ABSTRACT  Alzheimer's disease (AD) is characterized by progressive loss of memory and cognition. Our laboratory and many others have shown that the AD brain is under extensive oxidative stress. Numerous therapeutic approaches to AD therapy have been hypothesized. Among these are exogenous antioxidants. We suggest that the body’s own endogenous antioxidant systems should be mobilized against the oxidative stress inherent in AD brain. One of the most versatile antioxidants in the brain is glutathione. Glutathione is capable of protecting the cell against reactive oxygen species, redox metal ions, and reactive lipid peroxidation products and other electrophiles associated with AD. There are many ways to increase glutathione levels, and one or more of these ways to increase glutathione in the brain may be a promising therapeutic strategy for AD. Drug Dev. Res. 56:428–437, 2002. © 2002 Wiley-Liss, Inc.

Key words: glutathione; oxidative stress; Alzheimer’s disease; lipid peroxidation products

INTRODUCTION  Alzheimer’s disease (AD), characterized by progressive loss of memory and cognition, is an age-related neurodegenerative disorder currently affecting more than 4 million persons in the United States [Katzman and Saitoh, 1991]. The 40–42 amino acid peptide, amyloid β-peptide (Aβ), is produced in excess in AD brain, and many researchers opine that Aβ is central to the pathogenesis of this disorder [Selkoe, 2000]. Moreover, the AD brain is under extensive oxidative stress, manifested by, among other indices, lipid peroxidation, protein oxidation, free radical formation, DNA/RNA oxidation, protein-bound 3-nitrotyrosine, and advanced glycation endproducts [recently reviewed in Markesbery, 1997; Butterfield et al., 2001, 2002a; Butterfield and Lauderback, 2002].

Our laboratory combined these two concepts into a comprehensive model for neurodegeneration in AD brain: the Aβ-associated free radical model [Butterfield, 1997; Varadarajan et al., 2000; Butterfield et al., 2001, 2002a; Butterfield and Lauderback, 2002]. This model predicts that Aβ, perhaps in concert with other moieties, e.g., redox metal ions, causes oxidative stress that in turn causes neurodegeneration. We and many other laboratories have shown that, in ways inhibited by free radical antioxidants, Aβ causes lipid peroxidation, protein oxidation, free radical formation, and neuronal death. The single methionine residue at position 35 of the 42-mer [Aβ(1–42)] is essential for the oxidative stress and neurotoxic properties of the peptide [Yatin et al., 1999; Varadarajan et al., 2001; Butterfield and Kanski, 2002].

Contract grant sponsor: NIH; Contract grant numbers: AG 10836, AG-05119.

*Correspondence to: Professor D. Allan Butterfield, Department of Chemistry, Center of Membrane Sciences, and Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY 40506-0055, E-mail: dabcsns@uky.edu

Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/ddr.10095

© 2002 Wiley-Liss, Inc.
Given the oxidative stress, the excess Aβ production, and synapse loss in AD brain, therapeutic strategies to minimize or delay onset of symptoms of AD are being formulated [Irizarry and Hyman, 2001]. Some strategies involve inhibiting the production of Aβ by blocking the proteolytic cleavage of the amyloid precursor protein at the sites of Aβ formation. Others involve disruption of Aβ aggregation, while still others involve increasing the clearance of aggregated Aβ. Other strategies involve limiting neuroinflammation. Another strategy for AD therapy involves the use of exogenous antioxidants, both synthetic and dietary [Butterfield et al., 2002b,c]. In addition, we suggest that the body's own antioxidant systems can and should be mobilized against the oxidative stress inherent in AD brain. Among these endogenous antioxidant systems in brain is that associated with glutathione (GSH), and it is this approach to therapeutic intervention in AD brain that is the subject of this review.

**GLUTATHIONE**

The tri-peptide glutathione (γ-glutamyl-cysteinyl-glycine) (GSH) is an endogenous antioxidant of great importance. Glutathione (GSH) is required for the maintenance of the thiol redox status of the cell, protection against oxidative damage, detoxification of endogenous and exogenous reactive metal ions and electrophiles, storage and transport of cysteine, as well as protein and DNA synthesis, cell cycle regulation, and cell differentiation [Hammond et al., 2001].

Glutathione and glutathione enzymes play a key role in protecting the cell against the effects of reactive oxygen species (ROS). The key functional element of glutathione is the cysteinyl moiety, which provides the reactive thiol group. ROS are reduced by GSH in the presence of GSH peroxidase. As a result, GSH is oxidized to GSSG, which in turn is rapidly reduced back to GSH by GSSG reductase at the expense of NADPH (Fig. 1). The thiol-disulfide redox cycle also aids in maintaining reduced protein and enzyme thiols. Without a process to reduce protein disulfides, vulnerable cysteinyl residues of essential enzymes might remain oxidized, leading to detrimental changes in catalytic activity.

Nitric oxide (NO) has a number of physiological roles, but excessive production of NO is harmful to neurons. Excessive nitric oxide is capable of inducing heme oxygenase-1 [Foresti et al., 1997], a stress protein that degrades heme to carbon monoxide and biliverdin [Calabrese et al., 2001]. Intracellular glutathione concentrations appear to play an important factor in regulating the susceptibility of the cell to NO and its derivatives. Glutathione will react with NO to form hydroxylamine and oxidized glutathione (Fig. 2a).

Nitric oxide readily reacts with superoxide to form peroxynitrite (ONOO·) and the latter will also react with glutathione, leading to oxidized glutathione. If glutathione levels are compromised due to severe oxidative stress conditions, as found in AD, neurons become particularly sensitive to NO and ONOO· [Barker et al., 1996]. Peroxynitrite can nitrate tyrosine groups of proteins to form the stable compound 3-nitrotyrosine (3-NT). Nitration may impair protein function, leading to neuron degeneration. Immunohistochemical studies have demonstrated upregulation of 3-NT in AD brain [Smith et al., 1997; Su et al., 1997], specifically in the hippocampus and neocortical regions.
[Hensley et al., 1998]. Concentrations of 3-NT and the 3-nitrotyrosine/tyrosine ratio where found to be increased in the cerebrospinal fluid of AD patients [Toghi et al., 1999; Hensley et al., 1998] and increased significantly with decreasing cognitive function [Toghi et al., 1999]. Recently, a two- to three-fold increase in the lipid nitration product, 5-nitro-γ-tocopherol, was found in affected regions of the AD brain [Williamson et al., 2002]. These results indicate that NO-derived species are significant contributors to protein and lipid oxidation in the AD brain.

GLUTATHIONE AND AD

In AD, oxidized glutathione is increased [Benzi and Moretti, 1997], while the activity of glutathione S-transferase is decreased [Lovell et al., 1998b], consistent with the known increase in oxidative stress in AD [Markesbery, 1997; Butterfield, 1997; Varadarajan et al., 2000; Butterfield et al., 2001].

In addition to protection against ROS, glutathione is an excellent scavenger of lipid peroxidation products. Lipid peroxidation generates various reactive aldehydes, including 4-hydroxynonenal (HNE), malondialdehyde, and 2-propenal (acrolein) [Esterbauer et al., 1991; Butterfield and Stadtman, 1997]. All three markers of lipid peroxidation have been detected immunochemically in AD brain, particularly in neurofibrillary tangles, one of the major hallmarks of AD [Sayre et al., 1997; Yan et al., 1994; Calingasan et al., 1999; Lovell et al., 2001]. Nonreactive iso- and neuroprostanes, two other markers of lipid peroxidation, have been found in excess in AD brain and induced by Aβ [Montine et al., 1998, 1999; Mark et al., 1999]. These, in turn, under specific circumstances can form highly reactive γ-ketoaldehydes via an endoperoxide intermediate of iso- and neuroprostanes [Brame et al., 1999; Bernoud-Hubac et al., 2001]. These γ-ketoaldehydes can covalently add to and modify proteins [Brame et al., 1999; Bernoud-Hubac et al., 2001]. Glutathione reacts with epoxides, unsaturated carbon atoms (quinones, esters), and aromatic carbon atoms (aryl nitro compounds). This detoxification involves nucleophilic attack by GSH on an electrophilic carbon. This reaction can occur spontaneously, but most often is catalyzed by glutathione S-transferase. Thus, thiols, such as GSH, would inhibit the formation of endoperoxide intermediates, thereby preventing formation of these reactive γ-ketoaldehydes.

The strongly electrophilic, α-β-unsaturated aldehyde, acrolein, reacts rapidly with and depletes cellular glutathione [Ohno and Ormstad, 1985; Horton et al., 1997; Pocernich et al., 2001]. Acrolein covalently binds proteins, introducing a carbonyl group, a marker for protein oxidation [Uchida et al., 1998; Esterbauer et al., 1991; Butterfield and Stadtman, 1997]. Significant elevations of extractable acrolein in AD amygdala (2.5 ± 0.9 nmol/mg of protein) and hippocampus and parahippocampal gyrus (5.0 ± 1.6 nmol/mg of protein) compared to age-matched controls (0.3 ± 0.5 and 0.7 ± 0.1 nmol/mg of protein, respectively) were recently reported [Lovell et al., 2001]. HNE, also an α-β-unsaturated aldehyde, binds covalently to proteins and alters their structure and function [Subramaniam et al., 1997; Lauderback et al., 2001]. Glutathione is protective against HNE in neuronal systems [Bains and Shaw, 1997; Subramaniam et al., 1997]. As mentioned above, HNE is increased in AD [Montine et al., 1997; Sayre et al., 1997; Markesbery et al., 1998; Xie et al., 1998; Calingasan et al., 1999].

Mattson and colleagues [Mark et al., 1997a,b] showed that GSH protects cultured neurons against oxidative damage resulting from amyloid β-peptide and HNE. Aβ(1–42) has been shown to induce lipid peroxidation, specifically indexed by HNE production, and this alkenal was bound in excess to the glutamate-1 transporter (Glt-1) in synaptosomes after Aβ(1–42) treatment [Lauderback et al., 2001]. In AD brain the Glt-1 glutamate transporter has less activity [Masliah et al., 1996], and we showed that Glt-1 has excess bound HNE [Lauderback et al., 2001]. These may be related events: Aβ(1–42), produced in excess in AD brain, causes lipid peroxidation, which in turn, forms HNE, which, in turn, binds to Glt-1, which in turn inactivates the transporter [Butterfield et al., 2001; Lauderback et al., 2001]. Coupled to the loss of glutamine synthetase (GS) activity in AD brain [Hensley et al., 1995], excess glutamate-induced NMDA receptor-facilitated excitotoxicity could occur in AD brain with resultant neurodegeneration.

In addition, GSH can protect rodent synaptosomes from HNE impairment of glutamate and glucose transport [Keller et al., 1997a,b], from peroxynitrite-induced oxidative damage [Koppar et al., 1997a,b], and from hydroxyl free radical damage [Pocernich et al., 2000].

Glutathione also forms metal complexes via nonenzymatic reactions. GSH functions in the storage, mobilization, and delivery of metals between ligands, in the transport of metal across cell membranes, as a source of cysteine for metal binding, and as a reductant in redox reactions involving metals [Hammond et al., 2001]. The sulfhydryl group of the cysteine moiety of GSH has a high affinity for metal ions such as mercury, silver, cadmium, arsenic, lead, gold, zinc, and copper, forming a thermodynamically stable complex that can be eliminated.
ELEVATED GLUTATHIONE THERAPY IN AD

Redox metal ions such as iron (II) and copper (I) can catalyze free radical reactions and may contribute to oxidative damage observed in AD brain. Fe(II) and Cu(I) induce the Fenton reaction, producing an abundance of hydroxyl free radicals for lipid peroxidation. Iron and ferritin levels in AD are elevated significantly in cortical gray matter regions [Connor et al., 1992]. The iron binding protein p97 (melanotransferrin) is also elevated in serum, CSF, and brain regions in AD [Kennard et al., 1996]. Glutathione protects against oxidative damage [Mark et al., 1997a] and lipid peroxidation caused by iron toxicity [Milchak et al., 2002]. A significant decrease in Cu, and significant increases in Zn and Fe, were found in AD hippocampus and amygdala, areas showing severe histopathologic alterations in AD [Deibel et al., 1996]. In contrast, copper, iron, and zinc are all elevated in senile plaques of AD [Lovell et al., 1998a]. There is a predominant accumulation of Al within neurofibrillary tangles (NFT) AD with high levels detected in the nuclei of NFT-free and NFT-containing neurons [Lovell et al., 1993; Bouras et al., 1997].

STRATEGIES FOR INCREASING GSH LEVELS

Given the many advantageous aspects of increased GSH levels to combat oxidative stress in AD brain, different means of elevating brain GSH are outlined below. In principle, any and all of these approaches may be considered as potential therapeutic approaches for the oxidative stress in AD.

Whey Proteins

Whey proteins consist of α-lactoalbumin, β-lactoglobulin, immunoglobulin, serum albumin, and other unidentified proteins from pasteurized cow’s milk. There are many reports that whey proteins increase levels of glutathione. Rats on a diet in which the protein consisted of only whey proteins or β-lactoglobulin had a 40% increased reduced glutathione level in the liver [Zommara et al., 1998]. Mice fed whey protein diets at 84 weeks of age exhibited increased GSH levels to combat oxidative stress in AD [Kennard et al., 1996]. Glutathione protects against oxidative damage [Mark et al., 1997a] and lipid peroxidation caused by iron toxicity [Milchak et al., 2002]. A significant decrease in Cu, and significant increases in Zn and Fe, were found in AD hippocampus and amygdala, areas showing severe histopathologic alterations in AD [Deibel et al., 1996]. In contrast, copper, iron, and zinc are all elevated in senile plaques of AD [Lovell et al., 1998a]. There is a predominant accumulation of Al within neurofibrillary tangles (NFT) AD with high levels detected in the nuclei of NFT-free and NFT-containing neurons [Lovell et al., 1993; Bouras et al., 1997].

N-Acetylcysteine (NAC)

N-acetylcysteine (NAC), used for treatment of acetaminophen overdose and as an antimucolytic agent, also increases the levels of cysteine. NAC is metabolized by N-deacetylase to cysteine, thus leading to the increase of glutathione. The bioavailability of NAC given orally in humans is less than 20% [Borgstrom et al., 1986]. The absorption of NAC in the intestine allows for a greater increase in the synthesis of glutathione. NAC is virtually nontoxic at pharmaceutically relevant concentrations. Only at levels greater than 0.1 M has cytotoxicity been observed [Kharazmi et al., 1988], but because of the relatively low bioavailability it is highly improbable that these concentrations are achieved in humans [Borgstrom, 1986].

NAC has been shown to increase glutathione in many different cell systems and peripheral body areas.
Our laboratory has shown that i.p. injections of NAC into gerbils or rats increased total glutathione levels in the brain [Pocernich et al., 2000, 2001]. This increase in brain glutathione levels provided protection for synaptosomes against hydroxyl radicals [Pocernich et al., 2000], peroxynitrite [Koppal et al., 1999b], and the lipid peroxidation product acrolein at levels found in the AD brain [Pocernich et al., 2001]. In another animal model of an oxidative stress-related neurodegenerative disease, Huntington's disease (HD), the mitochondrial toxin 3-nitropropionic acid (3-NP), injected i.p. daily for 4 days, caused oxidative stress and HD-like striatal lesions [LaFontaine et al., 2000a]. GSH injection i.p. nearly abrogated both the oxidative stress and lesion formation in rat striatum [LaFontaine et al., 2000b].

Recently, NAC was reported to downregulate amyloid precursor protein (APP) gene transcription in human neuroblastoma cells [Studer et al., 2001]. The effect was reversible when cells were returned to NAC-free medium. The main component of amyloid plaques found in AD brains is Aβ, produced by proteolytic processing of APP. Regulating secretion of neurotoxic Aβ with NAC potentially could provide new therapeutic approaches to AD. As noted above, we and others showed that Aβ(1–42) produces a significant induction of oxidative stress and neurotoxicity. The effects are reversed when cells are pretreated with 30 mM NAC [Olivieri et al., 2001]. Cells exposed to H2O2, UV light, and Aβ25–35 secrete significantly higher amounts of Aβ1–40 and Aβ1–42 into the culture medium. Pretreatment with NAC inhibits the release of both Aβ1–40 and Aβ1–42 in Aβ25–35-treated cells [Olivieri et al., 2001]. NAC also lowered tau phosphorylation levels in the presence or absence of stress treatment. Recently, AD patients were given 50 mg/kg/day NAC for 6 months. Out of the 10 neurological and neurobehavioral tests given, only two, letter fluency task and Wechsler Memory Scale, showed significant improvements [Adair et al., 2001], although there was a favorable trend in all other measures taken. Taken together, the literature suggests that further research into NAC as a therapeutic approach to AD should be encouraged.

### Thiazolidine 2-Oxothiazolidine-4-Carboxylate (OTC)

Various thiazolidines have been used as cysteine precursors; however, the by-products have often limited their usefulness. Thiazolidine 2-oxothiazolidine-4-carboxylate (OTC), also called procysteine, has no harmful by-products. OTC is transported into most cells. The ring is opened by 5-oxoprolinase to an unstable intermediate 5-carboxycysteine, which rapidly forms cysteine. The by-product of this latter reaction is carbon dioxide, which can be exhaled.

OTC is reported to support the growth of rodents on a cysteine-deficient diet [Jain et al., 1995] and to increase glutathione levels in human erythrocytes [Bernard et al., 1997] and in whole blood in patients with chronic renal failure [Moberly et al., 1998]. The therapeutic effect of OTC has also been studied in human HIV cases. OTC had a moderate effect in increasing cellular GSH levels in HIV patients [Porta et al., 1991; Kalayjian et al., 1994; Barditch-Crovo et al., 1998].

The uptake and metabolism of OTC was compared with that of 35S-labeled cysteine and methionine in freshly isolated rat hepatocytes. All three substrates were metabolized to glutathione, but the rate of OTC metabolism was 30% less than that for cysteine or methionine. Similarly, the rate of uptake for OTC was also less than cysteine or methionine [Coloso et al., 1991].

Dringen and Hamprecht [1999] have shown NAC to be a more effective donor of cysteine for the synthesis of glutathione than OTC, and OTC is a more potent cysteine supplier in the liver than is NAC [Williamson et al., 1982].

### Glutathione Ethyl Ester and Glutathione Diethyl Ester

GSH is a tripeptide that is synthesized intracellularly by γ-glutamylcysteine synthetase and GSH synthetase, with ATP as a requirement. The limiting amino acid cysteine is present in micromolar levels in the brain, while glutamate and glycine are present in millimolar levels [Cooper, 1997]. γ-Glutamylcysteine synthetase catalyzes the formation of the dipeptide γ-glutamylcysteine, the limiting substrate in the biosynthesis of GSH, and is feedback-inhibited by GSH itself. GSH synthetase catalyzes the addition of glycine to this limiting substrate to form GSH.

GSH is transported into cells poorly and is most likely degraded to its amino acids extracellularly [Meister and Anderson, 1983]. Glutathione ethyl ester (GEE) and glutathione diethyl ester more effectively cross the plasma membrane than GSH does [Anderson and Meister, 1989; Anderson et al., 1985; Levy et al., 1993]. GEE, transported into a cell, is hydrolyzed by esterases to yield GSH and ethanol. GSH diethyl ester was found to be transported into human cells with more efficacy than GEE [Levy et al., 1993]. In human cells, it was found that GSH diethyl ester was transported into cells and subsequently degraded to GEE [Levy et al., 1993]. GEE has been administered in several cell types and systems to increase GSH levels.
and protect against oxidative stress systems [Anderson and Luo, 1998].

γ-Glutamylcysteine

γ-Glutamylcysteine administration has been proposed to increase GSH biosynthesis. There is evidence that γ-glutamyl compounds may cross the membrane by various transporters, thereby aiding the transport of γ-glutamylcysteine [Meister and Anderson, 1983]. If γ-glutamylcysteine crosses the membrane intact, the limiting substrate in GSH biosynthesis is provided and the feedback-inhibited γ-glutamylcysteine synthetase enzyme is bypassed, perhaps providing a more beneficial approach for GSH upregulation than a simple cysteine derivative. γ-Glutamylcysteine has been administered intracerebroventricularly to increase brain GSH levels [Pileblad and Magnusson, 1992]. γ-Glutamylcysteine and γ-glutamylcysteine were also found to increase renal GSH levels [Meister and Anderson, 1983].

In order to increase the efficacy of γ-glutamylcysteine transport across the membrane, several investigators have administered γ-glutamylcysteine ethyl ester (GCEE) in order to increase GSH levels in various cell types and systems, including ischemia/reperfusion in liver [Kobayashi et al., 1992; Ozaki et al., 1994] and in heart [Hoshida et al., 1994; Nishinaka et al., 1991], carbon tetrachloride hepatic injury [Nishida et al., 1998], and selenium-deficient heart [Okamoto et al., 1999]. In hepatocytes, GCEE was found to be transported into liver cells more readily than GSH and converted to GSH [Nishida et al., 1996]. Additionally, we have observed that GCEE has in vivo radical scavenging abilities against peroxynitrite similar to those of GSH [Drake et al., 2002]. Further, we found that GCEE increases GSH by providing the limiting substrate γ-glutamylcysteine and not by merely providing cysteine, thereby rendering synaptosomes isolated from GCEE-injected animals less susceptible to oxidative damage induced by peroxynitrite [Drake et al., 2002].

Cysteinylglycine

GSH is transported out of the cell with the aid of γ-glutamyltranspeptidase, transferring the γ-glutamyl residue to an acceptor, resulting in γ-glutamyl amino acid or glutamate and cysteinylglycine [Meister et al., 1981] that, in turn, can then be used again in synthesis of GSH. The dipeptide cysteinylglycine has been demonstrated to increase GSH levels in astroglial cells [Dringen et al., 1998], in neurons cocultured with astroglial cells [Dringen et al., 1999], and in neurons [Dringen et al., 2001]. In astroglial cells, cysteinylglycine may be transported into the cell by a peptidyl transporter [Dringen et al., 1998]. In neurons, the most likely pathway for cysteinylglycine utilization is the extracellular degradation of cysteinylglycine to its amino acids, which are then transported into the cell and used for GSH biosynthesis [Dringen et al., 2001].

Glutamine

In a kidney under oxidative stress, glutamine is rate-limiting for GSH synthesis in vitro [Welbourne, 1979]. In normal cells, glutamine may serve as a γ-glutamyl acceptor, encouraging uptake and breakdown and recycling of GSH [Rouse et al., 1995]. In cells under oxidative stress, glutamine may maintain or increase GSH levels by bypassing 5-oxoprolinase that is used by γ-glutamyl transferase [Rouse et al., 1995]. Glutamine can maintain or increase GSH levels in animals that received the chemotherapeutic agents methotrexate [Rouse et al., 1995] and doxorubicin [Cao et al., 1999]. Glutamine also maintains GSH levels in liver that received hepatic injury [Hong et al., 1992] and in intestinal GSH after intestinal ischemia/reperfusion [Harward et al., 1994]. Glutamine also increases glutathione levels of cultured rat astrocytes [Kamencic and Juurlink, 2001]. Glutamine supplementation, an alternative to cysteine-containing or cysteine precursor compounds, may have therapeutic relevance, as it maintains or increases GSH levels. Of course, deaminases could result in potentially excitotoxic glutamate, providing caution for this putative therapeutic approach to increasing GSH levels in AD.

CONCLUSIONS

AD brain is under intense oxidative stress, likely to involve the effects of Aβ [Butterfield et al., 2001, 2002a]. GSH is able to effectively scavenge the reactive aldehydic products of Aβ-induced lipid peroxidation [Subramaniam et al., 1997; Pocernich et al., 2001], the oxidative effects of peroxynitrite [Koppal et al., 1999a,b], and hydroxyl free radicals [Pocernich et al., 2000]. All these reactive molecules are implicated in the oxidative stress associated with both Aβ and AD [Butterfield et al., 2001, 2002a].

It is our view that a combination of exogenous and endogenous antioxidants should be pursued in AD therapy. Preventing the extremely damaging effects of Aβ-induced lipid peroxidation and protein oxidation with exogenous antioxidants (for example, vitamin E, polyphenols, etc.) [Butterfield et al., 2002b,c], coupled with endogenous antioxidants (e.g., GSH, melatonin, induction of heat-shock protein or heme oxygenase-1) [Motterlini et al., 2000; Poeggeler et al., 2001], may provide a more successful approach to combating the oxidative stress associated with AD. Studies to test this
combination approach in animal models of AD are in progress in our laboratory.

ACKNOWLEDGMENTS

NIH grants AG-10836 and AG-05119 were awarded to D.A.B.

REFERENCES


