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Proteomic analysis of rat brain mitochondria following exposure to dopamine quinone: Implications for Parkinson disease

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Oxidative stress and mitochondrial dysfunction have been linked to dopaminergic neuron degeneration in Parkinson disease. We have previously shown that dopamine oxidation leads to selective dopaminergic terminal degeneration *in vivo* and alters mitochondrial function *in vitro*. In this study, we utilized 2-D difference in-gel electrophoresis to assess changes in the mitochondrial proteome following *in vitro* exposure to reactive dopamine quinone. A subset of proteins exhibit decreased fluorescence labeling following dopamine oxidation, suggesting a rapid loss of specific proteins. Amongst these proteins are mitochondrial creatine kinase, mitofilin, mortalin, the 75 kDa subunit of NADH dehydrogenase, and superoxide dismutase 2. Western blot analyses for mitochondrial creatine kinase and mitofilin confirmed significant losses in isolated brain mitochondria exposed to dopamine quinone and PC12 cells exposed to dopamine. These results suggest that specific mitochondrial proteins are uniquely susceptible to changes in abundance following dopamine oxidation, and carry implications for mitochondrial stability in Parkinson disease neurodegeneration.

Keywords: Parkinson disease; Mitochondria; PC12 cells; Dopamine; Dopamine quinone; Proteomics; 2-D DIGE; Mitofilin; Mitochondrial creatine kinase

Introduction

Parkinson disease (PD) is a neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra (SN) pars compacta and the formation of Lewy bodies (Samii et al., 2004). Most PD cases are considered sporadic, and provide us with limited clues to causes of disease pathogenesis. However, increasing evidence implicates mitochondrial dysfunction and oxidative stress in PD (Betarbet et al., 2002; Dauer and Przedborski, 2003; Jenner, 2003; Pallanck and Greenamyre, 2006).

Decreased mitochondrial Complex I (NADH dehydrogenase) activity has been observed in both the SN (Janetzky et al., 1994; Orth and Schapira, 2002; Schapira et al., 1990) and periphery (Blandini et al., 1998; Shoffner et al., 1991) of PD patients. Deficiencies and inhibition of the mitochondrial electron transport chain (ETC), a known source of reactive oxygen species (ROS), can lead to increased mitochondria-generated free radicals and oxidative stress (Beal, 2003; Lenaz et al., 2002). Increased ROS may cause damage to macromolecules, such as increased oxidation of mitochondrial proteins, making them susceptible to accumulation or proteolytic degradation (Bota and Davies, 2001, 2002; Bulteau et al., 2006).

Although multiple brain regions are involved in PD, the degeneration of dopaminergic neurons under conditions of oxidative stress suggests dopamine (DA) may be contributing to PD pathogenesis (Greenamyre and Hastings, 2004; Ogawa et al., 2005; Stokes et al., 1999). Normal DA metabolism leads to the production of ROS, and DA not adequately stored in vesicles is prone to oxidation, forming the reactive DA quinone (DAQ) (Graham et al., 1978). Dopamine-induced toxicity, demonstrated both in cell culture (Jones et al., 2000; Koshimura et al., 2000) and *in vivo* (Hastings et al., 1996; Rabinovic et al., 2000), is dependent on DA oxidation and the formation of reactive DA metabolites. Post-mortem studies have found increased levels of cysteinyl-DA, the covalent modification of cysteine by DAQ, in SN of PD patients (Fornstedt et al., 1989; Spencer et al., 1998). Dopamine and DAQ exposure also alter mitochondrial respiration (Berman and Hastings, 1999; Cohen et al., 1997; Gluck et al., 2002) and trigger permeability transition (Berman and Hastings, 1999) in isolated rat brain mitochondria, suggesting modification of critical mitochondrial proteins, though specific proteins have yet to be identified. As previous proteomic studies have identified alterations in mitochondrial proteins in animal models of PD (Jin et al., 2005; Palacino et al., 2004; Periquet

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et al., 2005; Poon et al., 2005), it is of interest to identify and characterize the mitochondrial protein targets of DA oxidation. Such proteins could become therapeutic targets in PD.

In this study, we utilized 2-D DIGE techniques in combination with cysteine- and lysine-reactive fluorescent dyes as a non-biased approach to evaluate protein alterations in rat brain mitochondria immediately following *in vitro* exposure to DAQ. Differential fluorescent labeling indicated a significant loss following DAQ exposure of a subset of potentially critical proteins that were identified by subsequent mass spectrometric studies. Western blot analyses confirmed decreases in two of these proteins, mitochondrial creatine kinase and mitofilin, in isolated brain mitochondria exposed to DAQ and PC12 cells exposed to DA. These findings suggest that specific mitochondrial proteins are uniquely susceptible to oxidation-induced changes in abundance, and may have implications for PD pathogenesis.

Experimental procedures

Materials

Cysteine-reactive maleimide-conjugated Cy3/5 cyanine Ettan DIGE Saturation Labeling dyes (Cys-CyDyes) and lysine-reactive *N*-hydroxysuccinimide (NHS) ester-conjugated Cy3/5 cyanine Ettan DIGE Minimal Labeling dyes (Lys-CyDyes) were purchased from GE Healthcare (Piscataway, NJ). Sequencing Grade Modified Trypsin and Gold Mass Spectrometry Grade Modified Trypsin were purchased from Promega (Madison, WI). Solutions and stocks for in-gel trypsin digest and mass spectrometry procedures were prepared using HPLC-grade water from Fisher Biotech (Pittsburgh, PA) and HPLC-grade MeOH and acetonitrile from Sigma-Aldrich (St. Louis, MO). Protease inhibitor cocktail (cat # P2714), DA, mushroom tyrosinase, and most general chemicals for SDS–PAGE, buffers, and solutions were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Rabbit–anti-mitochondrial creatine kinase (MtCK) and rabbit–anti-mitofilin polyclonal antibodies were generated for our laboratory by GeneMed Synthesis, Inc. (South San Francisco, CA). All other general solutions and stocks were prepared using doubly distilled water (ddH_2O) from a Milli-Q system (Millipore Corp., Bedford, MA).

Mitochondrial isolation and respiration

All animal procedures were approved by the 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*. Mitochondria-enriched fractions were isolated from the brain tissue of adult male Sprague-Dawley rats via differential centrifugation by the method of Rosenthal et al. (1987) as previously described (Berman and Hastings, 1999; Berman et al., 2000), with elimination of the protease Nagarse. Final mitochondrial pellets were resuspended in mitochondrial isolation buffer (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mg/ml FA-free BSA, and 1 mM EGTA, pH 7.4) and kept on ice. Mitochondrial protein content was determined by the Bradford method (1976). Prior to experimental use, respiration rates based on oxygen consumption were measured in the mitochondrial preparations to ensure mitochondrial health, as previously described (Berman and Hastings, 1999). Mitochondrial health was determined by the ratio of respiration active state 3, induced by the addition of ADP, to resting state 4, induced by the addition of oligomycin. Only mitochondria with a coupled state 3/state 4 ratio above 6 were used for this study.

Exposure of isolated mitochondria to dopamine quinone

Mitochondria (2 mg total protein) were exposed to DA (150 μM) and tyrosinase (150 U) in modified mitochondrial isolation buffer with 25 mM HEPES minus BSA, pH 7.4 plus protease inhibitor cocktail (2.5 μl/mg protein; Sigma) for 15 min at room temperature (RT). Following incubation, mitochondria were placed on ice and immediately pelleted by centrifugation at 15,000×*g* for 15 min at 3 °C. Control mitochondria underwent the same procedure in the absence of DA. Pelleted mitochondria were lysed by rigorous pipetting in denaturing 2-D DIGE lysis buffer (9 M urea, 2% w/v CHAPS, and 30 mM Tris–base, pH 8.5) in a ratio of 100 μL buffer to 1 mg protein. Insoluble material was pelleted by centrifugation (16,000×*g* for 1–2 min at RT) and discarded. Protein concentrations of lysed control and DAQ-exposed samples were determined by Bradford (1976). Thiol reducing agents were excluded from the lysis buffer to maintain proteins in a non-reduced state.

Cys- and Lys-CyDye labeling

For cysteine-dye minimal labeling 2-D DIGE, migration-matched Cy3 and Cy5 Cys-CyDyes (GE Healthcare) were rehydrated in dimethylformamide (DMF) to a concentration of 0.5 mM, aliquoted, and stored at −20 °C with desiccation until use. Prior to use, an aliquot of dye was thawed to RT and diluted in DMF to a working concentration of 62.5 μM. Control and DAQ-exposed protein sample lysates were reacted with the indicated Cys-CyDye under non-reducing conditions at a ratio of 1 pmol dye per 2 μg protein. We used low concentrations of Cys-CyDyes to achieve a minimal labeling effect on non-reduced protein samples. Preliminary experiments using various dye concentrations identified 1 pmol dye per 2 μg protein, which is 0.125% of the ratio typically utilized for saturation labeling, as optimal for minimal Cys-CyDye labeling. This concentration provided sufficient labeling for detection and imaging while maintaining reproducible results across gels (data not shown).

Samples were labeled Cys-Cy5 control and Cys-Cy3 DAQ, or the reciprocal to control for differential dye affinity. Samples were gently vortexed and incubated in the dark for 45 min at RT. The reaction was quenched by adding an equal volume amount of 2-D DIGE sample buffer (9 M urea, 2% w/v CHAPS, 2% v/v 3–10 IPG ampholyte buffer, 130 mM dithiothreitol (DTT), and a trace of bromophenol blue in ddH_2O). Final DIGE samples were prepared by combining equal amounts of Cys-CyDye-labeled control protein and Cys-CyDye-labeled DAQ-exposed protein.

Lysine–dye minimal-labeling 2-D DIGE analysis was utilized to control for changes in protein abundance between control and DAQ-exposure groups in comparison to Cys-CyDye DIGE. Migration-matched Cy3 and Cy5 Lys-CyDyes were rehydrated in DMF to a concentration of 0.5 mM and stored at −20 °C with desiccation until use. Prior to use, dyes were thawed to RT and diluted 1:1 in DMF. Control and DAQ-exposed protein sample lysates were reacted with the indicated Lys-CyDye (Lys-Cy5 control and Lys-Cy3 DAQ, or the reciprocal) under non-reducing conditions at a ratio of 2 pmol dye per 1 μg protein in the dark for 30 min on ice. The reaction was quenched by the addition of free lysine to a final concentration of 385 μM and incubated 15 min on ice. Labeled samples were diluted 1:1 with 2-D DIGE sample buffer. Equal protein amounts of the Lys-