

Role of glutathione in intracellular amyloid- α precursor protein/ carboxy-terminal fragment aggregation and associated cytotoxicity

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Abstract

Alterations in glutathione (GSH) metabolism are associated with neurodegeneration in Alzheimer's disease (AD), and GSH depletion follows application of exogenous fibrillar amyloid β (A β) peptides in experimental systems; these results are commonly cited as evidence of oxidative damage in AD. We used MC65 human neuroblastoma cells that conditionally express carboxy-terminal fragments of the A β precursor protein (A β /CTFs) to directly test the hypothesis that GSH is part of the cellular response to stressors associated with A β /CTF accumulation and not simply a marker of oxidative damage. Our data showed that A β /CTFs accumulated by post-translational processes and were associated with progressive increases in oxidative damage and cytotoxicity. Ethyrcinic acid

(EA) or diethyl maleate (DEM), reagents that deplete GSH through non-specific thiol adduction, gave rise to dose-dependent cytotoxicity that was independent of A β /CTF expression and minimally responsive to α -tocopherol (AT). In contrast, buthionine sulfoximine (BSO), a selective inhibitor of GSH synthase, not only augmented A β /CTF-associated cell death but unexpectedly potentiated A β /CTF accumulation; both outcomes were completely suppressed by AT. These data suggest that antioxidants may serve as 'A β targeting' therapies that suppress toxic protein aggregation rather than simply acting as downstream radical scavengers.

Keywords: aggregation, α -tocopherol, Alzheimer's, amyloid, antioxidants, glutathione.

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Alzheimer's disease (AD), the major dementing disorder of the elderly, is characterized by deposition of amyloid β (A β) in brain tissue in the form of insoluble amyloid plaques. Genetic studies of early onset familial AD have led to the discovery of mutations in amyloid beta precursor protein (APP) and presenilin genes that provide the strongest evidence for a role for APP and its metabolism in the pathogenesis of AD. In addition, oxidative stress has been proposed to play a key role in the development of AD and, *in vitro*, the generation of free radicals by aggregated A β peptides suggests one mechanism by which oxidative stress may develop in diseased regions of brain of patients with AD (Mark *et al.* 1996; Huang *et al.* 1999; Varadarajan *et al.* 2000). Although other mechanisms have been proposed for A β peptide-mediated neurotoxicity, data supporting a role for A β -induced oxidative damage form part of the rationale for antioxidant therapy in patients with AD. Indeed, one clinical trial showed modest benefit from α -tocopherol (AT) supplementation in patients with established AD (Sano *et al.* 1997); trials evaluating this and other antioxidants at earlier stages of AD are underway.

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Abbreviations used: A β , amyloid beta; A β /CTFs, amyloid beta precursor protein carboxy-terminal fragments; AD, Alzheimer's disease; ADDLs, AD diffusible ligands; APP, amyloid beta precursor protein; AT, α -tocopherol; BHT, butylated hydroxytoluene; BSO, buthionine sulfoximine; C99, 99 carboxy-terminal residues (beginning at the α -secretase cleavage site) of APP; DEM, diethyl maleate; DTT, dithiothreitol; 6E10, monoclonal antibody directed against A β _{1–17}; EA, ethyrcinic acid; FBS, fetal bovine serum; GCL, glutamyl cysteine ligase; GSH, glutathione; GSHee, glutathione ethyl ester; HMWC, high molecular weight complexes; HPLC, high-performance liquid chromatography; IsoPs, F₂-isoprostanes; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; PVDF, polyvinylidene fluoride; SDS, sodium dodecyl sulfate; Tet, tetracycline; Tet⁻, cell culture medium without tetracycline; Tet⁺, cell culture medium containing 1 μ g/mL tetracycline.

Glutathione (GSH), a key intracellular antioxidant and scavenger of reactive intermediates, displays altered metabolism in affected regions of brain from AD patients and is decreased in experimental models of AD (Schulz *et al.* 2000; Aksenov and Markesbery 2001; Apelt *et al.* 2004). Indeed, this observation is widely cited as one of the earliest pieces of evidence that AD is associated with oxidative damage. One possibility is that decreased GSH simply is a marker of increased free radical stress in AD tissue but of limited pathophysiologic significance. Alternatively, decreased GSH may represent the signature of a failing attempt to respond to free radical stresses in AD. Evidence in support of a mechanistic role for GSH in AD pathogenesis is the associated induction of GSH reductase and GSH peroxidase transcripts in the hippocampus from AD patients (Karelson *et al.* 2001; Dedeoglu *et al.* 2004). In addition, one GSH transferase, *GST ω 1*, has been found in genetic studies to be correlated with the age of onset of AD as well as Parkinson's disease (Li *et al.* 2004). Application of exogenous A β fibrils to neurons in culture leads to intracellular GSH depletion (White *et al.* 1999); however, this does not inform us concerning any potential role for GSH in A β fibril formation. Moreover, recent attention has been focused on the role of smaller intracellular aggregates of A β (Lambert *et al.* 1998) that may be generated within affected neurons themselves. These species, denoted 'AD diffusible ligands' or 'ADDLs', are found in cerebrum in AD and are closely associated with the disease state (Klein 2002). Importantly, the interactions, if any, between intraneuronal expression and aggregation of amyloidogenic peptides and GSH homeostasis have not yet been described.

To address this gap in our knowledge, we employed a stably transfected human neuroblastoma cell line, denoted MC65, that expresses the first 17 amino-terminal residues (signal peptide) and the 99 carboxy-terminal residues (beginning at the α -secretase cleavage site and designated C99) of APP in a conditional manner (Fukuchi *et al.* 1992a, 1992b, 1993; Sopher *et al.* 1994, 1996; Jin *et al.* 2002; Woltjer *et al.* 2003). Removal of tetracycline from these cells results in the initial expression of a species that migrates at approximately 10 kDa, consistent with the expected molecular weight of the C99 construct, with subsequent processing to a smaller band that co-migrates with A β _{1–40}, but not A β _{1–42} (Woltjer *et al.* 2003). This species, although not completely characterized, is recognized by several antibodies to A β , including the 6E10 monoclonal antibody which recognizes the amino-terminal 17 residues of A β , and undergoes progressive aggregation to high-molecular weight bands that are also recognized by 6E10 in western blots (Woltjer *et al.* 2003). The expression of these aggregates is closely associated with the subsequent development of cytotoxicity that can be almost completely blocked by AT (Sopher *et al.* 1996). Hence, this cell line provides a convenient bioassay for treatments that modulate intracellular

A β expression, aggregation, and associated toxicity that may be relevant to AD. Here, we used this model to test the hypothesis that GSH is a key component in the cellular response to pathophysiological stresses initiated by intraneuronal A β /CTF aggregation.

Materials and methods

Chemicals and antibodies

Tetracycline, AT, buthionine sulfoximine (BSO), ethyrcinic acid (EA), diethyl maleate (DEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), glutathione-S-transferase from equine liver, monochlorobimane, butylated hydroxytoluene (BHT), EDTA, emetine, and cycloheximide were purchased from Sigma-Aldrich (St Louis, MO, USA). DEM, EA, and AT were prepared fresh in ethanol (VWR, Brisbane, CA, USA) immediately before their use, and other reagents were maintained as aqueous stocks frozen at -20°C . Mouse monoclonal 6E10 antibody, which recognizes A β _{1–17}, was purchased from Senetek (Napa, CA, USA). Anti-insulin receptor antibody (C-19) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-linked sheep anti-mouse immunoglobulin was from Amersham (Piscataway, NJ, USA). Minimum essential medium α (MEME α), OptiMEM medium without phenol red, 0.5% trypsin/EDTA solution for cell culture, phosphate-buffered saline (PBS) pH 7.4, and fetal bovine serum (FBS) were purchased from Gibco-BRL (Carlsbad, CA, USA). All materials used in protein electrophoresis were from Bio-Rad (Hercules, CA, USA).

Cell culture

MC65 cells were cultured and maintained as previously described (Sopher *et al.* 1994, 1996; Jin *et al.* 2002). Cell passages beyond passage 20 relative to the originally characterized line were not used. Conditional expression of the transfected construct is obtained upon withdrawal of tetracycline using the promoter system previously described (Gossen and Bujard 1992). Cells were grown to approximately 90% confluence in MEME α supplemented with 10% FBS and tetracycline, then trypsinized, washed with PBS, resuspended in OptiMEM with or without 1 $\mu\text{g}/\text{mL}$ tetracycline, and plated at 2.5×10^4 cells/cm² (Sopher *et al.* 1994, 1996; Jin *et al.* 2002).

Cell viability assays

MC65 cells were plated as described above in Nunc 48-well plates (Nalge Nunc, Naperville, IL, USA) in the presence (Tet+) or absence (Tet-) of tetracycline with various concentrations of experimental reagents. After 1.5 days, culture medium was removed and cell viability was determined by replacement of culture medium with 180 $\mu\text{L}/\text{well}$ of fresh OptiMEM containing 0.25 mg/mL MTT followed by incubation for an additional 2 h at 37°C and then solubilization of MTT formazan product by addition of 360 $\mu\text{L}/\text{well}$ 40 mM HCl in isopropanol followed by shaking at 23°C for 10 min. Cell viability was determined by spectrophotometric determination of absorbance at 570 nm, as described previously (Woltjer *et al.* 2003). As previously reported (Woltjer *et al.* 2003), this assay gives results for viability in this cell line that are similar to those using the LIVE/DEAD assay (Molecular Probes, Eugene, OR, USA). All

assays were carried out in at least triplicate wells for each condition, all experiments were repeated at least four times, and representative results are presented in figures.

Protein extraction, electrophoresis, and western blotting

For these assays, cells were plated in 100-mm dishes. Following the indicated treatment, cells were dislodged by scraping, transferred to 15-well conical tubes for centrifugation at 2000 *g*, rinsed by resuspension in PBS with subsequent recentrifugation at 2000 *g*, and solubilized by sonication in 125–200 μ L of Laemmli sample buffer [62.5 mM Tris pH 6.8, 2% sodium dodecyl sulfate (SDS), and 10% glycerol] without dithiothreitol (DTT) or bromophenol blue. After centrifugation at 14 000 *g* for 5 min, the protein concentration of lysate supernatants was determined using the BCA microassay method from Pierce (Rockford, IL, USA). Lysates were normalized to a standard protein concentration with lysis buffer, DTT was added to a final concentration of 50 mM, bromophenol blue was added to a final concentration of 0.01%, and 20 μ L lysate containing 20 μ g protein was electrophoresed in Bio-Rad Criterion (Bio-Rad) 16.5% acrylamide gels containing tricine. Electrophoresed proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA, USA) and probed with 6E10 monoclonal antibody as described (Jin *et al.* 2002) with subsequent treatment with horseradish peroxidase-linked anti-mouse secondary antibody and visualized using enhanced chemiluminescence as described (Jin *et al.* 2002). The procedure for detection of the insulin receptor was the same, except that Bio-Rad Criterion 4–15% gradient acrylamide Tris gels were used with probing of membranes by anti-insulin receptor antibody from Santa Cruz Biotechnology with subsequent treatment with horseradish peroxidase-linked anti-rabbit secondary antibody.

Determination of A β /CTF structure

MC65 cells were grown in OptiMEM in the absence of tetracycline for 3 days and harvested as described above, except that cellular pellets were sonicated in PBS containing 6 M urea. Insoluble A β /CTFs were collected by centrifugation at 14 000 *g* for 5 min and urea extraction of the resulting pellet was carried out an additional five times, followed by solubilization of A β /CTFs in Laemmli sample buffer containing DTT and bromophenol blue. The SDS-soluble extract was electrophoresed in tricine gels as described above, A β /CTFs were visualized as a unique band using BioSafe Coomassie stain (Bio-Rad), and excised from the stained gel. The excised band was diced, destained with 50% acetonitrile in 50 mM ammonium bicarbonate, shrunk using acetonitrile, and dried under vacuum centrifugation. The gel pieces were reswollen in 30 μ L of trypsin solution (12.5 ng/mL in 50 mM ammonium bicarbonate) for 60 min on ice. The supernatant was replaced by 50 mM ammonium bicarbonate and incubated overnight at 37°C. The supernatant was collected and gels extracted with 30 μ L of 50% acetonitrile. Combined solutions were dried down to near completeness and volume adjusted to approximately 20 μ L. Sample was then desalted on a C18 ZipTip (Millipore) and analyzed on a matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometer (Voyager DE-STR, Applied Biosystems, Foster City, CA, USA). The peptide mass fingerprint database search was performed using MASCOT (Matrix Sciences, Boston, MA) and peaks observed were also matched against a theoretical A β trypsin digest

using a GPMaw software package (Lighthouse Data, Odense, Denmark).

Cellular GSH determination

Cellular GSH was determined using a monochlorobimane fluorometric method developed by Kamencic *et al.* (2000) and utilized by several other laboratories (e.g. Nauen and Stumpf 2002; Gonzalez-Polo *et al.* 2003; Hamilton *et al.* 2003; Buccellato *et al.* 2004; Kudin *et al.* 2004). Kamencic *et al.* (2000) showed that this method gives identical results for GSH levels as a more commonly used high-performance liquid chromatography (HPLC) technique. In brief, cells were dislodged from 100-mm dishes, transferred to 15-well conical tubes, rinsed with PBS and lysed by sonication in 500 μ L of 50 mM Tris, pH 7.4, 5 mM EDTA, and 0.001% BHT. From these lysate suspensions, 100 μ L was removed for determination of protein content, and the remainder of the lysate was centrifuged at 14 000 *g* for 5 min at 4°C. To 180 μ L of the resulting supernatant, 22.5 μ L of 1 mM monochlorobimane in 50 mM Tris, pH 7.4, and 22.5 μ L glutathione-S-transferase (from equine liver, 10 U/mL in 50 mM Tris, pH 7.4) were added, mixtures were vortexed, and 200 μ L of the preparation was transferred to black 96-well plates (Costar, Cambridge, MA, USA). Standard solutions of known concentrations of GSH were prepared in 50 mM Tris, pH 7.4, in parallel. After incubation at 23°C for 30 min, fluorescence of samples and standards at 470 nm was determined upon illumination at 380 nm using the SPECTRAMax 250 microplate spectrophotometer system from Molecular Devices (Sunnyvale, CA, USA).

Cellular IsoP determination

MC65 cells were dislodged from 100-mm dishes, transferred to 15-well conical tubes, rinsed with PBS and lysed by sonication in 200 μ L of PBS. From the resulting lysate, 40 μ L was removed and added to 160 μ L Laemmli sample buffer without DTT or bromophenol blue, solubilized by sonication, and subjected to BCA protein assay as described above. IsoPs in the remaining sample were quantified in Folch extracts of cerebrum using gas chromatography with negative ion chemical ionization mass spectrometry with selective ion monitoring as previously described (Reich *et al.* 2001).

Statistical analysis

Statistical analyses were performed using Graph Pad Prism 3.0 software (GraphPad, San Diego, CA, USA).

Results

Our first set of experiments sought to characterize further the accumulation of intraneuronal A β /CTFs in MC65 cells. Figure 1 shows the time course of A β /CTF expression and aggregation that confirmed our previous results (Woltjer *et al.* 2003). Several species of variously spliced and cleaved endogenous APP are present in Tet⁺ cells. A new A β -immunoreactive species initially appeared at day 0.5 in Tet⁻ cells and migrated in tricine gels at approximately 10 kDa, all features of the C99 construct (Sopher *et al.* 1996; Woltjer *et al.* 2003). Subsequent processing yields a smaller

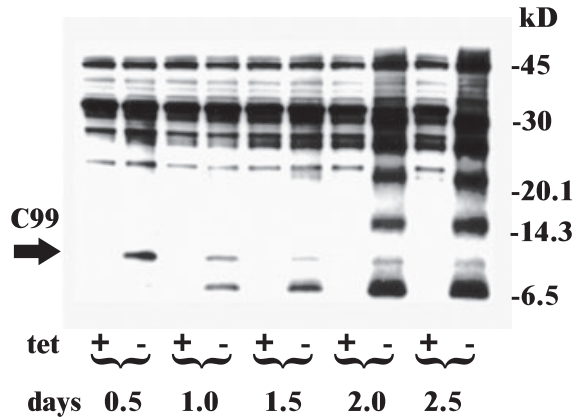


Fig. 1 Time course of A β /CTF expression and aggregation in MC65 cells. Cells were plated in 100-mm tissue culture dishes in the presence or absence of tetracycline for the indicated lengths of time. Whole cell lysates were prepared, and 20 μ g protein per lane were separated by electrophoresis in 16.5% tricine/SDS gels. After transfer to PVDF membranes, western blotting was performed using 6E10 antibody to the amino-terminal portion of A β . The first species expressed in the absence of tetracycline migrates at approximately 10 kDa, compatible with the predicted size of the C99 construct, with subsequent increasing expression of a 7.5-kDa species compatible with dimers of A β . This is accompanied by accumulation of progressively large immunoreactive species compatible with aggregates of A β /CTFs, with acceleration of this accumulation with the onset of cytotoxicity after 1.5 days incubation. These species are not apparent in MC65 cells cultured in the presence of tetracycline, although endogenous APP and its derivatives are present in the high-molecular weight range of the blot in cells grown in both the absence and presence of tetracycline.

band that co-migrates with synthetic A β_{1-40} (Woltjer *et al.* 2003); in our present experiments, this band was weakly present at day 0.5 and clearly present at day 1 in Tet $-$ cells. Here, for the first time, we purified this A β_{1-40} co-migrating band, digested it with trypsin, and analyzed the peptides by mass spectrometric analysis; we observed molecular masses corresponding to peptides derived from A β (HDSGYEVHHQK, corresponding to A β_{6-16} , as well as LVFFAEDVGSNK, corresponding to A β_{17-28}) but did not observe peptides either from the signal peptide of C99 or from those more carboxy-terminal to the γ -secretase cleavage site. Additional high-molecular weight A β -immunoreactive species that migrate in tricine gels at approximately 15 kDa, 22.5 kDa, and larger are compatible with aggregates of A β_{1-40} with itself and perhaps other CTFs; formation of these high molecular-weight complexes (HMWCs) has been repeatedly closely associated with cytotoxicity in this model (Sopher *et al.* 1996; Woltjer *et al.* 2003). The detailed genesis of such cell death-associated HMWCs has yet to be established in Tet $-$ MC65 cells.

We also pursued cellular changes that were temporally related to C99 translation, A β_{1-40} formation, and HMWC

accumulation, focusing on cytotoxicity, oxidative damage, and intracellular GSH concentration (Fig. 2). Figure 2(a) shows the time course for loss of MTT conversion activity, a measure of cytotoxicity that we have previously shown is equivalent to LIVE/DEAD assay in Tet $-$ MC65 cells (Woltjer

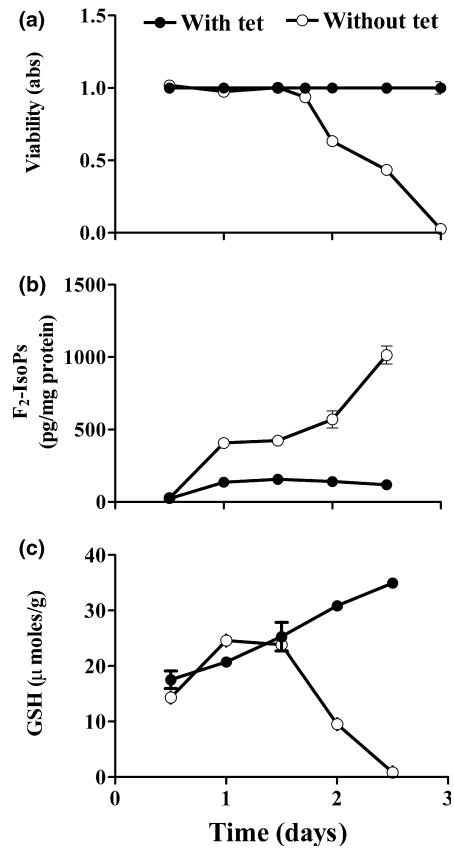


Fig. 2 Time course of cell viability, IsoPs, and GSH. Cells were plated in 48-well plates (a) or 100-mm (b and c) dishes in the presence or absence of tetracycline for the indicated length of time. At each time point, the appropriate endpoint was determined. For all assays, data are depicted as means \pm standard errors. Where error bars are not visible, errors were smaller than data symbols. Results depicted are an average of three determinations for each condition of time and tetracycline treatment. (a) Cell viability as determined by MTT assay, expressed as the ratio of the viability of Tet $-$ to Tet $+$ cells as reflected in MTT formazan product absorbance at 570 nm. Cell viability in Tet $-$ cells was preserved relative to Tet $+$ cells out to approximately 1.5 days in culture, after which cytotoxicity developed selectively in Tet $-$ cells with complete culture death by approximately 3 days. (b) Lipid peroxidation as determined by IsoP assay. Significant increases in lipid peroxidation were apparent by 1 day in Tet $-$ cells relative to Tet $+$ cells, well before the appearance of toxicity in MC65 cells, with a secondary marked increase that correlated with the development of cytotoxicity. (c) Cellular GSH content presented is normalized to protein content of harvested cells. GSH concentration increased linearly ($r^2 = 0.99$) over the course of the experiment as plated cells established active growth. In the absence of tetracycline, an increase in GSH was interrupted after approximately 1.5 days.

et al. 2003); note that the delayed onset of cytotoxicity followed accumulation of HMWCs (see Fig. 1). Figure 2(b) shows the time course for cellular F₂-isoprostanes (IsoPs), quantitative biomarkers of lipid peroxidation (Montine *et al.* 2002). No significant change in cellular F₂-IsoPs occurred in Tet⁺ cells over 2.5 days (ANOVA had $p > 0.05$). In contrast, cellular F₂-IsoPs were significantly greater in Tet⁻ cells compared to Tet⁺ cells at all time points except 0.5 days. These data show that onset of delayed oxidative stress was coincident with the appearance and initial processing of low molecular-weight A β /CTFs and then increased still further when HMWCs appeared. Figure 2(c) depicts the time course of cellular GSH concentration from MC65 cells. In Tet⁺ cells, GSH increased linearly ($r^2 = 0.99$) over the course of the first few days as cells recovered from plating and entered active growth. In Tet⁻ cells, this pattern was interrupted after approximately 1.5 days, with significant GSH depletion at both 2 and 2.5 days following Tet⁻ ($p < 0.0001$ for cellular GSH in Tet⁺ versus Tet⁻ MC65 cells at 2 days and beyond). Taken together, these changes in A β /CTF, viability, oxidative damage, and GSH are consistent with the following sequence: appearance of low molecular-weight A β /CTFs and the onset of partially compensated oxidative stress (day 0.5–1.0), progressive failure of antioxidant defenses and initial appearance of HMWCs (beginning around day 2), initial cell death (day 2.0–2.5), and then death of the culture (day 3.0).

To determine the mechanism(s) by which GSH depletion may contribute to this cascade of events in Tet⁻ MC65 cells, we employed multiple agents to deplete cellular GSH and

tested whether they specifically enhanced toxicity from C99 expression. We used three GSH depletors: EA and DEM, reagents that form adducts with GSH as well as other cellular thiols, and BSO, an inhibitor of γ -glutamyl cysteine ligase (GCL), the rate-limiting enzyme in *de novo* GSH synthesis (Anderson 1998). Intracellular GSH concentration was measured following 1-day exposure to varying concentrations of each compound and the EC₅₀ determined (Table 1). While the effectiveness at depleting GSH varied among compounds, it is interesting to note that in each case there was no difference in the EC₅₀ between Tet⁺ and Tet⁻, indicating no interaction with A β /CTF expression. Viability following the same treatments was measured at 1.5 days after tetracycline removal, a time at which there is not yet any loss of viability in untreated Tet⁻ cells; these EC₅₀ also are shown in Table 1. EA and DEM gave similar results in that both had EC₅₀ for GSH depletion that were approximately two- to threefold greater than the EC₅₀ for viability in both Tet⁺ and Tet⁻ cells. Taken together these data indicate that EA and DEM were toxic to cells through mechanisms other than those that involve depletion of GSH and not influenced by A β /CTF aggregation. In contrast, BSO had an EC₅₀ for GSH depletion that was substantially lower than the EC₅₀ for toxicity, consistent with the proposal that GSH depletion was central to toxicity from this compound. Importantly, BSO treatment showed an interaction with A β -specific toxicity, with no toxicity in Tet⁺ cells (represented as an EC₅₀ of '≥ 250 μ M') and significant toxicity in Tet⁻ cells ($p < 0.0001$). Under these conditions, toxicity was less than in EA- and DEM-exposed cells, consistent with the

Table 1 Comparing effects of GSH depletors on GSH and viability

Compound added	[GSH] EC ₅₀ (μ M)		Viability EC ₅₀ (μ M)		GSH EC ₅₀ / Viability EC ₅₀ (ratio)	
	Tet ⁺	Tet ⁻	Tet ⁺	Tet ⁻	Tet ⁺	Tet ⁻
EA	46.3 \pm 9	48.7 \pm 9	19.2 \pm 0.5	18.4 \pm 0.5	2.5 \pm 0.5	2.6 \pm 0.5
DEM	206.0 \pm 8	212.0 \pm 10	91.5 \pm 0.5	92.0 \pm 0.6	2.3 \pm 0.1	2.3 \pm 0.1
BSO	46.4 \pm 6.8	49.8 \pm 6	≥ 250*	168.0 \pm 7.7	n/a	0.30 \pm 0.05

*No toxicity (ANOVA had $p > 0.05$ compared to untreated Tet⁺ cells) was observed with BSO treatment up to 250 μ M. n/a, not applicable. For GSH, parallel cultures in triplicate 100-mm dishes were treated for 1 day in the presence or absence of tetracycline with increasing concentrations of EA, DEM, or BSO. GSH determinations were performed as in Fig. 3(c) and the EC₅₀ calculated for each compound. For viability, parallel cultures were plated in triplicate in 48-well tissue culture plates in the presence or absence of tetracycline with increasing concentrations of EA, DEM, or BSO. After 1.5 days incubation, cell viability was assessed by MTT assay as described for Fig. 3(a) and the EC₅₀ calculated for each compound. The ratio of GSH EC₅₀ to viability EC₅₀ was calculated to assess the relative effects of each compound on GSH metabolism and cytotoxicity. With EA or DEM (ratio > 1) cytotoxicity occurred at concentrations that did not alter GSH (e.g. 32 or 128 μ M, respectively, not shown), whereas BSO (ratio < 1) reduced GSH in Tet⁺ cells at subcytotoxic concentrations (to at least 256 μ M). Values are \pm SEM for all points; $n = 3$. There was no difference between Tet⁺ and Tet⁻ for EA or DEM (ANOVA had $p > 0.05$ in all cases). There was no difference in GSH between Tet⁺ and Tet⁻ for BSO up to 128 μ M (ANOVA had $p > 0.05$) despite significant differences in MC65 viability under these conditions (ANOVA had $p < 0.0001$).

conclusion that processes other than depleting GSH were contributing to the toxicity of the thiol adductors. These data showed that only the specific inhibitor of GSH synthesis, but neither of the thiol adductors, selectively influenced A β /CTF-associated cytotoxicity, demonstrating that GSH is critical in the response to stresses associated with C99 expression.

We confirmed a direct interaction between cellular GSH and post-translational processes that underlie A β /CTF aggregation in western blots of cells treated with BSO. The A β /CTF-associated toxicity in Tet⁻ cells exposed to 64, 128 or 256 μ M BSO (concentrations spanning the EC₅₀ for viability) was accompanied by increased accumulation of both low-molecular-weight A β /CTFs and HMWCs (Fig. 3). In contrast, concentrations of EA and DEM that approximated or were greater than their EC₅₀ for viability were associated with progressive loss of A β /CTFs, again consistent with cytotoxicity from these compounds occurring independently of A β /CTF formation (Fig. 3). As expected, no expression of A β /CTFs was observed in Tet⁺ cells, regardless of other cell treatments (not shown). To test the hypothesis that the intracellular accumulation A β /CTF aggregates resulted from post-translational events, we incubated cells in the presence of translational inhibitors, emetine or cycloheximide. Figure 4(a) confirms a decrease in synthesis of the insulin proreceptor (88% decreased with emetine and 77% decreased with cycloheximide) in

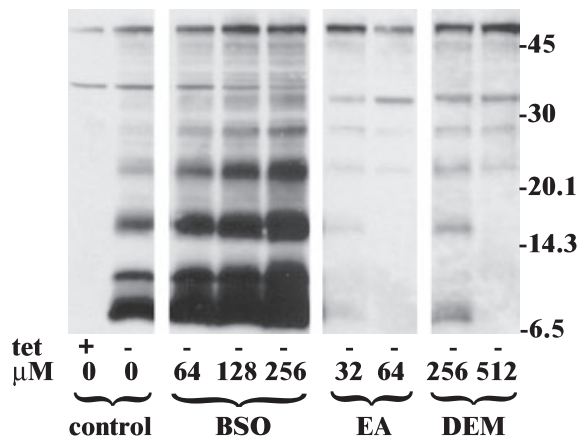


Fig. 3 Cells were cultured for 1.5 days in 100-mm dishes under the indicated conditions. Subsequently, cells were harvested, protein extracted, and samples electrophoresed and western blotted with 6E10 antibody directed against A β as described in the text. Treatment with BSO over a range of cytotoxic concentrations (64, 128 and 256 μ M resulting in 79, 56 and 31% viability, respectively) led to concentration-dependent increases in A β /CTF species and higher-molecular-weight aggregates. In contrast, markedly cytotoxic concentrations of EA and DEM (\geq EC₅₀ for each) were associated with progressive loss of A β /CTF expression; under no condition of treatment with EA or DEM was enhanced accumulation or aggregation of A β /CTFs observed.

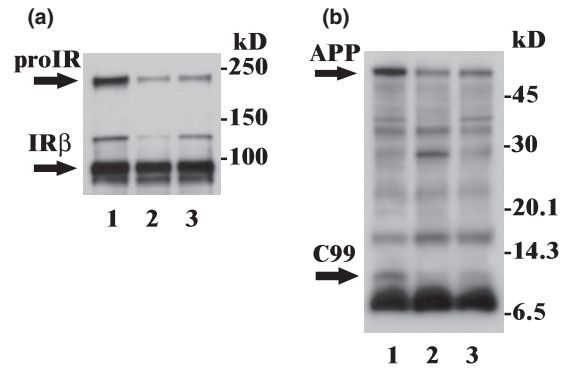


Fig. 4 MC65 cell cultures were plated in 100-mm dishes without tetracycline and with 128 μ M BSO. Lysates depicted in lane 1 were from cells that received no additional treatments for 1.5 days. In parallel, after establishment of cultures for 0.5 days, cells were treated with inhibitors of protein synthesis, either 10 μ g/mL emetine (lane 2) or cycloheximide (lane 3). After a total of 1.5 days in culture, cells were harvested, protein was extracted, and proteins were separated by electrophoresis. (a) Protein separation was carried out in a 4–15% acrylamide Tris/SDS gel followed by western blotting with antibody directed against the α -chain of the insulin receptor. There is a significant decrease in synthesis of the insulin proreceptor (proIR) in treated cells, and a less pronounced decrease in the mature insulin receptor α -chain (IR α), compared to control cells, confirming the efficacy of these inhibitors in MC65 cells. (b) Protein separation was carried out by tricine gel electrophoresis and western blotting with 6E10 antibody directed against A β as described above. Protein synthesis inhibition is apparent in decreases in both full-length endogenous APP as well as the induced C99 construct. However, treatment with neither emetine nor cycloheximide leads to diminished expression of the 7.5-kDa A β /CTF product or its aggregates.

treated cells, and a less pronounced decrease in the mature insulin receptor α -chain (19% decreased with emetine and 17% decreased with cycloheximide), compared to control cells, confirming the efficacy of these inhibitors under the conditions of our experiment. Similar to these other proteins, synthesis of endogenous APP (57% decreased with emetine and 48% decreased with cycloheximide), and the initially synthesized C99 (66% decreased with emetine and 58% decreased with cycloheximide), were both decreased by either emetine or cycloheximide (Fig. 4b). In contrast, inhibitors of protein synthesis did not diminish accumulation of low molecular-weight A β /CTFs (14% increased with emetine and 5% increased with cycloheximide) or the HMWCs. In summary, these data showed that, following translation of C99, low molecular-weight A β /CTFs and A β -immunoreactive HMWCs were formed by post-translational processes. This is apparent in the case of BSO-treated MC65 cells, as shown in Fig. 4(b), and also when MC65 Tet⁻ cells were cultured with protein synthesis inhibitors in the absence of BSO for longer time periods over which GSH is spontaneously lost (data not shown).

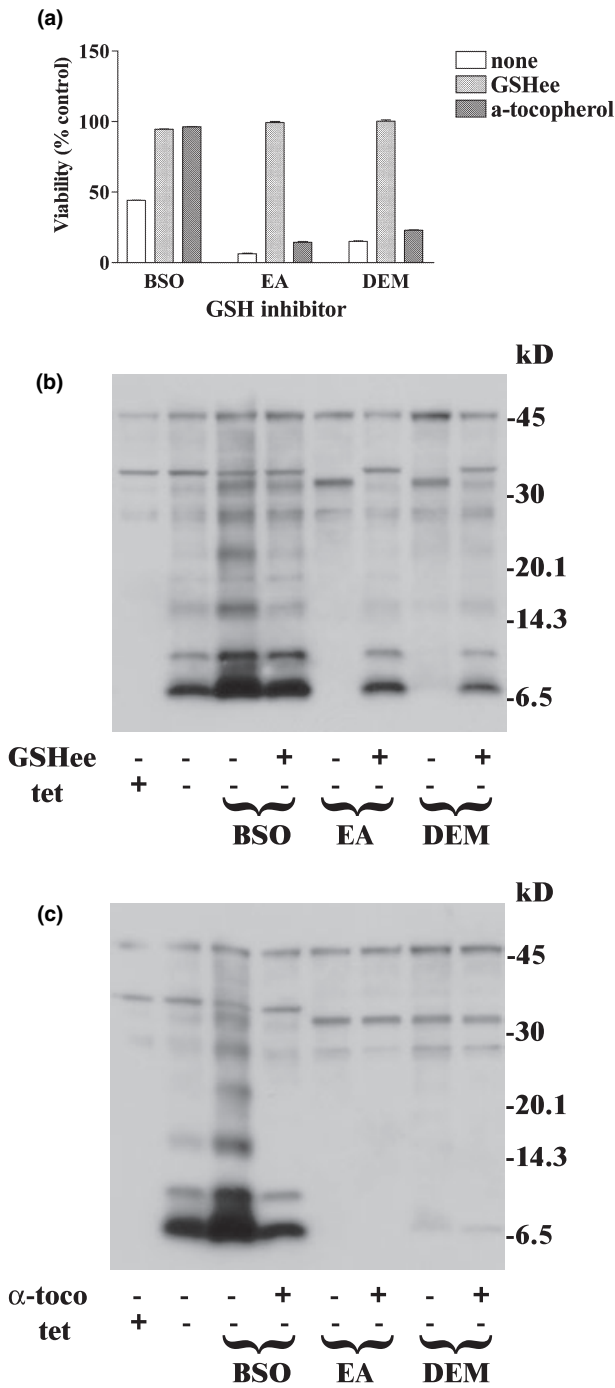


Fig. 5 (a) Cells were plated in 48-well tissue culture plates in the presence or absence of tetracycline and BSO (128 μM), EA (32 μM), or DEM (256 μM). For each compound, one group received no further treatment, while the others received either 1 mM GSHee or 10 μM AT. Because BSO did not alter toxicity in the presence of tetracycline, effects of GSHee or AT under this condition are not presented. After 1.5 days incubation, cell viability was assessed by MTT assay as described above. MTT absorbance was calculated and expressed as percentage control of MTT absorbance of Tet- cells with no other treatment; as seen in Fig. 3(a) there is no loss of viability due to tetracycline at this time. Where error bars are not visible, errors were smaller than data symbols. GSHee treatment was associated with a significant ($p < 0.0001$) degree of protection of BSO-treated MC65 cells from the effects of tetracycline removal, and two-way ANOVA demonstrated the significance ($p < 0.0001$) of an interaction between tetracycline removal and GSHee treatment in the presence of BSO. GSHee led to complete protection of MC65 cells from EA- and DEM-associated toxicity in both the presence and absence of tetracycline ($p > 0.05$ for difference between EA- or DEM-treated cells compared to untreated controls). (b and c) Parallel cultures in 100-mm dishes were treated with BSO (128 μM), EA (64 μM), or DEM (512 μM) in the presence or absence of 1 mM GSHee (b) or 10 μM AT (c). Cells were harvested after 1.5 days, protein extracted, and samples electrophoresed and western blotted with 6E10 antibody directed against Aβ as described above. Treatment with GSHee led to normalization of the increased expression and aggregation of Aβ/CTFs that was observed with BSO treatment to levels similar to those seen in untreated cells and to restoration to similar levels of Aβ/CTF expression that is lost with treatments with EA and DEM. Treatment with AT led to a similar normalization of increased expression and aggregation of Aβ/CTFs observed with BSO treatment, but had no effect on Aβ/CTF expression that was lost EA or DEM treatment.

We and others have shown previously that AT treatment at the time of Tet removal largely prevents Aβ/CTF-associated toxicity in MC65 cells (Sopher *et al.* 1996). Our current study showed that while GSH depletion occurred following an initial increase in oxidative damage (see Fig. 2), BSO-mediated depletion of GSH led to further oxidative events (see Fig. 3), suggesting two phases of oxidative damage following C99 expression. While GSH has multiple actions in cells, we hypothesized that its role as an antioxidant may

underlie its effect on Aβ/CTF-associated toxicity. To test this, we treated GSH-depleted cells with either a GSH precursor or AT and compared the results. GSH ethyl ester (GSHee), a membrane-permeable GSH precursor that restores GSH levels, was added along with EA, DEM, or BSO at the time of Tet removal. As expected, GSHee was associated with complete cytoprotection of EA-, DEM-, and BSO-exposed Tet- cells and reversal of all changes in the Aβ/CTF banding pattern associated with exposure to these compounds (Fig. 5). In contrast, while AT completely protected Tet- cells exposed to BSO and also reversed BSO-induced changes in the Aβ/CTF banding pattern, it had only a slight (but significant, $p < 0.0005$) effect on EA- or DEM-mediated toxicity (~10–15% increase in viability for each) and did not reverse alterations in Aβ/CTFs induced by these two compounds (Fig. 5). To confirm that the cytoprotective mechanism of AT involved effects on lipid peroxidation itself rather than indirect effects on GSH metabolism, we measured both F₂-IsoPs and GSH levels in BSO-treated cells with and without AT. We found that AT acted as potent antioxidant and more than compensated for the oxidative stress caused by GSH depletion by BSO, completely blocking the oxidative damage to lipids associated with conditional expression of

C99 (Fig. 6a). Consistent with an antioxidant mechanism, this protection occurred in the absence of any significant effect on intracellular GSH levels (Fig. 6b). Interestingly, treatment of cells with AT in the absence of BSO led to significant ($p < 0.005$) increases in intracellular GSH, suggesting that, although AT does not promote cell viability through preservation of GSH levels, it may have effects on GSH homeostasis that become apparent under milder conditions of non-pharmacologic spontaneous GSH depletion that occurs over time in Tet–MC65 cells. These results, taken together, support our hypothesis, as restoring the antioxidant component of GSH activity with GSHee, or providing other another antioxidant (AT) that is able to subsume a demonstrated effect of GSH on a specific marker of cellular oxidative damage (lipid peroxidation) specifically protected cells from A β /CTF-associated cytotoxicity. The results are also consistent with the additional hypothesis that cytotoxicity in MC65 cells is not due to GSH depletion *per se*, but rather to the failure of antioxidant defenses that leads

to lipid peroxidation, or to losses of cellular functions that correlate with lipid peroxidation.

Discussion

Free radical-mediated damage to nucleic acids, lipids, and proteins increases with age and still further in AD, where it is thought contribute to neuronal dysfunction and death. Several sources of increased free radical stress in AD have been proposed, but a major candidate is A β peptides; although initial investigations focused on extracellular A β fibrils, more recent data highlight the potential central importance of intraneuronal aggregates of A β . We have modeled this circumstance with MC65 cells that conditionally express C99 that is further cleaved to other CTFs of APP. Here, we added to our knowledge of this cell line by demonstrating by mass spectrometry the presence of sequence derived from residues 6–28 of A β in the initial cleavage product of C99, and that A β /CTF accumulation and aggregation proceeded in the presence of inhibitors of protein synthesis, suggesting impaired degradation as a mechanism of their accumulation. GSH is the most abundant cellular non-protein thiol, serves as an important antioxidant, and has been proposed to be important in the protection of cerebrum from oxidative damage. GSH has been reported to be decreased in cerebrum of aging rodents and humans (Hazelton and Lang 1980; Chen *et al.* 1989; Ravindranath *et al.* 1989; Iantomasi *et al.* 1993; Favilli *et al.* 1994; Martinez *et al.* 1995; Liu 2002), and alterations in GSH metabolism have been described in diseased regions of brain from AD patients (Aksenov and Markesbery 2001). Alterations in peripheral GSH metabolism have also been described in patients with mild cognitive impairment and AD; these are reported to involve altered activities of GSH peroxidase (Rinaldi *et al.* 2003). Despite these associations, little is known about the mechanisms of and consequences of GSH loss in AD. Here we addressed this gap in our knowledge.

We found that treatment of MC65 cells with BSO, an inhibitor of GSH synthase, led to marked depletion of GSH, followed by accelerated cytotoxicity that developed selectively in the absence of tetracycline. These data are consistent with the findings of others who used extracellular fibrillar A β , *viz.* that intraneuronal aggregation of A β /CTFs is cytotoxic, at least in part through enhanced free radical stress. We anticipated that free radical stress would be distal to A β /CTF aggregation. However, unexpectedly, selective depletion of GSH with BSO also was associated with enhanced A β /CTF aggregation. These effects were specific to GSH, as they were reversed by treatment of BSO-exposed with GSHee, a membrane-permeable analog of GSH. GSH has many functions in cells, so we pursued this finding further with another antioxidant, AT, and observed the same outcome. In combination with our F₂-IsoP data showing that

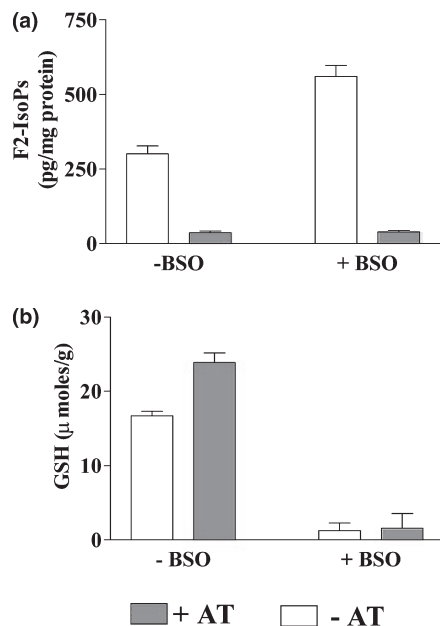


Fig. 6 Tet–MC65 cells were exposed to 128 μ M BSO alone or in combination with 10 μ M AT, and IsoPs (top panel) and GSH (bottom panel) were determined as described above. Results are depicted as means \pm standard errors. For IsoPs, two-way ANOVA had $p < 0.0001$ for BSO, $p < 0.0001$ for AT, and $p < 0.0001$ for interaction between these two. Bonferroni-corrected post-tests had $p < 0.001$ for +AT versus –AT and $p < 0.001$ for +BSO versus –BSO in cells not treated with AT; there was no significant difference between AT-treated cells with + or – BSO. In the case of GSH, in the absence of BSO, AT had a significant effect in preventing GSH loss ($p < 0.01$), whereas the effect appears relatively minimal in the presence of BSO, which depleted cellular GSH to levels nearly indistinguishable from background regardless of AT treatment status ($p > 0.05$ for difference with BSO present).

GSH and AT have the expected antioxidant activity in these cells, our results strongly suggest that free radical stress is not simply distal to A β /CTF aggregation but also contributes to A β /CTF aggregation. Indeed, our data suggest two phases of oxidative damage: one that is prior to and thus independent of GSH depletion and the other that follows GSH depletion. Previous studies with this model have shown that oxidative events that occur in the absence of pharmacologic GSH depletion are blocked by AT (Sopher *et al.* 1996; Maezawa *et al.* 2004). Here, we show that the increased oxidative damage associated explicitly with GSH depletion in C99-expressing MC65 cells also was completely suppressed by AT in the absence of a change in depleted GSH levels.

Although the precise details of the mechanisms involved in the interaction among A β /CTF aggregation, free radical damage, and cell death remain unclear, our data suggest a reinforcing cycle whereby intracellular A β /CTFs lead to early increase in free radical stress and oxidative damage to membranes that in turn enhances later A β /CTF HMWC formation. The demonstrated involvement of cellular membrane oxidation in Tet⁻ cells is intriguing given one proposed toxic mechanism of A β that has received substantial attention recently, *viz.* its capacity to form ADDLs, non-fibrillary oligomeric structures that bear functional similarity to micelles (Klein 2002). These may insert into cellular lipid membranes and disrupt vital membrane properties such as ion conductance that depend on the integrity of membrane lipid organization. In addition, aggregates of A β have been shown to promote free radical generation in *in vitro* models, and the association of ADDLs with cell membranes may contribute to the lipid peroxidation observed in Tet⁻ MC65 cells and in diseased regions of AD brain (Montine *et al.* 2002).

Similar to BSO, exposure to EA and DEM, agents that deplete GSH by thiol adduction, led to dose-dependent cytotoxicity in MC65 cells. In sharp contrast to BSO, toxicity from EA or DEM was not dependent on tetracycline removal and led to loss of A β /CTFs. This is similar to what we observed with the non-specific pro-oxidant hydrogen peroxide (data not shown). While toxicity from EA or DME was expectedly responsive to GSHee, cytoprotection from these agents by AT was relatively minimal. These findings are important because they show that accumulation and aggregation of A β /CTFs are not merely non-specific consequences of toxicity in this cell line. Moreover, through a negative association, these data further strengthen the association between increased cellular accumulation of A β /CTFs and MC65 cell death, as neither of these occur in the presence of AT.

The results presented here further describe the metabolism of intracellular A β /CTFs in the MC65 model of AD pathogenesis. Our findings suggest mechanisms that may underlie at least some aspects of the function and clinical

utility of antioxidants in the prevention of AD. Moreover, a common theme in neurodegenerative disease as a whole is the formation and toxicity of intraneuronal proteinaceous aggregates. A better understanding of the nature, trafficking, and effects of A β /CTFs and the association and interaction of these with the development of oxidative stress may illuminate directions of research that identify new targets and strategies for the prevention of not only AD, but other neurodegenerative disease as well.

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