

## IN VIVO PROTECTION BY THE XANTHATE TRICYCLODECAN-9-YL-XANTHOGENATE AGAINST AMYLOID $\beta$ -PEPTIDE (1–42)-INDUCED OXIDATIVE STRESS

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**Abstract**—Considerable evidence supports the role of oxidative stress in the pathogenesis of Alzheimer’s disease. One hallmark of Alzheimer’s disease is the accumulation of amyloid  $\beta$ -peptide, which invokes a cascade of oxidative damage to neurons that can eventually result in neuronal death. Amyloid  $\beta$ -peptide is the main component of senile plaques and generates free radicals ultimately leading to neuronal damage of membrane lipids, proteins and nucleic acids. Therefore, interest in the protective role of different antioxidant compounds has been growing for treatment of Alzheimer’s disease and other oxidative stress-related disorders. Among different antioxidant drugs, much interest has been devoted to “thiol-delivering” compounds. Tricyclodecan-9-yl-xanthogenate is an inhibitor of phosphatidylcholine specific phospholipase C, and recent studies reported its ability to act as a glutathione-mimetic compound. In the present study, we investigate the *in vivo* ability of tricyclodecan-9-yl-xanthogenate to protect synaptosomes against amyloid  $\beta$ -peptide-induced oxidative stress. Gerbils were injected *i.p.* with tricyclodecan-9-yl-xanthogenate or with saline solution, and synaptosomes were isolated from the brain. Synaptosomal preparations isolated from tricyclodecan-9-yl-xanthogenate injected gerbils and treated *ex vivo* with amyloid  $\beta$ -peptide (1–42) showed a significant decrease of oxidative stress parameters: reactive oxygen species levels, protein oxidation (protein carbonyl and 3-nitrotyrosine levels) and lipid peroxidation (4-hydroxy-2-nonenal levels). Our results are consis-

tent with the hypothesis that modulation of free radicals generated by amyloid  $\beta$ -peptide might represent an efficient therapeutic strategy for treatment of Alzheimer’s disease and other oxidative-stress related disorders. Based on the above data, we suggest that tricyclodecan-9-yl-xanthogenate is a potent antioxidant and could be of importance for the treatment of Alzheimer’s disease and other oxidative stress-related disorders. © 2005 Published by Elsevier Ltd on behalf of IBRO.

**Key words:** Alzheimer’s disease, amyloid  $\beta$ -peptide, antioxidant, D609, oxidative stress, glutathione.

Alzheimer’s disease (AD) is a progressive neurological disorder characterized by loss of memory cognition. Major pathological hallmarks of AD include loss of synapses and the presence of senile plaques and neurofibrillary tangles. The major protein component of the core of senile plaques is amyloid  $\beta$ -peptide ( $A\beta$ ).  $A\beta$  is formed upon proteolytic processing, by  $\beta$ - and  $\gamma$ -secretases (Haass and De Strooper, 1999), of the larger amyloid precursor protein (APP), a ubiquitously expressed transmembrane glycoprotein (Glenner et al., 1988; Glenner and Wong, 1984). The amyloid  $\beta$ -cascade hypothesis, suitably updated, postulates that  $A\beta$  is likely central to the pathogenesis of AD (Hardy and Allsop 1991; Selkoe 2001; Smith et al., 1999). The mechanisms involved in  $A\beta$ -mediated neurotoxicity are unknown, but there is evidence suggesting that oxidative stress plays a key role (Butterfield et al., 2001; Butterfield and Lauderback, 2002; Canevari et al., 2004). Growing attention has been focused to investigate the oxidative mechanism of  $A\beta$  toxicity and as well in the search for novel neuroprotective agents. Previous studies from our laboratory and others reported that  $A\beta$  peptide induces *in vitro* reactive oxygen species (ROS) production, protein oxidation, DNA and RNA oxidation and lipid peroxidation (Butterfield, 2002).

Because of the involvement of oxidative stress-mediated toxicity in neurodegenerative events and neuronal cell death (Good et al., 1996; Moreira et al., 2005), various experimental approaches for effective protection by antioxidants have emerged. In addition, antioxidant therapy is being discussed for Parkinson’s disease (Ebadi et al., 1996; Prasad et al., 1999), ischemia (Marczyn et al., 2003) as well as for AD (Gilgun-Sherki et al., 2003; Grundman and Delaney, 2002) and other age-related disorders (Ames, 2004). Numerous potential free-radical scavengers have been tested in different experimental paradigms of oxidative stress-induced cell death, such as Vitamin E (Behl et al., 1992), vitamin C, melatonin (Pappolla et al.,

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**Abbreviations:**  $A\beta$ , amyloid  $\beta$ -peptide; AD, Alzheimer’s disease; BSA, bovine serum albumin; CTR, saline/D609-injected gerbils; DCF, dichlorofluorescein; DCFH-DA, 2,7-dichlorofluorescein diacetate; DNPH, 2,4-dinitrophenylhydrazine; D609, tricyclodecan-9-yl-xanthogenate; GSH, glutathione; HNE, 4-hydroxynonenal; iNOS, inducible nitric oxide synthase; NO, nitric oxide; PBS, phosphate-buffered saline; PC-PLC, phosphatidylcholine-specific phospholipase C; ROS, reactive oxygen species; TBS-T, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20; 3-NT, 3-nitrotyrosine.

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1997), ginkgo biloba (Smith and Luo, 2003), steroid hormones (Behl et al., 1997; Goodman et al., 1996), *N*-acetylcysteine (Adair et al., 2001), etc. However, many clinical trials are still unsuccessful because all the antioxidants tested are poorly active in crossing the blood brain barrier (BBB).

Glutathione (GSH) is one of the major intracellular defense systems, and depletion of GSH is known to be involved in several neurodegenerative disorders (Benzi and Moretti, 1995; Butterfield et al., 2002; Markesbery, 1997). Many attempts have been made to develop antioxidant compounds able to “mimic” GSH as scavenger of ROS and to maintain the intracellular redox state. Increase in endogenous GSH levels by dietary or pharmacological intake of GSH precursors or GSH mimetics protects brain against oxidative stress (Anderson and Luo, 1998; Butterfield et al., 2001; Halliwell, 2001). Considering the importance of developing new antioxidant compounds and the relevance of their application in the treatment of neurodegenerative diseases, we focused our attention on tricyclocodecan-9-yl-xanthogenate (D609). D609 is an inhibitor of phosphatidylcholine-specific phospholipase C (PC-PLC) (Schutze et al., 1992; Wiegmann et al., 1994) and sphingomyelinase (Meng et al., 2004; Yu et al., 2000). D609 exhibits a variety of potent biological properties, including antitumor (Amtmann and Sauer, 1987), antiviral (Amtmann et al., 1987; Villanueva et al., 1991) and anti-inflammatory activities (Machleidt et al., 1996). The xanthate D609 is a reducing agent and it has been reported to protect rodents against UV-induced oxidative damage (Zhou et al., 2001). Previous studies from our laboratory have shown that D609 exerts its antioxidant properties acting as a GSH-mimetic compound (Lauderback et al., 2003). The free thiol group of the xanthate is oxidized to the corresponding disulfide (dixanthate) that is a substrate for GSH-reductase, regenerating D609 active form (xanthate). D609 can scavenge hydrogen peroxide and hydroxyl free radicals. In addition, D609 can bind directly to reactive alkenals, providing detoxication of these lipid peroxidation end-products thereby preventing oxidative damage of synaptic membranes (Lauderback et al., 2003). Recently, Sultana et al. (2004) demonstrated that D609 is protective against A $\beta$ -induced toxicity in primary neuronal cultures.

Based upon the mechanisms by which D609 scavenges free radicals, the aim of the present study was to investigate the ability of D609 to provide *in vivo* neuroprotection against A $\beta$ -induced oxidative stress. The results are consistent with the hypothesis that D609 is a potent antioxidant and could be beneficial in the treatment of AD and other oxidative stress-related disorders.

## EXPERIMENTAL PROCEDURES

### Materials

D609 was purchased from Biomol Inc. (Plymouth Meeting, PA, USA) and most other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The fluorescent indicator for ROS measurement, 2,7-dichlorofluorescein diacetate (DCFH-DA), was obtained from Molecular Probes (Eugene, OR, USA), and a fresh 10 mM stock solution was prepared in ethanol. Fresh D609 was

dissolved in phosphate-buffered saline (PBS). The OxyBlot™ oxidized protein kit was obtained from Intergen, Inc. (Purchase, NY, USA). Amyloid- $\beta$  peptide (1–42) (HPLC and MS certified quality) was purchased from Anaspec, Inc. (San Jose, CA, USA). For all experiments, A $\beta$  was incubated for 24 h in PBS at 37 °C before application to synaptosomes. Primary antibodies for 4-hydroxynonenal (HNE) and 3-nitrotyrosine (3-NT) were obtained from Chemicon (Temecula, CA, USA).

### Animals

For the present study, three month-old male Mongolian gerbils, approximately 100 g in size, were used to isolate synaptosomes. All the following protocols were approved by the University of Kentucky Animal Care and Use Committee, which mandates that all experiments involving animals conform to international guidelines on the ethical use of animals. Studies were conducted in such a way as to minimize the number of animals used. All the animals were kept under 12-h light/dark condition at University of Kentucky Animal Facility, and fed with standard Purina rodent laboratory chow *ad libitum*. The gerbils ( $n=12$ , 12 separate sets of experiments) were injected i.p. with freshly prepared D609 (50 mg/kg body weight) 1 h before killing. The dose and the time were chosen according to previous experiments performed in our laboratory (Joshi et al., 2005). Control animals were injected with saline solution for the same time ( $n=12$ ). The animals were killed with sodium pentobarbital to prevent any pain.

### Synaptosomal preparation

Synaptosomes were prepared according to the procedure described by Keller et al. (2000). The brain was isolated immediately after decapitation and placed in a 0.32 M sucrose isolation buffer containing 4  $\mu$ g/mL leupeptin, 4  $\mu$ g/mL pepstatin, 5  $\mu$ g/mL aprotinin, 20  $\mu$ g/mL trypsin inhibitor, 0.2 mM PMSF, 2 mM EDTA, 2 mM EGTA, 20 mM HEPES pH 7.4. Samples were homogenized with a Wheaton tissue homogenizer and centrifuged at 1500 $\times$ g for 10 min. The supernatant was collected and centrifuged at 20,000 $\times$ g for 10 min. The pellet was resuspended in 1 mL of 0.32 sucrose isolation buffer and layered onto discontinuous sucrose density gradients of 10 mL each of 0.85 M, pH 8.0; 1.0 M, pH 8.0; 1.18 M, pH 8.5 sucrose solution each containing 10 mM HEPES, 2 mM EDTA and 2 mM EGTA. The gradients were spun in a Beckman L7-55 ultracentrifuge at 82,550 $\times$ g for 1 h at 4 °C. The synaptosomal layer was collected at the 1/1.18 M sucrose interface, washed twice with PBS for 10 min at 32,000 $\times$ g, yielding synaptosomes. Protein concentrations of the purified synaptosomes were determined by the BCA assay (Pierce, Rockford, IL, USA). Synaptosomal preparations (1 mg/mL) were incubated with 10  $\mu$ M A $\beta$  (1–42) for 6 h at 37 °C.

### ROS measurements

ROS levels were measured by the dichlorofluorescein (DCF) assay. After incubation with A $\beta$  (1–42), synaptosomes (1 mg/mL) were washed with PBS and incubated with 10  $\mu$ M of non-fluorescent DCFH-DA for 30 min. Cytosolic esterases cleaved DCFH-DA, forming the anion DCFH that is trapped within the synaptosomes. The reaction of intracellular ROS with DCFH yields the fluorescent dye DCF. Synaptosomes were spun at 3000 $\times$ g in a tabletop Eppendorf centrifuge for 5 min at 4 °C. Synaptosomes were resuspended in 500  $\mu$ L of PBS and run in triplicate (100  $\mu$ L per well) in a black microtiter plate. The measurements were performed on a Molecular Devices SpectraMax microtiter plate reader with  $\lambda_{\text{ex}}=495$  nm and  $\lambda_{\text{em}}=530$  nm. Data are given as percentage of corresponding controls and are the mean of at least six independent experiments.

### Protein carbonyl measurement

Protein carbonyls are an index of protein oxidation and were determined as described previously (Berlett and Stadtman, 1997). Briefly, 5  $\mu$ L of synaptosome preparations (4 mg/mL) were incubated at room temperature with 10 mM 2,4-dinitrophenylhydrazine in the presence of 5  $\mu$ L of 12% SDS for 20 min at room temperature. The samples were neutralized with 7.5  $\mu$ L 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).

### 3-NT levels

Nitrotyrosine contents were determined by incubating 5  $\mu$ L of synaptosomes preparations with Laemmli buffer (0.125 M Trizma base, pH 6.8, 4% SDS, 20% glycerol) for 20 min. Two hundred fifty nanograms of protein were blotted onto nitrocellulose membranes and immunochemical methods were performed. The rabbit anti-3-NT primary antibody was incubated 1:200 in blocking buffer (BSA 3% in TBS-T) for 2 h. The membranes were washed three times with TBS-T and incubated with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (1:10,000). Densitometric analysis of bands in images of the blots was used to calculate levels of 3-NT.

### Lipid peroxidation measurement

HNE levels were measured as markers of lipid peroxidation. The samples (5  $\mu$ L) were incubated with 10  $\mu$ L Laemmli buffer for 20 min at room temperature. Two hundred fifty nanograms of protein samples were loaded in each well on nitrocellulose membrane in a slot blot apparatus under vacuum. The membranes were incubated with anti-HNE rabbit polyclonal antibody (1:5000) for 2 h, washed three times with TBS-T and then incubated with an anti-rabbit IgG alkaline phosphatase-conjugated secondary antibody (1:10,000). Controls in which the primary antibody was reacted with free HNE resulted in faint and non-specific binding of the antibody (data not shown). In addition, the specificity of primary and secondary antibodies was demonstrated by experiments previously performed in our laboratory (Perluigi et al., 2005; Sultana et al., 2004). Samples were developed using SigmaFast Tablets (BCIP/NBT substrate), and blots were scanned into Adobe Photoshop (Adobe System, Inc.) and quantitated with Scion Image (PC version of Macintosh compatible NIH Image).

### GSH assay

GSH levels were measured by a GSH Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA). Briefly, the cytosolic fraction isolated from brain homogenates was precipitated with 10% (w/v) metaphosphoric acid and then centrifuged for 5 min at 5000 $\times$ g. The supernatants were neutralized with 4 M triethanolamine and then analyzed according to manufacturer instructions. GSH concentration in the samples was calculated using the kinetic method

by measuring the absorbance at 405 nm with a Bio-Tek Powerwave X Microtiter Plate Reader (Bio, Inc.). A plot of the corrected absorbance vs. the concentration of GSH standards ( $\mu$ M) was utilized to calculate the average concentration of GSH present in the samples.

### Inducible nitric oxide synthase (iNOS) expression levels

Synaptosome samples (50  $\mu$ g) were mixed with sample loading buffer, denatured for 5 min at 100 °C and then loaded on 10% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membrane in 25 mM Tris, 192 mM glycine containing 20% (v/v) at 80 mA/gel for 2 h. For iNOS detection, membranes were probed first with 10 mL of blocking buffer (3% BSA in TBS-T) containing rabbit anti-iNOS polyclonal antibody (Santa Cruz Biotechnology Inc., CA, USA, 1:1000) for 2 h at 27 °C, followed by 10 mL of blocking buffer containing alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG from Sigma Chemical Co. (1:5000) for 1 h at 27 °C. Membranes were washed three times with T-TBS (0.5% Tween in TBS) and the bands were visualized using SigmaFast Tablets (BCIP/NBT) substrate. iNOS protein levels were normalized relative to the  $\beta$ -actin level in each sample.

### Statistical analysis

ANOVA was used for statistical evaluation of data followed by Student's *t*-test. *P* values <0.05 were considered significant for comparison between control and experimental data.

## RESULTS

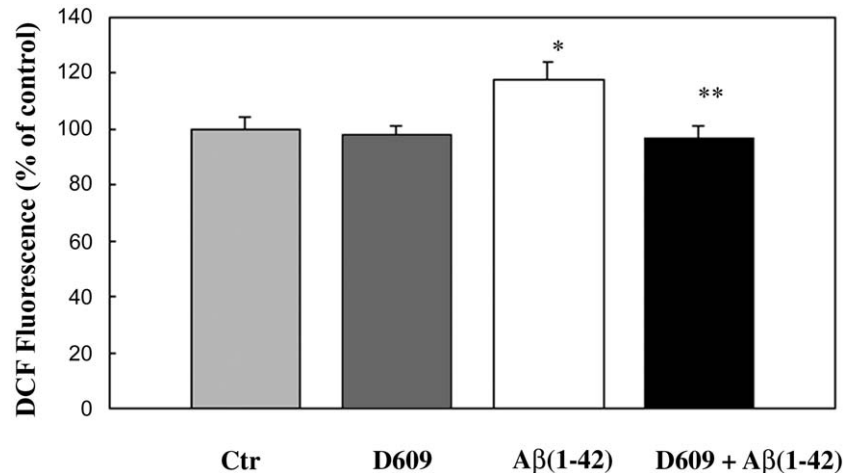
### D609 prevents *in vivo* ROS accumulation

The DCFH-DA method was used to monitor the ROS levels generated by A $\beta$  (1–42) in our experimental model. Fig. 1 shows the levels of ROS in synaptosomes isolated from saline/D609-injected gerbils (CTR), in synaptosomes isolated from saline-injected gerbils and treated *in vitro* with A $\beta$  (1–42) and in synaptosomes isolated from D609-injected gerbils and treated *in vitro* with A $\beta$  (1–42). In the absence of 10  $\mu$ M A $\beta$  (1–42), levels of ROS in synaptosomes from saline-injected gerbils did not show any significant difference compared with the levels measured in synaptosomes from D609-injected gerbils. Hence, both these groups can be referred as CTR. Thus, D609 itself does not reduce basal oxidation levels. Synaptosomes isolated from saline-injected gerbils and treated with 10  $\mu$ M A $\beta$  (1–42) for 6 h displayed an increased fluorescence, about 20% compared with control synaptosomes (untreated) (*P*<0.005). Synaptosomes isolated from D609-injected gerbils were not affected by A $\beta$  (1–42)-induced ROS accumulation (*P*<0.002). Thus, D609 is able to prevent ROS accumulation in synaptosomes.

### D609 *in vivo* protects against A $\beta$ (1–42)-induced protein oxidation and lipid peroxidation

Protein carbonyls and 3-NT levels were measured as markers of protein oxidation (Stadtman and Berlett, 1997). Collectively, ROS can lead to the oxidation of amino acid residue side chains, formation of protein-protein cross-linkages and oxidation of the protein backbones resulting in protein fragmentation. The presence of carbonyl groups in proteins has therefore been used as a marker of ROS-





**Fig. 1.** D609 prevents A $\beta$  (1–42)-induced ROS production. ROS levels were determined by the DCF fluorescence assay. Ctr=synaptosomes isolated from saline injected gerbils with no further treatment ( $n=6$ , six separate sets of experiments); D609=synaptosomes isolated from D609-injected gerbils with no further treatment ( $n=6$ ); A $\beta$  (1–42)=synaptosomes isolated from saline-injected gerbils and treated with 10  $\mu$ M A $\beta$  (1–42) for 6 h ( $n=6$ ); A $\beta$  (1–42)+D609=synaptosomes isolated from D609-injected gerbils and treated with 10  $\mu$ M A $\beta$  (1–42) for 6 h ( $n=6$ ). The data are the mean $\pm$ S.E.M of six independent experiments, expressed as percentage of control values. Statistical comparison was made using ANOVA test ( $n=6$  for each group). \*  $P<0.005$ , A $\beta$  (1–42) vs Control; \*\*  $P<0.002$ , A $\beta$  (1–42) vs A $\beta$  (1–42)+D609.

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 vitro* with A $\beta$  (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 A $\beta$  (1–42). D609 treatment protects synaptosomes against A $\beta$  (1–42)-induced oxidative protein damage ( $P<0.004$ ). As a control, synaptosomes were first treated with NaBH<sub>4</sub>, 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<sub>4</sub> 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 A $\beta$  peptides, A $\beta$  (1–40) and the nontoxic reverse of A $\beta$  peptide [A $\beta$  (42–1)] were used. Fig. 2b shows the carbonyl levels in various A $\beta$  peptide treated synaptosomes and in synaptosomes isolated from D609-injected gerbils subsequently treated with A $\beta$  (1–42), A $\beta$  (1–40) and A $\beta$  (42–1) respectively. As shown Fig. 2b, D609 treatment protects synaptosomes against A $\beta$  (1–42) and A $\beta$  (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 A $\beta$  (42–1).

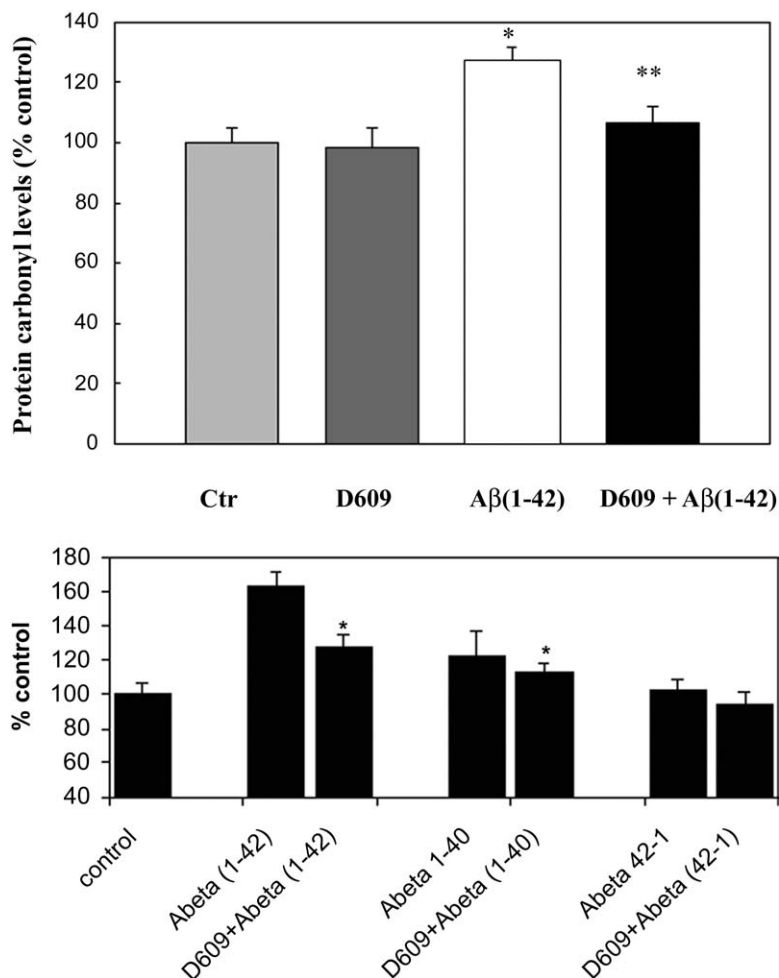
The antioxidant properties of D609 were further confirmed by measuring 3-NT levels, formed by reaction of reactive nitrogen species (RNS) with proteins (Castegna et al., 2003). Fig. 3 shows the protective effects of D609 on A $\beta$  (1–42)-induced formation of 3-NT. Synaptosomes isolated from saline-injected gerbils showed increased levels of 3-NT ( $P<0.005$ ) when treated *in vitro* with A $\beta$  (1–42),

while synaptosomes isolated from D609-injected gerbils were completely protected ( $P<0.05$ ).

Free radical attack on phospholipid PUFA leads to the formation of reactive aldehydes, among which one of the most neurotoxic is HNE (Esterbauer et al., 1991). This alkenal reacts with proteins forming stable covalent adducts to histidine, lysine and cysteine residues via Michael addition. The extent of this reaction can be measured immunochemically by quantifying the levels of HNE-bound proteins. Fig. 4 shows the HNE-bound protein levels in synaptosomes isolated from gerbils previously injected with D609 or with saline solution and incubated *in vitro* with 10  $\mu$ M A $\beta$  (1–42) for 6 h. Consistent with the protein oxidation results showed above, we observed *in vivo* protection by D609 against 10  $\mu$ M A $\beta$ -induced lipid peroxidation. HNE levels were found to be higher in A $\beta$  (1–42)-treated synaptosomes isolated from saline-injected gerbils, while A $\beta$  (1–42)-treated synaptosomes isolated from D609-injected gerbils showed reduced levels of HNE-bound proteins. These results are consistent with recent *in vitro* data obtained on primary neuronal cultures (Sultana et al., 2004), indicating that D609 acts as a potent antioxidant thus preventing protein oxidation and lipid peroxidation.

#### D609 modulates A $\beta$ (1–42)-induced iNOS expression levels

As shown in Fig. 5, iNOS levels were significantly increased (40%) in synaptosomes isolated from DMSO-injected gerbils treated with A $\beta$  (1–42) when compared with controls ( $P<0.01$ ). In contrast, we observed a decrease in expression of iNOS in synaptosomes isolated from D609-injected gerbils ( $P<0.05$ ) and subsequently treated with A $\beta$  (1–42), a result that is consistent with our findings of decreased levels of 3-NT. Several lines of evidence show



**Fig. 2.** (a) Protective effects of D609 on A $\beta$ -induced protein oxidation. Protein carbonyl content of synaptosomes was measured as described in "Experimental Procedures." Synaptosomes isolated from saline-injected gerbils and treated with 10  $\mu$ M A $\beta$  (1–42) demonstrate a higher level of protein carbonyls than that of untreated controls (Ctr and D609). \*  $P < 0.001$ , A $\beta$  (1–42) vs Control. Synaptosomes isolated from D609-injected gerbils were completely protected from A $\beta$ -induced oxidative modifications [A $\beta$  (1–42)+D609]. \*\*  $P < 0.004$ , A $\beta$  (1–42) vs A $\beta$  (1–42)+D609. (b) Protective effects of D609 on A $\beta$ -induced protein oxidation. Synaptosomes isolated from saline-injected gerbils and treated with 10  $\mu$ M A $\beta$  (1–42) or 10  $\mu$ M A $\beta$  (1–40) demonstrate a higher level of protein carbonyls than that of untreated controls, whereas 10  $\mu$ M A $\beta$  (42–1) did not induce any significant increase in protein carbonyls. Synaptosomes isolated from D609-injected gerbils were completely protected from A $\beta$  (1–42)- and A $\beta$  (1–40)-induced oxidative modifications. \*  $P < 0.05$ ,  $n = 5$ .

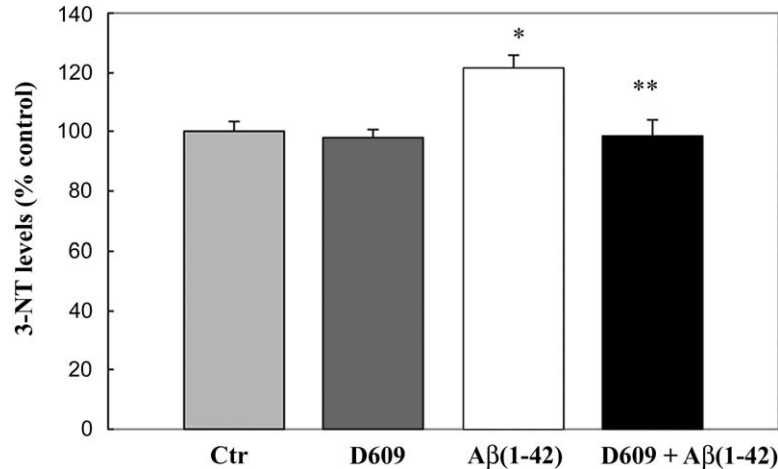
that iNOS expression is induced by oxidative stress and that antioxidant compounds suppress its expression either at gene level or at protein level (Ayasolla et al., 2004; Calabrese et al., 2004). In the present study, we observed that both iNOS and 3-NT levels were increased in A $\beta$  (1–42)-treated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

## DISCUSSION

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 A $\beta$  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 may be central to the A $\beta$ -driven neurodegeneration. Based on these notions, treatment with brain accessible antioxidants may be a promising approach for slowing disease progression to the extent that oxidative damage may be responsible for the cognitive and functional decline observed in AD.

Previous studies from our laboratory and others have shown that A $\beta$  (1–42) is associated with free radical generation leading to oxidative damage of proteins, lipids, DNA and RNA (Butterfield et al., 2001; Butterfield and Lauderback, 2002; Varadarajan et al., 2000). The oxidation of proteins by free radicals or free radical oxidation



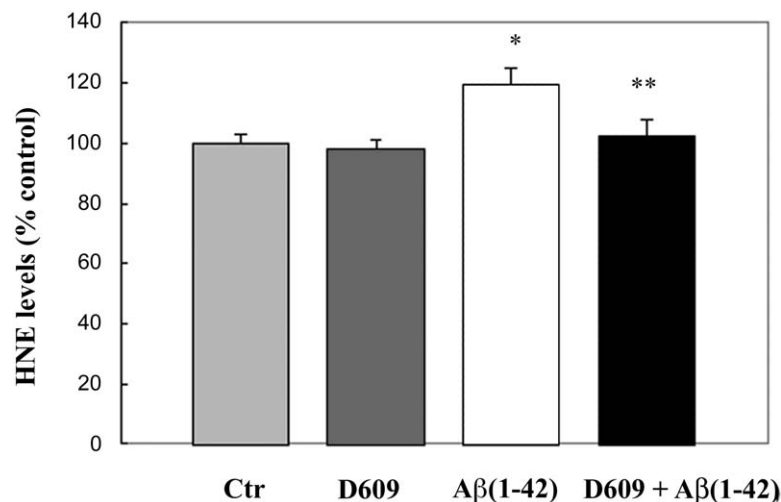
**Fig. 3.** Protective effect of D609 on A $\beta$ -induced 3-NT formation. 3-NT levels were determined as described in the “Experimental Procedures” section. Ctr=synaptosomes isolated from saline- or D609-injected gerbils with no further treatment; D609=synaptosomes isolated from D609-injected gerbils with no further treatment; A $\beta$  (1–42)=synaptosomes isolated from saline-injected gerbils and treated with 10  $\mu$ M A $\beta$  (1–42) for 6 h; A $\beta$  (1–42) +D609=synaptosomes isolated from D609-injected gerbils and treated with 10  $\mu$ M A $\beta$  (1–42) for 6 h. The data are the mean  $\pm$  S.E.M expressed as percentage of control values ( $n=6$ ). \*  $P<0.005$ , A $\beta$  (1–42) vs Control; \*\*  $P<0.05$ , A $\beta$  (1–42) vs A $\beta$  (1–42)+D609.

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

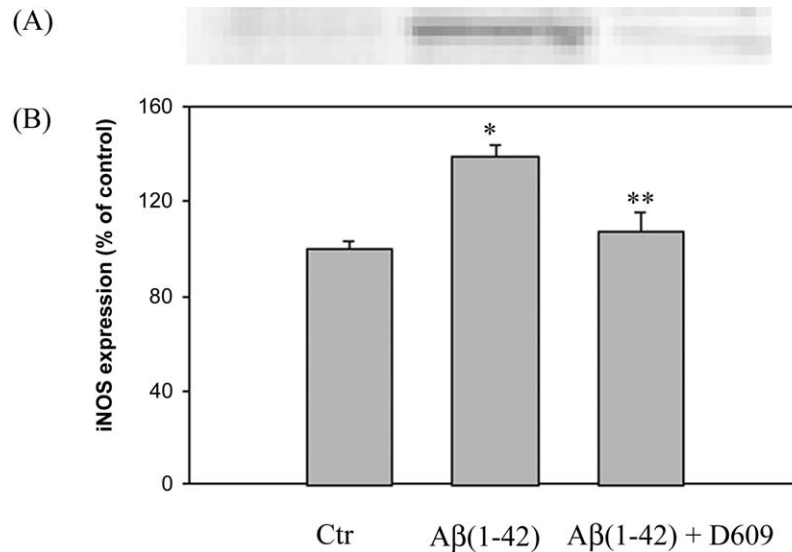
In the present study, we showed the ability of *in vivo*-injected D609 to provide neuroprotection against oxidative stress induced by A $\beta$  (1–42) on subsequently isolated synaptosomes. A variety of well-established and new potential antioxidant compounds is under investigation to prevent A $\beta$ -induced toxic effects (Behl, 2002; Grundman and Delaney, 2002). Among different classes of antioxidant drugs, much interest has been devoted to “thiol-delivering” compounds. Because decreased levels of GSH are associated with aging and neurodegeneration (Butterfield et al., 2002; Liu and Choi, 2000), therapeutic strate-

gies based on elevation of GSH levels have been shown to be protective against oxidative stress conditions of the brain (Butterfield et al., 2002). The age-related decrease of GSH may represent a key factor in the aging process and may underlie a number of changes occurring in normal aging and the onset of various diseases.

We have previously shown that the antioxidant properties of D609 are associated with the free thiol group of the xanthate (Lauderback et al., 2003). For example, methylated D609 is unable to protect cultured neurons from damage by A $\beta$  (1–42) (Sultana et al., 2004). D609 in its reduced form is oxidized to the corresponding disulfide (dixanthate) that is converted back to the xanthate by GSH reductase (Lauderback et al., 2003). D609 has been reported to protect against



**Fig. 4.** Protective effect of D609 on A $\beta$ -induced lipid peroxidation (HNE levels). HNE levels were determined as described in the “Experimental Procedures” section. Ctr=synaptosomes isolated from CTR; D609=synaptosomes isolated from D609-injected gerbils with no further treatment; A $\beta$  (1–42)=synaptosomes isolated from saline-injected gerbils and treated with 10  $\mu$ M A $\beta$  (1–42) for 6 h; A $\beta$  (1–42)+D609=synaptosomes isolated from D609-injected gerbils and treated with 10  $\mu$ M A $\beta$  (1–42) for 6 h. The data are the mean  $\pm$  S.E.M. expressed as percentage of control values ( $n=6$ ). \*  $P<0.005$ , A $\beta$  (1–42) vs Control; \*\*  $P<0.05$ , A $\beta$  (1–42) vs A $\beta$  (1–42)+D609.



**Fig. 5.** Western immunoblot analysis of synaptosomes for iNOS protein expression levels. Samples containing 50  $\mu$ g of protein were loaded onto 10% SDS-PAGE gels, and the blots were probed with the polyclonal anti-iNOS antibody (1:1000) for 2 h. Immunoblots were scanned by densitometry and all values were normalized to  $\beta$ -actin. A representative immunoblot (A) and densitometric values (B) are shown. Densitometric values represent the mean  $\pm$  S.E.M. obtained from three independent experiments. Significant differences were assessed by ANOVA. \*  $P < 0.01$ , control vs A $\beta$  (1–42); \*\*  $P < 0.05$ , A $\beta$  (1–42)+D609.

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 A $\beta$  (1–42) treatment.

The concept that A $\beta$  induces lipid peroxidation is a key component of the A $\beta$  (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  $\alpha,\beta$ -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 A $\beta$ -associated lipid peroxidation involves its direct binding to HNE thus providing an efficient tool for detoxification. Consistent with the A $\beta$ -associated free radical process, A $\beta$  (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 A $\beta$  are associated with the pathogenesis and/or progression of AD, and that some anti-inflammatory agents protect neurons against A $\beta$ -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 A $\beta$  plaques. Several studies have demonstrated that A $\beta$  stimulates microglial and astrocytic iNOS induction and subsequent NO production (Akama and Van Eldik, 2000). We observed an increase in 3-NT levels in synaptosomes isolated from DMSO-injected gerbils and treated with A $\beta$  (1–42) that coincides with a parallel increase in iNOS levels. Pre-treatment with D609 reduced significantly the levels of iNOS and of nitrated proteins. iNOS induction may reflect the degree of inflammation associated to A $\beta$  (1–42), and the ability of D609 to block this effect may represent a further additional protective mechanism in addition to the antioxidant potential of this xanthate.

The levels of oxidized GSH are consistently elevated in Alzheimer's patients as compared with their age-matched controls and correlate with cognitive dysfunction (Vina et al., 2004). Recent findings suggested that A $\beta$  could initiate a cascade of events resulting in a severe depletion of GSH (Abramov et al., 2003). Alteration of GSH homeostasis impairs neuronal viability because GSH depletion leaves the neurons vulnerable to damage by oxidative stress. Here, we demonstrated that D609 is able to provide neuroprotection against A $\beta$ -induced neurotoxicity acting as a GSH-mimetic compound, thus modulating the effects of GSH depletion. Previous studies from our laboratory have shown that *i.p.* administration of *N*-acetylcysteine and of gamma-glutamyl-cysteine ethyl ester (GCEE) protected synaptosomes against oxidative stress (Boyd-Kimball et al., 2005; Butterfield et al., 2002; Pocernich et al., 2001). These compounds led to an increase of brain GSH by serving as precursor for GSH biosynthesis. Because GSH itself penetrates the blood–brain barrier only poorly and cannot be taken up by neurons directly, other treatment options to increase brain concentration of GSH including

**Table 1.** D609 does not influence total brain GSH levels

Injection	GSH (mM)±S.E.M
Control	4.27±0.87
D609	3.98±0.54

Results presented are the mean±S.E.M,  $n=6$ . Total GSH levels, following i.p. D609 injection, were measured by a GSH assay kit as reported in the Experimental Procedures section.

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 A $\beta$ -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<sup>+2</sup> homeostasis through the production of ceramide. Since A $\beta$  may lead to altered Ca<sup>+2</sup> 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 A $\beta$ -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 A $\beta$  (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.

## CONCLUSION

In conclusion, the present study demonstrated the ability of D609 to act as a potent antioxidant *in vivo*, thereby providing neuroprotection against A $\beta$ -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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