Aggregation of α-Synuclein Induced by Oxidized Catecholamines as a Potential Mechanism of Lewy Body

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Lewy bodies (LBs) are neuronal inclusions that are closely related to Parkinson's disease (PD). The filamentous component of LB from patients with PD contains biochemically altered α-synuclein. We have investigated the effect of the oxidized products of catecholamines on the modification of α-synuclein. When α-synuclein was incubated with the oxidized 3,4-dihydroxyphenylalanine (L-DOPA) or dopamine, the protein was induced to be aggregated. The oxidized catecholamine-mediated α-synuclein aggregation was enhanced by copper ion. Radical scavengers, azide and N-acetyl cysteine significantly prevented the oxidized catecholamine-mediated α-synuclein aggregation. The results suggest that free radical may play a role in α-synuclein aggregation. Exposure of α-synuclein to the oxidized products of catecholamines led to the formation of dityrosine. Antioxidant dipeptides carnosine, homocarnosine and anserine significantly protected α-synuclein from the aggregation induced by the oxidized products of catecholamines.

Key Words : Lewy body, α-Synuclein, L-DOPA, Carnosine

Introduction

Parkinson's disease (PD) is the most common neurodegenerative disorder affecting brainstem extrapyramidal neurons. In PD, the synaptic protein α-synuclein was found to abnormally accumulate in Lewy bodies.[1-4] α-Synuclein is a major constitute of Lewy bodies in PD and senile plaque in Alzheimer's disease (AD).[5-7] It has been suggested that the aggregation of α-synuclein might be involved in the pathogenesis of PD.[8] Recently, it has been reported that α-synuclein was significantly aggregated by the copper-catalyzed oxidative reaction.[9] These findings support the contention that oxidative stress may play a critical role in the aggregation of α-synuclein. A variety of markers and indices in PD patients and animal models indicate involvement of oxygen-free radicals and oxidative stress in the pathogenesis of PD. These include lipid peroxidation,[10,11] reduced glutathione,[12] increased levels of iron and reduction of ferrritin concentrations in the substantia nigra pars compacta of PD.[13,14] Faults with the respiratory chain and dopamine metabolism have also been theorized to contribute to free radical production.[11]

Catechols are hydroquinones that may undergo oxidation to electrophilic semiquinones and quinines. Enzymatic oxidation of catechols followed by polymerization is a common form of pigmentation in animals. Also, protein-protein crosslinking by enzymatically oxidized catechols is a known mechanism of post-translational modification that occurs widely in nature.[15] Although such enzymatic activity is not present in human brain, endogenous brain catechols are oxidized spontaneously to quinoid compounds that polymerize to form neuromelanin in the substantia nigra and locus ceruleus.[16,17] It has been proposed that other quinone-mediated reactions beside polymerization may contribute to neurodegeneration in PD, e.g. catalytic transfer of reducing equivalents to molecular oxygen (redox cycling) and adduction to macromolecules.[18] The most widely used, and effective, treatment of PD since the late 1960s has been oral administration of L-DOPA. However, there has been considerable concern about the long-term effects of DOPA-induced cell apoptosis and cytotoxicity.[19-21] Futhermore, evidence has been presented for a potential role of catecholamine metabolites in the development and symptoms of schizophrenia.[22] L-DOPA and dopamine can be oxidized *in vitro* to generate semiquinones, $O_2^{-\cdot}$ and H_2O_2,[23] a process is greatly facilitated by the presence of transition-metal ions. In the case of iron and copper, $^{\cdot}OH$ will also be generated by Fenton reaction.[24] $^{\cdot}OH$ is the most powerful oxidizing species among several reactive oxygen radicals, and is able to oxidize most macromolecules like DNA, protein, lipid and carbohydrate. Recently, it has been reported that the modification and inactivation of human copper transport protein, ceruloplasmin, was induced by oxidized DOPA.[25]

In the present study, we investigated the effect of the non-enzymatically oxidized L-DOPA and dopamine on the modification of α-synuclein. Our results revealed that the aggregation of α-synuclein was induced by the products of oxidized catecholamines via the generation of free radicals. These results suggest that the oxidation of catecholamines may be involved in the oxidative stress-induced aggregation of α-synuclein in PD and related disorders.

Materials and Methods

Materials. Sodium azide, Dihydorxyphenylalanine (DOPA), dopamine, 5,5'-dimethyl 1-pyrolline *N*-oxide (DMPO), N-acetylcysteine, human erythrocytes Cu, Zn-SOD and catalase were purchased from Sigma (St. Louis, MO, USA). Chelex 100 resin (sodium form) was obtained from Bio-Rad

(Hercules, CA, USA). All solutions were treated with Chelex 100 resin to remove traces of transition metal ions.

Preparation of proteins. Recombinant DNA of human α-synuclein kindly provided by Dr. Jakes in pRK172 was transformed in *Escherchia coli* BL21(DE3) and the protein was prepared as described previously.[26] Bacterial cells (10 g) were lysed in 30 mL of 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl, 0.1 mM PMSF, 0.2 mg/ml lysozyme by the freeze (for 30 min in ice) and thaw method. Deoxycholic acid (40 mg) was added to the lysate and stirred at 4 °C. This solution was incubated at 37 °C for 30 sec. Streptomycin sulfate (0.3 g) was added to the solution and stirred at 4 °C. Following a 30 min centrifugation at 15,000 rpm, the supernatant was heated in a boiling water bath for 10 min. Following a further centrifugation the supernatant was applied to a Sephacryl S-100 column equilibrated in 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl (buffer I). Proteins were eluted with buffer I and the active fractions were applied to a DEAE-Sephacel equilibrated in buffer I. α-Synuclein was eluted with a linear gradient of 0.1-0.4 M NaCl in buffer I. All fractions containing α-synuclein were identified by SDS-PAGE. The fractionated α-synucleins were pooled and concentrated using an Amicon YM10 ultrafilter.

Immunoblot analysis of α-synuclein aggregation. α-Synuclein (0.25 mg/mL) in 10 mM potassium phosphate buffer, pH 7.4, was incubated at 37 °C for 4 h with different concentrations of catecholamines in a total volume of 20 μL. The samples were treated with seven μL of 4 X concentrated sample buffer (0.25 M Tris, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, 0.01% bromophenolblue) and were boiled at 100 °C for 10 min before electrophoresis. Each sample was subjected to SDS-PAGE and immunoblotting. For immunoblotting, the proteins on the polyacrylamide gel were electrophoretically transferred to nitocellulose membrane which was, in turn, blocked in 5% nonfat milk in Tris-buffered saline (TBS: 20 mM Tris, 0.2 M NaCl, pH 7.5) containing 0.05% tween-20 (TTBS). The membrane was incubated for 1 h at room temperature with anti-α-synuclein antibody (1 : 400) in TTBS. The membrane was washed by TTBS and incubated again with peroxidase labeled secondary antibody. The protein bands were visualized using enhanced chemiluminescence kit (ECL; Amersham).

Detection of o,o'-dityrosine. The reactions for the detection of o,o'-dityrosine were carried with α-Synuclein (0.25 mg/mL) and catecholamines in 10 mM potassium phosphate (pH 7.4). The fluorescence emission spectrum of the sample was then monitored in the 340-500 region (exitation, 325 nm) using Spectrofluorometer SMF 25 (Bio-Tek Instruments).

Results

Since free radicals can be generated in autoxidation of catecholamines,[23,24] we investigated whether catecholamines have any effects on the α-synuclein modification. Immunoblot analysis showed that the band shift was observed when α-synuclein was incubated with L-DOPA or dopamoine (Figure 1). The aggregation of α-synuclein became apparent at 10 mM catecholamines; the aggregation increased up to 50 μM catecholamines.

The effects of copper ion on catecholamine-mediated α-synuclein aggregation were investigated. Copper ions enhanced catecholamine-mediated α-synuclein aggregation (Figure 2). The participation of free radicals in the aggregation of α-synuclein by the oxidized catecholamines

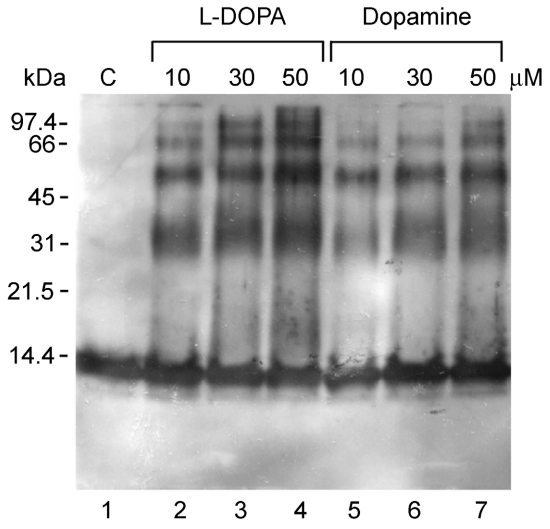

Figure 1. Aggregation of α-synuclein by oxidized catechols. α-Synuclein (0.25 mg/mL) was incubated in 10 mM phosphate buffer (pH 7.4) at 37 °C for 12 h under various conditions. Lane 1, α-synuclein control; lane 2-4, with 10, 30, and 30 mM L-DOPA; lane 5-7, with 10, 30 and 50 mM dopamine. The positions of molecular weight markers (kDa) are indicated on the left.

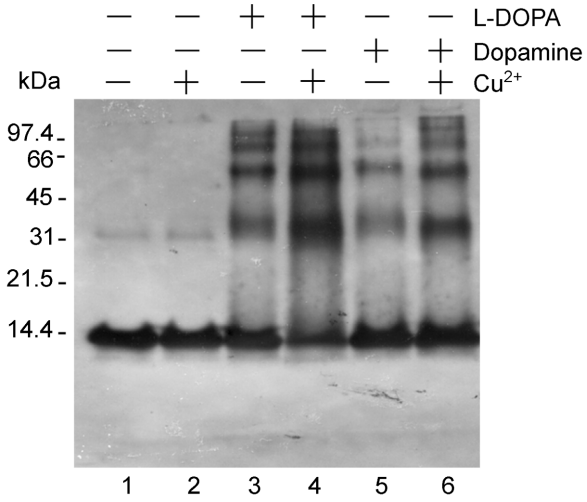

Figure 2. Effect of Cu^{2+} on the aggregation of α-synuclein. α-Synuclein (0.25 mg/mL) was incubated with 30 μM catechols with or without 500 μM Cu^{2+} in 10 mM phosphate buffer (pH 7.4) at 37 °C for 12 h. Lane 1, α-synuclein control; lane 2, with 500 μM $CuCl_2$; lane 3, with 30 μM L-DOPA; lane 4, lane 3 + 500 μM $CuCl_2$; lane 5, with 30 μM dopamine; lane 6, lane 5 + 500 μM $CuCl_2$. The samples were analyzed by immunoblotting. The positions of molecular weight markers (kDa) are indicated on the left.

was studied examining the protective effect of radical scavengers. The aggregation of α-synuclein was significantly suppressed in the presence of azide, N-acetyl-L-cysteine, catalase or DMPO (Figure 3). In contrast, Cu,Zn-SOD did not inhibited catecholamine-mediated α-synuclein aggregation. Spin-trapping agents have often been used to detect the presence of ˙OH radical in biological system. DMPO and PBN react with ˙OH radicals to form radical adducts. These results suggest that the autoxidation of catecholamines may lead to the generation of hydrogen peroxide and produce hydroxyl radical via transitional metal-catalyzed reaction (Fenton reaction). Thus, hydroxyl radical might play a critical role in the aggregation of α-synuclein by the oxidized catecholamines.

It has been reported that o,o'-dityrosine crosslink formation between dityrosine residues may play a part in the formation of oxidative covalent protein crosslink.[27] We investigated the formation of o,o'-dityrosine during catecholamines–induced aggregation of α-synuclein by measuring fluorescence emission spectrum between 340 and 500 nm with an excitation at 325 nm. When α-synuclein was incubated without catecholamines, the increase of fluorescence at 410 nm was not observed. In contrast, the incubation of α-synuclein with L-DOPA or dopamine dramatically increased the emission intensity (Figure 4). We investigated the effects of copper ion on the formation of dityrosine during the reaction of α-synucleins with catechols. The formation of dityrosine was enhanced in the presence of copper ions (Figure 5).

Carnosine (β-alanyl-L-histidine) and related dipeptides homocarnosine and anserine are present in several mammalian tissues, including skeletal muscle and brain at high concentrations (up to 20 mM in humans).[28-30] Many

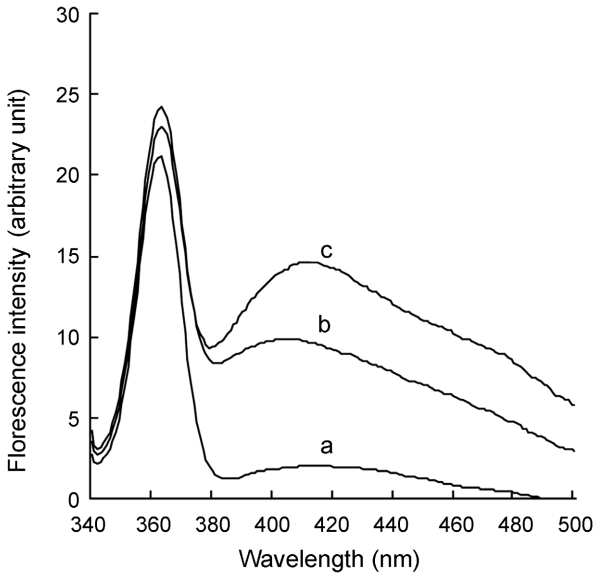

Figure 4. Dityrosine formation of the oxidized catechol-mediated α-synuclein aggregates. The fluorescence spectra of the oxidized catechol-mediated α-synuclein aggregates was observed after the reaction of α-synuclein with 30 μM catechols for 12 h. (a) α-synuclein control, (b) α-synuclein with dopamine, (c) α-synuclein with L-DOPA. The fluorescence emission spectrum of the sample was monitored in the 340-500 nm regions (excitation, 325 nm).

biochemical studies have suggested that these compounds possess copper chelation and free radical scavenging function which may partly explain its apparent homeostatic function.[31,32] Recently, it has been reported that carnosine could act as an antiglycation agent.[33] We investigated the effect of adding carnosine and related compounds to the aggregation of α-synuclein induced by the oxidized

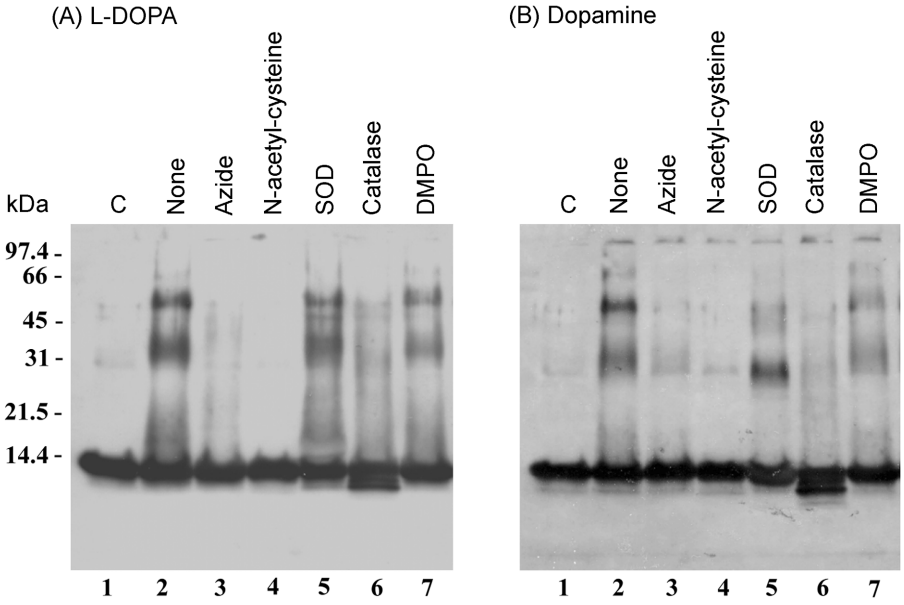

Figure 3. Effect of radical scavengers on the aggregation of α-synuclein by oxidized catechols. α-Synuclein (0.25 mg/mL) was incubated with 30 μM L-DOPA (**A**) or 30 μM dopamine (**B**) at 37 °C for 12 h in the presence of radical scavengers. Lane 1, α-synuclein control; lane 2, no addition of effectors; lane 3, 200 mM azide; lane 4, 50 mM N-acetyl cysteine; lane 5, SOD (0.2 mg/mL); lane 6, catalase (0.1 mg/ml); lane 7, 10 mM DMPO. The samples were analyzed by immunoblotting.

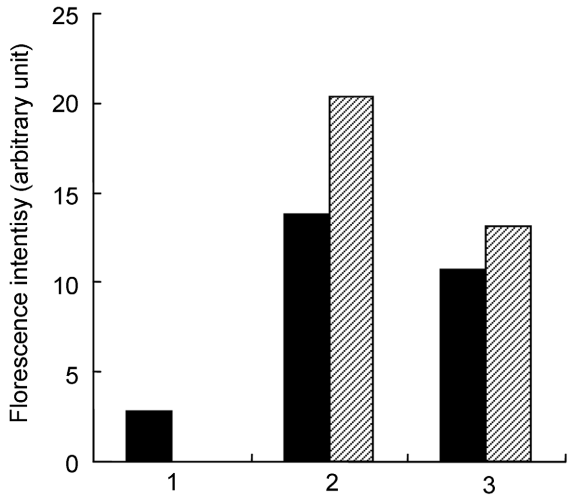

Figure 5. Effect of Cu^{2+} on dityrosine formation of the oxidized catechol-mediated α-synuclein aggregates. The fluorescence spectra of the oxidized catechol-mediated α-synuclein aggregates was observed after the reaction of α-synuclein with 30 μM catechols in the absence (black bar) or present (stripe bar) of 500 μM $CuCl_2$ for 12 h. (1) α-synuclein control (2) α-synuclein with L-DOPA (3) α-synuclein with dopamine.

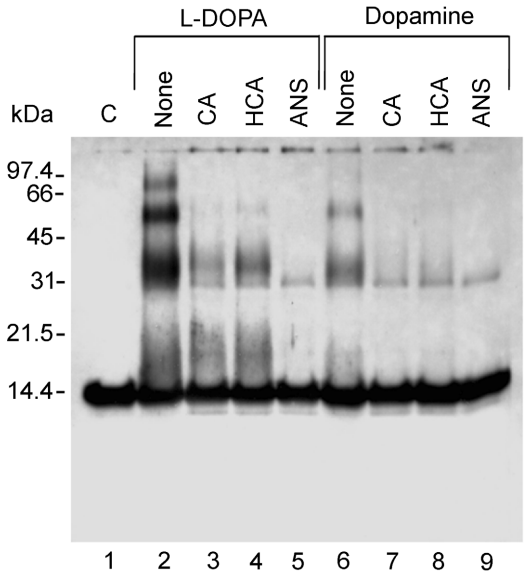

Figure 6. Effect of carnosine, homocarnosine and anserine on the aggregation of α-synuclein by oxidized catechols. Reaction mixture containing α-Synuclein (0.25 mg/mL), 30 μM L-DOPA or dopamine in the absence or present of carnosine, homocarnosine, and anserine. Reaction mixtures were incubated at 37 °C for 12 h and an aliquot was analyzed by immunoblotting analysis. Lane 1, α-synuclein control; lane 2, α-synuclein was incubated with 30 μM L-DOPA; lane 3, lane 2 + 20 mM carnosine; lane 4, lane 2 + 20 mM homocarnosine; lane 5, lane 2 + 20 mM anserine; lane 6, α-synuclein was incubated with 30 μM dopamine; lane 7, lane 6 + 20 mM carnosine; lane 8, lane 6 + 20 mM homocarnosine; lane 9, lane 6 + 20 mM anserine.

catecholamines. Carnosine, homocarnosine and anserine significantly prevented the catecholamine-mediated α-synuclein aggregation (Figure 6).

Discussion

α-Synuclein is a major component of aggregates forming amyloid-like fibrils in Lewy bodies (LBs) of Parkinson's disease (PD) and senile plaques of Alzheimer's disease (AD). Previous studies have suggested that oxidative stress might play a critical role in the pathogenesis of PD.[34-36] It has been shown that the iron-catalyzed oxidation reaction induced the aggregation of α-synuclein[37] and that copper (II) was the most effective metal ion affecting α-synuclein to form self-oligomers in the presence of coupling reagents.[38] Trace metals such as copper and iron, which are present in biological system, react with hydrogen peroxide and then produce the hydroxyl radicals. This reactive oxygen might lead to cross-linking and aggregation of amyloidogenic molecules.[39] The present study investigated the potential role of catecholamines in modification of α-synuclein. The toxicity of catechol may be augmented by its free radical-generating function in neurodegenerative disorder. It has been shown that the copper ions enhanced the production of hydroxyl radicals during the autoxidation of catechols.[40,41] Since the level of free radicals was reported to be increase in PD patients,[42,43] the oxidative modification of α-synuclein might have pathological significance. Our result showed that modification of α-synuclein was induced by the autoxidation of catecholamines.

L-DOPA and dopamine can be oxidized *in vitro* to generate semiquinones, $O_2^{\cdot-}$ and H_2O_2,[23] a process is greatly facilitated by the presence of transition-metal ions. In the case of iron and copper, $^{\cdot}OH$ will also be generated by Fenton reaction.[24] In this study, the catecholamine–mediated α-synuclein aggregation was inhibited by hydroxyl radical scavengers and spin-trapping agents. These results indicate that hydroxyl radicals may be involved in the aggregation of α-synuclein.

Trace metal such as iron and copper, which are variously present in biological systems, may interact with reactive oxygen species to damage macromolecules.[44-46] The cleavage of the metalloproteins by oxidative damage may lead to increases in the levels of metal ions in some biological cell.[47] It has been reported that copper concentration was significantly increased in the cerebrospinal-fluid of PD patients[48] and that in the cerebrospinal-fluid copper concentration was increased 2.2-fold in AD patients.[49] These results suggested that iron or copper-catalyzed oxidative reaction might contribute to the pathogenesis of PD. In the present study, copper ions led to enhancement of the aggregation of α-synuclein. These results indicate that copper ions may stimulate the catcholamine-mediated α-synuclein aggregation.

Many functions have previously been proposed for carnosine; these include antioxidant and free radical scavenger, physiological buffer, neurotransmitter, radioprotectant, metal chelator, and wound healing agent.[28-30] Our result showed that carnosine, homocarnosine, and anserine could protect α-synuclein from the aggregation by the oxidized catechols. One of the mechanisms by which antioxidants can protect

their biological targets from oxidative stress is the chelation of transition metals such as copper and iron, preventing them from participating in the deleterious Fenton reaction. Carnosine and anserine have been shown to be very efficient copper chelating agents.[31] These compounds might be able to bind Cu^{2+} and prevent some Cu^{2+}-dependent radical reaction.[28] Carnosine is active electrochemically as a reducing agent in cyclic voltammetric measurements, donating a hydrogen atom to the peroxyl radical.[28] It has been reported that carnosine and related compounds quench 50-95% of hydroxyl radicals produced in Fenton reaction.[32] Therefore, it can be assumed that carnosine, homocarnosine, and anserine may protect α-synuclein from the aggregation by the oxidized catecholamines through the scavenging of ·OH radicals. These results suggest that carnosine and related compounds may be explored as potential therapeutic agents for pathogenesis that involve the oxidative damage of protein mediated by active oxygen species.

In conclusion, the present results suggest that the modification of α-synuclein was induced by the autoxidation of catechols, involving ·OH generation from H_2O_2. This the catecholamine-mediated α-synuclein modification might therefore be involved in the pathogenesis of PD and related disorders.

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