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Derivatives of Xanthic Acid are Novel Antioxidants: Application to Synaptosomes

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Xanthic acids have long been known to act as reducing agents. Recently, D609, a tricyclodecanol derivative of xanthic acid, has been reported to have anti-apoptotic and anti-inflammatory properties that are attributed to specific inhibition of phosphatidyl choline phospholipase C (PC-PLC). However, because oxidative stress is involved in both of these cellular responses, the possibility that xanthates may act as antioxidants was investigated in the current study. Finding that xanthates efficiently scavenge hydroxyl radicals, the mechanism by which D609 and other xanthate derivatives may protect against oxidative damage was further examined. The xanthates studied, especially D609, mimic glutathione (GSH). Xanthates scavenge hydroxyl radicals and hydrogen peroxide, form disulfide bonds (dixanthogens), and react with electrophilic products of lipid oxidation (acrolein) in a manner similar to GSH. Further, upon disulfide formation, dixanthogens are reduced by glutathione reductase to a redox active xanthate. Supporting its role as an antioxidant, D609 significantly ($p < 0.01$) reduces free radical-induced changes in synaptosomal lipid peroxidation (TBARs), protein oxidation (protein carbonyls), and protein conformation. Thus, in addition to inhibitory effects on PC-PLC, D609 may prevent cellular apoptotic and inflammatory cascades by acting as antioxidants and novel GSH mimics. These results are discussed with reference to potential therapeutic application of D609 in oxidative stress conditions.

Keywords: D609; Xanthates; Glutathione; Antioxidant; Oxidative stress; Synaptosomes

Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; ROS, reactive oxygen species; NAC, *N*-acetyl cysteine; D609, tricyclodecan-9-yl-xanthogenate; PC-PLC, phosphatidyl choline phospholipase C; TA, terephthalic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PBS, phosphate buffered saline; RT, room temperature; TBARs, thiobarbituric acid reactive substances; EPR, electron paramagnetic resonance; Mal-6, 2,2,6,6-tetramethyl-4-maleimidopiperidin-1-oxyl; DNPH, 2,4-dinitrophenyl hydrazine; DNP, 2,4-dinitrophenyl hydrazone

INTRODUCTION

Glutathione (GSH) is a ubiquitously expressed tripeptide (L-γ-glutamyl-L-cysteinylglycine) and, at concentrations of 0.5–10 mM, is one of the most abundant intracellular thiols.[1] Among its many functions,[2] GSH scavenges reactive oxygen species (ROS) and maintains the intracellular redox state. Depletion of GSH is known to be damaging to cells and eventually leads to cell death.[3–7] Because GSH is a major defense against cellular oxidative injury, loss of GSH shifts the oxidant–antioxidant balance in favor of the oxidant, a condition known as oxidative stress.[8,9] Increased ROS generation induces oxidative modification to biomolecules such as proteins, lipids and DNA and results in cellular dysfunction.

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Therefore, maintenance of high intracellular GSH levels is of critical importance to cellular integrity.

Oxidative stress is implicated in a number of human diseases including many neurodegenerative disorders.[10,11] Oxidative damage is rampant in these disorders, and GSH deficiency is evident in Parkinson's disease[12] and correlates to decreased survival among HIV patients.[13] Addition of antioxidant compounds prevents oxidative damage and cell death in several models of neurodegeneration.[14–19] Further, clinical studies utilizing antioxidant therapeutics such as vitamin E or *N*-acetyl cysteine (NAC), a substrate for GSH synthesis, indicate the beneficial effects of these treatments.[13,20] These studies support the role of oxidative stress in these disorders and promote the hypothesis that compounds maintaining the redox state of cells, i.e. maintaining high GSH levels, might be beneficial in these disorders.[21]

Tricyclodecan-9-yl-xanthogenate (D609) is a derivative of xanthic acid[22] that has recently been reported to inhibit phosphatidylcholine-specific phospholipase C (PC-PLC). In doing so, D609 protects cells from ceramide-induced apoptosis and NF-κB-mediated transcription of inflammatory molecules.[23–25] Because oxidative stress is induced in both of these cascades, the antioxidant role of D609 was investigated in the current study. In this investigation, D609 and several D609 analogs were analyzed for their ability to interact with hydrogen peroxide and protect against oxidation-induced changes in synaptosomal lipid and protein oxidation and protein conformation. In addition, based upon the mechanism by which they scavenge free radicals, we propose that xanthates are novel antioxidants which can interact with both hydroxyl radicals and hydrogen peroxide.

MATERIALS AND METHODS

Materials

D609 was purchased from Biomol (Plymouth Meeting, PA) while the other xanthic acid derivatives (i.e. ethyl, isopropyl, cyclohexyl xanthates and methylated D609) were synthesized and purified as described[22] and characterized by NMR and mass spectral analysis. Terephthalic acid (TA) was purchased from Aldrich (Milwaukee, WI). Protease inhibitors used in the isolation buffer were purchased from ICN (Aurora, OH). Nitrocellulose membranes (0.45μm pore size) and transfer filter papers used for slot blotting were purchased from Bio-Rad (Hercules, CA). All other reagents were purchased from Sigma (St. Louis, MO).

Amplex Red-hydrogen Peroxide Assay

The Amplex Red-hydrogen peroxide/peroxidase assay kit (A-22188, Molecular Probes, Eugene, OR) was used to determine the hydrogen peroxide scavenging capabilities of D609. The enzyme-catalyzed oxidation of Amplex Red results in the formation of the fluorecent resorufin. A standard curve for hydrogen peroxide (0, 1, 2, 3, 4, 5, 10 and 25 μM) was performed concurrently with the D609 samples. D609 (25, 50, 75, 100 μM) was incubated with 10 μM hydrogen peroxide for 30 min at room temperature after the addition of the amplex red/horseradish peroxidase mixture. D609 alone did not change the fluorescence of resorufin. The fluorescence of resorufin was detected ($\lambda_{ex} = 563$ nm, $\lambda_{em} = 587$ nm) with a SpectraMax Gemini XS (Molecular Devices, Sunnyvale, CA) fluorescence plate reader.

Detection of Thiol Groups with 5,5′-Dithiobis (2-nitrobenzoic Acid) (DTNB)

DTNB is a commonly used reagent for the detection of thiol groups. DTNB was used in this assay to detect the presence of the thiol group of the xanthate derivatives, before and after UV-irradiation in the presence of H_2O_2. GSH was also tested as a positive control. Xanthates and GSH were prepared as 0.5 mM solutions in water. H_2O_2 and DTNB were prepared as 1 mM solutions in PBS. Xanthates (50 μM) or GSH (50 μM) were irradiated in the presence of 100 μM H_2O_2 in the microplate as indicated above. After 1 min of irradiation, a second set of samples were added to the plate and diluted exactly as the first set. DTNB was added in excess (200 μM) and the resulting absorbance at $\lambda = 412$ nm was monitored with a PowerwaveX (Biotek, Winooski, VT) absorbance plate reader.

To test the possibility that oxidized xanthates might be reduced by GSH reductase in a similar manner to oxidized GSH, the method of Griffith[26] was followed with slight modifications. After irradiation of 100 μM xanthates and GSH in the presence of 100 μM H_2O_2, 0.05 Units of GSH reductase were added to samples in a final volume of 200 μL. NADPH (200 μM) was added as a cofactor to the enzyme. DTNB (600 μM) reactivity of the samples was then monitored over time (13 min) at $\lambda = 412$ nm.

To monitor the reaction of the xanthates and GSH with acrolein, equimolar concentrations (100 μM) were incubated at RT for 15 min in a 96-well plate. DTNB reactivity was monitored as indicated above.

Preparation of Synaptosomes and Oxidative Treatment

Synaptosomes were prepared as previously described.[27] Briefly, cortices were isolated from

3 to 4 month old gerbils and homogenized by 12 passes with a motorized teflon pestle in isolation buffer (0.32 M sucrose, 4 μg/ml leupeptin, 4 μg/ml pepstatin, 5 μg/ml aprotinin, 20 μg/ml trypsin inhibitor, 0.2 mM PMSF, 2 mM EDTA, 2 mM EGTA and 20 mM HEPES). Homogenate was centrifuged at 3800 rpm (1500*g*) for 10 min at 4°C. Supernatant was removed and centrifuged at 14,800 rpm (20,000*g*) for 10 min at 4°C. The resulting pellet was mixed in isolation buffer and layered onto discontinuous sucrose gradients [10 ml each of 1.18, 1.0 and 0.85 M sucrose solutions each with 2 mM EDTA, 2 mM EGTA and 10 mM HEPES (pH 8.0 for 0.85 and 1.0 M solutions, pH 8.5 for 1.18 M solution)]. The gradients were centrifuged in a Beckman L7-55 ultracentrifuge at 22,000 rpm (82,500*g*) for 2 h at 4°C. Synaptosomes were removed from the 1.18 M/1.0 M interface, washed twice in PBS and the protein concentrations determined by the Pierce BCA method.

Fenton chemistry involves the reaction of H_2O_2 in the presence of a reduced redox-active metal ion, such as Fe^{2+}, generating hydroxyl radicals as a product. This reaction has been utilized previously to initiate the oxidation of lipid and protein components of synaptosomes.[28] Oxidation of synaptosomes was performed in the presence and absence of GSH or xanthate derivatives to determine protective effects of these compounds in the TBARs, EPR and protein carbonyl assays. Samples were oxidized with 50 μM Fe^{2+} and 1 mM H_2O_2 for 1 h at 37°C from stocks of 100 μM Fe^{2+} and 100 mM H_2O_2 prepared in PBS.

Synaptosomes (4 mg/ml) were treated with 50 nM acrolein in the presence or absence of 100 μM D609 for 30 min at room temperature. Samples were then analyzed for protein carbonyl content, using the immuno-slot blotting method.

Thiobarbituric Acid Reactive Substances (TBARs)

TBARs were determined in synaptosomes following treatment with 50 μM Fe^{2+} and 1 mM H_2O_2 for 1 h at 37°C. Aliquots of 250 μg protein were taken from each sample and precipitated with 0.4 ml of ice cold 10% TCA. Samples were centrifuged for 5 min at 6000 rpm (3000*g*), and 0.4 ml of the supernatant were incubated with 0.2 ml thiobarbituric acid (0.335% TBA in 50% glacial acetic acid) for 1 h at 100°C. Samples were cooled to RT before the addition of 0.4 ml of butanol. After mixing each sample with a pipette, the organic layer was allowed to separate from the aqueous layer, and 100 μl was immediately removed from the top organic phase and added to a 96 well plate. TBARs were detected by measuring their fluorescence ($\lambda_{ex} = 518$ nm and $\lambda_{em} = 588$ nm). Separate experiments demonstrated that there was no interference of D609 alone with the TBARS reagents or assay.

Spin Labeling and Electron Paramagnetic Resonance (EPR)

Mal-6, a protein-specific spin label, was used for the determination of oxidative stress-induced protein conformational changes in synaptosomal membranes. The label was dissolved in 100 μl of acetonitrile and diluted to a final concentration of 200 μM in 50 ml of lysing buffer (2 mM EDTA, 2 mM EGTA, 10 mM HEPES, pH 7.4). Labeling of synaptosomal proteins was achieved by incubating 12.5 μg Mal-6/mg of protein (50μM final concentration) overnight at 4°C.[29] Prior to EPR studies, synaptosomes were washed with lysing buffer to remove unbound spin label. Synaptosomes were pelleted by centrifugation, and the supernatant was discarded and replaced with fresh lysing buffer. After mixing, the cycle was repeated six times to ensure complete removal of all unbound spin label.

EPR spectra were acquired on a Bruker EMX spectrometer with the following instrumental parameters: microwave power, 20 mW; microwave frequency, 9.77 GHz; modulation amplitude, 0.3 G; modulation frequency, 100 kHz; receiver gain, 1×10^5; time constant, 1.28 ms.

Analysis of Protein Oxidation by Immuno-slot Blotting

An increase in DNPH-reactive sites on proteins, i.e. protein carbonyls, is indicative of protein oxidation.[30] To determine if protein oxidation was ameliorated in the presence of D609, sample aliquots (30–50 μg protein) were incubated with dinitrophenylhydrazine (DNPH) in the presence of SDS for 20 min at RT prior to neutralization. Samples with a concentration of 1 ng/μl were prepared, and 250 ng of protein were filtered under vacuum onto nitrocellulose. The membrane was subsequently blocked with PBS containing 3% BSA for 1 h at RT. The dinitrophenylhydrazone (DNP) adducts of synaptosomal proteins were determined by immunodetection using a rabbit antibody specific for DNP-protein adducts (1:150). Following a 1 h incubation with primary antibodies, membranes were washed and then incubated for 1 h with an alkaline phosphatase-conjugated secondary antibody (goat anti-rabbit, 1:15,000). SigmaFast was used as the colorimetric substrate for alkaline phosphatase. Blots were scanned into Adobe Photoshop and quantitated with Scion Image (PC version of Macintosh compatible NIH Image).

Statistical Analysis

ANOVA was used for the statistical evaluation of data and, except where indicated, significance is equivalent to $p < 0.01$.

RESULTS

D609 Interacts with Hydrogen Peroxide

D609 has been reported to be protective in models of apoptosis and inflammation,[4,23–25] two cellular responses that involve ROS generation. Consequently, studies were conducted to determine the ROS scavenging capabilities of D609 and several xanthate analogs (Fig. 1). Previous data[31] has shown that derivatives of xanthic acid scavenge hydroxyl radicals. D609 also interacts directly with hydrogen peroxide. When D609 was incubated with 10 μM H_2O_2, fluoresence of resorufin was decreased with increasing concentrations of D609 (Fig. 2). This suggests that D609 interacted with H_2O_2, preventing H_2O_2 from acting as a substrate for horseradish peroxidase. At 100 μM D609, H_2O_2 was virtually undetectable (Fig. 2), suggesting that D609 had scavenged H_2O_2.

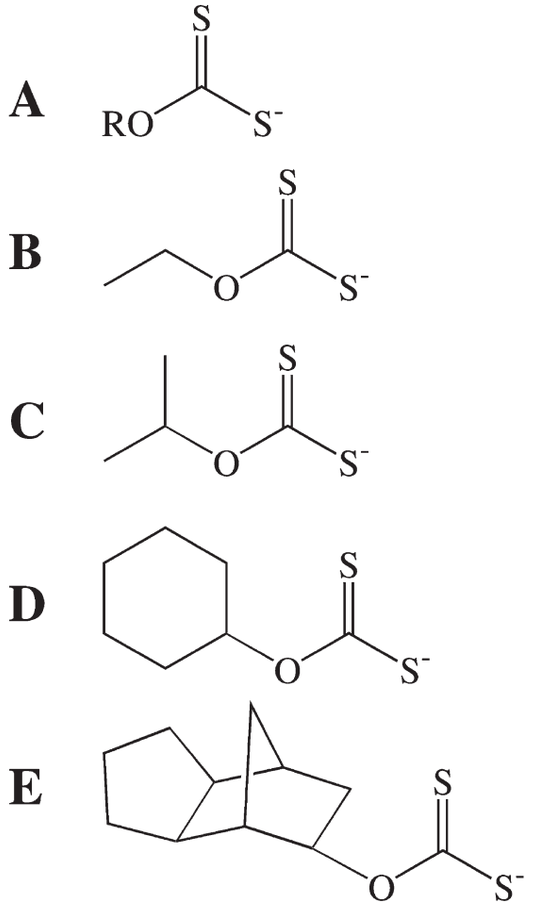

FIGURE 1 Structures of (A) a generic xanthate, where R = structure of the starting alcohol, and the (B) ethyl, (C) isopropyl, (D) cyclohexyl and (E) D609 xanthate structural analogs used in this study.

Xanthates Mimic Glutathione

Glutathione is well-known for its protection against cellular oxidative damage.[1] A cysteine-containing tri-peptide, GSH utilizes the sulfur atom of its cysteine residue for its molecular mechanism of protection, i.e. radical scavenging and nucleophilic attack of reactive products of lipid peroxidation. Because xanthates also contain a free sulfur atom, studies were designed to investigate GSH mimicry by the xanthates.

DTNB is a reagent widely used for the specific detection of free thiol groups. GSH reacts readily with DTNB to produce a strong absorbance at $\lambda = 412$ nm (Fig. 3A). However, oxidized GSH (GSSG) is unreactive toward DTNB. Derivatives of xanthic acid exhibit a similar pattern of DTNB reactivity. The xanthates used in this study also react with DTNB and show decreased DTNB reactivity after hydroxyl radical generation. This pattern of reactivity suggests disulfide bond formation by both GSH and the xanthates and is consistent with previous reports of disulfide bond formation by xanthates upon oxidation.[22] Further, upon oxidation to its disulfide (dixanthogen), there is a change in the UV-spectral profile of D609 (Fig. 3B) that is consistent with dixanthogen formation.[22]

GSSG is recycled to the reduced GSH by glutathione reductase.[26] In this manner, cells that are vulnerable to oxidative damage (e.g. neurons) have a renewable source of antioxidant protection. Because of hydroxyl radical-induced disulfide formation by the xanthates (i.e. dixanthogens), the potential reduction of the dixanthogens by GSH reductase was analyzed. Similar to GSSG, dixanthogen reactivity with DTNB increases with time in the presence of GSH reductase. The enzyme reduced all of the dixanthogen analogs studied (data not shown); however, among these xanthates, GSH reductase increased DTNB reactivity of the irradiated D609 the most (Fig. 4). GSH, which was also assayed as a positive control, was well off-scale in the amount of time used to assay the xanthates (data not shown). Taken together, these data suggest that upon oxidation and subsequent disulfide formation, the xanthic acid analogs studied can be reduced by GSH reductase into an active form.

By acting as a nucleophile, GSH can react with electrophilic aldehydes that are produced by the free radical-mediated oxidation of lipids.[21,32] Thus, GSH protects cells in two ways: free radical scavenging and removal of accessible lipid peroxidation products. Since the xanthates used in this

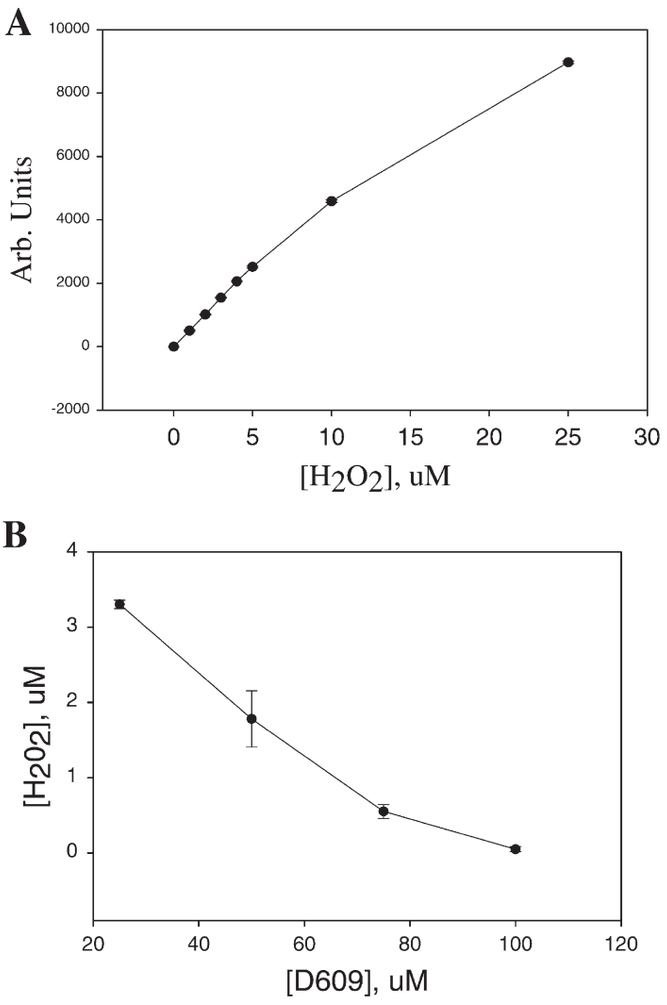

FIGURE 2 The ability of D609 to react with hydrogen peroxide was examined using the Amplex Red Hydrogen Peroxide assay (Top). A H_2O_2 standard curve (0, 1, 2, 3, 4, 5, 10, 25 μM) was performed. D609 (25, 50, 75, 100 μM) were incubated with 10 μM H_2O_2 with Amplex Red/horseradish peroxidase reagent for 30 min at room temperature in a black 96 well microtiter plate. The plate was protected from light during incubation and fluorescence determined ($\lambda_{ex} = 563$, $\lambda_{em} = 587$) (Bottom). Using, Beer's law, the H_2O_2 standard curve was used to calculate hydrogen peroxide remaining after co-incubation with D609.

study both scavenge radicals and form disulfide bonds in a similar manner to GSH, the reactions of D609 and GSH with acrolein, a toxic product of lipid peroxidation,[33] were monitored. If nucleophilic addition to the aldehyde by the free thiol of GSH or D609 occurred, a decrease in DTNB reactivity would result. Incubation of D609 with an equimolar concentration of acrolein for 15 min at RT resulted in a significant decrease ($p < 0.01$, 48%) in DTNB reactivity (Fig. 5) while the DTNB reactivity of GSH was completely eliminated (data not shown). This suggests that D609 is capable of detoxifying aldehydic products of lipid peroxidation by a mechanism similar to GSH, however, not with the same efficacy as the latter. D609 (100 μM) was also capable of significantly decreasing protein carbonyl formation ($p < 0.01$) caused by a physiologically relevant acrolein concentration (50 nM)[34] in synaptosomal membranes (data not shown).

Xanthates Prevent TBRAs Formation in a Structure-dependent Manner

TBARs are formed by free radical-mediated oxidation of unsaturated lipids[35] and are increased in oxidative-stress related disorders.[36] Because the xanthates used in this study scavenge hydroxyl radicals and hydrogen peroxide, their ability to prevent the oxidation of lipids was analyzed. As shown in Fig. 6, treatment of synaptosomes with 50μM Fe^{2+} and 1 mM H_2O_2 for 1 h at 37°C initiated lipid oxidation and significantly increased TBARs

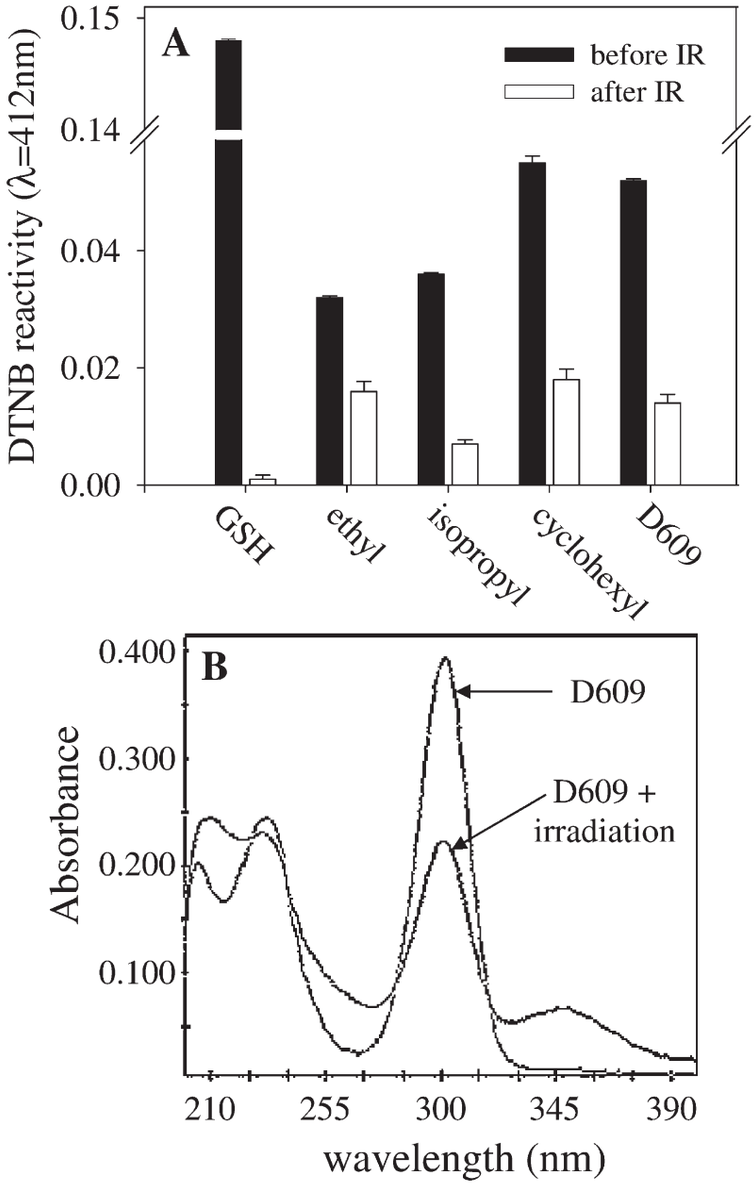

FIGURE 3 Derivatives of xanthic acid form disulfide bonds after scavenging hydroxyl radicals. (A) Before irradiation, xanthates contain a free thiol moiety that reacts with DTNB. However, after 1 min of UV-irradiation in the presence of 100μM H_2O_2, 50μM of each xanthate becomes less reactive to DTNB. These results mimic the reactivity of GSH toward DTNB and suggest that the xanthates form disulfide bonds upon radical scavenging in a manner similar to GSH. Data points are means and SEM from $n = 3$ replicate wells, from a single plate, and are typical of multiple assays. (B) The UV spectrum of D609 changes after 1 min of UV-irradiation in the presence of 100μM H_2O_2. A change in the UV-absorbtion profile of xanthates is consistent with disulfide bond formation.

($p < 0.01$, represented by dotted line). Incubation of the xanthate analogs in synaptosomes for 15 min prior to oxidation results in a dose- and structure-dependent inhibition of TBARs formation. At concentrations of 75 and 100μM, cyclohexyl xanthate significantly decreases TBARs ($p < 0.01$) while D609 eliminates the formation of TBARs at all of the concentrations tested. The ethyl- and isopropyl-xanthates, the least lipid soluble of the xanthates tested, followed the response of GSH and did not prevent TBARs formation at any concentrations.

D609 Protects Against Oxidative Stress-induced Protein Damage

Mal-6 is a protein-specific spin label that binds predominantly to thiol residues.[29] The protein-bound label can be classified into two environments with respect to the motion of the spin label: weakly (W)- and strongly (S)-immobilized. The motion of

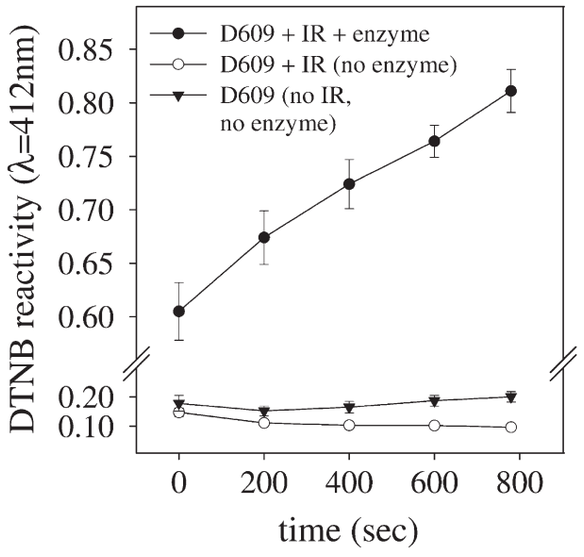

FIGURE 4 The disulfide formed upon oxidation of D609 is recycled by GSH reductase to a DTNB reactive species. Irradiation of 100μM D609 in the presence of 100μM H_2O_2 results in disulfide formation and minimal reactivity toward DTNB. However, when GSH reductase is added after irradiation, DTNB reactivity increases with time indicating the release of a thiol containing species. In the absence of enzyme, the DTNB reactivity of both D609 and that of the irradiated D609 remains constant with time. Data points are means and SEM from $n = 4$ replicate wells, from a single plate, and are typical of multiple assays.

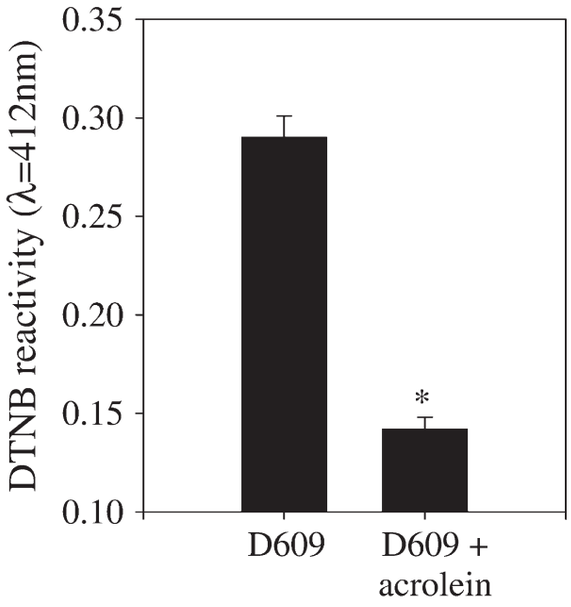

FIGURE 5 D609 scavenges acrolein, an α,β unsaturated aldehydic product of lipid peroxidation. D609 and GSH were incubated with equimolar concentrations of acrolein for 15 min before adding DTNB. Decreased DTNB reactivity results from the loss of thiol availability after a nucleophilic addition to acrolein. GSH reactivity toward DTNB was eliminated after incubation with acrolein. Data points are means and SEM from $n = 6$ replicate wells, from a single plate, and are typical of multiple assays (*$p < 0.01$; ANOVA).

Mal-6 bound to W sites is weakly restricted, and is manifested as narrow lines in the EPR spectrum. Alternatively, Mal-6 bound to S sites have strongly hindered motion, which results in broadened lines in the EPR spectrum. The resulting intensities of the respective W and S peaks of the $M_I = +1$ low field resonance lines yield the W/S ratio, a parameter that is highly sensitive to protein conformational changes.[29] Decreased values of the W/S ratio, which reflect an overall decreased motion of spin-labeled sites on proteins, arise from increased inter- and intra-molecular protein interactions, decreased segmental motion of spin labeled proteins, protein–protein crosslinking, or changes in the structure of the lipid bilayer.[29,37] Further, the W/S ratio is consistently lower in synaptosomes that are oxidized.[14,15,28,29,38,39] Oxidation of synaptosomes with 50μM Fe^{2+} and 1 mM H_2O_2 for 1 h at 37°C results in a significant decrease ($p < 0.01$, 21%) in the W/S ratio (Fig. 7). However, a 15 min incubation of 50 μM D609 with synaptosomes prior to oxidative insult results in a partial preservation of protein conformation. In the presence of D609, the change in W/S is significantly different ($p < 0.01$, 12%) than the change observed in untreated and oxidized samples. GSH (50 μM) had no protective effect against the oxidation-induced protein conformational changes.

Protein carbonyls are markers of protein oxidation[30] and their levels increase in various paradigms of oxidative injury.[14,15,28,29,38] Synaptosomal oxidation with 50 μM Fe^{2+} and 1 mM H_2O_2 for 1 h at 37°C resulted in a significant increase ($p < 0.01$) in the levels of protein carbonyls (Fig. 8). Measurement of protein carbonyls in oxidized samples that were pretreated with 50μM D609 resulted in a significant decrease ($p < 0.01$) in the levels of protein oxidation. However, consistent with the EPR results, 50μM GSH did not prevent the oxidation of proteins as determined by levels of protein carbonyls.

DISCUSSION

The results presented in this study suggest that derivatives of xanthic acid are good reducing agents and are capable of protection against neuronal oxidative stress. For example, the xanthates studied efficiently scavenge hydroxyl radicals generated in a cell-free environment. Although the *R*-groups of the various xanthates studied differ in structure (Fig. 1), the xanthate analogs maintain similar hydroxyl radical scavenging capabilities in solution. This structure-independent radical scavenging suggests that the xanthic acid functionality is responsible for this phenomenon and not the hydrocarbon side

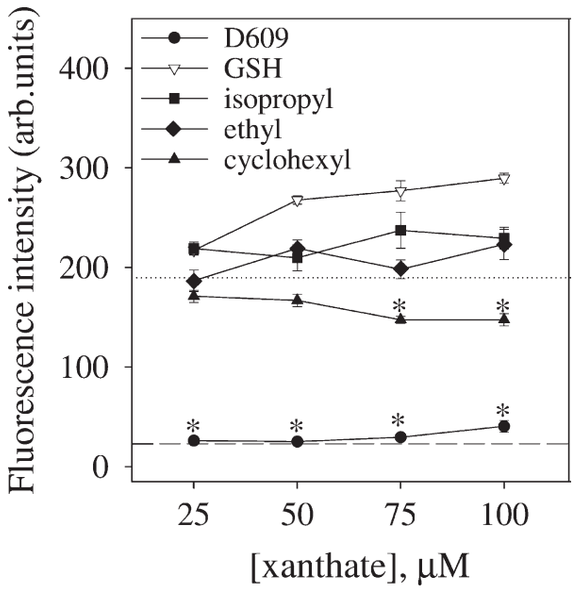

FIGURE 6 Xanthic acid derivatives prevent the formation of TBARS in synaptosomes in a structure dependent manner. Synaptosomes incubated with or without increasing concentrations of xanthic acid derivatives or GSH were treated with 50μM Fe^{2+} and 1 mM H_2O_2 for 1 h at 37°C. Increasing numbers of hydrocarbons in the side chain of each xanthic acid derivative increased the protective effect that was observed. Compared to oxidized control samples (represented as the dotted line), the protection against TBARs formation afforded by D609 was significant even at the lowest concentration tested, while the cyclohexyl xanthate protected against TBARs formation at higher doses. However, similar to the xanthates with a low number of side-chain hydrocarbons, GSH had no protective effect on TBARs formation. Data points are means and SEM from $n = 3$ synaptosomal preparations, assayed in duplicate or triplicate in 96 well plates ($^*p < 0.01$; ANOVA).

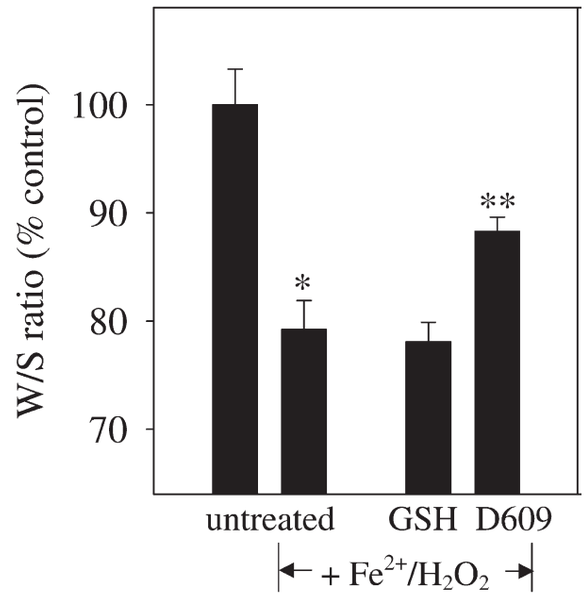

FIGURE 7 D609 decreases oxidative stress-induced synaptosomal protein conformational changes. Synaptosomes incubated with or without D609 or GSH were treated with 50μM Fe^{2+} and 1 mM H_2O_2 for 1 h at 37°C, and then labeled with Mal-6. Changes in the motion of Mal-6 spin labeled proteins are determined by changes in the W/S ratio and indicate changes in protein conformation. D609 significantly prevented oxidative stress-induced decreases in the W/S ratio, while GSH had no effect. Data points are means and SEM from $n = 8$ synaptosomal preparations ($^*p < 0.01$ compared to untreated and unoxidized control, $^{**}p < 0.01$ compared to untreated and oxidized control; ANOVA).

chain (*R*-group). Consistent with this notion the methylated-D609 failed to scavenge radicals. A structure-dependent prevention of TBARs formation in synaptosomal membranes was found. The larger and more hydrophobic R-groups prevented TBARs formation whereas the smaller side chains did not. While maintaining complete solubility in aqueous solutions, the larger and more hydrophobic side chains likely increase partitioning into the synaptosomal lipid bilayer. In this manner, xanthates with larger *R*-groups such as D609 may prevent the oxidation of lipids and may be beneficial in disorders where products of lipid oxidation play a role.

Previously we have demonstrated that D609 inhibits the formation of a PBN spin adduct generated by Fenton chemistry,[31] suggesting that D609 is a hydroxyl radical scavenger. However, the Amplex Red assay (Fig. 2) suggests that D609 may in addition be acting by reacting with hydrogen peroxide. Indeed, others have recently reported that D609 is also capable of scavenging H_2O_2.[40] These findings suggest that the novel antioxidant D609 may function not only as a hydroxyl radical scavenger but also a H_2O_2 scavenger.

Similar to GSH, xanthates form disulfide bonds upon oxidation.[22] In the present study, the UV spectrum of D609 changes after hydroxyl radical attack, consistent with the formation of the disulfide. Further, we demonstrated that xanthates react with DTNB, a thiol reagent, in a manner similar to GSH. Upon scavenging hydroxyl radicals generated by the UV-irradiation of H_2O_2, the DTNB reactivity of the xanthates is decreased, much like GSH (Fig. 3). However, the DTNB reactivity of unoxidized xanthates is much milder than that of GSH, and the formation of disulfide after oxidation does not seem to be complete as is the case with GSH. This is likely a function of resonance between the two sulfur atoms of xanthic acid. Distribution of the electron density between the two xanthic acid sulfur atoms would make either of the sulfurs only mildly nucleophilic. This is in contrast to GSH in that greater (i.e. not shared) electron density on its lone sulfur atom results in better reactivity with electrophiles. This not only explains the differences in initial DTNB reactivity between GSH and the xanthates, but also

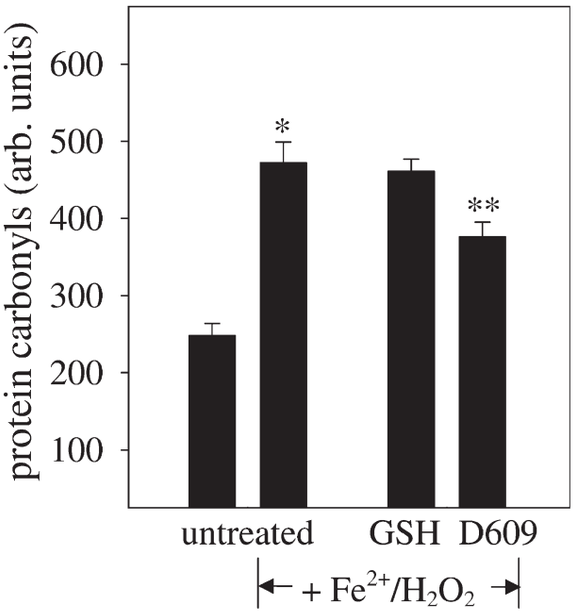

FIGURE 8 D609 decreases synaptosomal protein oxidation that occurs during incubation with Fe^{2+} and H_2O_2. Synaptosomes incubated with or without D609 or GSH were treated with 50 μM Fe^{2+} and 1 mM H_2O_2 for 1 h at 37°C, and the resulting protein carbonyls were determined. D609 significantly reduced the amount of protein carbonyls formed in synaptosomes after oxidative treatment, while the effect of GSH was insignificant. Data points are means and SEM from $n = 4$ synaptosomal preparations, assayed in triplicate (*$p < 0.01$ compared to untreated and unoxidized control, **$p < 0.01$ compared to untreated and oxidized control; ANOVA).

the differences in reactivity toward the electrophilic lipid peroxidation product acrolein (Fig. 5).

An unpaired electron resulting from hydroxyl radical-induced oxidation of a xanthate would also resonate between the two sulfur atoms of xanthic acid. This resonance would result in a much more stable radical than would be the case with a lone sulfur atom of GSH. Thus, upon oxidation of the xanthates and GSH by hydroxyl radicals, the xanthates still maintain some DTNB reactivity, whereas the DTNB reactivity of GSH is eliminated. Because xanthates may be more stable radicals than GSH, it would follow that xanthates would be better hydroxyl radical scavengers than GSH.

Based upon the radical scavenging and disulfide bond formation by the xanthates, we propose that xanthate derivatives mimic GSH. Because GSH reductase is responsible for the recycling of GSSG to GSH, the possibility that this enzyme also reduces dixanthogens was analyzed. Interestingly, in the presence of NADPH, GSH reductase increased the DTNB reactivity of D609, albeit much more slowly than the reduction of GSSG to GSH. Nonetheless, this result suggests that dixanthogens may be recycled to a redox active xanthate when GSH reductase is present. Therefore, D609 has the potential to substitute for GSH as a protector from intracellular ROS while maintaining GSH in the reduced state. In fact, treatment of lymphocytes with D609 inhibits radiation-induced losses of intracellular levels of reduced GSH.[31] In addition, D609 restores glutamate-induced depletion of GSH levels in a hippocampal cell line while preventing cell death.[4] Thus, as a GSH mimic, administration of D609 may protect against cellular ROS while maintaining GSH in the reduced state.

The shift in redox status of the cell following GSH depletion has been shown to induce neuron death.[3–7] Mitochondrial control of apoptosis is coupled to its redox status,[41] and activation of the mitochondrial permeability transition is associated with a decrease in matrix GSH.[42] Inhibition of GSH synthesis adversely affects mitochondria and results in losses of electron transport chain activities and increases in ROS generation[6,43] and induces morphological changes.[44] Further, decreased GSH sensitizes neuronal systems to such toxins as copper[7] and peroxynitrite.[45] Alternatively, the upregulation of GSH synthesis[13,46–48] or the overexpression of antioxidant biomolecules[16,47,49,50] protects against oxidative damage. Thus, compounds that mimic the protective action of GSH may also be protective. Analysis of synaptosomes treated with Fe^{2+} and H_2O_2 indicate that increases in lipid and protein oxidation could be attenuated with D609, but not GSH. This effect may be due to the partial lipid solubility of D609 and partial insolubility of GSH. Protection of synaptosomes with the GSH mono-ethyl ester has been demonstrated,[38,39,51,52] but not the acidic form of GSH. Also, because GSH is found at relatively high concentrations (e.g. 0.5–10 mM) within cells, another explanation for the lack of protection by GSH might be the low concentration (50 μM) of GSH used in these experiments.

The activity of PC-PLC has been implicated in several cellular processes including apoptosis and inflammation. PC-PLC hydrolysis of phosphatidyl choline releases diacylglycerol, which, in turn, activates acidic sphingomyelinase. D609 is reported to prevent apoptosis and inflammation by specifically inhibiting PC-PLC[4,23–25] resulting in the suppression of ceramide production by acidic sphingomyelinase. However, ROS are thought to play major roles in both of these events. For example, ceramide directly effects the mitochondrial electron transport chain resulting in ROS production,[53] and NF-κB, a protein transcription factor required for transcription of pro-inflammatory molecules, is activated in response to ROS.[18,54,55] Thus, by acting as a GSH mimic and free radical scavenger, it is plausible that D609 could prevent apoptosis and inflammation by regulating ROS in addition to inhibiting PC-PLC. In fact, it has been shown that radiation-induced activation of NF-κB is

significantly attenuated in the presence of D609.[31] Similarly, known antioxidants such as NAC and pyrrolidinedithiocarbamate prevent apoptosis and inflammation,[18] supporting an antioxidant mechanism of D609. It would be interesting to determine the effects of other derivatives of xanthic acid on PC-PLC activity.

The importance of maintaining intracellular levels of GSH and the redox status of the cell is clear. Elevation of GSH in disorders where neurodegeneration exists and oxidative stress is evident has proven to be beneficial. This report demonstrates that xanthic acids mimic GSH and suggests the possible use for these compounds in regulating GSH levels in oxidative stress-related models of neurodegeneration. Prevention of oxidative damage to the synapse, an area of the neuron where degeneration is likely to initiate,[56,57] is also critical. It was also demonstrated that D609 protects the synaptosome, a functional model of the synapse,[58] from free radical-induced injury. The results of this current investigation suggest that D609 or other xanthate analogs might also be effective in preventing neurodegeneration or other diseases where oxidative stress plays a role.

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