Role of Cytochrome c as a Stimulator of α-Synuclein Aggregation in Lewy Body Disease*

(Received for publication, June 1, 1999, and in revised form, July 28, 1999)

Makoto Hashimoto†, Ayako Takeda‡, Leigh J. Hsu, Takato Takenouchi, and Eliezer Masliah†¶

From the Departments of †Neurosciences and ¶Pathology, University of California San Diego, School of Medicine, La Jolla, California 92037-0624 and the ‡Department of Psychiatry, Yokohama City University, School of Medicine, 3-9 Fukaura, Kanazawa-ku, Yokohama 236, Japan

α-Synuclein is a major component of aggregates forming amyloid-like fibrils in diseases with Lewy bodies and other neurodegenerative disorders, yet the mechanism by which α-synuclein is intracellularly aggregated during neurodegeneration is poorly understood. Recent studies suggest that oxidative stress reactions might contribute to abnormal aggregation of this molecule. In this context, the main objective of the present study was to determine the potential role of the heme protein cytochrome c in α-synuclein aggregation. When recombinant α-synuclein was coincubated with cytochrome c/hydrogen peroxide, α-synuclein was concomitantly induced to be aggregated. This process was blocked by antioxidant agents such as N-acetyl-L-cysteine. Hemin/hydrogen peroxide similarly induced aggregation of α-synuclein, and both cytochrome c/hydrogen peroxide- and hemin/hydrogen peroxide-induced aggregation of α-synuclein was partially inhibited by treatment with iron chelator deferoxamine. This indicates that iron-catalyzed oxidative reaction mediated by cytochrome c/hydrogen peroxide might be critically involved in promoting α-synuclein aggregation. Furthermore, double labeling studies for cytochrome c/α-synuclein showed that they were colocalized in Lewy bodies of patients with Parkinson's disease.

Taken together, these results suggest that cytochrome c, a well known electron transfer, and mediator of apoptotic cell death may be involved in the oxidative stress-induced aggregation of α-synuclein in Parkinson’s disease and related disorders.

Recent studies have suggested a potential role for abnormal protein aggregation in neurodegenerative disorders (1). In PD, a neurodegenerative disorder associated with dopaminergic nerve cell loss and presence of neuronal inclusion bodies and dystrophic neurites in the substantia nigra and various other regions in the brain (2), the synaptic protein α-synuclein was found to abnormally accumulate in LBs (3–6). α-Synuclein is a major constituent of LBs in PD and related disorders, whereby as a whole or a partially truncated molecule was shown to be aggregated to form amyloid-like fibrils (7–9).

Although the mechanism by which α-synuclein is involved in neurodegeneration in PD is unknown, accumulating evidence suggests that aggregation of α-synuclein may play a critical role in the pathogenesis of PD (10). In vitro, recombinant α-synuclein is induced to form amyloid-like fibrils under certain conditions, such as long time lag and high temperature, providing a model system that α-synuclein by its full-length molecule acts as an amyloidogenic protein (11). In this respect, it was recently reported that the mutant α-synucleins (A53T and A30P) associated with rare form of familial PD tend to be more easily aggregated than wild type α-synuclein (12, 13). Furthermore, it was shown in vitro that aggregates of both wild type and mutant α-synucleins induce apoptotic cell death in a human neuroblastoma cell line (14). These findings support the contention that aggregation of α-synuclein might be centrally involved in the pathogenesis of LBD. However, since the great majority of cases are not associated with mutations within this molecule, then other factors might contribute to α-synuclein aggregation in sporadic forms of the disease. Indeed, it has been shown that the in vitro aggregation of α-synuclein is modulated by various factors, such as Aβ (15–17), non-Aβ component of Alzheimer’s disease amyloid peptide (17), aluminum (18), and lipids (19), although none of them are likely to explain the mechanism by which α-synuclein is preferentially aggregated in the PD brain.

More recently, we have shown that α-synuclein was significantly aggregated by the iron-catalyzed oxidative reaction in vitro (20). These aggregates displayed Thioflavine-S/Congo red positive filamentous structures, reminiscent of amyloid-like fibrils found in LBs of PD brain (20). In this regard, it has been well documented that free radical formation derived from the auto-oxidation of dopamine into neuromelanin may be related to the selective degeneration of dopaminergic neurons in the PD brain (21). Moreover, iron is known to exist abundantly in the substantia nigra and its increase in the PD brain has been consistently reported (22).

In this context, we hypothesize that cytochrome c, a heme protein, could be a source of iron and oxidative stress (22) that might trigger the pathological aggregation of α-synuclein. Since cytochrome c functions as an essential component of the mitochondrial electron transport chain (23), dysfunction of this molecule may trigger the production of superoxide in mitochondria, resulting in enhanced oxidative stress conditions. In addition, since cytochrome c acts as a mediator of apoptotic cell death signals (24), it might be involved in an as yet uncharacterized mechanism which may link apoptosis to amyloidogenesis and neurodegeneration. These notions prompted us to extend our earlier work to the current investigation to determine whether cytochrome c is involved in the aggregation of α-synuclein in PD and related disorders.

* This work was supported by National Institutes of Health Grants AG05131 and AG10689. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence and reprint requests should be addressed: Dept. of Neurosciences, University of California San Diego, La Jolla, CA 92037-0624. Tel.: 619-534-1576; Fax: 619-534-6292; E-mail: emasliah@ucsd.edu

‡ The abbreviations used are: PD, Parkinson's disease; LB, Lewy bodies; LBD, Lewy body disease; Aβ, amyloid β-protein; DLBD, diffuse Lewy body disease; LBV, Lewy body variant of Alzheimer's disease; PAGE, polyacrylamide gel electrophoresis.

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EXPERIMENTAL PROCEDURES

Materials—Human α-synuclein was produced using 6×His expression system (Life Technologies, Inc.), as described previously (11)). Bovine heart cytochrome c and hemin were purchased from Sigma. The latter was stocked as 10 mM stock solution in Me₂SO. N-Acetyl-L-cysteine and deferoxamine mesylate were obtained from Calbiochem.

Aggregation Assays in Vitro—Aggregation assays were performed, as described previously (11). Briefly, cytochrome c (1-100 μM) and/or α-synuclein (10 μM) proteins were incubated in a total volume of 20 μl, containing 100 mM Tris-HCl buffer (pH 7.5) with various reagents at 37 °C for 24 h. Protein preparations were then subjected to SDS-PAGE, immunoblotting, and electron microscopy.

Immunoblot Analysis—Immunoblotting was performed, as described previously (11)). Briefly, the protein preparations (1 μg) were then electrophoresed and blotted onto nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked with Tris-buffered saline containing 1% bovine serum albumin. The membrane was then incubated with 125I-labeled protein A (ICN, Costa Mesa, CA), followed by incubation with the biotinylated anti-rabbit IgG (Vector, 1:150) and visualized with Vector Blue Alkaline Phosphatase Substrate Kit III (Vector).

RESULTS

Cytochrome c Is Aggregated in the Presence of H₂O₂—Since the main objective of the present study was to determine whether the heme-containing protein cytochrome c promoted aggregation of α-synuclein under oxidative stress conditions, we first determined whether the structure of cytochrome c was affected by oxidation. For this purpose, bovine purified cytochrome c (100 μM) was incubated with various concentrations (∼up to 10 mM) of H₂O₂ under the pH 7.5 (Tris-HCl, 100 mM) conditions at 37 °C for 24 h. SDS-PAGE analysis (Fig. 1a) showed that cytochrome c was induced to be aggregated by H₂O₂ in a concentration-dependent manner. Extensive formation of the SDS-resistant oligomers/multimers were observed in addition to the monomeric band corresponding to 15 kDa (Fig. 1a). Electron microscopic analysis showed that the aggregates of cytochrome c displayed an amorphous electron-dense structure that, occasionally, appeared to form small prefibrillar structures (Fig. 2, a and b). However, these aggregates were different from the classical α-synuclein fibrillar structures formed after incubation at 65 °C (Fig. 2c).

Aggregation of α-Synuclein Is Stimulated in the Presence of Cytochrome c/H₂O₂—In order to determine whether the aggregation of cytochrome c has any effects on α-synuclein aggregation, recombinant human α-synuclein was incubated with various concentrations (0, 1, 10, and 100 μM) of cytochrome c either in the presence or absence of 100 μM H₂O₂ under pH 7.5 (Tris-HCl, 100 mM) conditions at 37 °C for 24 h. Immunoblot analysis (Fig. 1b) showed that α-synuclein was preferentially aggregated in the presence of both cytochrome c and H₂O₂. Formation of the SDS-resistant bands at approximately 32 kDa, and higher molecular bands were observed in addition to the decreased immunoreactivity of authentic monomeric band corresponding to 18 kDa, suggesting that α-synuclein was aggregated to form dimers and insoluble aggregates. In contrast, the bands were not observed when α-synuclein was treated by either cytochrome c or H₂O₂ alone. These results suggest that oxidation is essential for the cytochrome c-induced aggregation of α-synuclein. Consistent with this, the aggregation of α-synuclein by cytochrome c/H₂O₂ was significantly suppressed in the presence of the antioxidative reagent N-acetyl-L-cysteine (1 mM) (Fig. 1c). Electron microscopic analysis of the aggregates induced by cytochrome c/H₂O₂ showed that they displayed characteristic fibrillar structures (Fig. 2d).

Aggregation of α-Synuclein in the Presence of Hemin/H₂O₂ and Its Inhibition by Deferoxamine—We recently found that
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Fig. 3. Immunoblot analysis of α-synuclein aggregation in the presence of hemin/H₂O₂ and inhibitory effects of deferoxisamine. α-Synuclein was incubated at 37 °C for 24 h in the presence of 10 μM cytochrome c (lanes 2–5) or 10 μM hemin (lanes 6–9), either with (lanes 3, 5, 7, and 9) or without (lanes 1, 2, 4, 6, and 8) treatment of 100 μM H₂O₂. Lane 1, no treatment; lanes 4 and 5 and 8 and 9, coinubcation with 1 mM deferoxisamine. The positions of molecular mass markers are indicated on the left side (kilodaltons).

α-synuclein was induced to be aggregated by iron-catalyzed oxidative reactions (20). Because cytochrome c is a heme protein, it was predicted that iron derived from a heme group covalently attached to cytochrome c may be attributed to the cytochrome c/H₂O₂-induced aggregation of α-synuclein. To test this hypothesis, α-synuclein was incubated in the presence of either hemin/H₂O₂ or hematoporphyrin/H₂O₂. Immunoblot analysis showed that α-synuclein (10 μM/100 μM) was aggregated by hemin/H₂O₂ (10 μM/100 μM) (Fig. 3), but not by hematoporphyrin/H₂O₂ (not shown). As was observed in the cytochrome c/H₂O₂-induced aggregation of α-synuclein, a similar pattern of the formation of the SDS-resistant bands was observed in hemin/H₂O₂-induced aggregation of α-synuclein (Fig. 3). Furthermore, both cytochrome c/H₂O₂- and hemin/H₂O₂-induced aggregation of α-synuclein was significantly inhibited by treatment with the iron chelator deferoxisamine (1 mM) (Fig. 3). Taken together, these results suggest that iron-catalyzed oxidative reaction is critically attributed to the cytochrome c/H₂O₂-induced aggregation of α-synuclein.

**Cytochrome c Is Colocalized with α-Synuclein in the Nigral LBs**—In order to support the possibility that cytochrome c may be involved in the aggregation of α-synuclein in neurodegeneration in the human brain, we utilized double immunolabeling techniques to determine whether cytochrome c immunoreactivity was present in LBs and if it was colocalized with α-synuclein. Lewy bodies in the substantia nigra were intensely stained with cytochrome c antibody (Fig. 4). Consistent with previous reports (3, 7), the immunoreactivity of the cytochrome c was localized to the peripheral portion of the central core of LBs, whereas both the core and halo of LBs were robustly stained with anti-C-terminal α-synuclein antibody. Immuno-absorption of the cytochrome c antibody with aggregated (but not native) cytochrome c resulted in complete elimination of LB staining by the cytochrome c antibody (not shown). Frequencies of the cytochrome c-positive staining of LBs ranged from 40 to 80% of those of the α-synuclein-positive staining, but did not appear to be not significantly different among various forms of LBD, including sporadic PD, DLBD, and LBV (Table I). In striking contrast to LBs in the substantia nigra, no immunoreactivity was observed in neo-cortical LBs (not shown). Furthermore, the antibody against cytochrome c did not label neurofibrillary tangles or senile plaques of Alzheimer’s disease (data not shown).

**DISCUSSION**

The present study investigated the potential role of cytochrome c in the aggregation of α-synuclein. This study showed that cytochrome c was induced to be aggregated by H₂O₂ treatment. Because the aggregates of cytochrome c seems to be different from amyloid-like fibrils, it is currently unknown whether or not these aggregates in and of themselves exert cytotoxicity. The importance of cytochrome c may be augmented by its stimulatory effects on the aggregation of α-synuclein. In support of this hypothesis, this study showed that aggregation of α-synuclein was not induced by either cytochrome c or H₂O₂ alone, but rather was significantly stimulated in the presence of both. The cytochrome c/H₂O₂-induced aggregation of α-synuclein was partially inhibited by an antioxidant N-acetyl-l-cysteine and both cytochrome c/H₂O₂- and hemin/H₂O₂-induced aggregation of α-synuclein was significantly inhibited by treatment with a specific iron chelator, deferoxisamine, indicating that iron-catalyzed oxidative reactions (fenton reaction) may be attributed to the aggregation of α-synuclein. Thus, these results indicate that oxidation of cytochrome c is critical for the stimulation of α-synuclein aggregation. Similarly, previous studies have shown that hemin cross-links proteins such as apolipoprotein B, myosin, and erythrocyte cytoskeletal proteins (26–29). In addition, hemin

**TABLE I**

Assessment of percent Lewy bodies displaying α-synuclein and cytochrome c immunoreactivity

| Case       | Diagnosis* | α-Synuclein + LBs (%) | Cytochrome c + LBs (%) | % of total LBs |
|------------|------------|-----------------------|------------------------|---------------|
| 1          | DLBD       | 26                    | 14                     | 54.0          |
| 2          | DLBD       | 13                    | 5                      | 62.5          |
| 3          | DLBD       | 8                     | 5                      | 62.5          |
| 4          | LBV        | 16                    | 10                     | 62.5          |
| 5          | LBV        | 16                    | 13                     | 81.0          |
| 6          | LBV        | 33                    | 17                     | 51.5          |
| 7          | LBV        | 35                    | 21                     | 60.0          |
| 8          | PD         | 3                     | 2                      | 67.0          |
| 9          | PD         | 27                    | 13                     | 48.0          |
| 10         | PD         | 30                    | 19                     | 63.0          |
| 11         | PD         | 31                    | 13                     | 42.0          |
| 12         | C          | 0                     | 0                      | 0             |
| 13         | C          | 0                     | 0                      | 0             |
| 14         | C          | 0                     | 0                      | 0             |
| 15         | C          | 0                     | 0                      | 0             |

* C = normal controls; DLBD = diffuse Lewy body disease; LBs = Lewy bodies; LBV = Lewy body variant of Alzheimer’s disease; PD = Parkinson’s disease.
has been shown to cross-link Aβ (30). Taken together these studies support the contention that oxidative stress might lead to neurodegeneration by promoting cross-linking and aggregation of amyloidogenic molecules (31).

Further supporting the possibility that cytochrome c and α-synuclein interact in vivo in LB formation, double immunolabeling studies showed that approximately half of the α-synuclein-positive LBs were also cytochrome c-positive. A recent study showed that LBs in substantia nigra were more intensively stained with α-synuclein than ubiquitin, although quantitative evaluation was not described (7). Ubiquitin had been previously regarded as a major sensitive marker of detecting LBs (32), and disorder of the ubiquitin-proteasome degradation pathway may be in some way attributed to the aggregation of α-synuclein (33). Therefore, high frequency of cytochrome c-positive LBs in the substantia nigra suggests that it may play a crucial role in the aggregation of α-synuclein. Of considerable interest is that there seemed to be no specific difference of frequencies among various types of LBD, including sporadic PD, DLBD, and LBV, and that neo-cortical LBs and other pathological lesions, such as senile plaques and neuronal intranuclear inclusions, are closely related (34). Furthermore, such pathological lesions are known to act as an essential component of the complex that may be accounted for by severe mitochondrial dysfunction due to oxidation and/or some region-specific factors in this area. Alternatively, an epitope for the cytochrome c antibody might be altered by unknown mechanism in cortical LBs and other pathological lesions.

Cytochrome c is known to have two well defined physiological functions: regulation of the electron transfer in mitochondria and mediation of apoptosis (34), and we suppose that both of these physiological functions of cytochrome c are closely related to its pathological action as a stimulator of α-synuclein aggregation. Since cytochrome c has a specific function in transfer of electrons between complex III (ubiquinol:cytochrome oxidoreductase) and complex IV (cytochrome oxidase), a dysfunction of this molecule may trigger production of reactive oxidant species in mitochondria, which would deteriorate the intracellular oxidative stress conditions (23). In addition, cytochrome c is known to act as an essential component of the complex that activates apoptotic cell death signal pathway. Once cytochrome c is released by cell death signals from the intramembrane of mitochondria to cytoplasm, it can trigger the activation of caspase-3, hence activating the downstream of apoptotic cell death pathway (24). However, a recent study using microinjection experiment strongly suggests that release of cytochrome c is not enough to cause cell death in primary cultured rat dorsal neurons (35), indicating that some postmitotic neurons have a capacity to be resistant to cytochrome c-induced apoptosis. If this indeed is the case with dopaminergic neurons in the substantia nigra, it is possible that the extended period of time in which cytochrome c remains in the cytoplasm might lead to an interaction with α-synuclein and its aggregation under oxidative stress conditions. It is also interesting to determine whether structural change of cytochrome c caused by oxidation may affect the activity of apoptotic signal pathway.

In conclusion, our in vitro and in vivo data suggest that cytochrome c, a well known electron transfer in mitochondria as well as an apoptotic cell death mediator, may be involved in the stimulation of α-synuclein aggregation under pathological conditions. Further investigation of the precise role of cytochrome c in the aggregation of α-synuclein may clarify the fundamental mechanism of the amyloidogenesis and neuronal cell death in PD and related α-synucleinopathies.

Acknowledgments—We thank Drs. Leon Thal, Robert Katzman, and Akihiko Iwai for their continuous encouragement.

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