The Herbicide Paraquat Causes Up-regulation and Aggregation of α-Synuclein in Mice

PARAQUAT AND α-SYNUCLEIN*

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α-Synuclein-containing aggregates represent a feature of a variety of neurodegenerative disorders, including Parkinson’s disease (PD). However, mechanisms that promote intraneuronal α-synuclein assembly remain poorly understood. Because pesticides, particularly the herbicide paraquat, have been suggested to play a role as PD risk factors, the hypothesis that interactions between α-synuclein and these environmental agents may contribute to aggregate formation was tested in this study. Paraquat markedly accelerated the in vitro rate of α-synuclein fibril formation in a dose-dependent fashion. When mice were exposed to the herbicide, brain levels of α-synuclein were significantly increased. This up-regulation followed a consistent pattern, with higher α-synuclein at 2 days after each of three weekly paraquat injections and with protein levels returning to control values by day 7 post-treatment. Paraquat exposure was also accompanied by aggregate formation. Thioflavine S-positive structures accumulated within neurons of the substantia nigra pars compacta, and dual labeling and confocal imaging confirmed that these aggregates contained α-synuclein. The results suggest that up-regulation of α-synuclein as a consequence of toxicant insult and direct interactions between the protein and environmental agents are potential mechanisms leading to α-synuclein pathology in neurodegenerative disorders.

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The abbreviations used are: PD, Parkinson’s disease; Syn-1, anti-α-synuclein; PFA, paraformaldehyde; M.O.M., Mouse On Mouse Monoclonal; TH, tyrosine hydroxylase; ANOVA, analysis of variance; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

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In Vitro α-Synuclein Fibril Formation—Purified recombinant α-synuclein (10) solutions (140 μM, in 20 mM sodium phosphate buffer, pH 7.5) were incubated at 37 °C in a fluorescence plate reader (Fluoroskan Ascent) with shaking at 120 rpm with a 7-mm orbit. Thioflavine T fluorescence was used to monitor fibril formation (16). A volume of 135 μl of the mixture containing 10 μM thioflavine T was pipetted into a well of a 96-well plate (white plastic, clear bottom), and a one-eighth-inch diameter Teflon sphere (McMaster-Carr) was added. Each sample was replicated five times, and data from replicate wells were averaged before plotting fluorescence versus time. The fluorescence was measured at 30-min intervals (excitation at 450 nm and emission at 485 nm), with a sampling time of 40 ms. To examine the concentration-dependant effects of paraquat on the kinetics of fibril formation, the herbicide (Sigma) was co-incubated with α-synuclein. The kinetics of α-synuclein fibrillation are sigmoidal, defined by an initial lag phase, a subsequent growth phase in which the thioflavine T fluorescence increases, and a final equilibrium phase, when thioflavine T fluorescence reaches a plateau indicating the end of fibril formation. Thioflavine T fluorescence measurements were plotted as a function of time and fitted to a sigmoidal curve using the empirical approach described previously (17).
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sen Laboratories) received intraperitoneal injections of either saline or 10 mg/kg paraquat dichloride hydrate (dissolved in saline) once a week for 3 consecutive weeks. Animals were killed by cervical dislocation at days 2 and 7 following each injection. Experimental protocols were in accordance with the National Institutes of Health guidelines for use of live animals and were approved by the Animal Care and Use Committee at The Parkinson’s Institute.

**Immunoblotting**—Brain samples from ventral mesencephalon and frontal cortex were dissected on ice. The former brain region was chosen since it encompasses the substantia nigra, which is the primary site of PD pathology. Cerebral cortex (including frontal cortex) is one of the areas where inclusions are commonly observed in patients (18). Tissues were sonicated in lysis buffer with protease inhibitors, and after centrifugation, the supernatant fraction was decanted. Protein extracts (5 μg) were processed for SDS-polyacrylamide electrophoresis on 12% Tris-glycine gels (Novex) and transferred to nitrocellulose. Blots were blocked in 5% nonfat milk in 25 mM Tris-saline solution for 1 h at room temperature and incubated for 2 h with either 1:3,000 anti-α-synuclein (Syn-1; Transduction Laboratories), 1:1,000 anti-β-tubulin (Sigma), 1:1,600 anti-synaptophysin (Dako) or mouse IgG (to ensure specificity; Dako). Secondary anti-mouse IgG conjugated to horseradish peroxidase (1:6,000; Pierce) was applied for 1.5 h, and following rinsing, blots were incubated with a chemiluminescent substrate and exposed to Hyperfilm ECL (Amersham Biosciences, Inc.). Optical densities were determined using the ImageQuant program (Molecular Dynamics) and expressed as arbitrary units.

**Histochemistry**—Forebrain and midbrain blocks were immersion-fixed in 4% paraformaldehyde (PFA) and cryoprotected in sucrose. Serial coronal sections (40 μm) were cut on a cryostat, collected in cryopreservation, and stored. For α-synuclein immunostaining, midbrain sections were incubated initially in the Mouse On Mouse Monoclonal (M.O.M.) blocking reagent (Vector Laboratories) overnight at 4°C and then in Syn-1 antibody diluted 1:250 with M.O.M. protein solution. In some experiments, sections from control and paraquat-treated mice were stained with a second antibody against α-synuclein (1:150; Chemicon) that verified a comparable pattern of expression and distribution of the protein. After rinsing, sections were immersed in biotin-anti-mouse IgG secondary antibody for 1 h, treated with 0.3% H₂O₂ solution to quench endogenous peroxidase activity, and then incubated in avidin-biotin peroxidase complex (30 min). Visualization was performed using 0.03% 3,3'-diaminobenzidine for 3 min. Sections were mounted on gelatin-coated slides, counterstained in 0.5% Cresyl violet, dehydrated, and coverslipped. All steps were performed at room temperature unless otherwise indicated. For thioflavine S histochemistry, 48-h PFA-fixed tissue sections from frontal cortex and midbrain were stained in hematoxylin (2 min) and, following distilled water washes, in 0.25% thioflavine S (10 min). Sections were then immersed in 80% ethanol, rinsed, and mounted using a 50% glycerol solution. Dual label thioflavine S staining with either α-synuclein or tyrosine hydroxylase (TH) immunohistochemistry required midbrain blocks to be quick-frozen in isopentane on dry ice. Cryostat-cut sections (20 μm) were post-fixed in 4% PFA for 10 min at room temperature, blocked with 4% normal donkey serum for 1 h, and then incubated with the Syn-1 (1:100) or TH (1:100; Chemicon) antibody. Slides were covered with donkey anti-mouse secondary IgG conjugated to fluorescein (1:100; Chemicon) for 1.5 h, washed briefly, and incubated in 0.1% thioflavine S for 10 min. Slides were exposed to 80% ethanol solution for 5 min, rinsed, and coverslipped using 50% glycerol solution. Positive staining was observed using a Leica NT confocal microscope equipped for epifluorescence. In all immunohistochemical experiments, control sections were incubated with mouse IgG in lieu of the primary antibody.

**Statistical Analysis**—Differences among means were analyzed using one-way ANOVA. Newman-Keuls post-hoc analysis was employed when differences were observed in ANOVA testing (*p* < 0.05).

**RESULTS**

In Vitro Effects of Paraquat on α-Synuclein Fibrillation—

Concentrations of paraquat in the micromolar range significantly accelerated the rate of fibril formation of α-synuclein in a dose-dependent manner. As shown in Fig. 1A, increasing concentrations of paraquat led to decreased lag times and increased growth rates of fibril formation. The time to half-maximum fibrils (*t₅₀*) decreased in a log-linear fashion as the paraquat concentration increased (Fig. 1B). Similarly, the apparent first-order rate constant for fibril growth showed a linear increase with increasing paraquat concentration (Fig. 1C). Furthermore, if the paraquat concentration was fixed at 100 μM and the α-synuclein concentration varied, the accelerating effect of paraquat increased with increasing α-synuclein levels (data not shown).

**Paraquat-induced α-Synuclein Up-regulation in Mice**—

Interactions between α-synuclein and paraquat in vitro prompted us to test whether exposure to the herbicide affected α-synuclein levels and aggregation in the mouse model. Animals were treated with a weekly injection of 10 mg/kg paraquat for 3 consecutive weeks, and α-synuclein protein levels were assayed by Western immunoblot analysis in the ventral mesencephalon and frontal cortex. Denser α-synuclein-positive bands (at 19 kDa) were consistently observed in paraquat-treated versus saline-injected mice at 2 days post-treatment. This up-regulation occurred in both brain regions and contrasted with the lack of increase in the levels of β-tubulin (Fig. 2, A–C) and synaptophysin (data not shown) proteins. Interestingly, α-synuclein returned to basal values by 7 days after each herbicide treatment (see Fig. 2C for data at 7 days after the first injection). The effect of paraquat on α-synuclein tissue levels was also assessed by immunohistochemistry. Coronal sections from control and exposed mice were stained with the Syn-1 antibody 2 days following the third injection of either saline or paraquat. Microscopic examination showed enhanced immunoreactivity in the midbrain of paraquat-treated animals (Fig. 2, D and E), and at higher magnification of the substantia nigra pars compacta, a robust α-synuclein staining was seen in both the cytosolic and nuclei of neurons exposed to the herbicide (Fig. 2, F and G).

Paraquat-induced α-Synuclein Aggregation in Mice—

As suggested by the in vitro findings, α-synuclein-paraquat interactions may ultimately promote protein conformational changes and subsequent aggregation. To test this hypothesis in vivo, brain sections from control and paraquat-treated mice were stained with thioflavine S, a dye known to bind to amyloid fibrils (19). Thioflavine S staining was minimal in animals injected with saline, but pronounced at 2 days after the last paraquat administration in both midbrain and frontal cortex. Confocal microscopy showed neurons in the substantia nigra pars compacta in which thioflavine S-positive deposits were present in the cytosolic and nuclear regions (Fig. 3E). That
While the role of α-synuclein in PD and related disorders (recently referred to as “synucleinopathies”) is widely recognized, mechanisms involved in α-synuclein pathology remain elusive. In this study, interactions between α-synuclein and paraquat, an environmental agent implicated as a PD risk factor (12–15), were assessed both in vitro and in vivo as potential mechanisms leading to increased protein aggregation. In vitro findings extended previous observations (11) and demonstrated that paraquat can directly stimulate α-synuclein fibrillation. This effect was dependent upon both α-synuclein and paraquat concentrations. Quite remarkably, when mice were treated with the herbicide, α-synuclein levels were significantly enhanced in both substantia nigra and frontal cortex, and this up-regulation was accompanied by the formation of intraneuronal aggregates with histological properties of amyloid fibrils (i.e. thioflavine S staining) (19, 20). Co-localization of α-synuclein within these aggregates provided further evidence that, as predicted by the in vitro data, paraquat exposure triggers α-synuclein fibrillation in the mouse brain. Taken together, these results support the hypothesis that interactions between α-synuclein and environmental agents may contribute to neurodegenerative processes and, in particular, to the development of intraneuronal aggregates. The analogies between the in vitro and in vivo findings also provide a rationale for using both approaches for the screening of putative neurotoxicants, which, by affecting α-synuclein conformation and aggregation, may play a role in the pathogenesis of synucleinopathies.

The pattern of paraquat-induced α-synuclein up-regulation, as described here, suggests that increased expression of this protein is part of a neuronal response to toxic insults. Levels of α-synuclein were consistently enhanced at 2 days after each of three weekly paraquat administrations and returned to basal control values within 7 days post-treatment. Interestingly, a similar time course of α-synuclein up-regulation has recently been reported by Vila and colleagues following administration of mice with the parkinsonism-inducing neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (21). The significance and consequences of this α-synuclein response remain unknown. If one assumes a harmful role of α-synuclein, then its up-regulation would be expected to contribute to neuronal injury (22–24). On the other hand, work with cell cultures supports the hypothesis that overexpression of α-synuclein may actually delay cell death caused by toxic agents and protect against apoptotic stimuli (25, 26). If so, increased levels of the protein triggered by paraquat, MPTP, or other neurotoxicants may limit their damaging action. A third possibility may be consistent with both a protective and deleterious role of α-synuclein. The initial response may indeed represent an attempt by neurons to counteract and to limit injury. However, should the insult be particularly severe or persistent, or should the toxic agent be capable of interacting with α-synuclein (see below), protein up-regulation may ultimately result in pathological effects.

It could be argued that increased α-synuclein concentration alone may be sufficient to cause the protein aggregation observed in the brains of paraquat-exposed mice. However, results from other in vivo models indicate that higher levels of the protein are not necessarily associated with the development of thioflavine S-positive structures or α-synuclein-immunoreactive intraneuronal aggregates (21, 27). Therefore, it is most likely that, consistent with the in vitro work, α-synuclein assembly in our paraquat mouse model is a consequence of both protein up-regulation and α-synuclein-paraquat interactions. It is also possible that oxidative processes triggered by para-
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Paraquat administration may contribute to the development of α-synuclein pathology. Paraquat toxicity is known to involve the generation of free radicals via redox cycling reactions that transfer electrons to molecular oxygen (28, 29). This oxidative stress could facilitate α-synuclein association by altering the biophysical properties of the protein and/or impairing mechanisms of protein degradation within the neurons (30–32).

Several lines of evidence indicate that paraquat exposure results in neurotoxicity. Paraquat is accumulated into the brain through the blood-brain barrier, possibly via a carrier-mediated mechanism (33–35), and is capable of damaging nigrostriatal neurons when administered to mice either alone or in synergistic combination with other toxicants (36–38). Since protein deposition has been hypothesized to play a role not only in the pathogenesis of inclusions, but also in neuronal injury and degeneration (6, 32), it is conceivable that changes in α-synuclein assembly may ultimately contribute to paraquat neurotoxicity. Therefore, the animal model described in this study has the potential to provide critical insight into the relationship between α-synuclein aggregation and neurodegeneration and into mechanisms that may link these two critical features of neurodegenerative diseases.

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