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Mitochondria dysfunction of Alzheimer's disease cybrids enhances Aβ toxicity

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Abstract

Alzheimer's disease (AD) brain reveals high rates of oxygen consumption and oxidative stress, altered antioxidant defences, increased oxidized polyunsaturated fatty acids, and elevated transition metal ions. Mitochondrial dysfunction in AD is perhaps relevant to these observations, as such may contribute to neurodegenerative cell death through the formation of reactive oxygen species (ROS) and the release of molecules that initiate programmed cell death pathways. In this study, we analyzed the effects of beta-amyloid peptide (Aβ) on human teratocarcinoma (NT2) cells expressing endogenous mitochondrial DNA (mtDNA), mtDNA from AD subjects (AD cybrids), and mtDNA from age-matched control subjects (control cybrids). In addition to finding reduced cytochrome oxidase activity, elevated ROS, and reduced ATP levels in the AD cybrids, when these cell lines were exposed to Aβ 1–40 we observed excessive mitochondrial membrane potential depolarization, increased cytoplasmic cytochrome *c*, and elevated caspase-3 activity. When exposed to Aβ, events associated with programmed cell death are activated in AD NT2 cybrids to a greater extent than they are in control cybrids or the native NT2 cell line, suggesting a role for mtDNA-derived mitochondrial dysfunction in AD degeneration.

Keywords: Alzheimer's disease, beta amyloid, cell death, cybrids, mitochondria.

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Excessive accumulation of cortical 'plaques' is a key histopathologic feature of Alzheimer's disease (AD). β-Amyloid peptide (Aβ), the major component of senile plaques, is believed by some to play an important role in AD pathogenesis (Selkoe 1990; Smith 1998). Although it is not conclusively known how Aβ toxicity (if any) might be mediated *in vivo*, *in vitro* studies reveal Aβ peptides can cause cell demize by inducing oxidative stress (Beal 1995; Mark *et al*. 1996). Whereas it is not possible to conclude Aβ is etiologically responsible, considerable data do indicate oxidative stress occurs in AD. This is reflected by an increase in lipid and protein oxidation markers (Smith *et al*. 1991; Lovell *et al*. 1995).

Mitochondrial dysfunction is also observed in AD. The activity of several different mitochondrial enzymes appears reduced; the best studied and perhaps most important of these enzyme defects involves cytochrome oxidase, the activity of which is deficient in both degenerating and non-degenerating AD tissues (Swerdlow and Kish 2002). As oxygen radicals are a by-product of mitochondrial respiration, and mitochondrial dysfunction can cause an increase in radical production, these organelles may represent a relevant initiating or intermediate site for increased AD oxidative stress. Studies in our laboratory and others (Mark *et al*. 1996; Cardoso *et al*. 2001) found Aβ 25–35 and 1–40 peptides decrease the activity of mitochondrial respiratory chain complexes. Other studies, however, indicate Aβ is not prerequisite for mitochondrial dysfunction in AD (Swerdlow *et al*. 1997; Sheehan *et al*. 1997; Cassarino *et al*. 1998; Ghosh *et al*. 1999; Khan *et al*. 2000; Trimmer *et al*. 2000),

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Abbreviations used: Aβ, beta-amyloid peptide; AD, Alzheimer's disease; DCF, dichlorofluorescein; $DCFH_2$-DA, 2′,7′-dichlorodihydrofluorescein; $\Delta\psi_m$, mitochondrial membrane potential; mtDNA, mitochondrial DNA; PI, propidium iodide; PS, phosphatidylserine; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reactive substances.

and that mitochondrial dysfunction can increase Aβ levels (Khan *et al*. 2000), perhaps through altering the processing of its parent amyloid precursor protein molecule (Gabuzda *et al*. 1994). As links between Aβ and mitochondrial dysfunction appear to exist, the possibility of a positive toxic feedback loop requires consideration.

Further data implicating an association between Aβ and mitochondrial dysfunction derive from studies of apoptosis. Mitochondria are key players in an 'intrinsic' apoptosis pathway that may be particularly relevant to AD neurodegeneration. In *in vitro* studies, Aβ renders cells vulnerable to apoptosis and activates several caspase enzymes (Nakagawa *et al*. 2000; Troy *et al*. 2000; Allen *et al*. 2001; Xu *et al*. 2001; Cardoso *et al*. 2002).

To address the relevance and potential causes of the AD cytochrome oxidase defect, the cytoplasmic hybrid ('cybrid') technique, first described by King and Attardi (1989), has previously been applied. In this technique, mitochondria/mitochondrial DNA (mtDNA) from human AD and control platelets are transferred to culturable cells depleted of endogenous mtDNA (ρ0 cells). The resulting cybrids allow for the *in vitro* elucidation of mitochondrial differences. Using neuronal-like ρ0 nuclear backgrounds, Swerdlow *et al*. (1997) found that compared to control cybrids, AD cybrid cytochrome oxidase activity was depressed and reactive oxygen species (ROS) formation increased. Subsequently, a number of investigations using AD cybrids have revealed a number of downstream consequences of the transferred cytochrome oxidase defect (Swerdlow *et al*. 1997; Sheehan *et al*. 1997; Cassarino *et al*. 1998; Ghosh *et al*. 1999; Khan *et al*. 2000; Trimmer *et al*. 2000), although one very small AD cybrid study in which a cervical carcinoma nuclear background was used failed to show a cytochrome oxidase defect (Ito *et al*. 1999).

We now report the use of cybrid cell lines to examine the functional consequences of cytochrome oxidase dysfunction when cells are challenged with Aβ 1–40 peptide. We observed that alterations in AD cybrid oxidative status renders these cells vulnerable to Aβ-induced cell death because these cells manifest an excessive decrease in the mitochondrial membrane potential ($\Delta\psi_m$), excessive mitochondrial release of cytochrome *c*, and caspase enzyme activation. Our results support the view that at least under *in vitro* conditions, the adverse consequences of a primary mitochondrial respiratory chain defect and Aβ peptide can be synergistic.

Materials and methods

Study sample includes six patients, ages from 56 to 80 years, with probable Alzheimer's disease (AD), evaluated at the Neurological Unit of the University Hospital of Coimbra. Of this group, three cases (50%) had pre-senile dementia, with onset of illness before 65 years of age, and two had a positive family history of AD. The diagnosis of dementia was based on the guidelines of the Diagnostic and Statistical Manual of Mental Disorders – DSM-IV and the diagnosis of probable AD was established according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) (McKhann *et al.* 1994). All patients underwent extensive clinical evaluation including neurological/psychiatric examination, neuropsychological testing, and specific staging scales which provide objective information about severity of dementia in various domains: cognitive impairment was quantified using the Minimental-State Evaluation (MMSE) (Foltein *et al.* 1975) and the mean score of the patient group was 9.3 ± 5.4 (range 2–15). Global dementia severity was staged as moderate (four cases) and severe (two cases) in accordance with the Clinical Dementia Rating (CDR) (Berg 1988). Routine laboratory tests, including chemistry profile, complete blood count (CBC), thyroid function tests, vitamin B12 and folic acid level, syphilis and Lyme serology were also performed, as well as EEG, brain CT, CSF analysis and ApoE genotyping. This evaluation confirmed the probable diagnosis of AD and allowed further genetic classification. The genotype in this group was apo E4/3 (two patients), two patients were homozygotic for the apo E *4 allele and another one for the apo E *3 allele. Age-matched control subjects were free of any neurodegenerative disease, and the mean age was (n = 5) 60.67 ± 3.3 years. Phlebotomy was performed to obtain blood samples, platelets isolated via centrifugation, and cybrid cell lines successfully created.

Chemicals

β-Amyloid peptide fragment (Aβ 1–40 or Aβ 1–42) was obtained from Bachem (Bubendorf, Germany). Fetal calf serum was obtained from Biochrom KG (Berlin, Germany). Rhodamine 123, MitoTracker-Green, MitoTracker-Red, Prolong Antifade Kit, Alexafluor 594 and 488 goat anti-mouse IgG conjugate, and Vybrant Apoptosis assay Kit #2 were purchased from Molecular Probes (Leiden, Netherlands). Anti-cytochrome *c* antibody was purchase from PharMingen (San Diego, CA, USA). N-acetyl-Asp-6lu-val-Asp-P-milnoanilid (DEVD-pNA) and N-acetyl-Val-6lu-Jb-Asp-P-mitnuamilide (VEID-pNA) were from Calbiochem (Darmstadt, Germany). All other reagents were from Sigma Chemical Co. (St. Louis, MO, USA) or from Merck kgaA (Darmstadt, Germany).

Cell culture

Stock cultures of NT2 (human teratocarcinoma) ρ+ cells were purchased from Stratagene (La Jolla, CA, USA). mtDNA-deficient ρ0 cells, derived from the human NT2 teratocarcinoma line, were obtained as a generous gift from Professor W. D. Parker laboratory. The ρ0 cells have no detectable complex I or cytochrome *c* oxidase activity; they are autotrophic for pyruvate (Miller *et al*. 1996). ρ0 cells were repopulated with platelet mitochondria from either control or AD patients through fusion in the presence of polyethylene glycol (Swerdlow *et al*. 1997). Untransformed cells were killed through withdrawal of pyruvate from the culture medium (Miller *et al*. 1996; Swerdlow *et al*. 1996). The surviving cells were termed mitochondrial cybrids (i.e. cytoplasmic hybrids). Cells were grown routinely in 75 cm^2 tissue culture flasks in Optimen Medium that was supplemented with 10% heat inactivated fetal calf serum, penicillin (50 U/mL), and streptomycin (50 μg/mL). NT2, control and AD cybrids cell lines were grown and maintained at 37°C in a

humidified incubator containing 95% air and 5% CO_2. Cells were plated at 0.6×10^6/mL for ATP measurements, for reactive oxygen species determinations, and carbonyl groups quantification, 0.15×10^6/mL for membrane potential determinations, imunocytochemistry experiments and for apoptosis kit assay, and 2×10^6/mL for determination of cytochrome *c* oxidase activity, citrate synthase activity, for caspase activity and thiobarbituric acid-reactive substances (TBARS) quantification.

Twenty-four hours after seeding of cells the medium was aspirated and replaced with similar medium containing Aβ 1–40 (1 μM) peptide. Aged Aβ 1–40 was added from a 230 μM stock phosphate-buffered saline solution after incubation during 7 days at 37°C. For all conditions tested, control experiments were performed in which Aβ was not added; all other incubation parameters were unchanged.

Cytochrome *c* oxidase (complex IV) assay

Complex IV activity was measured according to the method of Wharton and Tzagoloff (1967) by measuring the decrease in absorbance at 550 nm that occurs as reduced cytochrome *c* is oxidized. Cytochrome *c* reduction was initially performed by adding ascorbate crystals to cytochrome *c* and placing the mixture in a dialysis membrane for 18–24 h against 0.01 M phosphate buffer, pH 7.0, at 4°C. Reduced cytochrome *c* concentration was then determined with 0.1 M ferricyanide. The reaction mixture contained 0.01 M potassium phosphate, pH 7.0, and 50 μM reduced cytochrome *c*. The reaction was initiated by the addition of the mitochondrial sample at 30°C. The pseudo first order rate constant (K) was calculated, because the reaction is of first order with respect to cytochrome *c*. Results are expressed as K/(min mg of protein).

Citrate synthase assay

Citrate synthase activity was determined by the method of Coore *et al*. (1971), which spectrophotometrically follows the formation of 5-thio-2-nitrobenzoate (412 nm). The reaction mixture contained 100 mM Tris, pH 8.0, 200 μM [5,5′-dithiobis(2-mitnobentoic acid)] (DTNB), 0.1% (v/v) Triton X-100, and the mitochondrial sample. The assay was initiated by the addition of 100 μM oxaloacetate at 30°C. Results are expressed as percentage of control cybrids.

Analysis of adenine nucleotides

After the incubation period, the medium was removed. Cells were extracted, on ice, with 0.3 M perchloric acid. The adenine nucleotides (ATP, ADP, and AMP) were assayed by separation in a reverse-phase HPLC, as described by Stocchi *et al*. (1985). The chromatographic apparatus used was a Beckman System Gold, consisting of a 126 Binary Pump Model and a 166 Variable UV detector controlled by computer. The column was a Lichrospher 100 RP-18 (5 μm) from Merck (Germany). An isocratic elution with 100 mM KH_2PO_4 buffer at pH 7.4 and 1% methanol was performed at a flow rat of 1.2 mL/min. The adenine nucleotides were detected at 254 nm for 6 min.

Monitorization of reactive oxygen species generation

ROS were measured according to Bass *et al*. (1983), following the oxidation of 2′,7′-dichlorodihydrofluorescein ($DCFH_2$-DA) to fluorescent dichlorofluorescein (DCF), which detects the formation of intracellular peroxides. Cells were loaded, in the dark, with 5 μM $DCFH_2$-DA for 20 min in a Na^+-medium containing: 132 mM NaCl, 1 mM KCL, 1 mM $MgCl_2$, 10 mM glucose, 10 mM Hepes-Tris, pH 7.4, at 30°C. After washing, DCF basal fluorescence was measured during 5 min in the same medium and H_2O_2 (10 μM and 100 μM) was added and measured for 5 min. DCF fluorescence was detected with excitation and emission wave lengths of 502 nm and 550 nm, respectively.

Quantification of lipid peroxidation

The extent of lipid peroxidation was determined by measuring TBARS, which include malondialdehyde, using the Thiobarbituric Acid Test according to a modified procedure described by Ernster and Nordenbrand 1967). The amount of TBARS formed was calculated using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and expressed as nmol TBARS/mg of protein.

Protein oxidation determination

Protein carbonyl content was determined as described by Levine *et al*. 1990), with slight modifications. Cell extracts were incubated with 0.5 mL of 10 mM dinitrophenylhydrazine in 2 N HCl (or 2 N HCl alone for the blanks), for 1 h at room temperature. The protein hydrazone derivatives were precipitated with 0.5 mL of 20% trichloroacetic acid and the precipitates were washed three times with 1 mL ethanol : ethylacetate (1 : 1). During each washing, the homogenized pellet was vortexed and left in the washing solution for 10 min at room temperature before centrifugation. The final pellet was resuspended in 6 M guanidine HCl, and incubated during 15 min at 37°C. The carbonyl content was determined spectrophotometrically at 360 nm on the basis of molar absorbance coefficient of 22 000 M^{-1} cm^{-1}, and expressed as nmol/mg of protein.

Visualization of mitochondrial membrane potential ($\Delta\psi$) by confocal microscopy

After treatment, cells grown on chambered coverglasses were washed twice with phosphate-buffered saline and incubated with MitoTracker Red (750 nM) for 1 h, at 37°C. Following a further wash with phosphate-buffered saline, cells were fixed with 4% formaldehyde for 15 min at room temperature. Cells were then washed with phosphate-buffered saline and incubated with glycine 20 mM in phosphate-buffered saline. Cells were gently washed with phosphate-buffered saline and permeabilized with saponine 0.1% in phosphate-buffered saline for 30 min. Cells were then incubated with anti-cytochrome *c* against its native form (dilution 1 : 100 in saponine solution) for 1 h at room temperature, in the dark. Excess antibody was removed by washing cells with saponine solution. The secondary antibody, Alexa Fluo 488-conjugated goat anti-mouse IgG, diluted 1 : 60 in saponine solution was added, and the cells were incubated for 30 min at room temperature. The coverslips were washed with saponine solution, and fixed with Antifade. Results were obtained by confocal microscopy (Biorad MRC 600 with laser, argon and krypton).

Visualization of cytochrome *c* and mitochondria by confocal microscopy

After treatment, cells grown on chambered coverglasses were washed twice with phosphate-buffered saline and incubated with MitoTracker Green (750 nM) for 1 h, at 37°C. Following a further

wash with phosphate-buffered saline, cells were fixed with 4% formaldehyde for 15 min at room temperature. Cells were then washed with phosphate-buffered saline and incubated with glycine 20 mM in phosphate-buffered saline. Cells were gently washed with phosphate-buffered saline and permeabilized with saponine 0.1% in phosphate-buffered saline for 30 min. Cells were then incubated with anti-cytochrome *c* against its native form (dilution 1 : 100 in saponine solution) for 1 h at room temperature, in the dark. Excess antibody was removed by washing cells with saponine solution. The secondary antibody, Alexa Fluo 594-conjugated goat anti-mouse IgG, diluted 1 : 60 in saponine solution was added, and the cells were incubated for 30 min at room temperature. The coverslips were washed with saponine solution, and fixed with Antifade. Results were obtained by confocal microscopy (Biorad MRC 600 with laser, argon and krypton).

Caspase activation assays

After treatment with Aβ 1–40 (1 μM), cells were harvested for assays of caspase activity, by the method described by Cregan *et al.* (1999) with slight modifications. Cells were washed twice in phosphate-buffered saline and collected in a buffer containing 25 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM EGTA, 2 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and 1 : 100 dilution of a protease inhibitor cocktail. Cells were scraped, homogenized on ice, and frozen three times on liquid nitrogen. The lysate was centrifuged at 15 800 *g* for 10 min, and the resulting supernatant was stored at −80°C. Lysates (50 μg of protein) were incubated at 37°C, during 90 min, in 25 mM HEPES, pH 7.5, containing 0.1% CHAPS, 10% sucrose, 10 mM dithiothreitol, and 40 μM DEVD-pNA or 40 μM VEID-pNA to determine caspase 3 or caspase 6 activation, respectively. Substrate cleavage was detected in a Ph L Mediators Luminometer (Vienna, Austria) at 405 nm.

Externalization of phosphatydilserine by confocal microscopy

After treatment, cells grown on chambered coverglass were washed twice with phosphate-buffered saline and incubated with 100 μL of a solution containing the annexin V conjugate, propidium iodide in a binding buffer, prepared according to manufacturer's instructions. Following a further wash with phosphate-buffered saline, cells were mounted onto slides and observed using a confocal microscope (Biorad MRC 600 with laser, argon and krypton).

Data analysis

Data were expressed as mean ± SEM of the indicated number of determinations, from at least three independent experiments. Statistical significance analysis was determined using the two-tailed Student's *t*-test (a p-value < 0.05 was considered significant).

Results

Mitochondrial respiratory chain in AD and control cybrids

Cytochrome *c* oxidase activity and ATP levels were evaluated in native NT2 cells, AD cybrids, and control cybrids to determine whether the alteration observed in AD platelets was maintained in AD cybrids (Cardoso *et al.* 2004). We found that cytochrome *c* oxidase activity was decreased in AD cybrids [3.15 ± 0.66 K/(min mg)] as compared to control cybrids [4.06 ± 0.57 K/(min mg)] and NT2 cells [3.95 ± 1.02 K/(min mg)] (Fig. 1). We also evaluated citrate synthase activity in order to abolish potential differences in mitochondrial enrichment between AD and control cybrids. As shown in Fig. 2, citrate synthase activity was comparable between groups (100 ± 6.9% in controls cybrids and 88.22 ± 4.7% in AD cybrids). The ratio between cytochrome *c* oxidase and citrate synthase activities in AD cybrids was decreased as compared to control cybrids (data not shown). Differences in mitochondrial mass are therefore unlikely to account for the observed decrease in complex IV activity. We further observed that this complex IV defect was associated with decreased ATP levels in AD cybrids (66.65 ± 5.32 nmol/mg for AD cybrids, as compared to 92.82 ± 8.06 nmol/mg in control cybrids and 91.48 ± 10.18 nmol/mg in the native NT2 cell line) (Table 1). However, the bioenergetic state of AD cybrids cells was not compromised, since under basal conditions mtDNA from AD patients had a minimal impact on cellular energy stores.

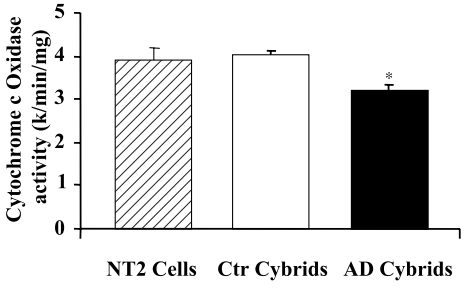


Fig. 1 Cytochrome *c* oxidase activity: influence of AD mtDNA. NT2 ρ+ cells, control and AD cybrids were used to determine cytochrome *c* oxidase activity, measured spectrophotometrically as described in Materials and Methods. Data are expressed as K/(min mg of protein), with the mean ± SEM derived from three to four independent determinations. *p < 0.05, significantly different as compared to control cybrids.

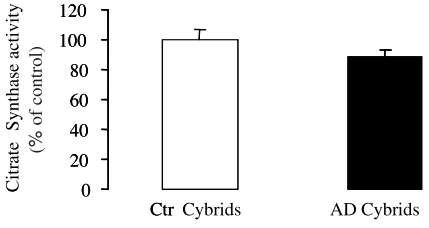


Fig. 2 Citrate synthase activity in platelet mitochondria. Data represent the mean ± SEM of five independent experiments and are expressed as percentage relatively to control cybrids.

Table 1 ATP levels and energy charge

	ATP levels (nmol/mg)	Energy charge
NT2 cells	91.48 ± 10.18	0.90 ± 0.02
Control cybrids	92.82 ± 8.06	0.95 ± 0.03
AD cybrids	66.65 ± 5.32*	0.91 ± 0.04

The results are the means ± SEM of duplicate determinations in five to six different experiments. *$p < 0.05$, significantly different as compared to control cybrids.

ROS production, lipid and protein oxidation in AD cybrids

As shown in Fig. 3(a), ROS production (measured by DCF fluorescence) in AD cybrids, was greater than that of control cybrids (84.5 ± 9.45 K and 37.5 ± 4.88 K, respectively).

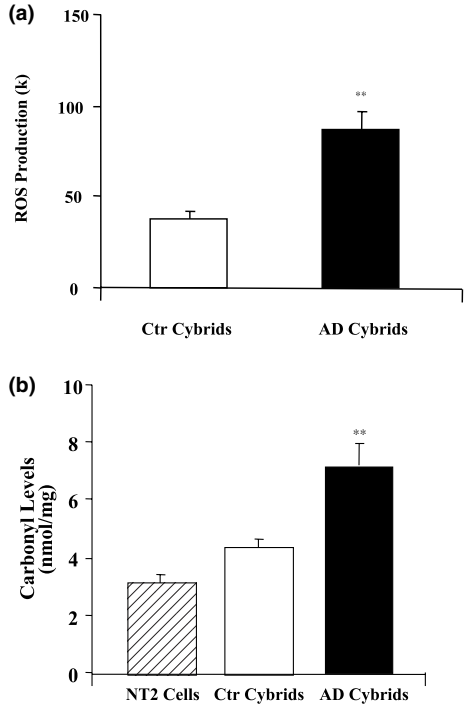


Fig. 3 The mtDNA-derived cytochrome *c* oxidase defect leads to increased oxidative stress. (a) ROS formation was detected using $DCFH_2$-DA oxidation and fluorescence as described in Materials and Methods. Results were expressed as the slope (*k*) obtained during 20 min, with the mean ± SEM derived from four to 10 independent experiments. **$p < 0.01$, significantly different as compared with control cybrids. (b) Protein oxidation was determined by detecting carbonyl groups formation as described in Materials and Methods. Results were expressed as nmol carbonyl groups per mg of protein, with the mean ± SEM derived from four to five independent experiments. **$p < 0.01$, significantly different as compared with control cybrids.

This observation is in accordance with our previous observations in AD platelets (Cardoso *et al*. 2004). We assessed whether this increase in ROS induced lipid and protein oxidation. AD cybrids showed evidence of both elevated lipid peroxidation (1.075 ± 0.109 nmol/mg in AD cybrids, 0.610 ± 0.022 nmol/mg in control cybrids, and 0.606 ± 0.020 nmol/mg in native NT2 cells) (Table 2) and protein carbonyl formation (7.126 ± 0.79 nmol/mg in AD cybrids, 4.400 ± 0.38 nmol/mg in control cybrids) (Fig. 3b). These findings are consistent with previous studies showing increased lipid and protein oxidation in AD brain tissue (Mecocci *et al*. 1994; Markesbery 1997).

Effects of mtDNA background and Aβ 1–40 on $\Delta\psi_m$, cytochrome *c* release, caspase activity, and phosphatidylserine externalization

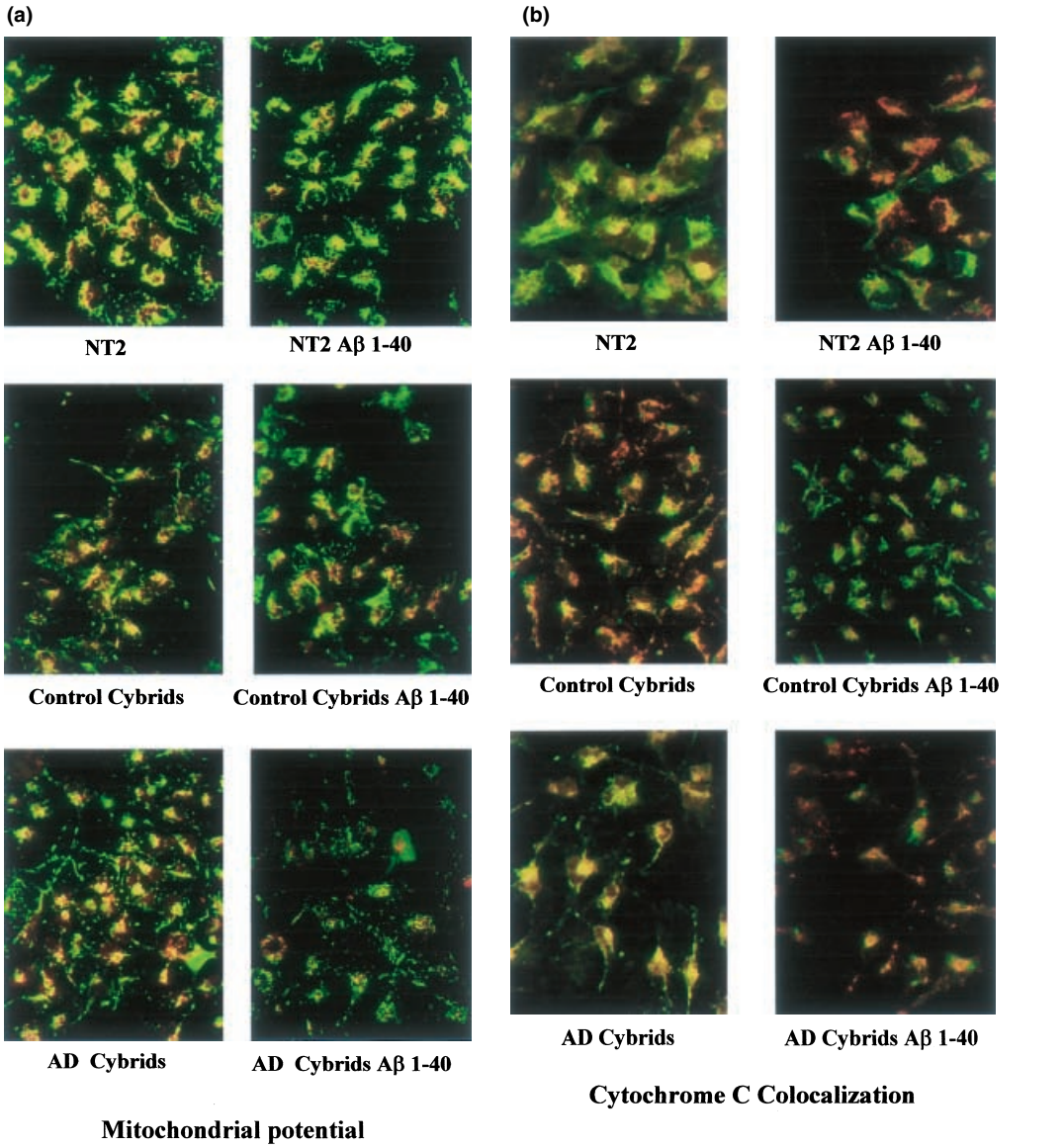
The disruption of mitochondrial membrane potential ($\Delta\psi_m$) appears to occur in cells undergoing apoptosis by allowing the release of cytochrome *c* (Cardoso *et al*. 2002). As shown in Fig. 4(a), $\Delta\psi_m$, detected using MitoTracker-red that accumulates in active mitochondria, decreased upon Aβ 1–40 exposure in NT2 and in cybrids, although the drop of $\Delta\psi_m$ was more pronounced in AD cybrids. In addition, decreased co-localization of cytochrome *c* with mitochondria, marked with Green-green, was observed upon Aβ 1–40 exposure in NT2 and cybrid cells (Fig. 4b). Once more, the release of cytochrome *c* from mitochondria was more marked in AD cybrids.

In many apoptotic systems, mitochondrial release of cytochrome *c* to the cytosol activates a proteolytic cascade, namely caspase-3. To determine whether caspase-3 activation was induced by Aβ 1–40 or by Aβ 1–42 we analyzed the cleavage of the peptide substrate DEVD-pNA. As shown in Fig. 4(c), both peptides induced caspase-3 activation in NT2 cells (2.28-fold increase and 2.13-fold increase, respectively), control cybrids (2.89-fold increase and 2.28-fold increase, respectively) and AD cybrids (2.7-fold increase and 1.82-fold increase, respectively). Nevertheless, caspase-3 activation was increased in AD cybrids in the absence of Aβ peptides (1.88-fold increase). Aβ 1–40 and Aβ 1–42 exposure induced an activation of caspase-6 in NT2 cells and in AD cybrids (Fig. 4d). However, no activation of caspase-6 was detected in control cybrids treated with Aβ (1–40 and 1–42) (Fig. 4d). When the brood range caspase

Table 2 Thiobarbituric acid-reactive substances (TBARS) levels

NT2 cells (nmol/mg)	0.606 ± 0.020
Control cybrids (nmol/mg)	0.610 ± 0.022
AD cybrids (nmol/mg)	1.075 ± 0.109**

The results are the means ± SEM of duplicate determinations in five to 10 different experiments. ** $p < 0.01$, significantly different as compared to control cybrids.



inhibitor, ZVAD-fmk, was added simultaneously we observed that caspase 3 and caspase 6 were not activated (data not shown).

Phosphatidylserine (PS) is normally located on the plasma membrane cytoplasmic surface. However, in apoptotic cells PS translocates from the inner to the outer membrane leaflet.

Fig. 4 Mitochondrial function upon Aβ treatment. (a) Mitochondria membrane potential was detected by MitoTracker-red and cytochrome *c* (green) co-localization, using confocal microscopy as described in Materials and Methods. Results show a representative experiment from three independent analyses. (b) Mitochondrial cytochrome *c* retention was detected by MitoTracker-green and cytochrome *c* (red) co-localization, using confocal microscopy as described in Materials and Methods, and shows a representative experiment from three independent analyses. (c) Caspase-3 activation was determined by Ac-DEVD-pNA cleavage. Results were expressed relative to the basal activity observed in the untreated cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. untreated NT2 cells. (d) Caspase-6 activation was determined by Ac-VEID-pNA cleavage. Results were expressed relative to the basal activity observed in the untreated cells. $^{**}p < 0.01$, vs. untreated NT2 cells.

Annexin V, a Ca^{2+}-dependent phospholipid binding protein has a high affinity for PS. Using fluorophore-labeled annexin V, we found that Aβ 1–40 increased PS externalization in AD cybrids more so than it did in control cybrids and NT2 cells (25% in AD cybrids, 15% in control cybrids and 6% in NT2 cells) (Figs 5a–d). In the later stages of apoptosis, plasma membrane compromise allows for internalization of the nucleic acid binding dye propidium iodide (PI). PI staining for AD and control cybrid cell lines was generally greater than it was in the native NT2 line (12% in AD cybrids, 6% in control cybrids and 3% in NT2 cells) (Figs 5a–d).

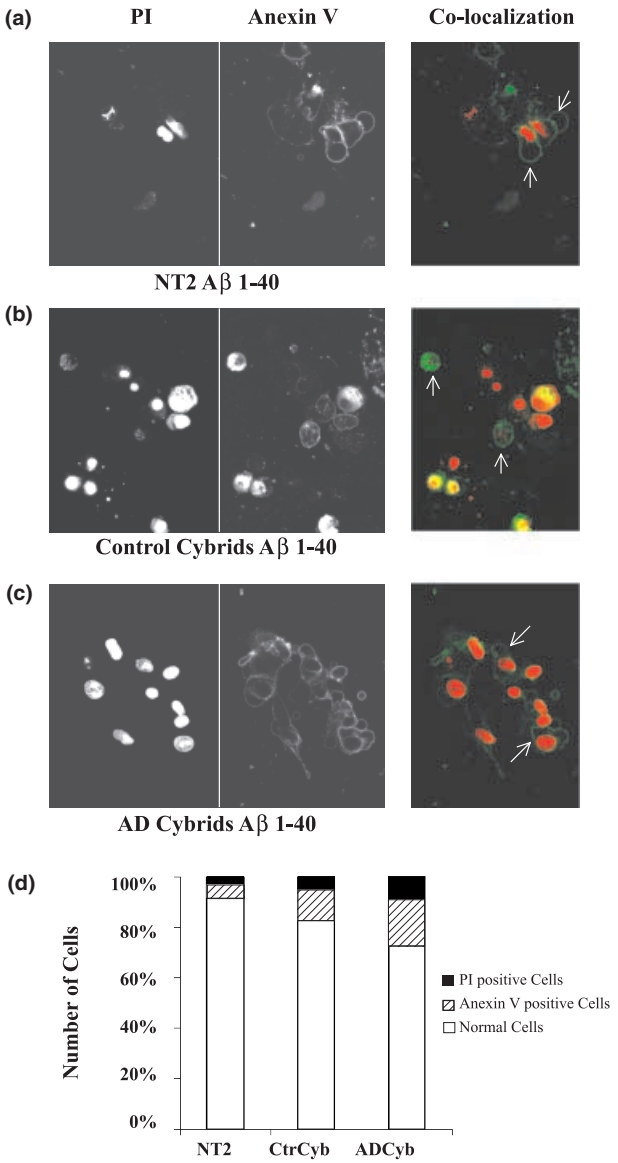


Fig. 5 Effect of Aβ 1–40 on phosphatidylserine externalization. Phosphatidylserine externalization (green) and propidium iodide internalization (red) in (a) NT2 cells, (b) control cybrids, (c) AD cybrids, and (d) representative graph, was determined using confocal microscopy as described in Materials and Methods. Results are representative of three independent analyses.

Discussion

These data confirm and extend the previous finding that expression of AD subject mtDNA within the context of the neuronal-like NT2 nuclear background has multiple functional consequences (Swerdlow *et al*. 1997). Mitochondrial function in these AD cybrids is altered in ways that recapitulate pathology observed in AD brain itself (reduced cytochrome *c* oxidase activity, increased oxidative stress) and in ways that promote events associated with programmed cell death. Our data support the view that functionally relevant mtDNA mutation exists in AD cybrids and by extension AD subjects, and at least partly accounts for the cytochrome *c* oxidase defect that is observed in multiple AD tissues.

It was previously proposed that the AD cytochrome *c* oxidase defect constitutes a genetically determined free radical generator (Swerdlow *et al*. 1997). Consistent with this, we observed ROS formation in AD cybrids was greater than it was in age-matched control cybrids. Additionally, Swerdlow *et al*. (1997) showed that ROS production was increased in AD cybrids despite the fact that ROS scavenging enzymes were up regulated, probably as a compensatory response. Dykens (1994) demonstrated that increased ROS production in isolated cerebral mitochondria from autopic AD brain could result from elevated basal cytosolic calcium levels. Further, data from SH-SY5Y cells show a correlation between cytochrome *c* oxidase inhibition and ROS formation (Miller *et al*. 1996). Functional interactions between mitochondrial function, calcium homeostasis, and ROS formation are potentially associated, and an imbalance of one of these parameters may have serious consequences at a cellular level (Beal 1992). Finally, we observed increased lipid and protein oxidation in AD cybrids, which likely reflects increased ROS

production in these cell lines. Based on the results of this study, we propose that in AD, ROS production could represent an important gain-of-function consequence of an mtDNA-derived cytochrome *c* oxidase defect.

Cytochrome *c* translocates from mitochondria to the cytosol when sufficient $\Delta\psi_m$ dissipation occurs (Mignotte and Vayssiere 1998). It is believed that defective mitochondria with reduced membrane potential may contribute to apoptosis by releasing cytochrome *c* (Liu *et al*. 1996). Once released, cytochrome *c* binds Apaf-1, and in the presence of dATP, activates caspase-9 (Zou *et al*. 1999). This in turn activates a series of downstream effector caspases, namely caspase-3 and -6 (Fernandes-Alnemri *et al*. 1995).

In this study we evaluated whether the cytochrome *c* oxidase defect and consequent ROS overproduction observed in AD cybrids increased susceptibility to further toxic insults. It has been shown that mitochondrial dysfunction can increase Aβ peptide secretion. Khan *et al*. (2000), using SH-SY5Y cell nuclear background, found AD cybrids secrete twice as much Aβ (1–40 and 1–42) as control cybrid lines, have increased intracellular Aβ 1–40, and develop distinct congo red-positive Aβ deposits that are not seen in control cybrid lines. In other tissue culture models, it was shown that cytochrome *c* oxidase inhibition shifts amyloid precursor protein degradation to a potentially insoluble form (Gabuzda *et al*. 1994).

Although we did not find NT2 AD cybrid cell lines to be overly sensitive to Aβ 1–40 in terms of viability measured by MTT reduction test (data not shown), we did find $\Delta\psi_m$ was reduced in our AD cybrids. This has been reported previously in studies using SH-SY5Y AD cybrids (Cassarino *et al*. 1998; Khan *et al*. 2000). Because $\Delta\psi_m$ depolarization is associated with mitochondrial cytochrome *c* release, we used our cell lines to analyze cytochrome *c* co-localization with mitochondria in the presence and absence of Aβ. Compared to control cybrids and native NT2 cells, Aβ 1–40 reduced cytochrome *c*-mitochondrial co-localization in AD cybrids. Under unstressed conditions, mitochondrial cytochrome *c* levels were decreased in NT2 AD cybrids, similar to the finding of Khan *et al*. (2000), a study in which SH-SY5Y AD cybrid cell lines were evaluated.

To study how Aβ 1–40 and Aβ 1–42 affected apoptotic induction in the cell lines under study, we evaluated caspase enzymes whose activation is dependent (caspase-3 and -6) of mitochondrial cytochrome *c* release. Both peptides activated caspase-3 in all cell lines, and the basal activity of this enzyme was also increased in AD cybrids. This observation is consistent with that of Khan *et al*. (2000) in studies of SH-SY5Y AD and control cybrid cell line caspase-3 activity. Caspase-6 activity was also increased in NT2 cells and AD cybrids with Aβ treatment. Nevertheless, a reduced sensitivity for Aβ peptides induced caspase-6 activity was observed in control cybrids. Moreover, the basal caspase-6 activity was increased in control and in AD cybrids.

To address the premise that cells with mitochondrial impairment might be vulnerable to Aβ-mediated apoptosis induction, we used PS externalization and PI internalization as experimental endpoints. With Aβ 1–40 exposure, PS externalization was observed in NT2 cells but this was not accompanied by PI internalization. In both AD and control cybrid cell lines, however, both PS externalization and PI nuclear staining occurred, suggesting these cells entered a more advanced apoptotic stage in which there was plasma membrane compromise. Aβ 1–40 is more toxic to cells with mtDNA alterations, even if these alterations arise as part of aging and are not temporally associated with AD phenotype manifestation. Still, both PS externalization and PI internalization were greater in AD than in control cybrids, after Aβ treatment, suggesting along with our cytochrome *c* oxidase data that if somatic mtDNA mutations do accumulate with age, these mutations are either quantitatively or qualitatively different between individuals with and without an AD phenotype.

We conclude that in AD cybrids there is a cytochrome *c* oxidase defect that derives from mtDNA, gives rise to oxidative stress, and activates pathways that are associated with cell death. Our results indicate that the cellular consequences of an increase in ROS production, lipid and protein oxidation, observed in untreated AD cybrids, renders them more vulnerable to an apoptotic cell death. While our data do not directly address the issue of neuronal demize in AD brain, expression of mtDNA from a non-degenerating AD subject tissue (platelets) is associated with events that are believed to intimately relate to neuronal loss and perhaps neurodegeneration (Agid 1995). As AD cybrids allow investigators to model (i) mitochondrial defects that are observed in AD subjects, and (ii) various processes that are associated with programmed cell death, it is worth considering the use of these cell lines for the elucidation of AD pathophysiology and potential therapeutic interventions.

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