

Oxidative Modification of Neurofilament-L by the Cytochrome *c* and Hydrogen Peroxide System

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As neurofilament proteins are major cytoskeletal components of neuron, abnormality of neurofilament is proposed in brain with neurodegenerative disorders such as Parkinson's disease (PD). Since oxidative stress might play a critical role in altering normal brain proteins, we investigated the oxidative modification of neurofilament-L (NF-L) induced by the reaction of cytochrome *c* with H₂O₂. When NF-L was incubated with cytochrome *c* and H₂O₂, the protein aggregation was increased in cytochrome *c* and H₂O₂ concentrations-dependent manner. Radical scavengers, azide, formate and N-acetyl cysteine, prevented the aggregation of NF-L induced by the cytochrome *c*/H₂O₂ system. The formations of carbonyl group and dityrosine were obtained in cytochrome *c*/H₂O₂-mediated NF-L aggregates. Iron specific chelator, desferoxamine, prevented the cytochrome *c*/H₂O₂ system-mediated NF-L aggregation. These results suggest that the cytochrome *c*/H₂O₂ system may be related to abnormal aggregation of NF-L which may be involved in the pathogenesis of PD and related disorders.

Key Words : Neurofilament-L, Cytochrome *c*, Oxidative stress, Parkinson's disease

Introduction

Many studies in Parkinson's disease (PD) have been shown that PD might be caused by protein aggregation due to aberrant protein folding or disturbed protein degradation.^{1,2} Lewy bodies (LBs) are cytoplasmic inclusions that are present consistently and with greatest frequency in neurons of the substantia nigra and locus ceruleus of patients with PD.³ The significance of brainstem LB lies in their intimate relation to neurodegeneration and PD. Indeed, the association between nigral LB formation and PD is so strong that the identification of a small number of nigral LB in asymptomatic patients has been interpreted by some as preclinical PD.^{4,5}

Detailed immunohistochemical studies indicate that neurofilaments are major components of LB.⁶ Neurofilaments are composed of three subunits, identified as light (NF-L), medium (NF-M) and heavy (NF-H). Neurofilaments are heteropolymers requiring NF-L together with NF-M or NF-H for polymer formation.⁷ All three subunits have been identified in LBs.⁸ Neurofilaments are the most abundant neuron-specific intermediate filaments⁹ and represent a major component of the neuronal cytoskeleton. It has been reported that peroxynitrite may nitrate tyrosine residues of NF-L, thereby altering NF assembly and causing neurofilament accumulation in neurons.¹⁰ Since NF-L is more abundant than the other two subunits in neurons, NF-L are more susceptible to oxidative stress.

Cytochrome *c* is known to have two well defined physiological functions: regulation of the electron transfer in mitochondria and mediation of apoptosis.¹¹ Because cytochrome *c* has a specific function in transfer of electrons between complex III and complex IV, a dysfunction of this molecule may trigger production of reactive oxygen species (ROS) in

mitochondria, which would deteriorate the intracellular oxidative stress condition.¹² It has been reported that mitochondrial dysfunction may be involved in a pathogenesis of neurodegenerative disorders.¹³ In addition, cytochrome *c* catalyses peroxidase-like reactions *in vitro*.¹⁴ Protein radicals induced by the peroxidative reaction of cytochrome *c* with H₂O₂ and other oxidants have been detected by ESR spin-trapping technique.^{15,16} Protein-derived radicals then can lead to oxidative damage of biological macromolecules.

In this study, we investigated whether the cytochrome *c* and H₂O₂ system is involved in the modification of NF-L. The present results revealed that the aggregation of NF-L was induced by cytochrome *c* and H₂O₂ is due to the oxidative damage resulting from free radicals generated by a combination of the peroxidase activity of cytochrome *c* and the Fenton reaction of free iron released from oxidatively damaged cytochrome *c*.

Materials and Methods

Materials. Cytochrome *c*, azide, formate, N-acetyl-cysteine, desferoxamine (DFX), ethylene glycol-bis(2-amino ethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), dithiothreitol (DTT), sodium dodecyl sulfate (SDS), 2,4-dinitrophenyl hydrazine (DNPH) and monoclonal anti-neurofilament 68 antibody (mouse) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Chelex 100 resin (sodium form) was obtained from Bio-Rad (Hercules, CA, U.S.A.).

Preparation of neurofilament-L. Protein expression and purification of NF-L were performed as previously described.¹⁷ A full-length cDNA clone of mouse NF-L in a pET-3d vector transfected into *E. coli* (BL21). Bacteria were grown in Luria broth supplemented with 1 mM isopropyl β -D-thiogalactopyranoside beginning at an OD₆₀₀ nm reading of

0.8. After a further 3 h at 37 °C, bacterial cells were harvested by centrifugation (4,000 × *g* for 10 min at 4 °C), resuspended in standard buffer (50 mM MES, 170 mM NaCl, 1 mM DDT, pH 6.25). The cells were disrupted with a French press at a pressure of 20,000 p.s.i. and centrifuged at 8,000 *g* for 15 min at 4 °C. The supernatant was incubated for 3 h at 37 °C and then was centrifuged at 100,000 × *g* for 20 min at 25 °C. The pellets containing the aggregated NF-L proteins were washed twice with standard buffer before they were dissolved in urea buffer (25 mM Na-phosphate, pH 7.5, 6 M urea, 1 mM EGTA and 1 mM DTT). The sample was loaded onto a DEAE-Sepharose column and was eluted with a linear 25–500 mM phosphate gradient in urea buffer and 80 ml NF-L eluted between 300 and 360 mM phosphate. These fractions were pooled and either used directly or stored at –80 °C for later experiments. Protein concentration was determined by the BCA method.¹⁸

Analysis of NF-L modification. Oxidative modification of NF-L was carried out by the incubation of NF-L (0.25 mg/mL) with 10 μM cytochrome *c* and 500 μM H₂O₂ in 10 mM phosphate buffer (pH 7.4) at 37 °C. After incubation of the reaction mixtures, the mixtures were stopped by freezing at –80 °C. The samples were treated with a 4 × concentrated sample buffer (0.25 mM Tris-HCl, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, 0.01% bromophenol blue) and heated in boiling water for 10 min. An aliquot of each sample was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli,¹⁹ using a 12% acrylamide slab gel. For immunoblotting, the proteins on the polyacrylamide gel were electrophoretically transferred to nitrocellulose 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 mouse monoclonal anti-neurofilament 68 antibody (1:400) in TTBS. The membrane was washed by TBS and incubated again with peroxidase labeled secondary antibody. The protein bands were visualized using enhanced chemiluminescence kit (ECL; Perkin-Elmer).

Detection of protein carbonyl compound. The carbonyl content of proteins was determined by immunoblotting with anti-DNP antibody as described elsewhere.³⁵ Both native and oxidized protein were incubated with 20 mM DNPH in 10% (v/v) trifluoroacetic acid at room temperature for 1 h. After incubation, a neutralization solution (2 M Tris) was added at room temperature for 15 min. After SDS-PAGE of the derivatized protein with 12% polyacrylamide gel, the proteins were transferred onto a nitrocellulose sheet and then probed with rabbit anti-DNP sera, used a dilution of 1:1000. The detection method used alkaline phosphatase-labelled goat anti rabbit IgG with the colorimetric detection system (Bio-Rad).

Detection of O,O'-dityrosine. The reactions for the detection of O,O'-dityrosine were carried with NF-L (0.25 mg/mL), 10 μM cytochrome *c* and 500 μM H₂O₂ in a total volume of 300 μL. The samples were diluted with 2.7 mL of Chelex 100-treated water and transferred to a cuvette (3

mL). The fluorescence emission spectrum of the sample was then monitored in the 340–500 region (excitation, 325 nm) using fluorescent spectrometer SMF 25 (Bio-Tek Instruments).

Replicates. Unless otherwise indicated, each result described in this paper is representative of at least three separate experiments

Results

We first investigated whether the cytochrome *c* can induce the aggregation of NF-L. For this purpose, NF-L was incubated with various concentrations of cytochrome *c* and H₂O₂ under the pH 7.4 (10 mM phosphate buffer) conditions at 37 °C for 2 h. Immunoblotting analysis showed that the intensity for the original protein was reduced and new high molecular weight material was visualized at the stacker/separator gel interface (Fig. 1). The aggregation of NF-L became apparent at 1 μM cytochrome *c* and 0.1 mM H₂O₂; the aggregation increased up to 30 μM cytochrome *c* and 1 mM H₂O₂, respectively.

The effect of radical scavengers on the aggregation of NF-L by the cytochrome *c*/H₂O₂ system was studied. The aggregation of NF-L by the cytochrome *c*/H₂O₂ system was significantly suppressed in the presence of azide, formate

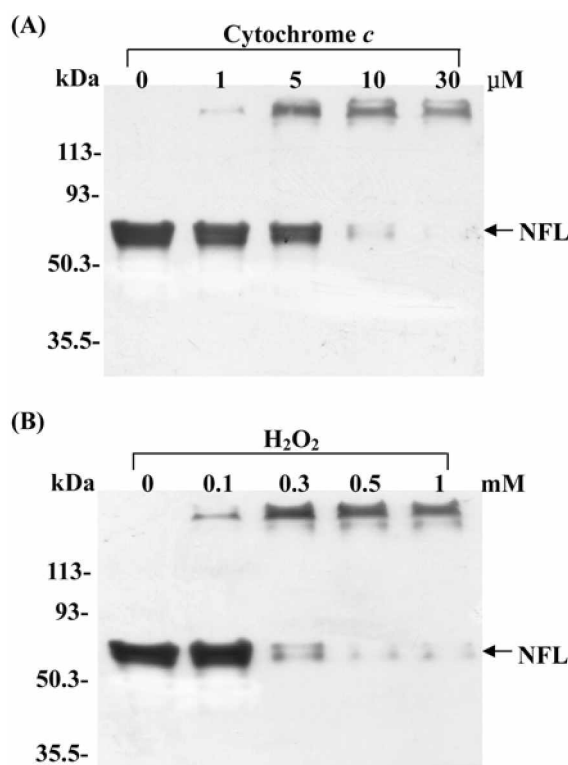


Figure 1. Aggregation of NF-L by the cytochrome *c* and H₂O₂ system analyzed by immunoblotting. NF-L (0.25 mg/mL) was incubated with various concentrations of cytochrome *c* and various concentrations of H₂O₂ in 10 mM phosphate buffer (pH 7.4) at 37 °C for 2 h. (A) NF-L was incubated with indicated concentrations of cytochrome *c* and 500 μM H₂O₂. (B) NF-L was incubated with 10 μM cytochrome *c* and indicated concentrations of H₂O₂. The positions of molecular weight markers (kDa) are indicated on the left.

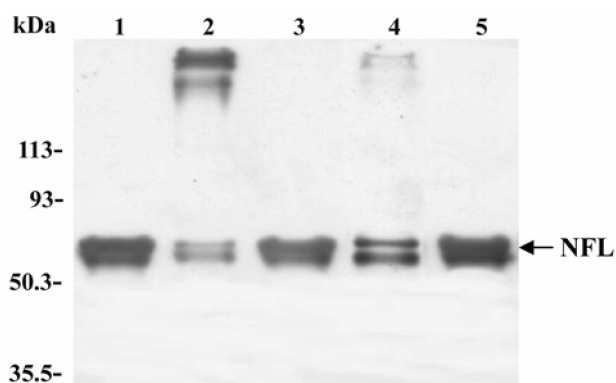


Figure 2. Effect of radical scavengers on cytochrome *c*/H₂O₂ system-mediated NF-L aggregation. NF-L (0.25 mg/mL) was incubated with 10 μ M cytochrome *c* and 500 μ M H₂O₂ in 10 mM phosphate buffer (pH 7.4) at 37 °C for 2 h in the presence of radical scavengers. Lane 1, NF-L control; lane 2, no addition; lane 3, 200 mM azide; lane 4, 200 mM formate; lane 5, 10 mM N-acetyl-L-cysteine.

and N-acetyl-cysteine (Fig. 2). The result suggests that free radical might play a critical role in the aggregation of NF-L by the cytochrome *c*/H₂O₂ system.

It has been shown that protein oxidation is accompanied the conversion of some amino acid residues into carbonyl derivatives.²⁰ The carbonyl content of protein can be measured using phenylhydrazine formation reaction. The method for detecting carbonyl-containing proteins employs derivatization with 2,4-DNPH followed by analysis with anti-DNP sera. Results obtained from the immunoblotting analysis of NF-L aggregates are shown in Figure 3. Carbonyl compounds were detected in the aggregates of NF-L induced by the cytochrome *c*/H₂O₂ system.

O,O'-dityrosine crosslink formation between tyrosine resi-

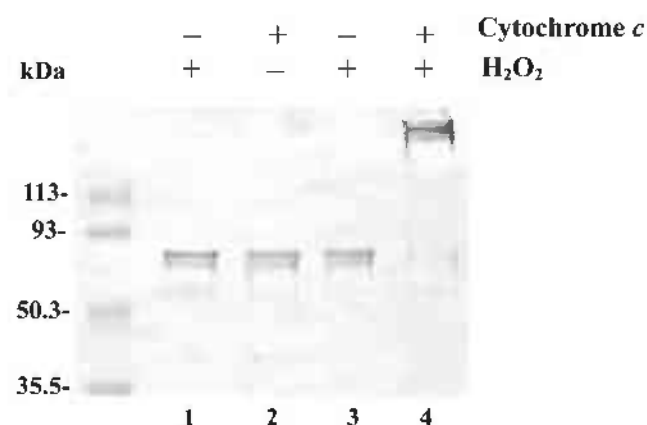


Figure 3. Immunoblotting analysis of carbonyl groups in NF-L after incubation of cytochrome *c* and H₂O₂. NF-L (0.25 mg/mL) was incubated in 10 mM phosphate buffer (pH 7.4) at 37 °C for 2 h under various conditions. After incubation, samples were derivatized DNPH as described under "Materials and methods". DNPH-derivatized proteins were subjected to SDS-PAGE for immunoblot with anti-DNP sera. Lane 1, NF-L control; lane 2, NF-L with 10 μ M cytochrome *c*; lane 3, NF-L with 500 μ M H₂O₂; lane 4, NF-L with 10 μ M cytochrome *c* and 500 μ M H₂O₂.

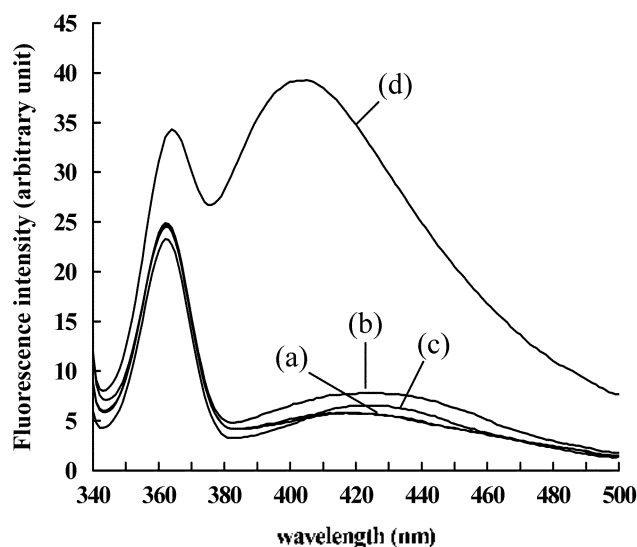


Figure 4. Fluorescence spectra of cytochrome *c*/H₂O₂ system-mediated NF-L aggregation. The fluorescence spectra of the formation of dityrosine was observed when NF-L (0.25 mg/mL) was incubated with or without 10 μ M cytochrome *c* and 500 μ M H₂O₂. (a) NF-L control (b) NF-L + cytochrome *c* (c) NF-L + H₂O₂ (d) NF-L + cytochrome *c* + H₂O₂.

dues may play a part in the formation of oxidative covalent protein crosslink.²¹ We investigated the formation of O,O'-dityrosine during the cytochrome *c*/H₂O₂ system-mediated NF-L aggregation by measuring fluorescence emission spectrum between 340 and 500 nm with an excitation at 325 nm. The reactions were carried out with NF-L in the presence or absence of cytochrome *c* and H₂O₂. As the reactions were proceeded, the emission peak at 410 nm due to the formation of O,O'-dityrosine crosslinks was increased (Fig. 4).

Because iron ion could be released from the oxidatively damage cytochrome *c* by H₂O₂,²² it was predicted that iron

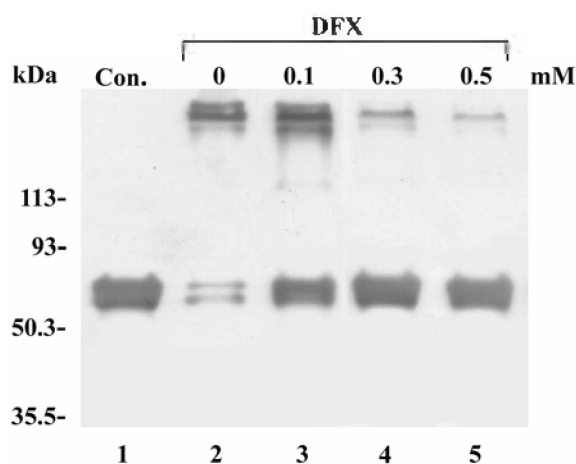


Figure 5. Effect of iron chelator on cytochrome *c*/H₂O₂ system-mediated NF-L aggregation. NF-L (0.25 mg/mL) was incubated with 10 μ M cytochrome *c* and 500 μ M H₂O₂ in 10 mM phosphate buffer (pH 7.4) at 37 °C for 2 h in the presence of iron chelator. Lane 1, NF-L control; lane 2, no addition; lane 3, 0.1 mM DFX; lane 4, 0.3 mM DFX; lane 5, 0.5 mM DFX. The positions of molecular weight markers (kDa) are indicated on the left.

may be contributed to the cytochrome *c*/H₂O₂-induced aggregation of NF-L. To test this possibility, we have investigated the effects of the iron chelators on the aggregation of NF-L by the cytochrome *c*/H₂O₂ system. The cytochrome *c*/H₂O₂-induced aggregation of NF-L was significantly inhibited by iron chelators, DFX (Fig. 5). These results suggest that iron ions are involved in the aggregation of NF-L by the cytochrome *c*/H₂O₂ system.

Discussion

NF-L, a major structural protein important to the survival of neurons, was modified by the cytochrome *c*/H₂O₂ system. Neurofilaments are susceptible to oxidation in part because they are among the most abundant proteins in a cell. Previous studies have suggested that oxidative stress might play a critical role in the pathogenesis of PD.^{23,24} Biochemical analysis of LB has shown them to be composed largely of a 68 kDa protein that was soluble in formic acid a result that almost certainly precludes covalent crosslinking of protein.^{25,26} It has been reported that free radicals were generated in the reaction of cytochrome *c* with H₂O₂, and that free radical formation was linear with respect to the concentrations of cytochrome *c* and H₂O₂.²⁷ Therefore, we suggest that free radicals formed by the cytochrome *c*/H₂O₂ may be involved in the aggregation of NF-L. Evidence that radical scavengers protected NF-L aggregation induced by the cytochrome *c*/H₂O₂ (Fig. 2) supports this mechanism. Cytochrome *c* forms tyrosyl radicals when exposed to H₂O₂,¹⁵ and these radicals can be transferred to tyrosine residues on other protein.²⁸ In this context, we hypothesize that tyrosines are key residues in the aggregation of neurofilament in the cytochrome *c*/H₂O₂ system. The data in Figure 4 shows that the formations of dityrosine are detected in NF-L aggregates. Therefore, we suggest that tyrosines in NF-L are required for NF-L aggregation.

Trace metal such as iron and copper, which are variously present in biological systems, may interact with ROS, to damage macromolecules.²⁹⁻³² The cleavage of the metallo-proteins by oxidative damage may lead to increases in the levels of metal ions in some biological cell.³³ Previous reports showed that a metal chelator inhibited the peroxidase activity of cytochrome *c*.²⁷ The participation of iron ions in the cytochrome *c*/H₂O₂-mediated NF-L aggregation was investigated by an examination of the protective effects of the iron chelator DFX. The results showed that DFX significantly prevented the aggregation of NF-L induced by the cytochrome *c*/H₂O₂ system (Fig. 5). It has been reported that iron ions released from the reaction of cytochrome *c* with H₂O₂. This is likely due to the peroxidase activity of cytochrome *c*. Interestingly, iron accumulation has been proposed as an important event in the nigral neurodegeneration of PD.³⁴ Since iron ions could stimulate Fenton reaction to produce hydroxyl radical, NF-L aggregation may be mediated in the cytochrome *c*/H₂O₂ system via the generation of free radicals.

In conclusion, the results presented here suggest that cytochrome *c*, a well known electron transfer in mitochondria, may be involved in the stimulation of NF-L aggregation under pathological conditions.

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