

Characterization of intracellular elevation of glutathione (GSH) with glutathione monoethyl ester and GSH in brain and neuronal cultures: Relevance to Parkinson's disease

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Abstract

Parkinson's disease (PD) is associated with loss of total glutathione (GSH) which may contribute to progressive cell death. Peripheral GSH administration has been used clinically with reported benefits. Despite this, there is little specific information to characterize its cellular uptake or clearance, brain elevation with peripheral delivery or neuroprotective efficacy in PD models. The current study was carried out to provide this information using in vitro and in vivo approaches. In rat mesencephalic culture, the monoethyl ester of GSH (GEE), but not GSH (1–10 mM, 24 h) produced a dose-dependent elevation in GSH. The half-life for clearance was 10.14 h and was not different in cells depleted of GSH prior to loading. Elevation of GSH with GEE protected neurons from oxidative stress with H₂O₂ or metabolic stress with the complex I and II inhibitors MPP⁺ and malonate, respectively. To determine if peripheral administration of GEE could elevate brain GSH levels, rats were administered 0.1–50 mg/kg/day GEE via osmotic minipump either subcutaneously (sc) or via a cannula placed into the left cerebral ventricle (icv) for 28 days. Only central delivery of GEE resulted in significant elevations of brain GSH. Elevation of brain GSH by icv infusion of GEE was examined for its neuroprotective effects against chronic central delivery of MPP⁺. Infusion of 0.142 mg/kg/day MPP⁺ for 28 days caused a selective ipsilateral loss of striatal dopamine. Co-infusion of MPP⁺ with 10 mg/kg/day GEE significantly protected against striatal dopamine loss. These findings show that the ethyl ester of GSH but not GSH per se can elevate intracellular GSH, that brain elevation of GSH requires central delivery of the ethyl ester and that this elevation provides neuroprotection against oxidative stress or chronic mitochondrial impairment.

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Introduction

The underlying etiology of sporadic Parkinson's disease (PD) remains elusive, although much evidence implicates oxidative stress in the process. The substantia nigra (SN) from autopsied brain material has been shown to contain increased levels of protein carbonyls (Alam et al., 1997), malondialdehyde or 4-hydroxynonenol (Dexter et al., 1994a; Yoritaka et al., 1996) and 8-hydroxy-2-deoxyguanosine (Sanchez-Ramos et al., 1994), indicative of oxidative damage to proteins, lipids and DNA, respectively. One of the earliest biochemical derangements observed in the SN of patients with PD is loss of total glutathione levels (Perry and Yong, 1986; Jenner et al., 1992;

Sian et al., 1994), without a corresponding increase in oxidized glutathione levels (Sian et al., 1994). This decrease is also observed in incidental Lewy body disease, a condition thought to presage PD (Jenner et al., 1992; Dexter et al., 1994b). Glutathione is a major antioxidant in the cell, and logically it would follow that the loss of intracellular GSH would contribute to the increase in free radical damage to cellular constituents. Thus, supplementation with antioxidants should be neuroprotective. Clinical studies, with vitamins E or C, however, have not supported this proposal (Fahn, 1992; Shoulson, 1998). More recent studies with co-enzyme Q10, have shown more promise (Shults et al., 2002), but it is unclear if protective benefits derive from its antioxidant properties or from other factors (Echtay et al., 2000, 2002; Papucci et al., 2003). In addition to direct removal of reactive oxygen species by GSH, a number of associated enzymes confer varied roles for

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GSH in the cell. Glutathione peroxidase, reductase, transferase(s) and glutaredoxin all utilize GSH in reactions that remove peroxide, as well as potential endogenous and exogenous toxins, control the redox state of the cell and regulate protein function through thiolation and dethiolation. This network of enzymes using GSH as substrate has been implicated in DNA synthesis and repair, protein synthesis, amino acid transport and enzyme activation or inactivation (Lomaestro and Malone, 1995). GSH may also act as a redox modifier of some ionotropic currents (Ogita et al., 1995). The multifaceted functions served by GSH raise the possibility that replacement therapy with an antioxidant other than GSH may not carry out all of the important functions of GSH per se. Thus, elevation of brain levels of GSH may be a more efficacious approach to neuroprotection in diseases such as PD.

One trial using GSH in patients with PD reported clinical improvement (Sechi et al., 1996), but the small number of patients and lack of controls limited the usefulness of the investigation. Anecdotal reports of improvement of rigidity, coordination, speech and depression with intravenous administration of GSH can also be found from non-journal reports (www.1whey2health.com/parkinsons_glutathione.htm). A short term, double blinded clinical trial of intravenous (iv) glutathione in PD patients is currently being conducted at the University of South Florida, and results are expected in 2006 (see *Official Journal of the National Parkinson Foundation*, vol. XVI, Fall 2005). Despite the use of peripheral administration of GSH clinically for PD, nothing is known regarding the ability of peripheral administration of GSH to elevate brain levels of GSH or the time for clearance of loaded GSH from cells. In addition, while a number of studies in vitro have indicated that elevation of intracellular GSH can protect against oxidative stress, little is known regarding its efficacy in vivo in chronic models of PD.

The present work was, therefore, carried out to determine if GSH or its ethyl ester crosses the plasma membrane of cells or the blood brain barrier to elevate intracellular or brain levels of GSH, the half-life for clearance of loaded GSH from cells and the ability of elevated intracellular GSH to protect against oxidative stress or mitochondrial dysfunction in vitro or in vivo.

Materials and methods

Mesencephalic culture and treatment conditions

Embryonic day 15 Sprague–Dawley rats (Charles River and Co.) were used for dissection and culturing of ventral mesencephalon as routinely done in the laboratory (Zeevalk and Bernard, 2005). Briefly, dissociated cells were plated at 2×10^5 cells/cm² onto polyornithine coated wells in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat inactivated horse and fetal bovine serums (DMEM/serum) and incubated at 37°C with 5% CO₂. GEE (1–10 mM) or reduced glutathione (GSH) was added to cultures on day 8 in vitro. To inhibit glutathione synthesis, buthionine sulfoximine (BSO, 1 μM) was added to some cultures on day 7 in vitro. Determinations for intracellular GSH content were carried out

immediately following loading with GEE or GSH or in some studies 1–48 h after loading. For toxicity studies, cultures were exposed to either 75 μM H₂O₂ for 6 h, 0.5 μM MPP⁺ for 24 h or 50 mM malonate for 24 h in the presence or absence of GEE. GEE, when present, was given 24 h before toxin treatment. At the end of exposure, the cultures were returned to conditioned medium and reincubated for 72 h prior to assessment of toxicity by either high affinity uptake or counts of TH immunoreactive cells as described below. Conditioned medium was obtained from untreated cultures grown in DMEM/serum for 7–9 days.

In vivo studies: pump implantation and cannula placement

Sprague–Dawley rats (300 g, Harlan; Indianapolis, IN) were housed in pairs and maintained on a 12 h light–dark cycle with food and water available *ad libitum*. Experiments were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Animals were anesthetized (ketamine 50 mg/kg; xylazine 2.5 mg/kg; acetyl promazine 0.625 mg/kg; im) prior to pump and cannula implantation. An Alzet osmotic minipump (Cupertino, CA; model 2ML4) was implanted subcutaneously on the back. For animals receiving infusions into the left lateral ventricle, cannula placement was made stereotaxically following the coordinates of Paxinos and Watson (1986): anterior +8.7, lateral left +1.4, depth –3.5. GEE (0.1–50 mg/kg/day) plus or minus MPP⁺ (0.142 mg/kg/day) was delivered at a rate of 2.5 μl/h for 28 days. At the end of the exposure, animals were sacrificed by decapitation, and 2 min prior to euthanasia, the animals received a tail vein injection of 3-mercaptopyruvate (160 mg/kg) to inhibit glutamic acid decarboxylase activity (Korf and Venema, 1983). The brains were rapidly removed and dissected. Wet weights were determined, and the tissues maintained at –80°C until analyzed for neurochemistry.

Toxicity: dopamine and GABA high affinity transport

The simultaneous measurement of transport of ³H-dopamine and ¹⁴C-GABA is as previously described (Zeevalk et al., 2000) with minor modifications. Cultures were incubated with 20 nM ³H-dopamine plus 5 μM ¹⁴C-GABA in the presence of 1 mM ascorbate, 100 μM pargyline (to prevent dopamine oxidation and metabolism), 10 μM aminoxyacetic acid (to preserve GABA) and 1 mM β-alanine (to block glial uptake of GABA) for 15 min at 37°C. Intracellular radioactivity was extracted with 95% ethanol and quantitated by scintillation counting. Uptake at 4°C was subtracted from other determinations to obtain energy dependent uptake.

Tyrosine hydroxylase immunocytochemistry and cell counts

Immunocytochemistry for TH was carried out as previously described (Zeevalk and Bernard, 2005). Cultures were immunostained with mouse monoclonal anti-TH (1:6000, Diasorin, Inc.). Primary antibody was detected with the Avidin Biotin Peroxidase system (Vector Labs). Cells were viewed and counted at 100× magnification. For counting, the bottom of

each well was marked at the center and in each of 4 quadrants. Marks were located, and immunopositive cells to the right and left of the mark were counted in a 1 mm² reticle by an observer blind to the treatment condition. Each condition was run in duplicate. Counts from 40, 1 mm² fields per condition (20 fields/well × 2 wells × 2 replicate experiments) were averaged and reported as mean counts per square mm ± SD.

HPLC determination of glutathione, glutamate and GABA

Amino acids and total GSH were measured by HPLC as previously published (Zeevalk et al., 2000). Cells were extracted with 0.2 N perchloric acid (PCA). For tissue samples, tissue was homogenized in 0.2 N PCA at a wet weight of 10 mg/ml. Samples were frozen at −80°C until used for measurement. The extract was neutralized to pH 5.8 with 2 M potassium carbonate, reacted with σ -phthalaldehyde and separated by reverse phase HPLC with fluorescent detection. Quantitation was obtained by comparison to known standards.

HPLC determination of dopamine and 5-HT

Tissue samples were homogenized in 0.2 N PCA on the day of measurement. Samples were centrifuged and the supernatant used for detection of monoamines with HPLC using electrochemical detection as described elsewhere (Yazdani et al., 2006).

Statistics

Most data were analyzed using a one-way analysis of variance (ANOVA, Prism 3.0) with the different treatments as the independent factor. Comparisons between means were tested by the Tukey–Kramer Multiple Comparisons post hoc test. In the case of left versus right differences from the same animal, data were analyzed using a two-tailed paired Student's *t* test. In all cases, significance was indicated by $p < 0.05$.

Results

Elevation of glutathione with glutathione ethyl ester in mesencephalic culture

Preloading mesencephalic cultures for 24 h with glutathione ethyl ester produced a dose-dependent increase in intracellular levels of GSH (Fig. 1A). Basal levels of GSH in the cultures were 1.75 ± 0.11 $\mu\text{mol}/100$ mg protein \pm SEM. GSH levels were elevated 66, 144 and 191% of basal with 2.5, 5 and 10 mM GEE, respectively. In contrast, preloading for 24 h with 1–10 mM reduced-GSH did not elevate intracellular glutathione levels (Fig. 1B). To better replicate conditions in Parkinson's disease brain with regard to lowered GSH levels which could alter loading characteristics, cultures were partially depleted of GSH by a 24 h pretreatment with buthionine sulfoximine (BSO, 1 μM) prior to GEE loading for 24 h in the continued presence of BSO. Treatment of cultures with 1 μM BSO in the absence of GEE reduced intracellular GSH levels from a basal level of

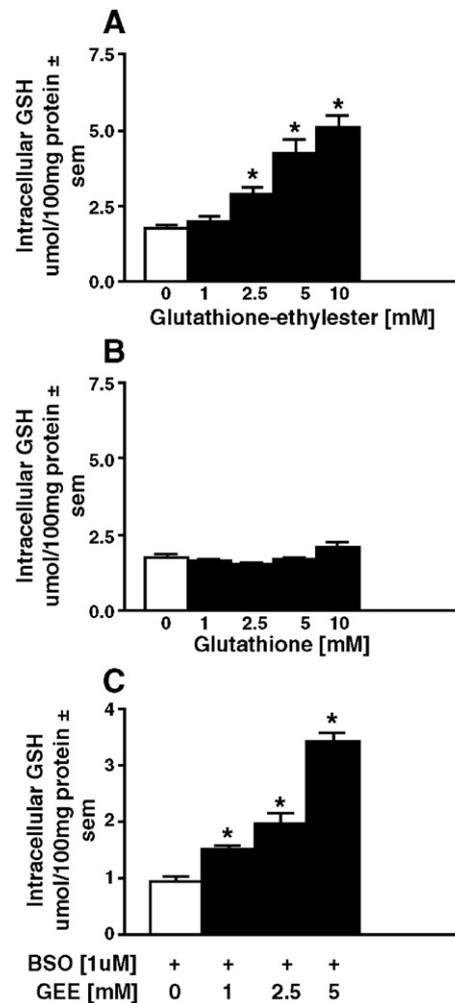


Fig. 1. Glutathione monoethyl ester but not glutathione elevates intracellular glutathione levels. Mesencephalic cultures were preloaded for 24 h with either the monoethyl ester of glutathione (A) or with reduced glutathione (B). (C) Some cultures had glutathione partially depleted prior to loading by treatment for 24 h with buthionine sulfoximine (BSO, 1 μM). GEE loading was carried out in the presence or absence of BSO. Following loading, cultures were washed, intracellular content extracted and glutathione quantified by HPLC. *n* is from 4 to 5 separate experiments run in duplicate. *Different from zero GEE addition.

1.62 ± 0.11 to 0.72 ± 0.10 $\mu\text{mol}/100$ mg protein \pm SEM, $n = 7$ –8 determinations per condition. This is a decrease of approximately 55% and similar to the 40–50% loss observed in PD (Jenner et al., 1992). When compared with GSH-depleted cells, GEE loading significantly elevated GSH levels with ≥ 1 mM GEE although absolute intracellular levels of GSH were always less than those observed in cultures not treated with BSO at comparable GEE concentrations (Fig. 1C and compare with Fig. 1A).

To determine whether there was any toxicity associated with GEE loading, cultures were loaded with 1–10 mM GEE for 24 h, switched to GEE free medium and allowed to recover for 72 h. Toxicity was determined by a functional assay for the high affinity transport of dopamine and GABA which allows assessment of toxicity in both these mesencephalic populations. Since toxicity is determined 3 days after removal of GEE, the results reflect irreversible damage and are not a pharmacological

consequence of treatment. GEE at 10 mM significantly decreased dopamine uptake by 38%, indicating toxicity to this population (Fig. 2A). There was a trend towards a decrease in GABA uptake, but this was not statistically significant (Fig. 2B). Loading with 10 mM reduced-GSH did not produce toxicity to dopamine or GABA mesencephalic populations, suggesting that the ethyl ester moiety was responsible for toxicity (data not shown).

Half-life for clearance of glutathione from preloaded glutathione ethyl ester

Cells closely regulate intracellular glutathione levels. We, therefore, wanted to determine how rapidly cells cleared elevated intracellular GSH when derived from exogenous sources. This would be important to know if trying to augment GSH levels in cells as a neuroprotective strategy. Studies were carried out to determine the half-life for clearance of GSH from preloaded GEE under normal and GSH-depleted conditions. Cultures were treated with 5 mM GEE or 5 mM GEE plus 1 μ M BSO for 24 h, switched to fresh medium and intracellular levels of GSH were monitored immediately following loading and at 8, 16, 24 and 48 h post loading. The half-life was determined from a one-phase exponential decay curve where $t_{1/2} = \ln 2 / \text{decay rate}$. The half-life for clearance of loaded GEE was 10.14 and 13.86 in normal and GSH-depleted cultures, respectively, and was not statistically different from one another (Fig. 3).

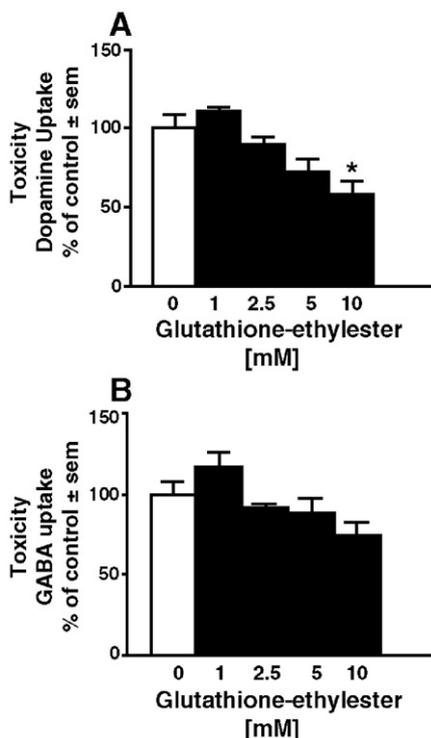


Fig. 2. Toxicity associated with glutathione monoethyl ester loading. Cultures were exposed to glutathione monoethyl ester (GEE) for 24 h then allowed to recover for 3 days prior to measuring toxicity by a functional assay of high affinity uptake for (A) ^3H -dopamine or (B) ^{14}C -GABA. Exposure to similar concentrations of GSH was not toxic. *n* is from 3 experiments run in duplicate. *Different from zero GEE.

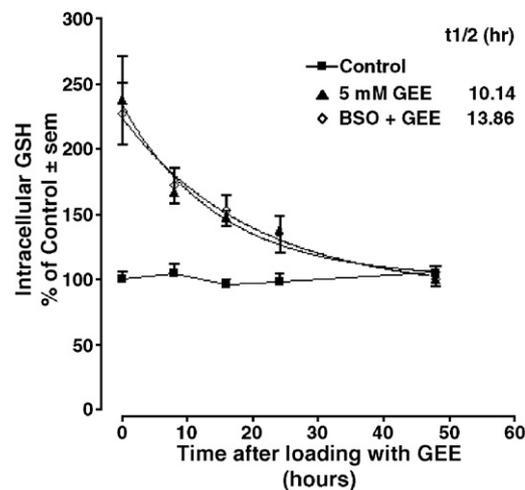


Fig. 3. Half-life for clearance of loaded glutathione. Mesencephalic cultures were treated with 5 mM glutathione monoethyl ester (GEE) plus or minus 1 μ M buthionine sulfoximine (BSO) for 24 h, switched to fresh medium plus or minus BSO and intracellular levels of GSH were monitored immediately following loading (0 time) and at 8, 16, 24 and 48 h post loading. The half-life was determined from a one phase exponential decay curve. *n* is from 4 experiments run in duplicate.

Glutathione ethyl ester preloading protects against mitochondrial inhibition and oxidative stress in vitro

In order to determine if preloading cells with glutathione prior to exposure to an oxidative challenge with H_2O_2 (75 μM , 6 h) or mitochondrial impairment with the complex II inhibitor malonate (50 mM, 24 h) or complex I inhibitor MPP^+ (0.5 μM , 24 h) protected mesencephalic neurons, cultures were exposed to GEE for 24 h prior to the toxin challenge. After 3-day recovery, toxicity was assessed by measurement of high affinity uptake for dopamine and GABA. GEE provided significant protection against H_2O_2 for both the dopamine and GABA populations with 2.5 and 5 mM GEE preloading (Fig. 4A). To provide further evidence that oxidative insult resulted in cell loss that was protected by GEE preloading, some cultures were examined for tyrosine hydroxylase (TH)-positive cell counts 3 days after exposure to H_2O_2 . As shown in Fig. 4B, 75 μM H_2O_2 produced a significant loss of TH-positive cells and this was fully protected by GEE preloading. Protection against malonate (Fig. 5A) and MPP^+ (Fig. 5B) in the dopamine population was also observed with ≥ 1 mM GEE. Significant toxicity in the GABA population was not observed with either mitochondrial inhibitor.

Elevation of brain glutathione in vivo

GSH is only modestly taken up across the blood brain barrier (BBB) (Cornford et al., 1978; Kannan et al., 1990). Little is known regarding the transport of GEE and if peripherally administered GEE can elevate brain tissue levels of GSH. To study this, GEE was delivered either centrally into the left cerebral ventricle or peripherally via subcutaneous administration for 28 days in Sprague–Dawley rats using an Alzet osmotic minipump implanted under the skin at the back. Prior to

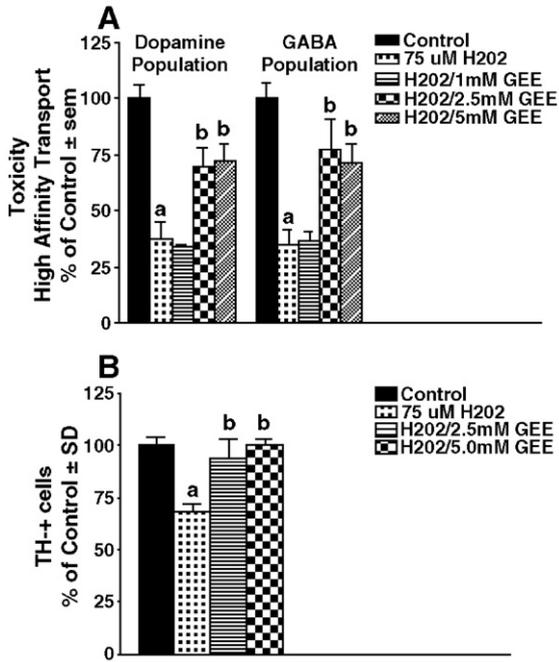


Fig. 4. Neuroprotection with glutathione loading against oxidative stress in vitro. Cultures were exposed to 1–5 mM GEE for 24 h prior to exposure to 75 μ M H₂O₂ for 6 h. Cells were allowed to recover for 3 days and toxicity assessed by (A) a functional assay for high affinity uptake for radiolabeled dopamine and GABA and (B) counts of tyrosine hydroxylase (TH) positive cells. *n* is from 3 to 4 experiments per condition. ^aDifferent from control; ^bdifferent from H₂O₂ alone.

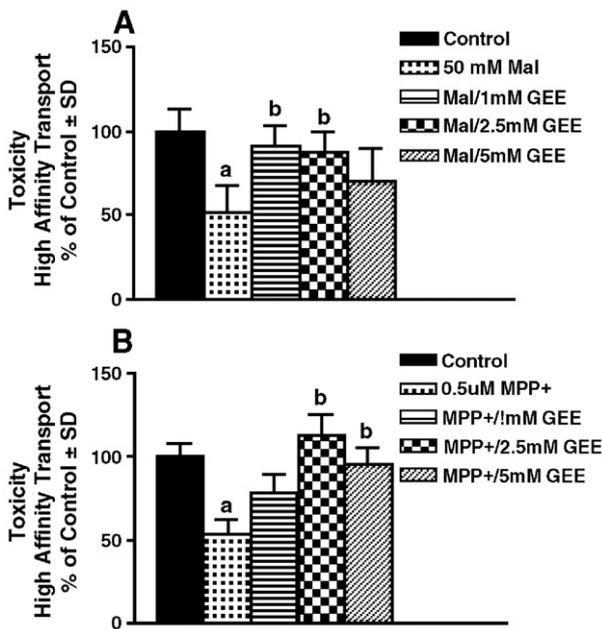


Fig. 5. Neuroprotection with glutathione loading against mitochondrial inhibition in vitro. Mesencephalic cultures were preloaded for 24 h with 1–5 mM glutathione monoethyl ester (GEE) prior to an additional 24 h incubation with (A) the complex II inhibitor malonate (50 mM) or (B) the complex I inhibitor MPP⁺ (0.5 μ M). Cells recovered for 3 days before assessing toxicity by measurement of the high affinity transport of dopamine. Toxicity was only observed in the mesencephalic dopamine population. *n* is from 3 to 4 experiments for A and 2 experiments for B run in duplicate per condition. ^aDifferent from control; ^bdifferent from mitochondrial inhibitor alone.

initiation of these studies, the stability of GEE was tested by incubating a 50 mg/ml solution of GEE in the dark at 37°C for 0–28 days and monitoring for the appearance of breakdown products by HPLC. No peaks for GSH, cysteine, glutamate, glycine or any dipeptides were observed at any time points, indicating that GEE remains stable over the course of the experiment. MPP⁺ stability was demonstrated previously (Yazdani et al., 2006). Intraventricular delivery of GEE significantly elevated brain levels of GSH on the side ipsilateral to infusion (Figs. 6A–C). GEE at ≥ 5 mg/kg/day significantly increased striatal levels of GSH, whereas cortical levels were elevated with 10 mg/kg/day. There was a trend towards increased GEE in hippocampus, but results were not statistically significant. The elevation of GSH was restricted to the side of infusion. In contrast, peripheral administration of 0.2–50 mg/kg/day GEE subcutaneously delivered by osmotic pump for 28 days did not result in elevation of GSH levels in any brain region examined (Figs. 7A–C).

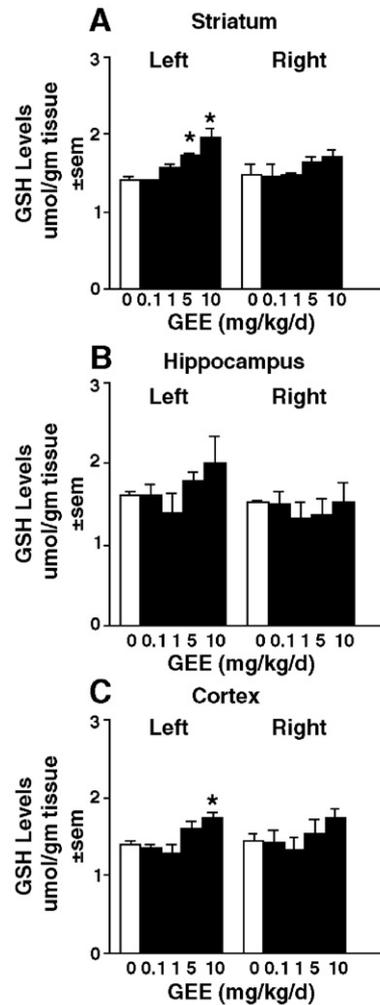


Fig. 6. Glutathione levels following icv infusion of GEE. Sprague–Dawley rats were implanted with an Alzet osmotic minipump under the skin at the back. A cannula was stereotaxically placed into the left cerebral ventricle (icv). Glutathione monoethyl ester (GEE) was dissolved in saline and delivered by the pump at 0.1–10 mg/kg/day. Animals were examined at the end of 28 days, and the indicated brain areas removed for HPLC determination of GSH. *n* is from 4 to 6 animals per group. *Different from saline treated controls.

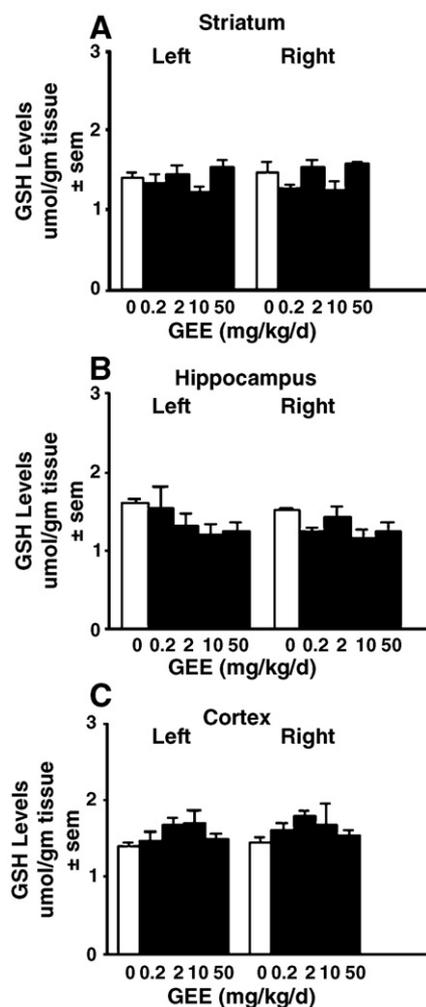


Fig. 7. Glutathione levels following subcutaneous infusion of GEE. Rats were implanted with osmotic minipumps under the skin at the back. Pumps were loaded with 0.2–50 mg/kg/day glutathione monoethyl ester (GEE) for delivery for 28 days. At the end of pump delivery, the brains were removed and left and right brain regions were analyzed for total GSH by HPLC. *n* is from 4 to 6 animals per group.

Glutathione ethyl ester provides protection against dopamine loss due to chronic mitochondrial impairment in vivo

Mitochondrial deficits are found in the sporadic PD population and are thought to be a contributing factor to dopamine cell degeneration. While acute models of mitochondrial impairment have been used in many past studies to model PD, chronic mitochondrial perturbation is thought to better replicate the ongoing pathophysiology in the disease. To this end, our laboratory has developed a chronic model of mitochondrial impairment in rat using central delivery of MPP⁺ (Yazdani et al., 2006). Chronic delivery of 0.142 mg/kg/day MPP⁺ for 28 days into the left lateral ventricle caused a selective loss of striatal dopamine with no significant loss of 5-HT, GABA or glutamate (Fig. 8A). As shown previously (Yazdani et al., 2006), this loss is only observed ipsilateral to infusion and results in the progressive degeneration of substantia nigra neurons. Striatal dopamine levels were 10.45±0.31 and 10.77±0.46 μg/g

tissue±SEM in left and right sides, respectively, in saline-I treated controls and 3.82±0.49 and 11.05±0.32 μg/g tissue±SEM in left and right striata, respectively, from 0.142 mg/kg/day MPP⁺ treated animals.

To determine if GEE could protect against striatal dopamine loss caused by chronic central MPP⁺ infusion, rats were given either saline-I (0.108 mg/kg/day), GEE (10 mg/kg/day), MPP⁺ (0.142 mg/kg/day) or GEE plus MPP⁺ for 28 days into the left cerebral ventricle (icv) via osmotic pump (Figs. 8B and C).

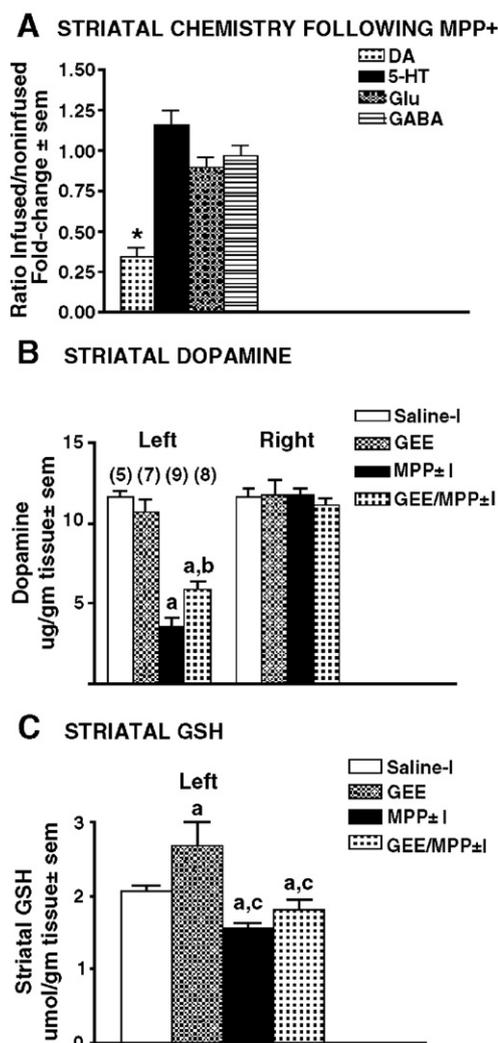


Fig. 8. Chronic MPP⁺ produces selective loss of striatal dopamine that is partially protected with co-infusion of GEE. Sprague–Dawley rats were administered either saline-I (0.108 mg/kg/day), glutathione monoethyl ester (GEE, 10 mg/kg/day), MPP⁺-I (0.142 mg/kg/day, free base), or GEE plus MPP⁺-I for 28 days into the left cerebral ventricle via an osmotic minipump. (A) Selectivity of damage was assessed by HPLC measurement of left and right striatal levels of dopamine (DA), 5-HT, glutamate (Glu) and GABA 28 days following MPP⁺-I infusion. Only DA levels were found to be reduced. *n* is from 9 animals. *Different from non-infused side and saline treated control. (B) Striatal DA on the contralateral (right) sides showed no differences with any of the treatments. MPP⁺-I infusion reduced DA on the ipsilateral (left) side. GEE co-infusion with MPP⁺ provided partial protection. The *n* for each group is given in parenthesis. ^aDifferent from saline control; ^bdifferent from MPP⁺ alone. (C) Striatal glutathione (GSH) levels were elevated following GEE infusion, but were significantly lower with MPP⁺ or MPP⁺ plus GEE. ^aDifferent from saline control; ^cdifferent from GEE alone.

After 28 days, MPP⁺ reduced striatal dopamine levels to 30% of saline-I treated controls ipsilateral to infusion. Concurrent administration of GEE with MPP⁺ provided partial protection, with dopamine levels 51% of control and 68% higher than with MPP⁺ alone. Dopamine levels on the contralateral side were unaffected (Fig. 8B). GSH levels were decreased by 25% on the left side with MPP⁺ (Fig. 8C). GEE alone enhanced GSH levels by 30% on the ipsilateral side. In animals treated concurrently with GEE and MPP⁺, GSH levels were 88% of controls. GSH levels in this group were not significantly different from either saline or MPP⁺, suggesting that GEE may have partially preserved GSH levels in MPP⁺ treated animals.

Discussion

The present work had 2 objectives. The first was to characterize the ability of GSH or its ethyl ester to elevate intracellular GSH, measure the half-life for clearance of preloaded GSH and determine the ability of peripherally delivered GSH to elevate brain levels. The second objective was to assess whether elevation of intracellular GSH in vitro and in vivo would protect dopamine neurons from damage due to oxidative stress or mitochondrial impairment. These issues are important to consider as GSH is currently in use by some clinicians as a therapeutic treatment for PD and there is little specific information on its uptake or clearance by neurons, although there is evidence in animal models to support a neuroprotective role.

With respect to the first objective, our findings show that the ethyl ester of GSH, but not GSH per se, significantly elevated intracellular GSH in mesencephalic cultures. In a variety of non-neuronal cell types, GSH is not elevated by exogenous addition (Dolphin et al., 1989) and our work in mesencephalic mixed cultures indicates that this is also true for neurons and glia. In contrast, the monoethyl ester of GSH readily increases intracellular GSH levels several fold. Some toxicity was noted when loading cultures with GEE. Toxicity was not found with equivalent amounts of GSH, suggesting that toxicity was related to the ethyl ester moiety. Toxicity with the ethyl esters of GSH has been noted previously (Anderson et al., 1985; Scaduto et al., 1988; Tsan et al., 1989; Gale et al., 1990) and may be due to the ethanol formed as GSH is released. One report attributes toxicity to impurities in the ester compound rather than to ethanol formation (Meister, 1991). The finding of toxicity with the ethyl ester of GSH indicates caution with its use in vivo.

Despite the rise in intracellular GSH levels with GEE, the issue of BBB permeability is also a potential constraint to elevation of brain levels of GSH in vivo. There is a carrier mediated transport of GSH across the BBB (Kannan et al., 1990), but it is found to be of low capacity (Cornford et al., 1978). Indeed, brain extraction of radiolabeled GSH after intracarotid injection was only 0.5% (Cornford et al., 1978). The issue of BBB permeability to GSH is somewhat moot given that extracellular GSH is incapable of elevating intracellular levels. The ability of peripherally administered GEE to elevate brain levels of GSH has not been examined previously. Findings reported here indicate that subcutaneous administration of up to

50 mg/kg/day for 28 days is not capable of increasing brain tissue levels of GSH. Only central delivery of GEE into the lateral ventricle was found to significantly elevate GSH levels. Elevation of GSH was only observed on the infused side and may be due to the anterior placement of the cannula into the ventricle (Yazdani et al., 2006).

The half-life for clearance of preloaded GSH was 10.14 h. This has implications for the timing of GSH administration and suggests that daily administration would be required to maintain an elevation in intracellular GSH. The half-life for clearance was not significantly different from cells that had undergone prior depletion of GSH with BSO. Basal levels of GSH in BSO treated cultures were approximately half that of control cultures throughout the experiment. These findings suggest that clearance of exogenously loaded GSH is not regulated by intracellular GSH concentrations unlike regulation of GSH synthesis by feedback inhibition of gamma-glutamylcysteine synthetase (Anderson and Lou, 1998).

Given our findings that GSH per se does not elevate brain or neuronal intracellular levels, what would account for the reported positive benefits of intravenous GSH in PD patients (Sechi et al., 1996; and see *Official Journal of the National Parkinson Foundation*, vol. XVI issue 4, Fall 2005)? Serum contains the GSH catabolizing enzyme, gamma glutamyltranspeptidase (Magnani et al., 1984). This would suggest that GSH is metabolized to cysteinylglycine and glutamate in the bloodstream. Cysteinylglycine may serve as a substrate for GSH biosynthesis (Anderson et al., 1985; Boyd-Kimball et al., 2005), but whether it crosses the BBB is unknown. The restriction of the BBB to small peptides, however, makes the uptake of cysteinylglycine unlikely (Cornford et al., 1978). Cysteinylglycine could be further metabolized by dipeptidases to cysteine and glycine. Cysteine is rate limiting in GSH biosynthesis (Wang and Ballatori, 1998). Cysteine may be transported across the BBB by the L-carrier system (Wade and Brady, 1981). A caveat to this is that elevation of cysteine in brain could pose a potential problem as cysteine can bind to glutamate receptors to produce an excitotoxicity (Olney et al., 1990; Puka-Sundvall et al., 1995). Additionally, it has been reported that exogenous GSH added to human plasma is rapidly oxidized to glutathione disulfide by the side oxidase activity of gamma glutamyltranspeptidase (Magnani et al., 1984), although it is unclear how this would relate to protection. Assuming that future evidence supports a protective role for peripherally administered GSH in PD, the fate of GSH and the mechanism underlying any benefit remain to be clarified.

A second objective of this study was to determine whether intracellular elevation of GSH would protect dopamine neurons both in vitro and in vivo from oxidative stress or mitochondrial impairment. In vitro, GEE preloading of mesencephalic cultures provided near complete protection of both the mesencephalic dopamine and GABA populations from oxidative stress due to H₂O₂ exposure. Significant protection was observed with 2.5 and 5 mM GEE. Mitochondrial impairment with the complex II inhibitor malonate or complex I inhibitor MPP⁺ was toxic to dopamine, but not GABA neurons. GEE preloading completely protected dopamine neurons against malonate toxicity and

MPP⁺ toxicity with 1 mM GEE and 2.5 mM GEE, respectively. There was less protection with 5 mM GEE preloading, and this could be due to toxicity from GEE per se at the higher concentration.

Acute *in vivo* models of mitochondrial impairment have been used extensively to model PD. Chronic, generalized mitochondrial deficits, however, are found in sporadic PD (Parker et al., 1989; Schapira et al., 1989). Chronic mitochondrial impairment may, therefore, better replicate the disease process and, importantly, may better predict clinical efficacy. Our laboratory has been developing a chronic model of central delivery of MPP⁺ in rats (Yazdani et al., 2006). Using this model, we examined whether co-administration of MPP⁺ with GEE provided protection. MPP⁺ delivered into the left lateral ventricle for 28 days at 0.142 mg/kg/day produced selective striatal loss of dopamine and, as we have reported previously, loss of tyrosine hydroxylase positive and Nissl positive neurons in the substantia nigra pars compacta (Yazdani et al., 2006). GEE at 10 mg/kg/day, a concentration found to significantly elevate GSH in the striatum, provided partial protection from loss of striatal dopamine. Protection in this chronic model of mitochondrial impairment, although significant, was not as robust as that observed in the acute *in vitro* treatments with an oxidative or mitochondrial insult. This likely reflects the greater challenge of a chronic mitochondrial perturbation. Alternatively, it is possible that the higher levels of intracellular GSH achieved *in vitro* (Fig. 1) as compared with *in vivo* (Fig. 6) provided more complete protection. Consistent with our findings, others have reported protection of dopamine terminals from acute MPTP toxicity with brain GSH elevation using *N*-acetylcysteine (NAC) (Munoz et al., 2004; Park et al., 2004). NAC, however, can also produce acidification (Gillissen et al., 2006) and damage to non-dopamine neurons (Munoz et al., 2004) and may, therefore, be of mixed benefit for enhancing GSH levels in cells.

Since GSH is a major antioxidant in cells, its depletion very likely contributes to the initial oxidative damage as well as the progressive damage during the latter stages of PD. The striking accumulation of oxidative damage in the SN of PD brain indicates a brain region under free radical attack. Enhancing antioxidant capabilities in the nigra, therefore, constitutes a rationale approach to preventing ongoing damage. These efforts for the most part have met with only modest success. High dose ascorbate plus α -tocopherol supplementation in one clinical trial delayed the need for L-Dopa therapy by 2–4 years in 75% of PD patients (Fahn, 1992). Treatment with α -tocopherol alone has been shown to have no effect (Shoulson, 1998). There have been some promising results with co-enzyme Q10. In PD patients treated for 16 months with 1200 mg per day of CoQ10, there was a 44% slowing of disability between the CoQ10 group and the placebo group. CoQ10 has known antioxidant properties, but may also enhance mitochondrial function, serve as a cofactor for uncoupling proteins (Echtay et al., 2000) or inhibit the activation of the mitochondrial permeability transition (Echtay et al., 2002). Which of these properties of CoQ10 contributes to protection is at present unclear.

Increasing GSH levels in brain would also be a rationale approach to antioxidant supplementation. This is compelling for 2 reasons. Firstly, there is a known deficit of GSH in the nigra in PD and, secondly, GSH serves many different roles in the cell in addition to it being an antioxidant. It is possible that loss of a number of functions of GSH in addition to its antioxidant capabilities contributes to dopamine cell susceptibility. Supplementation with an antioxidant other than GSH may overcome some, but not all of the consequences produced by loss of GSH. Thus, a strong case can be made for specifically enhancing GSH in brain. The best approach to doing this, however, is more problematic. GEE, although able to elevate intracellular levels of GSH, required central delivery and *in vitro* demonstrated some toxicity. Cysteine is taken up by the BBB and can be transported into neurons for GSH biosynthesis, but potentially can interact with glutamate receptors to produce excitotoxicity (Olney et al., 1990; Puka-Sundvall et al., 1995). NAC can elevate intracellular GSH levels, but its ability to cross the BBB is controversial (McLellan et al., 1995). In addition, NAC can cause acidification (Gillissen et al., 2006) and is also reported to cause neuronal toxicity (Munoz et al., 2004). Enhancing γ -glutamylcysteine synthetase expression and activity through the use of viral vectors may be one possible approach. The issue of how to best restore GSH levels requires further investigation.

In summary, similar to other cell types, GSH was not able to elevate intracellular levels in neuronal cells, whereas the ethyl ester of GSH resulted in significant increases in GSH concentration. Use of the ethyl ester to elevate GSH, however, was problematic in that it produced toxicity at the higher concentration used, needed to be delivered centrally in order to elevate brain GSH and had a half-life for clearance that would require daily supplementation in order to maintain elevated levels. Elevation of GSH was found to provide significant protection from dopamine cell damage due to oxidative stress or mitochondrial impairment both *in vitro* and *in vivo*. Therefore, despite the difficulty in optimizing the best approach to supplementation of GSH levels in neuronal cells, given the multifunctional roles served by GSH, this remains an avenue of significant therapeutic potential.

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