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The molecular mechanism of rotenone-induced α-synuclein aggregation: Emphasizing the role of the calcium/GSK3β pathway

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HIGHLIGHTS

- Rotenone-induced aggregation of α-synuclein in a concentration-dependent manner.
- Rotenone-induced α-synuclein aggregation was dependent on increased intracellular calcium.
- Rotenone-induced α-synuclein aggregation was associated with GSK3β activity.
- Rotenone-induced increases in intracellular calcium regulated GSK3β activity.

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ABSTRACT

Environmental toxin exposure is associated with the development of Parkinson's disease (PD), and environmental factors can influence the onset of the majority of sporadic PD cases *via* genetically mediated pathways. Rotenone, a widespread pesticide, induces Parkinsonism and the formation of Lewy bodies in animals; however, the molecular mechanism that underlies α-synuclein aggregation remains unclear. Here, we assessed the aggregation of α-synuclein in PC12 cells with or without cross-linking following rotenone exposure *via* a variety of methods, including western blotting, immunofluorescence and electron microscopy. We demonstrated that rotenone increased the intracellular calcium levels and induced the aggregation and phosphorylation of α-synuclein in a calcium-dependent manner. Aggregated α-synuclein is typically degraded by autophagy, and rotenone impaired this process. The attenuation of autophagy and α-synuclein alterations were reversed by scavenging calcium. Calcium regulates the activity of AKT-glycogen synthase kinase 3 (GSK3)β. We demonstrated that rotenone attenuated the phosphorylation of AKT and GSK3β, and the elimination of calcium reversed these phenomena. As a GSK3β inhibitor, lithium promoted autophagy and decreased the aggregation and phosphorylation of α-synuclein. GSK3β activation through overexpression depressed autophagy and increased the total protein level and phosphorylation of α-synuclein. These results suggest that rotenone-induced α-synuclein aggregation is mediated by the calcium/GSK3β signaling pathway.

1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disease that is characterized by the loss of dopamine neurons in the substantia nigra and the presence of abnormal intracellular protein inclusions referred to as Lewy bodies (LBs) (Schulz and Falkenburger, 2004); however, the molecular mechanisms that underlie the death of dopamine neurons and LB formation remain obscure. During the twentieth century, the general incidence of PD increased 1.63-fold, and this increase was most evident in developed countries, especially regions that use agrochemical compounds (Baldi et al., 2003; Freire and Koifman, 2012). Thus, there is strong evidence suggesting that pesticide exposure predisposes an individual to PD (Liew et al., 2014). However, the specific causative agents and the underlying mechanisms are not fully understood.

Abbreviations: PD, Parkinson's disease; LBs, Lewy bodies; mPTP, permeability transition pore; PI3K, phosphatidylinositol 3-kinase; GSK3, glycogen synthase kinase 3; DMSO, dimethyl sulfoxide; BAPTA/AM, 1,2-bis(2-aminophenoxy) ethane-*n,n,n,n*-tetraacetic acid acetoxymethyl ester; PCR, polymerase chain reaction.

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It is widely believed that an interaction between genetic and environmental factors influences susceptibility to PD, and environmental factors that affect PD-based genetic factors can lead to neuronal death (Dardiotis et al., 2013). Of the different pathogenic mechanisms involved in dopaminergic neuron degeneration in PD, the intraneuronal accumulation and aggregation of α-synuclein plays a central role in both genetic and sporadic cases (Maries et al., 2003). α-Synuclein is predominantly expressed in presynaptic terminals throughout the central nervous system, yet its physiological function remains elusive. There is evidence that α-synuclein modifies SNAP receptor (SNARE) complex assembly (Burre et al., 2010), the trafficking of neurotransmitter vesicles (Nemani et al., 2010), and the striatal release of dopamine(Senior et al., 2008). So, the abnormal aggregation of α-synuclein might be associated with several neurodegenerative diseases. A variety of pesticides could trigger *in vitro* α-synuclein accumulation (Chorfa et al., 2014), and multiple pathways that have been proposed to explain the observed neuronal cell death primarily involve α-synuclein aggregation (Lee et al., 2014), thus, α-synuclein aggregation may represent the bridge that connects environmental and genetic factors related to PD, but its molecular mechanism still need further research.

Rotenone is a widespread insecticide that is used worldwide, and rotenone-induced animal models display a parkinsonian-like phenotype with nigrostriatal degeneration and LB formation (Betarbet et al., 2000). Rotenone induced neurodegeneration and death through caspase activation (Sherer et al., 2003) and α-synuclein accumulation in experimental models of PD (Lee and Lee, 2002). However, the relationship between rotenone and α-synuclein aggregation is unclear. In this study, we investigated the signaling pathways involved in rotenone-induced α-synuclein aggregation.

2. Materials and methods

2.1. Cell culture and reagents

PC12 cells were grown in Dulbecco's Modified Eagle Medium (Invitrogen, Gibco, USA) supplemented with 5% heat-inactivated fetal bovine serum (Invitrogen, Gibco, USA) and 5% equine serum (Invitrogen, Gibco, USA). The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. The media were changed every two or three days, and the cells were passaged once or twice per week. Rotenone (Sigma–Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10^{-2} M. For cell or brain tissue exposure, rotenone stock solutions were diluted with PBS to obtain a final solution of 1‰ DMSO. PC12 cells were exposed to rotenone or the corresponding solvent for different times. 1,2-Bis(2-aminophenoxy) ethane-*n*,*n*,*n*,*n*-tetraacetic acid acetoxymethyl ester (BAPTA/AM) was used as a chelator of intracellular calcium. PC12 cells were pretreated with 10 μM BAPTA/AM or 10 mM lithium for 30 min prior to rotenone exposure. The total RNA and protein were extracted from the cells and used for subsequent analysis.

2.2. Animals

Adult male C57BL/6J mice were housed with a standard 12 h light/dark cycle. The animals were provided with food and water. All experiments were performed in accordance with the guidelines established by the National Institutes of Health for the care and use of laboratory animals and were approved by the Animal Care Committee of the Peking Union Medical College and Chinese Academy of Medical Sciences. Efforts were made to minimize animal suffering.

2.3. Intranigral injection of rotenone

The animals were anesthetized with chloral hydrate (400 mg/kg, intraperitoneal (i.p.)) and secured in a stereotaxic apparatus. The skull was exposed and a hole was drilled above the position of the substantia nigra pars compacta at −2.9 mm anterior, −1.3 mm lateral and −4.1 mm ventral to Bregma (Couch et al., 2011). Then, 1 μL of rotenone (10^{-6} mol/L in 1‰ DMSO) was injected using a graduated glass capillary tube (Drummond Scientific Company, Broomall, PA, USA) over 5 min (0.2 μL/min) followed by 2 min of rest to allow diffusion of the injected material prior to needle removal. The same volume of solvent was used as the vehicle in the contralateral nigra. The animals were anaesthetized and euthanized 48 h after exposure.

2.4. Immunofluorescence staining

PC12 cells were grown on coverslips coated with 0.01% poly-L-lysine (Sigma–Aldrich, USA). The cells were pretreated with the inhibitor 10 μM BAPTA/AM for 30 min prior to rotenone exposure. The PC12 cells were fixed in 4% paraformaldehyde for 20 min at room temperature and then washed three times in PBS. The cells were permeabilized in 0.1% Triton-X-100 (Sigma–Aldrich, USA) for 10 min, blocked in 3% goat serum for 1 h at room temperature, and subsequently labeled with an α-synuclein antibody (1:200, Santa Cruz, USA). After washes with PBS, the cells were incubated with an Alexa Fluor 488-conjugated antibody (1:200, Invitrogen) for 1 h at room temperature. Images were acquired with fluorescence laser scanning confocal microscopy (Leica TCS SP2). The confocal data were analyzed using Leica confocal software.

2.5. Real time-polymerase chain reaction (RT-PCR) and plasmid construction

Following rotenone exposure for 48 h, the PC12 cells were washed with PBS and lysed with TRIzol reagent. The total RNA was extracted according to standard methods. The RNA concentration was calculated from the optical density at 260 nm, and the purity was determined by the 260/280 nm absorbance ratio. The total RNA (300 ng) from each sample was analyzed by RT-PCR methods with a gene specific for α-synuclein (forward primer: 5′ ATGGATGTATTCATGAAAGG 3′, reverse primer: 5′ GGCTTCAGGTTCGTAGTCTTG 3′), GSK3β (forward primer: 5′ CTAGCTAGCGCATGTCAGGGCGGCCCAGAACCA 3′, reverse primer: 5′ CCGGATATCCTAGGTGGAGTTGGAAGCTGATG 3′), and β-actin (forward primer: 5′ATGGATGACGATATCGCTGC3′, reverse primer: 5′TTCTGACCCATTCCCACCATC 3′). The amplified cDNA was separated *via* electrophoresis in a 1% agarose gel, stained with ethidium bromide, and visualized using electrophoresis gel imaging analysis system QuantiGel software. The obtained GSK3β cDNA fragments and pIREShyg3 vector were cut by the endonuclease NheI/EcoRV and then ligated by T4 DNA ligase. The recombinant plasmid was verified *via* sequencing.

2.6. Measurement of intracellular calcium

The PC12 cells were seeded in complete growth medium in a 96-well plate precoated with poly-lysine. After 24 h, the cells were treated with 10 μM rotenone for 48 h following pre-incubation with or without 10 μM BAPTA/AM for 30 min. Six replicates for each treatment in the PC12 cells were completed. The cells were washed 3 times with PBS, loaded with 5 μM Fluo-3/AM (Sigma–Aldrich, USA) for 30 min at 37 °C in the dark, and washed once with PBS to remove the extracellular Fluo-3/AM. PBS was used in place of Fluo-3/AM as a negative control. The fluorescent

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