

Formation and Implications of Alpha-Synuclein Radical in Maneb- and Paraquat-Induced Models of Parkinson's Disease

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Abstract Parkinson's disease (PD) is a debilitating, progressive, neurodegenerative disorder characterized by progressive loss of dopaminergic neurons and motor deficits. Alpha-synuclein-containing aggregates represent a feature of a variety of neurodegenerative disorders, including PD; however, the mechanism that initiates and promotes intraneuronal alpha-synuclein aggregation remains unknown. We hypothesized protein radical formation as an initiating mechanism for alpha-synuclein aggregation. Therefore, we used the highly sensitive immuno-spin trapping technique to investigate protein radical formation as a possible mechanism of alpha-synuclein aggregation as well as to investigate the source of protein radical formation in the midbrains of Maneb- and paraquat-coexposed mice. Coexposure to Maneb and paraquat for 6 weeks resulted in active microgliosis, NADPH oxidase activation, and inducible nitric oxide synthase (iNOS) induction, which culminated in protein radical formation in the midbrains of mice. Results obtained with immuno-spin trapping and immunoprecipitation experiments confirmed formation of alpha-synuclein radicals in dopaminergic neurons of exposed mice. Free radical formation requires NADPH oxidase and iNOS, as indicated by decreased protein radical formation in knockout mice (P47phox^{-/-} and iNOS^{-/-}) and in mice treated with inhibitors such as FeTPPS (a peroxynitrite decomposition catalyst), 1400 W (an iNOS inhibitor), or apocynin (a NADPH oxidase inhibitor). Concurrence of

protein radical formation with dopaminergic neuronal death indicated a link between protein radicals and disease progression. Taken together, these results show for the first time the formation and detection of the alpha-synuclein radical and suggest that NADPH oxidase and iNOS play roles in peroxynitrite-mediated protein radical formation and subsequent neuronal death in the midbrains of Maneb- and paraquat-coexposed mice.

Keywords Protein radicals · Peroxynitrite · Parkinson's disease · Immuno-spin trapping · DMPO

Abbreviations

DMPO	5,5-Dimethyl-1-pyrroline <i>N</i> -oxide
MP	Maneb and paraquat
O ₂ ^{•-}	Superoxide
TH	Tyrosine hydroxylase
NO [•]	Nitric oxide
ONOO ⁻	Peroxynitrite

Introduction

Parkinson's disease (PD) is a movement disorder characterized by slow and progressive loss of dopaminergic neurons in the substantia nigra and manifests mostly as a sporadic condition [1, 2]. Although the cause of sporadic PD is not fully understood, various factors, including environmental toxins, heavy metals, and pesticides, have been implicated in disease pathogenesis [3, 2, 4]. An important aspect of human pesticide exposure is that it is usually to a mixture of pesticides rather than a single one, and it occurs over time [5]. Various chemicals, especially pesticides, and factors related to pesticide exposure (e.g., farming, well-water drinking, and rural

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living) have been proposed as potential risk factors for PD [6–9].

The fungicide Maneb (manganese ethylene-1,2-bisdithiocarbamate) and the herbicide paraquat (1,1-dimethyl-4,4-bipyridinium) are concurrently used in agriculture to treat a variety of crop pathologies [10, 11]. Paraquat crosses the blood-brain barrier through neutral amino acid transporters, preferentially targets the nigrostriatal pathway, inhibits mitochondrial complex I, undergoes redox cycling, and produces superoxide [12, 13]. Maneb and paraquat together are well documented as a trigger that induces the PD phenotype in mice [14]. Epidemiological studies also show that combined exposure to Maneb and paraquat strongly increases the risk of PD in humans, especially with coexposure [10, 11]. Considering the global use of pesticides and the presence of epidemiological evidence, the Maneb- and paraquat-induced model of PD seemed appropriate for studying the involvement of protein radicals in PD.

A common hallmark of several neurodegenerative disorders is the loss of specific populations of neuronal cells and the presence of abnormal protein aggregates containing specific modified proteins [15]. Because these modified proteins have a propensity to aggregate and interfere with cellular processes and signaling, either their production should be controlled or their removal must be quick enough to maintain cell survival. The ubiquitin-proteasome system is the primary pathway responsible for degradation of altered proteins [16, 17]. Conversely, an impaired ubiquitin-proteasomal system, as often seen in various models of PD, facilitates accumulation of modified proteins in the substantia nigra [18, 19]. The pathological features of PD include an abnormal accumulation of the protein alpha-synuclein in particular throughout various brain regions in the remaining dopaminergic neurons in the nigrostriatal pathway [20, 21].

Furthermore, inhibition of alpha-synuclein aggregation by Hsp90 renders it nontoxic and therefore affects neuronal survival [22]. Genetic ablation of alpha-synuclein has been shown to protect dopaminergic neurons from the environmental toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a precursor neurotoxin known to trigger the core neurological features of PD and specific dopaminergic neuronal death [23, 22]. Certain mutations in SNCA (the gene which encodes alpha-synuclein), posttranslational modifications, oxidative stress, and toxins interacting with oxidized dopamine are speculated to increase the propensity of alpha-synuclein to aggregate and accumulate [24]. Although protein aggregates and modified proteins such as alpha-synuclein [25, 26] play crucial roles in neuronal regulation during PD pathogenesis, the underlying initiating mechanism of aggregation remains unknown [27, 28]. It is generally accepted that protein radical formation might be a key mechanism that initiates

aggregation or radical-driven protein modification. However, the formation, detection, and consequences of protein radicals *in vivo*, especially in a neuronal context, have never been investigated.

Therefore, we aimed to investigate the underlying mechanism of the formation, detection, and consequences of the alpha-synuclein radical in dopaminergic neurons of Maneb- and paraquat-coexposed mice. Through various lines of evidence, we report for the first time that NADPH oxidase and iNOS play crucial roles in peroxynitrite-mediated alpha-synuclein radical formation in dopaminergic neurons of Maneb- and paraquat-coexposed mice. Alpha-synuclein radical formation appeared in concurrence with the neuronal death that occurs in the Maneb- and paraquat-induced PD-phenotype. Possible biochemical consequences of protein radical formation may involve changes in activity, gain or loss of function, alteration in protein assembly, and formation of aggregates [29, 30]. Understanding the protein radical-derived mechanisms in a pesticide-induced model of PD is important for defining protein radical-derived therapeutics that can *specifically* minimize or impair protein radical-mediated dopaminergic neurodegeneration.

Materials and Methods

Materials

Apocynin, bovine serum albumin, *N*-3-(aminomethyl) benzylacetamide 2HCl (1400 W), Maneb (manganese ethylene-1,2-bisdithiocarbamate), DAB, NADPH, paraquat (1,1-dimethyl-4,4-bipyridinium), paraformaldehyde, Triton X-100, and 5,10,15,20,-tetrakis(4-sulfonatophenyl) porphyrinato iron (III) chloride (FeTPPS) were from Sigma (St. Louis, MO). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was obtained from Dojindo Laboratories (Rockville, MD) and used without further purification. Chicken and rabbit polyclonal anti-DMPO antibodies were developed in our laboratory and used in the immuno-spin trapping studies. Mouse monoclonal anti-integrin α M (ox-42), rabbit polyclonal anti-iNOS, and P67 phox antibodies were from Santa Cruz Biotechnology (Dallas, TX). Mouse monoclonal anti-alpha synuclein and anti- β -actin antibodies were from Abcam (Cambridge, MA). Rabbit polyclonal anti-tyrosine hydroxylase antibody was from Millipore (Billerica, MA). Micro BCA Protein Assay Kit, Permunt, RIPA buffer, Surfact-Amps X-100, and Pierce Classic Immunoprecipitation Kits were from Thermo Scientific (Rockford, IL). Nitrocellulose membranes, Prolong Gold anti-fade reagent with DAPI, and Alexa Fluor secondary antibodies were from Invitrogen (Grand Island, NY). The Vectastain Elite ABC Kit was from Vector laboratories (Peterborough, UK).

Methods

Animal Treatment

Adult (8–10-week-old), pathogen-free male C57BL6/J mice (Jackson Laboratories, Bar Harbor, Maine) were housed one to a cage for 1 week before any experimental dosing. Mice that contained the disrupted iNOS (iNOS^{-/-}, stock number 002609) and P47 phox (P47 phox^{-/-}, stock number 004762) were treated identically. Age-matched mice of C57BL6/J origin that had normal iNOS and NADPH oxidase activity served as the control animals for knockout experiments. The animals were housed under standard conditions of temperature and humidity with a 12-h light/dark cycle and fed a standard pellet diet and water ad libitum. Animals were treated with normal saline (control), paraquat (10 mg/kg, i.p.), and Maneb (30 mg/kg, i.p.) with or without pretreatment with apocynin (10 mg/kg, i.p.), 1400 W (15 mg/kg, i.p.), or FeTPPS (10 mg/kg, i.p.) twice a week for 6 weeks. A minimum of 3–5 animals were used per group for biochemical, Western blot/ELISA, and immunohistochemical studies. All animals were treated in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals, and the animal study proposal was approved by the institutional review board.

HPLC and MS

5,5'-dimethyl-1-pyrroline *N*-oxide (DMPO) was administered to mice at a dose of 2 g/kg, i.p., followed by transcardial perfusion and sacrifice after 2 h. The livers (used as a positive control) and brains were dissected to analyze DMPO content in order to evaluate its entry into the brain. In brief, the nigrostriatal tissue and liver were homogenized in PBS containing 0.1 N HClO₄ followed by sonication and centrifugation at 21,000 × *g* for 30 min at 4 °C [13]. The supernatant was neutralized with sodium hydroxide to a pH of 7.0, centrifuged (14,000 rpm for 5 min at room temperature), and passed through a 0.2-μm filter. Samples thus obtained were loaded onto a column (Waters Atlantis T3, l=150 mm) pre-equilibrated with 0.1 % aqueous formic acid (flow rate, 0.2 ml/min). The fraction eluted at the retention time of 15 min was collected and investigated with mass spectroscopy for DMPO. MS and MS/MS experiments were carried out using a Waters Micromass Q-TOF (Waters, Milford, MA). Capillary voltage was 3300 V, sample cone voltage 30 V, extraction cone energy 4–5 eV, and collision energy 4–5 eV for MS and 15–20 eV for MS/MS. Helium was used as the collision gas, and the product ion mass was 114.09. For calibration, a solution of glu-fibrinopeptide B (500 fmol/μl) in water/ acetonitrile 80:20 (v/v) with 0.1 % formic acid and a mass of 785.8496 (2+) was used. Data analysis was accomplished using MassLynx software supplied by the manufacturer.

In Vivo Immuno-Spin Trapping Studies

DMPO was administered at a dose of 2 g/kg, i.p., 2 h after the final dose of Maneb and paraquat (MP) at the 6-week timepoint. Animals were sacrificed 2 h after DMPO injection, and the brain was dissected to isolate the nigrostriatal region. Tissues were homogenized in phosphate buffer containing a protease inhibitor cocktail and centrifuged at 10,000 rpm at 4 °C for 20 min. These samples were used for anti-DMPO ELISA [2].

Confocal Microscopy of Brain Tissue

For confocal microscopy of brain tissue, mice were anesthetized and perfused transcardially. Perfused mice brains were coronally dissected, postfixed, and cryoprotected in sucrose. The frozen sections containing the substantia nigra region (20 micron) were cryocut using a frozen tissue processor (Leica Instruments, Bannockburn, IL, USA) at the immunohistochemistry core facility at NIEHS. Tissue slices were then permeabilized (0.2 % Surfact-Amps X-100 in PBS) for 5 min and blocked overnight (2 % BSA in PBS with 0.1 % Surfact-Amps X-100). Afterward, slides were incubated for 2 h with the primary anti-DMPO (1:2000) and/or anti-alpha-synuclein (1:2000) and anti-tyrosine hydroxylase antisera (1:4000) at room temperature, followed by appropriate secondary Alexa Fluor antisera diluted 1:1000 for an hour. Slides were washed four times, dried, and mounted using Prolong Gold anti-fade reagent with DAPI. Confocal images were taken on a Zeiss LSM 510-UV meta microscope (Carl Zeiss Inc. Oberkochen, Germany) using a Plan-NeoFluar 40X/1.3 Oil DIC objective, in some cases with 3×zoom (Fig. 3a).

Immunoprecipitation of DMPO Nitroene Adduct and Alpha-Synuclein

Immunoprecipitation of DMPO nitroene adducts was carried out with the Pierce Classic Immunoprecipitation Kit with some modifications. Nigrostriatal homogenates were collected and protein concentrations measured with a Micro BCA Protein Assay Kit, and 1 mg of total protein was used for subsequent steps. The samples were precleared (1 h at room temperature) with 80 μl of control agarose slurry (50 %). The homogenate was incubated overnight with 10 μg of normal mouse IgG (control) and 10 μg of rabbit polyclonal anti-alpha-synuclein antibody, and this antigen-antibody mixture was then incubated overnight to form the immune complex. This immune complex was incubated with protein A/G slurry for an hour with gentle end-over-end mixing. Samples were then eluted with sample-reducing buffer according to the manufacturer's instructions, followed by Western blotting with detection using anti-DMPO primary antibody.

Western Blot Analysis

Following separation by SDS-PAGE, proteins were transferred electrophoretically (15 V for 40 min) to nitrocellulose membranes. Subsequently, membranes were blocked with 4 % nonfat dry milk in 100 mM (bi)carbonate buffer, pH 9.6, for 2 h. Membranes were washed once with washing buffer (0.05 % Tween-20 in Tris-buffered saline, pH 7.4) and then incubated overnight with mouse monoclonal anti-DMPO antibody (1:250)/anti-alpha synuclein antibody (1:3000)/anti-iNOS antibody (1:2000)/anti P-67 phox antibody (1:3000)/anti-tyrosine hydroxylase antibody (1:4000)/or anti- β -actin antibody (1:4000). Then, membranes were washed four times and incubated with appropriate fluorescent secondary antibody (1:10,000, diluted in washing buffer) labeled with infrared dyes for 2 h at room temperature in the dark. After incubation, traces of unbound antibody were eliminated by washing. An Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE) was used to acquire the images [2].

Enzyme-Linked Immunosorbent Assay (ELISA)

Rabbit polyclonal anti-DMPO antibody was used to detect DMPO-protein-derived nitron adducts using a standard ELISA as described previously [31]. In brief, brain homogenates (nigrostriatal region only) were immobilized by incubating reaction mixtures (2.5 mg/well) in 100 mM bicarbonate buffer, pH 9.6, for 90 min at 35 °C. The plate was washed once and blocked (4 % fish gelatinin PBS) for 90 min at 35 °C. Following another wash, rabbit polyclonal anti-DMPO antibody (1:5000) was added and incubated for 60 min. After incubation, plate wells were washed four times followed by incubation with anti-rabbit, IgG-alkaline phosphatase-conjugated secondary antibody (1:5000) for 60 min at 35 °C. After another four washes, CDP star (25 mM) was added, and the chemiluminescent product was detected using a Tecan Spectra Fluor-Plus multifunction plate reader with Xfluo software (Tecan US, Research Triangle Park, NC, USA).

Immunohistochemistry

Integrin α M (ox-42) and TH-immunoreactivity were performed by the method described previously [13]. In brief, mice were anesthetized, and the brain was perfused transcardially. Perfused mouse brains were coronally dissected, postfixed, and cryoprotected in sucrose. Sections (20 μ m thick) were cut serially using a cryostat, and the sections were then incubated with 0.5 % H_2O_2 in methanol to block the endogenous peroxidase activity. The sections were incubated in blocking reagent (5 % goat serum, 1 % BSA, and 0.1 % Triton X-100 in PBS) for 2 h followed by incubation with rabbit polyclonal anti-TH antibody (1:4000) and washed with PBS. The sections were then incubated with biotinylated anti-rabbit secondary antibody (1:300) for 2 h,

washed and incubated with avidin-biotin complex for 90 min, and developed using DAB. The sections were mounted permanently with Permount, and images were captured with a brightfield microscope at 10 \times magnification. Coded slides were used to ensure unbiased counting of TH-positive neurons in every fourth serial section. The number of TH-positive neurons was counted bilaterally using a Metamorph image analysis tool. A minimum of four animals per group was used for counting TH-positive neurons. The results are expressed as percent of the control. Counting of TH-positive neurons was also performed in the ventral tegmental area (VTA) region of the controls and the experimental groups that received Maneb and paraquat and other pretreatments. The same method was used for integrin α M (ox-42) immuno-staining using monoclonal anti-integrin α M antibody (1:2000).

Statistical Analyses

All in vivo experiments were repeated three times with a minimum of four mice per group ($n=4$; data from each group of four mice was pooled). One-way analysis of variance (ANOVA) was used for statistical analysis. The Newman-Keuls post-test was used for multiple comparisons. The results are expressed as mean \pm SEM. The differences were considered statistically significant when p values were less than 0.05.

Results

DMPO Detected in Brain after Intraperitoneal Injections

In order to trap protein radicals and facilitate their detection by immuno-spin trapping using the anti-DMPO antibody, DMPO must be shown to be present at the site of radical formation as a prerequisite. Typically, DMPO reacts with radicals on macromolecules such as proteins or DNA and forms a stable nitron adduct, which can be detected by the anti-DMPO antibody [32]. In order to verify that DMPO can enter the brain after intraperitoneal injection, DMPO was injected intraperitoneally, and the nigrostriatal tissue of the brain was analyzed for DMPO content using HPLC. Peaks resembling DMPO showed that DMPO reaches the brain after intraperitoneal injections (Fig. 1a) at a concentration almost equal to that in the liver (used as a positive control). To further confirm that the peaks were those of DMPO, the fractions eluted at the retention time of DMPO (15 min) were collected and investigated with mass spectroscopy. MS and MS/MS experiments showed a peak with m/z of 114.09, as expected for DMPO (Fig. 1b). Once the presence of DMPO in nigrostriatal tissue after intraperitoneal injections was confirmed, we further investigated protein radical formation in nigrostriatal tissue of mice coexposed to Maneb and paraquat.

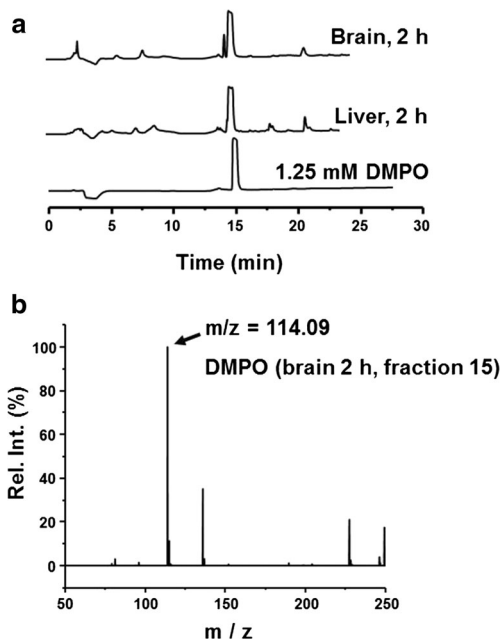
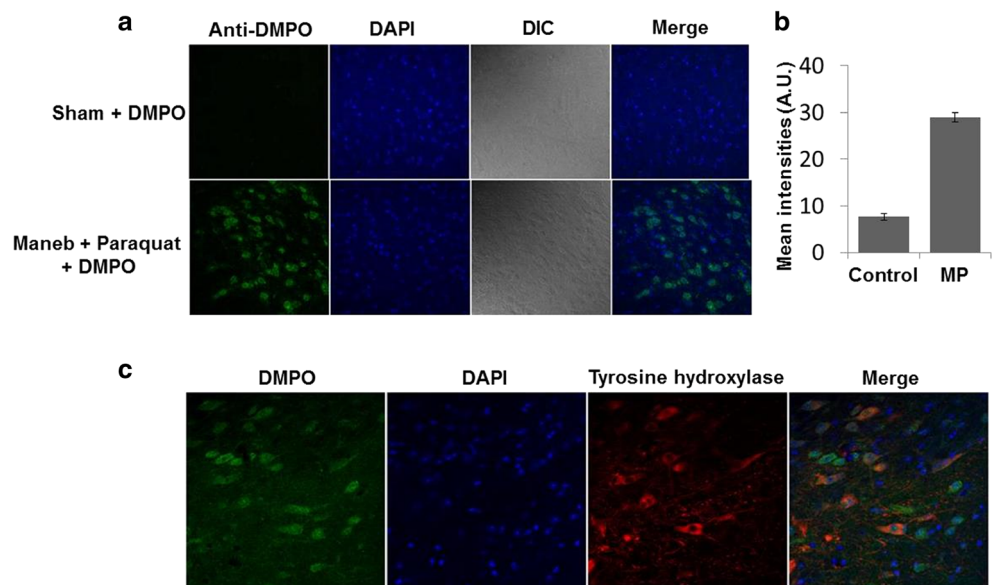


Fig. 1 DMPO enters the brain after intraperitoneal injections. **a** HPLC chromatogram showing a peak corresponding to DMPO in brain and liver homogenates after 2 h of DMPO injections (2 g/kg, i.p.). **b** MS spectra showing peaks with m/z of 114.09, indicating the presence of DMPO in brain homogenates after intraperitoneal injections

Maneb and Paraquat Coexposure Induces Protein Radical Formation in the Midbrain of Exposed Mice

Immuno-spin trapping experiments with midbrain sections of Maneb- and paraquat-coexposed mice showed intense anti-DMPO staining. This result gives strong evidence of protein radical formation in the midbrain of Maneb- and paraquat-coexposed mice (Fig. 2a, b). Colocalization of tyrosine hydroxylase with anti-DMPO further indicated formation of protein radicals in dopaminergic neurons. Though the anti-

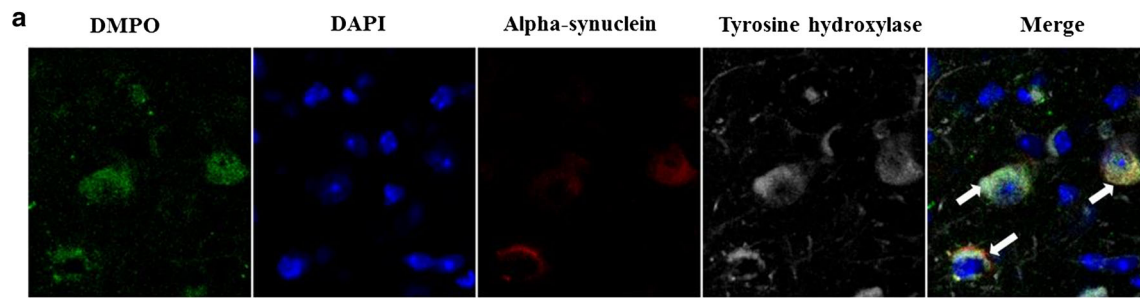
Fig. 2 Protein radical formation in the midbrain of mice after 6 weeks of Maneb (30 mg/kg, i.p.) and paraquat (10 mg/kg, i.p.) coexposure. **a** Confocal images showing the anti-DMPO staining in midbrain slices of Maneb- and paraquat-coexposed mice. **b** Fluorescence intensity quantification of anti-DMPO staining. **c** Confocal images showing the anti-DMPO staining in tyrosine hydroxylase-positive neurons and other adjacent cells



DMPO signal was quite apparent in dopaminergic neurons, it was also present in adjacent cells, possibly microglia (Fig. 2c). These results demonstrate the formation of protein radicals.

Detection of Alpha-Synuclein Protein-Centered Radicals In Vivo by Immuno-Spin Trapping

Once we confirmed the formation of radicals in the midbrain of Maneb- and paraquat-coexposed mice, we looked further for specific protein-centered radicals in dopaminergic neurons. Results with confocal microscopy (Fig. 3a) showed colocalization of anti-DMPO, alpha-synuclein, and tyrosine hydroxylase, which suggests radical formation on alpha-synuclein within dopaminergic neurons. To further confirm that the anti-DMPO signal was due to protein-centered radicals on alpha-synuclein, alpha-synuclein was immunoprecipitated and probed with anti-DMPO antibody. Proteins immunoprecipitated with normal mouse IgG (lane 1, Fig. 3b) did not show DMPO adducts. An anti-DMPO positive band on immunoprecipitated alpha-synuclein (Fig. 3b, lane 2) further indicated that alpha-synuclein-centered protein radicals were formed in dopaminergic neurons of MP-coexposed mice. Though Maneb and paraquat coexposure is known to trigger oxidative stress by complex 1 and/or complex 3 inhibition [5] and NADPH oxidase activation, a range of possibilities existed for how these protein radicals were formed. Because of a previous report that LPS-induced protein radical formation in BV2 microglial cells is peroxynitrite-mediated [2], subsequent experiments were designed to elucidate the underlying mechanism of peroxynitrite generation and protein radical formation in microglia and dopaminergic neurons of Maneb- and paraquat-coexposed mice.



b Anti-DMPO Western of immunoprecipitated alpha-synuclein

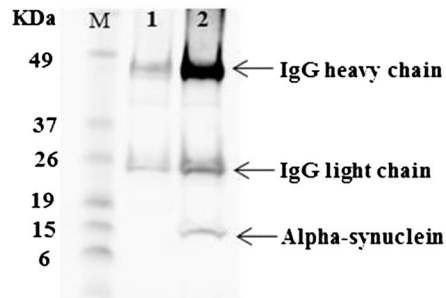


Fig. 3 Detection of alpha-synuclein-centered radicals in dopaminergic neurons. **a** Confocal images of cryocut midbrain sections from sham+DMPO- and Maneb+paraquat (MP)+DMPO-treated mice showing the colocalization of anti-DMPO staining with alpha-synuclein and tyrosine hydroxylase. **b** Immunoprecipitation of DMPO adducts. Proteins were

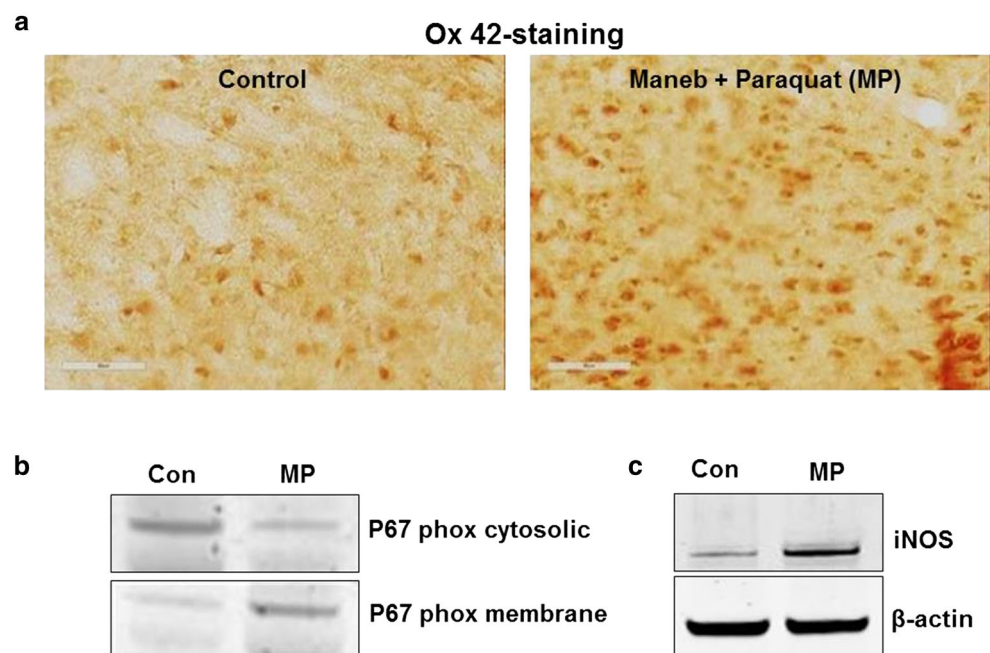
immunoprecipitated from nigrostriatal tissue homogenates using either normal mouse IgG as a control (*lane 1*) or with anti-alpha-synuclein antibody (*lane 2*) followed by Western blot analysis of immunoprecipitates using rabbit anti-DMPO antibody. Blots are representative of four different immunoblots from an equal number of experiments

Protein Radical Formation and Concurrent Microglial Activation

Coexposure to Maneb and paraquat induced active microgliosis, as evidenced by increased integrin α M (ox-42) immunoreactivity in the substantia nigra (Fig. 4a). Maneb and

paraquat coexposure triggered translocation of the P67 phox subunit of NADPH oxidase, which is considered to be the signature for its activation. An increase in P67 phox levels in the membrane fraction along with decreased cytosolic levels in Maneb- and paraquat-coexposed animals indicated that NADPH oxidase was activated in the substantia nigra of

Fig. 4 Maneb and paraquat (MP) induce active microgliosis in the midbrain of mice after 6 weeks of Maneb and paraquat coexposure. **a** Ox-42 staining in midbrain slices of sham and Maneb- and paraquat-coexposed mice. **b** Western blot analysis of P67 phox in the cytosolic and membrane fraction of the nigrostriatal tissue of mouse brain following 6 weeks of Maneb and paraquat coexposure. **c** Western blot analysis of iNOS in the nigrostriatal tissue of mouse brain following 6 weeks of Maneb and paraquat coexposure. Data show mean values \pm SEM from four independent experiments ($n=4$)



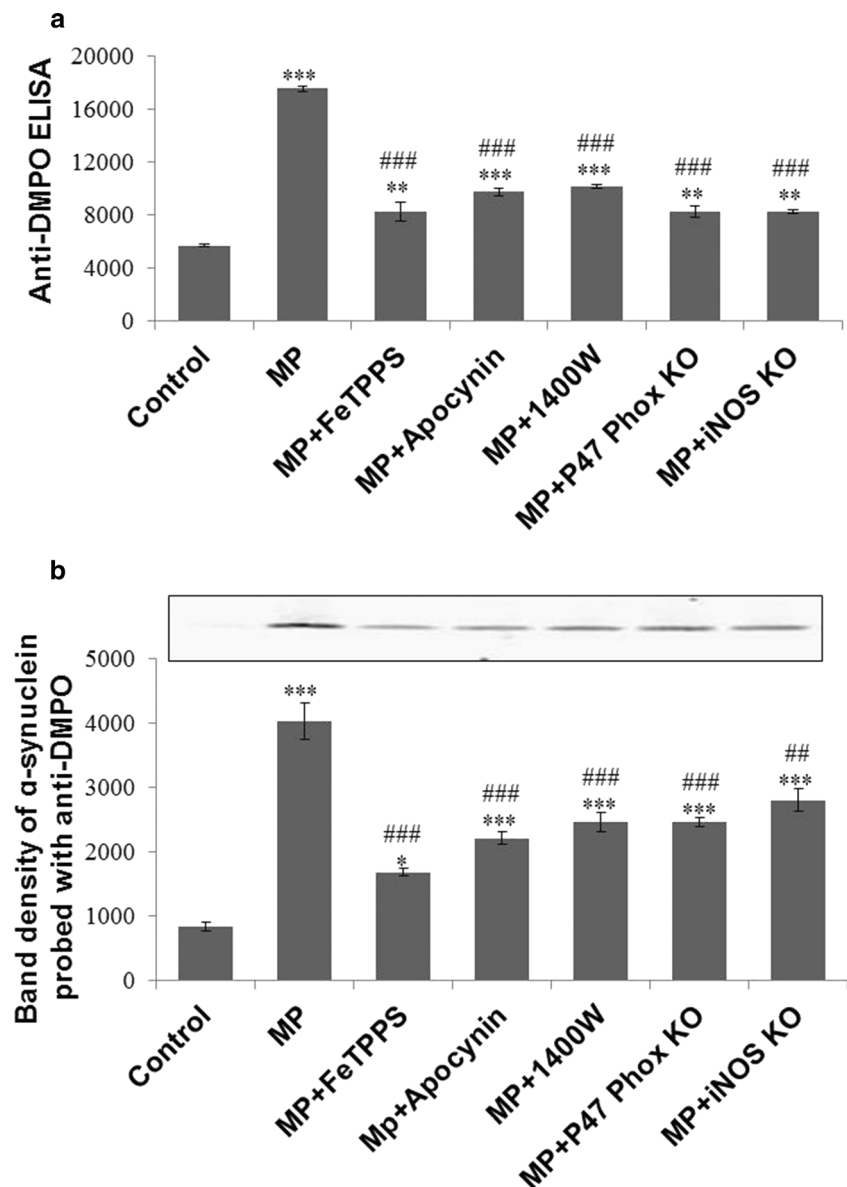
Maneb- and paraquat-coexposed animals (Fig. 4b). Furthermore, Maneb and paraquat coexposure also induced iNOS (Fig. 4c), which produces a significant amount of nitric oxide that can react with superoxide to form peroxynitrite. Being highly diffusible, peroxynitrite can easily diffuse into neurons in the immediate vicinity and may decompose to highly reactive radicals that can form protein-centered radicals.

Alpha-Synuclein Radical Formed by a Dual Role of NADPH Oxidase and Inducible Nitric Oxide Synthase in Maneb- and Paraquat-Coexposed Mice is Peroxynitrite-Mediated

To investigate the possible mechanism of alpha-synuclein radical formation as well as protein radical formation in general, animals received several pretreatments in conjunction with

Maneb and paraquat coexposure. Anti-DMPO ELISA showed increased protein radical formation in Maneb- and paraquat-coexposed animals, which was attenuated significantly in animals pretreated with a peroxynitrite decomposition catalyst (FeTPPS), an iNOS inhibitor (1400W), or an NADPH oxidase inhibitor (Apocynin) (Fig. 5a). Furthermore, mice lacking P47 phox (NADPH oxidase) and iNOS were used to confirm their role in Maneb- and paraquat-induced protein radical formation in the nigrostriatal region of the brain. In Maneb- and paraquat-coexposed knockout mice (P47 phox^{-/-} and iNOS^{-/-}), protein radical formation was significantly diminished compared to their wild-type littermates, as evident from anti-DMPO ELISA (Fig. 5a). Taken together, these results indicate the essential role of NADPH oxidase- and iNOS-mediated peroxynitrite in protein radical formation. To specifically quantify protein-centered radicals on alpha-synuclein,

Fig. 5 Effect of iNOS and NADPH oxidase inhibition/genetic ablation on Maneb- and paraquat-induced protein radical formation in the nigrostriatal tissue of mice after 6 weeks of Maneb and paraquat coexposure. **a** Anti-DMPO ELISA showing protein radical formation in Maneb- and paraquat (MP)-coexposed mice with or without pretreatment with FeTPPS (10 mg/kg, i.p.), apocynin (10 mg/kg, i.p.), or 1400W (15 mg/kg, i.p.), and in P47 phox or iNOS knockout mice. **b** Anti-DMPO Western blotting of immunoprecipitated alpha-synuclein from the nigrostriatal tissue of all experimental groups as in **a**. Data show mean values \pm SEM from four independent experiments ($n=4$). * $P<0.05$, ** $P<0.01$, *** $P<0.001$, with respect to control, and ### $P<0.01$, #### $P<0.001$, with respect to the Maneb- and paraquat-coexposed group



we immunoprecipitated alpha-synuclein from samples of all experimental groups. Band densitometry of individual bands obtained from samples of all experimental groups showed a consistent similarity with anti-DMPO ELISA (Fig. 5b). This concurrence between the alpha-synuclein radical and total protein radical, which was significantly attenuated by iNOS/NADPH oxidase inhibition/ablation as well as by a peroxynitrite decomposition catalyst, clearly indicates that NADPH oxidase and iNOS play a crucial role in peroxynitrite-mediated protein radical formation in general.

TH Immunoreactivity

To investigate the possible link between protein radicals and dopaminergic neuronal death, tyrosine hydroxylase immunoreactivity was investigated. The significant reductions in tyrosine hydroxylase immunoreactivity and the number of TH-positive neurons (Fig. 6a–c) were observed in the substantia nigra region of the brains of exposed mice after 6 weeks of exposure. Pretreatment with apocynin, 1400 W, or FeTPPS conferred significant neuroprotection to Maneb- and paraquat-coexposed mice. As observed with protein radical formation, genetic ablation of p47 phox or iNOS conferred neuroprotection with diminished neuronal death induced by Maneb and paraquat coexposure (Fig. 6). This concurrence between neuronal death and protein radical formation indicates a possible role of protein radicals in the neuronal death process.

Discussion

There has been considerable progress in understanding the disease process of PD, which has led to effective symptomatic treatment of its motor symptoms, initially with levodopa (L-DOPA) and then later with dopamine receptor agonists and deep brain stimulation. Yet, the efficacy of symptomatic therapy wanes after a few years, and patients develop worsened motor impairments and postural anomalies [33]. Most attention is currently focused on developing disease-modifying treatments. But, slowing or halting the underlying neurodegenerative process required an even better understanding of the mechanism of disease pathophysiology, tested in environmentally relevant animal models such as the Maneb- and paraquat-induced PD phenotype in coexposed mice.

Although various models of PD as well as autopsy reports show alpha-synuclein aggregates forming in the substantia nigra, the role that protein free radicals play in their formation in vivo is unknown, as is the more general question of the role of protein radicals in the context of PD [34, 24, 35, 2]. Knowing the central role of alpha-synuclein in PD pathogenesis, we have shown for the first time that alpha-synuclein radical is formed in the midbrain of Maneb- and paraquat-

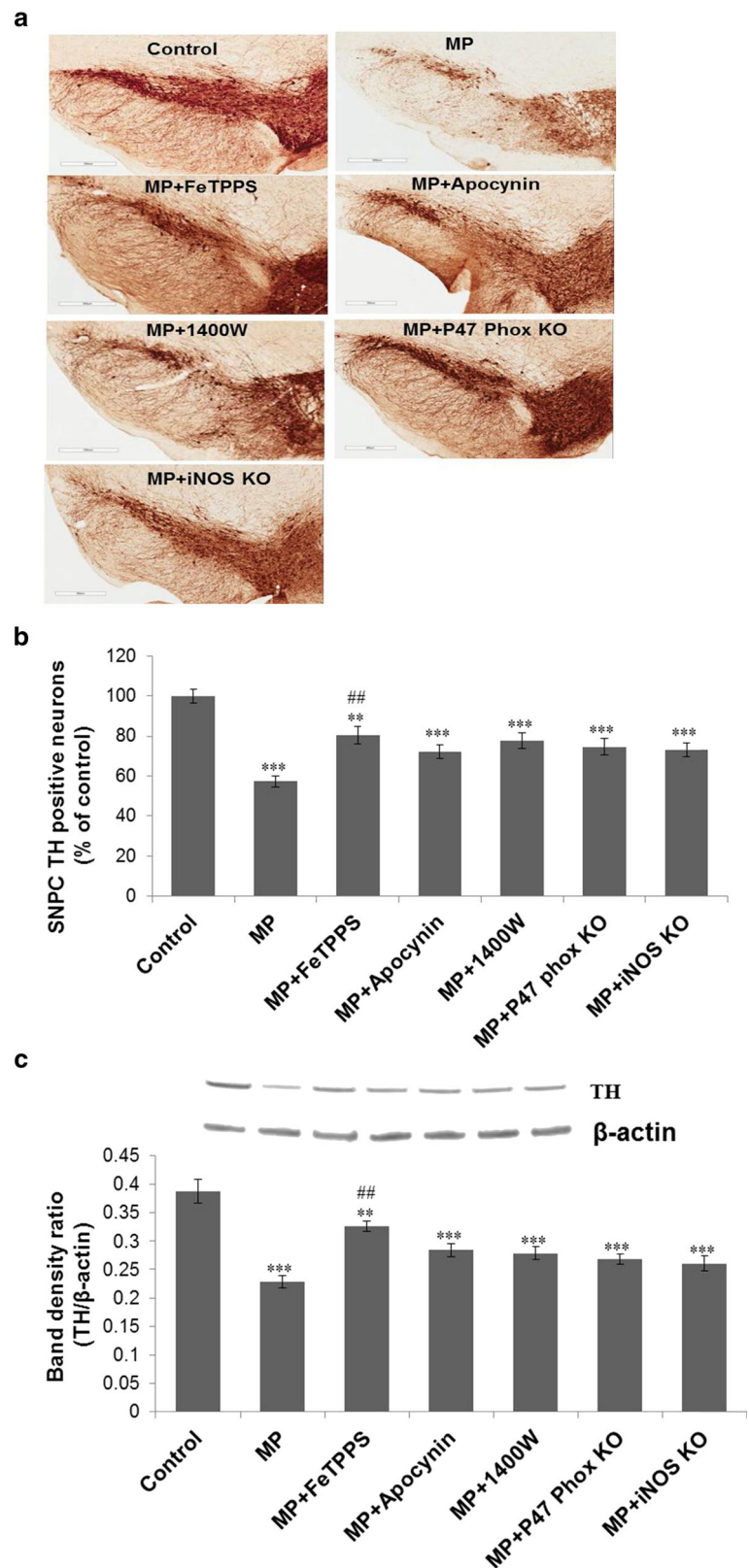
coexposed mice, which might be the key initial step that dictates its aggregation. This study also elucidates the roles of NADPH oxidase and iNOS, which play crucial roles in peroxynitrite-mediated protein radical formation in the dopaminergic neurons of Maneb- and paraquat-coexposed mice. The detection of protein radicals in dopaminergic neurons can be used to explore the possible implications of protein radicals, especially as the initiating mechanism for protein aggregation, which has implications in neurodegenerative diseases.

Aside from our report of protein radical formation in LPS-challenged BV2 cells [2], the only report showing involvement of protein radicals in a neuronal context shows mitochondrial protein radical adducts in an experimental model of amyotrophic lateral sclerosis [29]. With these two reports as proof of concept, the original goal of this study was to determine whether we could detect protein-radical formation in an animal model of PD to establish its involvement in the disease process. Since the presence of DMPO, a nitron spin trap, is a prerequisite to detecting nitron adducts using an anti-DMPO antibody, its entry into the brain after intraperitoneal injections was assessed with HPLC. Using known distributions of DMPO in different organs such as the liver, heart, and blood after intraperitoneal injections [36] and its partition coefficient, which indicates its low lipophilicity [37], we measured its presence in nigrostriatal tissue after intraperitoneal injections. Despite its low lipophilicity, a detectable amount of DMPO enters the brain (Fig. 1).

DMPO can permeate throughout the plasma membrane, enter into dopaminergic neurons and adjacent cells, bind to protein radicals, and enable their detection by immuno-spin trapping as evidenced by an intense anti-DMPO signal in midbrain slices of Maneb- and paraquat-coexposed mice (Fig. 2). The results obtained through this study not only confirm protein radical formation, but also identify for the first time a particular protein radical involved in PD pathogenesis (Fig. 3a). A combinatorial approach using immuno-spin trapping in conjunction with immunoprecipitation allowed us to ascertain that alpha-synuclein was adducted to DMPO (Fig. 3a) within the dopaminergic neurons of Maneb- and paraquat-coexposed mice. Parallel immunoprecipitation experiments using anti-alpha synuclein and anti-DMPO in conjunction with Western blotting showed a protein band that both immunoreacted to alpha-synuclein and coimmunoprecipitated with DMPO (Fig. 3b).

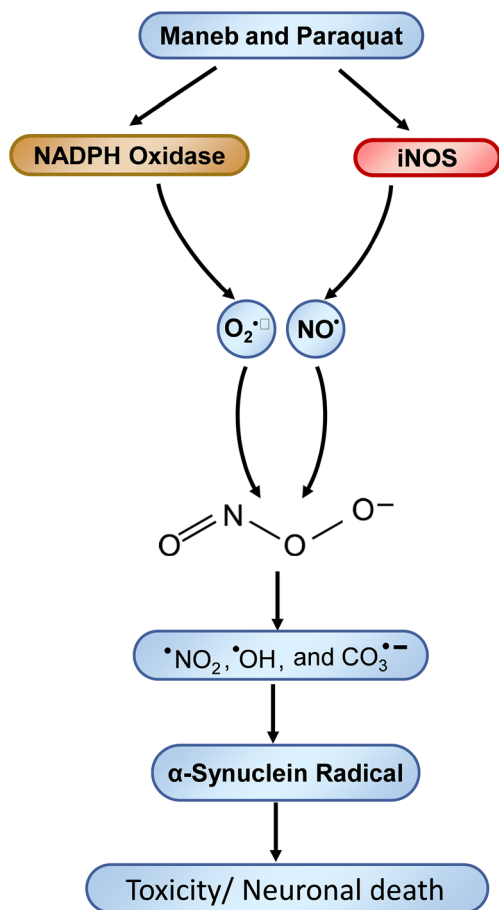
Whenever there is some disturbance in the brain's microenvironment, microglia infiltrate that region and become activated, which is called microgliosis. Microgliosis involves NADPH oxidase activation and iNOS induction (Fig. 4a–c), which leads to simultaneous production of superoxide anion and nitric oxide and, thereby, formation of peroxynitrite. Peroxynitrite, being a highly diffusible oxidant, diffuses through the plasma membrane and enters adjacent neurons [38]. Peroxynitrite can then decompose to form the reactive free radicals $\cdot\text{OH}$, $\text{CO}_3^{\cdot-}$, and $\cdot\text{NO}_2$,

Fig. 6 Tyrosine hydroxylase (TH) immunoreactivity in the substantia nigra of mouse brains following 6 weeks of Maneb and paraquat (MP) coexposure. **a** *Upper panel* shows TH immunoreactivity in frozen brain sections of control and treated animals with or without pretreatment with FeTPPS (10 mg/kg, i.p.), apocynin (10 mg/kg, i.p.), or 1400W (15 mg/kg, i.p.), and in P47 phox or iNOS knockout mice. **b** *Lower panel* shows the number of TH positive neurons in the substantia nigra pars compacta (SNPC) region of all experimental groups as in **a**. **c** Western blot analysis of TH protein expression in the nigrostriatal tissue of mouse brains following 6 weeks of Maneb and paraquat coexposure in the presence and absence of pretreatments as indicated in the graph. The *upper panel* depicts a representative blot of TH protein; the *lower panel* depicts a densitometric analysis of the same with β -actin as the reference. The data are expressed as mean \pm SEM ($n=3-5$). $**P<0.01$, $***P<0.001$ as compared with control and $##P<0.01$, as compared with the Maneb- and paraquat-coexposed group



which can induce protein radical formation within dopaminergic neurons as well as in adjacent cells (Scheme 1) [2]. Peroxynitrite can inhibit glutathione reductase and Cu, Zn, and Mn superoxide

dismutase enzymes [39, 40], which may further favor peroxynitrite formation due to the sustained availability of superoxide in the ambient cellular environment. This sustained



Scheme 1. Role of NADPH oxidase and iNOS in Maneb- and paraquat-induced peroxynitrite-mediated protein radical formation

peroxynitrite production may lead to increased protein radical formation and accumulation of protein aggregates, which may initiate or accelerate the process of neurodegeneration.

Inhibition or genetic ablation of NADPH oxidase/iNOS confers diminished total protein radical formation (Fig. 5a) as well as the formation of the alpha-synuclein radical (Fig. 5b). This decrease in alpha-synuclein radical formation when NADPH oxidase is inhibited or ablated concurs with reports that show that NADPH oxidase modulates alpha-synuclein aggregation in dopaminergic neurons [41]. Alpha-synuclein radical formation appears to be the initiating mechanism, which dictates its aggregation where NADPH oxidase and iNOS play crucial roles. These results, along with diminished protein radical formation in the presence of the peroxynitrite decomposition catalyst FeTPPS, demonstrate that protein radical formation is peroxynitrite-mediated and depends on both NADPH oxidase and iNOS. Experimental evidence from human and animal studies supports the theory that oxidative stress contributes to the pathogenesis of PD [42]. Tyrosine hydroxylase inactivation by peroxynitrite-mediated tyrosine nitration followed by impaired dopamine biosynthesis in PC12 cells indicates the role of posttranslational protein modification in PD [43]. This prompted us to

investigate a possible link between peroxynitrite-mediated protein radical formation and neuronal death through toxic aggregation of proteins like alpha-synuclein.

The loss of TH positive neurons and overall decrease in TH protein levels, which actually indicates dopaminergic neuronal death in the Maneb- and paraquat-coexposed model of PD (Fig. 6a–c), is in accordance with several reports so far [12, 44]. Additionally, pretreatment of Maneb- and paraquat-coexposed animals with the peroxynitrite decomposition catalyst FeTPPS or the inhibition/ablation of NADPH oxidase/iNOS conferred neuroprotection, further supporting our interpretation that peroxynitrite-mediated protein radical formation, which triggers protein aggregation, plays a causal role in neuronal death. The crucial implications of peroxynitrite-mediated protein radical formation in this study are supported by a study of methamphetamine-induced toxicity in which dopaminergic neurotoxicity was prevented by targeting peroxynitrite using a peroxynitrite decomposition catalyst [45].

In summary, this study provides evidence that NADPH oxidase and iNOS play roles in peroxynitrite-mediated alpha-synuclein radical formation, which might be the initiating mechanism for its aggregation. The detection of protein radicals and understanding the underlying mechanism of protein radical formation can be used to explore the possible implications of protein radicals specifically as the initiating mechanism for protein aggregation, which has implications for neurodegenerative diseases.

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Compliance with Ethical Standards Authors declare compliance with ethical standards.

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