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Dopamine quinone modifies and decreases the abundance of the mitochondrial selenoprotein glutathione peroxidase 4

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Abstract

Oxidative stress and mitochondrial dysfunction are known to contribute to the pathogenesis of Parkinson's disease. Dopaminergic neurons may be more sensitive to these stressors because they contain dopamine (DA), a molecule that oxidizes to the electrophilic dopamine quinone (DAQ) which can covalently bind nucleophilic amino acid residues such as cysteine. The identification of proteins that are sensitive to covalent modification and functional alteration by DAQ is of great interest. We have hypothesized that selenoproteins, which contain a highly nucleophilic selenocysteine residue and often play vital roles in the maintenance of neuronal viability, are likely targets for the DAQ. Here we report the findings of our studies on the effect of DA oxidation and DAQ on the mitochondrial antioxidant selenoprotein Glutathione Peroxidase 4 (GPx4). Purified GPx4 could be covalently modified by DAQ, and the addition of DAQ to rat testes lysate resulted in dose dependent decreases in GPx4 activity and monomeric protein levels. Exposing intact rat brain mitochondria to DAQ resulted in similar decreases in GPx4 activity and monomeric protein levels as well as detection of multiple forms of DA-conjugated GPx4 protein. Evidence of both GPx4 degradation and polymerization was observed following DAQ exposure. Finally, we observed a dose dependent loss of mitochondrial GPx4 in differentiated PC12 cells treated with dopamine. Our findings suggest that a decrease in mitochondrial GPx4 monomer and a functional loss of activity may be a contributing factor to the vulnerability of dopaminergic neurons in Parkinson's disease.

Parkinson's disease (PD) is a progressive neurodegenerative disease affecting approximately one million people in the United States. The primary movement symptoms of the disease: bradykinesia, resting tremor, and rigidity are attributed to a profound degeneration of the dopaminergic (DAergic) neurons of the nigrostriatal pathway. Although the exact

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mechanism underlying cell death remains unknown, it is clear that oxidative stress and mitochondrial dysfunction are contributing to the demise of the DAergic neurons [1].

Mitochondria are central figures in the regulation of cell death pathways. They release cytochrome c and apoptosis-inducing factor into the cytosol, activating caspase-dependent and caspase-independent cell death pathways, respectively [2, 3]. Mitochondria serve as calcium buffers, protecting neurons from excitotoxic cell death [4]. They also are one of the main sources of reactive oxygen species in the cell, which arise as byproducts of the electron transport chain [5]. Taken together, these roles suggest that preserving mitochondrial integrity is critical for neuronal survival.

Multiple lines of evidence exist that implicate mitochondrial dysfunction in the pathogenesis of PD. Familial forms of parkinsonism arise from mutations or deletions of the genes coding for the proteins DJ-1, PINK1, and Parkin [6-8]. All of which are thought to function in the maintenance of mitochondrial integrity [9-11]. Many patients with the sporadic form of PD have a systemic deficiency in complex I activity in the electron transport chain which is thought to result in excess superoxide production in mitochondria [12-14]. Notably, exposure to mitochondrial complex I inhibitors such as 1-methyl-4-phenylpyridinium and rotenone cause parkinsonian-like symptoms in primates and rodents [15, 16]. Recently, the rotenone model of PD has provided substantial evidence that chronic systemic inhibition of complex I is sufficient to cause selective degeneration of DAergic nurons in rodents [17-19]. However, the mechanism underlying the apparent sensitivity of DAergic neurons to mitochondrial dysfunction is not fully understood.

Dopamine (DA), the neurotransmitter in DAergic neurons, can spontaneously oxidize to an electron deficient DA quinone (DAQ), which readily forms a covalent bond with nucleophiles, such as the thiol group on the amino acid cysteine [20]. Irreversible modification of cysteine residues on proteins can alter the function of the protein, potentially jeopardizing the health of the cell. Exogenous DA has been shown to be toxic to SNpc neurons when injected into the striatum, while endogenous DA is toxic to SNpc neurons in mice that cannot sequester it into vesicles [21, 22]. Covalent modification of cysteinyl residues forming 5-cysteinyl-dopamine in both proteins and GSH is thought to be the mechanism underlying the toxicity of DA to these neurons [21].

Selenoproteins, a family of selenium-containing proteins, may be readily targeted by DAQ under physiological conditions. The selenol group on selenocysteine residues is a stronger nucleophile than the thiol of cysteine at physiological pH, due to its lower pKa, making it a likely target for DAQ modification [23]. The selenoprotein glutathione peroxidase 4 (GPx4, phospholipid hydroperoxide glutathione peroxidase, EC 1.11.1.12) is a critical antioxidant protein. $GPx4^{-/-}$ mice die at embryonic day 7.5, and the protein ranks high in the hierarchy of selenoproteins [24, 25]. GPx4 directly catalyzes the reduction of phospholipid hydroperoxides through an active site containing an essential selenocysteine residue (U46). Replacement of the selenocysteine with cysteine decreases activity 1000-fold, and alkylation of the residue with iodoacetamide completely abolishes activity [26]. Thus, oxidative modification of this protein is likely to alter its function.

GPx4 is highly expressed in the brain in comparison to most other selenoproteins [27]. In brain mitochondria, GPx4 primarily localizes to the inner membrane where it prevents the oxidation of membrane phospholipids such as cardiolipin [28]. Cardiolipin is an inner mitochondrial membrane phospholipid that plays multiple essential roles in the mitochondria. It is required for the proper formation of contact sites between the inner and outer mitochondrial membranes; it stabilizes the complexes of the electron transport chain; the activity of several mitochondrial enzymes (i.e. creatine kinase) depends on it; and it binds cytochrome c to the inner membrane which effectively prevents the induction of apoptosis (Reviewed in [29]). Maintaining cardiolipin in a reduced state is integral for the health of the mitochondria, suggesting adequate antioxidant defenses, such as GPx4 activity, are extremely important to the cell.

The role of selenoproteins in maintaining neuronal viability and mitochondrial homeostasis is rapidly emerging. Because DA readily forms electrophilic DAQ under oxidative conditions and selenoproteins represent an ideal target for quinone modification, we examined whether GPx4 would serve as a target for DAQ modification in a complex cellular environment such as isolated brain mitochondria.

EXPERIMENTAL PROCEDURES

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh and are in accordance with guidelines put forth by the National Institutes of Health in the *Guide for the Care and Use of Laboratory Animals*.

Chemicals and Reagents

All chemicals were purchased from Sigma-Aldrich unless otherwise noted. A polyclonal antibody for GPx4 was raised in rabbit against a peptide corresponding to the residues 178-KRYGMEEPQVIEKD-191 of full-length rat GPx4 by Genemed Synthesis Inc. (San Francisco, CA). The antibody was affinity purified and supplied in phosphate buffered saline (PBS) (1.6 μg/μL). For all experiments, protein content was determined by the Bradford assay [30]. Phosphatidylcholine hydroperoxide (PCOOH) was synthesized according to published procedures [31].

SDS-PAGE and Western Blot

Sample proteins were separated by 15% SDS-PAGE, and then transferred to 0.2 μm nitrocellulose membranes using a semi-dry trans-blot apparatus (Bio-Rad). Odyssey blocking buffer (LiCor Biosciences) diluted 1:1 with PBS was used to block membranes. Blots were incubated in primary antibody for 1hr at RT or overnight at 4 °C. Primary antibodies used were Genemed rabbit-anti-GPx4 (1:1000), mouse-anti-COX IV (Abcam Ab14744, 1:100,000), or rabbit-anti-HSP60 (Abcam Ab40993, 1:200,000). The secondary antibodies used were LiCor goat-anti-rabbit IRDye 800 (1:5,000-10,000) and LiCor goat-anti-mouse IRDye 680 (1:5,000). Following secondary antibody incubations (1hr, RT), the blots were washed extensively, and scanned using the Odyssey Infrared Imaging System (LiCor Biosciences). Band intensities were quantified using the Odyssey Application

Software Version 3.0, and the ratios of GPx4 band intensity to the loading control band intensity were used for statistical analyses.

GPx4 Activity Assay

GPx4 activity on PCOOH was measured using the standard glutathione reductase coupled spectrophotometric assay [31]. GPx4 activity in testes lysate was measured using 200 μg protein in GPx Assay Buffer [100mM Tris-HCl (pH 7.4), 5mM EDTA, 0.1% Triton X-100 (Roche, peroxide free)] in the presence of 3mM GSH, 0.2 mM NADPH, and 3U of glutathione reductase (Roche). Reactions were started by the addition of PCOOH and the decrease in NADPH absorbance at 340nm was recorded using a SPECTRAmax Plus384 spectrophotometer. The molar extinction coefficient for NADPH (6.22×10^3 $M^{-1} \cdot cm^{-1}$) was used to calculate specific activity (mol/min/mg protein) (1U = 1 μmol NADPH oxidized per minute) from the linear activity rates.

To increase the sensitivity of the GPX4 assay for brain mitochondrial lysates, we measured the change in NADPH fluorescence using a fluorometer (Shimadzu RF-5301PC), with the excitation wavelength set to 340nm and emission wavelength set to 460nm, recording every second. GPx4 activity was recorded in mitochondrial lysates (500μg protein) in GPx assay buffer in the presence of 3mM GSH, 0.075 mM NADPH, and 3U of glutathione reductase with constant stirring. After 5 min incubation at 37°C, PCOOH was added and decreases in NADPH fluorescence were measured continuously. Reaction rates were calculated by fitting linear regression lines to the data acquired 10s after the addition of PCOOH and continuing for at least 60s afterwards.

All samples were assayed in duplicate, and the mean specific activity from 3 separate experiments was used for statistical analyses.

Immunopurification of Testes GPx4 and ^{14}C-DAQ Exposure

Lewis rat testes were minced and homogenized in ice cold CHAPS lysis buffer [250mM sucrose, 100mM Tris-HCl, 12mM CHAPS, pH 7.4] (10mL/teste) containing protease inhibitor cocktail (PIC, Sigma) using a Dounce homogenizer. The homogenate was vortexed and remained on ice for 30 min prior to centrifugation (15 min, 20,000xg, 4°C) to pellet insoluble material. The resulting supernatant was applied, according to manufacturer's instructions, to an immunoaffinity column prepared using the Genemed GPx4 antibody and an Aminolink Plus Kit (Pierce). Partially purified protein was eluted using IgG elution buffer (Pierce) and collected in 1mL fractions. Eluted fractions containing GPx4 were concentrated using 10kDa centrifugal filters (Millipore). Partially purified protein (250 μg) was reacted with 150 μM ^{14}C-DA (MP Biosciences, 0.3 μCi) and tyrosinase (0.3U/μL) for 15 min at RT. When indicated, DTT was added to a final concentration of 1mM prior to the addition of DA/tyrosinase while an equivalent volume of buffer alone was added to the samples not treated with DTT. Reactions were stopped by the addition of SDS-PAGE sample prep buffer, boiled, and samples were immediately run on 1D SDS-PAGE followed by autoradiography and Western blot for GPx4.

Exposure of Testes Lysate to Dopamine Quinone

Lewis rat testes were lysed using CHAPS lysis buffer and aliquots were diluted to a final concentration of 2.0 μg/ L with GPx assay buffer containing the indicated amount of DA (0-150 μM). Control samples such as buffer alone, 150 μM DA alone, tyrosinase alone, GSH alone, and GSH/150 μM DA+ tyrosinase were also included. Reactions were started by the addition of tyrosinase (0.3U/μL) to convert DA to DAQ and were allowed to proceed for 15 min at RT before being quenched by the addition of 1mM GSH. Samples were then placed on ice and immediately assayed for GPx4 activity or prepared for SDS-PAGE followed by Western blot analyses.

Isolation of Rat Brain Mitochondria and Dopamine Quinone Exposure

Mitochondria were isolated from male Lewis rat brain by differential centrifugation as previously described [32]. The final mitochondrial pellets were resuspended in ice-cold isolation buffer [225 mM mannitol, 75mM sucrose, 5mM HEPES, 1mM EGTA, 1 μg/μL BSA, pH 7.4] and protein content was determined. For Western blot and GPx4 activity experiments, mitochondria (500 μg) were brought to a final protein concentration of 2.0 μg/μL in mitochondrial reaction buffer [225 mM mannitol, 75mM sucrose, 25mM HEPES, 1mM EGTA, pH 7.4] containing PIC (5μL/mg protein) and the indicated amount of DA (0-150μM). Some samples also contained GSH (1mM). DAQ was generated by the addition of tyrosinase (0.3U/μL), and reactions were allowed to proceed for 15 min at RT before the mitochondria were pelleted by centrifugation (15 min, 15,000xg, 4°C). The mitochondrial pellets were then vigorously resuspended in CHAPS lysis buffer using 200 μL buffer/mg protein. The mixtures were incubated on ice for 30 min with frequent vortexing before insoluble material was removed by centrifugation (20 min, 20,000xg, 4°C). The protein concentrations of the lysates were determined, and the lysates were either immediately assayed for GPx4 activity or prepared for SDS-PAGE by boiling in Laemli sample prep buffer for Western blot analyses.

Exposure of Rat Brain Mitochondria to ^{14}C-DAQ and 2D SDS-PAGE

Isolated rat brain mitochondria were exposed to 10 μM ^{14}C-DAQ (0.55μCi) as described above for non-radioactive DA experiments. Following 15 min incubation with DAQ at RT, the mitochondria were pelleted by centrifugation (15 min, 15,000xg, 4°C). Pellets were lysed by vigorous resuspension in 2D lysis buffer (500μL/mg protein) [100mM Tris-HCl, 8M urea, 100mM β-mercaptoethanol, 2% CHAPS, pH 7.4] containing 5M guanidine-HCl. Insoluble material was removed by centrifugation (15 min, 15,000xg, RT), and then low molecular weight contaminants (i.e. guanidine-HCl) were removed by three successive passes through Microcon Ultrafiltration Units (Millipore, 10,000 MWCO) replacing the buffer with 2D PAGE buffer [100mM Tris-HCl, 8M Urea, 2% w/v CHAPS, pH 7.4] following each pass. Protein samples (45-70μg) were isoelectrically focused using 7cm 3-10 pH Immobiline DryStrips (GE Healthcare) and a Multiphor II electrophoresis system (GE Healthcare). DryStrips were equilibrated using equilibration buffer [75mM Tris-HCl, 30% v/v glycerol, 6M urea, 1% w/v SDS, pH 6.8] containing 30mM DTT for 10 min at RT, followed by equilibration buffer containing 240mM iodoacetamide for 10 min at RT. Proteins were separated in the second dimension using 15% SDS-PAGE gels, and

transferred to 0.2 μm nitrocellulose membranes which were exposed to autoradiographic film (Kodak) for up to 2 weeks at -80°C. After autoradiography, Western blot analysis for GPx4 using the Genemed GPx4 antibody was performed as described above. Images from the autoradiograms and Western blots were digitally aligned based on the location of fluorescent marks placed on the blots, which could be observed in both images.

2D gels used for initial characterization of the GPx4 antibody were processed as described above using control rat brain mitochondrial protein (250μg) and 13cm 3-10pH IPG strips. Protein was labeled with a cysteine-reactive cy5 maleimide dye as previously described [32].

PC12 Cell Culture and Mitochondrial Isolation

PC12 cells were cultured and treated with DA as previously described by our laboratory [33]. Briefly, PC12 cells were differentiated with nerve growth factor (NGF, mouse, Fisher) for 6 days followed by the addition of DA to the media for 16hr. Following the DA incubation, mitochondrial rich fractions were obtained by differential centrifugation as described in [33]. The intact mitochondria were lysed in CHAPS lysis buffer (200μL buffer/mg protein) and lysates were immediately prepared for SDS-PAGE followed by Western blot analysis for GPx4.

RESULTS

A Novel GPx4 Polyclonal Antibody

At the beginning of this study, the commercially available antibodies against GPx4 were of poor quality in our hands. To address this issue, an outside company prepared a polyclonal antibody raised in rabbit. We chose an epitope near the C-terminus of GPx4 that was determined to be specific for the protein via a variety of sequence analysis tools (i.e. NCBI Blast, T-Coffee). Because most of the studies would be in rodent tissue and cell lines, we chose to use the sequence for rat GPx4.

To initially characterize the antibody, GPx4 was detected in rat testes lysate and rat brain mitochondria by Western blot (Figure 1A). Rat testes lysate was utilized because testes contain a very high concentration of the GPx4 protein. The antibody detects a band at 19kDa, consistent with the estimated molecular weight of the cytosolic and processed mitochondrial isoforms of GPx4. In an effort to further assess the specificity of the antibody, we separated rat brain mitochondrial protein labeled with a cysteine-reactive cy5 conjugated maleimide dye using 2D PAGE, followed by Western blot for GPx4 with our antibody (Figure 1B). The antibody detected a protein located at the predicted molecular weight and isoelectric point for mitochondrial GPx4 (MW = 19.34 kDa, PI = 8.30).

Covalent Modification of GPx4 by DAQ

To examine whether DAQ would bind to selenoproteins, we first demonstrated that DAQ could covalently bind to commercially available purified glutathione peroxidase 1 (GPx1) and reduce its activity (Supplemental Data, S1). Oxidant-induced changes in GPx1 activity have been reported by others [34, 35]. To test whether DAQ could also bind to GPx4, the

target of this study, we affinity purified GPx4 from rat testes using the Genemed GPx4 antibody and exposed the protein to 150μM ^{14}C-DAQ. Analysis of the samples by autoradiography and Western blot revealed that the purified GPx4 protein covalently bound ^{14}C-DAQ, and that it was necessary to perform the reaction in the presence of the reducing agent DTT to observe this modification (Figure 2A). If no DTT was present we observed a loss of monomeric GPx4 and the accumulation of a high-molecular weight species that was both GPx4 immunoreactive and radiolabelled with ^{14}C-DA (Figure 2A-B). In both samples, there was also a lower MW spot of radioactivity that was not immunoreactive for GPx4. This may represent a fragment of DAQ-modified GPx4 that has lost the antigenic site, but this was not formally evaluated. Alternatively, the spot may represent a polymerized product of oxidized ^{14}C-DA running close to the dye front.

Testes GPx4 Protein and Activity are Sensitive to DAQ

We next sought to investigate whether exposure to DAQ would affect GPx4 activity in testes lysate, a tissue chosen because it contains high amounts of GPx4 (Figure 3A). Exposure to either DA or tyrosinase alone had no effect on testes GPx4 activity. However, a significant dose-dependent decrease in GPx4 activity was observed following exposure to DAQ (25-150μM). The presence of the thiol containing peptide GSH (1mM) was able to completely prevent DAQ-induced loss of GPx4 activity, presumably by binding to the DAQ and blocking GPx4 modification.

Aliquots of DAQ-exposed testes lysate were analyzed by Western blot to determine if DAQ had an effect on levels of GPx4 protein (Figure 3B). Results showed that DAQ exposure led to a dose-dependent decrease in monomeric GPx4 protein (19kDa) levels. In DAQ exposed samples, the appearance of a slightly lower molecular weight GPx4 band and the diffuse GPx4 immunoreactivity across the high molecular weight region of the blot are indicative of the formation of a degradation product and polymerization/aggregation of the enzyme, respectively. The addition of GSH (1mM) prior to exposure to DAQ (150μM) completely prevented the loss of the 19kDa GPx4 protein and the formation of both lower and higher molecular weight GPx4 immunoreactive bands (Figure 3B). However, the effects on Gpx4 were not reversible by the addition of GSH or DTT after the DAQ reaction was complete (data not shown). Similar results were obtained using a different GPx4 antibody (Cayman) raised against an epitope located in the middle of the protein (as opposed to the C-terminus), indicating an actual loss of GPx4 protein and not a loss of epitope immunoreactivity (Supplemental Data, Figure S2).

DAQ Decreases Brain Mitochondrial GPx4 Protein and Activity

Previous proteomic studies completed in our laboratory indicated that a subset of proteins decrease in abundance following exposure of intact rat brain mitochondria to DAQ [32] . However, these studies did not identify GPx4 as part of that subset of proteins likely due to limitations in detecting low abundance proteins. Following our observation that GPx4 protein levels decreased in testes lysate after DAQ exposure, we sought to determine the effect of DAQ on GPx4 in intact rat brain mitochondria. We exposed intact rat brain mitochondria to DAQ, and then measured GPx4 protein levels by Western blot (Figure 4A). GPx4 band intensity was normalized to the mitochondrial loading control COX IV and

quantified. A significant dose-dependent decrease in monomeric GPx4 protein levels was observed, which began at concentrations as low as 5μM DAQ and continued until almost no protein was detected following 100μM DAQ (Figure 4A). The loss of GPx4 observed after DAQ (100μM) was completely blocked if GSH (1mM) was added to the mitochondrial suspension prior to DAQ generation, indicating that modification of the protein by DAQ was the underlying factor responsible for the loss of protein. Faint, immunoreactive bands could be detected just below the 19kDa GPx4 band following exposure to 5 and 10μM DAQ, representing a degradation product of GPx4 that had formed.

We next sought to determine if GPx4 activity was similarly decreased following DAQ exposure in intact brain mitochondria. Because of the low specific activity of GPX4 in rat brain mitochondria, we increased the sensitivity of the assay by measuring the change in NADPH fluorescence following the addition of the GPX4 substrate. Using this modified assay, we observed a significant decrease (-34%) in GPx4 activity in rat brain mitochondria after exposure to 10μM DAQ (Figure 4B).

Brain Mitochondrial GPx4 is Covalently Modified by DAQ

The loss of activity following exposure to DAQ could result from the loss of protein, covalent modification of active site residues by DAQ, or both. To determine if the protein was modified in the mitochondria by the DAQ, we exposed intact rat brain mitochondria to 10μM ^{14}C-DAQ. Using 2D PAGE, autoradiography, and Western blot for GPx4 we were able to detect ^{14}C-DA modified GPx4 in the mitochondrial lysate (Figure 5). Multiple forms of GPx4 were detected as a train of immunoreactive spots, the most acidic being the predominant form with three minor spots decreasing in intensity in the basic direction. The three basic GPx4 spots were associated with radioactivity, which increased in intensity from the acidic to basic direction. The addition of a DA molecule to a protein should result in a basic PI shift due to the added amine group. The inverse correlation amongst immunoreactivity and radioactivity that we observed with GPx4 suggests the addition of multiple DA molecules to the protein. Interestingly, we were only able to detect modified GPx4 protein if guanidine-HCl and β-ME were present in the buffer used to lyse the DAQ-exposed mitochondria. Others have noted that insoluble polymers of GPx4 found in the sperm mitochondrial capsule can only be solubilized completely in the presence of these two reagents [36].

PC12 Cell Mitochondrial GPx4 is Sensitive to DA Treatment

Finally, we examined the effects of reactive metabolites of DA on mitochondrial GPx4 in a cell culture model. Differentiated PC12 cells were exposed to DA (50μM and 150μM) for 16hr followed by isolation of mitochondrial-enriched fractions, as previously described [33]. Western blot analysis of the mitochondrial lysates using the mitochondrial loading control HSP60 revealed that monomeric GPx4 protein levels were significantly decreased by 40.5% and 84.5% after cells were exposed to 50 and 150μM DA, respectively (Figure 6).

DISCUSSION

Here, we report the results of our studies on the effect of reactive metabolites of DA, most notably DAQ, on the selenoprotein GPx4 *in vitro*, in isolated brain mitochondria, and in living cells. The focus of the study was to examine whether the electron deficient DAQ would target the selenoprotein GPx4 within a complex cellular environment that more closely resemble what might occur in vivo. Our results showed that GPx4 partially immunopurified from rat testes could be covalently modified by DAQ. The addition of DAQ to rat testes lysate resulted in dose dependent decreases in both GPx4 activity and monomeric GPx4 protein in conjunction with the formation of a possible GPx4 degradation product and high molecular weight polymerized GPx4. Exposing intact rat brain mitochondria to DAQ led to a similar dose-dependent decrease in the abundance of monomeric GPx4 protein along with the formation of a degradation product. Decreased GPx4 activity and multiple forms of DA-conjugated GPx4 were also detected in the lysates of brain mitochondria exposed to DAQ. Notably, brain mitochondrial GPx4 appeared more sensitive to DAQ induced alterations than GPx4 contained in testes lysate. Finally, we observed that exposing differentiated PC12 cells to DA, under conditions where intracellular DA oxidizes to DAQ, also resulted in a loss of mitochondrial GPx4 monomeric protein.

Although pathology exists in several brain regions in PD, the DAergic neurons of the SNpc show the most profound level of degeneration. One explanation for the apparent sensitivity of DAergic neurons is that they contain DA, a molecule which can easily oxidize and covalently modify nucleophilic residues in proteins. Multiple proteins with known links to PD, including alpha-synuclein, parkin, and DJ-1 can be modified and possibly functionally altered by DAQ [37-41]. Recent proteomic studies from our laboratory have identified both mitochondrial and endoplasmic reticular proteins that were affected following exposure to DA or DAQ [32, 33, 41]. Results showed that a subset of mitochondrial proteins, many of which were shown to be modified by DAQ, were also decreased in abundance following exposure to DAQ similar to our observation with GPx4 in this study [32, 41]. These findings suggest that DAQ-modification may target a protein for proteolytic degradation, although this has not been measured directly. Mitochondria contain proteases such as the Lon protease which is known to selectively degrade oxidized protein in the mitochondria [42, 43]. Thus, DAQ-modified proteins in mitochondria may be substrates for mitochondrial proteases. GPx4 was not detected in the previous studies likely due to its low abundance. However, our observations in this study suggest that GPx4 may be more sensitive to DAQ-induced modification and degradation than other mitochondrial proteins previously identified.

As hypothesized, exposure to electrophilic DAQ resulted in the presence of DA-conjugated GPx4 protein. Although the exact residue(s) was not determined, both the covalent modification and the loss of the protein likely contributed to the observed decrease in GPx4 activity. The crystal structure of the cysteine mutant of GPx4 has recently been determined and has revealed that GPx4 contains several surface exposed cysteine residues in addition to the active site selenocysteine (Cys2, Cys10, Cys66, Cys107, Cys148)[44], that may be targets for DAQ modification or oxidation and crosslinking. Several studies have shown that under conditions of oxidative stress or low GSH, GPx4 is prone to forming high molecular

weight polymers in a process mediated by a subset of these residues [24, 44]. Our data suggests that GPx4 may be modified by DA at more than one site, and that exposing GPx4 to DAQ results in the formation of high molecular weight polymers of the protein. Although GPx4 has a tendency to polymerize, high molecular weight aggregates of other proteins following DAQ exposure have also been reported [32, 37]. Regardless of the exact nature of the modification, GPx4 is clearly a target of DAQ that results in the loss of activity and GPx4 protein.

Recently, the Hastings' laboratory (unpublished observations) and others [45] have observed alterations in GPx4 immunoreactivity in human post-mortem PD substantia nigra as compared to control brains. Bellinger and colleagues reported that in control human SNpc neurons GPx4 localizes with neuromelanin and near Lewy bodies in PD brain [45].. The generation of DAQ in the cytosol is thought to be the first step in neuromelanin synthesis [46]. We propose that the unique changes in GPx4 immunoreactivity observed in PD brain may be due in part to the vulnerability of GPx4 to attack by DAQ.

The role that selenoproteins and selenium homeostasis play in the maintenance of neuronal viability has only recently begun to be appreciated [47]. Mice deficient in selenoprotein P, the selenium delivery protein for the brain, showed early evidence of neurodegeneration [48, 49]. While, mice that lack the tRNA molecule specific for selenocysteine insertion during translation are embryonic lethal [11]. The 25 selenoproteins coded for in the human genome have a variety of functions, including well established roles in protecting cells against oxidative stress (GSH peroxidases, thioredoxin reductases, selenoprotein R)[50]. The antioxidant activity is closely tied to the active site selenocysteine residue, which catalyzes redox reactions much faster than cysteine can [26]. The nucleophilic nature of the selenocysteine residue also renders these critical proteins more susceptible to covalent modification and inactivation by electrophilic compounds such as DAQ.

The consequences of the loss of GPx4 have begun to be unraveled using mouse models. A complete knockout of GPx4 results in embryonic lethality at day E7.5 [25]. Specific knockout of long-form GPx4 (predicted mitochondrial) does not result in embryonic death, neurodegeneration, or increased apoptosis [51]. However, it has recently been shown that long-form GPx4 is primarily localized to the testes and that the short-form (which does not contain an apparent mitochondrial trafficking sequence) is the isoform present in brain mitochondria [52]. Perhaps most telling are the results of conditional knockout of the protein. The knockout of GPx4 in neurons under the control of the CamKIIα promoter resulted in profound neurodegeneration in the cerebral cortex and hippocampus, where CamKIIα is preferentially expressed [53]. Whereas systemically knocking out the protein in adult animals resulted in the loss of hippocampal neurons and was lethal in two weeks or less [54].

GPx4 has recently been linked to processes occurring in Alzheimer disease. Mice that are heterozygous for GPx4 showed age-dependent increases in brain lipid peroxides, β-secretase activity, endogenous β-amyloid as compared to wild-type mice [55]. When heterozygote GPx4 (+/-) mice were crossed with transgenic mice overexpressing the amyloid precursor protein they showed increased amyloid plaque deposition as compared to the mice

overexpressing APP on a GPx4 wild-type background [55]. In a separate study, levels of GPx4 protein were reduced in mice overexpressing APP [56]. Altogether, there is mounting *in vivo* evidence indicating that GPx4 is a critical factor in maintaining neuronal viability and that alterations in GPx4 may be involved in neurodegenerative diseases.

The presence of GPx4 serves to limit lipid peroxidation and the formation of toxic aldehyde products which are found in neurodegenerative diseases. The unique ability of GPx4 to directly reduce phospholipid hydroperoxides places it in a position to control cell death through at least two different pathways. First, GPx4 is thought to protect against apoptosis and to maintain mitochondrial integrity by keeping cardiolipin in a reduced state, which in turn prevents cytochrome c release and stabilizes the electron transport chain [57]. GPx4 overexpression protects primary cortical neurons and RBL2H3 cells from oxidative stress-induced apoptosis [58, 59]. Accordingly, mitochondria from cells overexpressing GPx4 release less cytochrome c and are protected from permeability transition pore opening caused by oxidative stress [60, 61]. Secondly, it has been shown that GPx4 regulates the activity of 12/15-lipoxygenase by maintaining low levels of cellular lipid peroxides. High levels of lipid peroxides result in the oxidation and subsequent activation of 12/15-lipoxygenase leading to the release of apoptosis-inducing factor and cell death [53].

Increases in markers of lipid peroxidation have been consistently observed in the SNpc of sporadic PD patients and the brains of animal models of the disease [62-65]. Here, we have demonstrated that the protein primarily responsible for reducing phospholipid hydroperoxides, GPx4, is sensitive to modification by oxidative products of DA, and that this modification results in decreased GPx4 activity, GPx4 degradation, and the formation of high molecular weight polymers containing GPx4. Therefore, it is possible that a selective loss of GPx4 activity in DAergic neurons underlies the increases in lipid peroxides measured in PD brain and is a contributing factor in PD pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The abbreviations used are

DA	dopamine
DAergic	dopaminergic
DAQ	dopamine quinone
PCOOH	phosphatidylcholine hydroperoxide
PD	Parkinson's disease

SNpc substantia nigra pars compacta

REFERENCES

1. Hauser DN, Hastings TG. Mitochondrial dysfunction and oxidative stress in Parkinson's disease and monogenic parkinsonism. Neurobiol Dis. 2013; 51:35–42. [PubMed: 23064436]
2. Martinou JC, Youle RJ. Which came first, the cytochrome c release or the mitochondrial fission? Cell Death Differ. 2006; 13:1291–1295. [PubMed: 16763618]
3. Andrabi SA, Dawson TM, Dawson VL. Mitochondrial and nuclear cross talk in cell death: parthanatos. Ann N Y Acad Sci. 2008; 1147:233–241. [PubMed: 19076445]
4. Duchen MR. Mitochondria and calcium: from cell signalling to cell death. J Physiol. 2000; 529(Pt 1):57–68. [PubMed: 11080251]
5. Starkov AA. The role of mitochondria in reactive oxygen species metabolism and signaling. Ann N Y Acad Sci. 2008; 1147:37–52. [PubMed: 19076429]
6. Valente EM, Abou-Sleiman PM, Caputo V, Muqit MM, Harvey K, Gispert S, Ali Z, Del Turco D, Bentivoglio AR, Healy DG, Albanese A, Nussbaum R, Gonzalez-Maldonado R, Deller T, Salvi S, Cortelli P, Gilks WP, Latchman DS, Harvey RJ, Dallapiccola B, Auburger G, Wood NW. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science. 2004; 304:1158–1160. [PubMed: 15087508]
7. Bonifati V, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E, Dekker MC, Squitieri F, Ibanez P, Joosse M, van Dongen JW, Vanacore N, van Swieten JC, Brice A, Meco G, van Duijn CM, Oostra BA, Heutink P. Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science. 2003; 299:256–259. [PubMed: 12446870]
8. Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, Yokochi M, Mizuno Y, Shimizu N. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature. 1998; 392:605–608. [PubMed: 9560156]
9. Krebiehl G, Ruckerbauer S, Burbulla LF, Kieper N, Maurer B, Waak J, Wolburg H, Gizatullina Z, Gellerich FN, Woitalla D, Riess O, Kahle PJ, Proikas-Cezanne T, Kruger R. Reduced basal autophagy and impaired mitochondrial dynamics due to loss of Parkinson's disease-associated protein DJ-1. PLoS One. 2010; 5:e9367. [PubMed: 20186336]
10. Narendra DP, Jin SM, Tanaka A, Suen DF, Gautier CA, Shen J, Cookson MR, Youle RJ. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol. 2010; 8:e1000298. [PubMed: 20126261]
11. Thomas KJ, McCoy MK, Blackinton J, Beilina A, van der Brug M, Sandebring A, Miller D, Maric D, Cedazo-Minguez A, Cookson MR. DJ-1 acts in parallel to the PINK1/parkin pathway to control mitochondrial function and autophagy. Hum Mol Genet. 2011; 20:40–50. [PubMed: 20940149]
12. Krige D, Carroll MT, Cooper JM, Marsden CD, Schapira AH. Platelet mitochondrial function in Parkinson's disease. The Royal Kings and Queens Parkinson Disease Research Group. Ann Neurol. 1992; 32:782–788. [PubMed: 1471869]
13. Sipos I, Tretter L, Adam-Vizi V. Quantitative relationship between inhibition of respiratory complexes and formation of reactive oxygen species in isolated nerve terminals. J Neurochem. 2003; 84:112–118. [PubMed: 12485407]
14. Schapira AH, Cooper JM, Dexter D, Jenner P, Clark JB, Marsden CD. Mitochondrial complex I deficiency in Parkinson's disease. Lancet. 1989; 1:1269. [PubMed: 2566813]
15. Langston JW, Ballard P, Tetrud JW, Irwin I. Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science. 1983; 219:979–980. [PubMed: 6823561]
16. Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci. 2000; 3:1301–1306. [PubMed: 11100151]
17. Sherer TB, Kim JH, Betarbet R, Greenamyre JT. Subcutaneous rotenone exposure causes highly selective dopaminergic degeneration and alpha-synuclein aggregation. Exp Neurol. 2003; 179:9–16. [PubMed: 12504863]

18. Cannon JR, Tapias V, Na HM, Honick AS, Drolet RE, Greenamyre JT. A highly reproducible rotenone model of Parkinson's disease. Neurobiol Dis. 2009; 34:279–290. [PubMed: 19385059]
19. Greenamyre JT, Cannon JR, Drolet R, Mastroberardino PG. Lessons from the rotenone model of Parkinson's disease. Trends Pharmacol Sci. 2010; 31:141–142. [PubMed: 20096940]
20. Graham DG, Tiffany SM, Bell WR Jr. Gutknecht WF. Autoxidation versus covalent binding of quinones as the mechanism of toxicity of dopamine, 6-hydroxydopamine, and related compounds toward C1300 neuroblastoma cells in vitro. Mol Pharmacol. 1978; 14:644–653. [PubMed: 567274]
21. Hastings TG, Lewis DA, Zigmond MJ. Role of oxidation in the neurotoxic effects of intrastriatal dopamine injections. Proceedings of the National Academy of Sciences of the United States of America. 1996; 93:1956–1961. [PubMed: 8700866]
22. Caudle WM, Richardson JR, Wang MZ, Taylor TN, Guillot TS, McCormack AL, Colebrooke RE, Di Monte DA, Emson PC, Miller GW. Reduced vesicular storage of dopamine causes progressive nigrostriatal neurodegeneration. J Neurosci. 2007; 27:8138–8148. [PubMed: 17652604]
23. Huber RE, Criddle RS. Comparison of the chemical properties of selenocysteine and selenocystine with their sulfur analogs. Arch Biochem Biophys. 1967; 122:164–173. [PubMed: 6076213]
24. Low SC, Grundner-Culemann E, Harney JW, Berry MJ. SECIS-SBP2 interactions dictate selenocysteine incorporation efficiency and selenoprotein hierarchy. EMBO J. 2000; 19:6882–6890. [PubMed: 11118223]
25. Yant LJ, Ran Q, Rao L, Van Remmen H, Shibatani T, Belter JG, Motta L, Richardson A, Prolla TA. The selenoprotein GPX4 is essential for mouse development and protects from radiation and oxidative damage insults. Free Radic Biol Med. 2003; 34:496–502. [PubMed: 12566075]
26. Maiorino M, Aumann KD, Brigelius-Flohe R, Doria D, van den Heuvel J, McCarthy J, Roveri A, Ursini F, Flohe L. Probing the presumed catalytic triad of selenium-containing peroxidases by mutational analysis of phospholipid hydroperoxide glutathione peroxidase (PHGPx). Biol Chem Hoppe Seyler. 1995; 376:651–660. [PubMed: 8962674]
27. Zhang Y, Zhou Y, Schweizer U, Savaskan NE, Hua D, Kipnis J, Hatfield DL, Gladyshev VN. Comparative analysis of selenocysteine machinery and selenoproteome gene expression in mouse brain identifies neurons as key functional sites of selenium in mammals. J Biol Chem. 2008; 283:2427–2438. [PubMed: 18032379]
28. Panfili E, Sandri G, Ernster L. Distribution of glutathione peroxidases and glutathione reductase in rat brain mitochondria. FEBS Lett. 1991; 290:35–37. [PubMed: 1915888]
29. Houtkooper RH, Vaz FM. Cardiolipin, the heart of mitochondrial metabolism. Cell Mol Life Sci. 2008; 65:2493–2506. [PubMed: 18425414]
30. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72:248–254. [PubMed: 942051]
31. Zhang LP, Maiorino M, Roveri A, Ursini F. Phospholipid hydroperoxide glutathione peroxidase: specific activity in tissues of rats of different age and comparison with other glutathione peroxidases. Biochim Biophys Acta. 1989; 1006:140–143. [PubMed: 2804065]
32. Van Laar VS, Dukes AA, Cascio M, Hastings TG. Proteomic analysis of rat brain mitochondria following exposure to dopamine quinone: implications for Parkinson disease. Neurobiol Dis. 2008; 29:477–489. [PubMed: 18226537]
33. Dukes AA, Van Laar VS, Cascio M, Hastings TG. Changes in endoplasmic reticulum stress proteins and aldolase A in cells exposed to dopamine. J Neurochem. 2008; 106:333–346. [PubMed: 18384645]
34. Cho CS, Lee S, Lee GT, Woo HA, Choi EJ, Rhee SG. Irreversible inactivation of glutathione peroxidase 1 and reversible inactivation of peroxiredoxin II by H2O2 in red blood cells. Antioxidants & redox signaling. 2010; 12:1235–1246. [PubMed: 20070187]
35. Suryo Rahmanto A, Pattison DI, Davies MJ. Photo-oxidation-induced inactivation of the selenium-containing protective enzymes thioredoxin reductase and glutathione peroxidase. Free Radic Biol Med. 2012; 53:1308–1316. [PubMed: 22884457]
36. Ursini F, Heim S, Kiess M, Maiorino M, Roveri A, Wissing J, Flohe L. Dual function of the selenoprotein PHGPx during sperm maturation. Science. 1999; 285:1393–1396. [PubMed: 10464096]

37. Belluzzi E, Bisaglia M, Lazzarini E, Tabares LC, Beltramini M, Bubacco L. Human SOD2 modification by dopamine quinones affects enzymatic activity by promoting its aggregation: possible implications for Parkinson's disease. PloS one. 2012; 7:e38026. [PubMed: 22723845]
38. Conway KA, Rochet JC, Bieganski RM, Lansbury PT Jr. Kinetic stabilization of the alpha-synuclein protofibril by a dopamine-alpha-synuclein adduct. Science. 2001; 294:1346–1349. [PubMed: 11701929]
39. Girotto S, Sturlese M, Bellanda M, Tessari I, Cappellini R, Bisaglia M, Bubacco L, Mammi S. Dopamine-derived quinones affect the structure of the redox sensor DJ-1 through modifications at Cys-106 and Cys-53. J Biol Chem. 2012; 287:18738–18749. [PubMed: 22431735]
40. LaVoie MJ, Ostaszewski BL, Weihofen A, Schlossmacher MG, Selkoe DJ. Dopamine covalently modifies and functionally inactivates parkin. Nature medicine. 2005; 11:1214–1221.
41. Van Laar VS, Mishizen AJ, Cascio M, Hastings TG. Proteomic identification of dopamine-conjugated proteins from isolated rat brain mitochondria and SH-SY5Y cells. Neurobiol Dis. 2009
42. Bota DA, Davies KJ. Lon protease preferentially degrades oxidized mitochondrial aconitase by an ATP-stimulated mechanism. Nat Cell Biol. 2002; 4:674–680. [PubMed: 12198491]
43. Ngo JK, Pomatto LC, Bota DA, Koop AL, Davies KJ. Impairment of lon-induced protection against the accumulation of oxidized proteins in senescent wi-38 fibroblasts. J Gerontol A Biol Sci Med Sci. 2011; 66:1178–1185. [PubMed: 21868393]
44. Scheerer P, Borchert A, Krauss N, Wessner H, Gerth C, Hohne W, Kuhn H. Structural basis for catalytic activity and enzyme polymerization of phospholipid hydroperoxide glutathione peroxidase-4 (GPx4). Biochemistry. 2007; 46:9041–9049. [PubMed: 17630701]
45. Bellinger FP, Bellinger MT, Seale LA, Takemoto AS, Raman AV, Miki T, Manning-Bog AB, Berry MJ, White LR, Ross GW. Glutathione Peroxidase 4 is associated with Neuromelanin in Substantia Nigra and Dystrophic Axons in Putamen of Parkinson's brain. Molecular neurodegeneration. 2011; 6:8. [PubMed: 21255396]
46. Zecca L, Tampellini D, Gerlach M, Riederer P, Fariello RG, Sulzer D. Substantia nigra neuromelanin: structure, synthesis, and molecular behaviour. Mol Pathol. 2001; 54:414–418. [PubMed: 11724917]
47. Chen J, Berry MJ. Selenium and selenoproteins in the brain and brain diseases. J Neurochem. 2003; 86:1–12. [PubMed: 12807419]
48. Hill KE, Zhou J, McMahan WJ, Motley AK, Burk RF. Neurological dysfunction occurs in mice with targeted deletion of the selenoprotein P gene. The Journal of nutrition. 2004; 134:157–161. [PubMed: 14704310]
49. Valentine WM, Abel TW, Hill KE, Austin LM, Burk RF. Neurodegeneration in mice resulting from loss of functional selenoprotein P or its receptor apolipoprotein E receptor 2. Journal of neuropathology and experimental neurology. 2008; 67:68–77. [PubMed: 18172410]
50. Reeves MA, Hoffmann PR. The human selenoproteome: recent insights into functions and regulation. Cell Mol Life Sci. 2009; 66:2457–2478. [PubMed: 19399585]
51. Schneider M, Forster H, Boersma A, Seiler A, Wehnes H, Sinowatz F, Neumuller C, Deutsch MJ, Walch A, Hrabe de Angelis M, Wurst W, Ursini F, Roveri A, Maleszewski M, Maiorino M, Conrad M. Mitochondrial glutathione peroxidase 4 disruption causes male infertility. FASEB J. 2009; 23:3233–3242. [PubMed: 19417079]
52. Liang H, Yoo SE, Na R, Walter CA, Richardson A, Ran Q. Short form glutathione peroxidase 4 is the essential isoform required for survival and somatic mitochondrial functions. J Biol Chem. 2009; 284:30836–30844. [PubMed: 19744930]
53. Seiler A, Schneider M, Forster H, Roth S, Wirth EK, Culmsee C, Plesnila N, Kremmer E, Radmark O, Wurst W, Bornkamm GW, Schweizer U, Conrad M. Glutathione peroxidase 4 senses and translates oxidative stress into 12/15-lipoxygenase dependent- and AIF-mediated cell death. Cell Metab. 2008; 8:237–248. [PubMed: 18762024]
54. Yoo SE, Chen L, Na R, Liu Y, Rios C, Van Remmen H, Richardson A, Ran Q. Gpx4 ablation in adult mice results in a lethal phenotype accompanied by neuronal loss in brain. Free Radic Biol Med. 2012

55. Chen L, Na R, Gu M, Richardson A, Ran Q. Lipid peroxidation up-regulates BACE1 expression in vivo: a possible early event of amyloidogenesis in Alzheimer's disease. J Neurochem. 2008; 107:197–207. [PubMed: 18680556]
56. Yoo MH, Gu X, Xu XM, Kim JY, Carlson BA, Patterson AD, Cai H, Gladyshev VN, Hatfield DL. Delineating the role of glutathione peroxidase 4 in protecting cells against lipid hydroperoxide damage and in Alzheimer's disease. Antioxid Redox Signal. 2010; 12:819–827. [PubMed: 19769463]
57. Imai H, Nakagawa Y. Biological significance of phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) in mammalian cells. Free Radic Biol Med. 2003; 34:145–169. [PubMed: 12521597]
58. Nomura K, Imai H, Koumura T, Arai M, Nakagawa Y. Mitochondrial phospholipid hydroperoxide glutathione peroxidase suppresses apoptosis mediated by a mitochondrial death pathway. J Biol Chem. 1999; 274:29294–29302. [PubMed: 10506188]
59. Ran Q, Gu M, Van Remmen H, Strong R, Roberts JL, Richardson A. Glutathione peroxidase 4 protects cortical neurons from oxidative injury and amyloid toxicity. J Neurosci Res. 2006; 84:202–208. [PubMed: 16673405]
60. Imai H, Koumura T, Nakajima R, Nomura K, Nakagawa Y. Protection from inactivation of the adenine nucleotide translocator during hypoglycaemia-induced apoptosis by mitochondrial phospholipid hydroperoxide glutathione peroxidase. Biochem J. 2003; 371:799–809. [PubMed: 12534348]
61. Nomura K, Imai H, Koumura T, Kobayashi T, Nakagawa Y. Mitochondrial phospholipid hydroperoxide glutathione peroxidase inhibits the release of cytochrome c from mitochondria by suppressing the peroxidation of cardiolipin in hypoglycaemia-induced apoptosis. Biochem J. 2000; 351:183–193. [PubMed: 10998361]
62. Dexter DT, Carter CJ, Wells FR, Javoy-Agid F, Agid Y, Lees A, Jenner P, Marsden CD. Basal lipid peroxidation in substantia nigra is increased in Parkinson's disease. J Neurochem. 1989; 52:381–389. [PubMed: 2911023]
63. Dexter DT, Holley AE, Flitter WD, Slater TF, Wells FR, Daniel SE, Lees AJ, Jenner P, Marsden CD. Increased levels of lipid hydroperoxides in the parkinsonian substantia nigra: an HPLC and ESR study. Mov Disord. 1994; 9:92–97. [PubMed: 8139611]
64. Garcia E, Limon D, Perez-De La Cruz V, Giordano M, Diaz-Munoz M, Maldonado PD, Herrera-Mundo MN, Pedraza-Chaverri J, Santamaria A. Lipid peroxidation, mitochondrial dysfunction and neurochemical and behavioural deficits in different neurotoxic models: protective role of S-allylcysteine. Free Radic Res. 2008; 42:892–902. [PubMed: 18985488]
65. Palacino JJ, Sagi D, Goldberg MS, Krauss S, Motz C, Wacker M, Klose J, Shen J. Mitochondrial dysfunction and oxidative damage in parkin-deficient mice. J Biol Chem. 2004; 279:18614–18622. [PubMed: 14985362]

Highlights

Reactive metabolites of dopamine may contribute to cell death in Parkinson's disease.

The effects of dopamine quinone on the selenoprotein GPx4 were investigated.

Dopamine quinone covalently bound GPx4, leading to degradation and aggregation.

The loss of monomeric GPx4 protein was accompanied by the loss of GPx4 activity.

A selective loss of GPx4 activity in dopamine neurons may contribute to their death.

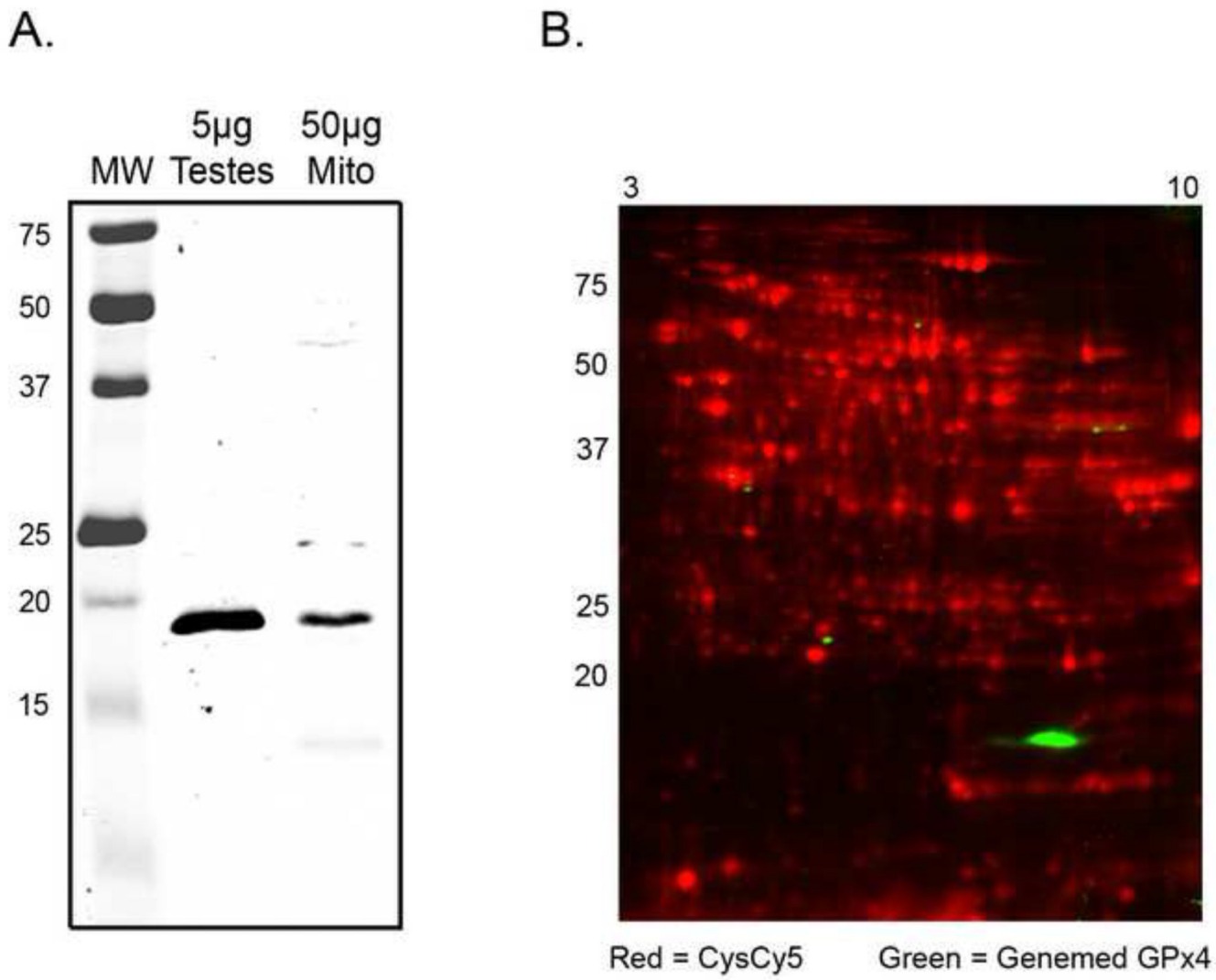

Fig 1.

Characterization of the GPx4 polyclonal antibody. (A) Western blot using the Genemed Rabbit-anti-GPx4 antibody on a 1D SDS-PAGE of rat testes lysate (5µg) and rat brain mitochondrial lysate (50µg). (B) Rat brain mitochondrial proteins were labeled with a cysteine-reactive Cy5 dye (red)and separated using 2D PAGE (3-10pH), followed by Western blot with the Genemed Rabbit-anti-GPx4 antibody (green).

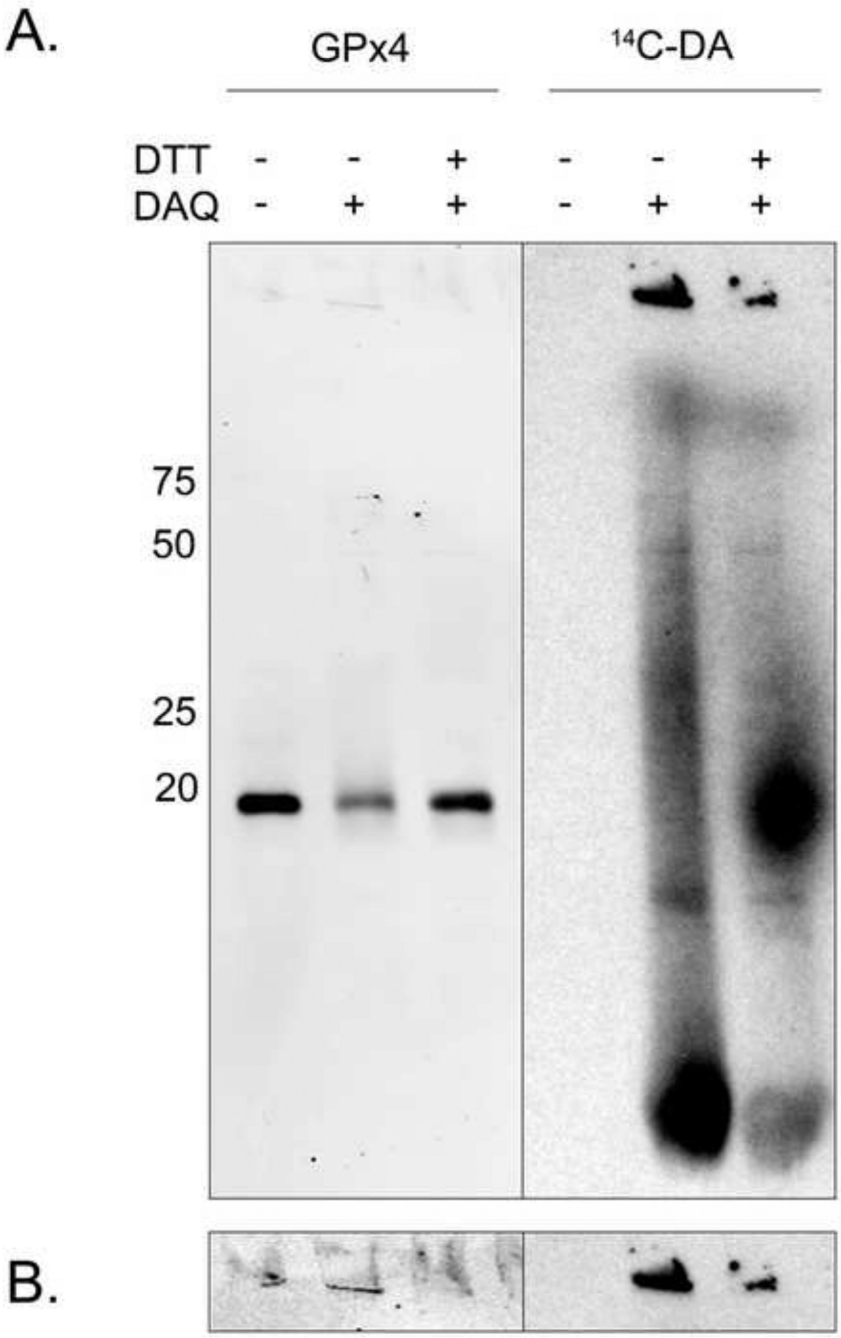

Fig. 2.

DAQ covalently binds to partially purified GPx4. (A) Immunopurified rat testes GPx4 was reacted with 150μM ^{14}C-DA/tyrosinase (DAQ) for 15 min and then run on 1D SDS-PAGE followed by autoradiography and Western blot for GPx4. Control samples received no DA, while some samples were reduced with 1mM DTT prior to the reaction with DAQ. The Western blot (left) shows GPx4 bands at 19kDa in all samples, with the nonreduced protein exposed to DAQ having decreased immunoreactivity. The autoradiogram (right) displays a prominent 19kDa band of radioactivity in the reduced (DTT- exposed) DAQ sample

corresponding to the GPx4 immunoreactive band (right lanes on both). (B) Digitally enhancing the contrast at the top of the Western blot shown in (A) reveals GPx4 immunoreactivity in the nonreduced DAQ exposed sample which corresponds to the high molecular weight band of radioactivity at the top of the gel.

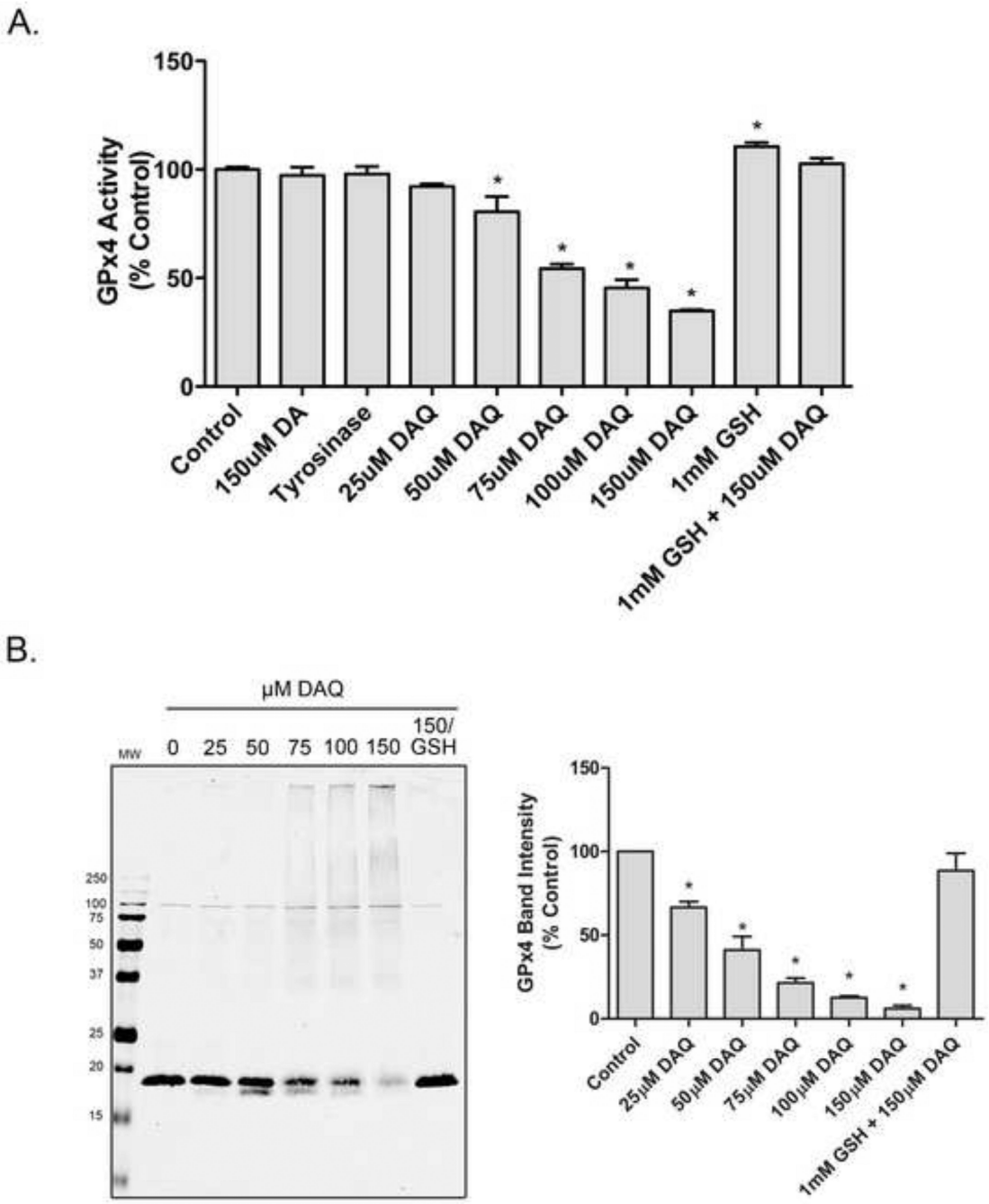

Fig. 3.

DAQ inactivates GPx4 and decreases GPx4 abundance in testes lysate. (A) Rat testes lysate was assayed for GPx4 activity following exposure to 25-150µM DAQ or other control conditions including DA alone, tyrosinase alone, GSH alone, and GSH + DAQ. Values listed as mean % Control GPx4 activity ±SEM, n=3-8. *, significantly different from control, $p<0.05$; ANOVA followed by Bonferroni's multiple comparison test. Control Specific Activity = 131.7±6.8 mU/mg. (B) GPx4 Western blot of DAQ exposed testes lysate

samples with quantitation of the 19kDa band. Values listed as % control ± SEM, n=3. *, significantly different from control, $p<0.05$; Z-test.

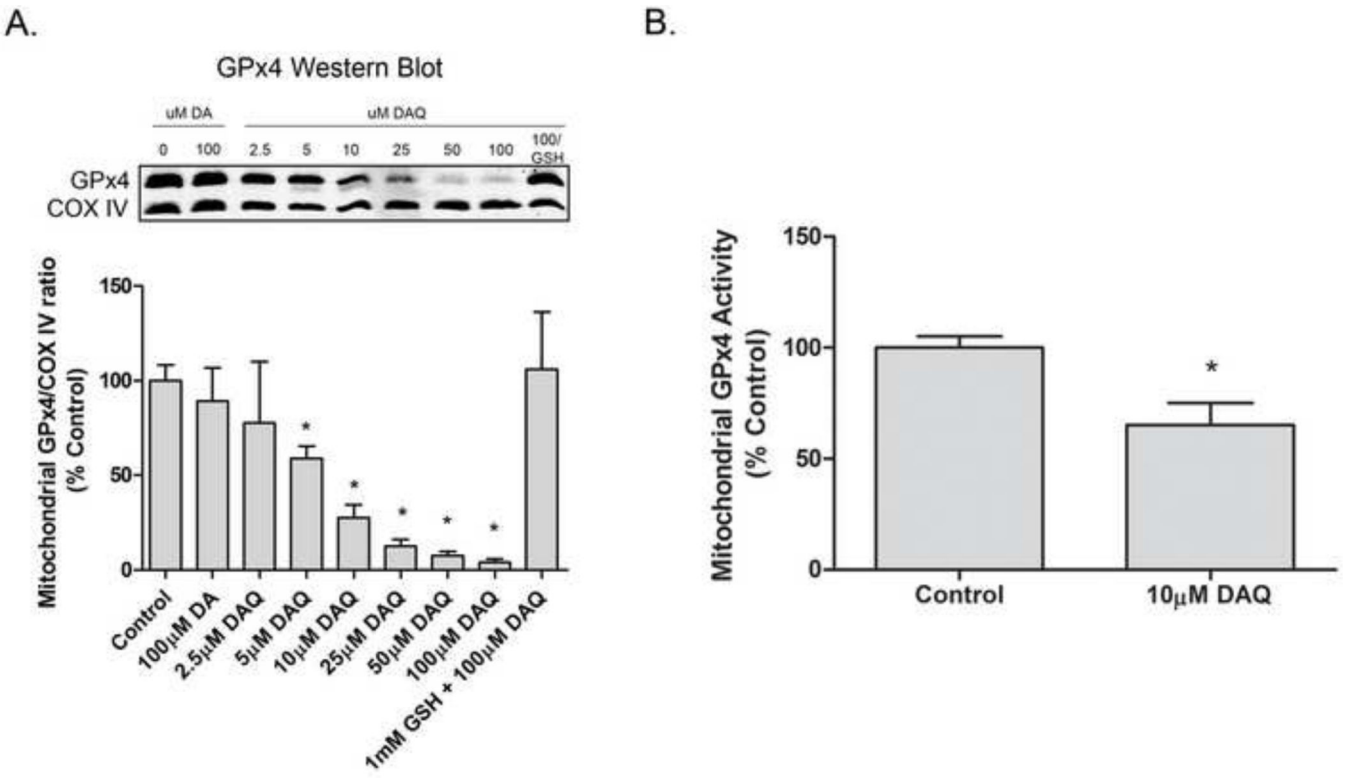

Fig. 4.

Exposure to DAQ decreases GPx4 abundance and activity in intact rat brain mitochondria. (A) Western blot of mitochondrial protein (40μg/lane) for GPx4 and COXIV following exposure of intact brain mitochondria to 2.5-100μM DAQ, DA alone, or 1mM GSH + 100μM DAQ. GPx4 bands were normalized to COXIV levels (loading control) and the values are graphed as Mean % control ± SEM, n=3. *, significantly different from control, $p<0.05$, ANOVA followed by Bonferroni's multiple comparison test. (B) GPx4 activity was measured in the lysates of intact rat brain mitochondria following exposure to 10μM DAQ. Activity is reported as the initial rate of change in NADPH fluorescence (340nm excitation, 460nm emission) per mg mitochondrial protein. Values listed as % control ± SEM, n=3. *, significantly different from control, $p<0.05$; Student's T-Test.

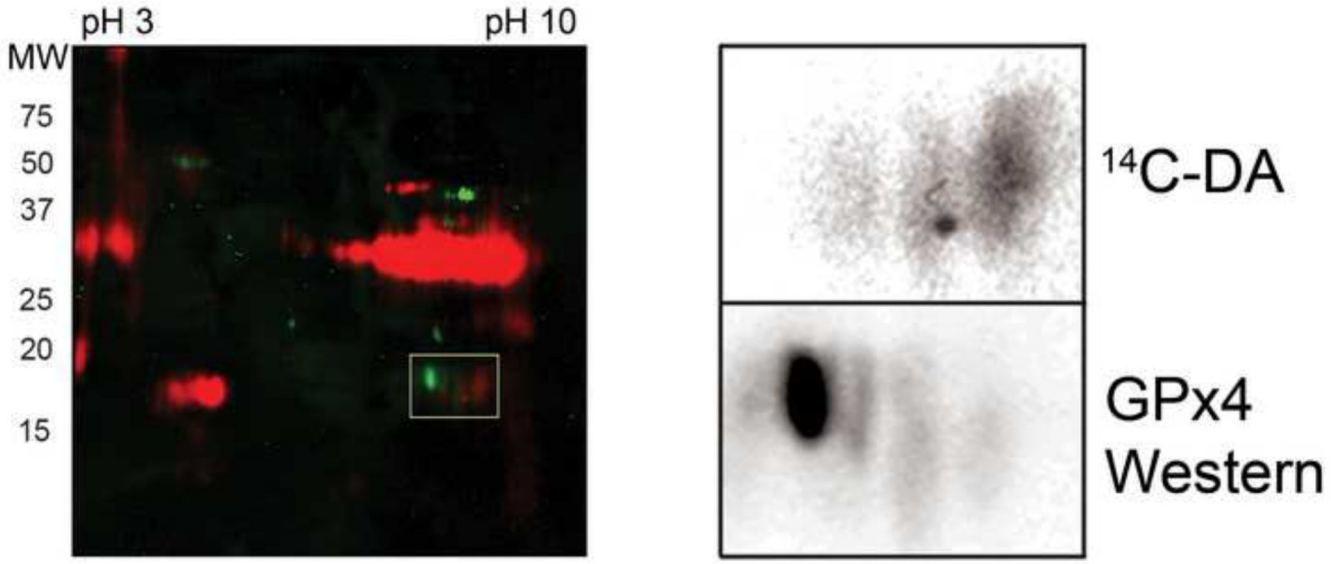

Fig. 5.

Covalent modification of GPx4 by 10μM ^{14}C-DA in intact rat brain mitochondria. Intact rat brain mitochondria exposed to 10μM ^{14}C-DAQ and were lysed with a buffer containing 5M Guanidine-HCl and 0.1M β-ME. Radiolabeled proteins (70μg) were separated by 2-D gel electrophoresis and then exposed to autoradiography (red) and GPx4 Western blot (green) analysis. Magnified images of the area of interest from the autoradiogram (top) and Western blot (bottom) are shown to the right of the full blot. The GPx4 Western blot showed a train of four spots of GPx4, decreasing in intensity in the acidic to basic direction. Three areas of radioactivity overlay with the three more basic GPx4 spots and increase in intensity in the acidic to basic direction. This is the expected observable spot pattern if GPx4 were modified multiple times by DA, which contains basic NH_3^+groups. These results are representative of two independent experiments.

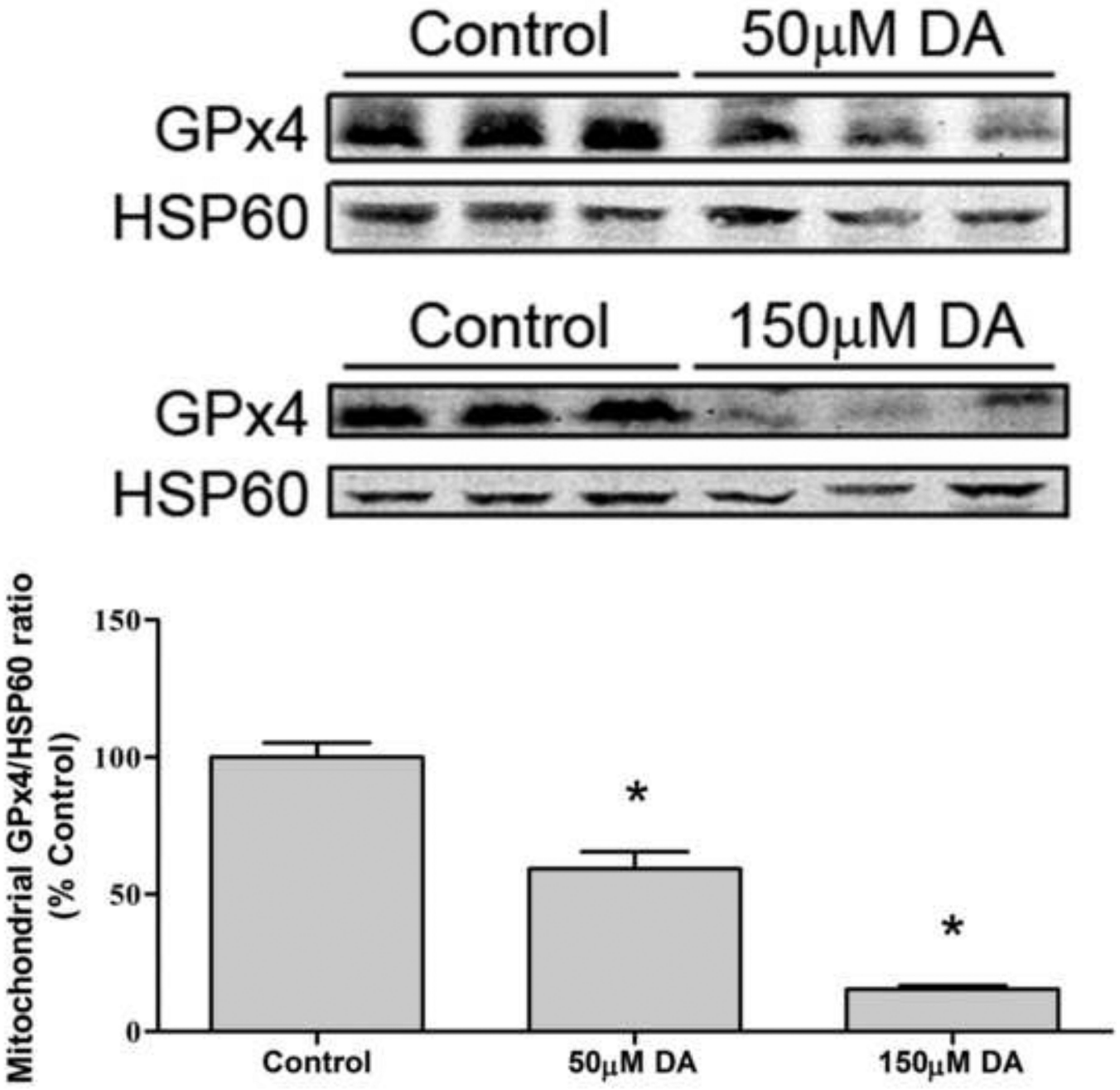

Fig. 6.

Exposure of differentiated PC12 cells to DA decreased mitochondrial GPx4 levels. Differentiated PC12 cells were exposed to DA (0, 50, 150μM) for 16hr. Mitochondria were isolated and GPx4 protein levels were determined by Western blot. Quantification of GPx4 protein bands normalized to the mitochondrial loading control HSP60 are expressed as Mean % control ± SEM, n=3-6. *, significantly different from control, $p<0.05$; ANOVA followed by Bonferroni's multiple comparison test.