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Silica nanoparticles induce alpha-synuclein induction and aggregation in PC12-cells

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ABSTRACT

Silica nanoparticles (SiO_2-NPs) are widely applied in diagnosis, imaging, and drug delivery of central nervous diseases. Previously, we found that SiO_2-NPs enter the brain and, more specifically, the dopaminergic neurons in the striatum. Whether SiO_2-NPs have neurotoxicity and contribute to development of Parkinson's disease (PD) remains unclear. In this study, we investigated the effect of SiO_2-NPs on PC12 cells, a dopaminergic neuron-like cell line. We showed that SiO_2-NPs up-regulated α-synuclein expression, and N-acetyl cysteine reduced α-synuclein. SiO_2-NPs inhibited 20S proteasome activity and decreased ubiquitin, Parkin, and ubiquitin carboxy-terminal hydrolase L1 (UCHL1) protein levels in the ubiquitin-proteasome system (UPS). SiO_2-NPs induced autophagy as shown by transmission electron microscopy, and elevated LC3-II and Beclin 1 levels in PC12 cells. SiO_2-NPs inhibited phosphorylation of PI3K, Akt, mTOR, and P70S6. These data suggest that SiO_2-NPs induce oxidative stress and α-synuclein aggregation by inhibiting the UPS. SiO_2-NPs also induce autophagy through inhibiting PI3K-Akt-mTOR signaling, which is known to negatively regulate autophagy. Amyloid aggregates of α-synuclein in dopaminergic neurons of the midbrain are considered the hallmark of PD. Our findings indicate that SiO_2-NPs exposure induces neurotoxicity and may be a significant risk factor for the development of PD.

1. Introduction

Silica nanoparticles (SiO_2-NPs) have excellent physicochemical properties and have extensive applications in central nervous system research, including targeted drug delivery, gene therapy, and molecular imaging [1]. However, such wide applications have also posed health concerns. Both *in vitro* and *in vivo* studies have shown that SiO_2-NPs can exert adverse effects in the brain [2–4]. SiO_2-NPs and silver nanoparticles can induce deposition of amyloid-β (Aβ) plaque and promote phosphorylation of tau protein in SK-N-SH and neuro-2a cells, which imply potential risks of nanoparticle application in Alzheimer's disease (AD) [5,6]. A recent study from our team demonstrated that SiO_2-NPs could translocate to brain tissues *via* the olfactory bulb and deposit specifically in the striatum, further triggering oxidative damage and apoptosis in dopaminergic neurons [4]. However, the effects of SiO_2-NPs exposure in PD development and the underlying mechanisms remain unclear.

PD pathology is characterized by the progressive degeneration and loss of dopaminergic neurons in the substantia nigra and the formation of Lewy bodies in neurons [7]. Alpha-synuclein is the major component of Lewy bodies, and α-synuclein mutations contribute to early-onset PD [7]. However, the effect of SiO_2-NPs exposure on α-synuclein aggregation remains unknown. Oxidative stress is considered an important pathway in initiating or promoting α-synuclein misfolding and aggregation [7]. In addition, oxidative stress plays an important role in inducing nanoparticle-associated injuries, and providing valid criteria for evaluating nanoparticles toxicity [8,9]. Our recent study showed that titanium dioxide nanoparticles (TiO_2-NPs) could impede the dynamic equilibrium between reactive oxygen species (ROS) and anti-oxidative enzyme activity in dopaminergic neurons and attack the cell membrane, inducing neuronal apoptosis and changes in cell cycle [10]. Therefore, we hypothesized that SiO_2-NPs may induce oxidative stress, which causes aggregation of α-synuclein and leads to consequential damage in dopaminergic neurons.

A number of studies have revealed that α-synuclein degradation may be mediated by the ubiquitin-proteasome system (UPS) [11]. Another pathway that may be involved in α-synuclein degradation is autophagy, a process mediating degradation of unnecessary and

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dysfunctional proteins in the lysosomes [12]. Damage to the UPS and autophagy-lysosomal pathway (ALP) is essential in the development and progression of PD [13]. Therefore, we hypothesized that SiO_2-NPs may interrupt the protein degradation function of the UPS and ALP, causing abnormal α-synuclein aggregation.

In this study, we investigated the effects of SiO_2-NPs exposure on oxidative stress, α-synuclein aggregation, the UPS, and the ALP in PC12 cells, a dopaminergic neuron-like cell line. We found that SiO_2-NPs induced oxidative stress and α-synuclein aggregation through inhibiting the UPS. In addition, SiO_2-NPs induced autophagy by inhibiting the PI3K-Akt-mTOR signaling pathway, which is known to negatively regulate autophagy development. Our findings elucidate the effects and molecular mechanisms of SiO_2-NPs exposure in the onset of PD. Specifically, SiO_2-NPs exposure involves impairment of α-synuclein degradation pathways, the UPS and autophagy.

2. Materials and methods

2.1. Reagents

Nerve growth factor (NGF)-2.5S, penicillin, streptomycin, N-acetyl cysteine (NAC), and anti-LC3 were purchased from Sigma Chemical Company (Saint Louis, MO, USA). High glucose Dulbecco's modified Eagle medium (DMEM), horse serum (HS) and fetal bovine serum (FBS) were purchased from Gibco-Invitrogen Company (Paisley, UK). RIPA lysis buffer was purchased from Beyotime Biotech (Nantong, China). Alpha-synuclein and red fluorescent Alexa Fluor 594 conjugated secondary antibodies were purchased from Abcam Company (Cambridge, UK). The anti-ubiquitin, anti-Parkin, anti-ubiquitin carboxy-terminal hydrolase L1 (UCHL1), Beclin 1, anti-phosphorylated-mTOR (p-mTOR), anti-mTOR, anti-phosphorylated-PI3K (p-PI3K), anti-PI3K, anti-phosphorylated-Akt (p-Akt), anti-Akt, anti-phosphorylated-P70S6 (p-P70S6), anti-P70S6, anti-β-actin, and the horseradish peroxidase-conjugated anti-rabbit secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Characterization of SiO_2-NPs

SiO_2-NPs were purchased from the Sigma Chemical Company (Saint Louis, MO, USA). The average size of the nanoparticles was confirmed by transmission electron microscopy (TEM, JEM-2010, JEOL Ltd., Tokyo, Japan). Nanoparticle size, agglomerates, and charge measurements in high glucose DMEM including 10% FBS were determined with dynamic light scattering (DLS) and zeta potential measurements using ZetaSizer (Malvern Instruments Ltd., Worcestershire, UK) at a concentration of 1 mg/mL. In order to improve the dispersion of nanoparticles in these fluids, the nanoparticle suspension was sonicated with a Hilscher UP200S (Hilscher Ultrasonics GmbH, Teltow, Germany), which generates ultrasonic pulses of 600 W at 20 kHz for 30 min. All analyzed measurements were obtained for at least three different spots or three times to provide consistent data.

2.3. Cell culture and SiO_2-NPs treatment

The PC12 cell line, which is derived from a rat adrenal medulla pheochromocytoma, was purchased from the cell bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. PC12 differentiation was induced by NGF. Differentiated cells displayed typical dopaminergic neuron characteristics with regards to form and function. PC12 cells were plated onto 6-well plates coated with poly-D-lysine/laminin at a density of 5.0×10^5 cells/well. Twenty-four hours after plating, the medium was replaced with high glucose DMEM containing 10% FBS, 100 U/ml penicillin, 100 μg/mL streptomycin and NGF (50 ng/mL) at 37 °C in an atmosphere of 5% CO_2. The culture medium was refreshed every two days. Cell morphology and neurite outgrowth was monitored every day under an inverted microscope (Olympus, Japan). After 7 days, the PC12 cells changed into neuron-like cells which had long neurite and contacted each other like a net. Mycoplasma was always negative during the cell culture. Then cells were plated into 75 cm^2 tissue culture flasks (Corning, US) and cultured overnight before SiO_2-NPs treatment.

SiO_2-NPs were sterilized by heating to 120 °C for 2 h and suspended in culture medium at concentrations of 25, 50, 100, and 200 μg/mL. Suspensions were sonicated for at least 30 min (600 W; 20 kHz) to generate a homogenous suspension right before use. After cells attached in the completed medium for 24 h, freshly dispersed serial dilutions of SiO_2-NPs suspensions were immediately applied to the cells and incubated for 24 h. Cells that received medium that did not contain SiO_2-NPs were used as negative controls.

To investigate the role of oxidative stress, we added NAC (20 mM, a well known antioxidant), and SiO_2-NPs (200 μg/mL) to the culture medium to compare the influence of SiO_2-NPs with or without NAC on α-synuclein expression in PC12 cells.

2.4. Western blotting

After treatment, cells from each group were washed twice with ice-cold phosphate buffered saline (PBS) and collected with 0.25% trypsin. After centrifugation at 1500 rpm for 5 min, the cell pellet was resuspended in RIPA lysis buffer and placed on ice for 30 min. The supernatant was collected after the lysate was centrifuged at 10,000*g* for 15 min at 4 °C and stored at −80 °C or for subsequent assays.

Protein concentrations were determined using the BCA method (Pierce, Rockford, IL, USA). A total of 30–40 μg proteins were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, U.S.). Membranes were blocked in a dilute solution of 5% non-fat dry milk for 1 h, and then incubated with a Tris-buffered saline and Tween 20 (TBST) solution containing desired primary antibodies (1:1000, antibodies targeting α-synuclein, β-actin, Parkin, UCHL1, LC3, Beclin1, p-PI3K, PI3K, p-Akt, Akt, p-mTOR, mTOR, p-P70S6, or P70S6) at 4 °C overnight. After rinsing the membranes with TBST three times, the membranes were incubated with respective secondary antibodies under gentle agitation for 1 h. After washing three times with TBST, an ECL chemiluminescence reagent (Millipore, USA) was applied to the membrane.

2.5. 20S proteasome activity assay

20S proteasome activity was measured using a kit (Millipore, U.S.), which detected the content of representative chymotrypsin according to the manufacturer's protocol. Briefly, the cells were collected and lysed using RIPA lysis buffer on ice for 30 min. After centrifugation at 10,000*g* 4 °C for 10 min, the supernatants were collected and protein concentrations were quantified using the BCA kit. A total of 30 μg protein was aliquoted and added to the assay buffer, and incubated at 37 °C for 1 h. Fluorescence intensity was measured at 380/460 nm on a fluorescence microplate reader (Thermo Labsystems, Helsinki, Finland).

2.6. Autophagy assay and transmission electron microscopy (TEM)

After treatment, cells were collected and centrifuged at 1500*g*