Free Radical Research, Volume 38 Number 5 (May 2004), pp. 449–458

Protective Effect of the Xanthate, D609, on Alzheimer's Amyloid β-peptide (1–42)-induced Oxidative Stress in Primary Neuronal Cells

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Accepted by Professor B. Halliwell

(Received 24 November 2003; In revised form 29 January 2004)

Tricyclodecan-9-yl-xanthogenate (D609) is an inhibitor of phosphatidylcholine-specific phospholipase C, and this agent also has been reported to protect rodents against oxidative damage induced by ionizing radiation. Previously, we showed that D609 mimics glutathione (GSH) functions and that a disulfide is formed upon oxidation of D609 and the resulting dixanthate is a substrate for GSH reductase, regenerating D609. Considerable attention has been focused on increasing the intracellular GSH levels in many diseases, including Alzheimer's disease (AD). Amyloid β-peptide [Aβ(1–42)], elevated in AD brain, is associated with oxidative stress and toxicity. The present study aimed to investigate the protective effects of D609 on Aβ(1–42)-induced oxidative cell toxicity in cultured neurons. Decreased cell survival in neuronal cultures treated with Aβ(1–42) correlated with increased free radical production measured by dichlorofluorescein fluorescence and an increase in protein oxidation (protein carbonyl, 3-nitrotyrosine) and lipid peroxidation (4-hydroxy-2-nonenal) formation. Pretreatment of primary hippocampal cultures with D609 significantly attenuated Aβ(1–42)-induced cytotoxicity, intracellular ROS accumulation, protein oxidation, lipid peroxidation and apoptosis. Methylated D609, with the thiol functionality no longer able to form the disulfide upon oxidation, did not protect neuronal cells against Aβ(1–42)-induced oxidative stress. Our results suggest that D609 exerts protective effects against Aβ(1–42) toxicity by modulating oxidative stress. These results may be of importance for the treatment of AD and other oxidative stress-related diseases.

Keywords: Aβ(1–42); Tricyclodecan-9-yl-xanthogenate; Protein carbonyl; Glutathione; Lipid peroxidation; Cortical neurons

INTRODUCTION

Alzheimer's disease (AD) neuropathology is characterized by loss of synapses and the presence of amyloid plaques and neurofibrillary tangles. Amyloid plaques have a central core of β-amyloid, a 39–43 amino acid peptide derived from the proteolytic cleavage of amyloid precursor protein (APP) by beta- and gamma-secretases. The exact mechanism by which amyloid β-peptide [Aβ(1–42)] causes induced neurotoxicity[1–4] is not known, but a number of previous studies from our laboratory and others have shown that the oxidative stress is induced by Aβ(1–42), assessed by excessive formation of reactive oxygen species and reactive nitrogen species (ROS/RNS).[5–9] ROS/RNS causes tissue damage resulting from a wide variety of insults like protein oxidation, lipid peroxidation, DNA and RNA oxidation and neuronal death.[5,6,8,10] Oxidative modification of proteins *in vivo* may affect a variety of cellular functions involving proteins: receptors, signal transduction mechanisms, transport systems and enzymes.

Glutathione (GSH) is the major intracellular thiol, participating in cellular redox reactions and thioether formation, and a decrease level of GSH may severely impair normal cellular functions.[11,12] Further, it has been shown that GSH levels are decreased in specific regions of the central nervous system of subjects

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/107157604200020647 8

affected by AD, and this may contribute to the oxidative stress mediated neuronal cell loss.[6,7,13–15] An imbalance in cellular defense systems may contribute to AD pathology and dementia.[6,7] One of the ways to combat the toxic effects of Aβ(1–42) is to augment or potentiate endogenous oxidative defense capacity through dietary or pharmacological intake of antioxidants.[16,17] Attempts have been made to increase GSH levels by injecting *N*-acetyl-cysteine (NAC) and GSH precursors, such as gamma-glutamyl-cysteine ethyl ester (GCEE).[15,18–22] Therefore, we tested the protective efficacy of tricyclodecan-9-yl-xanthogenate (D609), a GSH mimetic,[23] against Aβ(1–42)-induced oxidative stress in primary neuronal culture.

D609 is an inhibitor of phosphatidylcholine-specific phospholipase C [PC-PLC].[24,25] This xanthate has been reported to protect against oxidative damage induced by ionizing radiation and also shows antiviral and antitumor activity.[26,27] Previous studies from our laboratory and others have reported the GSH mimetic function of D609.[23] For example, D609 forms a disulfide upon oxidation, which is reduced to the xanthate by GSH reductase, an enzyme that converts oxidized GSH to GSH. D609 also has the ability to scavenge hydroxyl radicals and hydrogen peroxide.[23,28] In addition, D609 binds to and thus detoxifies reactive alkenals, thereby preventing the latter from damaging synaptosomes.[23]

Hence, the present study was designed to study the protective effect of D609 against Aβ(1–42) induced oxidative damage in neuronal culture. The results are consistent with the notion that D609 may be acting like GSH, thereby protecting the cells against Aβ(1–42) induced oxidative stress.

MATERIALS AND METHODS

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless stated otherwise. The oxidized protein detection kit was purchased from Intergen (Purchase, NY) and D609 from Biomol Inc. A fresh 10 mM stock solution of 2,7-dichlorofluoroscein diacetate (DCFH-DA) was prepared in ethanol. Fresh D609 (1 mg/ml) was prepared in phosphate-buffered saline (PBS). The cells were preincubated with D609 for 1 h before Aβ(1–42) was added. In some experiments, fresh media were added after 1-h preincubation of neuronal cells with D609. The protection afforded by D609 was the same whether the xanthate was present for 1 h only or for the entire 24-h period of Aβ(1–42) exposure. Assays for cell viability, protein oxidation, lipid peroxidation, apoptosis and ROS were performed 24 h after Aβ(1–42) treatment as previously described.[6,29,30]

Synthesis of Methylated D609 (MD609)

MD609 was synthesized according to Ref. [31]. In brief, D609 (0.19 mg, 71 μM), dissolved in tetrahydrofuran (THF) (15 ml) was reacted with methyl iodide (0.5 ml, 8.0 mM) under nitrogen at 0°C for 2 h. After 2 h the reaction was warmed to room temperature and stirred for another 2 h to complete the reaction. The reaction was quenched by water (20 ml) followed by extraction of the methylated product with ethyl acetate (3 × 20 ml). The ethyl acetate was removed *in vacuo*, and the remaining yellow liquid (16 mg, 65 μM) was verified as MD609 using ^{1}H NMR.

Determination of Cell Viability

Cortical neuronal cultures were obtained from 18-day-old Sprague–Dawley rat fetuses as described previously[29,30] and were plated in 48-well plates. Neuronal viability was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay. Briefly, 24 h after exposure of cells to Aβ(1–42), MTT stock solution in PBS was added to each well with final concentration of 1.0 mg/ml, and incubated for 1 h. The dark blue formazan crystals formed in intact cells were extracted with 200 μl of dimethyl sulfoxide (DMSO), and absorbance at 595 nm was measured with a microplate reader (Bio-Tek). Results were expressed as the percent of MTT reduction, assuming that the absorbance of control cells was 100%.

Measurement of Protein Carbonyls

Protein carbonyls are an index of protein oxidation and were determined as described previously.[32] Briefly, the cell extract (5 μg of protein) were derivatized 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). Derivatized protein samples were blotted onto nitrocellulose membrane with a slot-blot apparatus (250 ng/lane). Then the membrane was washed with wash buffer [10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20], blocked by incubation in the presence of 5% BSA, followed by incubation with rabbit polyclonal anti-DNPH antibody as primary antibody for 1 h. The membranes were washed with wash buffer and further incubated with alkaline phosphatase (ALP)-conjugated goat anti-rabbit antibody as secondary antibody for 1 h. Blots were developed using Sigma fast tablet (BCIP/NBT) and were quantified using Scion Image (PC version of Macintosh compatible NIH Image) software.

Measurement of 3-nitrotyrosine (3-NT)

Nitrotyrosine content was determined by incubating the sample with Laemmli sample buffer (0.125 M Trizma base, pH 6.8, 4% SDS, 20% glycerol) for 20 min. Then 250 ng of protein were blotted onto the nitrocellulose paper using the slot-blot apparatus and immunochemical methods as described above for protein carbonyls. The mouse anti-nitrotyrosine antibody was used as primary antibody and ALP-conjugated anti-mouse secondary antibody was used for detection. Controls in which the primary antibody was reacted with free 3-NT resulted in no detection of protein-bound 3-NT in Aβ-treated cells (data not shown). Densitometric analysis of bands in images of the blots was used to calculate levels of 3-NT.

Measurement of Lipid Peroxidation and HNE Levels

The thiobarbituric acid reactive substances (TBARS) method was used to assess membrane lipid peroxidation, as described previously.[33] Levels of HNE were quantified by slot-blot analysis as described previously.[34] Anti-HNE antibody raised in rabbit was used as the primary antibody. Controls in which the primary antibody was reacted with free HNE resulted in faint, non-specific binding of the antibody (data not shown). However, since both Aβ(1–42)- and Aβ(1–42) + D609-treated samples used the same antibody, background correction was identical in both samples.

Measurement of Intracellular ROS

Because it is a non-polar diester, DCFH-DA crosses the neuronal membrane, where cytosolic esterases cleave the ester functionality, forming an anion that is trapped within the neuron. Reaction of ROS, especially H_2O_2 or peroxyl free radicals, with DCFH yields fluorescent DCF. The intracellular accumulation of ROS in neuronal cell culture, following 24 h of Aβ(1–42) treatment in the presence and absence of D609, was measured in cells that were rinsed with Krebs' ringer solution (100 mM NaCl, 2.6 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 and 11 mM glucose), and 10 μM DCFH-DA was loaded. After 1 h incubation at 37°C, cells were examined under a confocal fluorescence microscope equipped with an argon laser (λ_{ex} 485 nm, λ_{em} 530 nm).

Analysis of DNA Fragmentation

Cultures were rinsed thrice using PBS, fixed with 4% paraformaldehyde for 10 min at 37°C, rinsed and stained with Hoechst 332584 (1 μg/ml) for 10 min at room temperature. The cells staining were visualized using a fluorescence microscope using a DAPI filter. The nuclear staining with Hoechst 33258 provided a morphological discrimination between normal and apoptotic cells as described by Ref. [35].

Protein Estimation

The amount of protein in the samples was measured by BCA method using bovine serum albumin as standard.[36]

Statistical Analysis

ANOVA was used to assess statistical significance. p-Values <0.05 were considered significant.

RESULTS

Effect of D609 on Cell Toxicity Induced by Aβ(1–42)

As shown in Fig. 1, exposure of neuronal cultures to Aβ(1–42) (10 μM) for 24 h reduced cell viability by 60% ($p < 0.005$). Pretreatment of neurons with D609 for 1 h significantly attenuated Aβ(1–42)-induced cytotoxicity ($p < 0.002$). D609 attenuated Aβ(1–42)-induced cell loss in a dose-dependent manner with maximal effects observed at 50 μM. Thus, this concentration was used in the subsequent experiments. By itself, D609 had no effect on cell viability.

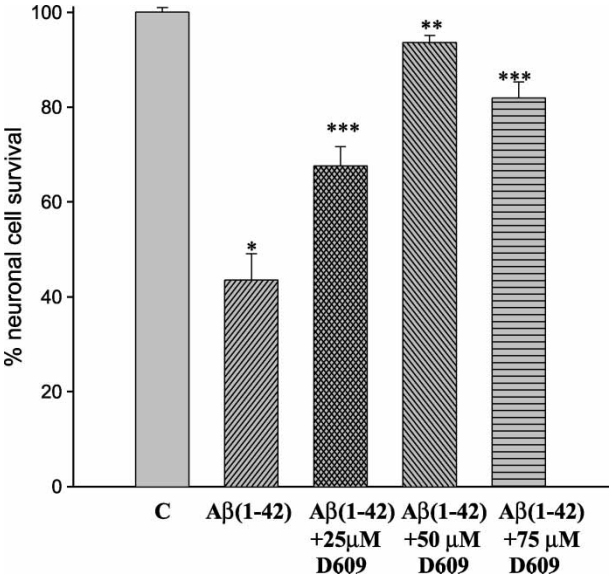

FIGURE 1 Effect of varying concentrations of D609 on cell viability induced by Aβ(1–42) in primary cultured rat cortico-hippocampal neurons. D609 was added to the culture 1 h prior to 10 μM Aβ(1–42) addition and the cells were incubated for 24 h. Cell viability was assessed using MTT reduction. The data are the mean ± SEM expressed as percentage of control values. Statistical comparison was made using ANOVA. ($n = 5$). (*) <0.005, Aβ(1–42) vs. Control; (**) < 0.002, Aβ(1–42) vs. Aβ(1–42) + D609, (***) <0.05.

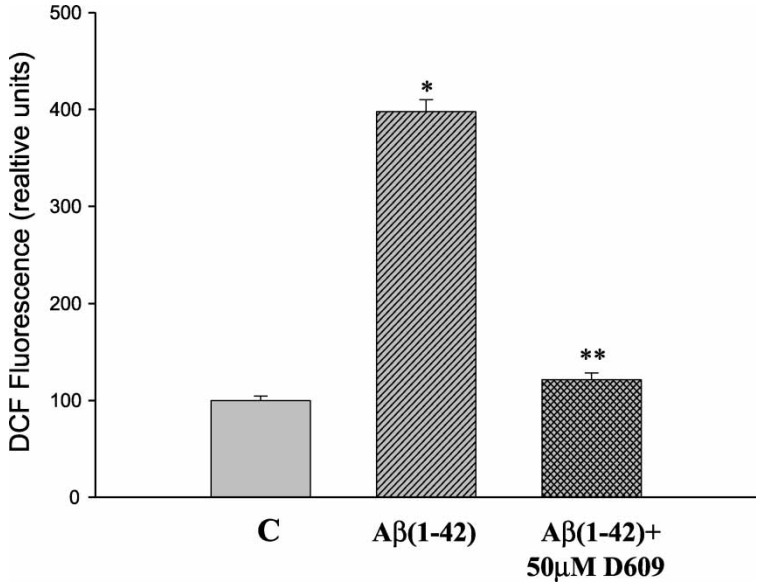

FIGURE 2 D609 prevents Aβ(1–42)-induced intracellular ROS accumulation. ROS levels were determined as described in the "Materials and methods" section. The treatment of cell culture is the same as described in legend of Fig. 1. The data are the mean ± SEM expressed as percentage of control values. Statistical comparison was made using ANOVA. ($n = 5$). (*) <0.005, Aβ(1–42) vs. Control; (**) < 0.002, Aβ(1–42) vs. Aβ(1–42) + D609.

When neurons were treated with the MD609 no significant protection against Aβ(1–42)-induced neurotoxicity was observed (data not shown).

D609 Inhibited Aβ(1–42)-induced Intracellular ROS Formation

Intracellular ROS was measured using cell permeable DCF-DA. Once inside the cell, this dye is hydrolyzed to DCFH by intracellular esterase activity, the subsequent anion prevents its removal from neuron. DCFH interacts with peroxides to form fluorescent 2′,7′-dichlorofluorescein (DCF). Aβ(1–42)-treated neuronal cells displayed increased fluorescence staining with the DCF dye compared to that of control ($p < 0.005$) (Fig. 2). D609 treatment significantly reduced the Aβ(1–42)-induced intracellular ROS accumulation ($p < 0.002$).

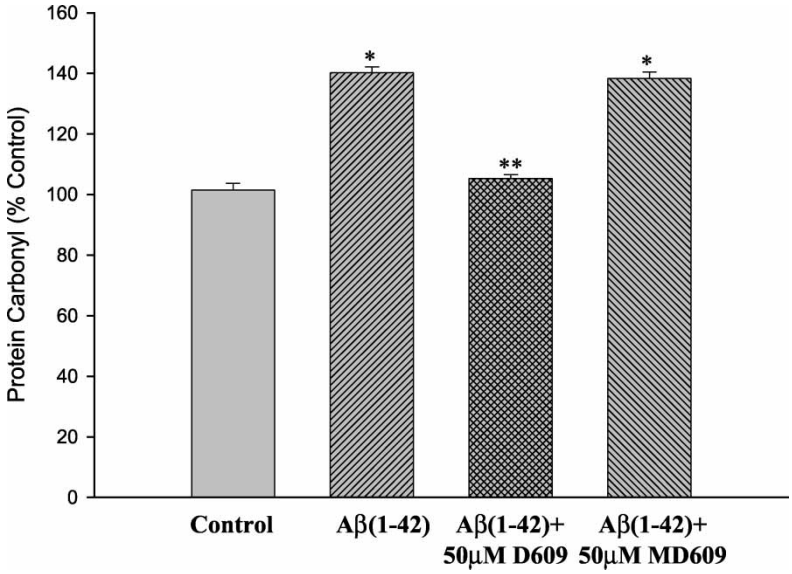

FIGURE 3 Protective effect of 50 μM D609 on Aβ(1–42)-induced protein oxidation (protein carbonyl). Protein carbonyl content was determined as described in the "Materials and methods" section. The treatment of cell culture is the same as described in legend of Fig. 1. The data are the mean ± SEM expressed as percentage of control values. Statistical comparison was made using ANOVA. ($n = 5$). (*) <0.005, Aβ(1–42) vs. Control, MD609 vs. Control; (**) < 0.002, Aβ(1–42) vs. Aβ(1–42) + D609.

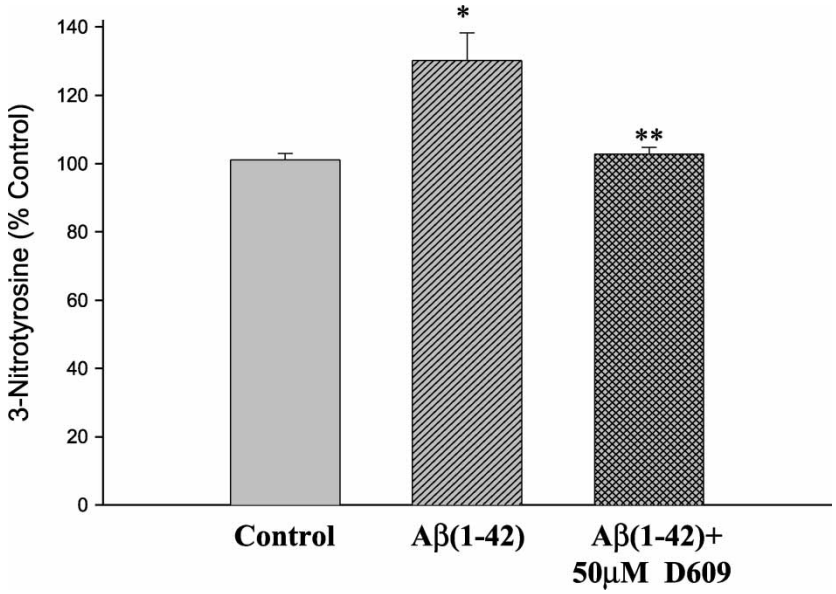

FIGURE 4 Protective effect of 50 μM D609 on Aβ(1–42)-induced 3-NT formation. 3-NT levels were determined as described in the "Materials and methods" section. The treatment of cell culture is the same as described in legend of Fig. 1. The data are the mean ± SEM expressed as percentage of control values. Statistical comparison was made using ANOVA. ($n = 5$). (*) < 0.005, Aβ(1–42) vs. Control; (**) < 0.002, Aβ(1–42) vs. Aβ(1–42) + D609.

D609 Attenuated Aβ(1–42)-induced Protein Carbonyl, 3-NT and Lipid Peroxidation

Figure 3 presents the protein oxidation (protein carbonyl) status in control, Aβ(1–42)-, D609 plus Aβ(1–42)-, and MD609 plus Aβ(1–42)-treated neuronal cells. The level of carbonyls was found to be significantly higher ($p < 0.005$) in Aβ(1–42)-treated cells, consistent with previous studies.[4,37] D609 treatment prior to addition of Aβ(1–42) significantly reduced the level of carbonyl formation ($p < 0.002$). However, MD609 did not protect neurons from Aβ(1–42)-induced protein oxidation (Fig. 3). Figure 4 shows the protective effect of D609 against Aβ(1–42)-induced formation of 3-NT, formed by reaction of RNS with proteins. 3-NT was found to be significantly increased in Aβ(1–42) treated cells ($p < 0.005$), but was significantly reduced by D609 ($p < 0.002$). Aβ(1–42) treatment significantly increased lipid peroxidation

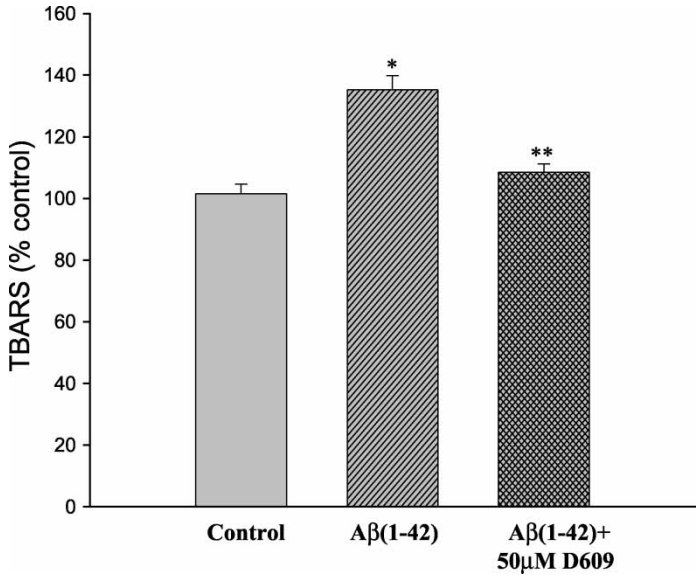

FIGURE 5 Protective effect of 50 μM D609 on Aβ(1–42)-induced lipid peroxidation. Lipid peroxidation assay was performed as described in the "Materials and methods" section. The treatment of cell culture is the same as described in legend of Fig. 1. The data are the mean ± SEM expressed as percentage of control values. Statistical comparison was made using ANOVA. ($n = 5$). (*) < 0.005, Aβ(1–42) vs. Control; (**) < 0.002, Aβ(1–42) vs. Aβ(1–42) + D609.

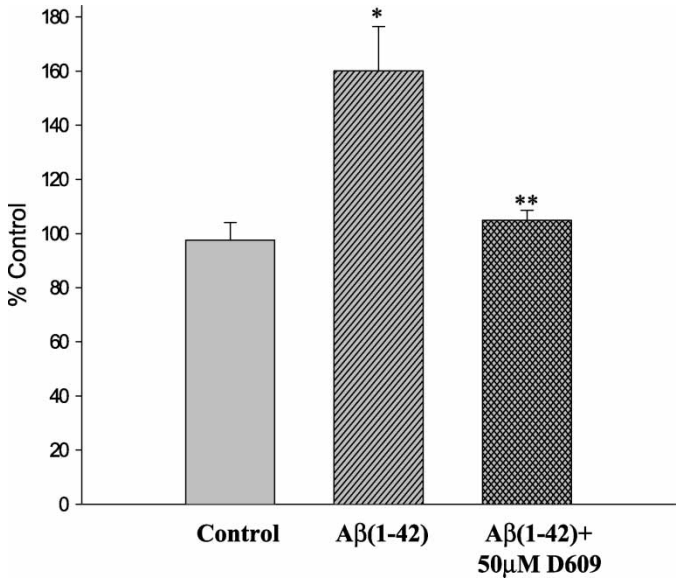

FIGURE 6 Protective effect of D609 on Aβ(1–42)-induced HNE. HNE levels were determined as described in the "Materials and methods" section. The treatment of cell culture is the same as described in legend of Fig. 1. The data are the mean ± SEM expressed as percentage of control values. Statistical comparison was made using ANOVA. ($n = 5$). (*) < 0.005, Aβ(1–42) vs. Control; (**) < 0.002, Aβ(1–42) vs. Aβ(1–42) + D609.

($p < 0.005$) as measured by TBARS (Fig. 5), and D609 pretreatment attenuated Aβ(1–42)-induced lipid peroxidation significantly ($p < 0.002$). Increased levels of HNE, a lipid peroxidation product,[8,34] was observed upon Aβ(1–42) ($p < 0.005$) treatment, confirming previous findings.[34,38] D609 significantly protected the neuronal cells against Aβ(1–42)-induced lipid peroxidation ($p < 0.002$) (Fig. 6). In contrast to these findings and consistent with the lack of protection against Aβ(1–42)-induced protein oxidation (Fig. 3), MD609 did not provide significant protection against Aβ(1–42)-induced lipid peroxidation or ROS, HNE and 3-NT formation (data not shown).

D609 Inhibited Aβ(1–42)-induced Apoptotic Cell Death

Figure 7 shows phase contrast photomicrographs of primary rat neuronal cells treated with 10 μM Aβ with and without 50 μM D609. Aβ-treated neurons (10 μM) demonstrated vacuolated soma and fragmented neurites, membrane blebbings and cell shrinkage. D609 mitigated morphological alterations

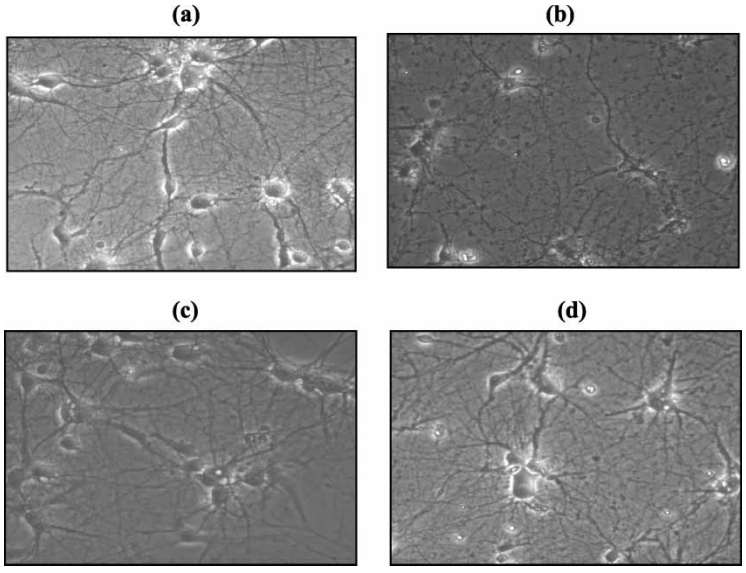

FIGURE 7 Protective effect of D609 on Aβ(1–42)-induced morphological alterations (c). Cells were photographed 24 h after the addition of Aβ(1–42). (a) shows normal neurons with extensive interneuronal connections. Numerous cell bodies in various states of condensation and degeneration are present in Aβ treated cultures (b) and (d) shows neurons treated with Aβ(1–42) + D609. Magnification, 100 ×.

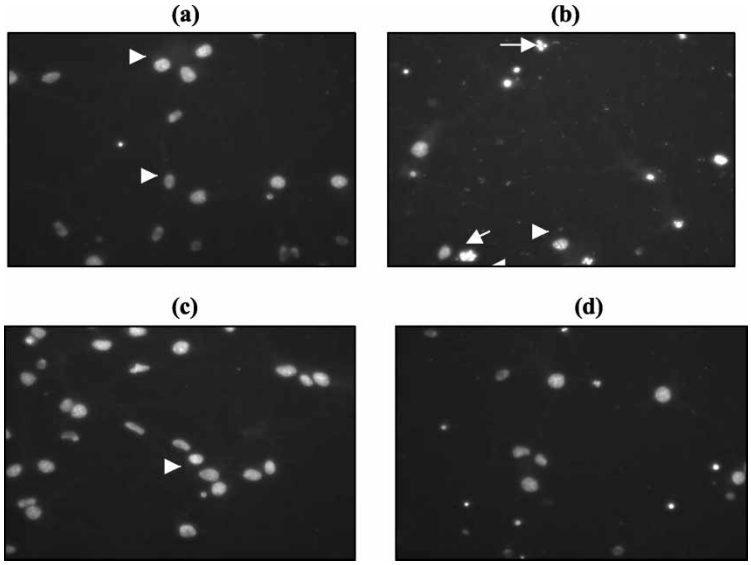

FIGURE 8 D609 protects against Aβ(1–42) induced DNA fragmentation as determined by Hoechst staining. (a) No treatment; (b) treated with Aβ(1–42) for 24 h; (c) D609 alone and (d) cells pretreated with D609 for 1 h + Aβ(1–42).

induced by Aβ(1–42). Hoechst staining was further used for detection of apoptotic bodies inside the cells. Treatment with Aβ(1–42) significantly increased the number of apoptotic bodies, which was reduced by D609 treatment (Fig. 8). MD609 did not protect against morphological alterations and apoptosis induced by Aβ(1–42) (data not shown).

DISCUSSION

Increasing evidence demonstrates that oxidative stress is an important contributor in the pathogenesis of a number of neurodegenerative disorders that involve neuronal degeneration and loss including AD, Parkinson's and Huntington's disease among others.[10] Oxidative stress is caused mainly by an imbalance in antioxidant and oxidant system. A shift towards oxidant levels cause a wide spectrum of cell damage, including protein oxidation, lipid peroxidation and DNA damage.[8,6,39–43]

One of the important non-protein thiols involved in cellular defense against oxidative stress is GSH. In cells, total GSH can be free or bound to proteins. Free GSH is present mainly in its reduced form, which is converted to the oxidized form during oxidative stress, and can be reverted to the reduced form by the action of the enzyme GSH reductase.[44,45] The redox status of neurons depends on the relative amounts of the reduced and oxidized forms of GSH (GSH/GSSG) and appears to be a critical determinant of cell viability.

GSH functions in the detoxification of hydrogen peroxide, other peroxides and free radicals.[46] It has been well established that a decrease of GSH concentration may be associated with aging and the pathogenesis of many diseases, including AIDS, AD, amyotrophic lateral sclerosis. Elevation of GSH may be a promising therapeutic strategy in AD.[47] GSH levels may be altered due to altered enzyme levels involved in GSH metabolism.[48,49] Gu *et al*.[50] provided consistent evidence of altered GSH levels in specific regions of CNS of subjects affected by AD. GSH has been shown to be protective against various oxidative stressors found in AD. One of the substrates for GSH is HNE[9,51] that was found to be increased in AD brain.[52–55] In addition, GSH can protect against peroxynitrite damage.[19,20,56]

Previously, replenishment of GSH has been achieved with GSH monoesters and diesters and with NAC or α-mercaptopropionylglycine.[57,19–22] Further, synthesis of GSH from these precursors requires the active enzymes involved in GSH biosynthesis, and when γ-glutamylcysteine ethyl ester (GCEE) is administered there is a chance of forming 5-oxo-L-proline and L-cysteine by the action of gamma-glutamylcyclotransferase on L-gamma-glutamyl-L-cysteine. D609 has been reported to protect against glutamate toxicity and ionizing radiation-induced oxidative stress in lymphocytes by increasing the intracellular levels of GSH. D609 has been reported to scavenge hydroxyl radical and hydrogen peroxide.[23]

The antioxidant property of D609 is associated with the free thiol group of xanthate.[23] Consistent with this previous observation, here we show that methylation of the free thiol of D609 ameliorated the antioxidant activity as indexed by protein carbonyl, HNE formation, and 3-NT production. Previous studies from our laboratory demonstrated the GSH mimetic function of D609 as evidenced by formation of the dixanthate that is

converted back to the xanthate by GSH reductase analogous to the case with GSH. As shown in the current study, protection of neuronal cells against Aβ(1–42)-induced oxidative stress and neurotoxicity with D609, but not with MD609, suggests that the thiol group of D609 plays an important neuroprotective role against Aβ(1–42). This suggestion is consistent with a protective mechanism of D609 being related to the GSH-mimetic properties of this xanthate.[23]

The results presented here demonstrated that Aβ(1–42) induces 60% reduction in neuronal viability as assessed by the MTT assay (Fig. 1). This finding is consistent with the previous data from our laboratory and others.[3,30,37,58] In addition, pretreatment of neuronal cells with D609 effectively protected neurons against Aβ(1–42)-induced oxidative stress as indexed by decreased protein oxidation and lipid peroxidation and ROS formation. The oxidation of proteins by free radicals may be responsible for damaging enzymes critical in neuronal function.[59] Protein carbonyl, 3-NT and HNE levels were found to be elevated in AD brains.[6–8,34] Previous studies from our laboratory identified oxidatively modified protein in AD brain by proteomics analysis.[41–43,59] Lipid peroxidation products presumably arising from oxidation of polyunsaturated fatty acids[60–62] have been identified in AD brain tissue.[54,63,64] These include F4-isoprostanes,[65] TBARS and reactive aldehyde species,[54,66] such as 4-hydroxynonenal (4-HNE), a highly reactive product of arachidonic acid that is believed to interfere normal cellular functions.[32,62] Neurons undergo apoptosis in response to oxidative stress, an effect that is enhanced after GSH depletion data.[67] Here we showed that treatment of neuronal cells with D609 protected neurons against Aβ(1–42)-induced apoptosis.

That the same degree of neuroprotection was afforded by D609 whether the xanthate was preincubated for 1 h with neurons followed by changing with fresh media or by incubation with neurons for the full 24 h of exposure to Aβ(1–42) is consistent with the notion that this xanthate provides neuroprotection by acting as a GSH mimetic and not by direct inhibition of aggregation of Aβ(1–42). The xanthate D609 is a specific inhibitor of PC-PLC. Thus, while our findings that D609, but not MD609, is protective against the oxidative stress and neurotoxicity associated with Aβ(1–42), are consisted with the GSH-mimetic properties of this xanthate as a principal mechanism of neuroprotection, we cannot completely rule out a role of inhibition of PC-PLC by D609 in these findings. Similarly, since Aβ disrupts Ca^{2+} homeostasis in neurons[68] and the action of PC-PLC may lead to altered Ca^{2+} dynamics, D609 potentially could be protective against oxidative stress in neurons in part by modulating the resulting Ca^{2+} dyshomeostasis.

In conclusion, this study has demonstrated that D609 protects neuronal cells from oxidative stress induced by Aβ(1–42), known to be important in the pathogenesis of AD. Neuronal cells pretreated with D609 displayed significant protection against Aβ(1–42)-induced protein oxidation, lipid peroxidation, ROS production and DNA damage. Based on the data from the present and previous studies,[23] the thiocarboxylic acid group present in D609 may be responsible for its potential antioxidant properties rather than drug-mediated inhibition of Aβ(1–42) aggregation. Thus, the antioxidant properties of D609 conceivably could be beneficial in the treatment of diseases related to oxidative stress. Studies to test this compound in animal models of AD and other conditions associated with oxidative stress are in progress.

Acknowledgements

This research was supported in part by grants from NIH [AG-10836; AG-05119].

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