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Original Contribution

In vivo administration of D609 leads to protection of subsequently isolated gerbil brain mitochondria subjected to in vitro oxidative stress induced by amyloid beta-peptide and other oxidative stressors: Relevance to Alzheimer's disease and other oxidative stress-related neurodegenerative disorders

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Abstract

Tricyclodecan-9-yl-xanthogenate (D609) has in vivo and in vitro antioxidant properties. D609 mimics glutathione (GSH) and has a free thiol group, which upon oxidation forms a disulfide. The resulting dixanthate is a substrate for glutathione reductase, regenerating D609. Recent studies have also shown that D609 protects brain in vivo and neuronal cultures in vitro against the potential Alzheimer's disease (AD) causative factor, Aβ(1–42)-induced oxidative stress and cytotoxicity. Mitochondria are important organelles with both pro- and antiapoptotic factor proteins. The present study was undertaken to test the hypothesis that intraperitoneal injection of D609 would provide neuroprotection against free radical-induced, mitochondria-mediated apoptosis in vitro. Brain mitochondria were isolated from gerbils 1 h post injection intraperitoneally (ip) with D609 and subsequently treated in vitro with the oxidants Fe^{2+}/H_2O_2 (hydroxyl free radicals), 2,2-azobis-(2-amidinopropane) dihydrochloride (AAPH, alkoxyl and peroxyl free radicals), and AD-relevant amyloid β-peptide 1–42 [Aβ(1–42)]. Brain mitochondria isolated from the gerbils previously injected ip with D609 and subjected to these oxidative stress inducers, in vitro, showed significant reduction in levels of protein carbonyls, protein-bound hydroxynonenal [a lipid peroxidation product], 3-nitrotyrosine, and cytochrome *c* release compared to oxidant-treated brain mitochondria isolated from saline-injected gerbils. D609 treatment significantly maintains the GSH/GSSG ratio in oxidant-treated mitochondria. Increased activity of glutathione S-transferase, glutathione peroxidase, and glutathione reductase in brain isolated from D609-injected gerbils is consistent with the notion that D609 acts like GSH. These antiapoptotic findings are discussed with reference to the potential use of this brain-accessible glutathione mimetic in the treatment of oxidative stress-related neurodegenerative disorders, including AD.

Keywords: Oxidative stress; Mitochondria; Cytochrome *c*; Alzheimer's disease; D609

Abbreviations: Aβ(1–42), amyloid β-peptide 1–42; AD, Alzheimer's disease; D609, tricyclodecan-9-yl-xanthogenate; DAG, diacylglycerol; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione S-transferase; HNE, hydroxynonenal; 3-NT, 3-nitrotyrosine; PBS, phosphate-buffered saline; PC-PLC, phosphatidylcholine-specific phospholipase C; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate.

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Introduction

Oxidative stress has been implicated in many neurodegenerative disorders, including Alzheimer's disease (AD) [1–4], AD is characterized clinically by progressive dementia and pathologically by extracellular amyloid protein deposits, intracellular neurofibrillary tangles (NFTs) (composed mostly of hyperphosphorylated tau protein), loss of synapses, mitochondrial dysfunction, and programmed cell death (PCD) [5,6]. The reduced energy metabolism in AD may be due to oxidative dysfunction of some of the key metabolic or mitochondrial enzymes [7–12], which may lead to increased reactive oxygen species (ROS) production.

It is well known that mitochondria are the major cellular site of energy production, and these organelles also play a key role in ROS generation, resulting in oxidative damage to neurons. Amyloid deposition, oxidative stress, mitochondria DNA deletion, and mitochondrial structural and functional abnormalities are prominent in AD [5,13,14]. Many proapoptotic signals and antiapoptotic defenses converge in the mitochondria [15]. Protein factors (cytochrome *c*, Apaf1, AIF, and SMAC/DIBLO) and Ca^{2+} released from mitochondria during oxidative stress activate caspase-dependent and/or caspase-independent mechanisms that lead to apoptotic cell death [15]. The role of mitochondria is not only as ATP producers but also as regulators of intracellular Ca^{2+} homeostasis and endogenous producers of ROS. Increased mitochondrial Ca^{2+} overload has been associated with the generation of superoxide and the release of proapoptotic mitochondrial proteins leading to cell death [16,17]. The alterations in Ca^{2+} homeostasis and ROS generation lead to increased susceptibility to cell death under circumstances that are otherwise not ordinarily toxic [17].

In AD brain ROS lead to protein oxidation [1,7–12], lipid peroxidation [2,3], DNA and RNA oxidation [18–20], and neuronal dysfunction or death. Recent studies indicate protein oxidation and lipid peroxidation in brain from mild cognitive impairment subjects [21,22], suggesting that oxidative stress is an early event in the pathogenesis of AD. ROS generation from mitochondria and its impact on neuronal systems hence become important in understanding oxidative stress and oxidative stress-related disorders, including AD.

Mitochondrial electron transport is a potential source of ROS production [23]. It is now well known that ROS such as superoxide anion (O_2^-), hydroxyl radical (OH), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$) contribute to neurodegeneration [21,24–28]. Mitochondrial membrane potential depolarization induces cytochrome *c* release into the cytoplasm and elevates the activity of caspase-3, suggesting a role for mtDNA-derived mitochondrial dysfunction in AD degeneration [5].

Glutathione is widely recognized as an endogenous nonenzymatic antioxidant, an oxyradical scavenger, thereby useful in protecting against oxidative damage by free radicals and inhibiting lipid peroxidation and DNA damage [29–33]. Glutathione has been implicated in a wide range of metabolic processes, including cell division, DNA repair, regulation of enzyme activity, activation of transcription factors, modulation of anion and cation homeostasis, and protection against oxidative damage [34]. The nervous system is particularly susceptible to oxidative insults, and dependent on its glutathione defense.

Tricyclodecan-9-yl-xanthogenate (D609) exhibits a variety of potent biological functions, including antiviral [35] and anti-inflammatory [36–38] activities. Most of these activities have been linked to the inhibitory effect of D609 on phosphatidylcholine-specific phospholipase C (PC-PLC) [35,39]. Such inhibition decreases production of the secondary messenger diacylglycerol (DAG) that activates protein kinase C (PKC) and acidic sphingomyelinase (aSMase) [40]. However, with a free thiol group, D609 may also possess strong antioxidant activity [41] with in vitro and in vivo radical scavenging properties and inhibition of free radial-induced oxidative stress [42–46].

The particular species of ROS that D609 can effectively scavenge is not clear, but this xanthate has the ability to scavenge hydroxyl radicals [42–46]. The reaction with other ROS is also possible since xanthates generally have high reductive potential [41]. D609 may protect intracellular GSH, which is an important intracellular defense molecule against oxidative stress in neurons and has been shown to play an important role in radiation protection [43,47,48]. Recently, we showed that D609, a glutathione mimetic [44], protects primary neuronal culture against amyloid β-peptide(1–42) [Aβ(1–42)]-induced oxidative stress and neurotoxicity in vitro [49] and in synaptosomes in vivo [46].

We performed the current study to test the hypothesis that in vivo loading with D609 had and antioxidant effect on isolated brain mitochondria exposed to various oxidants, including Aβ(1–42).

Materials and methods

Animals

For all studies male Mongolian gerbils (2–3 months of age), approximately 100 g in size, housed in the University of Kentucky Central Animal Facility under 12-h light/dark conditions and fed standard Purina rodent laboratory chow ad libitum, were used. The animal protocols were approved by the University of Kentucky Animal Care and Use Committee.

Materials

D609 was purchased from Biomol (Plymouth Meeting, PA) and all other chemicals were purchased from Sigma–Aldrich (St. Louis, MO). Fresh D609 (50 mg/kg body wt) was prepared in phosphate-buffered saline (PBS). The primary antibody for 4-hydroxynonenal (HNE) and 3-nitrotyrosine (3-NT) were purchased from Chemicon International.

Preparation of mitochondria

Brain mitochondria were isolated from gerbils previously injected ip with saline (control) or with D609 (50 mg/kg body wt), 60 min after injection. This time and level of D609 were chosen based on previous [42] dose–response experiments. The brain mitochondria were isolated according to the procedure of Sims [49] with minor modifications. Gerbils were decapitated and the whole brain was isolated on ice. Whole brain was

homogenized in ice-cold isolation buffer (250 mM sucrose, 10 mM Hepes, and 1 mM potassium EDTA, pH 7.2, 4 μg/ml leupeptin, 4 μg/ml pepstatin, 5 μg/ml aprotinin, 20 μg/ml trypsin inhibitor) with 6 passes of a Wheaton tissue homogenizer. The homogenate was centrifuged for 3 min at 1330*g* at 4°C, and the resulting pellet was resuspended in isolation buffer and centrifuged at 1330*g* for 3 min. The supernatants from both spins were combined and spun at 21,200*g* for 10 min at 4°C. The pellet was resuspended in 15% Percoll solution (v/v in isolation buffer) and layered onto discontinuous Percoll gradients of 23 and 40% Percoll (v/v in isolation buffer). Gradients were centrifuged at 30,700*g* for 5 min at 4°C. At the 23–40% Percoll interface, mitochondria were isolated and resuspended in respiration buffer (250 mM sucrose, 2 mM magnesium chloride, 20 mM Hepes, and 2.5 mM phosphate buffer, pH 7.2) and centrifuged at 16,700*g* for 10 min at 4°C. The pellet was resuspended in respiration buffer and centrifuged at 6900*g* for 10 min at 4°C, and the resulting pellet was washed in PBS at 6900*g* for 10 min at 4°C.

Protein estimation and treatment

The pellet was resuspended in 250 μl PBS and protein concentration determined by the Pierce BCA method [50], using bovine serum albumin (BSA) as a standard. The mitochondrial samples were divided into six aliquots and incubated at 37°C. The first sample set was incubated without treatment of any oxidant for 1 h; the second set of mitochondria was incubated with 30 μM Fe SO_4 and 2.0 mM H_2O_2, a process that induces hydroxyl radical formation [51]; the third and fourth set of samples were also incubated in same manner with/or without the oxidant AAPH (1 mM) for 1 h at 37°C. The fifth sample set was incubated without treatment of any oxidant for 6 h and the sixth sample set was incubated with Aβ(1–42) for 6 h. The mitochondrial samples were washed after incubation and resuspended in PBS/Tween.

Protein carbonyls

Protein carbonyls are markers of protein oxidation and were assessed by following the standard protocol described previously [42]. Samples (5 μl) (normalized to 4 mg/ml), 5 μl of 12% sodium dodecyl sulfate (SDS), and 10 μl of 10 times diluted 2,4-dinitrophenyl hydrazine (DNPH) from 200 mM stock were incubated at room temperature for 20 min. Samples were neutralized with 7.5 μl neutralization solution (2 M Tris in 30% glycerol). The resulting solution was loaded into each well on nitrocellulose membrane under vacuum using a slot-blot apparatus. The membrane was blocked in blocking buffer (3% bovine serum albumin) in PBS/Tween for 1 h and incubated with a 1:100 dilution of anti-DNP polyclonal antibody in PBS/Tween for 1 h. The membrane was washed three times in PBS/Tween and was incubated for 1 h with an anti-rabbit IgG alkaline phosphatase secondary antibody diluted in PBS/Tween in a 1:8000 ratio. The membrane was washed three times in PBS/Tween for 5 min and developed in Sigma Fast tablets (BCIP/NBT substrate). Blots were dried, scanned with Adobe PhotoShop, and quantified with Scion Image (PC version of Macintosh-compatible NIH Image). No nonspecific binding of antibody to the membrane was observed.

3-Nitrotyrosine

Samples (5 μl) (normalized to 4 mg/ml), 5 μl of 12% SDS, and 5 μl of modified Laemmli buffer containing 0.125 M Tris base, pH 6.8, 4% (v/v) SDS, and 20% (v/v) glycerol were incubated for 20 min at room temperature. Sample (250 ng) was loaded into each well on a nitrocellulose membrane in a slot-blot apparatus under vacuum. The membrane was blocked in blocking buffer (3% bovine serum albumin) in PBS/Tween for 1 h and incubated with a 1:2000 dilution of anti-3-NT polyclonal antibody in PBS/Tween for 1 h 30 min. The membrane was washed in PBS/Tween for 5 min three times after incubation. The membrane was incubated for 1 h, after washing, with an anti-rabbit IgG alkaline phosphatase secondary antibody diluted in PBS/Tween in a 1:8000 ratio. The membrane was washed three times in PBS/Tween for 5 min and developed in Sigma Fast tablets. Blots were dried, scanned with Adobe PhotoShop, and quantified with Scion Image as above. No nonspecific binding of antibody to the membrane was observed.

4-Hydroxynonenal (an index of lipid peroxidation)

Sample (5 μl) (normalized to 4 mg/ml), 5 μl of 12% SDS, and 5 μl of modified Laemmli buffer containing 0.125 M Tris base, pH 6.8, 4% (v/v) SDS, and 20% (v/v) glycerol were incubated for 20 min at room temperature. Sample (250 ng) was loaded into each well on a nitrocellulose membrane in a slot-blot apparatus under vacuum. The membrane was blocked in blocking buffer (3% bovine serum albumin) in PBS/Tween for 1 h and incubated with a 1:5000 dilution of anti-HNE polyclonal antibody in PBS/Tween for 1 h 30 min. The membrane was washed in PBS/Tween for 5 min three times after incubation. The membrane was incubated for 1 h, after washing, with an anti-rabbit IgG alkaline phosphatase secondary antibody diluted in PBS/Tween in a 1:8000 ratio. The membrane was washed three times in PBS/Tween for 5 min and developed in Sigma Fast tablets. Blots were dried, scanned with Adob PhotoShop, and quantified with Scion Image as above. A faint background staining due to the antibody alone was observed, but since each sample had a control, this minor effect was well controlled.

Estimation of cytochrome c release

Cytochrome *c* release was detected by the method of Yang et al. [52] with slight modification. After incubation and centrifugation of mitochondrial samples, the supernatant was used for Western blot analysis to cytochrome *c* release. The membrane was blocked in blocking buffer (3% bovine serum albumin) in PBS/Tween for 1 h and incubated with a 1:2000 dilution of anti-cytochrome *c* polyclonal antibody (C-5723; anti-sheep; Sigma) in PBS/Tween for 1 h 30 min. The membrane was washed in PBS/Tween for 5 min three times after incubation. The membrane was incubated for 1 h, after washing, with an anti-sheep IgG alkaline

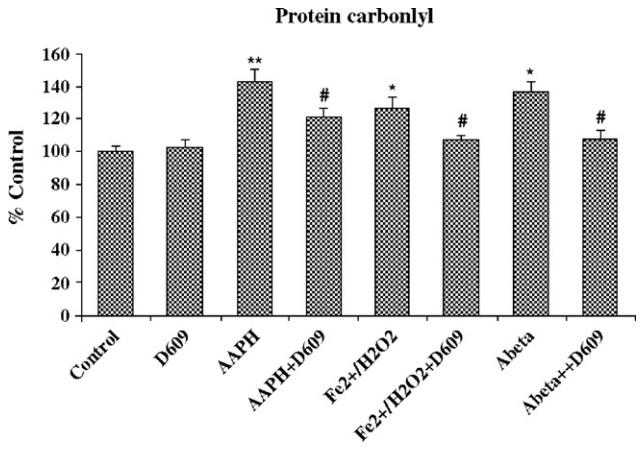

Fig. 1. The elevation in protein carbonyl formation in brain mitochondria isolated from saline-injected gerbils and treated with various oxidants [AAPH, Fe^{2+}/H_2O_2, or Aβ(1–42)] compared to control. The protective effects of D609 against protein carbonyl formation in brain mitochondria isolated from gerbils injected ip 1 h before sacrifice with D609 and treated with AAPH, Fe^{2+}/H_2O_2, and Aβ(1–42) also are shown. *$P<0.01$ and **$P<0.001$ compared to control and #$P<0.01$ compared to oxidant treatment. The data are presented as means ± SE expressed as percentage of control ($n=6$).

phosphatase secondary antibody diluted in PBS/Tween in a 1:8000 ratio. The membrane was washed three times in PBS/Tween for 5 min and developed in Sigma Fast tablets. Blots were dried, scanned with Adobe PhotoShop, and quantified with Scion Image as above.

Estimation of reduced glutathione (GSH)

Determination of GSH was performed by the method of Hissin and Hilf [53]. The reaction mixture containing 0.1 M sodium phosphate buffer (pH 8.0), 5.0 mM EDTA, 10 μl *o*-phthaldehyde (1.0 mg/ml), and 10 μl of sample. After incubation for 15 min at room temperature, fluorescence at emission 420 nm was recorded by excitation at 350 nm.

Estimation of oxidized glutathione (GSSG)

The estimation of GSSG was performed by the method of Hissin and Hilf [53]. The samples were incubated first with 0.04 M *N*-ethyleimide (NEM) for 30 min to interact with GSH present in sample. The reaction mixture containing 0.1 N NaOH, 5.0 mM EDTA, 10 μl *o*-phthaldehyde (1.0 mg/ml), and 10 μl of sample. After incubation for 15 min at room temperature, fluorescence at emission 420 nm was recorded by excitation at 350 nm.

Estimation of glutathione-S-transferase activity

GST (EC 2.5.1.18) activity was measured in a 96-well plate reader, with the reaction mixture consisting of 0.1 M phosphate buffer (pH 6.5), 1.0 mM reduced glutathione, 1.0 mM CDNB, and 0.1 μl of PMS in a total volume of 200 μl [54]. The changes in absorbance were recorded at 340 nm, and the enzymatic activity was calculated as nanomole CDNB conjugate formed per minute per milligram protein.

Estimation of glutathione peroxidase activity

GPx (EC 1.11.1.9) activity was measured in a 96-well plate reader at 37°C by a coupled assay system [55]. The reaction mixture consisted of 0.2 mM H_2O_2, 1.0 mM GSH, 0.14 U of GR, 1.5 mM NADPH, 1.0 mM sodium azide, 0.1 M phosphate buffer (pH 7.4), and 10 μl PMS in a total volume of 200 μl. The enzyme activity was calculated as nanomole NADPH oxidized per minute per milligram protein.

Estimation of glutathione reductase activity

The assay system to estimate GR (EC 1.6.4.2) activity consisted of 0.1 M phosphate buffer (pH 7.6), 0.5 mM EDTA, 1.0 mM oxidized glutathione, 0.1 mM NADPH, and 10 μl PMS in a total volume of 200 μl [56]. The enzyme activity was assayed in a 96-well plate reader by measuring the disappearance of NADPH at 340 nm and was calculated as nanomole NADPH oxidized per minute per milligram protein.

Statistical analysis

ANOVA was used for statistical evaluation of data followed by Student's *t* test. Results are presented as mean ± SE. *P* values less than 0.05 were considered significant.

Results

Protein oxidation and lipid peroxidation

Figs. 1, 2, and 3 show the levels of protein carbonyl, 3-NT, and HNE, respectively, in mitochondria isolated from brain of gerbils that had been previously injected ip with saline or D609. These isolated brain mitochondrial samples were subsequently treated

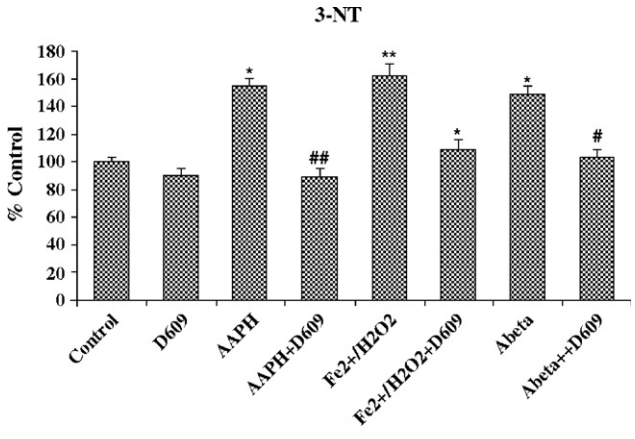

Fig. 2. The elevation in 3-NT levels in brain mitochondria isolated from saline-injected gerbils and subsequently treated with AAPH, Fe^{2+}/H_2O_2, or Aβ(1–42) compared to 3-NT levels in brain mitochondria isolated from saline-injected gerbil that received no treatment of any oxidant, *$P<0.01$ and **$P<0.001$. This figure also shows decreased 3-NT levels shows in brain mitochondria isolated from gerbils previously injected ip with D609 1 h before sacrifice and treated with AAPH, Fe^{2+}/H_2O_2, or Aβ(1–42) compared to the oxidant treatment but no prior injection of D609, #$P<0.01$. The data are presented as means ± SE expressed as percentage of control ($n=6$).

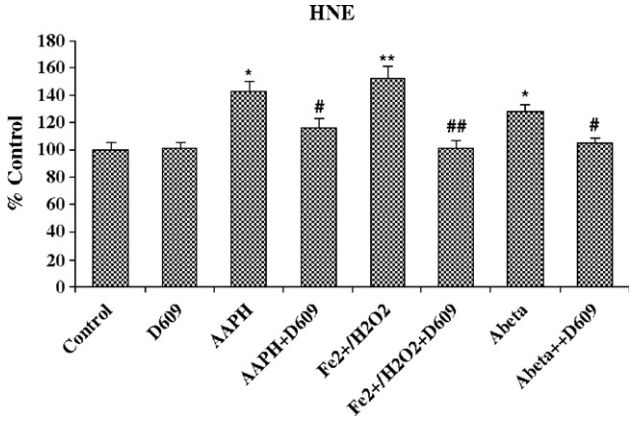

Fig. 3. The significantly elevated protein-bound HNE content in brain mitochondria isolated from saline-injected gerbils and treated with different oxidants [AAPH, Fe^{2+}/H_2O_2, or Aβ(1–42)]. The protective effects of D609 against HNE formation of protein-bound HNE in brain mitochondria isolated from gerbil injected ip with D609 1 h before sacrifice and treated with AAPH, Fe^{2+}/H_2O_2, or Aβ(1–42) also are shown. *$P<0.01$ and **$P<0.001$ compared to control, #$P<0.01$ and ##$P<0.001$ compared to oxidant treatment. The data are presented as means±SE expressed as percentage of control ($n=6$).

with Fe^{2+}/H_2O_2, AAPH, or Aβ(1–42) in vitro. The concentrations of these oxidants were chosen based on prior dose–response studies of the agents in in vivo investigations [42,46]. The levels of protein carbonyl, 3-NT, and HNE were found significantly increased in mitochondria isolated from saline-injected gerbil brain and subsequently treated with oxidants, in vitro, compared to control (without treatment of oxidants). Brain mitochondria isolated from gerbils previously injected ip with D609 and subsequent in vitro treatment with Fe^{2+}/H_2O_2, AAPH, and Aβ(1–42) showed significantly decreased protein carbonyl, 3-NT, and HNE levels as compared to brain mitochondria isolated from gerbils previously injected ip with saline and subsequently treated with Fe^{2+}/H_2O_2, AAPH, and Aβ(1–42), in vitro. The D609-alone sample had no significant effect on the levels of protein carbonyls, 3-NT, or HNE in brain mitochondria. It is clear that less protein oxidation and lipid peroxidation product, HNE, was found in Fe^{2+}/H_2O_2, AAPH, and Aβ(1–42)-treated mitochondria isolated from gerbils previously injected with D609.

Reduced glutathione

Fig. 4A shows the levels of GSH in mitochondria isolated from gerbil brain from saline- as well as D609-injected rodents.

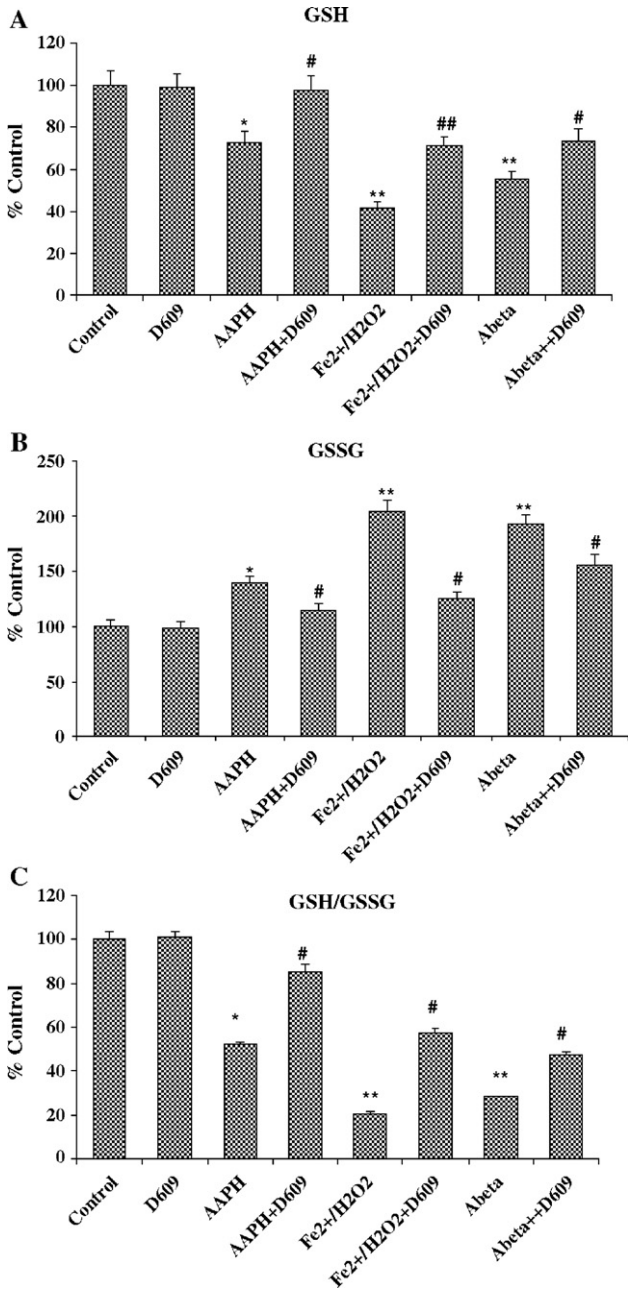

Fig. 4. (A) Shows a significant decrement in GSH levels in brain mitochondria isolated from saline-injected gerbils and subsequently treated with AAPH, Fe^{2+}/H_2O_2, or Aβ(1–42) compared to GSH levels in brain mitochondria isolated from saline-injected gerbils not subjected to treatment of any oxidant. Also shown is the protection of GSH levels in brain mitochondria isolated from gerbils previously injected ip with D609 1 h before sacrifice and treated with AAPH, Fe^{2+}/H_2O_2, or Aβ(1–42) compared to GSH levels in brain mitochondria isolated from saline-treated gerbils and then treated with oxidants. *$P<0.01$ and **$P<0.001$ compared to control, #$P<0.01$ and ##$P<0.01$ compared to oxidant treatment. The data are presented as means±SE expressed as percentage of control ($n=6$). (B) The increased level of GSSG in brain mitochondria isolated from saline-injected gerbils and subsequently treated with AAPH, Fe^{2+}/H_2O_2, or Aβ(1–42) as compared to GSSG levels in brain mitochondria isolated from saline-injected gerbils but not subjected to treatment of any oxidant. The reduction in GSSG level shows in brain mitochondria isolated from gerbils previously injected ip with D609 1 h before sacrifice and treated with AAPH, Fe^{2+}/H_2O_2, or Aβ(1–42) compared to GSSG levels in brain mitochondria isolated from saline-treated gerbil and then treated with oxidants. *$P<0.01$ and **$P<0.001$ compared to control, #$P<0.01$ and ##$P<0.01$ compared to oxidant treatment. The data are presented as means±SE expressed as percentage of control ($n=6$). (C) The ratio of GSH/GSSG decreased in brain mitochondria isolated from saline-injected gerbils and subsequently treated with AAPH, Fe^{2+}/H_2O_2, or Aβ(1–42), compared to the GSH/GSSG ratio in brain mitochondria isolated from saline-injected gerbils but not subjected to treatment of any oxidant. The increment in the ratio of GSH/GSSG in brain mitochondria isolated from gerbils previously injected ip with D609 1 h before sacrifice and then treated with AAPH, Fe^{2+}/H_2O_2, or Aβ(1–42) compared to this ratio determined in brain from mice treated with oxidant but no preinjection of gerbils with D609 is also shown. *$P<0.01$ and **$P<0.001$ compared to control, #$P<0.01$ and ##$P<0.01$ compared to oxidant treatment. The data are presented as means±SE expressed as percentage of control ($n=6$).

These brain mitochondrial samples were subsequently treated with Fe^{2+}/H_2O_2, AAPH, or Aβ(1–42) in vitro. The levels of GSH were found significantly decreased in mitochondria isolated from saline-injected gerbil brain and treated in vitro with oxidants compared to control. In contrast, brain mitochondria isolated from D609-injected gerbils and subsequently treated in vitro with Fe^{2+}/H_2O_2, AAPH, or Aβ(1–42) showed increased levels of GSH.

Oxidized glutathione

Fig. 4B shows significantly increased GSSG levels in brain mitochondria treated with different oxidants [Fe^{2+}/H_2O_2, AAPH, or Aβ(1–42)] that were isolated from saline-injected gerbils (control). In contrast, there were significantly decreased GSSG levels in mitochondria isolated from gerbils previously injected ip with D609 and treated with Fe^{2+}/H_2O_2, AAPH, or Aβ(1–42) in vitro.

Ratio of reduced and oxidized glutathione (GSH/GSSG)

Fig. 4C shows the significantly decreased GSH/GSSG ratio in brain mitochondria isolated from saline-injected gerbils (control) and treated with different oxidants in vitro. This GSH/GSSG ratio was significantly increased in brain mitochondria isolated from gerbils previously injected ip with D609 and treated with Fe^{2+}/H_2O_2, AAPH, or Aβ(1–42) in vitro.

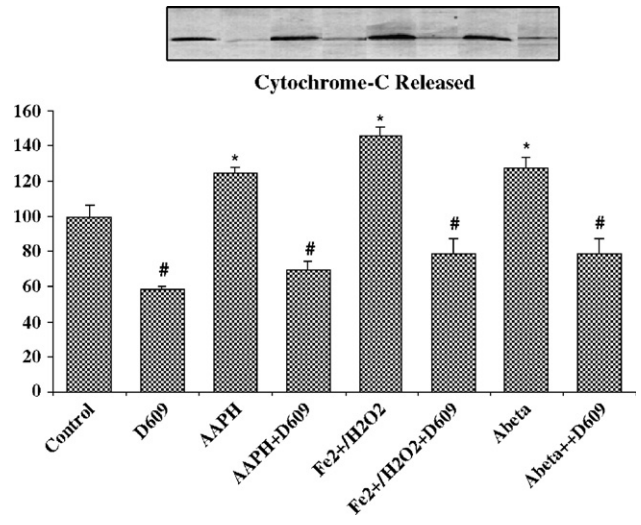

Fig. 5. The increased level of cytochrome *c* released from brain mitochondria isolated from saline-injected gerbils and treated with various oxidants (AAPH, Fe^{2+}/H_2O_2, or Aβ(1–42) as compared to cytochrome *c* released from brain mitochondria isolated from saline-injected gerbils but not subjected to treatment of any oxidant. Also shown is the decrement of cytochrome *c* release from brain mitochondria isolated from gerbils previously injected ip with D609 1 h before sacrifice and treated with AAPH, Fe^{2+}/H_2O_2, or Aβ(1–42) compared to that released from brain mitochindria isolated from gerbils subjected to oxidant treatment. The D609-only treatment shows significantly less cytochrome *c* release compared control. $^{*}P<0.01$ as compared to control, $^{\#}P<0.01$ compared to oxidant treatment. The data are presented as means±SE expressed as percentage of control ($n=5$).

Table 1
Activities of some GSH-related enzymes in brain homogenate obtained from gerbils that previously had been injected ip with saline (control) or D609

Enzyme	Brain from control group	Brain from D609-treated group
GST (nmol CDNB conjugate formed min^{-1} mg^{-1} protein)	38.4±3.44	61.5±4.74*
GPx (nmol NADPH oxidized min^{-1} mg^{-1} protein)	34.5±2.87	43.1±3.41$^{\#}$
GR (nmol NADPH oxidized min^{-1} mg^{-1} protein)	23.4±2.89	26.6±3.10

The protective effect of D609 on activity of glutathione *S*-transferase, glutathione peroxidase, and glutathione reductase isolated from gerbil barin from which mitochondria were obtained. The data are presented as mean±SE expressed as percentage of control ($n=6$). $^{*}P<0.01$ and $^{\#}P<0.05$ compared to control.

Cytochrome c release

Reactive oxygen species are produced by mitochondria that can cause the release of cytochrome *c* from the mitochondrial membrane. Fig. 5 shows the level of cytochrome *c* released from brain mitochondria isolated from saline-injected as well as D609-injected gerbils and subsequently treated with different oxidants in vitro. There was a significant reduction in cytochrome *c* release from brain mitochondria by prior ip D609 treatment compared to that in brain mitochondria isolated from saline-injected gerbils. Increased cytochrome *c* release from brain mitochondria isolated from control gerbils with subsequent in vitro treatment of Fe^{2+}/H_2O_2, AAPH, or Aβ(1–42) compared to control was observed. Brain mitochondria isolated from D609-injected gerbils and subsequently treated with Fe^{2+}/H_2O_2, AAPH, or Aβ(1–42), in vitro, showed a significant decrease in cytochrome *c* release compared to mitochondria isolated from saline-injected gerbils treated with oxidants (Fig. 5).

Activity of GSH-relevant enzymes

Table 1 shows the activity of some GSH-relevant enzymes in gerbil brain from which mitochondria were isolated. The activities of glutathione S-transferase and glutathione peroxidase were increased significantly in postmitochondrial supernatant from D609-injected gerbils. The activity of glutathione reductase also was increased, but not significantly. Thus, these results suggest that D609 has a role in the redox cycle of GSH (oxidation and reduction of GSH) and in the neuroprotection against free radicals.

Discussion

Oxidative stress reflects a marked imbalance between ROS and their removal by antioxidant systems. This imbalance may originate from an overproduction of ROS or from a reduction in antioxidant defenses or both [57]. An inverse relationship between lipid peroxidation and GSH and its dependent enzymes, along with the activities of catalase and superoxide dismutase, is well known [31,32]. A reduction in GSH may

impair H_2O_2 clearance and promote hydroxyl radical formation, thus increasing the free radical load, which triggers oxidative stress. Conversely, GSH is converted to GSSG, a process that in conjunction with the cofactor NADPH can reduce lipid peroxides, free radicals, and H_2O_2. NADPH also acts as a peroxynitrite reductant, thereby providing enzymatic defense against peroxynitrite [58]. Glutathione peroxidase and glutathione reductase protect the neurons from oxidative stress by catalyzing the reduction of H_2O_2 at the expense of glutathione [31,32]. Glutathine S-transferase plays a role in neuroprotection by catalyzing the formation of the GSH-HNE conjugate, which is then removed from neurons by the action of the multidrug-resistant protein-1 [59] In AD brain, both GST and MRP-1 are oxidatively modified and likely dysfunctional [59]. In our results GST (Table 1) and GPx have higher activities following in vitro oxidant treatment of brain homogenate isolated from gerbils previously injected with D609. GR activity in brain (Table 1) of D609-injected animals was not significantly elevated following subsequent in vitro oxidant treatment to isolated mitochondria relative to control.

Reduced levels of GSH (Fig. 4A) have been observed in oxidative stress-related disorders [60] in specific regions of the central nervous system of AD patients, and thus reduced GSH may contribute to the neuronal cell dysfunction and/or loss. In AD, increased levels of GSSG [61] have been observed. Studies have shown that an increase in endogenous GSH levels by dietary or pharmacological intake of GSH precursors or GSH mimetics or substrates for GSH synthesis protects the brain against oxidative stress [47,48,62–66]. The increase in the content of GSH and decrease in the extent of GSSG in mitochondria isolated from gerbil brain previously injected with D609 in our study is in agreement with earlier reports [46].

We earlier showed that D609 could effectively scavenge hydroxyl radicals [43]. The identification of D609 as a potent antioxidant implies that D609 may exert some of the reported activities that have been largely attributed to the inhibition of phosphatidylcholine-specific phospholipase C by its antioxidant properties. Among these activities are inhibition of LPS- and TNF-induced NF-kB activation and inflammatory cytokine production [35,39]. ROS-activated NF-kB causes a differential change in gene expression between neurons and astrocytes in the AD brain [67,68]. We earlier reported the anticarcinogenic activity of D609 might be due to its antioxidant property [43]. In vitro, D609 kills a variety of tumor cells but has limited toxic effects on normal cells [38]. Although D609 might be involved in alternate biochemical pathways, its glutathione mimetic property cannot be ignored. The free thiol group in D609 may act as substrate for hydroxyl radicals, or may act as electron acceptor from hydrogen peroxide to form a dixanthate, which forms the substrate for glutathione reductase to convert it back to xanthate. This property of D609 is yet to be explored.

Protein carbonyl, 3-NT, and HNE levels are elevated in AD and MCI brains [3,4,7–12,21,22]. HNE is a highly reactive product of arachidonic acid metabolism that is believed to interfere with normal cellular functions [3]. During oxidative stress several lipid peroxidation products are formed, including HNE, one of the most abundant and toxic lipid-derived aldehydes, and which can induce oxidative stress [69,70]. Lipid peroxidation products such as HNE and acrolein are known to cause damage to biomembranes, proteins, and other biomolecules in AD brain [70–72]. These alkenals form an immediate substrate for GSH [73] and these lipid peroxidation products are known to be involved in apoptosis, which can be initiated as a consequence of GSH depletion [74].

GSH is known to detoxify HNE and protect cultured neurons against oxidative damage resulting from amyloid β-peptide, iron, and HNE [74]. GSH can also protect brain from damage by peroxynitrite, hydroxyl free radicals, or reactive alkenals [63–66,75]. D609 may bind to reactive alkenals formed due to peroxidation of lipids and detoxify their effects [44].

HNE can alter pyruvate dehydrogenase (PD) [76], decrease cell survival (decrease MTT reduction), and cause inhibition of Na^+K^+ ATPase [74]. The activity of the adenine nucleotide transporter in mitochondria is inhibited by HNE, which directly results in ATP depletion [77]. Aβ increases lipid-derived free radical production [3,4,74,77,78]. HNE rapidly leads to decline in multiple sites of the mitochondrial respiratory chain and modified specific mitochondrial target proteins lead to apoptosis and cell death in AD [79].

Recently, we showed that the xanthate D609, a glutathione mimetic [44], protects primary neuronal culture against Aβ(1–42)-induced oxidative stress and neurotoxicity in vitro [45] and against Aβ(1–42) in vivo [46]. D609 has the ability to scavenge hydrogen peroxide and hydroxyl free radicals [44]. D609 can bind to reactive alkenals and detoxify their effect, thereby preventing these alkenals from damaging mitochondria [42,44]. AAPH is known to induce protein carbonylation and nitration [80] in mitochondria. In the current study, we report that in vivo delivery of D609 inhibits the damage to brain mitochondria caused by production of hydroxyl free radicals, alkoxyl and peroxyl radicals, and Aβ(1–42)-induced in vitro oxidative stress to decrease protein oxidation and lipid peroxidation.

Aβ disrupts Ca^{2+} homeostasis in neurons [81], and increased intracellular Ca^{2+} level can increase sphingomylinase activity to produce ceramide [82]. Activation of the apoptogenic sphingomyelin-dependent signaling pathway is mediated by ceramide [82] during oxidative stress to play a role in the pathogenesis of neuronal disease [83]. Apoptosis induced by the membrane-permeable second messenger ceramide, followed by the release of cytochrome *c* and Ca^{2+} from the mitochondria with the loss of mitochondrial transmembrane potential, has been observed [83]. Both cytochrome *c* release and rise of intracellular Ca^{2+} cause caspase-3 activation and nuclear condensation [83]. ROS-dependent and -independent pathways are initiated by caspase-8 activation and contribute to ceramide formation via the activation of both neutral and acid sphingomyelinases (SMases) in TNF-α-induced apoptosis of human glioma cells [83]. In one study, D609 has also been shown to inhibit sphingomyelin synthase in SV40-transformed human lung fibroblasts, but ceramide and DAG levels evolved in opposite directions [84] and partially protected against apoptosis [85].

In conclusion, this study has demonstrated that, similar to the case with elevated cytosolic GSH protecting brain mitochondria [65], ip injection of D609 protects brain mitochondria

(antiapoptotic) against oxidative stress induced in vitro by different oxidants, such as AAPH, Fe^{2+}/H_2O_2, or Aβ(1–42). In vivo delivery of D609 showed significant protection against protein oxidation, lipid peroxidation, and cytochrome *c* release in gerbil brain mitochondria. While a role for inhibition of PC-PLC by D609 in the protection of brain mitochondria cannot be excluded, based on the data from the present and previous studies [42,46], the increment in the GSH level and the activity of its dependent enzymes (GST, GPx, and GR) are strong indications to suggest that D609 has neuroprotective effects to brain mitochondria due in significant part to its antioxidant properties. Thus, in brain mitochondria the antioxidant properties of in vivo delivery of D609 conceivably could be beneficial in the treatment of diseases related to oxidative stress that involve mitochondria (AD, Huntington disease, Parkinson's disease, for example). This xanthate compound protects against in vitro treatment of Aβ(1–42); consequently, we suggest that D609 may be part of a promising therapeutic strategy for Alzheimer's disease. Studies to test this notion in rodent models of AD are in progress.

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