ANTIOXIDANTS & REDOX SIGNALING
Volume 7, Numbers 7 & 8, 2005

Forum Original Research Communication

Glutathione Depletion in a Midbrain-Derived Immortalized Dopaminergic Cell Line Results in Limited Tyrosine Nitration of Mitochondrial Complex I Subunits: Implications for Parkinson's Disease

SRINIVAS BHARATH and JULIE KAY ANDERSEN

ABSTRACT

Oxidative stress and mitochondrial dysfunction signify two important biochemical events associated with the loss of dopaminergic neurons in Parkinson's disease (PD). Studies using *in vitro* and *in vivo* PD models and in affected tissues from the disease itself have demonstrated a selective inhibition of mitochondrial complex I activity that appears to affect normal mitochondrial physiology leading to neuronal cell death. Earlier experiments from our laboratory have demonstrated that induced depletion of glutathione (GSH + GSSG) in cultured dopaminergic cells resulted in increased oxidative stress and a decrease in mitochondrial function. Furthermore, this dysfunction was linked to a selective decrease in mitochondrial complex I activity that appears to be due to oxidation of this complex. Glutathione depletion is the earliest detectable biochemical event during PD progression and occurs prior to complex I inhibition. Recent observations have also indicated that oxidative damage to complex I via naturally occurring free radicals such as peroxynitrite leads to modification of tyrosine and/or cysteine residues resulting in complex I inhibition. Using the sucrose gradient method, we detected in complex I-enriched fractions from a glutathione-depleted dopaminergic cell line two bands corresponding to ~25-kDa and ~30-kDa polypeptides that demonstrate anti-nitrotyrosine immunoreactivity, suggesting the possible involvement of protein nitration by peroxynitrite in glutathione depletion-mediated complex I inhibition. *Antioxid. Redox Signal.* 7, 900–910.

INTRODUCTION

OXIDATIVE STRESS appears to play a major role in the degeneration of dopaminergic neurons of the substantia nigra (SN) during Parkinson's disease (PD) (13). Dopaminergic neurons are particularly prone to oxidative stress due to dopamine oxidation producing reactive oxygen species (ROS) (17, 36), which can damage nearby biomolecules (12). It has been observed that the SN of early PD patients has significant depletion of total glutathione [reduced glutathione (GSH) + oxidized glutathione (GSSG)] (2). GSH, a tripeptide present in neurons and other cell types, is a major nonproteinaceous antioxidant and redox modulator within the brain. Glutathione depletion is the earliest reported biochemical change in the PD SN, preceding decreases in both mitochondrial complex I activity and dopamine levels (23, 31). Glutathione depletion in the brain is believed to promote mitochondrial damage via increased ROS production (16). Mitochondrial dysfunction appears to play a role in the neurodegeneration associated with PD (4, 5). Using an inducible glutathione-depleted dopaminergic cell model system, we previously demonstrated that glutathione depletion elicits a selective complex I inhibition resulting in mitochondrial dysfunction (18). These results suggest that the early glutathione depletion in the SN of PD patients could cause selective inhibition of complex I activity, resulting in subsequent mitochondrial dysfunction and neuronal cell loss. Complex I is in fact considered to be one of the most severely affected mito-

Buck Institute for Age Research, Novato, CA.

chondrial complexes by age-related increases in oxidative stress (19). Taken together, these data suggest that glutathione depletion may lead to oxidation of subunits within complex I that are important for its function, resulting in profound effects on mitochondrial performance (3).

Peroxynitrite (PN; $ONOO^-$) is a short-lived reactive nitrogen species (RNS) generated in the mitochondria by the reaction of superoxide (a by-product of mitochondrial oxidative phosphorylation) and nitric oxide ($NO^\bullet$) generated by the mitochondrial nitric oxide synthase. PN is highly reactive and can rapidly damage nearby biomolecules (7, 25). PN damages proteins by modifying tyrosine (nitration), cysteine (*S*-nitrosylation), and tryptophan (via formation of *N*-formylkynurenine) residues. PN-mediated oxidative damage to tyrosines may play a significant role in age-related neurodegeneration. Accumulation of 3-nitrotyrosine (3-NT)-modified proteins has been observed in Alzheimer's disease (30). In PD, there are reports of accumulation of 3-NT-modified brain proteins in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-injected mice (22) and 3-NT modification of α-synuclein protein isolated from the Lewy bodies of PD patients (14). Studies by Clementi *et al.* (10) have clearly demonstrated that prolonged exposure to NO inhibits cell respiration probably via PN-mediated complex I inhibition. Recently, Murray *et al.* (21) have shown that incubation of purified bovine heart mitochondria with PN results in 3-NT-modifications of specific subunits within complex I. This suggests that PN-mediated oxidative damage might play an important role in PD pathology, perhaps via damaging nitrative affects on mitochondrial complex I.

It is evident that the role of PN-mediated oxidative damage of mitochondrial complex I in a model physiologically relevant to PD needs to be explored. In this study, we have utilized both mouse brain mitochondria and the rat dopaminergic neuronal $1RB_3AN_{27}$ (N27) cell line to study the role of PN in mitochondrial complex I inhibition. As it has been established that GSH detoxifies PN, we have examined the role of glutathione depletion on PN-mediated damage to the complex (29). We have purified complex I from N27 mitochondria under conditions of glutathione depletion (18). Using anti-nitrotyrosine antibody, we have been able to detect two polypeptides within crude complex I preparations that are nitrated at tyrosine residues following glutathione depletion. The results from these experiments suggest the existence of specific targets of nitrative damage within complex I following an event known to occur early in the disease process in affected dopaminergic neurons that may mechanistically explain its subsequent inhibition.

MATERIALS AND METHODS

Materials

All tissue culture materials were procured from Life Technologies/Invitrogen (Carlsbad, CA, U.S.A.) or Cellgro (Kansas City, MO, U.S.A.). Anti-complex I mouse antibody against α subcomplex subunit 9 (NDUFA9; 39 kDa) and anti-nitrotyrosine antibodies were obtained from Molecular Probes (Eugene, OR, U.S.A.). Materials related to protein chemistry, such as gels and SYPRO Ruby stains, were obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.) and Sigma (St. Louis, MO, U.S.A.).

Cell line and tissue samples

Mouse brain and rat dopaminergic N27 neuronal cells were used as mitochondrial sources. N27 cells were derived from embryonic rat mesencephalic neurons via SV40 large T antigen immortalization. As such, N27 cells more closely emulate the cells that are lost in PD than other potential cellular models (24). N27 cells have been demonstrated to possess all the physiological and biochemical properties of dopaminergic neurons, including expression of tyrosine hydroxylase and the dopamine transporter, and to produce the neurotransmitter dopamine (1). N27 cells have, in addition, proven to be efficient in transplantation therapy in various animal models of PD (8, 9).

N27 cells were grown in RPMI medium 1640 containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml) and were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cells were subcultured once a week via trypsin treatment. For glutathione depletion, cells were incubated with different concentrations of buthionine sulfoximine (BSO), an inhibitor of the rate-limiting enzyme of GSH synthesis, gamma glutamyl cysteine ligase.

Preparation, PN treatment, and extraction of mitochondria

Mitochondria were prepared by the method of Trounce *et al.* (35). In brief, N27 cells were washed in buffer H (5 m*M* HEPES, 210 m*M* mannitol, 70 m*M* sucrose, 1 m*M* EGTA, and 0.5% bovine serum albumin) and resuspended in the same buffer. The cell suspension was homogenized and centrifuged at 800 *g* for 5 min at 4°C. The supernatant that was enriched in mitochondria was then centrifuged at 10,000 *g* for 20 min at 4°C. The resultant mitochondrial pellet was resuspended in buffer H and stored as aliquots at −80°C. For brain samples, freshly dissected tissue was washed and homogenized in ice-cold isolation buffer [320 m*M* sucrose, 5 m*M* TES (tris(hydroxymethyl)methylaminoethanesulfonic acid), 1 m*M* EGTA, pH 7.2]. The homogenate was centrifuged at 1,000 *g* for 5 min at 4°C, and then the supernatant was centrifuged at 8,500 *g* for 10 min at 4°C. The mitochondrial-enriched pellet was resuspended in isolation buffer, layered on top of 6% (wt/vol) Ficoll solution, and centrifuged at 75,000 *g* for 30 min at 4°C to remove myelin, which forms a layer at the top. The pellet was resuspended in reconstitution buffer (250 m*M* sucrose, 10 m*M* TES, pH 7.2) and stored as aliquots at −80°C. PN treatment of mitochondria was carried out according to Murray *et al.* (21). In brief, PN solution was placed on the wall of the tube containing mitochondria and vortex-mixed for a few seconds to ensure proper mixing before degradation of PN. These mitochondria were used in complex I assays and the sucrose gradient purification protocol.

For preparation of extracts, ~2 mg of mitochondria was washed with 20 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA and suspended in the same buffer containing protease inhibitor cocktail. This suspension was solubilized by adding *n*-dodecyl β-

D-maltoside (Sigma) to a final concentration of 1% at 5 mg/ml protein concentration and incubated for 30 min on ice. Insoluble material from this suspension was removed by centrifugation at 16,000 *g* for 30 min at 4°C.

Sucrose density gradient centrifugation

Sucrose gradient experiments were performed according to the method of Hanson *et al.* (15) and Taylor *et al.* (33). Mitochondrial extract prepared as above was subjected to a 10–35% sucrose step gradient consisting of 1-ml step fractions of 35, 32.5, 30, 27.5, 25, 22.5, 20, 17.5, 15, and 10% prepared in 10 m*M* Tris, pH 7.5, 1 m*M* EDTA, and 0.05% maltoside. The mitochondrial sample was loaded onto the gradient in 5% sucrose and centrifuged at 38,000 rpm for 16.5 h at 4°C in an Optima-XL 100 K ultracentrifuge (SW40 Ti swinging bucket rotor) from Beckman (Fullerton, CA, U.S.A.). Fractions of 1 ml were collected from the top, frozen immediately, and stored at −80°C. Each fraction was concentrated to 100 µl using a Microcon-100 concentrator (Millipore, Billerica, MA, U.S.A.). An aliquot of 20 µl of each concentrated fraction was analyzed by one-dimensional (1D) sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE), followed by western blotting with an anti-complex I monoclonal antibody against the 39-kDa complex I subunit NDUFA9 to ascertain fractions containing complex I.

Blue Native (BN) gel electrophoresis

Sucrose density gradient fractions that were positive for complex I as indicated by western analysis with the 39-kDa monoclonal antibody were pooled, concentrated, and subjected to BN PAGE analysis according to the method of Schagger and Pfeiffer (26) and Schagger and von Jagow (27). In brief, to 20 µl of concentrated pooled complex I fractions (20% of total sample volume), 20 µl of BN sample buffer (1.5 *M* aminocaproic acid, 0.05 *M* Bis-Tris, pH 7.0), 2.5 µl of 10% *n*-dodecyl β-D-maltoside, 2.5 µl of protease inhibitor cocktail, and 4 µl of gel loading buffer (5% Serva Coomassie Brilliant Blue G-250, 1 *M* aminocaproic acid) were added and loaded onto a 4–15% BN Tris gel (Bio-Rad Laboratories). A mixture of 50 m*M* Tricine, 15 m*M* Bis-Tris, pH 7.0, 0.02% Coomassie Blue was used as cathode buffer and 50 m*M* Bis-Tris, pH 7.0, as anode buffer. The gel was run at 4°C at 5 mA for the first 12 h and then at 10 mA until the dye marker reached the bottom of the gel. The gel was stained with 0.25% Coomassie Brilliant Blue G-250 (Serva Electrophoresis, Heidelberg, Germany) for 1 h, destained with 50% methanol/10% acetic acid for 1 h, and then destained with 10% methanol/10% acetic acid overnight. Alternately, the BN gel was subjected to silver staining as outlined later in this section.

SDS-PAGE

For 1D SDS-PAGE, complex I sucrose gradient fractions were separated on 10–20% precast SDS-PAGE, on 4–12% XT Bis-Tris SDS-PAGE, or on NuPAGE 4–12% Bis-Tris [2-morpholinoethanesulfonic acid (MES)] gel, respectively. MES gels were run in an X-Cell Sure Lock Mini Cell Apparatus (Invitrogen) with a SDS running buffer (50 m*M* MES, 50 m*M* Tris base, 0.1% SDS, 1 m*M* EDTA) at 100 V for 1.5–2 h. All SDS gels were fixed with 10% methanol, 7% acetic acid for 30 min, and subsequently stained with SYPRO Ruby, followed by destaining in 10% methanol/7% acetic acid.

Mitochondrial complex I assay

Complex I enzyme assays were carried out as described by Trounce *et al.* (35). In brief, the assay was initiated by addition of aliquots of mitochondria to 50 m*M* potassium phosphate, pH 7.4, 500 µ*M* EDTA, 1% bovine serum albumin, 200 µ*M* NADH, and 200 µ*M* decylubiquinone with and without 2 µ*M* rotenone in the presence of KCN with 0.002% dichloroindophenol as a secondary electron acceptor. The decrease in the absorbance at 600 nm was recorded as a measure of enzyme reaction rate at 30°C for 10 min, and specific activity was calculated. The results were plotted as relative rotenone-sensitive specific activity.

Cell viability assay

Cells were seeded in 96-well plates at a density of 10×10^3 per well. After BSO treatment (0, 25, and 50 µ*M* for 24 h), viable cells were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). In brief, 20 µl of 5 mg/ml MTT was added to cells and incubated at 37°C for 2 h. The medium was discarded, the dark blue formazan crystalline product was dissolved in dimethyl sulfoxide, and the absorbance was analyzed in a Spectramax plate reader (Molecular Devices) at 570 nm.

Estimation of total glutathione

Total cellular and mitochondrial glutathione estimation was carried out using a kit from OXIS Research, Inc. (Portland, OR, U.S.A.) as outlined by Bharat *et al.* (6). All estimations were conducted in triplicate and normalized per protein.

Anti-3-NT western blot

Proteins run on 1D SDS-PAGE were transferred onto nitrocellulose membrane and probed with anti-3-NT polyclonal antibody (Molecular Probes). Proteins from BN PAGE were transferred onto nitrocellulose membrane in native transfer buffer (25 m*M* Tris, 192 m*M* glycine, pH 8.3) in a Criterion blotter (Bio-Rad) at 400 mA for 1 h and probed with anti-3-NT antibody. In case of the BN PAGE, the Coomassie Blue concentration was decreased from 0.25% to 0.025% in the cathode buffer during the last half of the run.

Silver staining

Complex I-enriched samples were run on 4–15% BN PAGE as described earlier, and the proteins were silver-stained using the Gelcode SilverSNAP™ Stain Kit (Pierce, Rockford, IL, U.S.A.) according to the specifications provided by the manufacturer.

RESULTS

To assess the effects of elevated PN on complex I in neuronal tissues, we first analyzed the consequence of exoge-

nous PN addition on complex I activity in mitochondria isolated from whole mouse brain. Isolated mouse brain mitochondria were incubated with 0.375, 0.75, and 1.5 m*M* PN followed by mitochondrial complex I assay. As shown in Fig. 1A, a 50% inhibition of complex I activity was achieved at 0.75 m*M* PN, roughly the degree of inhibition observed in the PD SN (28). This concentration-dependent complex I inhibition by PN is consistent with previous reports (21). As a negative control, incubation of mitochondria with degraded PN did not inhibit complex I activity to a significant extent (Fig. 1B). Furthermore, preincubation with 250 μ*M* of the PN scavenger 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III), chloride (FeTPPS; Calbiochem, CA, U.S.A.) attenuated the inhibitory effects of PN incubation on complex I activity (Fig. 1B).

There are several possible mechanisms for inhibition of complex I by PN. PN can damage proteins by modifying tyrosines (nitration), cysteines (*S*-nitrosylation), or tryptophans (via formation of *N*-formylkynurenine). Nitrosylation of cysteines is reversible in the presence of reducing agents such as dithiothreitol (DTT). To test the possibility of cysteine nitrosylation as a mechanism for PN-mediated complex I inhibition, PN-treated mitochondria were incubated with 1 m*M* DTT. This did not appear to restore complex I inhibition to any significant degree, indicating that PN treatment does not inhibit complex I via nitrosation of cysteines (Fig. 1B).

To assess the role of protein nitration in PN-mediated complex I inhibition, we isolated complex I from mouse brain mitochondria and analyzed for the presence of nitrotyrosine modifications of its subunits. Mitochondrial complexes from mouse brain mitochondria incubated with or without 0.75 m*M* PN were isolated by using a sucrose gradient fractionation procedure (15, 33). Mitochondria were first solubilized with 1% maltoside and fractionated on a 10–35% sucrose gradient. Aliquots of the sucrose gradient fractions were collected and run on a 10–20% SDS-PAGE and probed with an anti-complex I antibody against the 39-kDa subunit to identify fractions containing complex I (Fig. 2A). Sucrose gradient fractions 6–10 in both untreated and PN-treated mitochondria (Fig. 2A, lanes 6–10 in both) were found to be highly enriched in complex I based on western blot analysis.

Sucrose gradient fractions containing complex I were analyzed by 1D SDS-PAGE (33). An aliquot of complex I-rich sucrose gradient fractions from both untreated and PN-

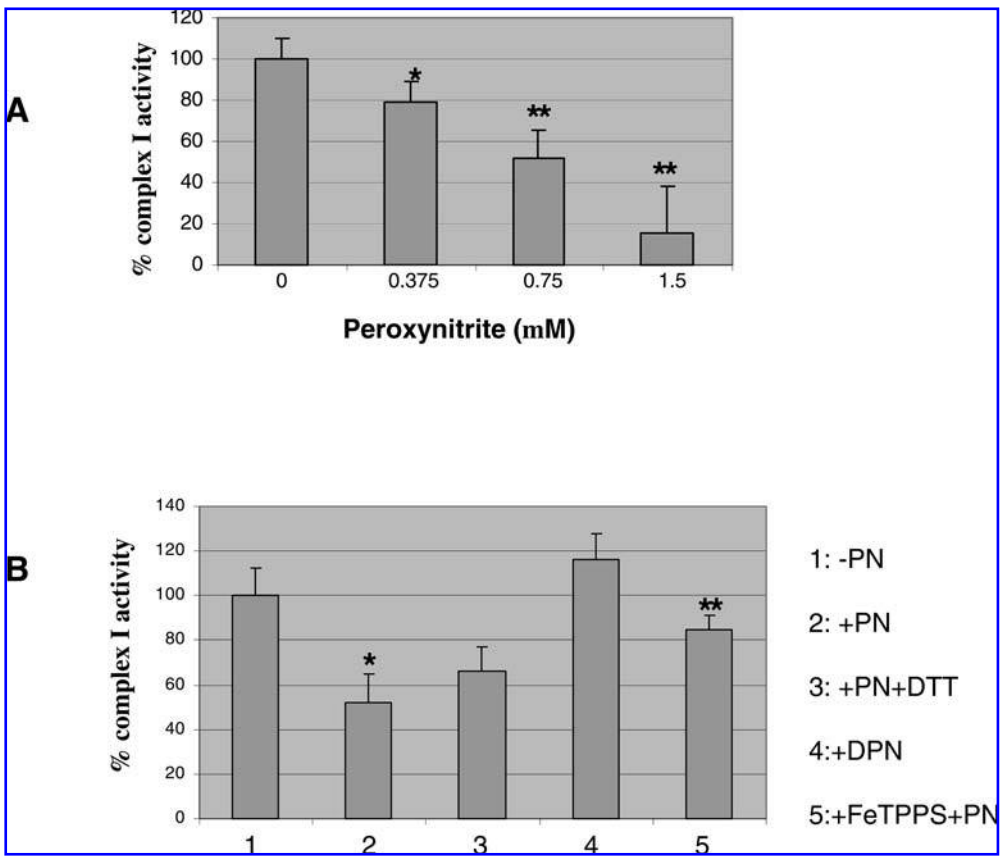

FIG. 1. PN inhibits complex I enzyme activity in a nitration-dependent manner. (**A**) Mouse brain mitochondria were incubated with increasing amounts of PN (0–1.5 m*M*) and immediately subjected to mitochondrial complex I assay; values are given as % untreated controls. **p* < 0.05, untreated versus 0.375 *M* PN-treated sample (*n* = 3); ***p* < 0.001, untreated versus 0.75 m*M* and untreated versus 1.5 m*M* treated samples (*n* = 3). (**B**) 0.75 m*M* PN-treated mouse brain mitochondrial complex I activity (**2**) was compared with that assayed in the presence of either 1 m*M* DTT (**3**) or preincubation with the PN scavenger (FeTPPS; 250 μ*M*) (**5**). As a negative control, degraded PN (DPN; 5 m*M*) (**4**) was used. Values are given as % untreated controls (**1**). **p* < 0.05, (1) versus (2) (*n* = 3); ***p* < 0.005, (2) versus (5) (*n* = 3).

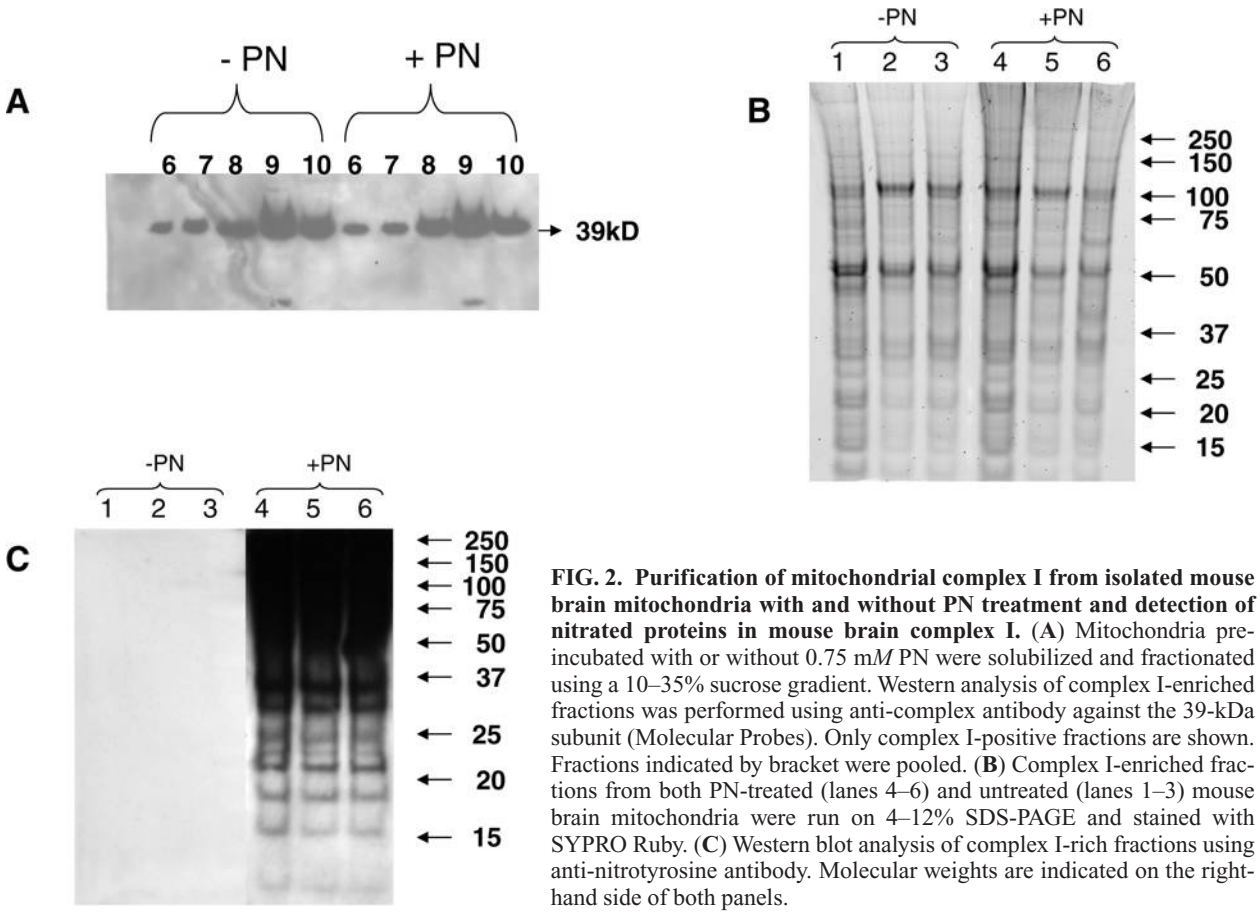

FIG. 2. Purification of mitochondrial complex I from isolated mouse brain mitochondria with and without PN treatment and detection of nitrated proteins in mouse brain complex I. (**A**) Mitochondria pre-incubated with or without 0.75 m*M* PN were solubilized and fractionated using a 10–35% sucrose gradient. Western analysis of complex I-enriched fractions was performed using anti-complex antibody against the 39-kDa subunit (Molecular Probes). Only complex I-positive fractions are shown. Fractions indicated by bracket were pooled. (**B**) Complex I-enriched fractions from both PN-treated (lanes 4–6) and untreated (lanes 1–3) mouse brain mitochondria were run on 4–12% SDS-PAGE and stained with SYPRO Ruby. (**C**) Western blot analysis of complex I-rich fractions using anti-nitrotyrosine antibody. Molecular weights are indicated on the right-hand side of both panels.

treated mitochondria was loaded onto a 10–20% SDS-PAGE and stained with SYPRO Ruby (see Fig. 2B).

We next examined whether PN-dependent complex I inhibition was correlated with increased nitrotyrosine modification of complex I subunits. For this, aliquots of complex I-containing sucrose gradient fractions from PN-treated and untreated mitochondria were run on SDS-PAGE and western blotted with anti-nitrotyrosine antibody. As evident in Fig. 2C, all three fractions (lanes 4–6) from PN-treated mitochondria showed intense signals in the 20–250-kDa range compared with untreated samples (lanes 1–3), confirming PN-dependent nitrotyrosine modification of many proteins.

We had reported earlier that oxidative stress due to glutathione depletion in dopaminergic PC12 cells elicits a selective complex I inhibition resulting in mitochondrial dysfunction (18). But the biochemistry underlying the oxidative damage-dependent complex I inhibition has not been fully explored. Several ROS and reactive RNS, including NO and PN, are candidates for the observed glutathione depletion-mediated complex I inactivation. Clementi *et al.* (10) have demonstrated that NO can inhibit complex I activity possibly via PN formation. It is possible that the complex I inhibition induced by glutathione depletion in dopaminergic cells may also be mediated by increased nitrosative stress. To test this possibility, we conducted experiments using the dopaminergic neuronal N27 cell line. In order to analyze the effects of glutathione (GSH + GSSG) depletion in this cell line, N27 cells were incubated with 0, 25, and 50 μ*M* BSO for 24 h and glutathione content measured. Following BSO treatment, there was a significant decrease in the levels of total cellular glutathione content with minimal (10–15%) loss in cell viability (Fig. 3A and B). For all subsequent experiments, addition of 50 μ*M* BSO for a time period of 24 h was used to maintain >50% depletion in total cellular glutathione content, similar to the depletion observed in the PD SN (23). Complex I enzyme assays were carried out using mitochondria from N27 cells incubated with or without 50 μ*M* BSO for 24 h. Figure 3C demonstrates that glutathione depletion in N27 cells impaired complex I activity by ~40%, as we have previously shown in other dopaminergic cell models (18).

In order to analyze the role of PN-mediated nitrosative stress during glutathione depletion and its possible involvement in subsequent complex I inhibition, we assessed nitrotyrosine modification of the complex in untreated versus BSO-treated N27 cells. Mitochondria prepared from untreated cells and N27 cells treated with 50 μ*M* BSO were solubilized and fractionated using the sucrose gradient isolation approach described above. Cells treated with PN were used as a positive control. Fractions from both samples were found to be enriched in complex I (Fig. 4A). Complex I-enriched fractions were run on SDS-PAGE, followed by either SYPRO Ruby staining (Fig. 4B) or anti-nitrotyrosine western (Fig. 4C). As

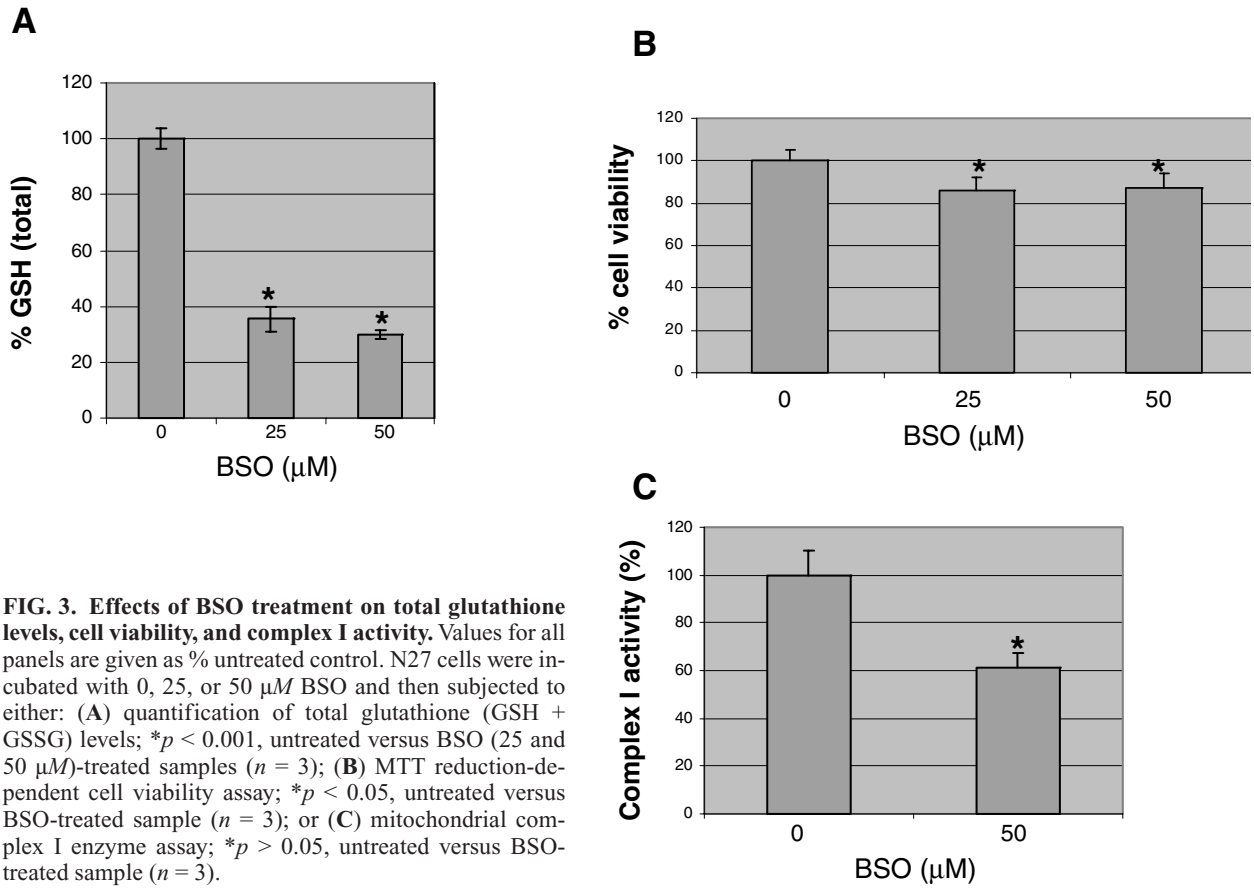

FIG. 3. Effects of BSO treatment on total glutathione levels, cell viability, and complex I activity. Values for all panels are given as % untreated control. N27 cells were incubated with 0, 25, or 50 μ*M* BSO and then subjected to either: (**A**) quantification of total glutathione (GSH + GSSG) levels; **p* < 0.001, untreated versus BSO (25 and 50 μ*M*)-treated samples (*n* = 3); (**B**) MTT reduction-dependent cell viability assay; **p* < 0.05, untreated versus BSO-treated sample (*n* = 3); or (**C**) mitochondrial complex I enzyme assay; **p* > 0.05, untreated versus BSO-treated sample (*n* = 3).

shown in Fig. 4C, two polypeptides corresponding to molecular weight ~25 kDa and ~30 kDa within fractions from the BSO-treated cells demonstrated nitrotyrosine reactivity not observed in complex I fractions from untreated N27 cells, suggesting that PN accumulation during BSO-mediated glutathione depletion results in selective nitration of complex I. This may explain in part its subsequent enzymatic inhibition.

It is possible that the two polypeptides that are positive for nitration in the BSO-treated N27 cell samples (Fig. 4C) might be trace components from other contaminating complexes. To investigate this, we carried out BN PAGE analysis of complex I-enriched fractions isolated from BSO-treated N27 cells and PN-treated mouse mitochondria, followed by either silver staining or western blot analysis using anti-3-NT antibody. Figure 5B demonstrates that in the case of PN-treated mouse brain complex I fractions, there is strong 3-NT immunoreactivity that extends as a streak from 700 kDa to 440 kDa, thus encompassing the region where complexes I, III, and V, but not IV, migrate, suggesting that all three complexes might contain subunits that have 3-NT modifications. This is consistent with an earlier published report by Murray *et al.* in isolated bovine heart mitochondria (21). In the case of complex I fractions from BSO-treated N27 cells, only a single band around 700 kDa was observed; this corresponds to the region of migration of complex I (Fig. 5A). This suggests that only complex I, but not other complexes, is affected by tyrosine nitration during glutathione depletion. Figure 5B demonstrates the corresponding silver staining of all complexes after BN PAGE.

DISCUSSION

Mitochondria play a key role in several cellular processes. The importance of this *in vivo* is emphasized by the fact that many neurological disorders are linked to mitochondrial dysfunction (5). Studies using postmortem tissue samples from PD patients or from *in vivo* or *in vitro* models of the disease have clearly indicated a correlation between complex I inhibition, and PD pathology (11). Earlier work from our own laboratory demonstrated experimental evidence linking oxidative stress, complex I inhibition, and mitochondrial dysfunction in a dopaminergic cell model (18). To date, there are many reports suggesting the role of modification of different complex I subunits by oxidative damage leading to loss of activity. Taylor *et al.* (34) have analyzed the distribution of *N*-formylkynurenine, a product of the dioxidation of tryptophan residues in proteins, throughout the human heart mitochondrial proteome. Their study demonstrates an overrepresentation of this oxidized amino acid in complex I subunits and other proteins involved in redox metabolism. Similarly, Murray *et al.* (21) have shown that PN, a predominant RNS, significantly inhibits the activities of complexes I, II, and IV. Using mass spectrometric approaches, they have demon-

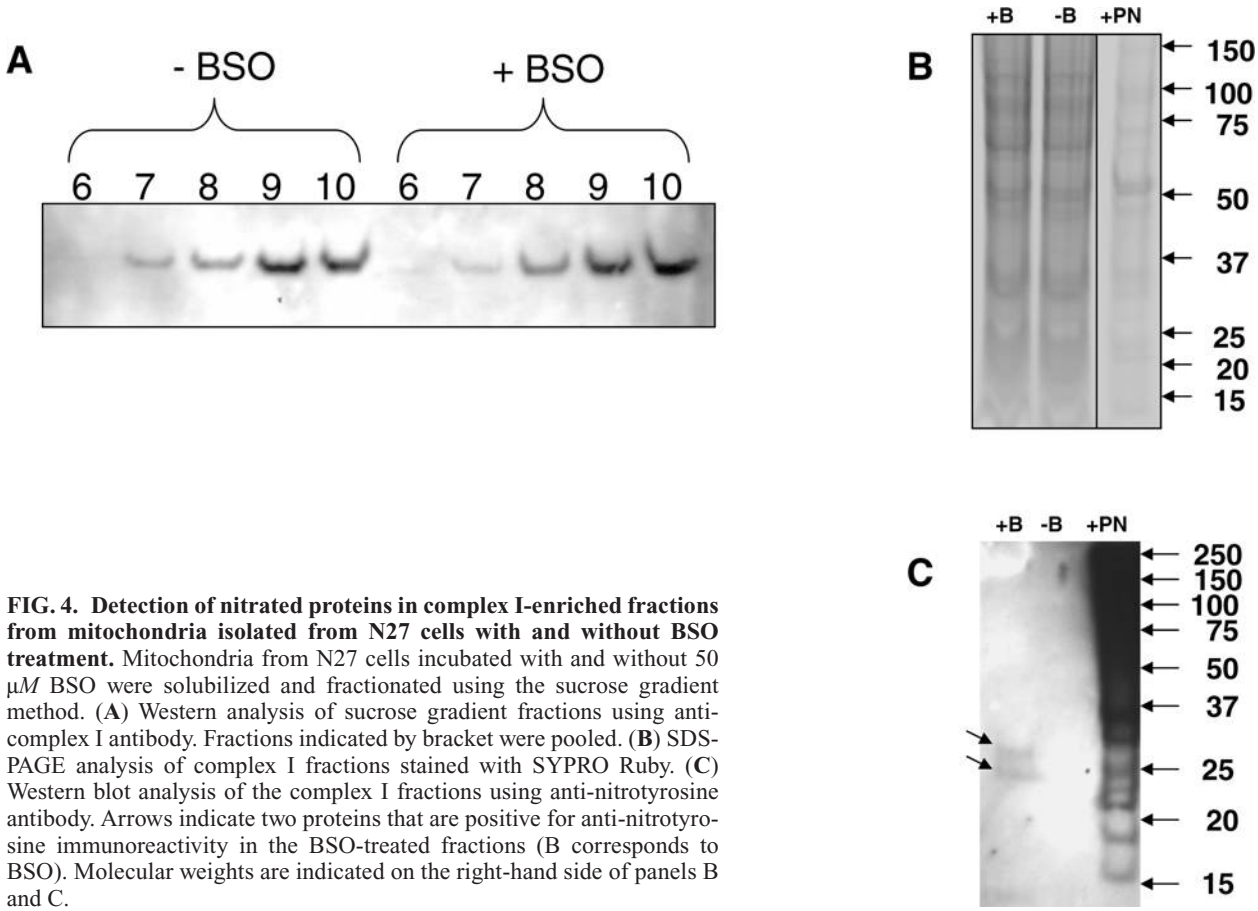

FIG. 4. Detection of nitrated proteins in complex I-enriched fractions from mitochondria isolated from N27 cells with and without BSO treatment. Mitochondria from N27 cells incubated with and without 50 μ*M* BSO were solubilized and fractionated using the sucrose gradient method. (**A**) Western analysis of sucrose gradient fractions using anti-complex I antibody. Fractions indicated by bracket were pooled. (**B**) SDS-PAGE analysis of complex I fractions stained with SYPRO Ruby. (**C**) Western blot analysis of the complex I fractions using anti-nitrotyrosine antibody. Arrows indicate two proteins that are positive for anti-nitrotyrosine immunoreactivity in the BSO-treated fractions (B corresponds to BSO). Molecular weights are indicated on the right-hand side of panels B and C.

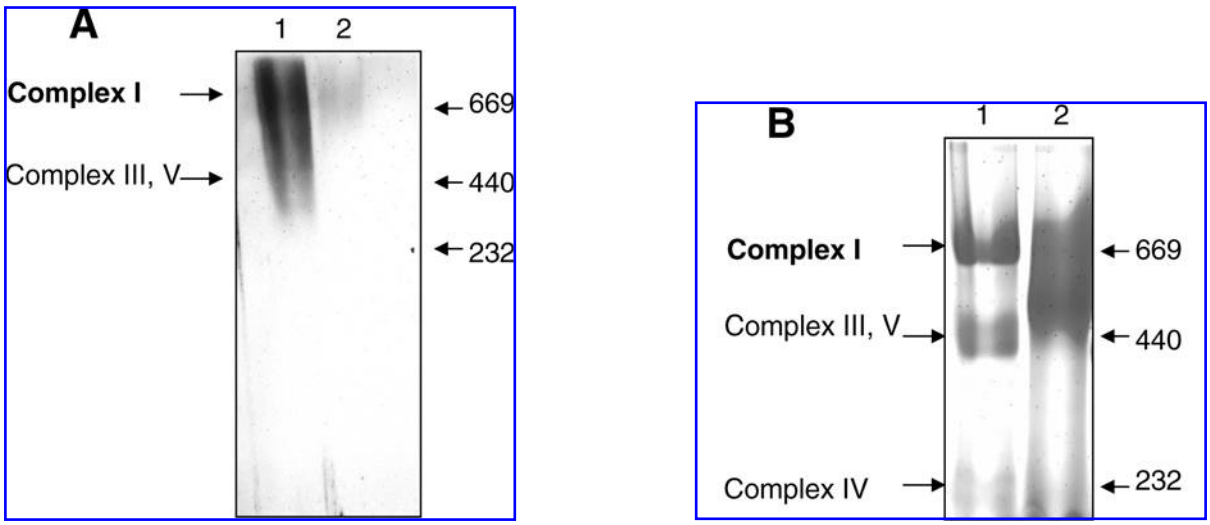

FIG. 5. Specificity of 3-NT immunoreactivity to complex I from glutathione-depleted N27 cells. (**A**) Complex I-rich sucrose gradient fraction from BSO-treated N27 cells was loaded on 4–15% BN PAGE, transferred onto nitrocellulose, and probed with anti-3-NT antibody (lane 2). Lane 1 shows PN-treated mouse brain complex I fraction as positive control. (**B**) Silver staining pattern of the complexes from glutathione-depleted cells (lane 1) and PN-treated mouse mitochondria (lane 2). Molecular weights are indicated on the right-hand side of both panels, and individual complexes are indicated on the left side.

strated PN modification resulting in a 3-NT signature predominantly in five subunits of complex I. Recent reports by Lin *et al.* (20) and Taylor *et al.* (32) have also demonstrated increased oxidation of complex I subunits under oxidative stress conditions. All of these studies indicate a correlation between complex I damage and mitochondrial dysfunction. However, most of these studies were performed on heart mitochondrial complex I preparations from various species.

We hypothesize that during oxidative stress conditions specifically known to occur in the parkinsonian SN such as glutathione depletion, there may be extensive oxidative modifications to the subunits of complex I affecting its activity and subsequent mitochondrial function. Nitrosative stress mediated via NO and PN might play a role in this phenomenon. In order to explore the possibility that nitrative modifications to complex I subunits may play a role in the disease, we have examined the effects of glutathione depletion in dopaminergic N27 neurons. We have compared the effects of glutathione depletion in these cells with the effects of exogenous PN addition to whole mouse brain mitochondria.

In whole mouse brain mitochondria, PN was found to inhibit complex I activity in a dose-dependent manner, and this correlated with extensive nitration of proteins in complex I-rich fractions (Figs. 1 and 2). We have treated mouse brain mitochondria with 0, 375, 750, and 1,500 μ*M* PN to assess the effect on complex I activity. This experiment was conducted as a proof of principle to correlate PN-mediated complex I inhibition with nitrotyrosine modifications of complex I subunits. We have incubated mitochondria and mitochondrial extracts with concentrations of PN lower than those indicated above and detected nitrotyrosine modification of total proteins (unpublished observations). But only in the concentration range of 375–1,500 μ*M* did we see direct correlation between PN-mediated inhibition of complex I activity and nitration of complex I-rich fractions of mitochondrial protein extracts. The point to be considered here is that PN is an extremely labile species that dissociates within a few seconds in neutral buffer. We presume that, because of this reason, nitration of complex I is detectable *in vitro* only at such high concentrations. Although such high levels of PN might not be present at any given time in the brain during pathological conditions, we predict that the constant exposure for longer periods to PN might play an important role in protein damage during disease. This is exemplified by our cell model using GSH depletion. It has been documented that during PD, there is an ~50% depletion of GSH within dopaminergic neurons in the SN region of the brain. We have used 50 μ*M* BSO in dopaminergic N27 neuronal cell line to deplete GSH levels by 60%, which is in the range directly relevant to PD. We have both published and unpublished data that show that during GSH depletion, there is increase in hydrogen peroxide, NO, 4-hydroxy-2-nonenal, *etc.* (18). We hypothesize that, in our cell culture system, GSH depletion up to 60% increases PN levels in the pathophysiological range that could be directly correlated with nitration of complex I.

Similar studies using comparable concentrations of PN have been performed in other systems relevant to PD. Pennathur *et al.* (22) have shown that, after MPTP treatment, there is an increase in the 3-NT levels in the stiatum and ventral midbrain of mice in the range of 0.2 and 0.3 mmol/mol 3-NT/tyrosine, respectively, compared with control (0.125 and 0.175 mmol/mol 3-NT/tyrosine, respectively). To prove the potential role of PN in 3-NT formation during MPTP treatment, the authors did *in vitro* experiments where they exposed normal brain total proteins to 1,000 μ*M* PN and detected 3-NT formation in the range of 5–10 mmol/mol 3-NT/tyrosine. Similarly, in their *in vitro* experiments, Murray *et al.* (21) incubated heart mitochondria with PN and found that there was ~50% complex I inhibition at 800 μ*M* PN. Also, this inhibition was linked to nitration of complex I subunits. We have used PN in our experiments in the concentration range 0–1,500 μ*M* based on the results obtained by these two groups. The concentrations of PN used here are not in the pathophysiological range relevant to PD, but were an aid in our *in vitro* experiments to prove the link between GSH depletion-mediated complex I inhibition and PN formation.

BSO treatment of midbrain-derived dopaminergic N27 cells, a model for the cells selectively lost in PD, resulted in glutathione depletion and complex I inhibition (Fig. 3). We observed that the complex I-enriched fractions from BSO-treated N27 cells displayed anti-nitrotyrosine immunoreactivity in only two polypeptides (molecular weight ~25 kDa and ~30 kDa) rather than the more extensive nitration observed following exogenous PN treatment of whole mouse brain mitochondria (Fig. 4). This could be due to either reduced levels of endogenous PN produced via glutathione depletion versus exogenous addition (the former being more physiological) or cell-specific differences in handling of PN (the former being more relevant to midbrain dopaminergic neurons).

Regarding the levels of PN in the cells during PD, it is very difficult to estimate PN levels in brain regions of either mouse models or human samples, due to its extremely short half-life (although it could be assumed that PN formation during PD and in *in vivo* models of the disease is likely in the pico- to nanomolar range). Due to this limitation, levels of nitrotyrosine formed during PN-mediated protein damage is often used as a cellular indicator for PN. It should also be considered that PN formation during PD is mostly intracellular, largely formed by increased mitochondrial superoxide formation and NO accumulation during disease.

This 3-NT modification seems to be specific to complex I based on two important results. Firstly, it has been demonstrated that glutathione depletion impairs the activity of complex I, whereas other complexes are largely unaffected. Secondly, complex I-rich fractions from BSO-treated cells displayed 3-NT immunoreactivity around 700 kDa and not in any other molecular weight regions. Our data suggest that two complex I subunits (~25 kDa and ~30 kDa) are targets for tyrosine nitration. The proteins in the 25-kDa range that are candidates for nitration following glutathione depletion include NDUFB10 (22 kDa), NDUFS8 (23 kDa), and NDUFV2 (24 kDa). NDUFS3 is the only likely candidate protein in the 30-kDa range. These proteins have many tyrosines in their sequences that could undergo nitration. The number and the positions of these tyrosines in the four polypeptides are listed in Table 1. Murray *et al.* (21) have reported that the PN-mediated 3-NT signature is predominantly specific to complex I subunits, although other complexes are also affected. The complex I subunits that were found to be nitrated in their study were NDUFS2 (49 kDa), TYKY (NDUFS8) (23 kDa),

Table 1. List of Mouse Complex I Subunits in the 25-kDa and 30-kDa Range that are Probable Targets for Nitration Showing the Number of Tyrosines and Their Positions in the Polypeptide Sequence

Subunit	*Other annotations*	*Number of tyrosines*	*Position of tyrosines*
NDUFB10	22 kDa PDSW	13	10, 32, 37, 62, 63, 64, 68, 86, 121, 139, 143, 149, 150
NDUFS8	23 kDa TYKY	11	2, 26, 38, 76, 86, 106, 145, 154, 185, 209, 211
NDUFV2	24 kDa NUHM	8	59, 69, 112, 118, 121, 129, 191, 192
NDUFS3	30 kDa NUGM	9	63, 71, 129, 145, 165, 190, 206, 211, 244

B17.2 (17.2 kDa), B15 (NDUFB4) (15 kDa), and B14 (NDUFA6) (15 kDa). They also demonstrated that subunits B15 (NDUFB4) and B14 (NDUFA6) contained the highest degree of nitration. The most reactive site in subunit B14 was Tyr^{122}, whereas the most reactive region in B15 contained three closely spaced tyrosines: Tyr^{46}, Tyr^{50}, and Tyr^{51}. Interestingly, only one of the subunits (TYKY/NDUFS8) that we have reported here overlaps with the subunits identified by Murray *et al.* This may be because glutathione-mediated nitration events are specific to complex I and occur at a very low concentration of endogenous PN.

The results from this study demonstrate that PN generation mediated by glutathione depletion in dopaminergic cells results in tyrosine nitration of at least two subunits of mitochondrial complex I. As PN is able to cause inhibition of complex I activity, this suggests that tyrosine modification within specific subunits of the complex might play a significant role in inhibition of complex I function following glutathione depletion similar to that observed in the PD SN. We speculate that there are several other reversible/irreversible modifications of complex I subunits that occur during glutathione depletion and, in general, during PD. Further analysis of these events would doubtless provide valuable insight into the molecular mechanism of complex I inhibition during the course of the disease. An interesting feature that needs to be addressed is the role of thiol antioxidant-based therapy to prevent protein damage during PD. We have not performed control experiments with thiol compounds such as glutathione ester to prove whether these agents are protective in our N27 cell model. However, we have shown earlier (6) that preincubation of dopaminergic PC12 cells with *R*-lipoic acid prevents BSO-mediated GSH depletion and restores complex I inhibition. This indicates that the presence of excess thiol reductant in the cellular milieu might prevent oxidative/nitrosative damage of complex I.

Perspectives

When there is increased cellular production of either ROS or RNS or a decrease in the levels of antioxidant defenses or both, this can result in toxic effects. Glutathione is not only a global antioxidant, but also a major cellular redox modulator. It is well documented that both glutathione depletion and oxidative/nitrosative stress are important precursors of PD pathology. Mitochondrial complex I inhibition is another primary feature noted during the cascade of events leading to neurodegeneration in this disease. In a cell, the structural and functional relationship of a protein/enzyme such as complex I could be disrupted due to oxidative/nitrosative stress leading to loss of function. Glutathione may normally act to prevent this not only by binding to and protecting protein thiol groups, but also by detoxifying toxic free radicals such as PN. Our data suggest that glutathione depletion in a midbrain-derived dopaminergic cell model results in increased tyrosine nitration of specific complex I subunits that may impact on complex activity. The consequence of glutathione depletion in dopaminergic midbrain neurons and subsequent complex I modification is likely to be of profound importance in understanding PD pathology.

ACKNOWLEDGMENTS

This project was supported by an NIH R21 grant to J.K.A. S.B. is a recipient of a postdoctoral fellowship from the National Parkinson Foundation. We thank Dr. Simon Melov, Buck Institute, for use of his double-beamed spectrophotometer.

ABBREVIATIONS

BN, Blue Native; BSO, buthionine sulfoximine; 1D, one-dimensional; DTT, dithiothreitol; FeTPPS, 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III), chloride; GSH, reduced glutathione; GSSG, oxidized glutathione; MES, 2-morpholinoethanesulfonic acid; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; N27 cells, $1RB_3AN_{27}$ rat dopaminergic cell line; NO, nitric oxide; 3-NT, 3-nitrotyrosine; PAGE, polyacrylamide gel electrophoresis; PD, Parkinson's disease; PN, peroxynitrite; RNS, reactive nitrogen species; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SN, substantia nigra; TES, tris(hydroxymethyl)methylaminoethanesulfonic acid.

REFERENCES

1. Adams FS, La Rosa FG, Kumar S, Edwards-Prasad J, Kentroti S, Vernadakis A, Freed CR, and Prasad KN. Characterization and transplantation of two neuronal cell lines with dopaminergic properties. *Neurochem Res* 21: 619–627, 1996.
2. Andersen JK. Do alterations in glutathione and iron levels contribute to pathology associated with Parkinson's disease? *Novartis Found Symp* 235: 11–20, 2001.
3. Balijepalli S, Annepu J, Boyd MR, and Ravindranath V. Effect of thiol modification on brain mitochondrial complex I activity. *Neurosci Lett* 272: 203–206, 1999.

4. Beal MF. Does impairment of energy metabolism result in excitotoxic neuronal death in neurodegenerative illnesses? *Ann Neurol* 31: 119–130, 1992.
5. Beal MF. Energetics in the pathogenesis of neurodegenerative diseases. *Trends Neurosci* 23: 298–304, 2000.
6. Bharat S, Cochran BC, Hsu M, Liu J, Ames BN, and Andersen JK. Pre-treatment with R-lipoic acid alleviates the effects of GSH depletion in PC12 cells: implications for Parkinson's disease therapy. *Neurotoxicology* 23: 479–486, 2002.
7. Byun J, Henderson JP, Mueller DM, and Heinecke JW. 8-Nitro-2′-deoxyguanosine, a specific marker of oxidation by reactive nitrogen species, is generated by the myeloperoxidase–hydrogen peroxide–nitrite system of activated human phagocytes. *Biochemistry* 38: 2590–2600, 1999.
8. Clarkson ED, Rosa FG, Edwards-Prasad J, Weiland DA, Witta SE, Freed CR, and Prasad KN. Improvement of neurological deficits in 6-hydroxydopamine-lesioned rats after transplantation with allogeneic simian virus 40 large tumor antigen gene-induced immortalized dopamine cells. *Proc Natl Acad Sci U S A* 95: 1265–1270, 1998.
9. Clarkson ED, Zawada WM, Adams FS, Bell KP, and Freed CR. Strands of embryonic mesencephalic tissue show greater dopamine neuron survival and better behavioral improvement than cell suspensions after transplantation in parkinsonian rats. *Brain Res* 806: 60–68, 1998.
10. Clementi E, Brown GC, Feelisch M, and Moncada S. Persistent inhibition of cell respiration by nitric oxide: crucial role of *S*-nitrosylation of mitochondrial complex I and protective action of glutathione. *Proc Natl Acad Sci U S A* 95: 7631–7636, 1998.
11. Dawson TM and Dawson VL. Molecular pathways of neurodegeneration in Parkinson's disease. *Science* 302: 819–822, 2003.
12. Dexter DT, Carter CJ, Wells FR, Javoy-Agid F, Agid Y, Lees A, Jenner P, and Marsden CD. Basal lipid peroxidation in substantia nigra is increased in Parkinson's disease. *J Neurochem* 52: 381–389, 1989.
13. Forno LS. Neuropathology of Parkinson's disease. *J Neuropathol Exp Neurol* 55: 259–272, 1996.
14. Giasson BI, Duda JE, Murray IV, Chen Q, Souza JM, Hurtig HI, Ischiropoulos H, Trojanowski JQ, and Lee VM. Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. *Science* 290: 985–989, 2000.
15. Hanson BJ, Schulenberg B, Patton WF, and Capaldi RA. A novel subfractionation approach for mitochondrial proteins: a three-dimensional mitochondrial proteome map. *Electrophoresis* 22: 950–959, 2001.
16. Jain A, Martensson J, Stole E, Auld PA, and Meister A. Glutathione deficiency leads to mitochondrial damage in brain. *Proc Natl Acad Sci U S A* 88: 1913–1917, 1991.
17. Jenner P. Oxidative stress in Parkinson's disease. *Ann Neurol* 53 Suppl 3: S26–S36, 2003.
18. Jha N, Jurma O, Lalli G, Liu Y, Pettus EH, Greenamyre JT, Liu RM, Forman HJ, and Andersen JK. Glutathione depletion in PC12 results in selective inhibition of mitochondrial complex I activity. Implications for Parkinson's disease. *J Biol Chem* 275: 26096–26101, 2000.
19. Lenaz G, Bovina C, Castelluccio C, Fato R, Formiggini G, Genova ML, Marchetti M, Pich MM, Pallotti F, Parenti Castelli G, and Biagini G. Mitochondrial complex I defects in aging. *Mol Cell Biochem* 178: 329–333, 1997.
20. Lin TK, Hughes G, Muratovska A, Blaikie FH, Brookes PS, Darley-Usmar V, Smith RA, and Murphy MP. Specific modification of mitochondrial protein thiols in response to oxidative stress: a proteomics approach. *J Biol Chem* 277: 17048–17056, 2002.
21. Murray J, Taylor SW, Zhang B, Ghosh SS, and Capaldi RA. Oxidative damage to mitochondrial complex I due to peroxynitrite: identification of reactive tyrosines by mass spectrometry. *J Biol Chem* 278: 37223–37230, 2003.
22. Pennathur S, Jackson-Lewis V, Przedborski S, and Heinecke JW. Mass spectrometric quantification of 3-nitrotyrosine, *ortho*-tyrosine, and *o,o′*-dityrosine in brain tissue of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice, a model of oxidative stress in Parkinson's disease. *J Biol Chem* 274: 34621–34628, 1999.
23. Perry TL and Yong VW. Idiopathic Parkinson's disease, progressive supranuclear palsy and glutathione metabolism in the substantia nigra of patients. *Neurosci Lett* 67: 269–274, 1986.
24. Prasad KN, Carvalho E, Kentroti S, Edwards-Prasad J, Freed C, and Vernadakis A. Establishment and characterization of immortalized clonal cell lines from fetal rat mesencephalic tissue. *In Vitro Cell Dev Biol Anim* 30A: 596–603, 1994.
25. Radi R, Beckman JS, Bush KM, and Freeman BA. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch Biochem Biophys* 288: 481–487, 1991.
26. Schagger H and Pfeiffer K. Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. *EMBO J* 19: 1777–1783, 2000.
27. Schagger H and von Jagow G. Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal Biochem* 199: 223–231, 1991.
28. Schapira AH, Cooper JM, Dexter D, Clark JB, Jenner P, and Marsden CD. Mitochondrial complex I deficiency in Parkinson's disease. *J Neurochem* 54: 823–827, 1990.
29. Sies H, Sharov VS, Klotz LO, and Briviba K. Glutathione peroxidase protects against peroxynitrite-mediated oxidations. A new function for selenoproteins as peroxynitrite reductase. *J Biol Chem* 272: 27812–27817, 1997.
30. Smith MA, Richey Harris PL, Sayre LM, Beckman JS, and Perry G. Widespread peroxynitrite-mediated damage in Alzheimer's disease. *J Neurosci* 17: 2653–2657, 1997.
31. Sriram K, Shankar SK, Boyd MR, and Ravindranath V. Thiol oxidation and loss of mitochondrial complex I precede excitatory amino acid mediated neurodegeneration. *J Neurosci* 18: 10287–10296, 1998.
32. Taylor ER, Hurrell F, Shannon RJ, Lin TK, Hirst J, and Murphy MP. Reversible glutathionylation of complex I increases mitochondrial superoxide formation. *J Biol Chem* 278: 19603–19610, 2003.
33. Taylor SW, Warnock DE, Glenn GM, Zhang B, Fahy E, Gaucher SP, Capaldi RA, Gibson BW, and Ghosh SS. An alternative strategy to determine the mitochondrial proteome using sucrose gradient fractionation and 1D PAGE on highly purified human heart mitochondria. *J Proteome Res* 1: 451–458, 2002.

34. Taylor SW, Fahy E, Murray J, Capaldi RA, and Ghosh SS. Oxidative post-translational modification of tryptophan residues in cardiac mitochondrial proteins. *J Biol Chem* 278: 19587–19590, 2003.
35. Trounce IA, Kim YL, Jun AS, and Wallace DC. Assessment of mitochondrial oxidative phosphorylation in patient muscle biopsies, lymphoblasts, and transmitochondrial cell lines. *Methods Enzymol* 264: 484–509, 1996.
36. Zhang Y, Dawson VL, and Dawson TM. Oxidative stress and genetics in the pathogenesis of Parkinson's disease. *Neurobiol Dis* 7: 240–250, 2000.

Address reprint requests to:
Julie Kay Andersen, Ph.D.
Buck Institute for Age Research
8001 Redwood Blvd.
Novato, CA 94945

E-mail: jandersen@buckinstitute.org

Received for publication August 15, 2004; accepted January 19, 2005.

This article has been cited by:

1. George K. Shinomol, M. M. Srinivas Bharath, Muralidhara. 2012. Neuromodulatory Propensity of Bacopa monnieri Leaf Extract Against 3-Nitropropionic Acid-Induced Oxidative Stress: In Vitro and In Vivo Evidences. *Neurotoxicity Research* **22**:2, 102-114. [CrossRef]
2. George K. Shinomol, M.M. Srinivas Bharath, Muralidhara. 2012. Pretreatment with Bacopa monnieri extract offsets 3-nitropropionic acid induced mitochondrial oxidative stress and dysfunctions in the striatum of prepubertal mouse brain. *Canadian Journal of Physiology and Pharmacology* **90**:5, 595-606. [CrossRef]
3. Joanne DiFrancisco-Donoghue, Eric M. Lamberg, Ely Rabin, Ahmed Elokda, Enrico Fazzini, William G. Werner. 2012. Effects of Exercise and B Vitamins on Homocysteine and Glutathione in Parkinsons Disease: A Randomized Trial. *Neurodegenerative Diseases* **10**:1-4, 127-134. [CrossRef]
4. Philip Y. Lam, Kam Ming Ko. 2012. Schisandrin B as a Hormetic Agent for Preventing Age-Related Neurodegenerative Diseases. *Oxidative Medicine and Cellular Longevity* **2012**, 1-9. [CrossRef]
5. Prashanth Chandramani Shivalingappa, Huajun Jin, Vellareddy Anantharam, Anumantha Kanthasamy, Arthi Kanthasamy. 2012. N-Acetyl Cysteine Protects against Methamphetamine-Induced Dopaminergic Neurodegeneration via Modulation of Redox Status and Autophagy in Dopaminergic Cells. *Parkinson's Disease* **2012**, 1-11. [CrossRef]
6. Rajeswara Babu Mythri, C. Venkateshappa, G. Harish, Anita Mahadevan, Uday B. Muthane, T. C. Yasha, M. M. Srinivas Bharath, S. K. Shankar. 2011. Evaluation of Markers of Oxidative Stress, Antioxidant Function and Astrocytic Proliferation in the Striatum and Frontal Cortex of Parkinson's Disease Brains. *Neurochemical Research* . [CrossRef]
7. Claire Maller , Ewald Schröder , Philip Eaton . 2011. Glyceraldehyde 3-Phosphate Dehydrogenase is Unlikely to Mediate Hydrogen Peroxide Signaling: Studies with a Novel Anti-Dimedone Sulfenic Acid Antibody. *Antioxidants & Redox Signaling* **14**:1, 49-60. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
8. Ahmed Elokda, Joanne DiFrancisco-Donoghue, Eric M. Lamberg, William G. Werner. 2010. Effects of exercise induced oxidative stress on glutathione levels in Parkinson's disease on and off medication. *Journal of Neurology* **257**:10, 1648-1653. [CrossRef]
9. Linjuan Sun, Shengli Xu, Ming Zhou, Chaodong Wang, Yanchuan Wu, Piu Chan. 2010. Effects of cysteamine on MPTP-induced dopaminergic neurodegeneration in mice. *Brain Research* **1335**, 74-82. [CrossRef]
10. Raquel Requejo, Thomas R. Hurd, Nikola J. Costa, Michael P. Murphy. 2010. Cysteine residues exposed on protein surfaces are the dominant intramitochondrial thiol and may protect against oxidative damage. *FEBS Journal* **277**:6, 1465-1480. [CrossRef]
11. Montserrat Marí , Albert Morales , Anna Colell , Carmen García-Ruiz , José C. Fernández-Checa . 2009. Mitochondrial Glutathione, a Key Survival Antioxidant. *Antioxidants & Redox Signaling* **11**:11, 2685-2700. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
12. Vijay Kumar, Kiran Dip Gill. 2009. Aluminium neurotoxicity: neurobehavioural and oxidative aspects. *Archives of Toxicology* **83**:11, 965-978. [CrossRef]
13. Yasuko Fujisawa, Kazunobu Kato, Cecilia Giulivi. 2009. Nitration of tyrosine residues 368 and 345 in the #-subunit elicits F o F 1 -ATPase activity loss. *Biochemical Journal* **423**:2, 219-231. [CrossRef]
14. S CHINTA, J ANDERSEN. 2008. Redox imbalance in Parkinson's disease. *Biochimica et Biophysica Acta (BBA) - General Subjects* **1780**:11, 1362-1367. [CrossRef]
15. Guey Shuang Wu, Narsing A Rao. 2008. Photoreceptor mitochondrial oxidative stress in uveitis. *Expert Review of Ophthalmology* **3**:3, 299-310. [CrossRef]
16. Steven R. Danielson, Julie K. Andersen. 2008. Oxidative and nitrative protein modifications in Parkinson's disease. *Free Radical Biology and Medicine* **44**:10, 1787-1794. [CrossRef]
17. G ZEEVALK, R RAZMPOUR, L BERNARD. 2008. Glutathione and Parkinson's disease: Is this the elephant in the room?. *Biomedicine & Pharmacotherapy* **62**:4, 236-249. [CrossRef]
18. S. Vali, R.B. Mythri, B. Jagatha, J. Padiadpu, K.S. Ramanujan, J.K. Andersen, F. Gorin, M.M.S. Bharath. 2007. Integrating glutathione metabolism and mitochondrial dysfunction with implications for Parkinson's disease: A dynamic model. *Neuroscience* **149**:4, 917-930. [CrossRef]
19. Derek A. Drechsel, Li-Ping Liang, Manisha Patel. 2007. 1-Methyl-4-phenylpyridinium-induced alterations of glutathione status in immortalized rat dopaminergic neurons. *Toxicology and Applied Pharmacology* **220**:3, 341-348. [CrossRef]

20. Rajeswara Babu Mythri , Balusamy Jagatha , Nityananda Pradhan , Julie Andersen , M. M. Srinivas Bharath . 2007. Mitochondrial Complex I Inhibition in Parkinson's Disease: How Can Curcumin Protect Mitochondria?. *Antioxidants & Redox Signaling* **9**:3, 399-408. [Abstract] [Full Text PDF] [Full Text PDF with Links]
21. Rajeswara Babu Mythri, Balusamy Jagatha, Nityananda Pradhan, Julie Andersen, M. M. Srinivas Bharath. 2006. Mitochondrial Complex I Inhibition in Parkinson's Disease: How Can Curcumin Protect Mitochondria?. *Antioxidants & Redox Signaling*, ahead of print061221112325012. [CrossRef]
22. Shankar J. Chinta, Julie K. Andersen. 2006. Reversible inhibition of mitochondrial complex I activity following chronic dopaminergic glutathione depletion in vitro: Implications for Parkinson's disease. *Free Radical Biology and Medicine* **41**:9, 1442-1448. [CrossRef]
23. K. Prabhakaran, L. Li, J.L. Borowitz, G.E. Isom. 2006. Inducible nitric oxide synthase up-regulation and mitochondrial glutathione depletion mediate cyanide-induced necrosis in mesencephalic cells. *Journal of Neuroscience Research* **84**:5, 1003-1011. [CrossRef]
24. Renu A. Kowluru, Vibhuti Kowluru, Ye Xiong, Ye-Shih Ho. 2006. Overexpression of mitochondrial superoxide dismutase in mice protects the retina from diabetes-induced oxidative stress. *Free Radical Biology and Medicine* **41**:8, 1191-1196. [CrossRef]
25. Salvador Moncada, Juan P. Bolanos. 2006. Nitric oxide, cell bioenergetics and neurodegeneration. *Journal of Neurochemistry* **97**:6, 1676-1689. [CrossRef]
26. Philip Eaton , Michael J. Shattock . 2005. Protein S-Thiolation: Emphasis on Cell Signaling and Gene Expression. *Antioxidants & Redox Signaling* **7**:7-8, 839-840. [Citation] [Full Text PDF] [Full Text PDF with Links]