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The plasma membrane redox system is impaired by amyloid β -peptide and in the hippocampus and cerebral cortex of 3xTgAD mice

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Abstract

Membrane-associated oxidative stress has been implicated in the synaptic dysfunction and neuronal degeneration that occurs in Alzheimer's disease (AD), but the underlying mechanisms are unknown. Enzymes of the plasma membrane redox system (PMRS) provide electrons for energy metabolism and recycling of antioxidants. Here, we show that activities of several PMRS enzymes are selectively decreased in plasma membranes from the hippocampus and cerebral cortex of 3xTgAD mice, an animal model of AD. Our results indicate the decreased PMRS enzyme activities are associated with decreased levels of coenzyme Q₁₀ and increased levels of oxidative stress markers. Neurons overexpressing the PMRS enzymes (NQO1 or cytochrome b5 reductase) exhibit increased resistance to amyloid β -peptide (A β). If and to what extent A β is the cause of the impaired PMRS enzymes in the 3xTgAD mice is unknown. Because these mice also express mutant tau and presenilin-1, it is possible that one or more of the PMRS could be adversely affected by these mutations. Nevertheless, the results of our cell culture studies clearly show that exposure of neurons to A β 1-42 is sufficient to impair PMRS enzymes. The impairment of the PMRS in an animal model of AD, and the ability of PMRS enzyme activities to protect neurons against A β -toxicity, suggest enhancement PMRS function as a novel approach for protecting neurons against oxidative damage in AD and related disorders.

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Keywords

Alzheimer's disease; Plasma membrane redox system; Oxidative stress; Coenzyme Q; Amyloid β -peptide

Introduction

Alzheimer's disease (AD) involves the dysfunction and degeneration of neurons in brain regions that play critical roles in learning and memory (Caselli, et al., 2006). Excessive production/secretion and impaired clearance of cytotoxic transitional oligomeric forms of the amyloid β -peptide ($A\beta$) is believed to play a pivotal role in AD (Thinakaran and Koo, 2008). Indeed, mutations in the β -amyloid precursor protein (APP) and presenilins-1 and -2 that cause early-onset familial AD increase the production of $A\beta_{1-42}$, a particularly cytotoxic form of $A\beta$. Aging is a major risk factor for AD and, together with genetic and environmental factors, may promote $A\beta$ accumulation and toxicity as a result of increased oxidative stress and impaired cellular energy metabolism (Jo, et al., 2008, Mattson, 2004). When $A\beta$ is in the process of aggregation on the cell surface it induces membrane lipid peroxidation (Bruce-Keller, et al., 1998, Cutler, et al., 2004, Mark, et al., 1997), possibly by interacting with Fe^{2+} and/or Cu^{+} to generate H_2O_2 and $OH^{\bullet}$ (Huang, et al., 1999, Lynch, et al., 2000, Rottkamp, et al., 2001). The membrane-associated oxidative stress (MAOS) may then perturb cellular ion homeostasis and energy metabolism, thereby contributing to synaptic dysfunction and neuronal degeneration (Mattson, 2004).

Recent findings suggest important roles for the plasma membrane redox system (PMRS) in aging and neuronal survival (Hyun, et al., 2006). There are at least three basic components for the PMRS: membrane associated quinone reductases (e.g. cytochrome b5 reductase (b5R), NADH-quinone oxidoreductase 1 (NQO1), etc), lipophilic antioxidants (Coenzyme Q (CoQ) and α -tocopherol) and the cytosolic electron donor NAD(P)H (Hyun, et al., 2006, Navas, et al., 2007). The PMRS produces NAD^+ for glycolytic ATP production by transferring electrons from intracellular reducing equivalents to extracellular acceptors, and reduces oxidative stress. Neurons exhibit trans-PM electron transport which is critical for their survival in culture (Wright and Kuhn, 2002). Multiple enzymes of the PMRS are expressed in neural cells and are up-regulated in response to mitochondrial impairment to preserve energy metabolism and protect the cells against oxidative stress (Hyun, et al., 2007, Rodriguez-Aguilera, et al., 2000, Villalba and Navas, 2000).

There has been considerable emphasis on the involvement of perturbed mitochondrial function in the pathogenesis of AD, with excessive free radical generation and reduced ATP production being a major focus in studies of AD patients and experimental models (Cottrell, et al., 2002, Mattson, et al., 2008, Parker, et al., 1994). In contrast, nothing is known of whether and in what ways the PMRS is altered in AD. The PMRS may play a particularly important role in protecting neurons against oxidative stress and energy impairment during aging because multiple PMRS enzymes are down-regulated in brain cells during normal aging in mice, and long-term dietary energy restriction in adult life preserves PMRS function and reduces oxidative damage to brain cells (Hyun, et al., 2006). However, the increased amount of MAOS that occurs in cells in vulnerable brain regions of AD patients (Markesbery, et al., 2005, Subbarao, et al., 1990) and animal and cell culture models of AD (Mark, et al., 1997, Montine, et al., 1999, Pratico, et al., 2001), suggests that one or more PMRS enzymes are likely to be altered. To elucidate the involvement of a perturbed PMRS in AD, we quantified activity levels of five different PMRS enzymes, CoQ and markers of oxidative stress in PMs isolated from brain regions with abundant or sparse $A\beta$ and neurofibrillary pathologies in the triple transgenic mouse model of AD (3xTgAD) compared with nontransgenic control mice. We show that the activities of

multiple PMRS enzymes are selectively reduced in brain regions with a high A β load (hippocampus and cortex), and that overexpression of NQO1 and b5R can protect neurons from being damaged by A β .

Methods

Animals

Eighteen male non-transgenic C57BL/6 mice, and 18 male triple transgenic (3xTgAD) AD mice harboring PS1_{M146V}, APP_{Swe}, and tau_{p301L} transgenes (Oddo, et al., 2003) that had been backcrossed to C57BL/6 mice for seven generations, were maintained in cages (4–5 five per cage) under a 12 hr light and dark cycle according to NIH guidelines and under a protocol approved by the National Institute on Aging Animal Care and Use Committee. As A β deposits and cognitive deficits become evident by the age of 10 months (Nelson et al., 2007), we euthanized 12 month-old 3xTgAD mice and age-matched non-transgenic mice by anesthetic overdose and five different brain regions (cerebral cortex, hippocampus, striatum, cerebellum and brain stem) were removed by dissection. The brain tissues were immediately flash-frozen and were stored at -80°C . Tissues from three different mice were pooled for the isolation of PMs resulting in 6 PM samples for control and 3xTgAD mice.

Isolation and characterization of the plasma membrane fraction

PMs were isolated from the dissected brains by using a two-phase partition, as described (De Cabo, et al., 2004, Hyun, et al., 2007). Immunoblotting and enzyme activity assays using markers for PMs, mitochondria, and ER were performed to establish the purity of the isolated fractions as described (De Cabo, et al., 2004). For immunoblotting, anti-Na⁺/K⁺-ATPase α -subunit monoclonal antibody (1:1,000 dilution; Affinity BioReagents, Golden, CO), anti-cytochrome *c* oxidase subunit I monoclonal antibody (1:1,000 dilution; Molecular Probes, Eugene, OR), and anti-ribophorin antibody (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) were used.

Measurement of PM CoQ₁₀ levels

CoQ₁₀ levels were assessed, as described previously (de Cabo, et al., 2003). Briefly, lipid fractions were extracted from the isolated PMs using hexane and then analyzed by HPLC with a reverse phase C₁₈ column (Supelcosil, 25 cm $\times$ 4.6 mm, 5 mm particle size, Supelco, Bellefonte, PA) and quantified by comparing integrated peak area with an internal standard.

Measurement of activities of PMRS enzymes

NADH-ascorbate free radical (AFR) reductase, which is mainly associated with b5R (Gomez-Diaz, et al., 1997), and NQO1 activities were determined *in vitro* using different electron donors as described previously (de Cabo, et al., 2003). Briefly, the reactions were carried out using a buffer containing the following: 50 mM Tris-HCl buffer, pH 7.6, 0.2 mM NADH and 0.4 mM of fresh ascorbate (NADH-AFR reductase) or 50 mM Tris-HCl buffer, pH 7.6, containing 0.08% Triton X-100, 0.2 mM NAD(P)H, 10 μM menadione and 76 μM cytochrome *c* (NQO1). Changes in absorbance were measured at 340 nm (NADH-AFR reductase) and 550 nm (NQO1). The extinction coefficients used for the specific activity calculation were 6.22 (NADH-AFR reductase) and 29.5 (NQO1) $\text{mM}^{-1}\text{cm}^{-1}$. Levels of b5R and NQO1 proteins were assessed by immunoblot analysis using antibodies against b5R (1:2,000, gifted from Jose Manuel Villalba, Universidad de Córdoba, Spain) and NQO1 (1:1,000, provided by David Ross, University of Colorado at Denver).

Activities of NADH-ferricyanide (FeCN) reductase, NADH-CoQ reductase and NADH-cytochrome *c* oxidoreductase were examined in a buffer containing the following: 50 mM Tris-

HCl pH 7.6, 0.2 mM NADH and electron donors (0.1 mM potassium FeCN, 0.2 mM CoQ₁₀ or 20 μ M cytochrome c). The reaction was started after the addition of NADH and followed by measuring changes in absorbance at 420 nm (for NADH-FeCN and -CoQ reductase) or 550 nm (NADH-cytochrome c oxidoreductase). The extinction coefficients used for the specific activity calculation were 1 (NADH-FeCN reductase), 0.7 (NADH-CoQ reductase) and 29.5 mM⁻¹cm⁻¹ (NADH-cytochrome c oxidoreductase).

Determination of levels of oxidative stress markers

Lipid peroxidation levels were assessed using the 8-Isoprostane Assay Kit (OxisResearch, Portland, OR). Briefly, PM fractions (100 μ l) were added to a 96-well plate and incubated with 100 μ l horse radish peroxidase-conjugated antibody at room temperature for 1 h, and 200 μ l substrate was added to the plate and it was incubated for 30 min. Absorbance was read at 450 nm after stopping the reaction by adding 50 μ l 3 M sulphuric acid. Protein carbonyl content was determined as described previously (Lyras, et al., 1997), except the final PM protein pellets were dissolved in 1 ml of 6 M guanidinium hydrochloride. Carbonyl content was calculated as nmol/mg protein (Reznick and Packer, 1994). Measurement of protein-bound nitrotyrosine (NT) content of isolated PM was performed using the Nitrotyrosine Assay Kit (OxisResearch).

Overexpression of PMRS proteins and A β -cytotoxicity assay

b5R and NQO1 proteins were overexpressed in human SH-SY5Y neuroblastoma cells using plasmid vectors (pCB6 for b5R and pBE8 for NQO1). More than 5 clones were selected and the protein levels were confirmed by immunoblot analysis. For cytotoxicity assays the cells (2×10^4) were cultured in a 96-well plate for 2 days and treated with a variety of concentration of oligomeric A β (1-42) (0 nM, 1 nM, 10 nM, 100 nM, 1 μ M and 10 μ M) or vehicle in serum-free media and incubated for 24 hours. Cell viability was measured by trypan blue exclusion and by using a Cell Counting Kit-8 (Donjindo, Kumamoto, Japan), as performed previously using SH-SY5Y cells (Nakagawa, et al., 2002; Park, et al., 2009); CCK-8 solution (10 μ l) was added to each well, and after a 30 minute incubation at 37°C the absorbance at 450 nm was measured.

Statistical analysis

Statistical differences were determined by one-way ANOVA. Pairwise comparisons were performed using a *post-hoc* Bonferroni *t*-test. Values are the mean and SEM; n=4-6. *p<0.01 compared with the value for wild-type (WT).

Results

Purity of the isolated plasma membranes

To determine the purity of the PM fractions, an immunoblot analysis using markers for PM, mitochondria and endoplasmic reticulum (ER) was performed. Following the two-phase partition process, the PM fractions were immunoreactive against a PM-specific Na⁺/K⁺-ATPase α -subunit antibody, whereas cytochrome c oxidase (mitochondrial protein) and ribophorin (endoplasmic reticulum protein) were not detected (Supplementary Fig. 1).

Plasma membrane CoQ₁₀ levels in brain regions of 3xTgAD mice

Levels of PM CoQ₁₀ were relatively higher in the cerebral cortex (CTX), hippocampus (HIP) and striatum (STR) compared with the cerebellum (CER) and brain stem (BS) (Fig. 1). PM CoQ₁₀ levels were significantly lower in the CTX and HIP of brains of 3xTgAD mice compared with nontransgenic control mice. In contrast, the PM CoQ₁₀ levels were not significantly different in STR, CER and BS of 3xTgAD and control mice.

Activities of NADH-dependent plasma membrane redox enzymes in brain regions of 3xTgAD mice

In nontransgenic control mice, NADH-AFR reductase activity was relatively higher in HIP and STR, compared with CTX, CER and BS (Fig. 2A). However, NADH-AFR reductase activity was significantly decreased in the PMs isolated from CTX and HIP of 3xTgAD mice compared with control mice. In contrast, this redox enzyme activity was not changed in STR, CER and BS of 3xTgAD mice compared to control mice (Fig. 2A). Immunoblot analysis revealed lower levels of NADH-AFR reductase protein in the HIP and CTX of 3xTgAD mice compared with control mice (Fig. 2A), indicating that the reduced enzyme activity levels was due, at least in part, to reduced levels of the enzyme.

In nontransgenic control mice, dicoumarol-sensitive PM-associated NQO1 activity was greater in the HIP compared with the other 4 brain regions examined (Fig. 2B). NQO1 activity was markedly lower in the CTX and HIP of 3xTg AD mice compared with control mice. There was a non-significant trend towards decreased NQO1 activity in the STR of 3xTgAD mice. Immunoblot analysis revealed that NQO1 protein content was lower in HIP and CTX PM fractions from 3xTg AD mice compared with control mice (Fig. 2B).

Activities of the other three NADH-dependent enzymes evaluated (NADH-FeCN reductase, NADH-CoQ reductase and NADH-cytochrome c oxidoreductase), were significantly greater in the HIP compared with the other four brain regions. Activity levels of these three enzymes were significantly reduced in the CTX and HIP (but not in the STR, CER and BS) of the AD mice compared with control mice (Fig. 3 A, B and C).

Markers of plasma membrane oxidative stress in brain regions of 3xTgAD mice

Levels of 8-isoprostane in PM, a marker for lipid peroxidation, were similar among all five brain regions of nontransgenic control mice (Fig. 4A). Levels of 8-isoprostane were significantly greater in the HIP and CTX of 3xTgAD mice compared with control mice (Fig. 4A). Levels of protein carbonyls, a biomarker of protein oxidation, were significantly greater in the PMs from CTX and HIP of AD mice compared with control mice, whereas protein carbonyl levels were not significantly different in PMs from the STR, CER and BS of 3xTgAD and control mice (Fig. 4B). Levels of nitrotyrosine (NT), a marker of reactive nitrogen species (RNS) activity, were similar among all five brain regions of control mice. NT levels were significantly higher in HIP and CTX PMs from 3xTgAD mice compared with control mice, whereas NT levels were similar in the STR, CER and BS of 3xTgAD and control mice (Fig. 4C).

Enhancement of the PMRS protects neuronal cells against amyloid β -peptide toxicity

If a deficit in the PMRS contributes to neuronal dysfunction and degeneration in AD, then enhancement of the PMRS would be expected to protect neurons against insults relevant to AD, including $A\beta$ toxicity. We found that human neuroblastoma cells overexpressing either NQO1 or b5R (Supplementary Fig. 2) were more resistant to being killed by $A\beta$ 1-42 than were mock-transfected control cells (Fig. 5).

Levels of PMRS enzymes are reduced in neural cells exposed to $A\beta$ 1-42

To determine if exposure of neurons to $A\beta$ affected PMRS enzymes in the 3xTgAD mice, we treated cultured human neuroblastoma cells with increasing concentrations of $A\beta$ 1-42 (from 1 nM – 10 μ M) for 16 hours and then measured levels of b5 reductase and NQO1. Levels of b5R were decreased in cells exposed to the highest two concentrations of $A\beta$ 1-42 (1 and 10 μ M) (Fig. 6A). Levels of NQO1 were markedly decreased in cells exposed to concentrations

of A β 1-42 from 10 nM to 10 μ M (Fig. 6B). These results suggest that exposure of neurons to A β 1-42 is sufficient to account for a decrease in PMRS function in AD.

Discussion

In AD, the PM is believed to suffer considerable oxidative damage to lipids and proteins as a result of aging combined with aberrant aggregation of A β at the cell surface (Cutler, et al., 2004, Mark, et al., 1997, Mark, et al., 1997, Rowan, et al., 2005). We found that activity levels of multiple enzymes of the PMRS (NQO1, AFR reductase, FeCN reductase, CoQ reductase and cytochrome c reductase) were impaired in brain regions of 3xTgAD mice previously shown to have major A β accumulation (CTX and HIP) compared with brain regions with minimal A β (STR, CER and BS) (Hirata-Fukae, et al., 2008, Nelson, et al., 2007, Oddo, et al., 2003) and with the hippocampus and cerebral cortex of nontransgenic control mice. Because PMRS enzyme activities are sensitive to inactivation/proteolysis during even relatively short (hours) postmortem intervals (Kozik, 1981, Yamazaki and Wakasugi, 1994), it has not been possible to determine whether these enzyme activities are reduced in vulnerable brain regions of AD patients prior to their degeneration. It is also difficult to interpret differences in levels of proteins in AD brain tissue samples compared with control brain tissue samples because of the neuronal loss that occurs in vulnerable brain regions. In contrast, the 3xTgAD mice exhibit A β accumulation and synaptic dysfunction, but no detectable neuronal death, mainly in the hippocampus and cerebral cortex (Halagappa, et al., 2007, Hirata-Fukae, et al., 2008, Nelson, et al., 2007, Oddo, et al., 2003), and so provided us the opportunity to establish the status of the PMRS in a model relevant to early stages of the AD process.

Interestingly, the activity levels of all five PMRS enzymes examined were higher in the hippocampus compared with the other four brain regions in control mice, and were reduced in the hippocampus of 3xTgAD mice to levels similar to those of the non-vulnerable brain regions. Previous studies showed that NQO1 protein levels were increased in brain regions closely related to AD pathology (Raina, et al., 1999, SantaCruz, et al., 2004). However, they measured levels of whole NQO1 protein, which is mainly located in the cytosol and translocated into the inner surface of the PM in response to oxidative stress (Li and Jaiswal, 1992, Rushmore, et al., 1991). This could explain why NQO1 protein levels were decreased in the PMs of affected brain regions of 3xTgAD mice even though cytosolic NQO1 levels were still higher than age-matched control groups. The high activity level of the PMRS in the hippocampus suggests a particularly important role for the PMRS in protecting neurons in this brain region against membrane-associated oxidative stress and maintaining cellular energy levels. It was reported that treatment with nicotinamide can ameliorate hippocampus-dependent cognitive deficits in 3xTgAD mice (Green, et al., 2008). While the latter study proposed a mechanism based upon sirtuin inhibition, it is possible that by supplying NAD, nicotinamide also enhances function of the PMRS. Indeed, it has been reported that neuronal NADH levels are decreased in aging (Parihar and Brewer, 2007). In addition, evaluation of protein-bound NADH in the CA1 pyramidal layer of presymptomatic APP mutant mice, using fluorescence lifetime sensitive spectroscopy, revealed a blue shift in the NADH spectrum suggesting reduced levels of protein bound NADH (Buchner, et al., 2002).

When taken together with previous findings, our data suggest that an impaired PMRS may contribute to the pathogenesis of AD both upstream and downstream of oxidative stress. Previous studies have provided evidence that oxidative stress and cellular energy deficits contribute to neuronal dysfunction and death in AD (see (Butterfield, 2003, Parihar and Brewer, 2007) for review). It was previously reported that markers of lipid peroxidation are increased and levels of α -tocopherol are decreased in the brains of 3xTgAD mice (Resende, et al., 2008). We found that CoQ₁₀ was depleted in PMs isolated from the hippocampus and cerebral cortex of 3xTgAD mice, suggesting a role for reduced PM antioxidants in membrane lipid

peroxidation in this mouse model. CoQ₁₀ depletion may contribute to A β pathology and synaptic dysfunction because previous studies have shown that dietary supplementation with CoQ₁₀ resulted in reduced A β pathology in a mouse model (Yang, et al., 2008). Consistent with a role for A β in the impaired PMRS in 3xTgAD mice, an electron microscopic 3D reconstruction analysis in 3xTgAD mice revealed an intimate spatial relationship of A β deposits with dendrosomatic PMs (Nuntagij, et al., 2009). In the present study, we showed that PMRS enzymes can protect neurons against degeneration and death; cells overexpressing either NQO1 or b5R were resistant to being killed by relatively high concentration of A β 1-42, even though very low concentration of A β can affect mitochondrial function (Eckert, et al., 2008, Iversen, et al., 1995, Saito, et al., 2001). If and to what extent A β is the cause of the impaired PMRS enzymes in the 3xTgAD mice is unknown. Because these mice also express mutant tau and presenilin-1, it is possible that one or more of the PMRS could be adversely affected by these mutations. Nevertheless, the results of our cell culture studies clearly show that exposure of neurons to A β 1-42 is sufficient to impair PMRS enzymes. Collectively, our findings demonstrate that multiple components of the PMRS are impaired in a brain region-specific manner in an animal model of AD, and further suggest that enhancement of PMRS function can suppress the neurodegenerative process in AD.

Supplementary Material

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Acknowledgments

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Fig. 1.

CoQ₁₀ is selectively depleted in PMs isolated from the hippocampus and cerebral cortex of 3xTgAD mice. CoQ₁₀ in the PMs from the indicated brain regions of WT and 3xTgAD mice were analyzed by HPLC. Values are the means and SEM; n=6, by using pooled tissue from three mice per group. *p<0.01 compared with the value for WT. CTX, cerebral cortex; HIP, hippocampus; STR, striatum; CER, cerebellum; BS, brainstem.

Fig. 2. Levels and activities of plasma membrane redox enzymes NADH-AFR and NQO1 are reduced in PMs from brains of 3xTgAD mice. The PM fractions isolated from the indicated brain

regions were used to measure activity and expression levels of NADH-ADR reductase (A) and NQO1 (B). Immunoblot analysis using NADH-AFR or NQO1 was performed and representative blots were shown. Values are the means $\pm$ SEM; n=6, by using pooled tissue from three mice per group. *, $P<0.01$ compared with the value for WT. CTX, cerebral cortex; HIP, hippocampus; STR, striatum; CER, cerebellum; BS, brainstem; W, WT; T, 3xTgAD.

Fig. 3.

Levels and activities of NADH-dependent redox enzymes are reduced in PMs isolated from the brains of 3xTgAD mice. The PM fractions isolated from indicated brain regions were used to measure activities of NADH-FeCN reductase (A), NADH-CoQ reductase (B) and NADH-cytochrome c oxidoreductase (C). Values are the mean and SEM; n=6, by using pooled tissue from three mice per group. *p<0.01 compared with the value for WT. CTX, cerebral cortex; HIP, hippocampus; STR, striatum; CER, cerebellum; BS, brainstem.

Fig. 4.

Levels of oxidative damage to lipids and proteins are increased in PMs from brains of 3xTgAD mice compared with control mice. PM fractions isolated from the indicated brain regions were used to measure levels of isoprostanes (A), protein carbonyls (B) and nitrotyrosine (C). Values are the mean and SEM; n=6, by using pooled tissue from three mice per group. *p<0.01 compared with the value for WT. CTX, cerebral cortex; HIP, hippocampus; STR, striatum; CER, cerebellum; BS, brainstem.

Fig. 5.

NQO1 and cytochrome b5 reductase protect neural cells against Aβ-cytotoxicity. Clones of human neuroblastoma cells overexpressing NQO1 or b5R, and mock-transfected control cells were exposed to a variety of concentration of Aβ1-42 (1 nM, 10 nM, 100 nM, 1 μM and 10 μM) or vehicle for 24 hours and neuronal survival was quantified by measuring the level of absorbance of CCK-8 solution (see Methods). Values are the mean and SEM of 5 separate experiments. *p<0.05 compared to the corresponding value for mock-transfected control cells.

Fig. 6.

Exposure of human neuroblastoma cells to Aβ1-42 decreases levels of the PMRS enzymes cytochrome b5 reductase and NQO1. Cultured human SH-SY5Y neuroblastoma cells were exposed to saline (control) or the indicated concentrations of aggregating Aβ1-42 for 16 hours. Levels of cytochrome b5 reductase (panel A) and NQO1 (panel B) proteins were then evaluated by immunoblot analysis. Similar results were obtained in a separate experiment.