BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **248,** 168–173 (1998)
ARTICLE NO. RC988866

Cyclosporin A Increases Resting Mitochondrial Membrane Potential in SY5Y Cells and Reverses the Depressed Mitochondrial Membrane Potential of Alzheimer's Disease Cybrids

David S. Cassarino,*,†,‡ Russell H. Swerdlow,‡,§ Janice K. Parks,‡,§
W. Davis Parker, Jr.,†,‡,§,¶ and James P. Bennett, Jr.†,‡,§,‖,**

**Medical Scientist Training Program, †Neuroscience Graduate Program, ‡Center for the Study of Neurodegenerative Diseases, §Department of Neurology, ¶Department of Pediatrics, ‖Department of Psychiatric Medicine, and **Department of Pharmacology, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908*

Received May 13, 1998

Alzheimer's disease (AD) brains exhibit oxidative stress and a biochemical defect of complex IV (cytochrome oxidase, COX) of the mitochondrial electron transport chain (ETC). This defect can be transferred through mitochondrial DNA (mtDNA) into clonal SY5Y cells depleted of their mtDNA. The resulting cytoplasmic hybrids or "cybrids" retain the complex IV defect and exhibit oxidative stress. We measured the mitochondrial membrane potential ($\Delta\psi_m$) in AD and control cybrids via H^3-tetraphenylphosphonium ion (H^3-TPP^+) accumulation. AD cybrids exhibited a significant (about 30%) decrease in H^3-TPP^+ accumulation relative to controls. Acute treatment of normal SY5Ys with azide, a COX inhibitor, moderately decreased H^3-TPP^+ retention and strongly inhibited COX activity in a dose-dependent manner. As the mitochondrial transition pore (MTP) can be activated by reactive oxygen species and ETC inhibitors, and its opening causes $\Delta\psi_m$ dissipation, we tested the effects of the MTP inhibitor cyclosporin A (CsA) on TPP^+ accumulation. 5mM CsA increased basal $H^{3\text{-}TPP^+}$ accumulation in SY5Y cells about 10-fold, corresponding to about a 2-fold increase in $\Delta\psi_m$. In the AD cybrids, CsA increased the apparent $\Delta\psi_m$ to the same final levels as it did in controls. These results indicate that low-conductance MTP activity contributes significantly to resting $\Delta\psi_m$ in SY5Y cells. We propose the novel hypothesis that the COX defect and resulting oxidative stress in AD may pathologically activate the MTP, resulting in lower $\Delta\psi_m$ and the release of mitochondrial factors involved in apoptosis. © 1998 Academic Press

Key Words: **Alzheimer's disease; mitochondria; mitochondrial membrane potential; Cyclosporin A; mitochondrial permeability transition pore.**

Abbreviations used: AD, Alzheimer's disease; ETC, electron transport chain; mtDNA, mitochondrial DNA; TPP^+, tetraphenylphosphonium ion; $\Delta\psi_m$, mitochondrial membrane potential; ROS, reactive oxygen species; MTP, mitochondrial transition pore; PT, permeability transition; CsA, cyclosporin A; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; ATR, atractyloside; MAO, monoamine oxidase; 5-HT, 5-hydroxytryptamine.

Alzheimer's disease is a commonly occurring and devastating neurodegenerative disorder that causes progressive loss of cognitive abilities in those afflicted. Although a small subset of AD cases are clearly familial, involving mutations in the presenilin 1 and 2 (chromosomes 14 and 1, respectively) or amyloid precursor protein (chromosome 21) genes (1), the majority of AD cases are late onset and appear to be "sporadic", not showing any evidence of autosomal inheritance (1, 2).

Mounting evidence suggests that mitochondrial dysfunction is prominent in AD and may underlie the "sporadic" cases of the disease (2–8). A mitochondrial defect of complex IV or cytochrome oxidase (COX) of the electron transport chain (ETC) is found in multiple tissues, including brain, in sporadic AD (2, 3, 4, 6). Transfer of platelet mitochondrial DNA (mtDNA) from AD patients into clonal host cells (creating cytoplasmic hybrids, or "cybrids") recreates the COX defect in these cells, demonstrating its mtDNA origin (6). Impaired COX activity increases reactive oxygen species (ROS) production and antioxidant enzyme activities (6) and disrupts mitochondrial Ca^{2+} handling (7) in the AD cybrids.

Mitochondrial damage is increasingly being implicated in human disease (2–9). At the subcellular level, many different insults lead to mitochondrial depolarization, increased permeability of the mitochondrial membrane, and release of soluble factors involved in

0006-291X/98 $25.00

cell death (9–13). The mitochondrial permeability transition (PT) occurs due to high-conductance opening of a CsA-sensitive megachannel, the mitochondrial transition pore (MTP) which spans the inner and outer mitochondrial membranes (10, 11, 14). The PT is inhibited by low concentrations of CsA (10, 14) and induced by ETC inhibitors (10, 11, 15, 16), elevated ROS and Ca^{2+} (9–11, 15, 16), and the adenine nucleotide translocator ligand atractyloside (ATR) (10, 11). PT leads to dissipation of the mitochondrial membrane potential ($\Delta\psi_m$), loss of ATP production, and release of mitochondrial stores of Ca^{2+}, glutathione, and cytochrome c (9–13, 16, 17). Hence, it is not surprising that the MTP seems to be a controlling factor in many cases of apoptosis, including those involving ETC inhibition (9, 15, 16), ROS (9, 18–20), and elevated calcium (9, 16, 20). Low-conductance opening of the MTP can also occur, and may allow changes in $\Delta\psi_m$ and release of small molecules without the mitochondria undergoing the PT and large-amplitude swelling (10, 17).

We have recently shown that the MTP can be opened by the parkinsonian neurotoxin MPP^+ in isolated mitochondria (21, 22, and in review), and have proposed a model implicating the MTP in apoptosis in those neurodegenerative diseases (including Parkinson's and Alzheimer's), which involve ETC defects and oxidative stress (22, and in review). To begin to examine the possible role of this channel in AD, we measured the CsA-sensitive accumulation of the lipophilic cation tetraphenylphosphonium (TPP^+), which measures $\Delta\psi_m$ (23), in normal SY5Y human neuroblastoma cells and in control and AD cybrids created from SY5Y cells.

MATERIALS AND METHODS

Preparation of cybrids. SY5Y cybrids were prepared from mtDNA-depleted cells as previously described (24). Cells were grown at 37°C and 5 % CO_2 in DMEM (Gibco BRL) supplemented with 10% fetal bovine serum, 1× penicillin/streptomycin, and 50 μg/ml pyruvate. Cells were grown to confluence in 6-well plates (Corning) prior to TPP^+ assays.

TPP^+ assay. The cell media was replaced with DMEM containing .5 μCi/ml [H^3]TPP^+. Where indicated, 5 μM CsA, 25 mM carbonyl cyanide m-chlorophenyl hydrazone (CCCP), or 25 mM ATR was present in the media. Cells were incubated for 1 hour at 37°C and 5 % CO_2, then washed with the same media for 10 minutes to remove nonspecific [H^3]TPP^+-binding. Remaining nonspecific binding was calculated from incubations of [H^3]TPP^+ with 100 mM unlabeled TPP^+, and these counts were subtracted. Cells were then washed with 2 ml PBS and harvested in 625 ml PBS. Two separate 100 ml aliquots were counted for [H^3]TPP^+ using liquid scintillation counting. Background counts were subtracted from all sample readings.

Monoamine oxidase (MAO) assay. MAO-A was assayed according to Paterson et al. (25), using .25 mCi/ml [C^{14}]-5-hydroxytryptamine (5-HT) bioxalate. Briefly, 400 ml cell homogenates were mixed with 100 mM 5-HT in PBS, and the reaction was run for 2 hours at 37°C. The acid product of 5-HT oxidation was extracted with ethyl acetate:toluene (1:1, V/V) and aliquots of the organic phase were counted in duplicate. Under these conditions less than 10% of the [C^{14}]-5-HT bioxalate was consumed, ensuring linearity. Nonspecific activity was determined separately for each sample by preincubation with 50 μM pargyline and was subtracted.

Cytochrome oxidase (COX) assay. SY5Y cells were harvested in trypsin-EDTA and transferred to 15 mL conical tubes in maintenance media. The cells were spun down, resuspended and washed by centrifugation in PBS, and then resuspended in Hanks buffered saline solution. COX activity was determined spectrophotometrically as the apparent first-order rate constant and referenced to protein as previously described (4), except that the reaction was followed for 10 minutes.

Statistics. All statistical analyses were performed using Sigma Stat software (Jandel Scientific).

RESULTS

SH-SY5Y cells incubated for 1 hour with [H^3]TPP^+ accumulated this lipophilic $\Delta\psi_m$-marker (Fig 1A). Treatment with 25 mM CCCP, which uncouples mitochondrial oxidative phosphorylation and collapses $\Delta\psi_m$, demonstrates the predominant mitochondrial-localization of [H^3]TPP^+ (Fig 1A). In order to test the effects of the MTP inhibitor CsA on [H^3]TPP^+ accumulation, we included 5 mM CsA in the 1-hour incubation. Surprisingly, CsA substantially increased [H^3]TPP^+ retention approximately 10-fold above baseline (Fig 1B). This corresponds to about a 2-fold increase in $\Delta\psi_m$ according to the Nernst equation.

This effect was completely prevented by inclusion of CCCP or the adenine nucleotide translocator ligand and PT-inducer ATR (Fig 1B), demonstrating that CsA's effect on $\Delta\psi_m$ is due to actions on the mitochondria and the MTP. We also sought to find the optimal concentration of CsA to use for our experiments by creating a concentration-response curve (Fig 1C). The maximal increase in [H^3]TPP^+ accumulation in response to CsA was obtained at about 5 μM, above which concentration the response decreases (Fig 1C). Hence, we chose a concentration of 5 μM CsA for our experiments.

In order to verify that the effect of CsA on [H^3]TPP^+ retention was due prevention of MTP-opening and subsequent washout of [H^3]TPP^+ (as opposed to increasing [H^3]TPP^+ uptake or other effects), we observed the CsA-dependent washout of accumulated [H^3]TPP^+. We found that 5 μM CsA substantially reduced the washout rate of accumulated [H^3]TPP^+ from SY5Y cells over 40 minutes following a 1-hour accumulation (Fig. 2).

We next compared [H^3]TPP^+ accumulation in SY5Y cybrid cells made with mitochondria from either control or AD patients. The AD cybrids exhibit significantly depressed complex IV activities compared to control cybrids (.0186 versus .0236, $p = .001$; Fig 3). We found that [H^3]TPP^+ accumulation was significantly decreased in the AD compared to control cell lines ($p = .01$; Fig. 4A). We normalized [H^3]TPP^+ counts to MAO-A activity to account for potential differences in mitochondrial mass between AD and control cells. MAO-A is a nuclear-encoded mitochondrial membrane protein.

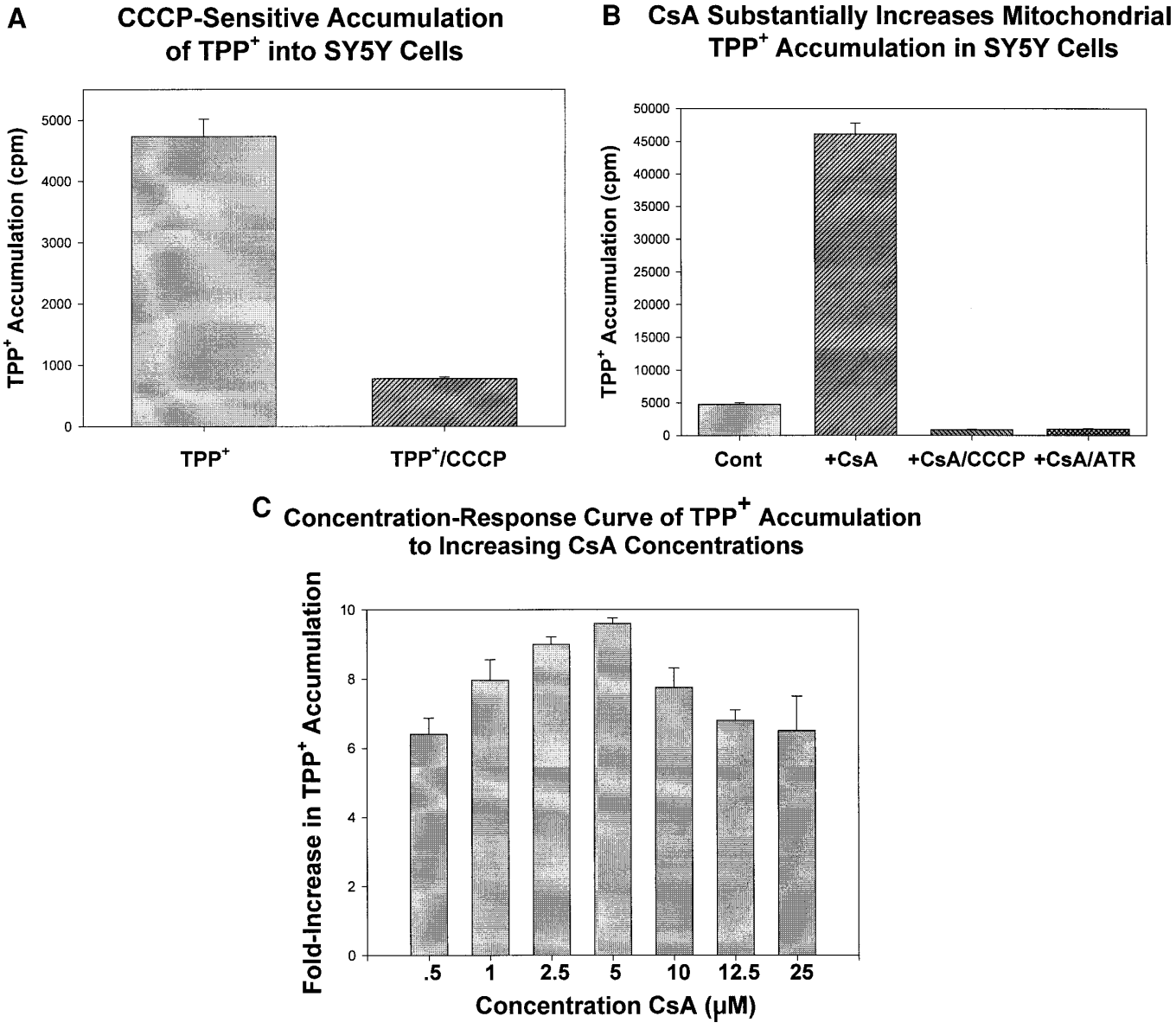

FIG. 1. (A) CCCP-sensitive accumulation of TPP^+ into SY5Y cells. SY5Y cells were incubated for 1 hour in the presence of .5 μCi/ml TPP^+ alone or with 25 mM CCCP, which uncouples oxidative phosphorylation and dissipates $\Delta\psi_m$. Cells were then washed for 10 minutes in DMEM, and washed and harvested in PBS for counting. (B) CsA substantially increases mitochondrial TPP^+ accumulation in SY5Y cells. SY5Y cells were incubated for 1 hour in the presence of .5 μCi/ml TPP^+ alone or with 5 μM CsA, 5 μM CsA plus 25 μM CCCP, or 5 μM CsA plus 25mM ATR. Cells were then washed for 10 minutes in DMEM, and washed and harvested in PBS. (C) Concentration-dependence of CsA's effect on TPP^+ accumulation. Cells were incubated for 1 hour in the presence of .5 μCi/ml TPP^+ with .5-25 μM CsA. Data presented are from at least 3 independent experiments, and represent the mean value +/− sem.

Since the cybrids are derived from a clonal cell line, the nuclear background is identical in each of them.

Addition of CsA to the incubation resulted in an intense increase (about 17-fold) in $[H^3]TPP^+$ accumulation by the AD cybrids (Fig. 4B), which was greater than the increase (about 10-fold) seen in controls ($p = .01$). There was no difference in the final TPP^+/MAO-A ratios between disease and control cybrids treated with CsA ($p = .49$). Hence CsA had a greater relative effect on $\Delta\psi_m$ in AD than in control cells, but increased $\Delta\psi_m$ to similar absolute values.

As the primary defect in the AD cybrids appears to be complex IV dysfunction, we attempted to model this in normal SY5Y cells with the complex IV inhibitor, sodium azide. The effects of a 3-day incubation with 50-300 μM azide on complex IV activity, as measured by a modification of the method of Wharton and Tzagaloff (26) are shown in Fig. 5. Azide inhibited complex

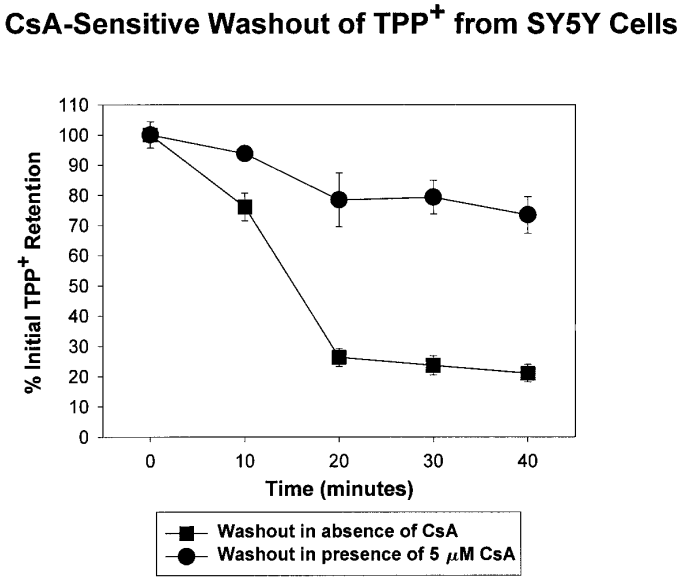

FIG. 2. CsA-sensitive washout of TPP^+ from SY5Y cells. Cells were incubated for 1 hour in the presence of .5 μCi/ml TPP^+ alone or with 5 μM CsA. Cells were then washed for 10 minutes in DMEM which was then replaced with fresh DMEM. Cells were then washed and harvested in PBS at the indicated times. Data presented are from at least 3 independent experiments, and represent the mean value +/− sem.

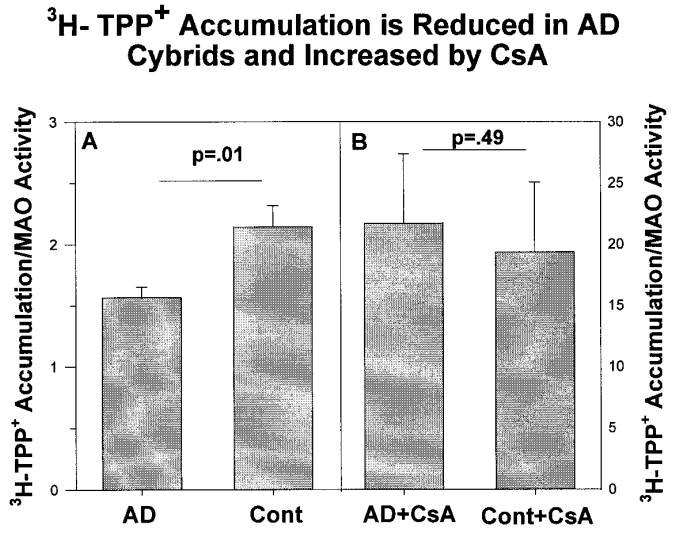

FIG. 4. (A) Accumulation of TPP^+ by AD and control cybrids. TPP^+ counts were normalized to MAO-A oxidase activity, a stable mitochondrial membrane marker. AD cybrids (n = 6) exhibited significantly lower (p = .01, unpaired t-test) TPP^+/MAO-A ratios and a greater increase in response to CsA than control cybrids ($P < .05$, unpaired t-test). (B) CsA treatment increased the TPP^+/MAO-A ratios in AD and control cybrids to similar levels (p = .49). Each cybrid line was grown up and assayed independently at least twice.

IV activity significantly at 50 mM, and caused a dose-dependent inhibition at higher doses. However, the effects of the same azide treatments on TPP^+ accumulation were much less dramatic, with 3 days of 300μM azide only leading to about a 30% decrease in TPP^+ accumulation (Fig. 5).

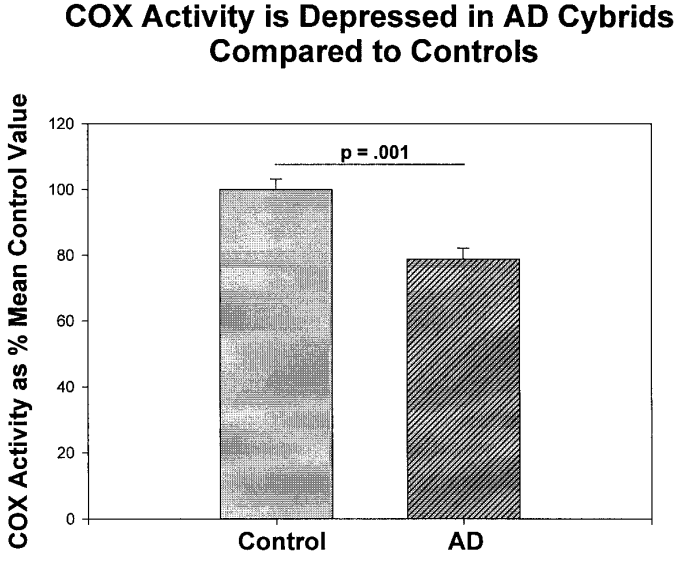

FIG. 3. Cytochrome oxidase activity in AD (n = 6) and control (n = 6) cybrids, expressed as a percentage (plus or minus sem) of the mean control value. The AD cybrids exhibited a significant (p = .0011) decrease in COX activity. Each cybrid line was grown up and assayed independently at least twice.

DISCUSSION

By measuring [H^3]TPP^+ accumulation, we have shown that SY5Y cybrids made with mitochrondria from AD patients exhibit significantly decreased $\Delta\psi_m$ compared to control cybrids (Fig. 4A). This decrease was reversed by CsA, which increased the retention of TPP^+ in both control and AD cybrids (by about 10- and 17-fold, respectively; Fig. 4B). This action of CsA occurs at the level of the mitochondria and most likely derives from inhibition of the low-conductance state of the MTP, as it is reversible by CCCP (uncoupling of oxidative phosphorylation) and ATR (opening of the MTP), respectively (Fig. 2). This dramatic effect of CsA was not an artifact of the cybrid technique, as native SH-SY5Y cells exhibited the same response to CsA as did the control cybrids. These results raise the novel and intriguing possibility that there is a high level of MTP activity in resting cells, which significantly contributes to basal $\Delta\psi_m$. We have also observed a similar effect of CsA on an independent measure of $\Delta\psi_m$ in SY5Y cells (tetramethylrhodamine fluorescence; C. P. Fall and J. P. Bennett, in review), excluding the possibility that this effect is peculiar to TPP^+.

Our results with azide indicate that acute and highly substantial inhibition of complex IV of the ETC does not replicate the mitochondrial membrane potential de-

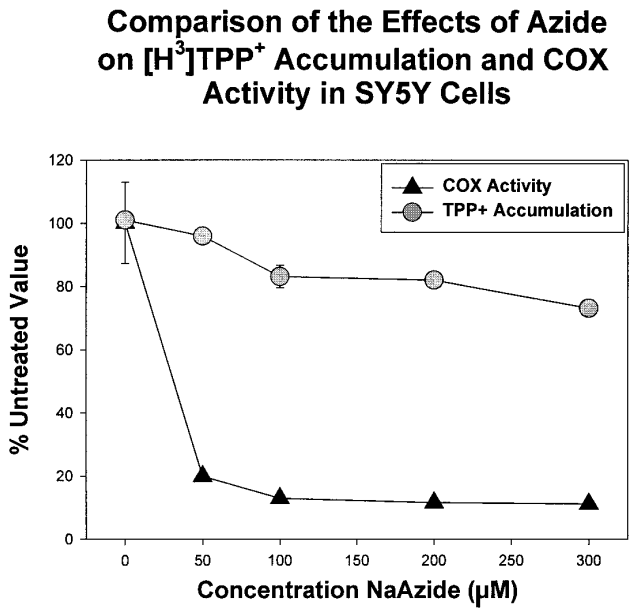

FIG. 5. Effects of azide on the accumulation of TPP^+ and COX activity in SY5Y cells. Cells were incubated for 3 days with 50 to 300 μM Na azide. COX activity was measured as described in the methods by following the oxidation of cytochrome c at 30° C. Each azide concentration was assayed at least twice separately.

fect seen in AD cybrids (Fig. 5). Thus, the decrease in COX activity present in the AD cybrids does not directly explain their decreased $\Delta\psi_m$. This might be expected if other, long-term effects of chronic partial inhibition of complex IV, as occurs in AD and in the AD cybrids, are responsible for the depressed $\Delta\psi_m$. We hypothesize that accumulating oxidative damage to mitochondria and mitochondrial membrane components, particularly the redox-sensitive sites on the MTP, may account for the difference in apparent $\Delta\psi_m$ between AD and control cybrids.

Pathological MTP-opening has been implicated in many processes including apoptosis (9, 15, 16, 18–20), and loss of $\Delta\psi_m$ appears to be required for apoptosis in many model systems (9). The possibility that the MTP may be involved in cell death in neurodegenerative diseases is just beginning to be explored (20–22). Our results represent the first evidence that mitochondria from AD patients may have a higher rate of opening of the MTP. This defect must ultimately result from mtDNA differences, as the AD cybrids differ from the control cybrids only in the source of their mtDNA, negating the influence of any nuclear or environmental factors (6).

Hence, a model for neuronal cellular injury and death in AD may be proposed in which an ETC defect in complex IV results in elevated ROS production and progressive cellular oxidative damage. Oxidation of redox-sensitive sites on the MTP (eg, the thiol site(s) on the adenine nucleotide translocator) (10, 11) would increasingly lead to pathological pore-opening and release of signals including Ca^{2+} and cytochrome c which are known to be capable of inducing apoptosis (9, 12, 13, 16, 17, 27). This theory is consistent with findings that ETC inhibitors and ROS can induce the mitochondrial PT and apoptosis (9, 15, 16, 18–20). Furthermore, others have demonstrated decreased mitochondrial membrane fluidity in the AD brain (28), decreased mitochondrial membrane-associated metabolic capacity in AD fibroblasts (29), and increased oxidative stress and oxidative mtDNA damage in the brains of AD patients (5, 28–30). These findings suggest that antioxidants, mitochondrial transition pore inhibitors, and mitochondrial membrane potential stabilizers may be of therapeutic value in treating AD patients.

ACKNOWLEDGMENT

This work was supported by a grant from the National Parkinson Foundation, and by NS35325 (JPB). DSC is supported by the University of Virginia Medical Scientist Training Program. We would like to thank John N. Davis II and Dr. Jill D. Bernstein for proofreading the manuscript.

REFERENCES

1. Levy-Lahad, E., and Bird, T. D. (1996) *Annals Neurol.* **40,** 829–840.
2. Parker, W. D., Filley, C. M., and Parks, J. K. (1990) *Neurology* **40,** 1302–1303.
3. Mutisya, E. M., Bowling, A. C., and Beal, M. F. (1993) *J. Neurosci.* **63,** 125–131.
4. Parker, W. D., Parks, J. K., Filley, C. M., and Kleinschmidt-DeMasters, B. K. (1994) *Neurology* **44,** 1090–1096.
5. Mecocci, P., MacGarvey, U., and Beal, M. F. (1994) *Annals Neurol.* **36,** 747–751.
6. Swerdlow, R. H., Parks, J. K., Cassarino, D. S., Maguire, D. J., Maguire, R. S., Bennett, J. P., Jr., Davis, R. E., and Parker, W. D., Jr. (1997) *Neurology* **49,** 918–925.
7. Sheehan, J. P., Swerdlow, R. H., Miller, S. W., Davis, R. E., Parker, W. D., and Tuttle, J. B. (1997) *J. Neurosci.* **17,** 4612–4622.
8. Beal, M. F. (1996) *Current Opin. In Neurobiol.* **6,** 661–666.
9. Zamzani N., Hirsch T., Dallaporta B., Petit P. X., and Kroemer G. (1997) *J. Bioenerg. Biomemb.* **29,** 185–193.
10. Zoratti, M., and Szabo, I. (1995) *Biochim. Biophys. Acta* **1241,** 139–176.
11. Bernardi, P. (1996) *Biochim. Biophys. Acta* **1275,** 5–9.
12. Liu X., Kim C.N., Yang J., Jemmerson R., and Wang X. (1996) *Cell* **86,** 147–157.
13. Kantrow, S. P., and Piantadosi, C. A. (1997) *Biochem. Biophys. Res. Commun.* **232,** 669–671.
14. Crompton, M., Costi, A., and Hayat, L. (1987) *Biochem. J.* **255,** 357–360.
15. Pastorino, J. G., Snyder, J. W., Serroni, A., Hoek, J. B., and Farber J. L. (1993) *J. Biol.Chem.* **268,** 13791–13798.
16. Pastorino, J. G., Snyder, J. W., Hoek, J. B., and Farber, J. L. (1995) *Am. J. Physiol.* **268,** C676–C685.
17. Reed, D. J., and Savage, M. K. (1995) *Biochim. Biophys. Acta* **1271,** 43–50.
18. Marchetti, P., Decaudin, D., Macho, A., Zamzani, N., Hirsch, T., Susin, S. A., and Kroemer, G. (1997) *Eur. J. Immunol.* **27,** 289–296.

19. Nieminen, A.-L., Byrne, A. M., Herman, B., and LeMasters, J. L. (1997) *Am. J. Physiol.* **272,** C1286–C1294.
20. Wong, A., and Cortopassi, G. (1997) *Biochem. Biophys. Res. Commun.* **239,** 139–145.
21. Parks, J. K., Cassarino, D. S., Smith, T. S., Trimmer, P. A., Parker, W. D., Jr., and Bennett, J. P., Jr. (1997) *Soc. Neurosci. Abst.* **23,** 740.1.
22. Cassarino, D. S., Fall, C. P., Smith, T. S., Bennett, J. P., Jr. (1998) *J. Neurochem.*, in press
23. Rugolo, M., and Lenaz, G. (1987) *J. Bioenerg. Biomemb.* **19,** 705–718.
24. Swerdlow, R. H., Parks, J. K., Miller, S. W., Tuttle, J. B., Trimmer, P. A., Sheehan, J. P., Bennett, J. P., Jr., Davis, R. E., and Parker, W. D., Jr. (1996) *Ann. Neurol.* **40,** 663–671.
25. Paterson, I. A., Juorio, A. V., Berry, M. D., and Zhu, M. Y. (1991) *J. Pharmacol and Exper. Ther.* **258,** 1019–1026.
26. Wharton, D. C., and Tzagaloff, A. (1969) *Methods Enzymol.* **10,** 245–250.
27. McConkey, D. J., and Orrenius, S. (1997) *Biochem. Biophys. Res. Commun.* **239,** 357–366.
28. Mecocci, P., Beal, M. F., Cecchetti, R., Poliori, M. C., Cherubini, A., Chionne, F., Avellini, L., Romano, G., and Senin, U. (1997) *Molec. Chem. Neuropathol.* **31,** 53–64.
29. McClure, R. J., Kanfer, J. N., Panchalingam, K., Klunk, W. E., and Pettegrew, J. W. (1994) *Annals N.Y. Acad. Sci.* **747,** 110–124.
30. Markesberry, W. R. (1997) *Free Rad. Biol. Med.* **23,** 134–147.