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OXIDATIVE STRESS AND DOPAMINE DEPLETION IN AN INTRASTRIATAL 6-HYDROXYDOPAMINE MODEL OF PARKINSON'S DISEASE

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Abstract—Although the etiology of Parkinson's disease (PD) is unknown, a common element of most theories is the involvement of oxidative stress, either as a cause or effect of the disease. There have been relatively few studies that have characterized oxidative stress in animal models of PD. In the present study a 6-hydroxydopamine (6-OHDA) rodent model of PD was used to investigate the *in vivo* production of oxidative stress after administration of the neurotoxin. 6-OHDA was injected into the striatum of young adult rats and the production of protein carbonyls and 4-hydroxynonenal (HNE) was measured at 1, 3, 7, and 14 days after administration. A significant increase in both markers was found in the striatum 1 day after neurotoxin administration, and this increase declined to basal levels by day 7. There was no significant increase found in the substantia nigra at any of the time points investigated. This same lesion paradigm produced dopamine depletions of 90–95% in the striatum and 63–80% in the substantia nigra by 14–28 days post-6-OHDA. Protein carbonyl and HNE levels were also measured in middle-aged and aged animals 1 day after striatal 6-OHDA. Both protein carbonyl and HNE levels were increased in the striatum of middle-aged and aged animals treated with 6-OHDA, but the increases were not as great as those observed in the young adult animals. Similar to the young animals, there were no increases in either marker in the substantia nigra of the middle-aged and aged animals. There was a trend for an age-dependent increase in basal amounts of oxidative stress markers when comparing the non-lesioned side of the brains of the three age groups. These results support that an early event in the course of dopamine depletion following intrastriatal 6-OHDA administration is the generation of oxidative stress.

Key words: reactive oxygen species, protein carbonyls, 4-hydroxynonenal, striatum, substantia nigra, aging.

Parkinson's disease (PD) stems from the loss of dopamine (DA) caused by the degeneration of the dopaminergic neurons of the substantia nigra. The nature of this degeneration remains unclear, although current theories suggest that reactive oxygen species (ROS) are involved in some capacity early in the disease process (Jenner, 2003). One of the earliest changes in patients with PD and incidental Lewy body disease is the loss of glutathione (Sian et al., 1994; Owen et al., 1996). Postmortem tissue from PD patients has been shown to contain elevated levels of the oxidative stress products 4-hydroxynonenal (HNE) (Yoritaka et al., 1996), protein carbonyls (Alam et al., 1997a; Floor and Wetzel, 1998), 8-hydroxy-2-deoxyguanosine and 8-hydroxyguanine (Alam et al., 1997b; Zhang et al., 1999), and 3-nitrotyrosine (Good et al., 1998; Giasson et al., 2000).

The administration of 6-hydroxydopamine (6-OHDA) into the brain of the rat produces a well-established model of PD (Kirik et al., 1998; Blum et al., 2001; Betarbet et al., 2002; Deumens et al., 2002). Many investigators have demonstrated that 6-OHDA induces oxidative stress (Kumar et al., 1995; Soto-Otero et al., 2000, 2002; Seth et al., 2002; Mazzio et al., 2004), which can lead to the induction of apoptosis and cellular loss (Choi et al., 1999; Marti et al., 2002; Seth et al., 2002; Liang et al., 2004). The effects of 6-OHDA are age-dependent as there is a greater effect seen in aged animals compared with young animals, particularly with lower doses of 6-OHDA (Marshall et al., 1983; Cass et al., 2002).

To identify oxidative stress that has occurred *in vivo*, it is more feasible to detect the products of oxidative damage as opposed to the causative agents, which exist only transiently. The measurement of protein carbonyls is one of the most widely accepted techniques to measure oxidative damage (Stadtman, 2002). HNE, the most abundant cytotoxic molecule generated under oxidative stress, is both a product of oxidative damage and a causative agent of cellular damage (Uchida, 2003). Both protein carbonyls and HNE are in abundance under oxidative conditions, and reliable methods of detection are available for each (Yatin et al., 1999; Poon et al., 2004; Theodore et al., 2006).

The purpose of the present study was to determine the time course of the development of oxidative stress that is generated in a striatal 6-OHDA lesion model of PD, and to ascertain if there are any age-related differences involving the generation of oxidative stress in this model. Establishing this time course in the rodent model will allow for the evaluation of experimental therapies on an early event in the neurodegenerative process. Young adult animals were lesioned with an intrastriatal injection of 6-OHDA. Protein carbonyls and HNE were assayed in both the striatum and substantia nigra in order to determine a time course of the

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Abbreviations: ANOVA, analysis of variance; DA, dopamine; DNP, 2,4-dinitrophenylhydrazine; HNE, 4-hydroxynonenal; HPLC, high pressure liquid chromatography; I:C ratio, ratio of the ipsilateral to contralateral side; PD, Parkinson's disease; ROS, reactive oxygen species; TTBS, Tris-buffered saline with Tween 20; 6-OHDA, 6-hydroxydopamine.

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generation of oxidative stress induced by 6-OHDA. Additionally, because 6-OHDA has been shown to have a greater effect on the DA systems of aged animals (Marshall et al., 1983; Cass et al., 2002), we examined the generation of protein carbonyls and HNE in middle-aged and aged animals to determine if there was an age-related effect in 6-OHDA-induced generation of oxidative stress.

EXPERIMENTAL PROCEDURES

Animals

Young adult (3–4 months old) (212 g–336 g), middle-aged (13–14 months old) (402 g–498 g) and aged (22–23 months old) (372 g–524 g) male Fischer-344 rats (Harlan Sprague–Dawley, Indianapolis, IN, USA) were used for all experiments. Animals were housed in groups of two under a 12-h light/dark cycle with food and water freely available. All animal use procedures were in strict accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee at the University of Kentucky. Every effort was made to minimize the number of animals used and their suffering.

6-OHDA lesions

The rats were anesthetized with isoflurane (2.0–2.5% as needed) and placed into a stereotaxic frame. The skull was exposed and 2 small burr holes were drilled in the skull above the right striatum (0.5 mm posterior to bregma, 4.2 mm right of midline; 0.5 mm anterior to bregma, 2.5 mm right of midline). The dura was cut and a SGE syringe with a 26 gauge blunt-tipped needle was slowly lowered to a depth of 5.0 mm below the surface of the brain. Two microliters of either vehicle (0.9% saline with 0.1% ascorbic acid, pH 5.5) or 10 μg of 6-OHDA (Sigma-Aldrich, St. Louis, MO, USA) dissolved in vehicle was injected at a rate of 0.4 μl/min for 5 min into each of the two sites. The needle was left in place for an additional 5 min following each injection and then slowly withdrawn. The burr holes were filled with Gelfoam and the incision was closed with wound clips. The animals were placed in a heated recovery chamber until they recovered from the anesthetic, after which they were transferred back to their home cages.

Tissue collection for HPLC and slot blots

The animals were rendered unconscious with CO_2, decapitated and the brains quickly removed and chilled in ice-cold saline. A coronal slice 2 mm thick was removed at the level of the striatum using a chilled brain mold (Rodent Brain Matrix; ASI Instruments, Warren, MI, USA). The left and right striata were then dissected from the slice. A similar 2 mm coronal slice was made through the midbrain and the substantia nigra removed from both sides of the brain. All tissue samples were placed in pre-weighed vials, weighed and frozen on dry ice. The samples were stored at −80 °C until analysis.

High pressure liquid chromatography (HPLC) analysis

Tissue samples were analyzed for DA using HPLC with electrochemical detection as described previously (Cass et al., 2003). The retention times of standards were used to identify peaks, and the peak heights were used to determine amount of recovery of internal standard (dihydroxybenzylamine) and amounts of DA.

Quantification of markers for oxidative stress

Sample preparation for slot blots. Crude synaptosomes were made from the striatum. Tissue was homogenized with a Teflon pestle in cold 0.32 M sucrose buffer containing protease inhibitors (Complete, Mini; Roche Diagnostics, Indianapolis, IN, USA) and centrifuged for 15 min at 1000×*g*. The supernatant was transferred to a clean vial and centrifuged a second time for 15 min at 12,500×*g*. The supernatant was discarded and the resulting crude synaptosomes were resuspended in 50 μL of buffer. The substantia nigra samples were homogenized in cold 0.32 M sucrose buffer containing protease inhibitors using a sonic dismembrator (Fisher Model 50 Sonic Dismembrator; Fisher Scientific, Pittsburgh, PA, USA). Protein concentrations of all samples were determined using the Pierce BCA method for protein quantification (Pierce Biotechnology, Rockford, IL, USA).

Protein carbonyls. The methods for protein carbonyls and HNE by slot blots were as described previously (Poon et al., 2004) with slight modification. Crude synaptosomes (striatum) or whole tissue homogenate (substantia nigra) (15 μg protein/5 μl), 12% SDS, and 2,4-dinitrophenylhydrazine (DNP; OxyBlot Protein Oxidation Detection Kit; Chemicon International, Temecula, CA, USA) were incubated at room temperature for 20 min, after which the reaction was stopped with a neutralizing solution. Derivatized proteins (250 ng) were applied to a nitrocellulose membrane via vacuum filtration (Bio-Dot SF; BioRad, Hercules, CA, USA). The membrane was blocked with 3% BSA for 1 h then rinsed three times with Tris-buffered saline with Tween 20 (TTBS) (Sigma-Aldrich). The membrane was then incubated with rabbit anti-DNP (1:150) for 1 h, rinsed three times with TTBS, followed by goat anti-rabbit conjugated to alkaline phosphatase (Sigma-Aldrich) (1:15,000) for 1 h followed by rinsing three times with TTBS. An alkaline phosphatase substrate (SigmaFast; Sigma-Aldrich) was used to visualize the resulting bands. After drying, the developed membrane was scanned into Scion Image (Scion Corporation, Frederick, MD, USA) and the bands quantified by densitometry.

HNE. Crude synaptosomes or tissue homogenate (15 μg protein/5 μl) with 12% SDS was applied to a nitrocellulose membrane via vacuum filtration. The membrane was blocked with 3% BSA for 1 h then rinsed three times with TTBS. The membrane was then incubated with rabbit anti-HNE (Alpha Diagnostic, San Antonio, TX, USA) (1:10,000) for 2 h, rinsed three times with TTBS, followed by goat anti-rabbit conjugated to alkaline phosphatase (Sigma-Aldrich) (1:15,000) for 1 h followed by rinsing three times with TTBS. An alkaline phosphatase substrate (SigmaFast; Sigma-Aldrich) was used to visualize the resulting bands. After drying, the developed membrane was scanned into Scion Image, converted to a gray scale image, and the bands quantified by densitometry.

Data analysis

Tissue levels of DA are expressed as ng/g wet weight of tissue, or as a ratio of the side ipsilateral to the lesion to the side contralateral to the lesion (I:C ratio). Data from the slot blots for both the protein carbonyl and HNE levels were expressed as a ratio of the side ipsilateral to the lesion to the side contralateral to the lesion (I:C ratio), or as a percentage of the results from young adult, naïve animals that had no surgical procedures. Results are expressed as mean±S.E.M. All data were analyzed by one-, two-, or three-way analysis of variance (ANOVA) followed by a Newman-Keuls test for post hoc comparisons. *P*-values ≤0.05 were considered statistically significant.

RESULTS

DA content of striatum and substantia nigra in young, middle-aged and aged rats

Tissue levels of DA in the striatum and substantia nigra were measured 3½ to 4 weeks after intrastriatal saline or