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Up-regulation of γ -glutamyl transpeptidase activity following glutathione depletion has a compensatory rather than an inhibitory effect on mitochondrial complex I activity: Implications for Parkinson's disease

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Abstract

Up-regulation of activity of γ -glutamyl transpeptidase (GGT) has been reported to occur in the Parkinsonian substantia nigra, the area of the brain affected by the disease. Increased GGT activity has been hypothesized to play a role in subsequent mitochondrial complex I (CI) inhibition by increasing cysteine as substrate for cellular uptake. Intracellular cysteine has been proposed to form toxic adducts with dopamine which can be metabolized to compounds which inhibit CI activity. We have demonstrated that in addition to CI inhibition, GGT activity is up-regulated in dopaminergic cells as a consequence of glutathione depletion. Inhibition of GGT rather than resulting in increased CI inhibition results in exacerbation of this inhibitory effect. This suggests that increased GGT activity is likely an adaptive response to the loss of glutathione to conserve intracellular glutathione content and results in a compensatory effect on CI activity rather than in its inhibition as has been previously widely hypothesized.

Keywords

Parkinson's disease; Glutathione; γ -Glutamyl transpeptidase; Reactive oxygen species; Mitochondria; Free radical

Introduction

The pathogenesis underlying the selective loss of dopaminergic midbrain neurons in Parkinson's disease (PD) is not fully understood but multiple lines of evidence implicate the role of mitochondrial dysfunction in this process. Depletion in levels of the thiol reducing agent glutathione (GSH) is one of the earliest reported biochemical events to occur in the affected substantia nigra (SN) of PD patients [1]; decreases in total glutathione (GSH + GSSG) levels appears to occur prior to the selective loss of mitochondrial complex I (CI) activity associated with the disease which is believed to contribute to subsequent dopaminergic cell death. GSH depletion is not observed in any other neurodegenerative disorders of the basal ganglia but is

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unique to PD [2,3]. GSH is synthesized by a two-step reaction involving the enzymes γ -glutamylcysteine ligase (GCL) and glutathione synthetase (GS). GCL is the rate-limiting enzyme in this process and brain GSH levels appears to arise primarily through synthesis from its constituent amino acids via this enzyme. GCL is a heterodimer consisting of a heavy catalytic subunit (GCLC) and a light modulatory subunit (GCLM) [4]. GSH is synthesized in the cytosol and transported into the mitochondria via an energy-dependent transporter. Decreased GSH availability in the brain has been demonstrated to promote morphological mitochondrial damage [5]. We have previously demonstrated that GSH depletion via down-regulation of GCL activity in dopaminergic cells *in vitro* results in a rather selective inhibition of CI activity which is rate-limiting with regard to mitochondrial function [6,7].

A marked increase in the activity of γ -glutamyl transpeptidase (GGT, EC 2.3.2.2), a membrane-bound enzyme that is responsible for extracellular cleavage of the γ -glutamyl bond in the GSH molecule, has been reported in the post-mortem PD SN [8]. GGT hydrolyzes extracellular GSH into cysteinyl-glycine, which is further broken down by membrane-bounded dipeptidases to cysteine and glycine. These amino acids can be taken up into cells by specific amino acid transporters and used as substrates for *de novo* intracellular glutathione synthesis via GCL. GGT can also transfer the γ -glutamyl moiety of GSH to extracellular cystine to form γ -glutamylcystine which can also be taken up into cells, reduced to γ -glutamylcysteine, and used, for scavenger glutathione synthesis via GS. GGT has, therefore, a role in cellular GSH homeostasis and may be of significant importance when the level of cyst(e)ine is reduced [9]. Increased GGT levels have been hypothesized to contribute to dopaminergic neurodegeneration associated with PD by increasing the amount of cysteine available for transport into dopaminergic neurons where it can interact with dopaminergic quinones formed during dopamine oxidation. The presence of such dopaminergic-cysteine (S-S-cysDAQ) conjugates has been reported in the PD SN [10]. Furthermore, metabolites of these conjugates have been reported to inhibit mitochondrial CI activity [11].

We recently found that GSH depletion in midbrain-derived dopaminergic cell lines results in an up-regulation of GGT activity. This raised the possibility that the observed increase in GGT activity could be responsible for the inhibition of CI activity which we previously observed following dopaminergic GSH depletion *in vitro* [7]. To test this, we assessed whether the selective inhibition of GGT in dopaminergic neurons protected against the GSH-depletion-mediated inhibition of CI in our cell system.

Materials and methods

Chemicals and reagents

All chemicals used in this study were obtained from Sigma unless otherwise noted. Glycylglycine was from ICN Biochemical (Aurora, OH, USA). L- γ -Glutamyl 7-amino-4-methylcoumarin (γ -Glu AMC) was purchased from Bachem Bioscience (King of Prussia, PA, USA). TRIzol reagent was from Life Technologies (Grand Island, NY, USA). DNA-free reagents were from Ambion (Austin, TX, USA). TaqMan reverse transcription reagent and SYBR Green PCR Master Mix were from Applied Biosystems (Foster City, CA, USA). All chemicals used were at least analytical grade.

Creation of doxycycline-inducible anti-GCL N27 cell lines

Permanent anti-GCL N27 cell lines were produced as described previously except lipofectamine 2000 (Invitrogen) was used for all cell transfection experiments [6].

Growth of anti-GCL cell lines

Cells were grown on poly-L-lysine-coated plates (Greiner) in medium containing RPMI 1640 (Cellgro), 10% tetracycline (tet) system-approved fetal bovine serum (Clontech), 200 µg/ml geneticin (G418), 200 µg/ml hygromycin B, and 10 ml/L of antibiotic antimycotic solution (Cellgro). Doxycycline (dox) was then added to the medium at 20–40 µg/ml for 24 h to induce down-regulation of total glutathione levels. For dox removal experiments, cells were washed with Hank's balanced saline solution and grown for 24 h in dox-free media. Cells were subcultured once a week via trypsin treatment.

GCL activity

Cell homogenates resuspended in 100 mM Tris-Cl, pH 8.0, were used to assay GCL activity levels as described previously [12]. Assays were run in the absence of α-aminobutyrate as a blank. GCL values were normalized per protein using Bio-Rad reagent.

Glutathione levels

Total glutathione levels were measured using the Bioxytech GSH/GSSG 412 assay kit (Oxis Research) per the manufacturer's instructions.

GGT activity

Cells were rinsed with cold phosphate-buffered saline before being harvesting. GGT activity was measured according to the method described by Forman et al. [13] with slight modifications for use on a fluorescence microplate reader. Specificity of GGT activity was confirmed by acivicin, a specific GGT inhibitor.

Mitochondrial preparations

Cells were washed and resuspended in ice-cold isolation buffer (320 mM sucrose, 5 mM 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid, and 1 mM EGTA, pH 7.4) followed by homogenization in a Dounce homogenizer. Homogenates were centrifuged at 1000g for 5 min at 4°C. Supernatant containing the mitochondria was saved and pellet resuspended in isolation buffer and rehomogenized. Centrifugation of the resuspended homogenate was repeated and the supernatants were pooled. The pooled supernatant was centrifuged at 10,000g for 10 min at 4°C. The pellet containing the mitochondrion was resuspended in 50 µl isolation buffer for mitochondrial complex I enzyme activity assay.

Complex I activity

Activity was assayed in homogenates from isolated mitochondria as rotenone-sensitive NADH dehydrogenase activity by measuring 2,6-dichlorophenol-indophenol reduction in mitochondrial extract following addition of 200 µM NADH, 200 µM decylubiquinone, 2 mM KCN, and 0.002% 2,6-dichlorophenolindophenol in the presence and absence of 2 µM rotenone [14]. The reaction was performed at 600 nm at 30 °C. Values for all the assays were normalized per protein by using Bio-Rad reagent.

Amplex red assay for peroxidase levels

Cells were plated at 5×10^3 per well. After being allowed to settle for 4–5 h, the cells were induced for 24 h with 40 µg/ml dox. Amplex red reagent (Molecular Probes) was added in a reaction buffer containing peroxidase [15]. The resulting increase in fluorescence over time was measured at 563 nm excitation/587 nm emission in a Spectramax fluorometer (Molecular Devices). Values represent-catalase sensitive oxidant levels.

4-hydroxynonenal 4-HNE assay

4-Hydroxynonenal levels were measured by use of the Bioxytech LPO-586 kit (Oxis Research) per the manufacturer's instructions.

GGT mRNA assay $\pm$ actinomycin D and cycloheximide

Total RNA was extracted using TRIzol reagent and treated with DNA-free reagent according to the manufacturers' protocols. DNA-free RNA samples were reverse transcribed using the TaqMan reverse transcription system (Applied Biosystems) and real-time PCRs were run with a Cepheid 1.2 real-time PCR machine. Briefly, 5 μ l of reverse transcription reaction product was added to reaction tubes containing 12.5 μ l SYBR Green PCR Master Mix and primer pair specific for total or types of GGT mRNA; the total PCR sample was 25 μ l. GAPDH was used as internal control (25 μ l PCR: 2.5 μ l RT reaction, 12.5 μ l SYBR Green PCR Master Mix, primers, and water). Specificity of PCR products was confirmed by DNA sequencing.

Statistical analyses

For relative mRNA quantitation, we used the comparative $\Delta\Delta C_T$ method. The relative quantification of target, normalized to an internal control (GAPDH), and an untreated sample is given by relative quantitation = $2^{-\Delta\Delta C_T}$, $\Delta\Delta C_T$ being defined as the difference of mean ΔC_T (treated sample) and mean ΔC_T (untreated sample) and ΔC_T as the difference in mean C_T (GGT) and C_T (GAPDH) as the internal control. Threshold cycles (C_T) were selected in the line in which all samples were in logarithmic phase. All other data are expressed as mean $\pm$ SD, for the number ($n = 3$) of independent experiments performed. Differences among the means for all experiments described were analyzed using one-way analysis of variance (ANOVA).

Results

Generation of N27 anti-GCL cells with inducibly reduced GCL and GSH levels

We previously reported the ability to reduce both cellular and mitochondrial levels of total glutathione (GSH + GSSG) within dopaminergic PC12 cells via antisense expression of the catalytic subunit of GCLC [6,7]. We chose in this current study to use N27 cells; this cell line was derived from dopamine-producing neurons of the rat fetal midbrain via SV40 large T antigen immortalization and therefore consists of cells comparable to the midbrain dopaminergic neurons selectively lost in PD [16]. As such, they constitute a superior cellular model system for exploring possible mechanisms involved in the disorder.

As previously demonstrated in our anti-GCL PC12 cell lines, dox treatment of N27 antiGCL cells for 24 h resulted in decreases in GCL activity in a dose-dependent manner (data not shown) with maximum depletion observed at 40 μ g/ml (Fig. 1A). The dependence of levels of total cellular glutathione (GSH and its oxidized form, GSSG) on dox concentration was seen to parallel that observed for GCL activity (Fig. 1B). Treatment with 40 μ g/ml dox for 24 h resulted in a decrease in GSH levels of approximately 50%, a similar decrease to that previously reported to occur in the SN of early PD brains [2,5]. Consequently, this dosage regime was used for all subsequent experiments.

Effect of glutathione depletion on GGT activity

GGT plays a key role in GSH homeostasis by providing substrates for both its intracellular *de novo* and scavenger synthesis. Furthermore, its activity has been demonstrated to be elevated in postmortem PD brains. We therefore examined the effect of dopaminergic GSH depletion in our antiGCL N27 cell lines on GGT activity. Dox treatment which induces antisense expression of GCLC and thus decreases GSH levels was found to significantly enhance the

catalytic activity of GGT by nearly 1.6-fold compared with that of non-dox-treated controls (Fig. 2). Increased GGT activity was found to be reversible upon removal of dox from the medium.

Blockage of GGT induction exacerbated GSH-depletion-mediated inhibition of CI activity

If increased GGT activity is responsible for selective CI inhibition following dopaminergic GSH depletion, then its inhibition should result in an attenuation of this effect. To test this hypothesis, we measured CI activity in the presence and absence of acivicin, a selective GGT inhibitor (Fig. 3). CI inhibition had previously been determined to not be due to a decrease in subunit protein levels (data not shown). Rather than being restored, CI activity was actually further decreased following acivicin treatment in our dopaminergic GSH-depleted cell lines. Acivicin treatment alone had no significant statistical effect on CI activity. Inhibition of complex I was furthermore partially restored by the addition of cystine to the medium as a source of substrate for *de novo* synthesis of GSH that would bypass GGT inhibition. CI inhibition was also attenuated when the cells were supplemented with exogenous GSH. These data suggest that, rather than being responsible for CI inhibition, increased GGT activity is actually a compensatory event acting to help restore cellular GSH homeostasis following its depletion. To test this, we assessed GSH levels in the absence and presence of acivicin in GSH-depleted N27 cell line (Fig. 4). GGT inhibition resulted in a further decrease in GSH levels, while acivicin alone had no significant effect. GSH levels could be partially restored via either exogenous GSH addition to the media or cystine addition in the presence of acivicin. In addition, levels of 5cys-DAQ compounds measured via HPLC in the GSH-depleted cells were not significantly increased (data not shown). This suggests that the GSH-depletion-mediated GGT increase acts to compensate for the loss in GSH levels rather than contributing to CI inhibition via the formation of dopaminergic quinone adducts as previously hypothesized.

Reduced GSH levels result in increases in oxidants previously demonstrated to induce increases in GGT activity via increases in its mRNA levels

Previous studies in lung epithelial cells have demonstrated that oxidants including H_2O_2 and 4-HNE can result in elevation in GGT activity via induction of GGT transcription and increases in its mRNA levels [17,18]. We have previously demonstrated that GSH depletion results in elevation in both of these oxidant species in dopaminergic PC12 cells [7]. We examined the impact of glutathione depletion in our current N27 cell model and found, as previously reported for our PC12 cell lines, that levels of both H_2O_2 and 4-HNE were increased following GSH depletion (Figs. 5A and 5B). Furthermore, as previously reported following H_2O_2 or 4-HNE exposure of lung epithelial cells, GGT mRNA content was found to be significantly increased following GSH depletion in these cells.

To determine whether the increase in the GGT mRNA requires new protein synthesis, the effect of cycloheximide on GGT mRNA levels was studied. Cycloheximide did not inhibit the GSH-depletion-mediated increase in GGT mRNA content. This suggests that the increase in GGT mRNA is a direct rather than secondary response to GSH depletion in our cells. To confirm this, dopaminergic N27 cells were pretreated with actinomycin D, an RNA transcription inhibitor, for 1 h before addition of doxycycline. In the presence of actinomycin D, dox treatment has no effect on GGT mRNA levels (Fig. 6). These results suggest that glutathione depletion increases GGT mRNA by stimulating gene transcription.

Discussion

It has been suggested that the noted increase in GGT activity in the affected Parkinsonian midbrain acts to increase cysteine uptake which in turn contributes to formation of toxic 5cys-DAQ compounds. These dopamine adducts undergo further oxidation to form agents that can

cross the outer mitochondrial membrane and irreversibly inhibit complex I activity [19], a major hallmark of the disease. However in our study, blocking GSH-depletion-induced elevations in GGT activity within midbrain-derived dopaminergic cell line by the specific GGT inhibitor acivicin resulted in an exacerbation rather than an improvement in CI activity. Furthermore, addition of either cystine in the presence of GGT inhibition by acivicin or exogenous GSH to the medium resulted in partial attenuation of CI inhibition associated with dopaminergic GSH depletion. In addition, levels of 5cysDA adducts were not significantly elevated following glutathione depletion nor blocked by acivicin treatment (data not shown). Elevations in GGT activity therefore appear to act to attenuate the GSH-depletion-mediated decrease in CI activity rather than contribute to it. This suggests that the increase in GGT activity observed in dopaminergic PD midbrain is likely a compensatory response to the loss in cellular GSH levels in an attempt by the cell to attenuate mitochondrial dysfunction associated with GSH depletion. This is born out by assessment of GSH levels in GSH-depleted cells following treatment with either acivicin where levels are further reduced or cystine in the presence of acivicin or exogenous GSH addition to the media which both acted to attenuate the loss in GSH levels.

The facts that GGT mRNA and subsequent activity have previously been demonstrated to be up-regulated in lung epithelial cells challenged with either quinones or 4-HNE as an adaptive response to oxidative stress [18,20] and that levels of these oxidants are also elevated following GSH depletion in our cells suggested to us that the observed up-regulation of GGT activity may occur via a similar mechanism. GGT mRNA levels are found to be elevated in response to GSH depletion. To determine whether the increase in GGT mRNA levels was a primary or secondary response to the production of reactive oxygen species, we treated the cells with cycloheximide (a protein synthesis inhibitor) and actinomycin D (an RNA transcription inhibitor) in presence of doxycycline. It was found that cycloheximide did not inhibit the GSH-depletion-mediated increase in GGT mRNA content whereas actinomycin D blocks the increased GGT mRNA levels. These results suggest that the GSH depletion induced increases in GGT mRNA levels which therefore were due to increased transcription as previously reported in lung epithelial cells.

Previous studies with nitric oxide (NO) donors have demonstrated that the activity of the GGT enzyme in colon carcinoma cells is induced after exposure to NO and that the increase in GGT helped maintain intracellular GSH levels in cells grown in cystine-depleted medium by increasing substrate available for uptake into the cells [21]. Increased GGT activity thus acted to protect these cells against nitrosative stress. Earlier studies in the GGT^{enu1} mouse demonstrated that GGT deficiency has a dramatic impact on glutathione homeostasis. Genetic GGT deficiency in the GGT^{enu1} mouse impairs intracellular glutathione metabolism and results in systemic glutathionemia and oxidant stress in the kidney [22]. Previous studies with lung epithelial cells also demonstrated that when cells were exposed to quinones as an oxidant-induced stress, GGT would be increased as part of the adaptation of cells to oxidative stress by enhancing the utilization of extracellular GSH [23].

The results from the current study demonstrate that GGT activity may be up-regulated in dopaminergic cells as a consequence of acute GSH depletion and that this is likely an adaptive response to the loss of GSH to conserve intracellular glutathione content (Fig. 7). Furthermore, it results in a compensatory effect on CI activity rather than in its inhibition as has been previously hypothesized. Prolonged elevation of GGT activity *in vivo*, however, may function in both adaptive and maladaptive manners, something which is experimentally testable and being actively pursued in our laboratory.

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Abbreviations

PD	Parkinson's disease
GGT	γ -glutamyl transpeptidase
GSH	glutathione
ROS	reactive oxygen species
GCL	γ -glutamylcysteine ligase
HNE	4-hydroxy-2-nonenal
SN	substantia nigra
CI	complex I
GS	glutathione synthetase
tet	tetracycline
dox	doxycycline

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Fig. 1. GCL activity and total cellular glutathione levels in N27 anti-GCL cells treated with or without dox. (A) GCL activity following 24 h dox treatment. Values are reported as % of untreated control (100% GCL activity, 2.6 ± 0.3 nmol/min/mg of protein), $*p < 0.01$ compared to no dox control. (B) Total glutathione levels following 24 h dox treatment (100% GSH value, 20.0 ± 2.0 nmol/mg protein), $*p < 0.01$ compared to no dox control. Data represent triplicate values from three separate experiments. N27 anti-GCL cells were treated with/without 40 μ g/ml dox for 24 h before assays were performed. Con, no dox control; dox, 40 μ g/ml dox.

Fig. 2.

Effect of GSH depletion on GGT activity in anti-GCL N27 cells. Cells were treated with 0 or 40 $\mu\text{g/ml}$ dox for 24 h. Specific activity of GGT is reported as % of untreated control (100% GGT value, 32 ± 2.3 units/min/mg protein). DR represents dox removal for 24 h. Each bar represents a mean $\pm$ SE from four separate experiments; * $p < 0.01$ compared with no dox control; ** $p < 0.01$ compared with dox-treated group.

Fig. 3.

Effect of increased GGT activity on mitochondrial complex I activity. Rotenone-sensitive NADH dehydrogenase activity was measured in mitochondrial preparations from dox-treated vs control cells in the presence of acivicin (200 μ M), cystine (200 μ M), and glutathione (1 mM); $n = 5$ per condition from three separate experiments. Values are expressed as % untreated control (100% value, 260 ± 15 nmol/min/mg of mitochondrial protein); * $p < 0.01$ compared with untreated control; ** $p < 0.05$ compared with dox-treated; *** $p < 0.01$ compared with dox + aci-treated cells. Con, no dox control; dox, 40 μ g/ml dox; Aci, acivicin.

Fig. 4.

Measurement of intracellular glutathione levels in anti-GCL N27 cells. Glutathione was measured after 24 h incubation in dox-treated anti-GCL N27 cells in the presence of acivicin, cystine, and glutathione. Values are reported as % untreated controls (100% GSH value, 21.5 ± 2.0 nmol/mg protein); $p < 0.01$ compared with untreated control; $**p < 0.05$ compared with dox-treated; $***p < 0.05$ compared with dox + Aci-treated.

Fig. 5. Glutathione depletion results in increased H₂O₂ and HNE levels in dox-treated anti-GCL N27 cells. Anti-GCL N27 cells were incubated with 0 and 40 μ g/ml dox for 24 h and then H₂O₂ (A) and 4-HNE (B) levels were determined. Values are reported as % untreated controls (100% HNE value, 1.26 ± 0.03 μ M); * $p < 0.01$ compared to no dox control. Data represent triplicate values from three separate experiments. Con, no dox control; dox, 40 μ g/ml dox.

Fig. 6.

Glutathione depletion results in increased GGT mRNA levels. Cells were treated with 0 or 40 $\mu\text{g/ml}$ dox $\pm$ cycloheximide (1 $\mu\text{g/ml}$) and dox + actinomycin D (1 $\mu\text{g/ml}$) for 24 h and GGT mRNA content was determined by quantitative PCR analysis. The identity of the PCR product was confirmed by DNA sequencing. * $p < 0.01$ compared to no dox control; Con, no dox control; dox, 40 $\mu\text{g/ml}$ dox; CHX, cycloheximide; Act D, actinomycin D.

Fig. 7.

Schematic representation of the possible role that GGT increase may play during oxidative stress induced by GSH depletion on mitochondrial complex I activity. A depletion in glutathione levels (2) following GCL inhibition (1) results in increased H₂O₂ and HNE levels (3) which in turn induces increased GGT transcription, GGT mRNA, and enzyme activity levels (4). Increased GGT activity in the presence of extracellular transpeptidases leads to the enhanced metabolism of extracellular GSH into cys and glu-cys (5) which can be taken up by glutamate-cyst(e)ine transporters into the cell (6). Within dopaminergic cells, either they can hypothetically conjugate with dopamine (DA) (7) to form cys-DA-Q whose metabolites have been proposed to inhibit mitochondrial complex I (CI) activity (8) or they can act to provide increased substrates for *de novo* (1) GSH synthesis via GCL (cys) or scavenger synthesis (9) via GS (glu-cys).