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ORIGINAL PAPER

Liposomal-Glutathione Provides Maintenance of Intracellular Glutathione and Neuroprotection in Mesencephalic Neuronal Cells

Gail D. Zeevalk · Laura P. Bernard · F. T. Guilford

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Abstract A liposomal preparation of glutathione (GSH) was investigated for its ability to replenish intracellular GSH and provide neuroprotection in an in vitro model of Parkinson's disease using paraquat plus maneb (PQMB) in rat mesencephalic cultures. In mixed neuronal/glial cultures depleted of intracellular GSH, repletion to control levels occurred over 4 h with liposomal-GSH or non-liposomal-GSH however, liposomal-GSH was 100-fold more potent; EC_{50s} 4.75 μM and 533 μM for liposomal and non-liposomal-GSH, respectively. Liposomal-GSH utilization was also observed in neuronal cultures, but with a higher EC_{50} (76.5 μM), suggesting that glia facilitate utilization. Blocking γ-glutamylcysteine synthetase with buthionine sulfoxamine prevented replenishment with liposomal-GSH demonstrating the requirement for catabolism and resynthesis. Repletion was significantly attenuated with endosomal inhibition implicating the endosomal system in utilization. Liposomal-GSH provided dose-dependent protection against PQMB with an EC_{50} similar to that found for repletion. PQMB depleted intracellular GSH by 50%. Liposomal-GSH spared endogenous GSH during PQMB exposure, but did not require GSH biosynthesis for protection. No toxicity was observed with the liposomal preparation at 200-fold the EC_{50} for repletion. These findings indicate that glutathione supplied in a liposomal formulation holds promise as a potential therapeutic for neuronal maintenance.

Keywords Glutathione · Neurodegeneration · Autism · Schizophrenia · Parkinson's disease

G. D. Zeevalk (✉) · L. P. Bernard
Department of Neurology, UMDNJ-Robert Wood Johnson Medical School, Staged Research Building, 661 Hoes Lane, Piscataway, NJ 08854, USA
e-mail: zeevalgd@umdnj.edu

F. T. Guilford
Your Energy Systems, LLC, Palo Alto, CA 94301, USA

Introduction

Oxidative stress and free radical damage to cells is a consequence of life in an aerobic environment. Many cellular defenses have evolved to protect cells. Glutathione and its associated enzymes form one of the major antioxidant defense systems in all cells. A strong antioxidant defense is particularly important in the brain as this organ relies solely on aerobic metabolism which can generate significant reactive oxygen species (ROS). In addition, neurotransmitters such as dopamine generate ROS during their metabolism and can form reactive quinones [1] that can conjugate with proteins and alter protein function. Most neurodegenerative diseases involve oxidative damage [2, 3]. Furthermore a number of neuropathological and neuropsychiatric conditions have been shown to specifically present derangements in the glutathione system. In Parkinson's disease (PD), there is a 40–50% decrease in total GSH [4–6]. The decrease in GSH is found in the brain region most affected in the disease, i.e., the substantia nigra and is thought to be one of the earliest dysfunctions in the disease process [5]. GSH levels in the prefrontal cortex of patients with schizophrenia are decreased by 52% [7]. Evidence indicates that this is due to a polymorphism in the catalytic subunit of glutamylcysteine ligase, the first enzyme involved in the synthesis of GSH [8]. Plasma levels of GSH were recently shown to be decreased in patients with

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autism spectrum disorders [9, 10] and in the blood of children with Down syndrome [11].

Given the contribution of GSH in defense of cells from oxidative stress, as well as its more recently delineated role in protein modification and cell signaling [12, 13], maintenance of intracellular GSH is of significant import to cell function and viability. Neurons, like many other cell types, however, do not have a transport mechanism for uptake of exogenous GSH [14]. Other strategies have, therefore, been tried in an effort to elevate or maintain intracellular GSH. Cysteine is the rate limiting amino acid in GSH biosynthesis and the L-amino acid uptake system aids to transport cysteine across the blood brain barrier to maintain adequate supplies to the brain [15]. Cysteine supplementation can replenish GSH [16], however, elevations in extracellular cysteine can damage neurons via an excitotoxic process [17, 18]. *N*-acetyl-cysteine (NAC), a derivative of cysteine has been shown in animal studies to provide neuroprotection in an ischemia model and in a 6'OHDA model of Parkinson's disease [19, 20], but can cause acidification [21] and neuronal toxicity [19] as well as induce astroglial and microglial activation [19]. Ethyl ester derivatives of glutathione readily increase intracellular GSH both in vitro and in vivo [14]. The ethanol formed following esterase cleavage of the ethyl ester, however, can cause toxicity to neurons [14]. Elevations in extracellular GSH per se can also enhance neuronal toxicity through modulation of NMDA receptors [22] and enhancement of excitotoxicity and ischemic damage [23, 24].

Here we examine a liposomal formulation of GSH encapsulated in lipid vesicles made from lecithin and glycerol as a safe alternative for maintenance of neuronal GSH. This formulation of GSH was recently shown to have antioxidant and anti-atherogenic properties [25]. Apolipoprotein-E deficient mice fed daily for 2 mo with 50 mg/kg/d liposomal-GSH showed a significant reduction in lipid peroxides, macrophage cholesterol mass and a reduction in atherosclerotic lesion development. Using mesencephalic neuronal cultures, the present study examined the ability of liposomal-GSH to replenish intracellular GSH following its depletion and to provide neuroprotection in the paraquat plus maneb environmental model of Parkinson's disease [26]. In addition, studies were conducted to provide a more mechanistic understanding of the utilization of liposomal-GSH for neuronal repletion and protection.

Materials and methods

Materials

Acivicin was purchased from Enzo Life Sciences Inc. (Plymouth, PA). Maneb was obtained from ChemService (West Chester, PA). The purity of maneb is reported to be >95%. N2 supplement was from Invitrogen (Carlsbad, CA). Liposomal-glutathione was provided by *Your Energy Systems* (Palo Alto, CA). The liposomal preparation of glutathione consists of reduced glutathione encapsulated in hydroxylated lecithin (1.5%) and suspended in water containing 15% glycerol and 0.1% potassium sorbate as preservative. Glutathione content per ml of undiluted stock was 82 mg. Stock solutions of liposomal-GSH and non-liposomal-GSH were pH adjusted to 6.0 prior to use. All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Mixed Mesencephalic Cultures

Timed pregnant Sprague–Dawley rats were obtained from Charles River (Wilmington, MA). The ventral mesencephalon from embryonic day 15 rat fetuses were dissected, pooled, dissociated and plated as described previously [27]. Briefly, cells were mechanically dissociated by trituration and plated at 2.5×10^5 cells/cm^2 onto polyornithine and serum coated substrate in 48 well trays. Cultures were incubated at 37°C in a 95% air/5% CO_2 humidified atmosphere. Cultures were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5 mM glucose, 2 mM glutamine, 2.2 g/L bicarbonate plus 10% fetal bovine and horse serums (DMEM/serum). 5-Fluoro-2-deoxyuridine (13 μg) plus uridine 33 μg) was added from days 7–9 in vitro to reduce glial growth. Under these conditions, cultures contain approximately 70% neurons and 30% glia. The medium was supplemented with 5 mM glucose every 72 h until the conclusion of the studies.

Mesencephalic Neuronal Enriched Cultures

Neuronal enriched cultures were established as described previously [28]. Mesencephalic cells were isolated from embryonic day 15 rat brains as described above. Within 4–6 h after plating in DMEM/serum, the medium was replaced with DMEM supplemented with serum-free N2 (Invitrogen, Eugene, OR) as described by [29]. N2 supplement containing 8.6 μM insulin, 1 mM human transferrin, 2 μM progesterone, 10 mM putrescine and 3 μM selenite promotes the growth of post-mitotic neurons, but not astroglial cells.

Glutathione Measurement

Total glutathione was measured by HPLC as we have previously described [30]. Briefly, just prior to extraction of intracellular contents with 0.2 N perchloric acid, cultures were rinsed 3 times with warm bicarbonate buffered

Krebs–Ringer (KRB; 119 mM NaCl, 4.8 mM KCl; 25 mM 3-[*N*-morpholino]propane sulfonic acid; 1.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5.5 mM glucose, 23.8 mM sodium bicarbonate, pH 7.4). The acid extract was neutralized to—pH 5.0 with K_2CO_3 derivatized with σ—phthalaldehyde and separated on a C18 column using a gradient of sodium acetate and methanol and a Beckman System Gold HPLC with fluorometric detection. Quantification was by comparison with a standard of glutathione.

General Cell Viability Assay

Overall cell viability was determined using the Cell Titer-Blue™ Cell Viability Assay kit (Promega, Madison, WI). Viability is determined by the ability of cells to reduce resazurin into resorufin. The procedure followed that supplied with the kit using a 1 h incubation. Product formation was determined in a CytoFluor 4000 microplate reader (PerSeptive Biosystems) set at an excitation/emission of 530/580. Two vehicles were used to control for nonspecific antioxidant or neuroprotective effects. Vehicle 1 was a solution of liposomal constituents, lecithin plus glycerol. Vehicle 2 was liposomal vesicles made of hydroxylated lecithin surrounding water. Vehicles 1 and 2 were diluted to the same concentration as that found in the final concentration of liposomal-GSH used for treatment. Neither vehicle showed any antioxidant or neuroprotective efficacy and results from toxicity assays report the combined results from both vehicles.

Viability of Dopamine Neurons

Toxicity to the dopamine neuronal population in the mesencephalic cultures was determined by a functional assay for the high affinity transport of a ^{3}H-labeled dopamine as routinely reported [14, 30, 31]. ^{3}H-dopamine (final concentration 37.5 nM) uptake was carried out in a HEPES buffer (25 mM HEPES, 5.6 mM glucose, 125 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$ containing 1 mM ascorbate, and 100 μM pargyline) for 15 min at 37°C. Following extensive washing, radioactivity in the cell extract was quantified by scintillation counting. Values were corrected for background binding determined in samples incubated at 4°C.

γ-Glutamyltranspeptidase Assay

γ-Glutamyltranspeptidase (γ-GT) was determined by a spectrophotometric assay that measures the release of *p*-nitroaniline from γ-glutamyl-*p*-nitroanilide. Cultures were put into 1 ml of KRB containing 2 mM γ-glutamyl-*p*-nitroanilide plus 20 mM glycinylglycine and incubated for 1 h in a 5% CO_2 incubator, 37°C. Some wells contained the γ-GT inhibitor acivicin (1 mM). For competition studies, 2 mM liposomal-GSH or 2 mM non-liposomal-GSH was added to the wells just prior to the addition of γ-glutamyl-*p*-nitroanilide. After incubation, 0.6 ml media was added to 0.15 ml glacial acetic acid and the absorbance read at 410 nm. Quantification was by comparison to a standard curve derived from *p*-nitroaniline.

Glutathione S-Transferase Assay

Glutathione S-transferase (GST) activity was determined via a spectrophotometric assay that measures the conjugation of glutathione to chlorodinitrobenzene (CDNB). The assay was carried out in 0.1 M phosphate buffer to which was added various concentrations of non-liposomal-GSH or liposomal-GSH (0.1–1 mM) and CDNB (1 mM). The reaction rate was monitored at 340 nM for 2 min. The reaction was initiated by the addition of 50 μl of cytosol from rat brain homogenate and the reaction monitored for an additional 2 min. Any background rate was subtracted from the rate with brain homogenate. Results are reported as change in absorbance per minute.

Peroxide Assay

Peroxide in solution was determined using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Eugene, OR) as we have published on previously [28]. The Ampled Red reagent (10-acetyl-3,7-dehydroxyphenoxazine) in the presence of horseradish peroxidase (HRP) reacts with peroxides to generate a fluorescent product. Wells containing 5 μM peroxide in HEPES buffer were incubated with various concentrations of liposomal or non-liposomal-GSH or vehicle for 10 min at room temperature. Reaction mix containing Amplex Red plus HRP was then added and the plate incubated an additional 5 min at room temperature. The amount of peroxide remaining in solution was determined in a CytoFluor 4000 multiwell plate reader at 530/580 ex/em. Quantification was by comparison to a peroxide standard curve.

Statistics

Data were analyzed for statistical significance by ANOVA (GraphPad Instat 3.0) with Tukey post-hoc treatment. A P value of <0.05 was considered statistically significant. EC_{50s} were calculated using Prism 2.01 and a sigmoidal dose–response equation. For each set of experiments where EC_{50s} were derived for intracellular GSH repletion, Top and bottom values were set as follows: the top value was the intracellular GSH level in untreated control cultures incubated in Krebs–Ringer buffer for 4 h; the bottom value was the intracellular GSH level in DEM treated cultures

further incubated in Krebs–Ringer buffer for 4 h without supplementation.

Results

Exogenous Liposomal-GSH is More Efficacious than Non-Liposomal GSH for Replenishment of Intracellular Glutathione Levels

A model for GSH depletion and optimal time for repletion was determined in mixed mesencephalic cultures. Intracellular levels of GSH were transiently reduced by a 30 min treatment with diethylmaleate (DEM), a compound that readily chelates with GSH for removal. This treatment reduced intracellular GSH by approximately 70% (Fig. 1; intracellular GSH 2.01 ± 0.23 and 0.65 ± 0.12 µmol/100 mg protein ± sem for untreated control and DEM treated cultures, respectively). Optimal repletion was carried out in Krebs–Ringer supplemented with cysteine, glycine and glutamine. Glutamine was used as the gamma-glutamyl donor instead of glutamate since glutamate causes an excitotoxicity in the mesencephalic cultures [32]. Four hours were required to return GSH levels to those found in controls not treated with DEM but incubated in Krebs–Ringer for 4 h. (Fig. 1a). Consistent with cysteine being the rate limiting amino acid for GSH repletion [16], cysteine alone was sufficient to replenish intracellular GSH in 4 h. No repletion occurred in the absence of supplements (Fig. 1a). A similar lack of repletion was found with supplementation with glutamine or glycine in the absence of cysteine (data not shown), and is consistent with findings reported by others [16].

The ability of liposomal-GSH was compared with non-liposomal-GSH for replenishment of intracellular levels of the antioxidant following DEM treatment. Both compounds restored intracellular levels by 4 h, however, liposomal-GSH was 100-fold more potent in serving as a source for intracellular GSH repletion; EC_{50s}, 4.75 µM ± 1.38 and 533 µM ± 1.35 (mean ± sem) for liposomal-GSH and non-liposomal-GSH, respectively (Fig. 1b). Cultures exposed to 1 mM liposomal-GSH for 24 h showed no toxicity as assessed by the Cell-Titer Blue viability assay. At 1 mM liposomal-GSH, viability was not statistically different from control (100 ± 6.5 and 93 ± 5.0 percent of control ± sem, $n = 3$ for control and 1 mM liposomal-GSH, respectively).

Mixed mesencephalic cultures contain both neurons and glia. To determine if liposomal-GSH could be utilized by neurons per se, neuronal enriched cultures were established as described previously [28]. Four hours after DEM treatment, liposomal-GSH supplemented KRB restored

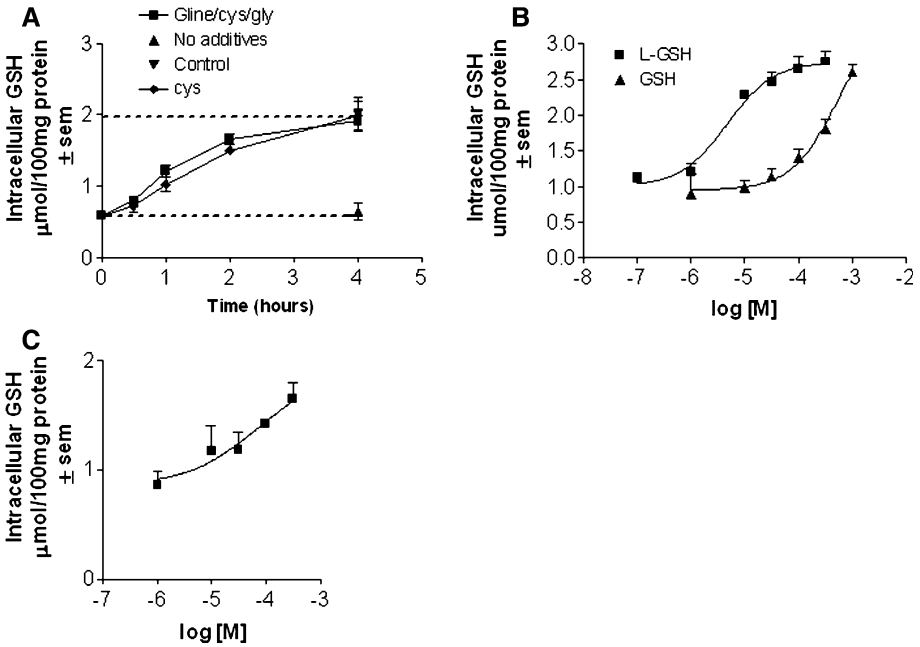

Fig. 1 **a** Replenishment of intracellular GSH was studied in mesencephalic cultures consisting of neurons and glia. Cultures were depleted of intracellular GSH with a 30 min treatment with 0.5 mM diethylmaleate (DEM) on day 8 in vitro. Repletion was followed over time in a balanced bicarbonate buffered Krebs–Ringer (KRB) supplemented with either 200 µM cysteine, 200 µM glycine and 1 mM glutamine (Gline/cys/gly); 200 µM cysteine (cys) or no additives. **b** Mixed mesencephalic cultures or **c** neuronal enriched mesencephalic cultures were depleted of GSH with DEM treatment. Cells were then incubated in KRB supplemented with various concentrations of liposomal-GSH or non-liposomal-GSH as indicated. Intracellular GSH levels were measured at 4 h by HPLC as described in Methods. The n is from (**a**) 3, (**b**) 3–5 and (**c**) 5 determinations per condition with all samples run in duplicate

intracellular GSH to 86% of controls. The potency for repletion of intracellular GSH by liposomal-GSH in neuronal enriched cultures was approximately 15-fold less than in mixed neuronal/glial cultures; EC_{50} 76.5 μM ± 1.26 (Fig. 1c).

Liposomal-GSH Requires Catabolism and Resynthesis Prior to Repletion

The mechanism by which liposomal-GSH was utilized by mesencephalic neuronal cells was investigated. To accomplish this, replenishment with liposomal-GSH in mixed mesencephalic cultures was carried out with glutathione synthesis blocked with buthionine sulfoxamine (BSO, 10 μM), an inhibitor of γ-glutamylcysteine synthetase. Liposomal-GSH returned intracellular GSH to control levels by 4 h (Fig. 2a). In contrast, in the presence of BSO, repletion was completely blocked indicating that liposomal-GSH needed to be catabolized and its constituent amino acids utilized for resynthesis of intracellular GSH.

Since catabolism and resynthesis of the GSH packaged in the lipid vesicles was required prior to its utilization for intracellular replenishment, we investigated whether acivicin, an inhibitor of the ectoenzyme γ-GT, thought to be responsible for the extracellular breakdown of GSH to a γ-glutamyl amino acid plus cysteinylglycine, would prevent liposomal-GSH utilization in the cultures. A modest, but statistically significant decrease of 32% in liposomal-GSH utilization was observed (Fig. 2b) indicating that while some GSH was processed extracellularly, the majority (~70%) of the liposomal-GSH was not processed in this manner. Studies were then conducted to determine whether liposomal-GSH would serve as substrate for γ-GT. This investigation would also address the integrity of the liposomal preparation with regards to the packaging of GSH within the lipid vesicle as leakage of GSH from vesicles might account for extracellular metabolism by γ-GT. Equimolar concentrations of liposomal-GSH or non-liposomal-GSH (2 mM) were tested for their ability to compete with 2 mM γ-glutamyl-p-nitroanilide in a γ-GT activity assay (Fig. 2c). While non-liposomal-GSH effectively

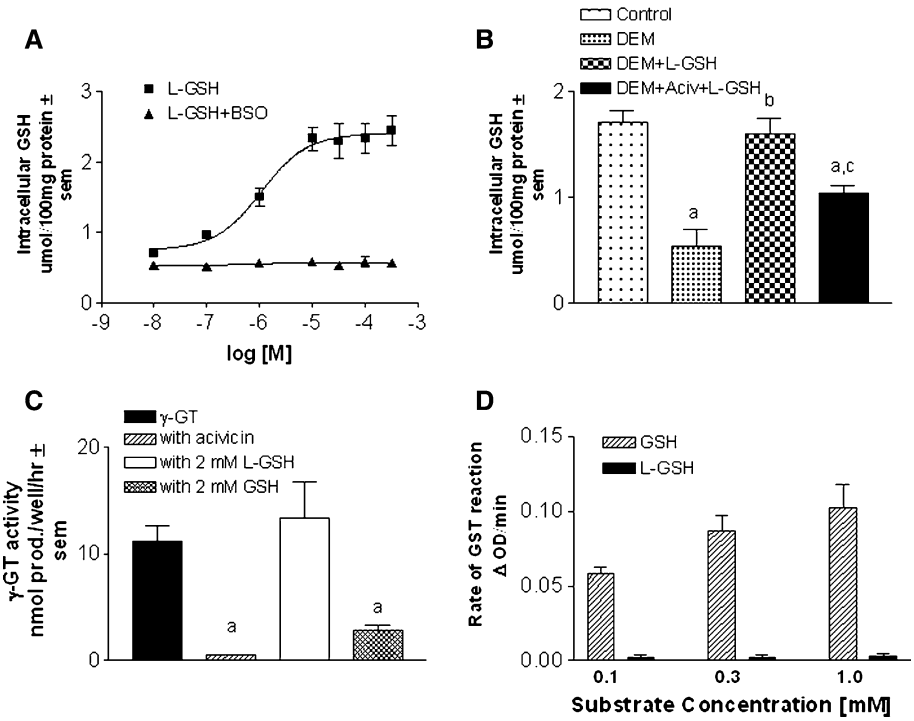

Fig. 2 **a** Depletion of intracellular GSH with DEM treatment was carried out in mixed mesencephalic cultures on day 8 in vitro as described in Methods. Cultures were then incubated in KRB supplemented with various concentrations of liposomal-GSH (L-GSH) in the presence or absence of 10 μM buthionine sulfoxamine (BSO). At 4 h, cell content was extracted and GSH levels were determined by HPLC. The *n* is from 3–5 determination per condition run in duplicate. **b** Cultures were treated as in A to deplete GSH and then incubated for 4 h with 100 μM liposomal-GSH plus or minus 1 mM acivicin. The *n* is from 3–4 determinations per condition. [a]Different from control; [b]different from DEM; [c]different from DEM + L-GSH. **c** γ-glutamyltranspeptidase (γ-GT) activity was measured in mixed mesencephalic cultures as described in Methods. Liposomal-GSH (2 mM) and non-liposomal-GSH (2 mM) were used to compete with the assay substrate, 2 mM γ-glutamyl-*p*-nitroanilide. The *n* is from 3 determinations per condition. [a]Different from control γ-GT activity. **d** GST activity was measured in rat brain cytosolic homogenates in the presence of different concentrations of either GSH or liposomal-GSH as described in Methods. Only GSH could act as substrate for GST. The *n* is from 3 determinations per condition

competed with γ-glutamyl-*p*-nitroanilide to reduce the rate of product formed by γ-GT by 75%, liposomal-GSH was without effect demonstrating that the GSH in the liposomal preparation was not available as a substrate for γ-GT. As expected, acivicin, an inhibitor of γ-GT, completely blocked activity. The lack of extravesicular GSH in the liposomal preparation was further verified by studies to examine if liposomal-GSH could directly serve as substrate for GST. Similar to what was found for γ-GT, non-liposomal-GSH, but not liposomal-GSH supported GST activity (Fig. 2d).

Utilization of Liposomal-GSH in Mesencephalic Cultures

The finding with acivicin indicated that most of the liposomal-GSH was not processed extracellularly. Liposomes can be taken up into cells via an endocytic process [33]. Once inside the cell, endosomes can fuse with lysosomes for intravesicular degradation and subsequent reutilization of constituent components [34]. To determine if the endosomal/lysosomal system was involved in the utilization of liposomal-GSH for intracellular repletion, liposomal-GSH utilization was followed in the presence of phenylarsine oxide (PAO), an endosomal inhibitor [35], or concanavalin A (ConA), shown to inhibit endosomal/lysosomal fusion [36]. PAO (1 μM) and ConA (1–30 μM) significantly attenuated liposomal-GSH utilization (Fig. 3a, b). PAO can also serve as a metabolic inhibitor [35] and could cause cell toxicity. Even though cell loss was not evident at the time of intracellular GSH determination (i.e. following 2 h of exposure), toxicity studies when assayed after a 72 h recovery indicated severe cell loss (Fig. 3c) bringing to question if blockage of GSH repletion at 2 h could have been due to compromised cells. ConA, however, showed only modest loss of cell viability at the highest dose tested (32% loss at 30 μg/ml). At 1 and 10 μg/ml, ConA was not toxic (Fig. 3d), yet produced a 48 and 60% loss, respectively, of liposomal-GSH supported repletion. Repletion of intracellular GSH by cysteine was not affected by ConA treatment. Repletion was 92 ± 2.8 percent of control with 200 μM cysteine and 89 ± 6.2 percent of control with cysteine in the presence of 10 μg/ml ConA (mean ± sem, $n = 3$ with duplicate runs). These findings provide evidence for utilization of liposomal-GSH via the endosomal/lysosomal pathway.

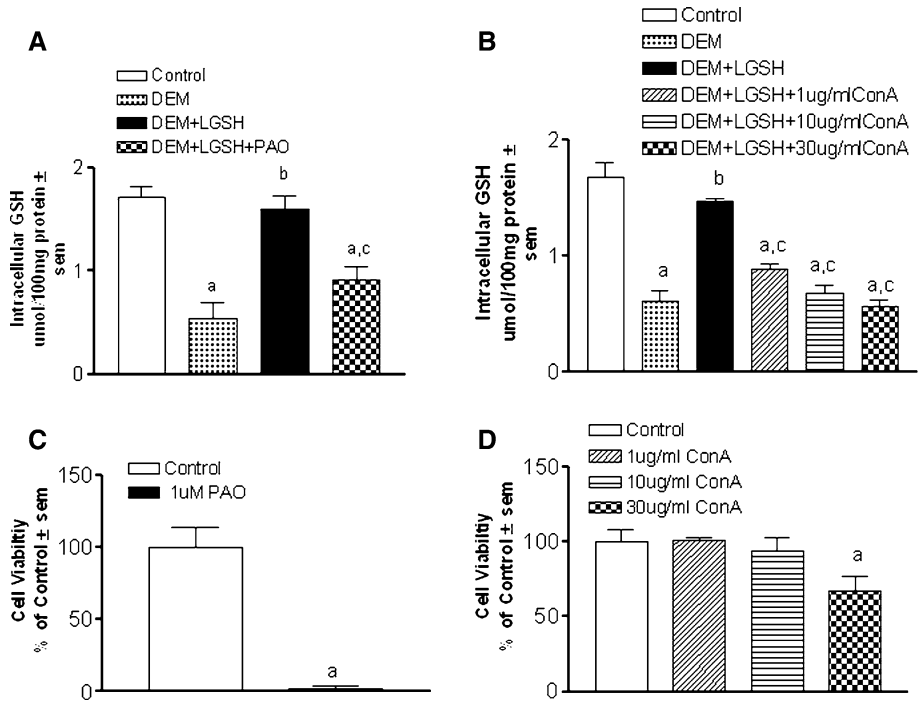

Fig. 3 The effect of endosomal inhibition on intracellular GSH repletion with liposomal-GSH was tested in mixed mesencephalic cultures transiently depleted of GSH with DEM treatment. Replenishment was carried out for 2 h in KRB containing 100 μM liposomal-GSH in the presence or absence of **a** 1 μM phenylarsine oxide (PAO) or **b** 1–30 μg/ml concanavalin A (ConA). Intracellular GSH content was measured by HPLC from cell extracts. PAO and ConA significantly inhibited liposomal-GSH supported repletion. Toxicity of **c** 1uM PAO or **d** 1–30 μg/ml ConA was determined by the Cell Titer Blue Viability Assay in cultures treated for 2 h with the agents followed by 72 h of recovery in growth medium. This viability assay measures the ability of cells to reduce resazurin to resorufin. The *n* is from 3 separate experiments with each parameter run in duplicate. [a]Different from control; [b]different from DEM; [c]different from DEM plus L-GSH

Liposomal-GSH Provides Neuroprotection in an Environmental Model of Parkinson's Disease

Perturbation of intracellular glutathione is associated with a number of disease states including PD and is thought to be a contributing factor to the loss of midbrain dopamine neurons in PD [5, 37]. Paraquat plus maneb are widely used pesticides that have been linked to PD [38, 39]. In animal models, paraquat plus maneb causes loss of dopamine neurons [26] via a mechanism that involves oxidative stress and the GSH system [14, 40]. Here we investigated if liposomal-GSH provided neuroprotection in an in vitro paraquat plus maneb model. Mixed mesencephalic cultures were exposed to 45 μM paraquat plus 45 μM maneb for 4 h in KRB in the presence or absence of various concentrations of liposomal-GSH. After exposure, cultures were returned to their growth medium and allowed to recover for 72 h. Control cultures were incubated in Krebs–Ringer buffer for 4 h and then returned to growth medium for a 72 h recovery. General cell viability was then assessed. Treatment with paraquat plus maneb reduced overall cell viability by approximately 80%. Liposomal-GSH provided dose-dependent protection with an EC_{50} for protection of 14.1 μM ± 1.75 (Fig. 4a). Vehicle control was without affect.

Mesencephalic cultures contain a small population of presumptive midbrain dopamine neurons which are representative of the population of neurons that degenerate in PD. In order to determine if paraquat plus maneb was toxic to dopamine neurons and if liposomal-GSH protected these neurons from the toxic insult, viability was assessed in the dopamine population by a functional assay that monitors the high affinity transport of dopamine. Our past work has shown that assessment of toxicity in the dopamine population via monitoring of DAT activity parallels that observed with counts of tyrosine hydroxylase positive cells when a recovery period of 48–72 h is allowed between treatment and toxicity assessment [31]. Paraquat plus maneb caused a 75% loss of dopamine cell viability which was similar to the extent of loss in the total cell population. Liposomal-GSH dose-dependently provided protection of dopamine neurons with an EC_{50} of 10.5 μM ± 1.08. The

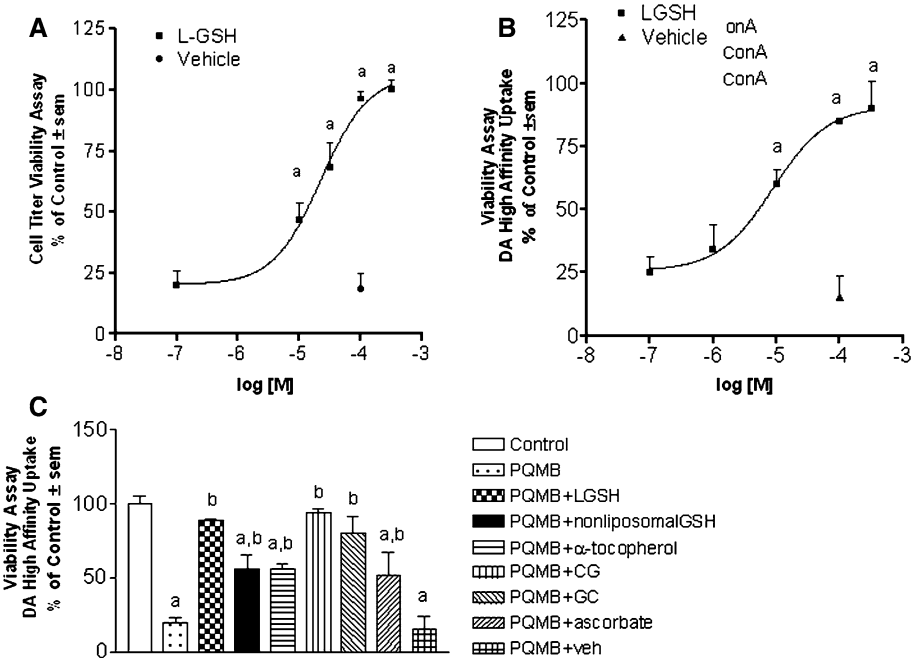

Fig. 4 Neuroprotection by liposomal-GSH was tested in mixed mesencephalic cultures using an environmental model of Parkinson's disease. Cultures were treated on day 8 in vitro with 45 μM paraquat plus 45 μM maneb plus or minus various concentrations of liposomal-GSH for 4 h in KRB. Following treatment, cultures were returned to complete medium and allowed to recover for 72 h. **a** Overall toxicity in the cultures was determined by the Cell Title Blue Viability assay. **b** Toxicity specific to the dopamine neuronal population in the cultures was determined by a functional assay that measured the high affinity transport of ^{3}H-labeled dopamine. **c** Comparison of protection from paraquat plus maneb exposure in dopamine neurons by several agents known to have antioxidant properties was carried out in mesencephalic cultures on day 8 in vitro. Cultures were treated with paraquat plus maneb as described in 3A in the presence or absence of liposomal-GSH, non-liposomal-GSH, α-tocopherol, cysteinylglycine and glutamylcysteine at 100 μM, ascorbate at 400 μM and combined liposomal vehicles 1 and 2 at a concentration of empty liposomes or lecithin plus glycerol equivalent to that found in 100 μM liposomal-GSH. The *n* is from 3–5 for **a** and **b** and for the antioxidant compounds shown in **c**. The *n* is from 6–8 for combined control and paraquat plus maneb data shown in **c**. **a** and **b**: [a]different from paraquat plus maneb; **c**: [a]different from control, [b] different from paraquat plus maneb

vehicle for lipid vesicles, i.e. lecithin plus glycerol provided no protection (Fig. 4b).

Protection by liposomal-GSH was compared with several other compounds with known antioxidant properties. Liposomal-GSH at 100 μM was compared with equimolar concentrations of non-liposomal-GSH, alpha-tocopherol, and the precursor GSH dipeptides cysteinylglycine and glutamylcysteine. Ascorbate was tested at 400 μM to approximate reported brain levels. While all the antioxidants provided significant neuroprotection, only the GSH precursor dipeptides showed a similar potency to liposomal-GSH to provide complete protection at 100 μM (Fig. 4c).

Liposomal-GSH Spares Endogenous GSH during Paraquat Plus maneb Exposure but Protection does not Require GSH Biosynthesis

In order to determine if liposomal-GSH provided protection by maintaining intracellular GSH levels, mixed mesencephalic cultures were treated with paraquat plus maneb and liposomal-GSH while GSH biosynthesis was blocked with BSO. As shown in Fig. 5a, paraquat plus maneb treatment caused a 79% toxicity in the dopamine population. Liposomal-GSH provided significant protection. Inhibition of GSH biosynthesis with BSO during paraquat plus maneb exposure did not prevent neuroprotection (Fig. 5a) suggesting that the utilization of liposomal-GSH for GSH biosynthesis was not required for protection.

Intracellular GSH levels were significantly decreased following 4 h of treatment with paraquat plus maneb to approximately 50% of control. The presence of liposomal-GSH during paraquat plus maneb exposure spared intracellular GSH as levels remained similar to controls (Fig. 5b). When BSO was added with paraquat plus maneb, intracellular GSH was further reduced to 25% of control values (control shown in Fig. 5b), indicating the active depletion and resynthesis of GSH during paraquat plus maneb exposure (Fig. 5c). In the presence of liposomal-GSH with BSO and paraquat plus maneb, intracellular levels of GSH were depressed as compared with control, but were significantly higher than with BSO plus paraquat and maneb in the absence of liposomal-GSH.

Liposomal-GSH has Direct Antioxidant Properties

The finding of protection by liposomal-GSH from paraquat plus maneb exposure in the presence of BSO suggested that liposomal-GSH may have direct antioxidant properties. To

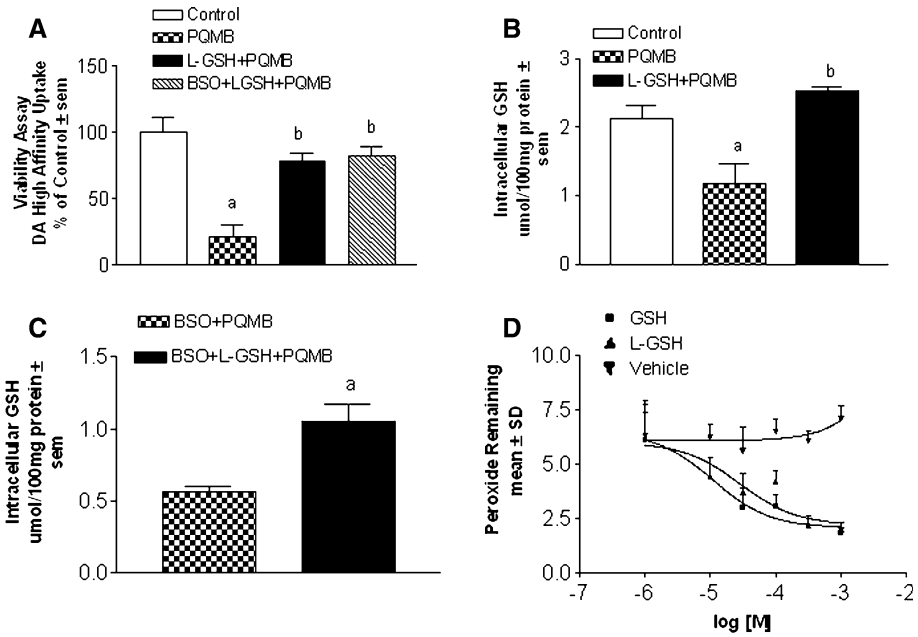

Fig. 5 **a** The effect of inhibition of GSH biosynthesis on neuroprotection by liposomal-GSH was studied in mixed mesencephalic cultures treated with 45 μM each paraquat plus maneb for 4 h in KRB. Following a 3d recovery in complete medium, viability in the dopamine population was determined as described in Methods. The *n* is from 4 determinations per condition run in duplicate. [a]Different from control, [b]different from paraquat plus maneb. **b** Intracellular GSH levels in mesencephalic cultures were determined following treatment for 4 h with paraquat plus maneb. The *n* is from 3 determinations run in duplicate. [a] different from control, [b] different from paraquat plus maneb. **c** Cultures were treated with paraquat plus maneb for 4 h in the presence of 10 μM BSO plus or minus 100 μM liposomal-GSH and intracellular GSH content was determined by HPLC immediately following treatment. The *n* is from 3 determinations per condition. [a] different from BSO with paraquat plus maneb. **d** Liposomal-GSH was tested for its ability to remove H_2O_2 from a cell-free solution using the Amplex Red peroxide detection kit as described in Methods. The *n* is from 3 determinations run in duplicate

test this, the ability of liposomal-GSH to remove H_2O_2 from a cell-free solution was tested using an Amplex red peroxide detection kit (Molecular Probes). Liposomal-GSH was found to remove H_2O_2 from solution in a concentration-dependent manner (Fig. 5d). By comparison, non-liposomal-GSH, as expected, also removed peroxide, whereas liposomal vehicle equivalent to the equimolar concentration of lecithin plus glycerol found in the different concentrations of liposomal-GSH was without effect (Fig. 5d).

Discussion

Two major findings result from these studies: firstly that liposomal-GSH can be utilized for repletion and maintenance of intracellular GSH in neuronal cells and secondly, that liposomal-GSH can provide significant protection to neurons in a model system relevant to Parkinson's disease. Glutathione plays multifunctional and diverse roles in neuronal cells including peroxide and toxin removal, maintenance of the redox state of proteins and protein signaling via glutathionylation [13, 37, 41]. In animal studies, disturbances in glutathione can result in damage to neurons per se [42] or in enhancement of toxicity due to metabolic or oxidative stress [23, 24, 43]. In humans, a number of neurodegenerative and neuropsychiatric conditions are associated with disturbances in glutathione [3, 5, 7, 9, 11, 44]. Repletion and maintenance of neuronal GSH is, therefore, important to cell health and viability and could provide therapeutic benefit in situations when GSH is deficient. Repletion of neuronal GSH, however, has met with difficulty. Neurons, like most other cells, do not possess transport mechanisms for GSH. In addition, elevation of extracellular GSH may pose potential toxicity problems that increase neuronal vulnerability during ischemia [23] or enhance toxicity involving NMDA receptors [24]. Other approaches such as the use of cysteine, NAC and ethyl esters of glutathione while effective in repleting and in the case of the ethyl ester, elevating intracellular GSH, have limited usefulness due to potential toxicities [14, 17–19, 21]. Encapsulation of GSH into lipid vesicles may avoid the potential toxicity to neurons associated with extracellular GSH elevation and may facilitate drug delivery to cells as has been shown for other liposomal preparations [45, 46].

Facilitation of intracellular GSH repletion was greatly enhanced by liposomal delivery. The concentration needed for half maximal repletion in mixed mesencephalic cultures containing approximately 70% neurons and 30% glia (unpublished observations) was 100-fold less when GSH was encapsulated into liposomal vesicles (4.75 μM for liposomal-GSH versus 533 μM for non-liposomal fully reduced GSH). Neuronal enriched cultures also directly utilized liposomal-GSH for intracellular repletion, but with an EC_{50} 15-fold higher than in the mixed cultures (76.5 μM). Glia are known to support GSH utilization by neurons. In vitro studies using enriched cultures of neurons and glia have shown that glia can efflux GSH [47, 48] which can then be metabolized by the ectoenzyme γ-GT and dipeptidases to supply neurons with the substrates for GSH biosynthesis. While the findings demonstrate direct neuronal utilization of liposomal-GSH, they also suggest that glia facilitate liposomal-GSH utilization by neurons. This would be consistent with a significant, but modest inhibitory effect of acivicin on liposomal-GSH utilization in the mixed mesencephalic cultures and with liposomal-GSH not serving as a substrate for γ-GT. Since exogenous liposomal-GSH was found not to be available for catabolism by γ-GT (Fig. 2c), only liposomal-GSH taken-up into cells would be further metabolized and its amino acid products used for GSH resynthesis. This is in accord with the finding that BSO, an inhibitor of the first GSH biosynthetic reaction completely prevented intracellular GSH repletion with exogenous liposomal-GSH administration as well as with the modest effects of acivicin and lack of metabolism of liposomal-GSH by γ-GT.

If liposomal-GSH is not available for extracellular catabolism by γ-GT, how is the liposomal-GSH utilized? Liposomal-GSH appears to gain access to the cytosol and its constituent amino acids utilized for GSH resynthesis via endosomal uptake and lysosomal degradation. Liposomes have been shown to gain entry into cells through macropinocytosis or phagocytosis [33, 45]. Endosomes can then fuse with lysosomes for digestion of intravesicular contents [34]. PAO, an inhibitor of endosomal uptake significantly attenuated liposomal-GSH utilization, however, PAO was also toxic and it is not clear if non-specific cell compromise or endosomal inhibition was responsible for the block of GSH repletion. The lectin, ConA, on the other hand, has been shown to inhibit endosomal/lysosomal fusion [36] and can inhibit degradation of endocytosed molecules [49]. ConA at concentrations that were not toxic to cells, caused a dose dependent reduction in the ability of liposomal-GSH to replenish intracellular GSH (Fig. 3b, d). Cysteine is taken up into neurons predominately via the glutamate transporter system X_{AG^-} [50, 51]. Consistent with this, ConA had no effect on the ability of cysteine to replenish intracellular GSH. In total, the data provide evidence for uptake of liposomal-GSH into endosomes and fusion with lysosomes where GSH is hydrolyzed to its constituent amino acids. The amino acids can then be used by the cell for further GSH biosynthesis. Neurons could accomplish this independent of glia. Glia could also utilize liposomal-GSH in this manner and in addition, continue to efflux GSH for extracellular metabolism and utilization of

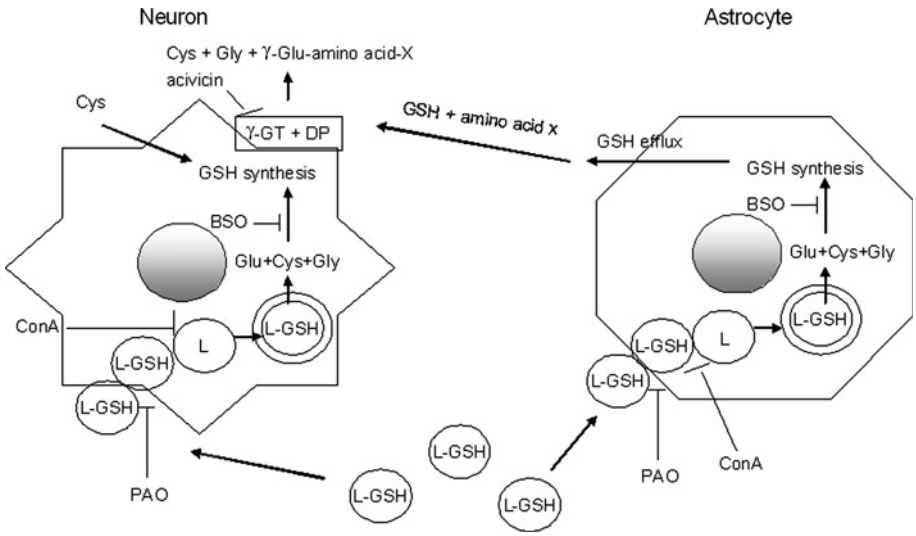

Fig. 6 A schematic summary of the findings for liposomal-GSH (L-GSH) utilization in the mixed mesencephalic cultures is shown. L-GSH is taken up into neurons and astrocytes via an endosomal process inhibited by phenylarsine oxide (PAO). Once inside the cell, endosomes containing L-GSH can fuse with lysosomes (L), where hydrolysis and release of GSH to its constituent amino acids, glutamate (Glu), cysteine (Cys) and glycine (Gly) occur. ConA can inhibit the formation of the phago-lysosomes. Hydrolyzed amino acids released from the lysosomes can be used for GSH biosynthesis, the first step of which is inhibited by buthionine sulfoxamine (BSO). Some efflux of GSH occurs by astrocytes and can be metabolized extracellularly by the ectoenzyme γ-glutamyltranspeptidase (γ-GT) and dipeptidases (DP). Acivicin inhibits γ-GT activity. The amino acids derived from the extracellular breakdown of GSH, and in particular cysteine, can be taken up by neurons to further supply substrates for GSH synthesis

its metabolites by neurons. It is this latter component that would be inhibited by acivicin (Fig. 2b). A schematic representing the postulated utilization of liposomal-GSH is shown in Fig. 6.

The lack of extracellular catabolism of the GSH in the liposomal preparation is important since catabolism to constituent amino acids, in particular, glutamate and cysteine can overstimulate glutamate receptors on neurons and lead to an excitotoxicity [17, 18]. In addition, there appears to be little leakage of GSH from the liposomal vesicles as the GSH in the preparation was neither a substrate for γ-GT nor glutathione S-transferase. This is also a point of interest as elevations in extracellular GSH may pose a toxicity risk as well [23, 24]. The mesencephalic cultures exposed to a concentration of the liposomal preparation >200-times the EC_{50} for GSH repletion did not cause toxicity. This finding attests to the relative safety of the liposomal preparation.

Mesencephalic cultures contain a small population of presumptive midbrain dopamine neurons. These neurons are representative of the midbrain neurons that degenerate in Parkinson's disease and are a commonly used in vitro system to model Parkinson's disease [14, 31]. Sporadic Parkinson's disease is thought to result from a combination of genetic susceptibility and environmental factors [52]. Exposure to pesticides is a risk factor for the development of Parkinson's disease [38, 39, 53]. Paraquat plus maneb are widely used pesticides and their combination has been shown in animal models to cause selective loss of substantia nigra pars compacta dopamine neurons [26]. In vitro, paraquat plus maneb caused loss of dopamine neurons, however, the extent of cell loss observed in the dopamine population was similar in magnitude to the loss observed in the general population which consists predominately of midbrain GABAregic neurons. The difference in the relative vulnerability of dopamine neurons to paraquat plus maneb in vivo versus in vitro could be due to a chronic dosing paradigm in vivo (5–6 weeks) in comparison with the acute exposure used in vitro (4 h). Chronic versus acute exposure in other Parkinson's disease models in vitro, i.e. rotenone, can alter the relative vulnerability of GABergic and dopaminergic mesencephalic neurons [31].

Regardless of differences in neuronal vulnerability, liposomal-GSH provided complete neuroprotection of dopamine neurons and the general mesencephalic population with EC_{50s} in the low μM range (14.1 and 10.5 μM for the general population and dopamine neurons respectively). Thus liposomal-GSH may be of therapeutic benefit not only in Parkinson's disease, but in other CNS conditions where there is perturbation of the glutathione system. While the EC_{50s} for protection were similar to those observed for intracellular GSH repletion, protection was not dependent on the utilization of liposomal-GSH for repletion since the same degree of protection was observed in the presence or absence of BSO. This suggested that the liposomal preparation had direct antioxidant properties and this was confirmed in a cell free assay where liposomal-

GSH was demonstrated to remove H_2O_2 from solution. This is possible as the readily diffusible H_2O_2 can traverse the lipid vesicle to access intravesicular GSH. There was no contribution of the lecithin and glycerol used for the liposomal preparation as lecithin plus glycerol were unable to remove H_2O_2. The direct removal of ROS by liposomal-GSH would account for the sparing of endogenous intracellular GSH during paraquat plus maneb exposure. This sparing effect has also been noted for other antioxidants such as ascorbate [54] as some of the burden for ROS removal by endogenous GSH is shared. The antioxidant property of liposomal GSH could provide a dual benefit as a potential therapeutic agent, firstly, in that it can be metabolized to supply substrates for GSH synthesis particularly when there is increased oxidative stress and increased demand for substrates and secondly, liposomal-GSH can serve to directly remove ROS.

In a limited study in 9 Parkinson's patients, i.v. administration of 600 mg GSH twice daily for 30d provided significant improvement in disability as assessed using a modified Columbia University Rating Scale [55]. Due to the open label design of the study and the limited number of patients, however, conclusions from this study should be viewed with caution. A controlled, double-blinded clinical trial is planned for study of i.v. glutathione administration in the treatment of Parkinson's disease [56]. Non-liposomal-GSH requires i.v. administration to avoid the hydrolysis and gut absorption that follows oral administration. An advantage of the liposomal preparation over non-liposomal-GSH is that it can be administered orally [25] thus allowing self-administered daily application.

One enigmatic issue at present, is whether liposomal-GSH crosses the blood brain barrier. There is some evidence for non-liposomal-GSH transport across the blood brain barrier [57], but this is thought to be of low capacity [58]. Direct transport of non-liposomal-GSH or liposomal-GSH into brain may not be necessary, however, to support brain maintenance of GSH. The reasons underlying low brain GSH levels in neurodegenerative diseases such as Parkinson's disease are unknown. In contrast with Schizophrenia [7, 8], synthesizing enzymes for glutathione function normally in Parkinson's disease [59]. Cysteine can serve as an antioxidant and is the rate limiting enzyme in the production of glutathione [16, 60]. High levels of oxidative stress as well as elevations in cysteine conjugates such as cysteinyldopamine have been reported in Parkinson's disease brain [61, 62]. These findings point towards an imbalance in supply and demand with low levels of glutathione due to limiting amounts of cysteine. Peripheral GSH and/or liposomal-GSH could serve as a source of peripherally generated cysteine which readily traverses the blood brain barrier via the L-system carrier [15]. Thus benefit from peripherally administered GSH could derive from GSH per se or from a peripherally generated metabolite. Further studies on the blood brain barrier permeability of the liposomal preparation and its neuroprotective potential in vivo are needed.

In summary, the studies presented here show that a liposomal preparation of GSH is 100-fold more potent than non-liposomal-GSH in providing substrates for maintenance of intracellular glutathione in neuronal cells and provides complete protection of neurons in an environmental model of Parkinson's disease. These findings provide a rationale for in vivo studies of liposomal-GSH in a variety of disease models where oxidative stress and/or perturbations in the glutathione system are thought to be involved.

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