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In vivo protein targets for increased quinoprotein adduct formation in aged substantia nigra

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ABSTRACT

The selective vulnerability of dopaminergic neurons in the substantia nigra pars compacta in Parkinson's disease, a late age onset neurodegenerative disorder, indicates the involvement of dopamine metabolism in the pathogenesis. Dopamine oxidation produces dopamine *o*-quinone, which covalently modifies cysteinyl proteins forming quinoprotein adduct. Although quinoprotein formation correlates with increased dopaminergic neurotoxicity, the *in vivo* protein targets for quinone modification remain unclear. Using two-dimensional gel electrophoresis and nitroblue tetrazolium/glycinate redox-cycling staining, we compared quinoprotein adducts in the substantia nigra of 2- and 15-month old rats and for the first time identified the *in vivo* protein targets with increased quinone modification in aged substantia nigra. Interestingly, several key enzymes in energy metabolism and mitochondrial function were selectively modified by quinone during aging. *In vitro* analyses confirmed that two of identified enzymes, L-lactate dehydrogenase (LDH) and malate dehydrogenase (MDH), were readily conjugated by dopamine *o*-quinone, resulting in a significant reduction in enzyme activity. Since the proteomic approach to detect quinoprotein adducts represents a single analysis comparing pools of substantia nigra from young or old rats, these findings need to be verified in the future. Nonetheless, our results reveal that the enzymatic activity of LDH and MDH can be compromised by quinone modification, suggesting a role of energy metabolism impairment in the selective vulnerability of aged substantia nigra dopaminergic neurons in Parkinson's disease.

1. Introduction

Parkinson's disease (PD) is the second most common age-dependent neurodegenerative disorder, affecting 1–2% of the population over the age of 65 (Beitz, 2014; Eriksen et al., 2003). Aging has been identified as the most critical risk factor for PD (Bennett et al., 1996; Collier et al., 2011; Morens et al., 1996; Tanner and Goldman, 1996). Thus far, the pathogenic mechanism of PD remains obscure. Pathologically, PD is characterized by the selective loss of dopaminergic neurons in the substantia nigra (SN) pars compacta, which has led to the hypothesis that dopamine (DA) metabolism is a major contributing factor to the pathogenesis of PD (Hastings, 2009).

DA is usually sequestered in the monoaminergic synaptic vesicles by vesicular monoamine transporter (VMAT2) (Staal et al., 2004). When DA is present in the cytosol instead of its proper vesicular storage, it is prone to oxidation (Linert et al., 1996; Segura-Aguilar et al., 2014; Sulzer and Zecca, 2000), resulting in the generation of reactive dopamine *o*-quinone (DAQ), reactive oxygen species (ROS) (Graham et al., 1978; Hastings, 1995; Sulzer and Zecca, 2000) and neurotoxicity (Caudle et al., 2007; Chen et al., 2008; Taylor et al., 2011). DAQ and its derivatives (aminochrome and 5,6-indolequinone) are electrophiles, which are readily susceptible to the attack by cellular nucleophiles, particularly the reduced sulfhydryls on reduced glutathione (GSH), free cysteine and cysteinyl proteins (Segura-Aguilar et al., 2014; Tse et al., 1976), leading to the formation of thiol–quinone adduct (Fornstedt et al., 1986; Graham et al., 1978; Hastings et al., 1996). Since cysteine residues

Abbreviations: PD, Parkinson's disease; DA, dopamine; DAQ, dopamine quinone; SN, substantia nigra; NBT, nitroblue tetrazolium; GSH, reduced glutathione; LDH, lactate dehydrogenase; MDH, malate dehydrogenase.

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are generally critical for biological activities, DAQ modification of cysteinyl proteins often leads to compromised protein function (Hastings, 2009).

Previous studies of tocopherol quinones clearly demonstrated that compared to ROS, thiol–quinone adduct formation is much more detrimental to the viability of mammalian cells (Cornwell and Ma, 2007, 2008; Wang et al., 2006). Accordingly, thiol-DAQ adduct formation in the aged dopaminergic neurons may play a more important role in the pathogenesis of PD than ROS generated by redox cycling of DAQ. We have shown that quinoprotein adduct selectively accumulates in the substantia nigra in an age-dependent manner, whereas no significant change of lipid peroxidation was detected during aging process (Wang et al., 2011). In cultured dopaminergic neuronal cells, the formation of quinoprotein adducts directly correlates with cytotoxicity (Wang et al., 2011). These observations are consistent with the notion that quinoprotein adduct formation plays a critical role in age-dependent selective vulnerability of SN dopaminergic neurons.

To understand the molecular insight of neurotoxicity associated with quinoprotein adduct formation, identifying the protein targets for DAQ conjugation, particularly those proteins susceptible to an increased DAQ conjugation during aging, is critical, which may reveal the molecular basis of the selective SN dopaminergic neurodegeneration in PD. Several attempts have been made to identify the protein targets for DAQ modification. By incubating isolated rat brain mitochondria with radiolabeled DAQ or incubating differentiated SH-SY5Y cells with ^{14}C-DA, Van Laar et al. identified various protein targets susceptible to DAQ conjugation, including chaperonin, ubiquinol-cytochrome c reductase core protein 1, glucose regulated protein 75/mitochondrial HSP70/mortalin, mitofilin, and mitochondrial creatine kinase (Van Laar et al., 2009). Interestingly, two proteins responsible for the pathogenesis of genetic PD, ubiquitin carboxy-terminal hydrolase L1 and DJ-1, were also identified as targets for DAQ conjugation (Van Laar et al., 2009). In a separated study, LaVoie et al. have shown that parkin, another protein involved in the pathogenesis of genetic PD (LaVoie et al., 2005), is covalently modified by DAQ, resulting in a functional inactivation of parkin (LaVoie et al., 2005).

So far, the *in vivo* protein targets for DAQ conjugation, particularly those with increased DAQ modification during aging, still remain unknown. In this study, we isolated proteins from the SN region of 2- and 15-month old wild-type rats, separated total proteins by two-dimensional gel electrophoresis, stained quinoproteins with the nitroblue tetrazolium (NBT)/glycinate redox-cycling staining (Paz et al., 1991), and identified proteins with increased quinone modification during aging by mass spectrometry. DAQ modification and the subsequent inactivation of two of these protein targets were verified by *in vitro* analyses. Our findings provide novel insights into the selective vulnerability of aged SN dopaminergic neurons.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats were purchased from SLRC Laboratory Animals Co. (Shanghai, China). The dissection of SN region from 2-month and 15-month old rats was described previously (Wang et al., 2011). The rat experiments were performed according to the Guidelines on the Humane Treatment of Lab Animals established in 2006 by the Ministry of Science and Technology of China [policy (2006)398] and approved by the Institutional Animal Care and Use Committee of East China Normal University.

2.2. Two-dimensional (2-D) gel electrophoresis

Dissected SN region was snap frozen in liquid nitrogen and homogenized in a lysis buffer containing 7 M Urea, 2 M thiourea, 4% CHAPS, 65 mM DTT, 0.2% Bio-lytes. Tissue lysates were centrifuged at 15,000 *g* for 15 min at 4 °C and the supernatants were subjected to two-dimensional (2-D) gel electrophoresis. Protein concentration was determined by the BCA protein assay kit (Pierce).

A total of 500 μg of proteins was loaded onto a pH 3–10 ReadyStrip IPG strip (Bio-Rad) for isoelectric focusing, and the strip was actively rehydrated in a protean IEF cell (Bio-Rad) for 16 h (h) at 50 V. The isoelectric focusing was performed in the protean IEF Cell (Bio-Rad) according to manufacturer's instruction, consisting of running at 250 V for 0.5 h linearly; 500 V for 0.5 h rapidly; 4000 V for 3 h linearly, 4000 V for 5 h rapidly and 500 V for 24 h rapidly. All processes described above were carried out at room temperature. The strip was stored at −20 °C until a second dimension electrophoresis was performed.

For second dimension gel electrophoresis, IPG Strips were equilibrated for 10 min (min) in a buffer containing 375 mM Tris–HCl (pH 8.8), 6 M urea, 2% sodium dodecyl sulfate (SDS), 20% glycerol, and 2% dithiothreitol, and then re-equilibrated for 15 min in the same buffer containing 2.5% iodoacetamide in place of dithiothreitol. Twelve percent SDS-polyacrylamide gels were used for second dimension electrophoresis.

For total protein stain, the gels were washed three times with deionized water and stained with Bio-Safe Coomassie Blue (Bio-Rad).

2.3. Nitroblue tetrazolium (NBT)/glycinate redox-cycling staining

After one- or two-dimensional SDS-polyacrylamide gel electrophoresis, the proteins in the gel were transferred onto nitrocellulose membrane (Millipore) using a Transblot-Blot SD Semi-Dry Transfer Cell (Bio-Rad). After transfer, membranes were washed once with deionized water and stained with buffer containing 0.24 mM NBT in 2 M potassium glycinate, pH 10 for 1 h (Paz et al., 1991). Afterward, the blot was washed with 0.1 M sodium borate, pH 10 and scanned by a scanner (UMAX, Powerlook 2100XL-USB).

2.4. Mass spectrometry (MS) analysis

Mass spectrometry analyses to identify *in vivo* protein targets and confirm the presence of dopamine modification were performed by Shanghai Applied Protein Technology, Co., Ltd. Briefly, the gel particle recovered from Coomassie Blue stained gels was smashed and destained in a buffer containing 100 mM NH_4HCO_3 and 30% acetonitrile. After lyophilization, the gel spots were digested with 10 ng/μL modified trypsin (Promega) at 37 °C for 20 h, and then mixed with equal volume of a-cyano-4-hydroxytrans-cinnamic acid (5 mg/mL) and analyzed by a MALDI-TOF–TOF instrument (4800 proteomics analyzer; Applied Biosystems). The MS spectra were recorded in reflector mode in a mass range from 800 to 4000 with a focus mass of 2000. Combined peptide mass fingerprinting (PMF) and MS/MS queries were performed by using the MASCOT search engine 2.2 (Matrix Science, Ltd.) on the IPI (International Protein Index) database with the following parameter settings: 100 ppm mass accuracy, trypsin cleavage one missed cleavage allowed, carbamidomethylation set as fixed modification, oxidation of methionine oxidation of methionine and dopamine modification of cysteine (cysteine + 151.0633 Da) was allowed as variable modification, and MS/MS fragment tolerance was set to 0.4 Da. A GPS Explorer protein confidence index ≥95% was used for further manual validation.

Mass spectrometry analyses to confirm the presence of dopamine modification in recombinant hMDH1 after dopamine treatment were performed by the laboratory of functional proteomics (East China Normal University). Briefly, proteins were solubilized in 8 M urea containing 10 mM of dithiothreitol and incubated at 37 °C for 30 min, and then alkylated with 15 mM of iodoacetamide. The solution was diluted to a final urea concentration of 2 M, and digested with sequence grade modified trypsin (Promega Inc.) for 16 h. The digested peptides were desalted with StageTip (Waters Corporation) solid-phase extraction material, and loaded through autosampling device on an EASY-nanoLC1000 UHPLC system (Thermo Inc.) onto a 2 cm C18 trapping column that is connected to a 15 cm micro HPLC column (75 μm ID,