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Protein carbonyls are an index of protein oxidation and were determined as described previously (Berlett and Stadtman, 1997). Briefly, 5 μL of synaptosome preparations (4 mg/mL) were incubated at room temperature with 10 mM 2,4-dinitrophenylhydrazine in the presence of 5 μL of 12% SDS for 20 min at room temperature. The samples were neutralized with 7.5 μL of the neutralization solution (2 M Tris in 30% glycerol). Two hundred fifty nanograms of protein sample was loaded into the wells of the slot blot apparatus. Proteins were transferred directly to nitrocellulose paper under vacuum pressure and standard immunochemical techniques were performed. Membranes were blocked in the presence of 3% bovine serum albumin (BSA) in TBS-T (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 h, followed by incubation with rabbit polyclonal antibody anti-DNP (1:100) for 1 h. The membrane were washed three times with TBS-T and incubated with alkaline-phosphatase (AP)-conjugated secondary antibody for 1 h. The specificity of primary antibodies has been previously demonstrated by experiments performed in our laboratory (Aksenov et al., 2001). Samples were developed using SigmaFast Tablets (BCIP/NBT) substrate, and blots were scanned into Adobe Photoshop (Adobe System, Inc., Mountain View, CA, USA) and quantitated with Scion Image (PC version of Macintosh compatible NIH Image).

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mediated protein oxidation. Fig. 2a shows the carbonyl levels in synaptosomes isolated from saline-injected gerbils and from D609-injected gerbils, and then treated *in vi ro* with Aβ (1–42). The level of carbonyls was found to be significantly higher ($P<0.001$) in synaptosomes obtained from saline-injected gerbils and treated with Aβ (1–42). D609 treatment protects synaptosomes against Aβ (1–42)-induced oxidative protein damage ($P<0.004$). As a control, synaptosomes were first treated with $NaBH_4$, which reduces carbonyls to alcohols. Reaction with 2,4-dinitrophenylhydrazine (DNPH) is expected not to occur; hence, no anti-DNP hydrazone antibody binding is expected. We demonstrated this result previously (Sultana et al., 2006), and confirmed this finding in the present study (data not shown). Thus, $NaBH_4$ pretreatment resulted in no antibody binding, demonstrating the specificity of the immunochemical detection of carbonyls by our procedures. In order to demonstrate the specificity of D609, two other Aβ peptides, Aβ (1–40) and the nontoxic reverse of Aβ peptide [Aβ (42–1)] were used. Fig. 2b shows the carbonyl levels in various Aβ peptide treated synaptosomes and in synaptosomes isolated from D609-injected gerbils subsequently treated with Aβ (1–42), Aβ (1–40) and Aβ (42–1) respectively. As shown Fig. 2b, D609 treatment protects synaptosomes against Aβ (1–42) and Aβ (1–40)-induced oxidative protein damage ($P<0.05$). There was no significant increase in protein carbonyl levels in synaptosomes isolated from saline-injected and D609-injected gerbils subsequently treated with Aβ (42–1).

The antioxidant properties of D609 were further con-

that iNOS expression is induced by oxidative stress and that antioxidant compounds suppress its expression either at gene level or at protein level (Ayasolla et al., 2004; Calabrese et al., 2004). In the present study, we observed that both iNOS and 3-NT levels were increased in Aβ (1–42)-treated synaptosomes and that D609 treatment showed protection against the Aβ (1–42)-induced increase of iNOS and 3-NT levels.

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Oxidative damage is present in the brains of patients with AD, and is observed within every class of biomolecules, including nucleic acids, proteins, lipids and carbohydrates (Butterfield et al., 2001; Butterfield and Lauderback, 2002; Castegna et al., 2003; Good et al., 1996; Aliev et al., 2004; Lue et al., 2005). Our laboratory has suggested a comprehensive model for neurodegeneration in AD combining two established notions: i) elevated oxidative stress in AD brain; ii) centrality of Aβ in the cause and consequences of this dementing disorder (Butterfield and Lauderback, 2002; Castegna et al., 2003). Many additional studies from different laboratories have supported the view that oxidative stress maursL7.4.25

products may be responsible for damaging enzymes critical in neuronal function (Butterfield et al., 2003; Varadarajan et al., 2000).

In the present study, we showed the ability of *in vivo*-

glutamate toxicity and ionizing radiation-induced oxidative stress in lymphocytes by maintaining intracellular GSH homeostasis (Zhou et al., 2001). The results presented in this paper demonstrated that *in vivo* injection of D609 was effective in reducing protein oxidation, lipid peroxidation and ROS production induced by Aβ (1–42) treatment.

The concept that Aβ induces lipid peroxidation is a key component of the Aβ (1–42)-associated free radical model for neurodegeneration in AD (Butterfield 1997; Lauderback et al., 2001; Varadarajan et al., 2000). HNE alters the conformation of transmembrane and cytoskeletal synaptosomal proteins (Esterbauer et al., 1991; Subramaniam et al., 1997). GSH blocks the damaging effects of this unsaturated aldehyde on synaptosomal proteins (Pocernich et al., 2000, 2001). As noted above, D609 binds to α,β-unsaturated aldehydes to prevent their toxicity (Lauderback et al., 2003). Taken together, these data support the notion that the ability of D609 to exert its protective effects against Aβ-associated lipid peroxidation involves its direct binding to HNE thus providing an efficient tool for detoxication. Consistent with the Aβ-associated free radical process, Aβ (1–42) induces protein oxidation, indexed by the increase of carbonyl levels and of nitrotyrosine residues. Oxidative modification of crucial proteins results in alteration of their structural and functional properties, eventually leading to synapse loss and neurodegeneration.

There is compelling evidence supporting that enhanced pro-inflammatory activities induced by Aβ are associated with the pathogenesis and/or progression of AD, and that some anti-inflammatory agents protect neurons against Aβ-induced neurotoxicity (Breitner, 1996). One of the principal enzymes that plays a pivotal role in mediating an inflammatory response is iNOS. iNOS is mainly localized in astrocytes and microglia, and catalyzes the oxidative deamination of L-arginine to produce nitric oxide (NO), a potent pro-inflammatory mediator. In Alzheimer's tissue, pro-inflammatory iNOS is notably up-regulated and colocalized in Aβ plaques. Several studies have demonstrated that Aβ stimulates astrocytic iNOS induction

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GSH analogs, mimetic or precursors have been used in patients or animal models. Based on the results presented in the current paper and on our previous studies (Sultana et al., 2004), we hypothesized that D609 is a potential brain-accessible GSH-mimetic compound. This hypothesis that D609 is a GSH mimetic that is itself not GSH is further supported by the finding that D609 treatment does not lead to an increase of brain GSH levels (Table 1).

The ability of *in vivo* D609 to prevent Aβ-induced oxidative stress could also be related to its property as an inhibitor of PC-PLC and sphingomyelinase. Most of D609 biological activities (antitumor, antiviral, anti-inflammatory) have been largely attributed to the inhibition of PC-PLC and sphingomyelinase. However, the identification of D609 as a potent antioxidant implies that D609 may exert some of the reported activities by its antioxidant properties. The biological activity of PC-PLC and sphingomyelinase involves regulation of Ca^{+2} homeostasis through the production of ceramide. Since Aβ may lead to altered Ca^{+2} homeostasis in neurons (Mattson et al., 1993), it is reasonable to argue that the protective effects of D609 could rely also on its inhibitory activity on PC-PLC or sphingomyelinase. Thus, we suggest that multiple biological functions of D609 could potentially contribute to counteract Aβ-driven neurotoxicity in the brain. The presence of the free thiol group in the molecule confers to the xanthate a strong reducing property (Lauderback et al., 2003; Rao, 1971; Sultana et al., 2004) that is undoubtedly responsible for the antioxidant activity of D609.

Considering that Aβ (1–42) is a potent inducer of oxidative stress and that the deposition of this peptide can induce the cascade of pathological changes occurring in AD, many attempts to test effective protection by antioxidants are currently under investigation. However, many clinical trials are unsuccessful due to a low brain-accessible capability of the antioxidant compounds tested. Based on these notions, searches for new potential antioxidant compounds could be of relevance for future directions of AD treatments.

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In conclusion, the present study demonstrated the ability of D609 to act as a potent antioxidant *in vivo*, thereby providing neuroprotection against Aβ-induced oxidative stress. Further studies are required to gain insight into the potential use of D609 in the treatment of AD and other oxidative stress-related disorders. Investigations of the use of D609 on animal models of AD are in progress.

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