sequence in the forward and reverse directions.

Viability assays. Cells were grown in 6-well Falcon plates to near confluence and treated as follows: cells received hydrogen peroxide (H_2O_2), 50-750 μM, made fresh daily, and were exposed for 6 hours. Cells receiving 1 μM cyclosporin A (Sandimmune) were pre-treated with the drug for 30 minutes before the addition of H_2O_2. Cells used in the calcium-free (Ca^{2+}-free) experiments were grown in Ca^{2+}-free media (DMEM, calcium-free, supplemented with uridine, FBS and sodium pyruvate) 24 hours prior to use. The cells were given fresh Ca^{2+}-free media before the addition of H_2O_2 and/or CsA. After 6 hours, the cells were harvested by brief exposure to trypsin-EDTA and resuspended in PBS. To derive survival data, 3-20 replicates at each dose were carried out, and 150 cells of the 100,000 on the dish were scored as either alive or dead by trypan blue exclusion.

Statistical analysis. Student's t-test was used to determine significance of survival data at individual data points. Two-way ANOVA (i.e. mutant vs. control) was used to analyse variation in survival data wholistically, i.e. at all 5 doses in aggregate.

RESULTS

LHON, MERRF, and MELAS Are Sensitive to H_2O_2

A wide range of H_2O_2 doses and cell survival times was surveyed, and a dose range of 0 to 750 μM for six hours was determined to give a large change in cell viability. Paired dose-response curves for mutant vs. control cell lines appear in Figure 1. MELAS, LHON, and MERRF mutant cells were significantly more sensitive to H_2O_2 than their non-mutant parental cell, $p < 0.001$ for each (2-way ANOVA). The maximum difference in sensitivity at any particular dose assayed was 29% at 250 μM H_2O_2 for MELAS cells, 19% in LHON cells at 100 μM H_2O_2, and 6% at 100 μM for MERRF cells. Significant differences between mutant and control cells at each dose were determined by the Student's t-test. The relative rank order of oxidant sensitivity of cells to oxidant stress was MELAS > LHON > MERRF > control.

Ca^{2+} Depletion Protects Cells from Oxidant-Induced Death

If H_2O_2 exerts its toxicity by inducing a rise in toxic intracellular Ca^{2+} (25), then depletion of Ca^{2+} would be expected to protect cells from oxidant-induced death. Mutant and control cells were grown in Ca^{2+}-free media 24 hours prior to receiving H_2O_2. The cells were given fresh (Ca^{2+}-free) media before the addition of H_2O_2. All cells were significantly protected from oxidative stress under Ca^{2+}-depleted conditions (Figure 2).

Both control lines, 143B, and Da1B, were significantly protected from H_2O_2 stress by Ca^{2+} depletion (Figure 2A and B). Ca^{2+} depletion caused striking protection of 143B cells from oxidant stress. For example at 750μM H_2O_2, Ca^{2+}-depleted 143B cells had 100% survival, compared to 19% survival at this dose in Ca^{2+}-rich medium. Da1B cells were also protected from oxidant stress by Ca^{2+} depletion, but less so than 143B; the maximal difference in protection observed at any particular dose was 22% at 500 μM H_2O_2; protections were significant for both control cell lines, $p < 0.0001$ (2-way ANOVA).

Depletion of Ca^{2+} from medium also significantly protected LHON, MERRF, and MELAS cells from oxidant stress, but less so than their parental control lines. LHON and MERRF cells were most protected from oxidant stress by Ca^{2+} depletion ($p < 0.0001$, 2-way ANOVA), and the major contributor was protection in the doses 100 and 500 μM H_2O_2 for LHON and at 50-500 μM H_2O_2 for MERRF cells. MELAS cells were the least protected by Ca^{2+} depletion, and the ANOVA p value was marginal ($p < 0.05$); also considered on a dose-by-dose basis there was no statistically significant protection at any particular dose (e.g. $p = 0.06$-0.96). The reduced protection of MELAS cells by Ca^{2+} depletion likely reflects the different parentage of the cell lines, in that Ca^{2+} depletion protected 143B cells (parental lines of LHON and MERRF) more than Da1B. In every case, depletion of Ca^{2+} protected mutant cells less than their parental controls from oxidant stress.

Protection of LHON, MERRF, and MELAS Transmitochondrial Cell Lines from H_2O_2 Induced Death by Cyclosporin A

Given that oxidants often induce a toxic rise in intracellular Ca^{2+}, and that Ca^{2+} depletion protected both the control and mutant cells from oxidant-induced death, we searched for a potential downstream Ca^{2+}-inducible target. The Mitochondrial Permeability Transition (MPT) is induced by Ca^{2+} and other agents (reviewed in ref. 33), and is inhibitable by CsA, Cyclosporin A (34-37). The MPT has been inferred to be important in the control of cell death (38-43), and we (44) and others (45) have recently demonstrated that conditions which trigger MPT also cause release

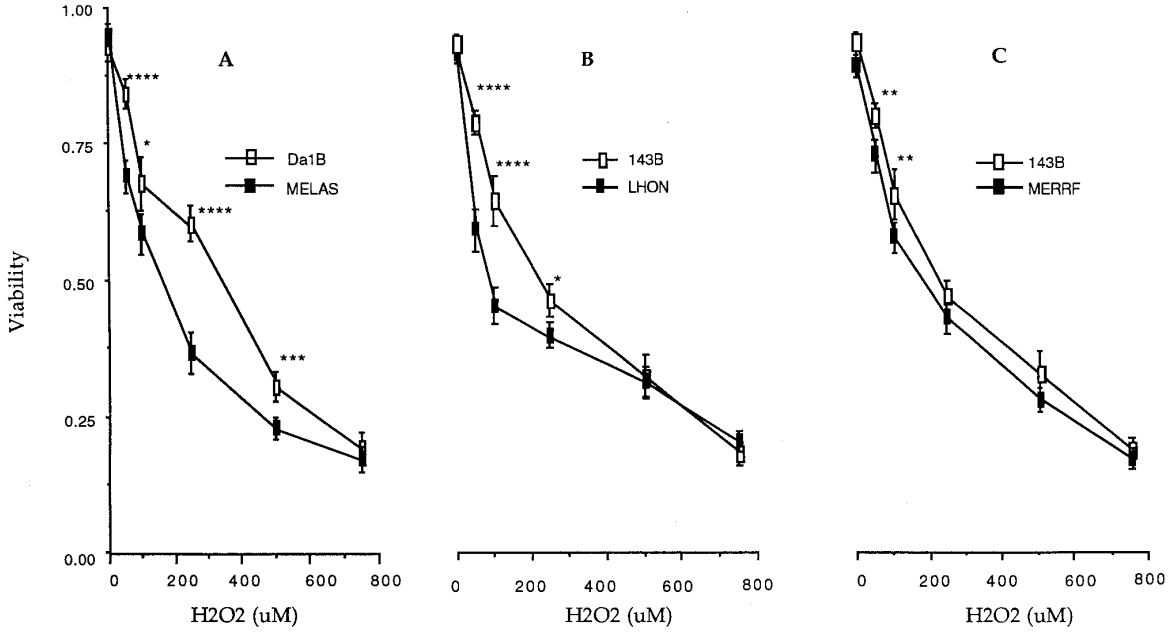

FIG. 1. Sensitivity of cells bearing mitochondrial DNA mutations exposed to hydrogen peroxide (H_2O_2). Cells were exposed to increasing concentrations of H_2O_2 for 6 hours, after which the cells were harvested and viability of live cells was determined by the trypan blue exclusion assay. The MELAS cybrid is shown in comparison to its parental cell line, Da1B, in A. Transmitochondrial cell lines derived from patients with LHON or MERRF are compared to their parental cell line, 143B, in B and C, respectively. The open squares represent controls, the filled squares represent mutant cells. Significance levels at individual doses are represented by asterisks, * = $p < 0.05$, **$p < 0.005$, ***$p < 0.001$, and ****$p < 0.0001$. The deviation of the overall survival of mutants vs. controls by two-way ANOVA was significant, $p < 0.001$. Means of 20 independent experiments are shown, and bars represent two standard errors of the mean.

of the apoptotic factor, cytochrome c, and that CsA inhibits release of cytochrome c from mitochondria (44). Thus we determined whether CsA had a protective effect on H_2O_2-mediated death in mutant and control cells (Figure. 3). One μM cyclosporin A significantly rescued MELAS, LHON, MERRF and control cells from H_2O_2-mediated death, $p < 0.0001$ (2-way ANOVA).

Perhaps more interestingly, CsA protected mutant MELAS, LHON, and MERRF cells significantly more than their parental controls ($p<0.001$, 2-way ANOVA). It was observed that the greater protection of mutant cells vs. controls occurred primarily at lower doses of H_2O_2, (e.g. LHON, $p < 0.0001$ at 50-250 μM H_2O_2, and $p = 0.49$ and 0.42 at 500 and 750 μM H_2O_2, by t-test at individual doses, respectively) CsA alone, i.e. in the absence of H_2O_2 did not affect viability.

DISCUSSION

This is the first analysis of the sensitivity of cells bearing mitochondrial mutations to oxidative stress. The major observations are 1) that mitochondrial mutations responsible for MELAS, LHON, and MERRF disease confer oxidant sensitivity to their host cells; 2) that the sensitivity is partially rescued by depletion of Ca^{2+}, implicating Ca^{2+} in the transmission of the death signal; and 3) that the MPT inhibitor CsA protects MELAS, LHON, and MERRF cells death induced by oxidant stress.

Cells bearing pathogenic mtDNA mutations are sensitized to oxidant stress. Since the nuclear DNA of transmitochondrial cell lines bearing mutant mtDNA is of identical origin as those bearing normal mtDNAs, all pathophysiological differences observed between mutant and control cells are attributable ultimately to differences in mtDNA. All three pathogenic mtDNA mutations conferred significant oxidant-sensitivity to their host cells, with the MELAS mutation conferring the greatest amount of oxidant-sensitivity, followed by LHON, and MERFF (Figure 1).

Calcium depletion rescues cells from death induced by oxidative stress. Depletion of Ca^{2+} significantly rescued all cells from oxidative stress, the magnitude of rescue was 143B > LHON > Da1B > MERRF > MELAS. This strongly implicates Ca^{2+} as a messenger in the death signal. Secondly, the greater rescue of LHON and MERRF cells than MELAS by Ca^{2+} depletion presumably reflects the origin of the LHON and MERRF lines, 143B, which exhibited the largest amount of rescue; whereas MELAS cells are derived from the Da1B line in which Ca^{2+} depletion exhibited a smaller amount of rescue. In each case the amplitude of rescue mediated by Ca^{2+}-depletion was less for the mutant than control line, this observation is consistent

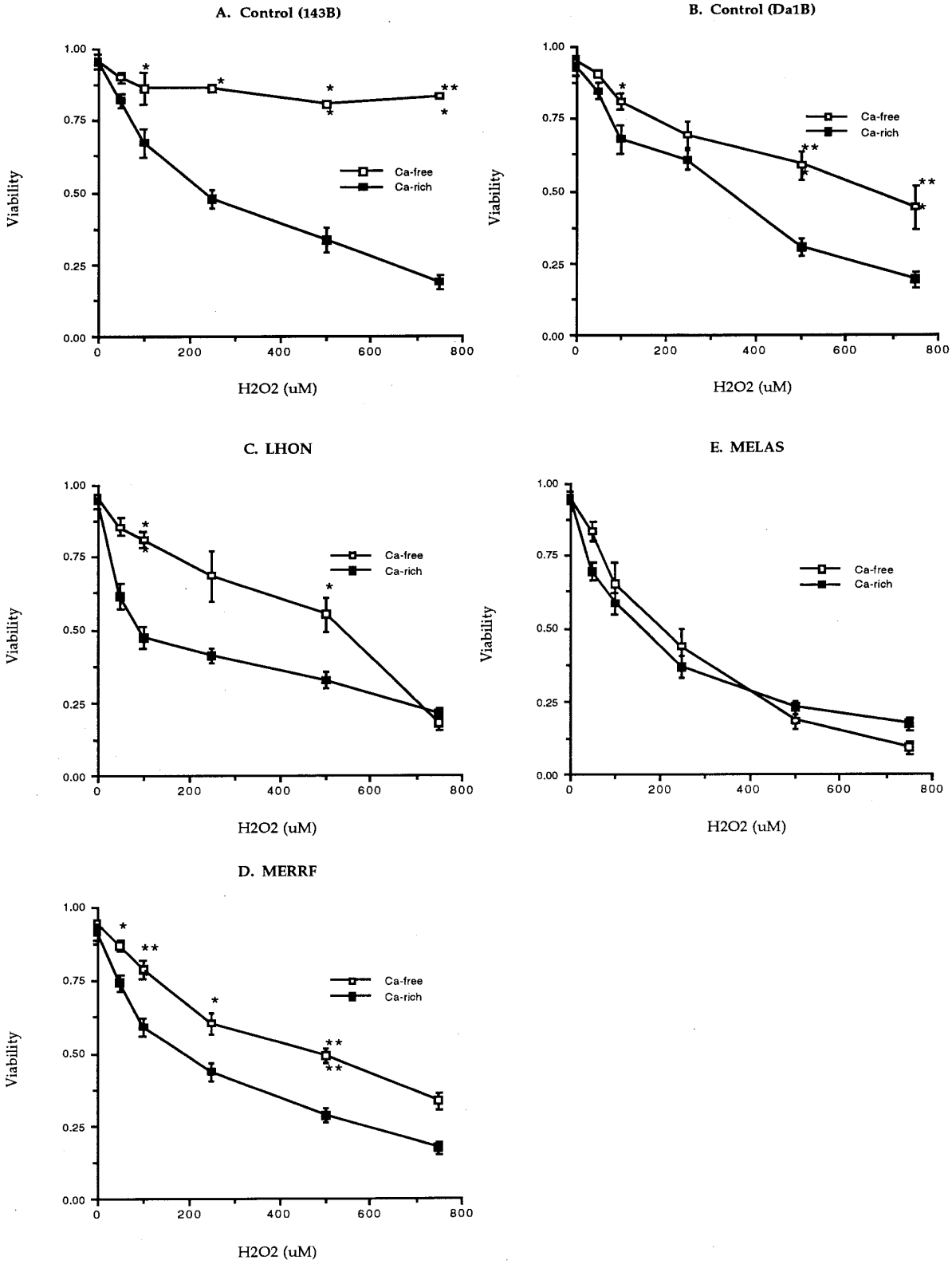

FIG. 2. Ca^{2+}-free media protects control cells and mutants from H_2O_2 induced death. Cells were grown in Ca^{2+}-free media for 24 hr., and fresh Ca^{2+}-free media was added to the cells before the addition of H_2O_2. Cells were harvested 6 hours later and cell viability was determined by the trypan blue exclusion assay. A-143B, B- Da1B, C-LHON, D-MERRF, and E-MELAS. Open squaresells in Ca^{2+}-free media; filled squares=cells in Ca^{2+}-rich media. Significance levels at individual doses are represented by asterisks, * = $p < 0.05$, **$p < 0.01$, **$p < 0.005$, and ****$p < 0.0001$. Ca^{2+}-depletion caused significant protection of cells at all doses in aggregate by two way ANOVA, $p < 0.001$. Averages from 3-20 independent experiments are shown, with bars representing two standard errors of the mean.

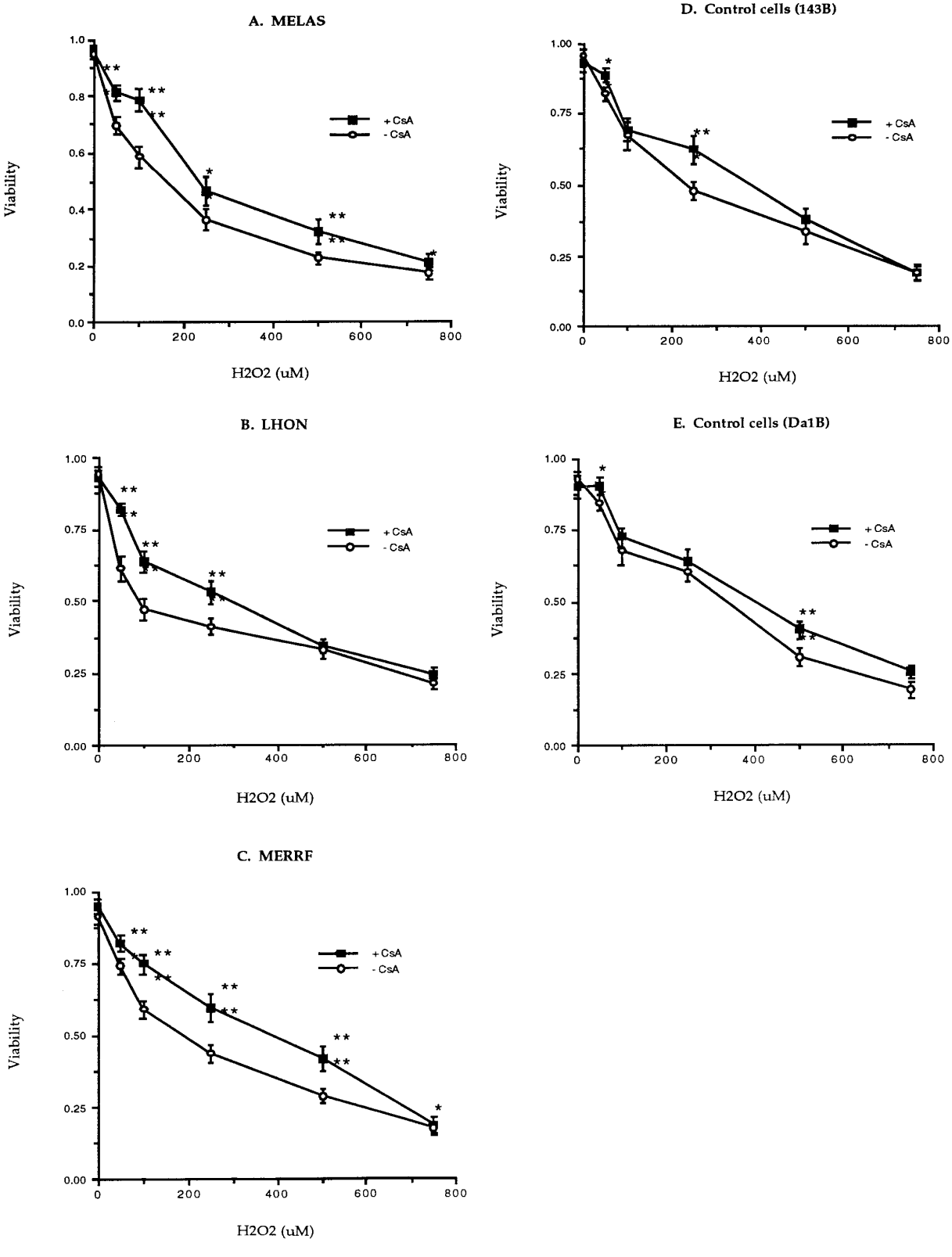

FIG. 3. Cyclosporin A protects mutant and control cells from oxidant stress. Cells (A-MELAS, B-LHON, C-MERRF, D-143B, and E-Da1B) were pre-treated with cyclosporin A (CsA) for 30 minutes before addition of H_2O_2. After 6 hours, the cells were harvested and percent viability was determined by the trypan blue exclusion assay. The following symbols represent the incubation conditions for each cybrid: filled-symbols=cells + CsA; and open symbols=cells. Means from 20 independent experiments are shown. Significance levels at individual doses are represented by asterisks, * = $p < 0.05$, **$p < 0.005$, ***$p < 0.001$, and ****$p < 0.0001$. CsA-mediated protection of controls and mutants was also significant ($p < 0.001$) by two-way ANOVA analysis of each mutant by control pair at all five doses, and CsA protected mutant cells better than control, $p < 0.0001$. Bars represent two standard errors of the mean.

with the hypothesis that mutant cells are defective at a point distal to the Ca^{2+} signal.

Cyclosporin A protects mutant mitochondria from oxidant-induced death. CsA protected both mutant and control cells from oxidant-induced death (Figure 3). Since the only genetic difference between the mutant and control cells is a mitochondrial one, the major biochemical-pathophysiological differences between mutant and control cells are likely to be mitochondrial ones. CsA has been demonstrated to inhibit the mitochondrial permeability transition (38-43), and to inhibit the release of the apoptotic factor cytochrome c from Ca^{2+}-treated mitochondria (44,45). Given the mitochondrial specificities of the mutations and CsA, it is possible that the protective effect of CsA on mutant and control cells is mediated by inhibition of MPT, and that the significantly greater protection of mutant vs. control cells by CsA ($p < 0.001$) is the result of rescue of a process (MPT) that is a major factor in the death of the mutant cells.

Data summary. We observe that the MELAS, LHON, and MERRF mutations confer sensitivity to death induced by oxidant stress, and that this death is partially rescued by Ca^{2+} depletion, which rescues controls significantly better than mutant cells, and by Cyclosporin A, which rescues mutants significantly better than control cella. The observations are consistent with a hypothesis in which oxidant stress induces a rise in intracellular Ca^{2+} which triggers MPT which triggers cell death, and that cells bearing mitochondrial mutations are compromised at the MPT step of this pathway. The hypothesis is subject to further tests, which are underway, and the observations may potentially be relevant to the understanding of mitochondrial disease pathophysiology and therapy.

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REFERENCES

1. Wallace, D. C., Lott, M. T., Shoffner, J. M., and Brown, M. D. (1992) *J. Inherited Metabolic Dis.* **15,** 472–479.
2. DiMauro, S., and Moraes, C. T. (1993) *J. Bioenerg. Biomembr.* **50,** 1197–1208.
3. Schon, E. A., Hirano, M., and DiMauro, S. (1994) *J. Bioenerg. Biomembr.* **26,** 291–299.
4. Chomyn, A., Martinuzzi, A., Yoneda, M., Daga, A., Hurko, O., Johns, D., et al. (1992) *Proc. Natl. Acad. Sci.* **89,** 4221–4225.
5. King, M. P., Koga, Y., Davidson, M., and Schon, E. A. (1992) *Mol. Cell Biol.* **12,** 480–490.
6. Muller-Hocker, J., Hubner, G., Bise, K., Forster, C., Hauck, S., Paetzke, I., et al. (1993) *Arch. Pathol. Lab. Med.* **117,** 202–210.
7. Goto, Y., Horai, S., Matsuoka, T., Koga, Y., Nihei, K., Kobayashi, M., et al. (1992) *Neurol.* **42,** 545–550.
8. James, A. M., Wei, Y. H., Pang, C. Y., and Murphy, M. P. (1996) *Biochem. J.* **318,** 401–407.
9. Moudy, A. M., Handran, S. D., Goldberg, M. P., Ruffin, N., Karl, I., Kranz-Eble, P. et al. (1995) *Proc. Natl. Acad. Sci.* **92,** 729–733.
10. Enriquez, J. A., Chomyn, A., and Attardi, G. (1995) *Nature Genetics* **10,** 47–55.
11. Hanna, M. G., Nelson, I. P., Morgan-Hughes, J. A., and Harding, A. E. (1995) *J. Neurol. Sci.* **130,** 154–160.
12. Byrne, E., Trounce, I., Marzuki, S., Dennett, X., Berkovic, S. F., Davis, S., et al. (1991) *Acta. Neuropathol.* **81,** 318–323.
13. Masucci, J. P., Davidson, M., Koga, Y., Schon, E. A., and King, M. P. (1995) *Mol. Cell Biol.* **15,** 2872–2881.
14. Villani, G., and Attardi, G. (1997) *Proc. Natl. Acad. Sci.* **94,** 1166–1171.
15. Hofhaus, G., Johns, D. R., Hurko, O., Attardi, G., and Chomyn, A. (1996) *J. Biol. Chem.* **271,** 13155–13161.
16. Majander, A., Finel, M., Savontaus, M.-L., Nikoskelainen, E., and Wilstrom, M. (1996) *Eur. J. Biochem.* **239,** 201–207.
17. Jun, A. S., Trounce, I. A., Brown, M. D., Shoffner, J. M., and Wallace, D. C. (1996) *Mol. Cell Biol.* **16,** 771–777.
18. Choi, D. W. (1988) *Trends Neurosci.* **11,** 465–469.
19. Beal., M. F. (1995) *Ann. Neurol.* **38,** 357–366.
20. Beal., M. F. (1996) *Current Opin. Neurobiol.* **6,** 661–666.
21. Bowling, A. C., and Beal, M. F. (1995) *Life Sciences* **56,** 1151–1171.
22. Duchen, M. R., McGuinness, O., Brown, L. A., and Crompton, M. (1993) *Cardiovas Res.* **27,** 1790–1794.
23. Fleckenstein, A., Frey, M., and Fleckenstein-Grun, G. (1983) *Eur. Heart J.* **4,** 43–50.
24. Orrenius, S., Ankarcrona, M., and Nicotera, P. (1996) *Adv. Neurol.* **71,** 137–149.
25. Orrenius, S., Burkitt, M. J., Kass, G. E., Dypbukt, J. M., and Nicotera, P. (1992) *Ann. Neurol.* **32,** S33–S42.
26. Smith, M. A., Richey Harris, P. L., Sayre, L. M., Beckman, J. S., and Perry, G. (1997) *J. Neurosci.* **17,** 2653–2657.
27. Owen, A. D., Schapira, A. H., Jenner, P., and Marsden, C. D. (1996) *Ann. NY Acad. Sci.* **786,** 217–223.
28. Sheehan, J. B., Swerdlow, R. H., Miller, S. W., Davis, R. E., Parks, J. K., et al. (1997) *J. Neurosci.* **17,** 4612–4622.
29. Sheehan, J. P., Swerdlow, R. H., Parker, W. D., Miller, S. W., Davis, R. E., and Tuttle, J. B. (1997) *J. Neurochem.* **68,** 1221–1233.
30. King, M. P., and Attardi, G. (1989) *Science* **246,** 500–503.
31. Chomyn, A., Meola, G., Bresolin, N., Lai, S. T., Scarlarto, G., and Attardi, G. (1991) *Mol. Cell Biol.* **11,** 2236–2244.
32. Shoubridge, E. A. (1995) *Biochem. Biophys. Res. Comm.* **213,** 189–195.
33. Zoratti, M., and Szabo, I. (1995) *Biochim. Biophys. Acta.* **1241,** 139–176.
34. Fournier, N., Ducet, G., and Crevat, A. (1987) *J. Bioenerg. Biomembr.* **19,** 297–303.
35. Crompton, M., Ellinger, H., and Costi, A. (1988) *Biochem. J.* **255,** 357–360.
36. Broekemeier, K. M., and Pfeiffer, D. R. (1989) *Biochem. Biophys. Res. Commun.* **163,** 561–566.
37. Halestrap, A. P., and Davidson, A. M. (1990) *Biochem. J.* **268,** 153–160.
38. Nazareth, W., Yafei, N., and Crompton, M. (1991) *J. Mol. Cell Cardiol.* **23,** 1351–1354.
39. Broekemeier, K. M., Carpenter-Deyo, L., Reed, D. J., and Pfeiffer, D. R. (1992) *FEBS Lett.* **304,** 192–194.

40. Snyder, J. W., Pastorino, J. G., Attie, A. M., and Farber, J. L. (1992) *Biochem. Pharmacol.* **44,** 833–835.

41. Imberti, R., Nieminen, A.-L., Herman, B., and LeMasters, J. J. (1993) *J. Pharmacol. Exp. Ther.* **265,** 392–400.

42. Griffiths, E. J., and Halestrap, A. P. (1993) *J. Mol. Cell Cardiol.* **25,** 1461–1469.

43. Pastorino, J. G., Snyder, J. W., Serroni, A., Hoek, J. B., and Farber, J. L. (1993) *J. Biol. Chem.* **268,** 13791–13798.

44. Yang, J. C., and Cortopassi, G. In press, *Free Radical Biology and Medicine.*

45. Kantrow, S., and Piantadosi, C. A. (1997) *Biochem. Biophys. Res. Commun.* **232,** 669–671.